The isolation of useful bioproducts remaining from the large-scale fermentation of penicillium chrysogenum

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A Thesis Entitled

The Isolation of Useful Bioproducts Remaining From the Large-Scale Fermentation of *Penicillium chrysogenum*

Submitted by

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Statement of Copyright

Declaration

The work described in this thesis was carried out in the Department of Chemistry at Durham University between October 2004 and October 2007. All the work was carried out by the author, unless otherwise stated, and has not previously been submitted for a degree at this or any other university.

This work has been presented at:

- 8th International Conference of the European Chitin Society, Antalya, Turkey, Oral Presentation by Dr. David R.W. Hodgson
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The following journal articles are currently under preparation:

- H.R. Watson, D.C. Apperley, R. Edwards, D.P. Dixon, L.R. Hutchings, R. Johnson, J. Davies, and D.R.W. Hodgson, *" An Efficient Method for ^{15}N-labeling of Chitin in Fungi."*
- H.R. Watson, D.C. Apperley, R. Edwards, D.P. Dixon, L.R. Hutchings, R. Johnson, J. Davies, and D.R.W. Hodgson, *" Whole-Cell ^{13}C and ^{15}N ssNMR Studies on the Chitinous Materials in Fungi."*

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Abstract

Chitin, a homopolymer of β-(1-4) linked *N*-acetyl D-glucosamine units, and its deacetylated derivative chitosan have unique properties that may allow their utilisation in a diverse array of high-value applications. Currently chitinous materials are commercially produced from the waste products of the seafood processing industry, this supply is seasonal and the extraction procedures required harsh, resulting in products with heterogeneous characteristics. In this work novel methods of extraction of chitinous material from the dry fungal biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* in the penicillin manufacturing industry were investigated, with the aim of avoiding or minimising the harsh chemical treatments. This work was carried out in partnership with Angel Biotechnology, who produce penicillin commercially and provided the waste biomass. It was determined that the chitinous material present in this biomass was too intractable for this to be a suitable commercial source of chitin, as large quantities of non-chitinous polysaccharide impurities remained in the product. Attempted enzymatic degradations of the fungal cell wall did not increase the level of purity of the extract. Comparison to other fungal sources of chitinous material indicated that *P. chrysogenum* does not provide the most efficient source of chitinous material.

During the course of these studies it became apparent that there is no agreed literature procedure for the determination of the degree of deacetylation (DDA) of chitinous material, this characteristic is essential in determining the physiochemical properties of the polymer. In reviewing the procedures available we concluded that ^{15}N solid-state NMR offered the most reliable method, however, its use was limited by the low natural abundance of ^{15}N. We therefore developed a novel, efficient and directed strategy for the ^{15}N labelling of chitinous material in fungal cells walls. This allows the direct determination of the DDA of chitinous material in whole fungal cells without the need for lengthy extraction procedures. The whole cell CPMAS ssNMR techniques developed may find many applications, such as monitoring cell wall biogenesis in response to varying nutrient conditions. Additionally, this may allow the rapid screening of fungal species to determine the concentration and DDA of chitinous material.

Contents

Abbreviations

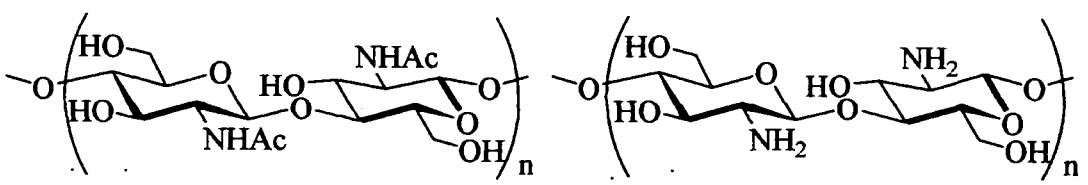
AcCoA	Acetyl Coenzyme A
AcOH	Acetic Acid
AIM	Alkali Insoluble Material
CoASH	Coenzyme A
CP	Cross-Polarisation
CPMAS	Cross-Polarisation Magic Angle Spinning
DA	Degree of Acetylation
DCC	Dicyclohexylcarbodiimide
DDA	Degree of Aeacetylation
DIPEA	*N,N*-diisopropylethylamine
DMAc	*N,N*-dimethylacetamide
DMF	*N,N*-dimethylformamide
DP	Direct-Polarisation
EtOH	Ethanol
$FeCl_3$	Iron (III) Chloride
Fmoc-Cl	Fluorenylmethoxycarbonyl-chloride
Fru-6-P	Fructose-6-phosphate
FTIR	Fourier Transform Infrared Spectroscopy
GlcN	Glucosamine
GlcNAc	*N*-acetyl Glucosamine
GPC	Gel Permeation Chromatography
GPI	Glycosyl Phophotidylinositol
HCl	Hydrochloric acid
HPDEC	High-Power ^1H Decoupling
HPLC	High-Performance Liquid Chromatography
IR	Infrared spectroscopy
KOH	Potassium hydroxide
LC-MS	Liquid Chromatography/Mass Spectrometry
L-Gln	L-Glutamine
L-Glu	L-Glutamate
LiCl	Lithium Chloride
LS	Light Scattering
MAS	Magic Angle Spinning
MBTH	3-Methyl-2-Benzothiazolone Hydrazone
NaOH	Sodium Hydroxide
$NaNO_3$	Sodium Nitrate
$(NH_4)_2SO_4$	Ammonium Sulphate
NMR	Nuclear Magnetic Resonance
PAA	Phenylacetic Acid
Pd	Palladium
RALS	Right-Angle Light Scattering
RI	Refractive Index
SEC	Size Exclusion Chromatography
SD	Standard Deviation

ssNMR	Solid-State Nuclear Magnetic Resonances
UDP	Uridine Diphosphate
UTP	Uridine Triphosphate
UV	Ultraviolet

Chapter 1 – Introduction

1. Introduction

Chitin, a homopolymer of β-(1-4) linked *N*-acetyl D-glucosamine units (Figure 1.1), is a natural polysaccharide of major importance. This biopolymer is synthesised by fungi and invertebrates and is second only to cellulose in the amount produced annually by biosynthesis.[1] It is structurally similar to cellulose, with an acetamide group in place of the C2 hydroxyl group of cellulose. This similarity in structure is reflected in the similar roles played by the two polymers in nature, both acting as structural and defensive materials. Chitin occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods[2] (crustaceans, insects and spiders) and in the cell walls of fungi.[3] It is also produced by a number of other living organisms in the lower plant and animal kingdoms, serving many functions where reinforcement and strength are required.[4]



Chitin Chitosan

Figure 1.1 - β-(1-4) linked N-acetyl D-glucosamine (Chitin) and β-(1-4) linked D-glucosamine (Chitosan)

The principal derivative of chitin is the fully deacetylated form, known as chitosan, which contains β-(1-4) linked glucosamine units. The names chitin and chitosan actually refer to a family of compounds with varying degrees of deacetylation (DDA). The DDA dictates the behaviour and the physiochemical properties of the polymer such as its solubility,[5, 6] biological activity[7] and reactivity.[8] As the DDA increases to 50 % the material becomes increasingly acid soluble and is known as

chitosan, which is a polycationic polymer.[4] The natural occurrence of chitosan is much less widespread than that of chitin, however, it does occur naturally in some fungi.[9]

Chitinous materials' lack of toxicity and allergenicity along with their biocompatibility[10], biodegradability[11] and interesting bioactivities, described in sections 5.1, 5.2 and 5.3, make them very useful materials for pharmaceutical and medical applications.[12] Chitin can be used for biodegradable sutures and can act as second skins for burns victims,[13] or be woven into bandages that stop bacterial infection and bleeding.[14] Research has shown that chitinous materials can promote healing of wounds[7], and they have also been used for systemic and local delivery of drugs and vaccines.[15-17] Chitosan is also used in the paper and textiles industry to produce certain surface properties.[18] The presence of amine groups in chitosan confers an ability to chelate heavy metals and bind organic molecules, removing them from aqueous solution. Therefore chitosan can be used to treat drinking water, separating organic compounds and heavy metals.[19, 20]

In the following chapter a brief introduction to chitinous materials, their structure, commercial sources and applications, will be outlined. Further introduction to more specific areas of interest in this project, such as the role and nature of chitinous materials in fungal cell walls and the proposed methods of analysis of chitinous materials, is given at the start of each of the relevant chapters.

1.1 History of Chitin

Chitin was first isolated in 1811 by Braconnot as the residue remaining from the treatment of *Agaricus volvaceus* and other mushrooms with warm dilute alkali.[21] Braconnot termed this product 'fungine' and demonstrated that it contained nitrogen and its degradation yielded acetic acid. We now know that fungine most likely contained an equal mixture of chitin and a non-nitrogenous polyglucan. Braconnot described several chemical reactions of this extracted product and clearly stated that it

was distinct from polysaccharides present in plants. The name Chitin (Greek for tunic or covering) was suggested by Odier in 1823 who isolated chitin from the elytrum of the cockchafer beetle, or May bug, by repeated treatments with hot KOH solutions.[22] He commented that 'it is most remarkable to find out in the insect structure the same substance that forms the structure of the plants'. Odier identified chitin as being present in demineralised crab carapace and suggested that it is the basic material of the exoskeletons of all insects. He also noted the similar protecting roles of cellulose and chitin in plants and animals respectively. The following year Children published an English translation of Odier's work together with some additional results from his own studies which included information on the elemental composition of chitin.[23] Consideration of the extraction procedures employed and the elemental analysis of the products indicates that it is probable that both Odier and Children extracted chitosan rather than chitin, however, chitosan was not recognised and described until 1859 when Rouget reported that treatment of chitin with concentrated KOH under reflux conditions yielded a 'modified chitin' which was soluble in dilute solutions of organic acids.[24] In 1894 Hoppe-Seyler heated chitin from the shells of crustaceans in concentrated KOH at 180 °C to yield a product which was readily soluble in acetic acid and hydrochloric acid solutions, the author proposed the name chitosan for the product.[25]

For much of the nineteenth century there was confusion between cellulose, chitin and chitosan and it was some time before the structures of chitin and chitosan were assigned. Today it is generally accepted that chitin, from both animal and fungal sources, is predominately poly[β-(1-4)-2-acetamido-2-deoxy-D-glucopyranose]. D-glucosamine residues may also be present, the mole fraction and distribution of which is dependent on the chitin source.

1.2 Physical Structure of Chitin

The ability of chitin to perform structural roles in nature is primarily due to its high tensile strength and poor solubility; these properties arise from the extensive

crystalline nature of the material. X-ray diffraction studies of chitin, which indicate that it contains a high degree of local order as well as areas of long-range disorder[26], have led researchers to the conclusion that chitin chains associate by hydrogen bonding to form areas of highly ordered crystalline structure.[27-29] In these crystalline regions chitin has been found in three polymorphic forms which differ in the packing and polarities of the adjacent polymer chains.[27, 30] In α-chitin the chains are arranged anti-parallel, in β-chitin they are parallel and in γ-chitin there are two chains 'up' for each chain 'down', see Figure 1.2. IR studies claim to reinforce these findings by establishing differences in the hydrogen-bonding network present in these polymorphs, notably in the amide I (C=O) vibration mode.[27, 30, 31]

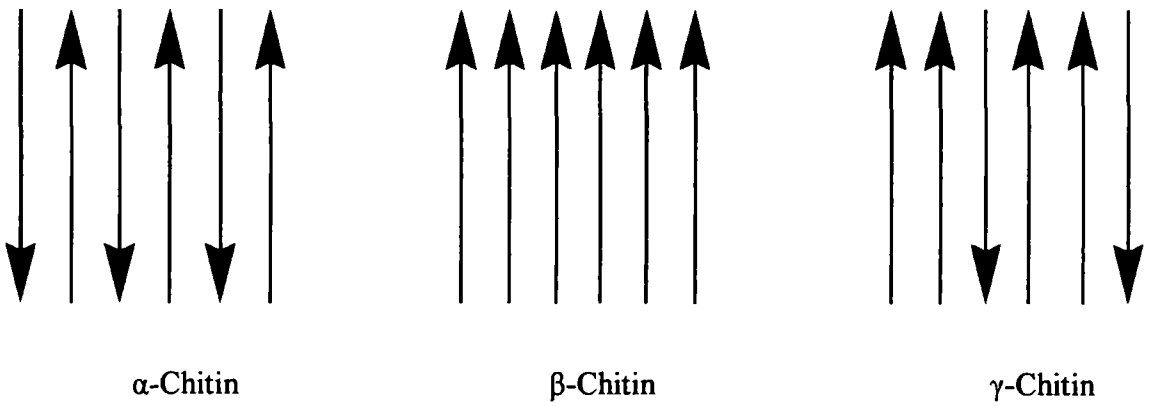


Figure 1.2 – Arrangement of the chitin chains found in the three polymorphic forms

The polymorphic form of chitin has been related to its source, that is the taxonomic group from which it comes, for example β-chitin appears to be absent in the fungal kingdom. Although the source does affect the form of chitin present to some extent, all three polymorphic forms have been found in one organism indicating that the form relates to the function of the chitin rather than its source.[27] The chain composition determines the characteristics of the chitin, for example its toughness, solubility and reactivity. α-Chitin is the most crystalline and compacted form as it has the most rigid network of hydrogen bonding and so is found where extreme hardness is required, for example in the cell walls of fungi and the shells of crustaceans. β and γ chitin have a less rigid framework and so are found where flexibility, mobility and toughness are required, for example β-chitin is found in squid pens and γ-chitin in the cocoon fibres

of beetles and the stomach lining of squid. The lower degree of intermolecular bonding found in β-chitin imparts a higher degree of solubility to the material and confers the ability to swell. This means that its reactivity, for example towards deacetylation, is higher. Elucidation of the 3D structure of chitin has aided our understanding of its role in nature and enabled us to tune its physical properties to suit the requirements of the roles we wish it to perform. The most abundant polymorphic form is α-chitin, which also appears to be the most stable as both β and γ chitin can be transformed into the α-form by destroying and reforming the crystal structure, for example by precipitating from solution in formic acid or by the formation of an alkali chitin complex.[32] Therefore, in the following sections the hydrogen bonding network present in all three polymorphs will be discussed, however, much greater attention will be paid to α-chitin.

1.3.1 α-chitin

As the most common polymorph, the structure of α-chitin is the most extensively studied with the first crystallographic studies dating from the 1920s.[33] The unit cell dimensions were established early on, however, it was not until 1957 that a detailed structural analysis of the unit cell was proposed.[28] Carlstrom proposed a unit cell containing a disaccharide unit of two chains which are arranged anti-parallel. This structure was based on a bent chain, which Carlstrom argued would allow intramolecular hydrogen bonding between the endocyclic pyranose oxygen and the C3 hydroxyl group, as shown in Figure 1.3, which would stabilise the chain and give a repeat distance that agrees with X-ray diffraction results.

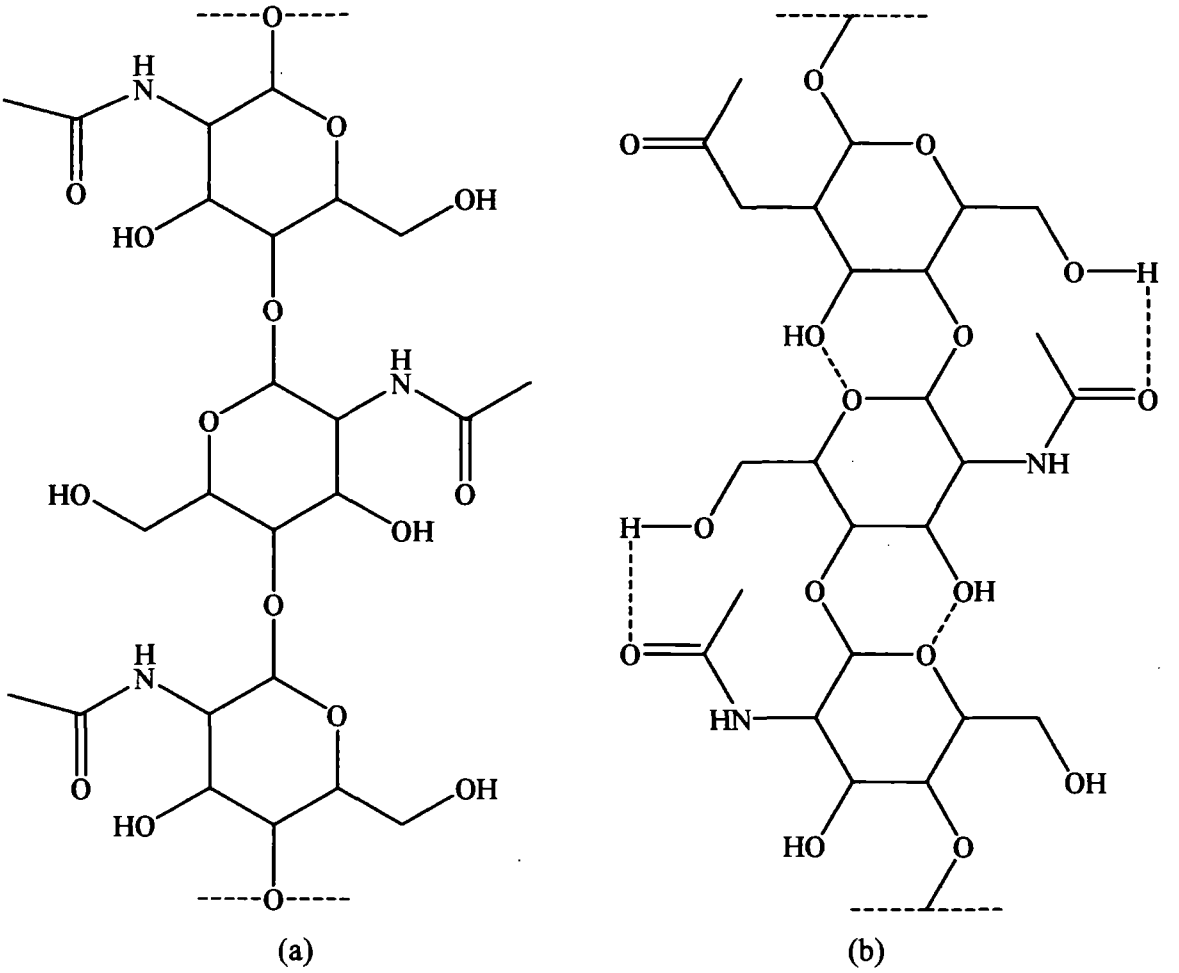


Figure 1.3 - Straight (a) and bent (b) conformations of the repeat disaccharide unit in the chitin chain.

According to Carlstrom's model the structure of chitin can be seen as a series of hydrogen bonded sheets formed by chitin chains arranged in an anti-parallel sense. Successive chains of the same sense along the A axis, perpendicular to the sheets (see Figure 1.4), are linked by hydrogen bonds between the carbonyl oxygen and the hydrogen of the amide group, forming hydrogen bonds between the sheets. Comparing the observed and theoretical X-ray diffraction intensities, Carlstrom's model gave reasonable agreement. However, the inability of the model to match the characteristics indicated by the IR analysis of this material presented some major criticisms. Namely, IR analysis indicates that all of the hydroxyl groups should be participating in hydrogen bonding and, importantly, the IR spectra suggest that the amide groups appear to be present in two environments, as evident by the splitting of

the amide I peak. Additionally, chitin's inability to swell in water suggests that there must be interchain hydrogen bonding in the C direction, in the plane of the sheets perpendicular to the direction of the chains. These factors are not accounted for by Carlstrom's model.

Blackwell *et al.* proposed an improved model (see Figure 1.4) in which the intramolecular hydrogen bonding between the carbonyl oxygen and the C6 hydroxyl group is present in only fifty percent of the chains.[29] The authors suggest a 50/50 statistical mixture exists between this intramolecular bonding and intermolecular hydrogen bonding that involves the CH_2OH residues of two adjacent chains in the C direction. These bonds are marked * in Figure 1.4. In addition they suggested that there is full intramolecular bonding between the C3 hydroxyl group and the endocyclic oxygen, shown in, and full intermolecular bonding between the N-H of an amide group on one chain and the carbonyl oxygen of an amide group on a chitin chain in an adjacent sheet in the A direction (not shown).

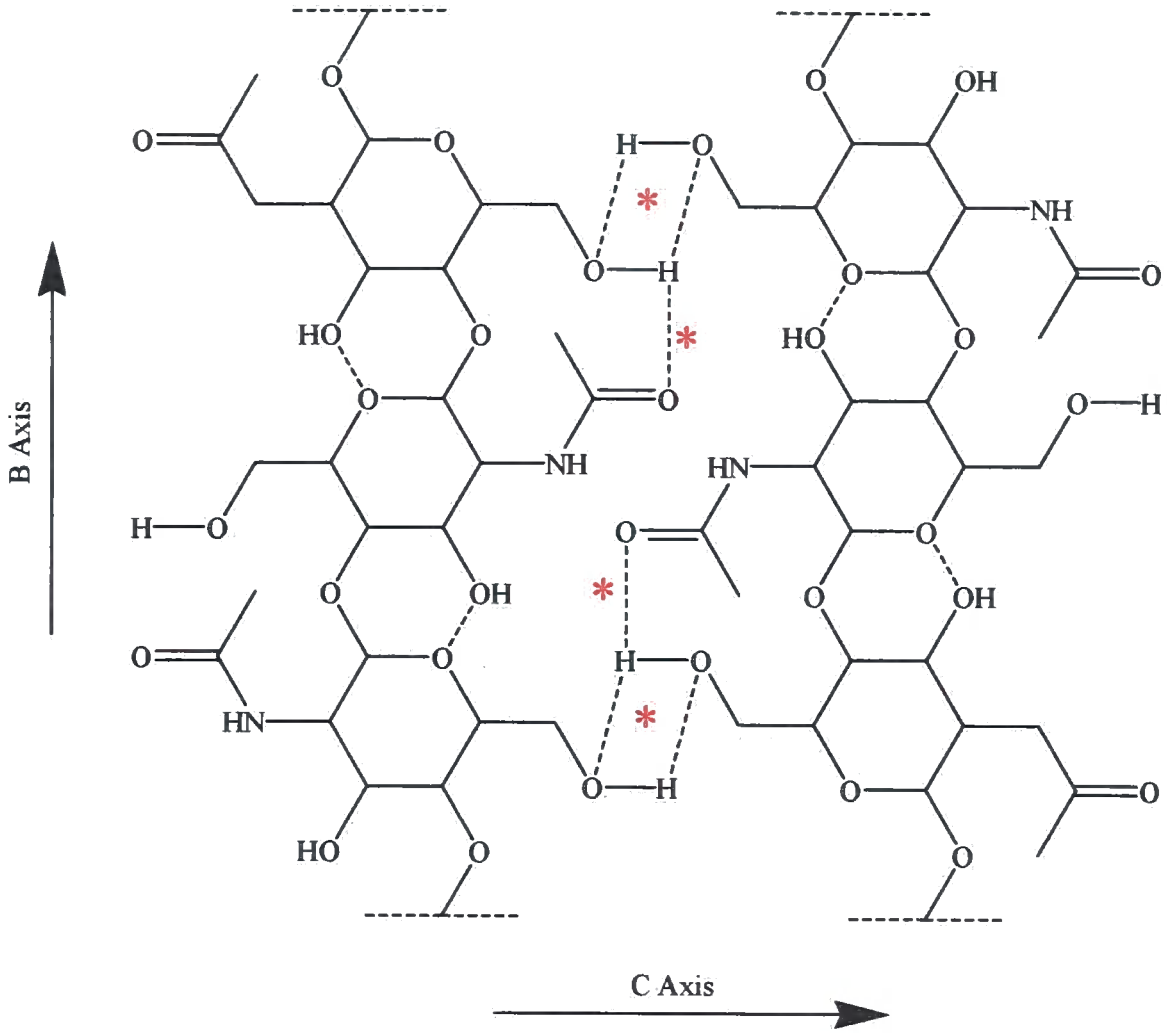


Figure 1.4 - Intermolecular hydrogen bonding present in α-chitin (A axis is perpendicular to the plane) Dashed lines represent hydrogen-bonds. * Represents hydrogen bonds present in a 50:50 statistical mix.

This model accounts for the splitting of the amide band in IR spectroscopy as the amide group is involved in two hydrogen-bonding environments, all of the amide groups are involved in intermolecular hydrogen bonding in the A direction and half are involved in intramolecular bonding in the B axis. Chitin's lack of ability to swell is also explained, as hydrogen bonds are present along all three axes forming a rigid network. The third criterion is also met, as all of the hydroxyl groups are now involved in hydrogen bonding. Many authors have since verified this proposed hydrogen-bonding structure by means of FT-IR, FT-Raman and ^{13}C CPMAS ssNMR spectroscopy and X-ray diffraction studies.[30, 34, 35]

1.3.2 β-chitin

As discussed previously, X-ray diffraction and IR spectroscopy studies indicate that in the β-chitin polymorphic form chitin chains arrange in a parallel fashion. Determination of the exact nature of the hydrogen-bonding network in this structure has been complicated by the various hydrated forms in which β-chitin can appear. Blackwell *et al.* proposed that in β-chitin, as in α-chitin, intrachain hydrogen-bonds between the C3 hydroxyl group and the sugar ring oxygen are present, as are the intermolecular hydrogen bonds between the amide protons and carbonyl functionalities in the A direction.[36] Additionally, the authors proposed that the carbonyl group is involved in interchain bonding with the CH_2OH group along the A axis. The increase in the unit cell dimensions in the A direction combined with the increase in the frequency of the N-H stretching band indicate that these interchain bonds are longer than those found in α-chitin. In contrast to α-chitin, this structure predicts that all of the amides are in the same environment. This is seen in the IR spectrum, as the amide I peak is no longer split in β-chitin.[30] The most significant difference between α and β-chitin is that in β-chitin the hydrogen bonding is either intrachain or along the A axis, there are no hydrogen bonds along the C axis. This accounts for β-chitin's ability to swell, forming hydrates containing either one or two water molecules per residue, which are hydrogen-bonded between the C6 and C3 hydroxyl groups of two adjacent chains along the C axis. Again, this proposed hydrogen bonding structure has been verified by subsequent authors who have studied the polymorphic form of chitin *via* X-ray diffraction, IR and ^{13}C CPMAS ssNMR spectroscopy.[35, 37, 38]

1.3.3 γ-chitin

Relatively few studies have been carried out into the structure of γ-chitin other than to assign its unit cell dimensions.[18] Kyeong *et al.* investigated and compared the

physiochemical characteristics of α, β and γ-chitin by means of X-ray diffraction, FTIR and ^{13}C CPMAS ssNMR.[30] The authors also studied average molecular weights, relative viscosities and thermal decomposition activation energies of the three polymorphs and concluded that the characteristics of γ-chitin most closely resembled those of α-chitin. Owing to its low natural abundance, little else has been done to elucidate the structure of γ-chitin.

1.4 Physical Structure of Chitosan

The greater importance of chitin over chitosan in nature means that considerably less research has been carried out into the structure of chitosan, however, the growing use of chitosan as a speciality polymer has led to an increased interest in this area. The study of the structure of chitosan is complicated by the various routes possible for its preparation and the variety of starting materials available. As α-chitin is the most dominant polymorph of chitin, it is generally used as the starting material for deacetylation to form chitosan. However, if β or γ chitin were used it is plausible that they would produce different crystalline structures of chitosan.[18]

It is doubtful that there is one unique crystal structure for chitosan; instead there are a number of structures of differing stabilities and extents of hydration. Four major conformations of chitosan have been found; the most abundant polymorph is that of a hydrated crystal.[39] In this polymorph chitosan is packed in an antiparallel fashion; the chains form intermolecular hydrogen bonds between the C6 hydroxyl and the carbonyl groups of adjacent chains to form sheets. As in chitin, there are also intramolecular bonds between C3-OH and C5-O. This structure is stabilised by the presence of water molecules between these stacked sheets, which form hydrogen bonds to the chitin chains in the A direction. This reflects the major difference between the structure of chitosan and that of chitin. As a result of the deacetylation, the intersheet hydrogen bonding between the amide protons and carbonyl groups in the A direction is no longer present, this allows the chains to move further apart in this direction. As the hydrogen-bonding network is no longer present in all three

directions, chitosan is free to adsorb water and has increased solubility characteristics. In reality chitin and chitosan exist as heteropolymers of acetylated and deacetylated monomer units. The deacetylation pattern, which is not usually uniform,[18] will govern the structure and properties of the material; therefore, determination of the DDA of the material is vital to determining its physiochemical properties.

1.5 Applications of Chitin and Chitosan

Chitin and chitosan exhibit an unusual combination of physiochemical and biological properties that make them attractive speciality materials and have found them numerous applications in various fields such as waste and water treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement, and food processing.[40] In addition to the lack of toxicity and allergenicity[41] displayed by these materials their biocompatibility, biodegradability[42] and bioactivity, which is described in sections 1.5.1 and 1.5.2, also make them very attractive substances for such diverse applications as biomaterials in pharmaceutical and medical fields.[12] The ability of chitinous materials to form fibres also enables them to be utilised in many applications, such as textile materials and forming wound dressings or surgical sutures. In order to spin fibres the raw material must be dissolved in a suitable solvent and then extruded, or 'wet' spun, into a non-solvent to yield continuous filaments. In the case of chitin, lithium chloride-*N,N*-dimethylacetamide (LiCl:DMAc) solvent systems have been proposed as a true non-derivatising solvent,[43] and has been utilised in the formation of chitin fibres,[44] this solvent system is discussed more fully in 3.7.2. Chitosan is soluble in mild acids and therefore fibres can be spun using acetic acid as a solvent.[45]

As the fields in which chitinous materials can be employed are diverse and numerous, it is not possible to extensively review each possible application here. Therefore, in the following sections a selection of the current and most promising possible future applications of chitin and chitosan will be discussed.

1.5.1 Wound Dressings and Surgical Sutures

Chitin and chitosan have many distinctive biomedical properties; perhaps the most significant of which is their ability to promote wound healing, combined with their non-allergenic nature and antimicrobial activity this makes them ideal candidates for wound healing products, such as dressings, surgical sutures[44] and artificial skins for burns victims. In comparison to other polysaccharides currently used in wound dressing materials, modified chitosans possess useful biomedical properties in that they promote ordered tissue reconstruction, vascularization and result in little scar formation. It is thought that this is due to their susceptibility to the hydrolytic action of lysozyme and *N*-acetyl-β-D-glucosaminidase enzymes. These enzymes degrade the material, forming chito-oligomers which are capable of macrophage stimulation and exert a favourable influence on collagen deposition.[7] The presence of chitin containing materials has been shown to correlate with the proliferation of fibroblasts which produce connective tissue such as collagen,[13] and regenerate the skin tissue around the area of the wound.[46] Oligomeric and monomeric species are also expected to be incorporated into extracellular matrix components which further assist in the rebuilding of physiologically valid tissue. Chitin-based materials have been shown to have a high affinity for the skin surface: surgical dressings made of chitosan-gelatin complexes have been shown to adhere strongly to subcutaneous fat. Minagawa *et al.* studied the effect of molecular weight and DDA of chitin and chitosan on their abilities to promote wound healing. Their results suggest that although the polymers and oligomers of chitin and chitosan enhance wound healing, the higher the DDA of the chitinous material the more effective it is.[47] Biagini *et al.* developed an *N*-carboxy-butyl chitosan dressing for the treatment of plastic surgery donor sites.[48] This dressing was found to promote ordered tissue regeneration, better histoarchitectural order, better vascularization and the absence of inflammatory cells. Numerous wound dressings composed of chitin, chitosan or chitosan derivatives have been proposed and patented. A chitin-based wound dressing, comprised of a non-woven fabric manufactured from chitin filaments, is currently available in Japan.[46]

In addition to the requirements for wound healing, it is important to control any infection of a wound under the dressing; this is where the bioactivity of chitosan is important. The antimicrobial activity of chitosan has been recognised against several bacteria, the efficiency of which is dependent upon its physical properties, such as DDA and molecular weight. The antibacterial activity of chitosan is dependent upon the strain of bacterium, as chitosan generally exerts stronger effects against gram-positive bacteria than gram negative, and the pH of the solution, with greater activity shown in acidic conditions.[49] It is thought that in dilute solutions the positive charge of chitosan interferes with the negatively charged residues of phospholipids at the bacterial cell surface, presumably by competing with Ca^{2+} for electronegative sites on the membrane without conferring dimensional stability. This in turn renders the membrane leaky.[50] Alternately, Tokura *et al.* propose that the anti-microbial activity of chitosan arises from the ability of chitosan oligomers to suppresses the metabolic activity of bacteria by blocking nutrient permeation through the cell wall.[14] The antimicrobial activity of chitosan may also be used in other applications, for example, in the replacement of chemical preservatives in food stuffs. To this end, Begin *et al.* describe the production of a chitosan film that possesses antimicrobial activity.[50]

1.5.2 Drug Delivery Systems

Chitin and chitosan as biocompatible and biodegradable polymers are very attractive candidates for the development of matrices for controlled drug release. The use of drug delivery vehicles has attracted increasing interest in the past few decades as a method of decreasing the toxicity of pharmacons, prolonging their circulation time in the body and altering their bio-distribution. In addition, drug delivery system may be designed to result in controlled release or targeted delivery of the drug, reducing the frequency of administration and the possibility of toxic side-effects.

A variety of chitin and chitosan based delivery vehicles have been described for the delivery of genes, proteins, peptides, vaccines, DNA and polar drugs.[49] Chitosan and chitin are useful in these applications as they do not promote any inflammatory or allergic reaction, they are also biodegradable, the degradation resulting in the release of amino sugars which can be incorporated into metabolic pathways and excreted. There are a variety of ways in which polymers can be used to form drug delivery vehicles, for example they can form hydrogels or microparticles, which contain the drug moiety, see sections 1.5.2.1 and 1.5.2.2, alternatively, polymer-drug conjugates may be formed with specific biodegradable linkages designed to release the drug at a particular site in the body, as discussed in 1.5.2.3. It is not possible to fully review every application of chitinous materials in drug delivery systems within the restraints of this chapter, therefore the general concepts will be outlined and illustrated with suitable examples.

1.5.2.1 Hydrogels

Hydrogels are highly swollen, hydrophilic polymer networks that can absorb large amounts of water and drastically increase in volume. These three-dimensional networks, which consist of polymer backbones cross-linked to produce a complex network, can be used as a drug delivery vehicle. A variety of chitin and chitosan

based hydrogels have been explored; these are reviewed by Kumar[46]. The hydrogels can be designed to swell and contract with response to external stimuli such as temperature and pH in an attempt to achieve controlled release of drugs at specific sites.[51] These hydrogels commonly involve chitin or chitosan in an interpenetrating polymer network with, for example, polyethers, poly(ethylene glycol) and gelatine, which are crosslinked by glutaraldehyde.[46]

Chen *et al.* investigated the use of carboxymethyl derivatised chitin as protein carrier matrices.[52] This derivative is an amphoteric polyelectrolyte, the charge density of which can be controlled by varying the DDA and degree of substitution of the chitin chain. Crosslinking with glutaraldehyde forms a gel, which swells to incorporate the solvent that it is in, if the gel is swollen in the presence of a protein the protein can be loaded into the gel. The swelling of the gel is pH dependent: at a low pH (typically below pH 2.0) the dominant charge is the protonated amino group. at higher pH (above pH 4.0) the dominant charge is the unprotonated carboxyl groups. In these regions the gel swells as a result of the mobile ions present, however, in between these pH values the polymer is at its isoelectronic point. We therefore see intra-ionic attractions, which decrease the mobile ion concentration and cause the gel to shrink. This offers the opportunity for the oral administration of drugs that would ordinarily be degraded in the low pH of the stomach. In the higher pH of the intestine the gel will swell rapidly releasing the drug.

1.5.2.2 Microparticulate Drug Delivery Systems

Much research has focused on the development of microencapsulation systems for the controlled delivery of drugs, proteins, genes and DNA delivery. As a natural, positively-charged biopolymer, chitosan has been the focus of a great deal of research for this role.[15] Microparticulate systems can be classified as microspheres, in which the drug is uniformly dispersed in the chitosan network, and microcapsules, in which the drug forms a solid core surrounded by a polymeric membrane.[53] Chitosan membranes, which have been cross-linked to improve the integrity and strength of the

membrane, have been applied in microencapsulation systems.[54] These systems have been used to encapsulate DNA for biological protection during gastrointestinal transit.[55] Chitosan-alginate beads, which resist the low pH conditions and high pepsin concentration in the human stomach, disintegrate when transferred to the conditions of the intestine, and thus release the drug content.[56] Chitosan nanoparticles have also been used to entrap the anti-cancer drug doxorubicin. This was done by forming a dextran sulphate-doxorubicin complex, which could then be entrapped in the positively charged chitosan nanoparticle.[57] The authors proposed that the use of a positively charged carrier favours cell adhesion, and potentially cell uptake, owing to their attraction to negatively charged cell membranes. The anti-inflammatory drug indomethacin was incorporated into chitosan microspheres cross-linked with citric acid.[54] This acidic drug would normally have a low plasma concentration, however in this system it was released in a controlled manner. The drug is released at a reasonable rate in the high pH conditions of the gastrointestinal tract but in the presence of low pH conditions (pH 2.0-4.0) its release rate decreases to negligible levels.

In a review of work on nasal delivery systems for vaccines, Illum *et al.* describe the use of chitosan microspheres[58] as delivery system for nasally administrated vaccines.[17] The authors describe how the increased absorption of drugs in the nasal cavity results from the bioadhesive effect of chitosan. As the amine groups of chitosan are positively charged in solution they exhibit strong electrostatic attractions to the negatively charged sialic acid residues of mucin in mucus. This increases the half time of the clearance from the nasal passage, thus increasing absorption of the drug. Illum and co-workers have exploited this concept for delivery of many pharmacological agents, including peptide and protein drugs such as insulin,[59] low molecular weight drugs such as morphine[60] and vaccines against influenza[61] and diphtheria.[62]

Chitosans have also been shown to be effective in gene delivery.[16] This requires that the system is delivered to the target cell, can be transported through the cell membrane and can be taken up and degraded by the endolysosomes. The system must

also form a stable complex that will protect against DNase degradation. Cationic polymers have received much interest as non-viral delivery vectors as they can interact electrostatically with negatively charged DNA and form self-assembling complexes. As a polycationic, biocompatible polymer chitosan can be used to effectively condense plasmid DNA and protect it from degradation. Chitosan can also be derivatised to contain ligands for specific cell interactions, such as galactose, which may result in targeting of the polymer-DNA complex.[63]

1.5.2.3 Chitin/Chitosan – Drug Conjugates

The pharmacodynamic and pharmacokinetic properties of drugs may be improved by conjugation to an appropriate polymer. This may alter the rate of excretion of the drug by increasing its molecular weight, or masking metabolic markers, or protecting functional groups that are susceptible to enzymatic attack. Additionally, the potential toxicity of the drug may be reduced and the antigenic sites may also be masked. The polymer chain may be further functionalised with sugar moieties, which act as cell specific recognition devices, this may result in targeting of the polymer-drug conjugate. For example hepatocytes in the liver are known to express a galactose specific receptor.[63] The polymer-drug conjugate may also be designed to result in controlled release of the drug at a specific site. For example a drug-chitosan conjugate in which a drug moiety is conjugated to the amide functionality of the chitosan polymer via a linkage that is designed to be degraded at a specific site *in vivo,* such as peptide linkers which have specific sequences of amino acids corresponding to specific intra cellular enzymes that are capable of degrading the link and releasing the drug.

The carrier-mediated approach using chitin, chitosan and their related compounds has been attempted using several anti-tumour agents.[64] An example of this is the formation of a chitosan laminin anti-tumour drug conjugate. Laminin is known to be involved in the metastasis of tumour cells, a peptide sequence corresponding to a partial sequence of laminin was identified and found to inhibit angiogenesis and thus

depress tumour growth. A conjugate of chitosan and this peptide sequence was prepared and found to display anti-metastatic activity, with a higher inhibitory rate than the parent peptide alone.[12]

1.5.3 Food and Nutrition

Chitin and chitosan also have potential use as food supplements in animal feed. It was considered that a highly nutritious animal feed may be formulated containing chitinous products in conjunction with a high lactose cheese whey, which is a by-product of the cheese making industry.[44] At present the high quantity of lactose in whey prevents its use due to lactose intolerance. Alkyl glycosides of *N*-acetyl-D-glucosamine have been shown to promote the growth of bifidobacteria; this bacteria limits the growth of other types of micro-organisms and generates lactase, which is required for the digestion of lactose.[65] In studies on rats and chickens, it was shown that animals fed with whey containing chitinous products had higher weights than those without, suggesting it could be used in animal feed.[44]

Chitosan may also be used to lower serum cholesterol levels as the capacity of chitosan to bind fatty acids and bile acids imparts a hypocholesterolemic action in animals.[65] Chitosan which contains protonated amine groups, can bind fatty acids to form the corresponding salts, due to hydrophobic interactions these salts can then bind additional lipids. As this complex passes through the gastrointestinal tract the hydrolysis of these lipids and fatty acids is prevented, resulting in their excretion.[66] The extent of the hypocholesterolemic effect has been shown to increase with increasing molecular weight and DDA of the chitosan polymers.[67]

1.5.4 Metal Complexation

The ability of chitosan to form complexes with metal ions, particularly transition metal and post-transition metal ions is well documented.[18, 68, 69] The chelating

ability of chitosan provides it with a wide-scale application, enabling it to be used for the removal of metallic impurities in wastewater, for example pollutants such as Cd^{2+}, Cr^{3+}, Cr^{6+}, Hg^{2+} and Pb^{2+}. As a general rule chitosan will not form complexes with alkali and alkaline earth metals. Chitosan has been shown to be more effective at scavenging heavy and toxic materials than other natural materials having a binding capacity of more than 1 mmol/g for heavy and toxic metals.[68] Toxic heavy metal pollution produced in industrial wastewaters is a great environmental problem. Conventional methods for removing dissolved heavy metal ions include chemical precipitation, chemical oxidation or reduction, filtration, ion exchange, electrochemical treatment and the application of membrane technology. However, these processes often do not result in complete removal of the metal ions, they can require expensive equipment and can generate toxic sludges.[70] The removal of these heavy metal ions by binding to a biopolymer offers advantages over these processes due to the reduced cost of materials, ease of operation and selectivity over the alkaline materials. Chitosan's high metal retention capacity may be particularly suitable in this application. For example, chitosan microspheres have been reported for the removal of heavy metal ions from coal mining effluents.[71] The sorption behaviour of chitosans is not limited to heavy metal removal, chitosan has also been employed for the removal of dyes from effluent solutions.[46, 72] Chitin has also been quoted as having metal chelating abilities, however, this is probably due to deacetylated units in the chitin chain as chitin rarely has a DDA of zero.

Using the concept of hard and soft acids and bases, chitosan would be classified a hard base as the groups most likely involved in the complexation are R-NH_2 and R-OH. Therefore we would expect chitosan to form more stable complexes with hard acids such as Cr(III) and Mn(II), however, chitosan appears to complex to soft acids such as Pd(II), Hg(II) and Pt(IV) more readily.[18] This suggests that complexation is not as straightforward as simple ion exchange. The exact nature of the interactions of metal ions and chitosan are difficult to elucidate, as competition between adsorption, ion-exchange and chelation most probably occurs. It is generally accepted that at $pH < 6$ chitosan acts as a poly(monodentate) ligand via the amine groups. At higher pH

values it behaves as a poly(bidentate) ligand forming chelates via the involvement of two or more amine groups from the same or different chitosan chains.[18]

The formation of the chitosan-metal ion complex occurs primarily through the hydroxyl and amine groups; therefore, the DDA of the sample is a critical factor in the level of metal ion uptake. Additionally, it is not only the concentration of the amine groups but also their accessibility that affects the metal ion uptake, chitosans containing highly crystalline regions will therefore have reduced binding efficiencies.[73] Partial destruction of this crystallinity may be achieved by acetylating the chitosan to some extent, or by cross-linking the chitosan chains with a polyfunctional reagent. Koyama *et al.* describe the increase in the binding capacity of chitosan for copper from 74 to 96%, when it was crosslinked with glutaraldehyde, at an aldehyde to amide ratio of 0.7.[74] However, the authors found that increasing the degree of cross-linking further decreased the binding capacity, this may be either due to a loss of amino binding sites or the restricted diffusion of molecules through the polymer network. The physical state of chitosan – flake, ground powder, or film – also appears to affect the metal ion uptake,[75] the low surface area and porosity of flake and powder forms of chitosan rendering them unsuitable for use as adsorbants, highly porous chitsosan beads and resins are far more effective.[76] Attempts to increase the adsorption capacity of cross-linked chitosans have involved the synthesis of metal complexed chitosans. Here the metal ions act as a template, the metal-chitosan complexes are then cross-linked and the metal ions removed. This results in templated, crosslinked, chitosan that exhibits a high specificity for the desired metal ion.[77]

A great many derivatives of chitosan have been investigated for their chelating ability, these are extensively reviewed by Varma *et al.*[68] Chitosan mercaptans which contain thiol groups have been shown to have increased affinities for mercury ions.[19] Histidine functionalised chitosan membranes of high porosity have also been prepared, this showed significant increased binding capacity for copper ions.[78] Much of the work on metal ion adsorption has been carried out on chitosan from crustacea exoskeletons. Muzzarelli *et al.* have studied the metal ion complexing

ability of the mixed polysaccharide, chitosan-glucan complexes, which is obtained by treating waste mycelia with 40% NaOH to convert the chitin component to chitosan.[20] The authors showed that the presence of other polysaccharides with chitosan does not prevent its ability to complex metal ions. Therefore chitosan would not need to be purified for this application.

Despite a large number of studies on the use of chitosan for metal ion recovery, this research area has so far failed to find practical application at the industrial level. This is most likely due to the variability in the characteristics of commercially available chitosans and the lack of availability of the material. Techniques for the commercially viable production of chitosan, with controlled characteristics, may result in the commercial application of chitosan's in the treatment of wastewater.

1.5.5 Other Uses of Chitin and Chitosan

The possible applications of chitin and chitosan are far ranging, this brief review only covers a select few. In addition to those uses mentioned above chitosan has found uses in many other applications. For example, chitosan has been reported to impart wet strength to paper.[46, 79] Chitosan may also find use as a coating to improve the quality of textiles, their mechanical properties and their staining behaviour.[80] Chitosan also shows promise in ultrafiltration membranes that are used to separate and concentrate proteins.[81] There is a large body of research into the use of chitin and chitosan based materials for enzyme immobilisation. Chitosan's affinity for proteins, availability of reactive functional groups for chemical modification, biocompatibility, biodegradability, mechanical stability and rigidity makes it an ideal candidate for enzyme immobilisation.[82]

The biocompatibility and bioactivity of chitin and chitosan result in a plethora of biomedical applications for which they may find many potential uses. For example, sulphated chitins have been investigated for anti-coagulant activity, these derivatives may inhibit the enzyme thrombin, which is responsible for converting fibrinogen into

its insoluble form fibrin.[83] Phosphorylated chitin derivatives have also received attention as possible anti-inflammatory agents in the treatment of acute respiratory distress syndrome.[84] Early experiments indicate that chitosan may also accelerate the regeneration of bone.[79] Li *et al.* report the use of biodegradable porous chitosan-alginate hybrids as scaffolds for bone tissue regeneration.[85] Freier *et al.* investigated the use of chitin hydrogel tubes as biodegradable nerve guides and determined that chitin and chitosan support nerve cell adhesion and neurite growth, making these materials potential candidates for matrices in neural tissue engineering.[86] Chitosan possesses all the characteristics required for making an ideal contact lens: optical clarity, mechanical stability, sufficient optical correction, gas permeability, wettability and immunological compatibility.[46] Contact lenses made from chitosan extracted from squid pens are tough and clear, and possess the required physical properties such as tear strength and oxygen permeability. Chitosan is also used in cosmetic applications and has been used for many years as a component of hair care products.[46] It can also be used in skin care products as it improves the skin compatibility of cosmetic emulsions.[87]

1.6 Commercial Sources of Chitin and Chitosan

As one of Nature's most important biopolymers, chitin is very abundant particularly in exoskeletons of arthropods and the cell walls of fungi. One form of shellfish alone, the copepods, are known to produce 10^9 tons of chitin annually by biosynthesis. The accessibility of this chitin to extraction is limited. A study in 1991 by Allan into the potential sources of chitin estimated the total amount of chitin accessible, from crustacean and fungal sources, globally as only 150×10^3 tons.[18] Advances in chitin science since this study may have increased the amount of accessible chitin, however, there is still a large volume of chitin which is inaccessible. This inaccessibility is in part due to commercial pressures, the use of crustacea, such as shrimp and crab, as a chitin source is limited by the demand for these products for other uses such as luxury food items. The difficulty in extracting high quality chitinous products due to the large quantities of inorganic materials in crustacea, and

polysaccharides in fungi, is also a limiting factor in the amount of, high-quality, chitin that can be extracted.

1.6.1 Crab and Shrimp Sources of Chitinous Materials

Currently all commercially produced chitin would appear to be from the exoskeletons of crab and shrimp, which is obtained as a waste product from the seafood processing industry. It is estimated that the crab and shrimp shell wastes from the United States of America alone could produce around 6,000 and 39,000 tons, respectively, of chitin annually.[88] With the global annual production of shell wastes from crab, lobster, shrimp, krill and clam/oyster at 1.44 million tons, estimated in 1991, and the value of chitin currently at $10,000 a ton,[89] with world demand currently outstripping supply, this provides a profitable means of disposing of a waste product. However, this source of chitin is restricted due to the problems of seasonal and limited supply in some countries. Additionally, the matrix of interactions in which chitin is involved requires harsh chemical extraction procedures, which are environmentally undesirable and can degrade the chitin chains and result in chitinous products with heterogeneous characteristics.

The cuticles that form the exoskeletons of crustacea and insects can contain up to 40% chitin, depending on the species and cuticle type. This material is invariably found complexed with proteins and functions as a light, but mechanically strong, scaffold material. The extent of the interaction between chitin and protein molecules differs within a given sample and the structure may be hardened or sclerotised by crosslinking with poly-hydroxyphenols. The exact nature of the bonding between the chitin and protein chains is unknown.[90] The role of the protein is thought to be a biological defence mechanism against the action of chitinase enzymes which degrade the polymer chain, sclerotised protein may also prevent excess hydration. Chitin is also closely associated with minerals, mainly $CaCO_3$, pigments and lipids. In order to achieve the high purity, necessary for biomedical applications, these impurities must be quantitatively removed. Extraction of chitinous materials from these sources

therefore requires demineralisation and deproteinisation steps. Demineralisation is conventionally accomplished by extraction with dilute hydrochloric acid.[91] This acidic treatment may result in degradation of the chitin chain as the β(1→4) glycosidic linkages are susceptible to acid hydrolysis. The deproteinisation step typically consists of an alkali treatment, the preferred reagent in the literature being NaOH.[18] Again this can result in degradation and deacetylation of the polymer chain, this is discussed further in section 4.4.1.2. Finally, the increasing interest in chitosan as a function biopolymer, see section 1.5, has increased the demand for this deacetylated product, however, chitosan is not known to occur naturally in these sources, therefore, a further deacetylation step may also be required. The methods of deacetylation are discussed more fully in section 4.4.1.2, however, efficient deacetylation generally requires harsh alkali conditions at high temperatures and can result in polymers of varying molecular weight and deacetylation patterns.

1.6.2 Fungal Sources of Chitinous Materials

The production of chitin and chitosan from true fungal sources has gained increased attention in recent years due to the potential advantages offered over the current crustacean sources. Fungal sources importantly do not have the issues of seasonal and geographically limited supply seen in shell-food waste, as they can be cultivated throughout in the year. Furthermore, fungal sources contain lower levels of inorganic materials compared to crustacean sources, therefore, the demineralisation step seen in the extraction of chitinous materials from these sources is not required.

The role of chitin in true fungi is as the principal structural component of the cell wall. The structure of the fungal cell wall is described in more detail in section 4.2, in brief, fungal cell walls vary in composition between species but are generally comprised of fibrillar polysaccharides embedded in a matrix of amorphous components such as polysaccharides, lipids and proteins. The fibrous polymers constitute the structural skeleton of the cell wall and the matricial gel-like polymers act as interconnecting molecules. The fibrillar structure arises from multiple sugar chains that associate to

form microfibrils, which then in turn associate to form larger fibrils. This structure imparts rigidity on the cell wall, which is essential in order to protect the protoplast from the harmful extracellular environment. Chitin forms the principal, fibrillar, structural component of fungal cell walls and is predominant in the fungal kingdom where it appears in the vast majority of fungal taxa.[92] The exact composition of fungal cell walls is species specific, therefore the content and DDA of the chitinous material present depends upon the taxonomy of the fungus in question. In general, despite its structural importance, chitin is considered to be a relatively minor component of the fungal cell wall accounting for 1-2 % of the yeast cell wall by dry weight and 10-20 % of the cell walls of filamentous fungi, such as *Aspergillus* and *Penicillium*.[93]

Whereas chitin is present in the majority of fungi, the natural abundance of chitosan is much lower. It is probable that incompletely acetylated chitin exists in most fungi, however, chitosan is a characteristic component of only one fungal taxonomic group, the *Zygomycetes,* where its incorporation can exceed that of chitin.[9] However, little is known of the role of chitosan in fungal cell walls. Chitosan is easily hydrated and the interchain hydrogen bonding is weaker than that of chitin, indicating that it does not play a structural role in the same manner as chitin.[39] Chitosan is a polycation and it is probable that it complexes with the polyphosphate and polyuronides that are abundant in *Zygomycetes* cell walls.[92] It has been hypothesised that chitosan protects chitin from hydrolytic attack by chitinases.[18] It also possible that chitosan fulfils the interconnecting role performed by glucans in the cell walls of other fungi, as aside from the cell wall of spores, glucans are not present in *zygomycetes*.

The extraction of chitinous materials from fungal sources principally requires the removal of the protein and polysaccharide impurities, however, unlike crustacean sources a demineralisation step is not required. The extraction techniques commonly reported in the literature are described in detail in section 4.4. As for crustacean sources, deproteinisation typically involves an alkali treatment. In the case of the *zygomycetes* the alkali insoluble material (AIM) contains chitosan, which can be removed by an acid extraction step, and acid insoluble chitin. In all other fungal taxa

the chitinous material has a lower DDA and is present as acid insoluble chitin alone, which is embedded in a matrix of β-(1,6) and (1,3) glucans. Removal of these non-chitinous polysaccharides has been reported by chemical and enzymatic techniques,[94] this is discussed in section 4.6. Chitin may also be deacetylated *in situ*, affording chitosan, which can be extracted in high purity by an acid extraction step. As for crustacean sources, the deacetylation of chitin currently requires harsh alkali treatments, which can result in degradation of the polymer chains and an undefined acetylation pattern.

1.6.3 Biosynthesis of Chitin and Chitosan in Fungi

The biosynthesis of chitin is mediated by chitin synthases, which are integral membrane enzymes that catalyse the transfer of *N*-acetylglucosamine from uridine diphosphate (UDP)-*N*-acetylglucosamine to a growing chitin chain.[95] Chitin synthesis is based on the regulation of distinct chitin synthase isoenzymes, whose number ranges from one to seven in some filamentous fungi.[96] Each chitin synthase isoenzyme performs specific roles in chitin synthesis, such as the cell wall repair or the formation of primary septa in dividing yeast cells.[97] The elongation of the chitin polymers occurs *via* vectorial synthesis, so that the nascent chains are extruded through the plasma membrane as they are made.[98] Hydrogen bonding between the newly formed polymers of chitin results in microfibril formation and subsequent crystallisation of chitin in the extracellular space immediately adjacent to the plasma membrane. In filamentous fungi, localised areas of cell wall synthesis occur at the hyphal apex, or growing tip.

The biosynthesis of chitosan occurs in a tandem process with that of chitin. This was first noted by Araki *et al.*, who demonstrated the presence of the enzyme chitin deacetylase in *M. rouxii*, a *zygomycete* that contain native chitosan. The authors hypothesised that this enzyme was involved in chitosan formation.[99] Davis *et al.* went on to show that this enzyme was effective only on nascent chitin chains and demonstrated that the biosynthesis of chitosan occurs by two consecutive enzymic

reactions: glycosyl transfer from UDP-*N*-acetylglucosamine to growing chitin chains, catalysed by chitin synthase, followed by the subsequent deacetylation of the nascent chains by the enzyme chitin deacetylase to form chitosan.[100]

1.6.4 Fungi as Commercial Sources of Chitin and Chitosan

Fungal sources of chitinous materials overcome some of the issues presented by crustacean sources. The conditions required for the extraction of chitin from fungal sources are not as harsh as those from crustacean sources and may result in a more a homogeneous product as the acid demineralisation step, which may affect hydrolysis of the chitin chain, is not required.[101] The increased demand for chitosan as a biomaterial is currently met by the chemical deacetylation of chitin, resulting in chitinous polymers of varying molecular weight and with uncontrolled acetylation patterns, which can render the material unsuitable for some high-value biomedical applications.[102] Additionally, this procedure is not environmentally appealing as large volumes of alkali waste containing high concentrations of salts are produced. Fungal species containing naturally high levels of chitosan, which can be extracted in high purity, offer an alternative source that does not require this deacetylation step.[103, 104] Fermentation of filamentous fungi for the production of chitinous material may also offer the opportunity to manipulate and standardise the physiochemical properties of the materials by controlling the parameters of fermentation.[101] For example, Arcidiacono *et al.* demonstrated that modification of the culture composition and pH resulted in the production of chitosans of different molecular weights.[105] The chitin content of fungal cell walls can also be influenced by the composition and quantity of carbon and nitrogen sources in the growth medium, the acidity of the medium, the aeration rate and the age of the culture.[106]

Although the production of chitin and chitosan by fungal fermentation has great potential, at present the high costs of the fermentation methods limit its use. However, considering the significant amounts of fungal-based waste materials accumulated in the mushroom production and fermentation industries, and the expenses involved in

managing these wastes, production of highly functional value-added products may provide a profitable solution to the industry. For example, Wu *et al.* describe the extraction of chitin from the waste accumulated during commercial mushroom production.[107] The authors state that in the USA alone approximately 50,000 tonnes of waste fungal material is produced per year from the farming of *Agaricus bisporus*, the most popular mushroom world-wide; this waste currently has no commercial use.[104]

The use of fungi for the production of commercial products is an ancient science dating from the age of the Pharoahs and the fermentation of alcoholic beverages, however, with the advent of more sophisticated submerged culture techniques used in the penicillin fermentation its use has increased rapidly over the last half century. Today submerged culture systems of filamentous fungi are employed industrially for the production of a variety of metabolites of enormous social and economic importance.[108] The range of commercially exploited products is diverse and is expanding, and includes the production of enzymes, β-lactam antibiotics, such as the penicillins and cephalosporins, and citric acid, which is in heavy demand as a bulk chemical for food and other industries.[109] The annual world requirements for citric acid are estimated at 400,000 tons, which results in approximately 80,000 tons of *Aspergillus niger* mycelium waste per year.[104] This mycelium contains extractable chitin, which could produce a viable commercial source of chitinous material.[110]

If a suitable extraction procedure could be identified, which minimises the environmental issues presented by the chemical extraction of chitinous materials and produces high quality materials of defined characteristics, waste fungal biomass could provide an extremely viable source of chitin. Therefore, the main objective of this work was to investigate the extraction of chitinous material from the solid residues remaining from the large-scale fermentation of *Penicillium chrysogenum* in the penicillin manufacturing industry. With the current world market for β-lactam antibiotics estimated to be approximately $15 billion, penicillin producing filamentous fungi are cultured on massive scales.[111] The production of penicillin results in large volumes of waste fungal biomass, which is currently either used as an animal

feed or discarded.[112] This waste biomass consists mainly of *Penicillium* mycelia, which is potentially a valuable source of lipids, proteins and biopolymers, such as chitin. The presence of chitin in the cell walls of *Penicillium* strains has been identified[113, 114] and the extraction of chitosan from *Penicillium chryosgenum* mycelium by chemical means has already been demonstrated.[115]

1.7 Commercial Penicillin Production

Penicillins are natural products of filamentous fungi that are produced as secondary metabolites. These β-lactam antibiotics are produced industrially by the fed-batch cultivation of high-yielding strains of *Penicillium chrysogenum* in stainless steel tank reactors of 30,000 to 100,000 gallon capacity.[111] The cultivation procedures have been studied extensively in order to maximise penicillin production.[116, 117] In a largely automated process, temperature, pH, dissolved oxygen, carbon dioxide, sugar, ammonia and many more variables are measured constantly throughout the fermentation process in order to maintain the optimal conditions. Additionally, precursors are added and their uptake monitored in order to stimulate the production of the correct form of penicillin, phenylacetic acid in the case of penicillin G and phenoxyacetic acid for penicillin V. Penicillin is excreted into the medium, which undergoes a process of ultra-filtration; acidification of the filtrate and the addition of potassium acetate then affords the penicillin derivative as a crystalline potassium salt. The recovered penicillins may be used directly or converted into intermediates that are used for the production of semi-synthetic penicillins and cephalosporins. The solid residue remaining from the filtration of the whole broth is then dried at 113 °C. This dry biomass contains the fungal mycelia from which we will attempt the extraction of chitinous material.

1.8 Conclusion

Chitin and its deacetylated derivative chitosan have unique properties that make them useful for a variety of applications. Much research has been undertaken into the possible applications of chitinous products and their derivatives, however, at present, the only viable commercial source of chitin is the waste products of the seafood industry. Chitin and chitosan extracted from the exoskeletons of crustacea are heterogenous in composition and DDA, which renders them unsuitable for some biomedical applications. Fungal sources do offer an alternative source of chitin, however, the cost of the fermentation procedure precludes this from being a viable source. If chitin and chitosan could be extracted efficiently from a fungal waste source, such as the by-product from the penicillin manufacturing industry, this would provide a cost effective method of producing a more defined chitin product. There is therefore potentially great utility in developing new extraction methods for isolating chitin/chitosan from *Penicllium* biomass, which avoid or minimise harsh chemical treatments to produce a reliable and uniform product of high quality. In addition this could potentially allow for other valuable biomolecules to be isolated as by-products in the course of chitosan extraction.

Chapter 2 - Aims and Objectives

The initial aims and objectives of this study were to investigate novel methods of extraction of chitinous material from fungal sources, more specifically the dry fungal biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* in the penicillin manufacturing industry. This work was carried out in partnership with a local company, Angel Biotechnology, who are a commercial producer of penicillin and provided the waste biomass. Current chemical extraction procedures require harsh acid and alkali conditions which can result in degradation of the polymer chain and heterogeneous deacetylation patterns, rendering them unsuitable for many high-value biomedical applications. We therefore wished to develop new extraction methods for isolating chitin/chitosan from *Penicllium* biomass, which avoid or minimise the harsh chemical treatments to produce a reliable and uniform product of high quality from what is currently a waste product.

The first objective was to develop analytical methods for determining the key characteristics of chitin and chitosan, namely the level of purity, the degree of deacetylation, and the molecular weight. In order to do this, the literature methods of analysis currently available were reviewed and the most appropriate methods were selected. Where appropriate methods were not available new techniques were developed. Commercial samples of chitin and chitosan were analysed in order to evaluate each proposed analytical technique. The quality and yield of chitinous materials extracted using traditional methodologies were then assessed, the analysis of this extracted material providing a standard against which all other extraction methodologies were compared. Alternative extraction methods were then explored, such as partial digestion of the biomass by commercially available enzymes in order to aid chemical extraction. Finally, comparative extraction of chitinous materials from other fungal species known to be producers of chitin and chitosan were carried out in order to assess the suitability of *Penicillium chrysogenum* as a source of these materials.

Chapter 3 - Methods of Analysis of Chitinous Materials

3.1 Introduction

When considering the analysis of chitinous materials it must be remembered that there is no definitive 'standard' material for either chitin or chitosan. Therefore analysis of these materials seeks to characterise the material rather than confirm whether or not it conforms to a definite structure. The characteristics to be analysed are determined partially by the intended end use of the material. For example materials intended for medicinal use must be analysed for residual protein content, which may be allergenic, and heavy metal content, which may be toxic. The characteristics most frequently assayed are the purity of the material, the molecular weight of the polymer and the degree of deacetylation (DDA). It is this latter property that is perhaps the most significant as it governs the physicochemical properties of the material, in particular its solubility and tensile strength, this is discussed further in section 3.6. There are many proposed literature procedures for determining the DDA, however there is little agreement upon an absolute strategy and many procedures provide inconsistent and unreliable results. This is discussed in section 3.6. Analysis of chitinous materials is hampered by their inherent insolubility[30, 41] and hygroscopic nature.[118]

For the purposes of this study it was decided that the most important qualities to be analysed are the purity of the chitinous materials extracted, the DDA and molecular weight. The literature procedures available to assay each of these characteristics are evaluated in this chapter. In initial investigations commercial sources of chitin (Sigma) were used to examine the feasibility of each procedure, using the information gathered from these investigations the most suitable strategies for assaying each characteristic were chosen. Commercial samples of chitin and chitosan were supplied by Sigma-Aldrich UK, product numbers C9752 and C3646 respectively. No specifications are available as to the molecular weight or purity of the samples. Commercial chitosan was prepared by the partial deacetylation of chitin in hot alkali and it is stated as having a DDA of greater than 85 %, no details were available on the

DDA of chitin. This chapter aims to define a set of analytical procedures applicable to samples of chitinous materials extracted from fungal sources. Additionally, work carried out into the analysis of the whole fungal cell to monitor the effects of nutrient composition and growth stage on the chitinous component of the fungal cell wall by ^{13}C and ^{15}N CPMAS ssNMR will be discussed.

3.2 Assaying the Purity of Chitinous Materials by Elemental Analysis

As discussed in section 4.1, chitin is found in a matrix of interactions in the fungal cell wall. Extraction procedures are designed to break down these covalent interactions to yield pure chitin or chitosan. However, the chosen extraction process may not be completely efficient and the resulting chitinous materials may still contain residual glycoproteins and glucans. If the extracted chitinous materials are to be used for commercial purposes the composition, or level of purity, must be ascertained. Elemental analysis is a widely accepted technique for the determination of the composition of samples and there is literature precedent for its use in analysing chitinous materials.[102, 119, 120] Elemental analysis is advantageous as it does not require the sample to be solubilised and can therefore be used to analyse chitinous materials that span a wide range of DDA, however, the presence of any adsorbed water will interfere with the analysis. Additionally, as the elemental analysis of chitinous materials varies with the DDA this value must be determined in order to assess the level of purity of the sample in this way.

Elemental analysis was performed, in triplicate, on commercial samples of chitin and chitosan (Sigma) see Table 3.1. The theoretical values of the elemental composition of samples of chitin with a DDA of 0 % and chitosan with a DDA of 100 and 85 % are shown for comparison, the commercial chitosan sample is stated as having a DDA of not less than 85 %. The results for both chitin and chitosan do not agree with the theoretical values to within the generally accepted margin of error (± 0.3 % see Table 3.1). Notably, the results are all below the calculated values. This disagreement may infer that the samples contain impurities, however, it may also be explained by the

hygroscopic nature of chitinous materials.[118] The retention of moisture in the samples results in increased values for the content of hydrogen and oxygen and consequently decreased values of carbon content. We may negate this interference by studying the ratio of the carbon to nitrogen content of the samples instead of the absolute values. Analysing the commercial sample of chitin in this way we can see that the ratio of carbon to nitrogen agrees with the theoretical value of 6.86 to within ± 0.18 %. Comparison of the results from the commercial sample of chitosan to the theoretical samples with DDAs of 100 % and 85 % indicates that the sample has a DDA of approximately 85 % as the carbon to nitrogen ratio is in agreement to within ± 0.21 %.

	C (%)	H (%)	N (%)	C/N
Theoretical Chitin DDA = 0 %	47.29	6.45	6.89	6.86
Commercial Chitin*	42.59 (0.8)	6.24 (0.2)	6.10 (0.1)	6.98 (0.06)
Theoretical Chitosan DDA = 100 %	44.7	6.88	8.69	5.14
Commercial Chitosan*	40.90 (0.7)	6.62 (0.1)	7.43 (0.2)	5.51 (0.06)
Theoretical Chitosan DDA = 85 %	45.09	6.82	8.42	5.35

Table 3.1 - Elemental analysis of commercial samples of chitin and chitosan compared to the theoretical compositions. *Standard deviation shown in brackets, taken from 3 repetitions.

We can conclude that using elemental analysis to determine the ratio of carbon to nitrogen in a sample of chitinous material gives a reasonable indication of the level of purity of the sample, providing the DDA is known. Unfortunately, analysis in this way cannot give a quantitative estimation of the chitin content of a sample, as the composition of the impurities is not known. Therefore in order to more accurately determine the chitin content of samples of chitinous material extracted from fungal sources in this study, further analysis is required. Preferably, this analysis should be independent of the DDA of the sample.

3.3 Glucosamine Determination as an Assay of Purity

Elemental analysis can provide an indication of the level of purity of chitinous materials, as discussed in section 3.2, however, it does not give a quantitative determination of the chitin content of a sample and is dependent upon the DDA of the sample. A more quantitative determination of the chitin content of a sample may be achieved by completely hydrolysing and deacetylating the sample to produce free glucosamine residues, which can then be subsequently assayed. If a sample is 100 % chitinous it will contain 100 % glucosamine units. This method is advantageous as it is independent of the DDA of the chitinous material. The most widely quoted procedures for the determination of hexosamines and *N*-acyl hexosamines are those derived from the Elson-Morgan[121] and Morgan-Elson[122] methods, respectively. In the Elson-Morgan procedure glucosamine hydrochloride is condensed with acetylacetone to form a pyrrole, which in the presence of alcohol and Ehrlich's reagent (*p*-dimethylamino-benzaldehyde) develops a stable red colour. The Morgan-Elson procedure involves warming *N*-acetyl-glucosamine in dilute alkali to form an oxazole derivative, which condenses with Ehrlich's reagent to give a reddish purple colour. The concentration of hexosamine or *N*-acyl hexosamine can then be determined spectrophotometrically. Many modifications of these assays have been carried out to improve their sensitivity, reproducibility and specificity, however, the interference of sugars and amino acids in the quantitative determination of hexosamines reduces their appeal.[123]

Alternative spectrophotometric methods for determining the glucosamine, or chitosan, content of samples have been proposed in the literature. The use of ninhydrin to quantify the content of amino-sugars has been described, however, this is not specific to amino-sugars as amino acids will interfere with the assay.[124] Muzzarelli *et al.* describe a colorimetric determination of chitosan content which involves the use of anionic dyes, however, this requires accurate determination of the DDA of the material.[125] Gummow *et al.* reported that solutions of chitosan in dilute acetic acid induce a metachromic effect in the visible spectra of the anionic dye 2'-hydroxy-1,1'-azonaphthalene-4-sulphonate.[126] Yamaguchi *et al.* demonstrated the interference of

glucosamine in the colour reaction between *o*-hydroxyhydroquinonepthalein and Pd(II) by the formation of a Pd(II) glucosamine complex.[127] Unfortunately these interactions are not specific to hexosamines and therefore cannot be used as a definitive assay.

Techniques other than spectrophotometry have been proposed, for example liquid and gas chromatography. Zhu *et al.* described the use of high-performance liquid chromatography (HPLC) to determine the chitin content of samples.[128] This involves the hydrolysis of chitin to form glucosamine units, which are subsequently derivatised with fluorenylmethoxycarbonyl-chloride (Fmoc-Cl). The resulting compound absorbs in the wavelength range useful for HPLC with ultraviolet detection. However, the assay is dependent upon the optimisation of both the hydrolysis and the derivatisation reaction, which may introduce errors. Methods of gas chromatography have also been described, however, these also require derivatisation of the amino sugar.[129]

Tsuji *et al.* proposed a simple, specific, and sensitive method of determining hexosamine content that involves the deamination of glucosamine residues (produced upon complete hydrolysis of chitinous materials) by nitrous acid to form anhydromannose, which in the presence of $FeCl_3$ forms a blue complex with 3-methyl-2-benzothiazolone hydrazone (MBTH).[130] The mechanism proposed by Tsuji *et al.* for this colour reaction, which produces a highly conjugated product that gives rise to an absorbance at 650 nm, is shown in Figure 3.5.[131] It is possible that the reaction actually proceeds *via* the direct coupling of the intermediates B and C followed by oxidation to produce the charged species shown in Figure 3.5, as the proposed intermediate D would be highly unstable. The authors demonstrated that neutral sugars, *N*-acylglucosamines, ascorbic acid, gluroronic acid and most amino acids produce negligible absorbances under the reaction conditions. Those amino acids that do absorb, tryptophan, threonine and methionine, do so with an intensity of approximately 5 % that of hexosamines.[130] The use of the MBTH assay is widely quoted in the literature, more recently MBTH has been used successful applied as a means of determining the reducing end content of chito-oligosaccharides.[132]

The MBTH assay of Tsuji *et al.* described above was chosen for use in this project because of its specificity to hexosamines and ease of use. Additionally, the assay is independent of the DDA of the chitinous material. The MBTH assay gives a positive result for any amino sugars and is not specific to glucosamine. However, the concentration of other amino sugars in purified chitinous extracts will be low compared to chitin and therefore produce negligible errors. The major impurity thought to be present in chitinous materials extracted from fungal sources are β-glucans, as discussed more fully in chapter 4. A commercial sample of β-glucan was shown to produce a negative response to the assay and will therefore not interfere with the analysis of the chitin content.

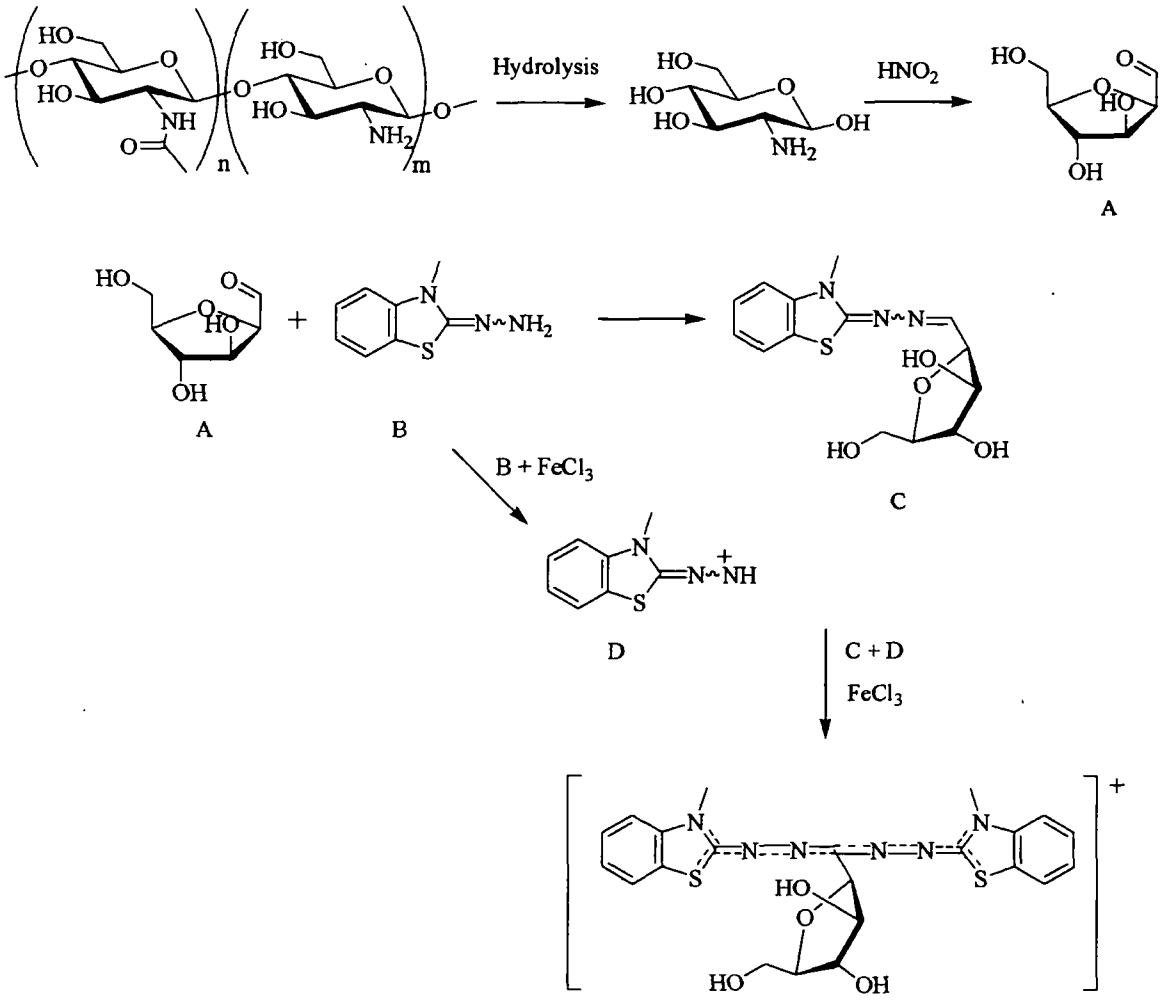
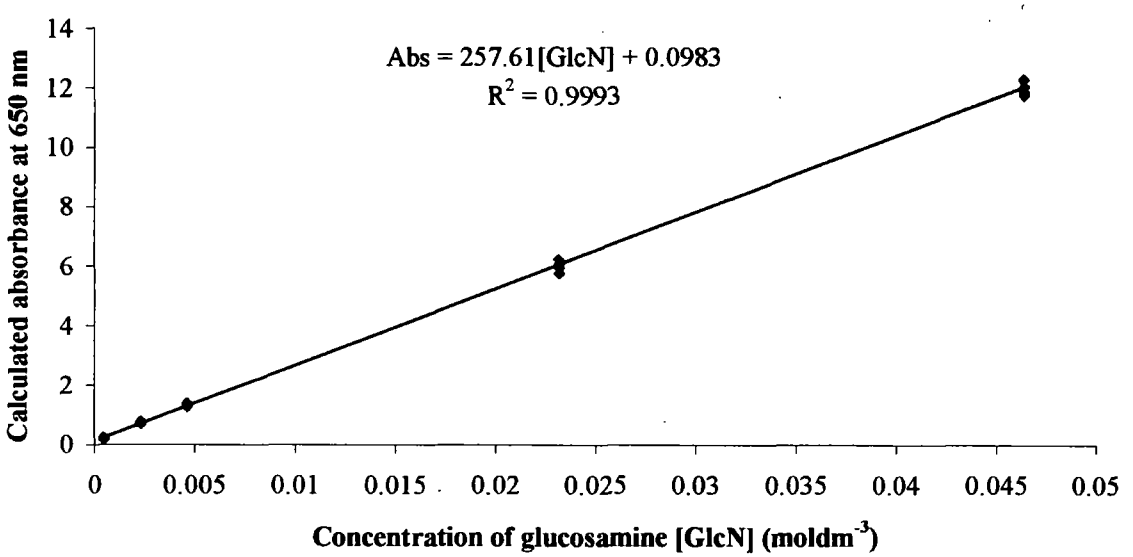


Figure 3.5 - Proposed mechanism for formation of the complex that absorbs at 650 nm in the assay of glucosamine.[132]

3.4 Standardisation of the Glucosamine Assay

Using the spectrophotometric method of determining the concentration of glucosamine described above, a standard plot was constructed using known concentrations of commercial glucosamine hydrochloride (Sigma) in 6 M HCl. The absorbance of the resulting compound at 650 nm forms a linear relationship with the concentration of glucosamine in the region of 10 to 0.01 $mgml^{-1}$ (0.046 to 0.00046 $moldm^{-3}$). See Graph 3.1. The calculated absorbances were acquired by diluting the analyte solutions with distilled water before the absorbance was measured in order to achieve results in the range of 0 to 1. The absorbance was then multiplied by the dilution factor in order to construct the standard plot.



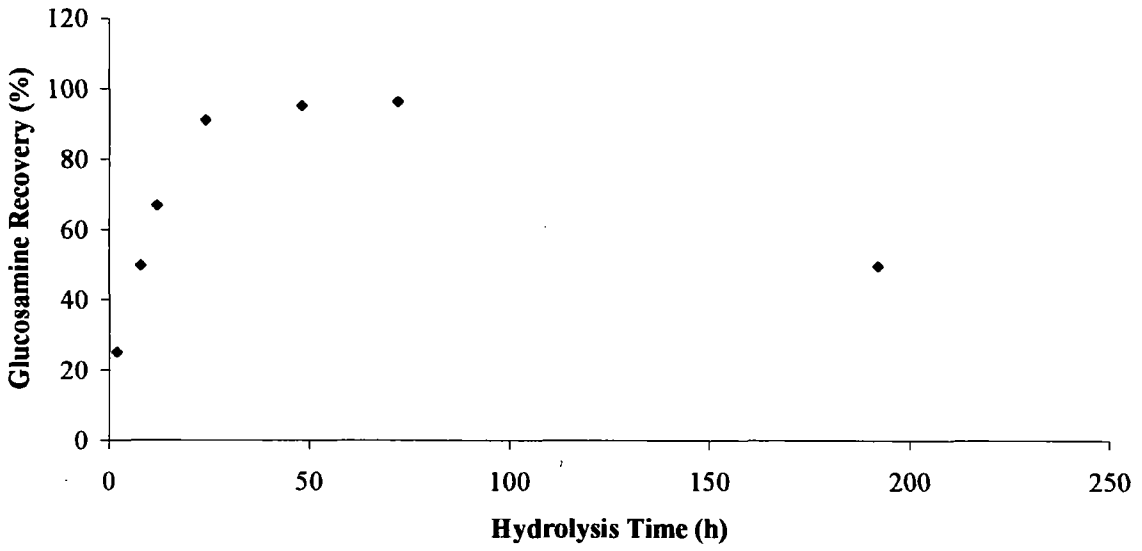
Graph 3.1 – Standard plot of glucosamine concentration against absorbance at 650 nm following the assay proposed by Tsuji et al.[131]

Studies were carried out in order to optimise the deamination time of the reaction and to study the absorption profile at 650 nm after the addition of $FeCl_3$, these results are not shown. It was found that the deamination reaction and complex formation proceed to completion within the timeframes outlined in the literature.

3.5 Glucosamine Determination of Chitinous Samples

Complete hydrolysis of chitinous samples is required in order to analyse the glucosamine content. Investigation of the characteristics of glucosamine release under acid, alkali and enzymatic conditions in the literature suggests that acid hydrolysis is the preferred method.[133] This results in cleavage of the glycosidic bonds between (acetyl)glucosamine molecules and deacetylation of the resulting *N*-acetyl-D-glucosamine units to produce free glucosamine residues. The amount of glucosamine recovered is dependent upon the type and concentration of acid, the reaction temperature and the reaction time. Extended crystallinity of a sample can reduce its accessibility to acid hydrolysis, therefore the nature of the material also affects the reaction conditions required to ensure full hydrolysis. This procedure must be optimised as under the harsh hydrolysis conditions liberated glucosamine monomers may be degraded reducing the apparent concentration seen by the assay.[128]

In order to determine the optimum hydrolysis conditions, we studied glucosamine recovery as a function of hydrolysis time. Glucosamine recovery is defined as the yield of glucosamine released from a sample of chitinous material. In this study, commercial chitin was used and therefore it was assumed that the material contains 100 % chitin. Chitin was hydrolysed in 6 M HCl at a temperature of 100 °C and the glucosamine concentration was assayed, as described in section 7.2.2, regularly at hydrolysis times between 2 and 72 hours. Additionally, a time point was taken after an 8-day treatment. The data, shown in Graph 3.2, indicates that the glucosamine recovery increases rapidly to a reaction time of 24 hours. After this point the recovery plateaus. This indicates that a hydrolysis time of 24 hours is adequate to ensure sufficient glucosamine recovery, after this time 91 % glucosamine recovery was seen, this only rose to 96 % after 72 hours. The results indicate that after extended periods of hydrolysis glucosamine recovery falls to approximately 50 %, this may be explained by the acid catalysed formation of glycosidic bonds, resulting in the presence of disaccharides. This would reduce the concentration of glucosamine monomer units available to form the required anhydromannose intermediate.



Graph 3.2 - Effect of hydrolysis time on recovery of glucosamine from commercial chitin. Assuming that commercial chitin is 100 % glucosamine.

The hydrolysis time has been optimised for commercial samples of chitin which are extracted from crustacean sources, however, it is possible that the crystallinity of these samples will vary from those produced from fungal sources. Extended crystallinity can reduce the accessibility of the material and therefore alter the conditions required to ensure complete hydrolysis. In order to address this, a sample of chitin extracted and purified from dry fungal biomass, as described in sections 7.4.3 and 7.4.4, was analysed. The results, not shown, indicate that hydrolysis at 100 °C in 6 M HCl for 24 hours is sufficient to ensure maximum glucosamine recovery from chitinous materials extracted from fungal sources.

The optimal hydrolysis conditions defined in this study are in disagreement with literature procedures. According to Cousin, the optimum hydrolysis conditions for chitinous materials are 6 M HCl for 2 hours at 110 °C,[133] however, this was insufficient for complete hydrolysis of the commercial chitin samples used in this study. Wu *et al.* proposed that, in 6 M HCl at 110 °C, complete hydrolysis takes 3 and 12 hours for chitosan and chitin respectively,[107] however, this was also found to be unsatisfactory. As discussed above, this disagreement may be due to a difference in the degree of crystallinity of the chitinous materials.

We can conclude that a quantitative determination of the glucosamine, and hence chitin/chitosan, content of a sample may be achieved by hydrolysing the sample in 6 M HCl at 100°C for 24 hours followed by the subsequent MBTH based assay proposed by Tsuji *et al*, described in section 7.2.2.

3.6 Determination of the Degree of Deacetylation (DDA)

Chitinous materials do not exist in nature as homopolymers, instead the names chitin and chitosan actually refer to a family of heteropolymers that have varying degrees of deacetylation (DDA). The DDA of the polymer is crucial as it governs the physiochemical properties of the polymer[134, 135], its solubility[5, 6] and hence reactivity, its biodegradability[8, 11, 136] and immunological activity.[137] Therefore, the determination of the DDA is one of the routine analyses performed for the quality control of chitinous materials. The search for a quick, low-cost and accurate method to determine the DDA has been a major objective for many decades, however, the inherent complexity of chitinous materials turns this apparently simple analytical problem into a very difficult one. Agreement on an absolute method for determining the DDA is much argued over in the literature and many procedures have been proposed.[40, 138, 139] For ease of discussion, these methods have been classified into three groups in the literature: conventional methods (e.g. titration and conductometry); destructive methods (e.g. elemental analysis, acid or enzymatic hydrolysis followed by chromatographic analysis); and spectroscopic methods (e.g. IR, NMR and UV).[139] These methods all have associated drawbacks, many yielding non-reproducible results, and many are limited to samples with a narrow range of DDA. Several of the proposed methods rely upon calibration to either a sample of known DDA or a calibration curve obtained using an alternate method. Some of these techniques require the sample to be of high purity, as impurities such as minerals, proteins and pigments may create interference and yield large variations in experimental results. The majority of the characterisation techniques rely upon the determination of either the amine, or amino group content of the sample. At the extreme values of DDA this may result in errors in the calculation, as the

concentration of the group assayed may be lower than the level of sensitivity of the assay. A more comprehensive assay should consider both the amine and amino group content in order to produce reliable results across the whole range of DDAs. A significant hindrance in the determination of the DDA of chitin is its inherent insolubility;[26] the use of spectroscopic methods can circumvent this problem. The hygroscopic nature of chitin can also induce difficulties in determining the DDA in some techniques, such as IR. Additionally, chitinous materials extracted from fungal source often contain impurities, mainly polysaccharides such as β-glucans, this is discussed in chapter 4. The presence of these impurities may also hamper the determination of the DDA.

In the following sections the literature methods of determining the DDA are discussed. The most suitable procedures were applied to a series of samples of commercial chitin that were deacetylated to varying extents, as described in section 7.5. Using this data the most reliable method of determining the DDA will be evaluated for use in further studies. This section aims to identify a fast and reliable method of determining the DDA of chitinous samples, which does not rely upon any reference standard of known DDA, or calibration curve obtained by another technique and is tolerant of impurities such as β-glucans.

3.6.1 Determination of DDA by Elemental Analysis

The determination of the DDA by elemental analysis has been proposed in the literature.[4, 140-142] Pure chitin, with a DDA of 0 %, should have a nitrogen content of 6.89 % by weight whilst pure chitosan, with a DDA of 100 %, should contain 8.69 % nitrogen by weight. Thus in theory it is possible to determine the DDA of a sample from its nitrogen content. This could similarly be carried out using information on the carbon content of the sample. However, the relative change in nitrogen content upon deacetylation is much larger than that of carbon. Elemental analysis requires minimal sample preparation and, unlike many other methods of determination of DDA, may be carried out across the whole range of DDA. Problems

in this method of analysis occur due to the hygroscopic nature of chitin, this was discussed in section 3.2, this may be avoided by investigating the ratio of carbon to nitrogen, which remains unchanged upon hydration. The use of the carbon to nitrogen ratio also negates the interference from any inorganic impurities. Using this value Xu *et al.* proposed that the degree of acetylation may be calculated by the following equation[142]:

$$DA = [(C/N - 5.14)/1.72] \times 100$$

Where DA is the degree of acetylation and C/N is the ratio (w/w) of carbon to nitrogen content of the sample. The DDA can be obtained by subtracting this number from 100. In order to use this method of determination it is crucial that the sample is pure and contains no residual proteins or glucans. This may present a problem in samples extracted from fungal sources.

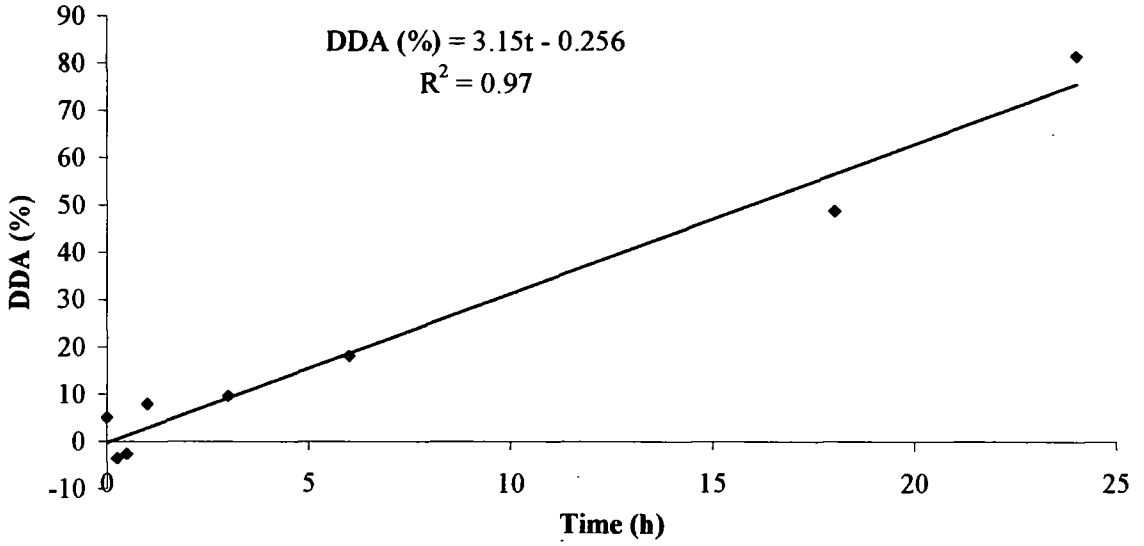
We performed elemental analysis on a series of samples of commercial chitin deacetylated in 40 % NaOH (w/v) at 100 °C under nitrogen for varying lengths of time, as described in section 7.5. The resulting data was used to calculate the ratio of carbon to nitrogen in each sample, this data is shown in Table 3.2, the theoretical values for chitin and chitosan with DDA of 0 and 100 % respectively are shown for comparison. A commercial source of chitosan (Sigma), claimed to have a DDA of ≥ 85 % was also analysed in this way. The carbon to nitrogen ratios were then used to estimate the DDA of the sample, using the method of Wu *et al.* described above.

Sample	C/N	DDA* (%)
Theoretical chitin DDA = 0 %	6.86	-0.21
Theoretical chitosan, DDA = 100 %	5.14	99.8
Commercial chitosan	5.56	75.3
Commercial chitin deacetylated		
T = 0 h	6.77	5.03
T = 0.25 h	6.92	-3.60
T = 0.5 h	6.91	-2.69
T = 1 h	6.72	7.97
T= 3 h	6.70	9.56
T = 6 h	6.55	18.0
T = 18 h	6.02	48.7
T = 24 h	5.46	81.4

Table 3.2 – The C/N calculated from the elemental analysis of a series of samples of commercial chitin deacetylated as described in section 7.5. for varying lengths of time, T. * DDA, calculated as described by Xu *et al.* using the C/N. The theoretical values of chitin and chitosan with DDA of 0 and 100 % respectively and a commercial sample of chitosan with DDA ≥ 85 % are shown for comparison

Calculation of the DDA of theoretical samples of chitin and chitosan, with DDA of 0 and 100 % respectively, demonstrates that this method has an inherent error of approximately ± 0.2 %. The calculated DDA values of a series of deacetylated samples of commercial chitin are shown in Graph 3.3. These results, which appear to follow a linear trend with deacetylation time, do not seem reasonable as several have negative values that do not fall within the estimated error limits of ± 0.2 %. This could be explained by the presence of impurities such as proteins and polysaccharides, which alter the C to N ratio as seen by elemental analysis and give inaccurate DDA. Without an accurate identification of the level and identity of these impurities it is impossible to accurately assign the DDA of the sample. Analysis of a commercial

sample of chitosan suggests that the DDA is much lower (75.3 %) than the manufacturer's claim of ≥ 85 %.



Graph 3.3 - DDA of samples of commercial chitin deacetylated for varying lengths of time, calculated from the C:N as determined by elemental analysis,

We can conclude that elemental analysis is not a suitable method for determining the DDA of chitinous materials, as the presence of any impurities will affect the results seen. This is of particular importance when considering chitinous materials extracted from fungal sources, as they are known to be associated with other polysaccharides, such as β-glucans. This will result in high C to N ratios and inaccurate determination of the DDA.

3.6.2 Hydrolytic Techniques

Hydrolysis of the *N*-acetyl groups of chitinous materials under either acid or alkali conditions can be followed by the determination of the acetic acid produced. This information can then be used to determine the DDA of the sample. The most common example in the literature involves acid hydrolysis of the polymer and the subsequent analysis of the acetic acid that is produced by HPLC. As 1 M of acetic acid will be released from the hydrolysis of 1 M of *N*-acetylglucosamine the quantitative

determination of the amount of acetic acid released can be used to calculate the DDA of the sample. Literature examples of the determination of the DDA by HPLC vary mainly in the hydrolysis conditions of the chitinous polymer. Niola *et al.* reported an acid hydrolysis-HPLC method that allows the determination of the whole range of the DDA.[143] However, this method of analysis requires a vacuum hydrolysis system, which has limited availability in many laboratories. The hydrolysis procedure was simplified and improved upon by Ng *et al.* who modified the reaction temperature, time and acid concentrations.[144] The authors state that this presents a suitable methodology for the determination of the whole range of DDA of chitinous materials. The reliance of this method upon the total hydrolysis of the chitinous material presents a drawback, as this can be a lengthy procedure and insufficient hydrolysis will result in inaccurate values of DDA. The conditions required for total hydrolysis are dependent upon the nature of the sample, as a highly crystalline sample will require more severe hydrolysis conditions. The crystallinity of the sample is, to some degree, dependent upon the DDA of the material, therefore it may not be possible to apply standard hydrolysis condition across a range of DDA. The presence of any impurities will also affect the determination, as an accurate weight of chitin is required. As acetic acid is a volatile compound, it may be lost due to evaporation, which would result in overestimated values of DDA. We can therefore conclude that acid hydrolysis of chitinous materials followed by HPLC analysis of the acetic acid produced is not a suitable method of determining the DDA.

3.6.3 Determination of the Amine Group Content

The DDA of chitinous materials can be achieved by determining either the amine or amide group content of a sample. A more comprehensive method of assaying the DDA would take into account both species, however, more commonly the amine group content is assayed. There are several literature procedures available to do this, acid-base titrations[18], colloid titrations[145], metachromic titration[146], periodate oxidation[147], dye adsorption[148], picric acid adsorption[149] and conductometry[150], the most popular of which will be discussed here.

3.6.3.1 Acid-Base Titrations

Acid-base titrations involve the dissolution of the sample in a known excess of acid, this solution is then titrated potentiometrically with a known, standardised, alkali, for example NaOH. This produces a titration curve with two inflection points, the difference between the volumes of these two points corresponds to the alkali consumed for the conversion of the amine group to the salt. The volume of alkali can be used to calculate the DDA of the sample, however, this method is only applicable to chitinous materials of high DDA, which are soluble.[18]

3.6.3.2 Picric Acid Adsorption

It is proposed in the literature that the DDA of a wide range of chitinous polymers can be determined by the adsorption of picric acid.[149] This involves the binding of picric acid to the amino groups of a known weight of chitinous material. Following adequate washing, the picric acid is then subsequently quantified by treatment with methanolic *N,N*-diisopropylethylamine (DIPEA). The concentration of DIPEA picrate in the resulting eluent being calculated from the absorbance of the solution at 358 nm. The disadvantage of this assay is that it requires an accurate determination of the weight of the sample and therefore the level of purity must be known. Any protein impurities present will interfere with the assay, as they will react with the picric acid. Additionally, this assay poses a safety hazard as picric acid is a shock sensitive explosive.

3.6.3.3 Dye Adsorption

Maghami *et al.* reported in 1988 the heterogeneous adsorption of anionic dyes at acidic pH to protonated amine groups, which act as dye sites, in chitin and chitosan.[151] The authors demonstrated that quantitative determination of the extent of adsorption can be achieved by monitoring the change in the absorbance of the dye

solution and hence monitoring the change in concentration of the anionic dye. This information can then used to determine the concentration of protonated amine groups, and hence the DDA, of chitinous materials.[151] This technique requires that the extent of diffuse adsorption of dye ions to be negligible and assumes that the stoichiometry of the ionic interaction between the dye ions and protonated amine groups is known. A 1:1 stoichiometry was shown to exist between protonated amine groups on the polymer chain and negatively charged sulphonic acid groups on a series of dyes.[152] Maghami *et al.* studied the adsorption of three anionic dyes with chitin and chitosan under acidic conditions using this methodology and compared the data for the DDA acquired to those from FTIR determination, concluding that equilibrium adsorption measurements may be used for the analysis of chitin and chitosan. Of the three dyes studied, the authors state that C.I. Acid Orange 7 (4-(2-hydroxy-1-napthylazo)benzensulphonic acid, sodium salt) attained equilibrium the most rapidly. Therefore, the authors concluded that this is the preferred dye for routine use in such analysis.[151]

It had been previously demonstrated that protonated amine groups induce a metachromic effect in the visible spectrum of a number of anionic dyes. Quantitative use of this metachromic effect was been used to determine the amino group concentration, and hence the DDA, *via* metachromic titrations.[126] However, this method of determination requires the sample being analysed to be soluble and is therefore unsuitable for chitinous material with a low DDA. The dye adsorption method is heterogeneous and therefore can be carried out on samples with a low DDA, the authors demonstrated that chitosan, although soluble in dilute acetic acid, will not dissolve if the acetic acid solution contains an excess of an anionic dye. However, this method of analysis does present drawbacks, importantly it requires that all the dye sites should be accessible to the dye ions, in a highly crystalline sample of chitin with a low DDA this may not be the case, this will introduce errors into the determination. Additionally, an accurate weight of the sample to be analysed must be determined, therefore the moisture and impurity content must be known. Any impurities present that contain dye sites will also introduce error in to the calculations.

We determined the DDA of a series of samples of commercial chitin, deacetylated to varying extents by the dye adsorption method, as described by Roberts *et al.*, see section 7.2.3.[148] The decrease in the absorbance of the dye solution at 484 nm upon stirring with known weights of chitinous material at 60 °C for 16 hours was used to calculate the DDA of the chitinous material. These results are shown in Table 3.3.

Deacetylation Time (h)	DDA (%)
0	3.0
0.25	4.0
0.5	4.0
1	3.8
3	2.9
6	2.1
18	2.3
24	3.3

Table 3.3 - DDA of a series of commercial samples of chitin deacetylated in 40 % NaOH at 100 °C under nitrogen for varying lengths of time. DDA calculated by the dye adsorption method of Roberts.[148]

The DDAs, as calculated by the dye adsorption method, remain largely unchanged across a series of samples of commercial chitin that have been deacetylated for varying lengths of time, Table 3.3. This suggests that either the sample has not been deacetylated under these conditions, or that the assay is flawed. It is improbable that no deacetylation has taken place, and more likely that a high degree of crystallinity may have rendered the dye sites inaccessible to the dye ions. Therefore the dye will not be efficiently adsorbed, producing inaccurately low DDA. As it is not possible to ensure that all dye sites are available for adsorption, we can conclude that dye adsorption is not a practical method for determining the DDA of chitinous materials.

3.6.4 Spectroscopic Methods of Determining the DDA

There are a number of spectroscopic methods available in the literature for evaluating the DDA of chitinous materials, namely, IR, UV, solution state ^{1}H and ^{13}C NMR and solid state ^{13}C and ^{15}N NMR. These methods will be reviewed in the following sections. Many of the spectroscopic methods of determination offer the advantage that they do not require an accurate mass of the sample being analysed, with the exception of UV, this means that the presence of impurities in the sample is not as detrimental to the determination of the DDA. With the exception of solution state NMR and UV spectroscopy, the spectroscopic methods do not require the sample to be solubilised, therefore spectroscopic methodologies are applicable to a wider range of DDA.

3.6.4.1 FT-IR

IR spectroscopy is one of the most widely studied methods for determining the DDA of chitinous materials. This method involves the determination of the absorbance of a probe band that changes in intensity with DDA, such as the amide I or amide II absorbance bands, relative to that of an internal reference band. The use of IR has a number of advantages. It is reasonably rapid and importantly does not require the samples to be solubilised, which means that it can be applied to chitinous materials with a wider range of DDA. Additionally, an accurate mass of the sample is not required, therefore the purity of the sample does not need to be determined, providing that the impurities do not interfere with the probe and reference absorbance bands.

A number of absorption band ratios have been proposed in the literature for use in determining the DDA, differing either in the probe band or internal reference band chosen. Each of the ratios proposed has certain limitations, particularly with respect to the range of DDA values for which it is suitable. The most commonly used probe bands are the carbonyl stretching (amide I) band at approximately 1655 cm^{-1} and the

NH bending (amide II) band at approximately 1550 cm^{-1}. The most commonly used reference bands are the OH stretching band at approximately 3450 cm^{-1} and the CH stretching band at 2877 cm^{-1}. These bands are marked on the FTIR spectrum of a commercial sample of chitin shown in Figure 3.6. The literature convention is to acquire the IR spectra in the absorbance mode as it is the intensity of the absorbance that is assayed.[153, 154]

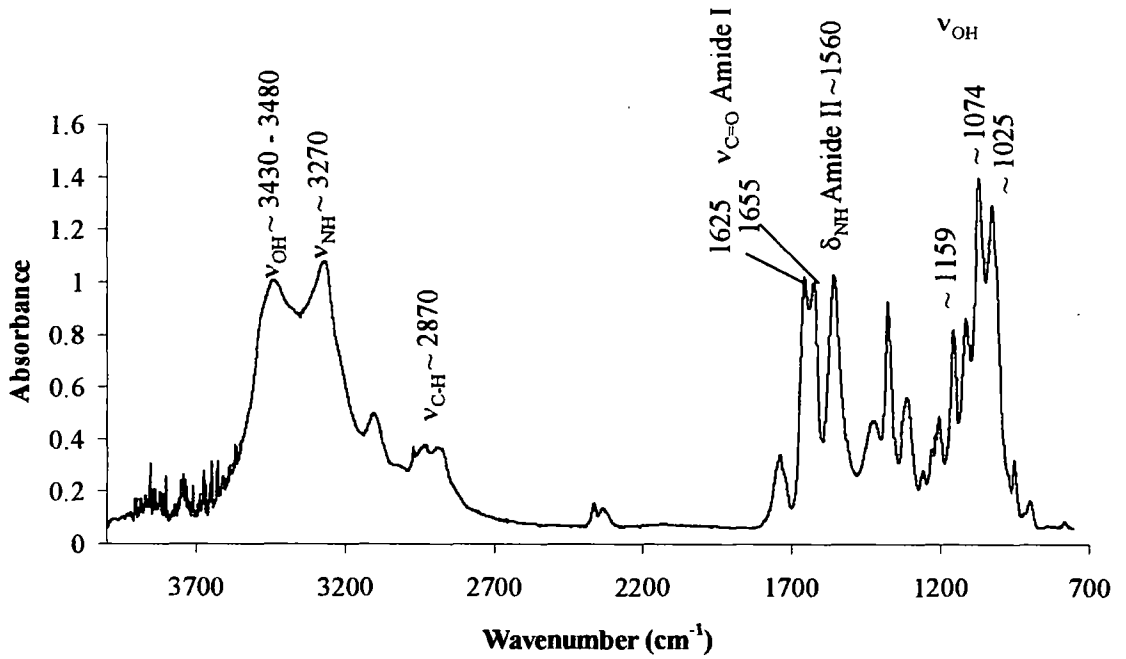


Figure 3.6 - FTIR spectrum of a commercial sample of chitin (Sigma) obtained in absorbance mode. The most commonly used probe and reference absorption bands used in the determination of the DDA in the literature are marked.

Use of the two C-O stretching bands at 1074 and 1025 cm^{-1} as internal reference bands has also been proposed in the literature.[153] The C-O stretching band at 1159 cm^{-1} arises from the asymmetric stretching of the bridging oxygen, the absorbance of this band may change if the sample is depolymerised therefore this band will not be discussed any further.[155]

The available absorbance ratios have been extensively reviewed in the literature,[153, 155, 156] the most common ratios used are A_{1655}/A_{3450}, A_{1550}/A_{2878} and A_{1655}/A_{2867}. The $A_{1655/3450}$ ratio has received the most interest in the literature as it presents a number of advantages over other proposed ratios.[157, 158] The band at

approximately 3450 cm^{-1} is prominent and relatively isolated, whereas the band around 2870 cm^{-1} lies in a complex spectral region where at least five bands have been observed due to symmetric and asymmetric stretchings of CH groups in the pyranose ring and from CH_2OH and CH_3 groups. Importantly, the intensity of the internal reference band at approximately 3450 cm^{-1} is not dependent upon the level of *N*-acetylation, whereas the band at approximately 2870 cm^{-1} arises from C-H stretching and therefore will vary with a variation in the DDA. Thus the use of this latter absorption band as an internal reference peak requires the absorption ratio values to be calibrated by another technique. The A_{1655}/A_{3450} ratio should not require such calibration and therefore, in principle, represents an absolute technique. However, the use of the absorption band at 3450 cm^{-1} is complicated by the presence of water, which will contribute to the hydroxyl absorption band in this region. As chitin is hygroscopic this may present a problem, as discussed in the following section. The use of the amide I band at 1655 cm^{-1} as a probe band over the amide II band was proposed by Miya *et al.* as they found the former to give more reliable results for samples with a high DDA.[156] Duarte *et al.* went on to describe the appearance of a band at 1597 cm^{-1} with increasing DDA, which they state is probably due to the increasing concentration of primary amines, this may interfere with the amide II band and complicate its use as a probe band.[155]

Baxter *et al.* demonstrated the reliability of the absorbance ratio A_{1655}/A_{3450} for determining the DDA of chitinous materials that have a DDA between 45 and 100 %.[158] The authors determined that the relationship between the absorbance ratio and the degree of acetylation DA can be described as:

$$\% \text{ DA} = (A_{1655}/A_{3450}) \times 115$$

The choice of this ratio as the most reliable for use has been reinforced by other authors who have demonstrated that the values of DDA produced agree with other techniques for determining the DDA.[126, 151, 157] However, Shigemase *et al.* demonstrated that this ratio is unsuitable for materials with a low DDA[153] as the amide I absorption band contains two components: one centred around 1655 cm^{-1} due

to singly hydrogen bonded amide groups and one centred around 1630 cm^{-1} due to amide groups involved in two hydrogen bonds. Considering both absorption bands at 1630 and 1655 cm^{-1}, referenced to the absorption band at 3450 cm^{-1}, provides a method which Roberts states is applicable to the whole range of DDA, the DA is calculated using the following equation:[159]

$$\% \text{ DA} = [(A_{1655}/A_{3450}) + (A_{1630}/A_{3450}) - 0.13] \times 85.5$$

The absorbances of these bands are calculated using the baseline method; the choice of baseline introduces another point for debate in the literature. In a review of this discussion Roberts states that the absorbance band at 3450 cm^{-1} should be measured using the baseline from 4000 to 2500 cm^{-1}.[138] The absorbance bands at 1655 and 1630 cm^{-1} are measured using the baselines 1800 to 1500 cm^{-1} and 1650 to 1600 cm^{-1} respectively.[159] When considering the absorbance band at 1655 cm^{-1} alone as the probe band a baseline of 1800 to 1600 cm^{-1} is suggested.[158] These baselines are shown in Figure 3.7.

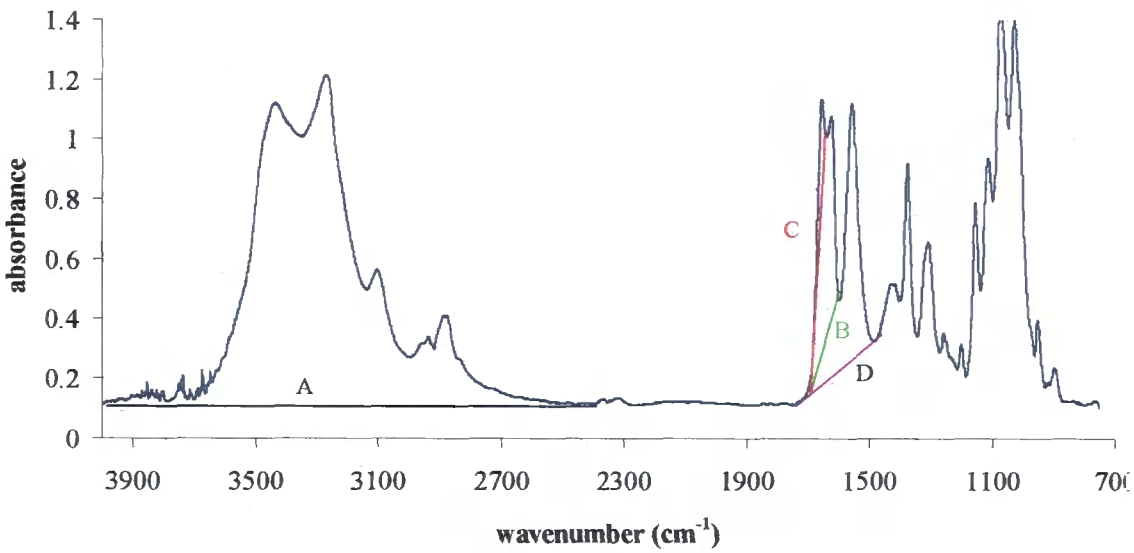


Figure 3.7 - FTIR spectrum of a commercial sample of chitin. The proposed baselines used to calculate the absorption of the bands at, A - 3450 cm^{-1}, B - 1655 cm^{-1}, C - 1630 cm^{-1} and D - 1655 cm^{-1} are marked.

IR is probably the most studied method for the determination of the DDA as it is relatively fast and can assay insoluble samples. It is often used as standard technique against which alternatives are often compared.[18] However, the use of this technique does present a number of issues, not least of these is the disagreement in the literature as to the identification of suitable baselines. Also chitinous materials are extremely hygroscopic and therefore must be dried extensively to eliminate moisture that could contribute to the hydroxyl band intensity and lead to incorrect DDA determination. The presence of water may also interfere with the absorbance band assigned as the amide I doublet, as the OH bending of water has an absorbance band at approximately 1640 cm^{-1}. The absorbance band at 3450 cm^{-1} may suffer from interference from a second O-H stretching band at approximately 3480 cm^{-1}, due to another type of hydrogen bonding in which the CH_2OH groups are involved in α-chitin. At low DDA the N-H stretching band may also interfere with this absorption. Additionally the presence of residual proteins, which may be bound to the chitin and chitosan matrix, may affect the position and intensities of probe bands.

We obtained IR spectra, as described in section 7.2.4, of a series of commercial samples of chitin that had been previously deacetylated to varying extents, see section 7.5. The IR spectra of the untreated commercial chitin and a sample that has been deacetylated for 24 hours are shown in Figure 3.8. The absorbance of the amide I and amide II bands are noticeably decreased in the deacetylated samples, indicating that deacetylation has taken place. Notably, the two amide I peaks can no longer be resolved; this may be due to a change in the hydrogen bonding network of the sample. As the sample is deacetylated from chitin to chitosan the nitrogen-containing group is only involved in one type of hydrogen bond resulting in a single absorbance in the IR spectrum, this is discussed in section 3.8.2.1. The peak at approximately 3430 cm^{-1} is no longer well resolved in the spectrum of the deacetylated sample, therefore its use as a reference peak may introduce errors.

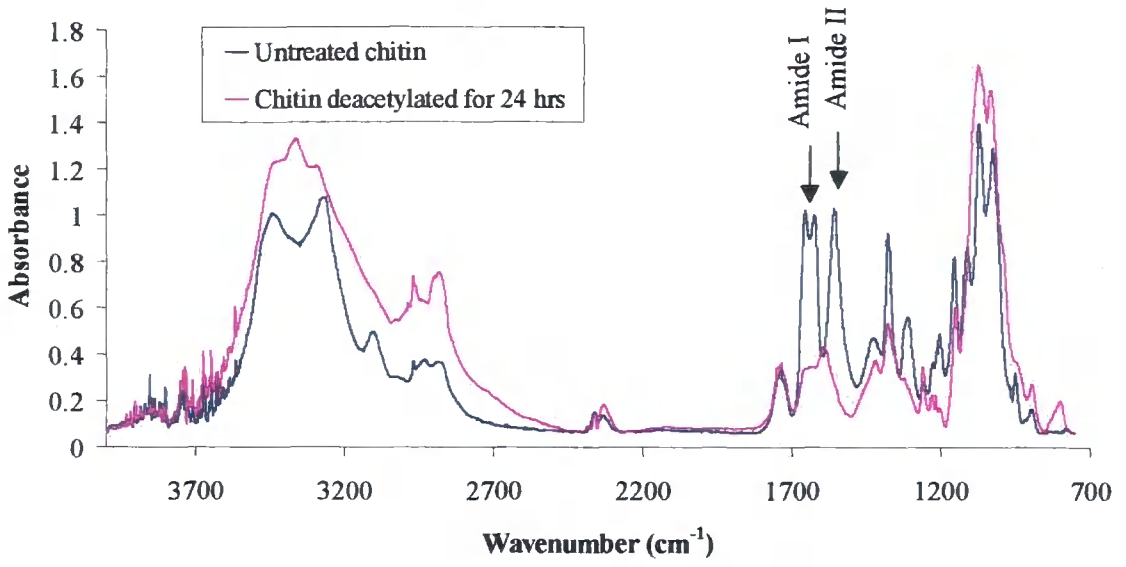


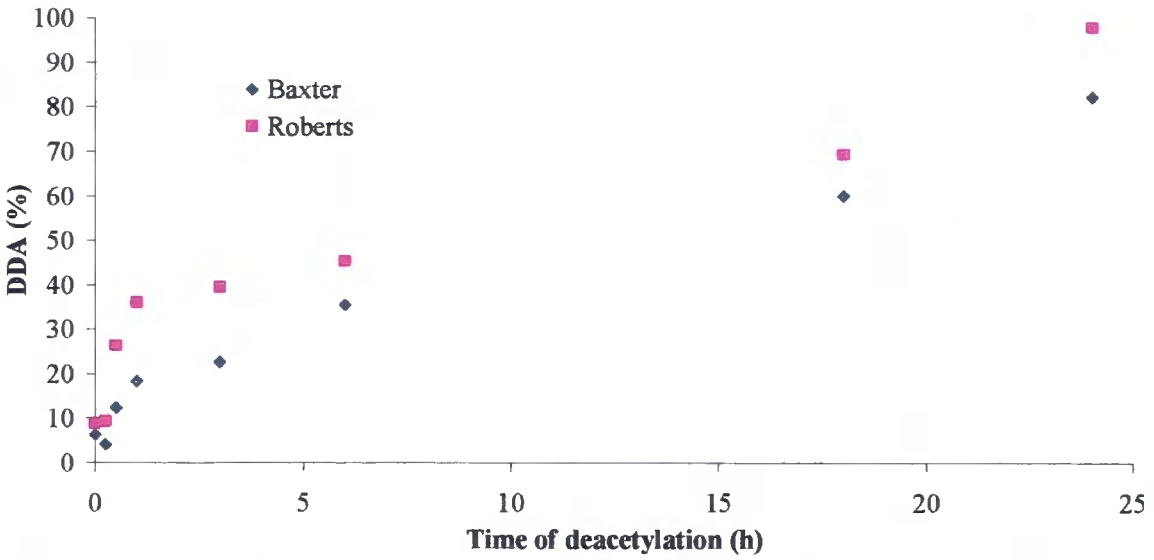
Figure 3.8 - IR spectra of commercial chitin, untreated and deacetylated in 40 % NaOH at 100 °C under nitrogen for 24 hours.

The IR spectra of the full range of deacetylated samples were used to determine the DDA of the samples by both the method of Baxter *et al.*[158] and Roberts [159]. Both methods require the determination of the absorbance of the band at approximately 1655 cm^{-1}, however, this is determined using two separate baselines, 1800 to 1600 cm^{-1} and 1800 to 1500 cm^{-1} for the Baxter and Roberts methods respectively. The absorbance of the band at 1630 cm^{-1} was determined using the baseline from 1600 to 1650 cm^{-1} for use in the determination by Roberts' method. The absorption band at 3450 cm^{-1} was used as a reference band in both methods. The results are shown in Table 3.4.

Deacetylation Time (h)	DDA (%) (A_{1655}/A_{3450})*	DDA (%) (A_{1630}/A_{3450} + A_{1655}/A_{3450})**
T = 0	6.3	8.8
T = 0.25	4.1	9.3
T = 0.5	12	26
T = 1	23	39
T = 3	18	36
T = 6	35	45
T =18	60	69
T = 24	82	98
Commercial chitosan	87	97

Table 3.4 - DDA of a series of commercial samples of chitin deacetylated in 40 % NaOH at 100 °C for varying lengths of time. The DDA are calculated from the absorbance ratios stated by the methods of *Baxter[158] and **Roberts.[159]

These results are presented in Graph 3.4. The DDA follow a similar trend when determined by both methods, however, the values vary greatly by between 2 and 18 %. In the highly deacetylated samples it was not possible to visualise the absorbance band at 1630 cm^{-1}, however, this does not account for the variance in samples with low DDA in which the band at 1630 cm^{-1} could be resolved.



Graph 3.4 - The DDA of a series of samples of commercial chitin deacetylated for varying lengths of time. DDA is determined from the ratio of the absorbance of the amide I band to the OH stretching band at 3450 cm^{-1} using the methods of Baxter[158] and Roberts.[159]

The variation between the results produced when determining the DDA by the methods of Baxter and Roberts highlights the major issue with using IR, the choice of baseline and absorbance ratio greatly affects the results produced. Currently there is no absolute agreement in the literature as to which should be used to calculate the DDA. Additionally, as previously mentioned, the hygroscopic nature of chitin may also preclude IR as a method of determining the DDA as the OH bending of water produces an absorption band at 1640 cm^{-1} which may interfere with the amide I band. Hydroxyl stretching at 3480 cm^{-1} may also interfere with the reference band at 3450 cm^{-1}. We can therefore conclude that IR spectrometry is not a suitable method for determining the DDA of chitinous materials.

3.6.4.2 UV Spectroscopy

Castle *et al.* were the first to report the quantitative use of UV spectroscopy to analyse chitinous materials.[160] They recorded the UV spectra of chitin dissolved in *N,N*-dimethylacetamide-lithium chloride (DMAc-LiCl) and observed an absorption band attributable to the *N*-acetyl group at 274-278 nm. However, this method was quickly

disregarded as the intensity of this band was found to be dependent upon the age of the solution, the authors explained this as being due to the setting up of complex equilibria which also led to a non-linear relationship between the concentration and absorbance.[160] Additionally, the intensity of the absorption band may be complicated by interference from the absorbance of DMAc, which is known to have an absorption maxima at 280 nm. This method is therefore not suitable for the quantitative determination of the DDA of chitinous materials.

Muzzarelli later reported a method for determining the DDA using first derivative UV spectroscopy which proved more viable. This method involves the dissolution of the samples in solutions of acetic acid and observation of the resulting absorbance. Muzzarelli reported that at 199 nm the absorbance of *N*-acetylglucosamine is linearly dependent upon concentration and is not influenced by the presence of acetic acid. He noted that glucosamine concentrations do affect the calibration but devised simple correction factors.[161] However, the range of DDA which can be assayed in this way is limited as the method requires the samples being analysed are soluble in solutions of acetic acid, which limits the range of DDA which can be assayed to below 40 %.[162] Reliable determination of the DDA by first derivative UV spectroscopy within this range has been reported in the literature,[163] however, our goal is to define a methodology which is applicable to the whole range of DDA values, therefore first derivative UV spectroscopy is not suitable.

3.6.4.3 Solution-State NMR

NMR spectroscopy has been used to characterise polymers for more than 50 years. Following the introduction of Fourier transform methods, the availability of superconducting magnets with higher magnetic fields and the development of multidimensional methods, high resolution liquid-state NMR has become the technique of choice for the determination of the molecular structure of polymers and biopolymers in solution.

Characterisation of carbohydrate structure by NMR is simple, rapid and so suitable for routine analysis. The methyl signal of the *N*-acetyl group (GlcNAc) and the CH-1 resonances (GlcN and GlcNAc) appear at the highest and lowest field respectively and are well discriminated from the other resonances in both the ^{1}H and ^{13}C NMR spectra. Thus the DDA can be estimated from the relative intensities of these resonances, referenced to those of the sugar backbone. NMR offers the advantage that it does not require accurate weighing of the sample being analysed, therefore extensive drying of the sample is not required and the purity of the sample does not need to be determined, providing that the impurity peaks do not overlap with the relevant peaks of the chitinous material. Sample preparation is simple and there is no need for any calibration curve or reference sample of known DDA. The DDA can be calculated using different combinations of peaks in order to verify that the method is consistent. However liquid phase NMR is limited by the solubility of the sample.

Early work in the characterisation of the DDA by ^{1}H NMR was carried out by Varum *et al.* in 1991.[164] The authors assigned the ^{1}H NMR spectrum of chitosan and determined the DDA, they also studied the distribution of acetylated monomer units within the polymers using ^{1}H NMR. However, in order to do this, they partially deacetylated chitin samples in alkali conditions and then partially depolymerised the resulting chitosans, by treating with nitrous acid in order to solubilise the samples in D_2O. Among the various conditions proposed in the literature for determining the DDA of chitosan by ^{1}H NMR those by Hirai *et al.* using 20 % DCl solutions at 70 °C have been widely accepted.[165] Lavertu *et al.* used these conditions to study chitinous materials with DDA ranging from 48 % to 100 %. At 70 °C, the authors state that the solvent peak does not interfere with the sample peaks. DDA values were calculated using the intergrals of several peak combinations and were shown to be internally consistent.[166] Shigemasa *et al.* also reported the use of 20 % DCl as an NMR solvent for chitinous samples with low DDA.[153]

Solution state NMR appears to offer a fast, reliable and easy method of determining the DDA of chitinous samples, which have a high DDA and are therefore increased in solubility. However, it may not be applied to samples across the whole range of DDA

owing to the insolubility of chitinous materials with low DDA. Additionally many of the proposed NMR solvent systems involve the use of DCl, usually at 70 °C; under these conditions it likely that the chitinous material will undergo deacetylation and depolymerisation, therefore the results may not be reliable.

We performed initial investigations into the use of ^{1}H solution-state NMR to determine the DDA of chitinous materials using the conditions proposed by Hirai *et al.*[165] A commercial source of chitin (Sigma) was stirred in 20 % DCl in D_2O at 70 °C. As described in section 7.2.5, an internal standard, 3-(trimethylsilyl)-1-propane sulphonic acid, was added to the solution to enable referencing of the peaks. However, addition of the standard produced additional peaks in the chemical shift range of the methyl group of chitin (spectra not shown). This complicated the interpretation of the spectra; therefore this standard was used to assign the shift of the solvent peak, which was then used as a reference for all subsequent spectra. The resulting ^{1}H NMR spectrum is shown in Figure 3.9.

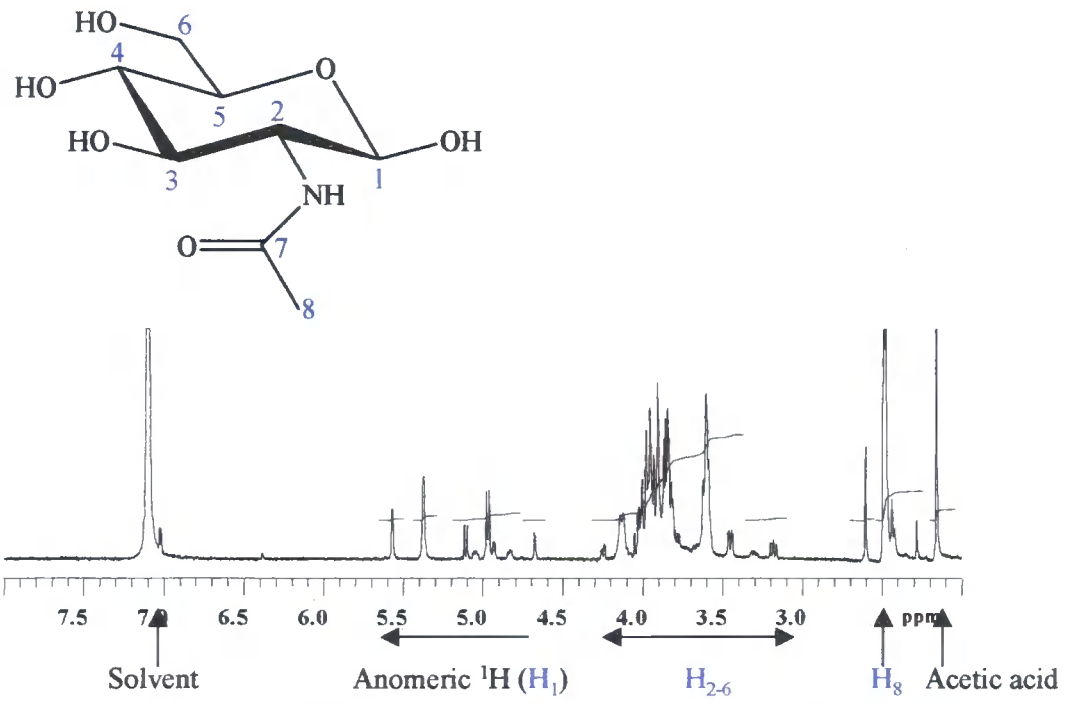


Figure 3.9 - ^{1}H NMR spectrum of a commercial source of chitin (sigma) in 20 % DCl in D_2O.

The spectrum in Figure 3.9 displays six doublets in the region between 4.64 and 6.50 ppm, which are assigned as anomeric protons coupled to H_2 protons. Consideration of the relative coupling constants of α and β anomers and comparison of the spectrum to that of commercial glucosamine has allowed assignment of these six doublets, shown in Figure 3.10. We can conclude that under the harsh NMR conditions, 20 % DCl at 70 °C, the glycosidic linkages of the chitinous materials have been hydrolysed, producing glucosamine and *N*-acetyl glucosamine units. Owing to the equilibria between the pyranose and linear forms of sugars, these monomer units will be present as the α and β anomers. Additionally, there appears to be some residual polymeric material in the acetylated and deacetylated forms.

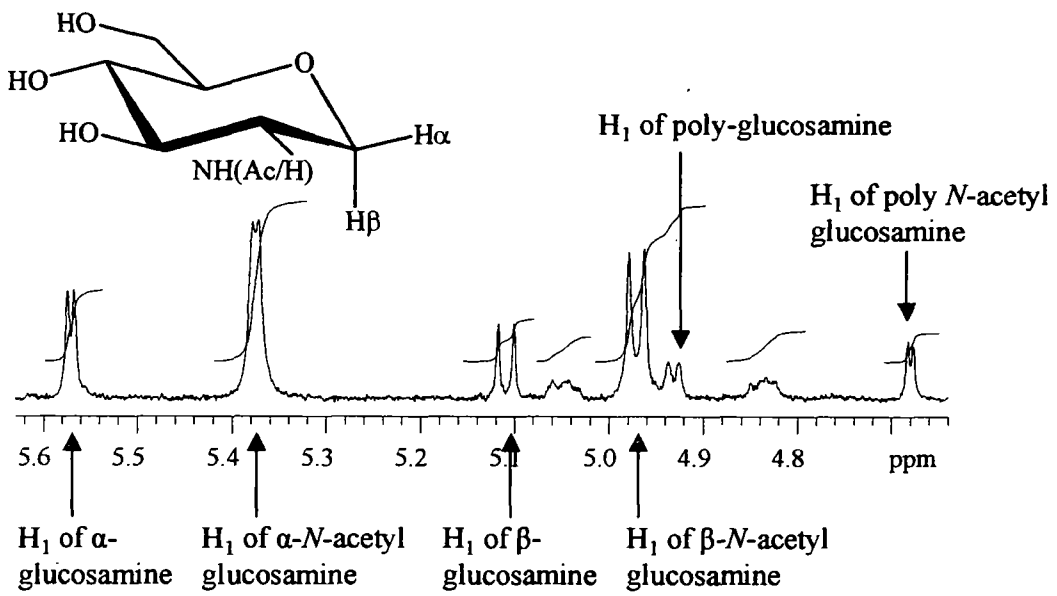


Figure 3.10 - Possible assignment of the anomeric protons at 4.64 to 5.60 ppm in the ^{1}H NMR spectrum of a commercial sample of chitin acquired in 20% DCl in D_20.

Owing to their similar chemical shifts, the identities of the $H_{2\text{-}6}$ can not be resolved, see Figure 3.9. Resolution of these peaks is further complicated by the hydrolysis of the glycosidic bonds, which will affect the chemical shift of H_4. An additional peak, of relatively high intensity, can be seen in the region of the chemical shift of the methyl protons at approximately 2.1 ppm. This may be accounted for when we consider that under the harsh NMR conditions is it probable that some deacetylation has taken place. This would result in the presence of acetic acid, which has a

chemical shift of 2.1 ppm. The amide and hydroxyl protons are not represented in the spectrum due to fast proton exchange.

We can estimate the DDA by comparing the integral of the methyl peak, H_8, with those of the sugar ring, $H_{2\text{-}6}$, which suggests a DDA of 45.4 %. However, any deacetylation that has taken place in the NMR solvent is not accounted for. This may be corrected for by including the integral of the acetic acid peak in the determination. This results in an estimated DDA of 23.6 %, which seems unreasonably high for a commercial source of chitin. We have demonstrated that the harsh conditions required to solubilise the sample result in depolymerisation and deacetylation of the sample, therefore these results are unreliable. We can conclude that ^{1}H solution-state NMR is not a suitable method for determining the DDA of chitinous materials across the whole range of DDA.

3.6.4.4 ^{13}C CPMAS ssNMR

As described in section 3.6.4.3 Solution-State NMR is the method of choice for determination of the molecular structure of polymers and biopolymers in solution. However, chitinous materials with a low DDA are insoluble in common NMR solvents. Solid-state NMR (ssNMR) offers an alternative, as this method does not require the sample to be solubilised, additionally it does not require an accurate mass of the sample and therefore the sample does not need to be extensively dried. Also the presence of impurities, providing their chemical shifts do not interfere with those of chitin, does not affect the analysis. The ^{13}C ssNMR spectra of solid samples are generally recovered using magic-angle spinning (MAS), cross-polarisation (CP) and high-power ^{1}H decoupling (HPDEC) techniques. These techniques are discussed more fully in section 3.8.2, in brief MAS averages out dipolar interactions and chemical shift anisotropy and HPDEC removes the ^{1}H-^{13}C dipolar interactions. CP increases the sensitivity of ^{13}C NMR and reduces the relaxation delay. ^{13}C CPMAS ssNMR has been used widely to study the conformation and structure of chitinous materials, this is further discussed in sections 3.8.2.1-3, the following paragraphs will

discuss the application of CPMAS ssNMR to the study of the DDA of chitinous materials.

The use of ssNMR for the determination of the DDA was first reported by Pelletier *et al.* in 1990[167] and subsequently by Raymond *et al.* in 1993.[150] Both groups calculated the DDA of samples of chitinous materials by comparing the area of the CH_3 resonance, which has a chemical shift of approximately 23 ppm, to the resonances of the glucose carbons, which have chemical shifts in the range of 50 to 110 ppm. Alternatively, the integral of the carbonyl peak, at approximately 174 ppm, could be used to calculate the DDA. There are many examples of the use of ^{13}C CPMAS ssNMR as a reliable strategy for the determination of the DDA of chitinous materials in the literature and it is often quoted as an absolute technique against which alternative techniques are calibrated.[139, 143, 144, 168, 169]

Typical ^{13}C CPMAS ssNMR spectra of commercial sources of chitin and chitosan are shown in Figure 3.11, these spectra have previously been assigned in the literature.[170, 171] The methyl and carbonyl peaks appear at the highest and lowest fields respectively and are well resolved from the other peaks in the ^{13}C spectra. Therefore analysis of the integrals of these peaks, referenced to the integral of the resonances of the carbons of the sugar ring, which act as an internal standard, should allow direct determination of the DDA.

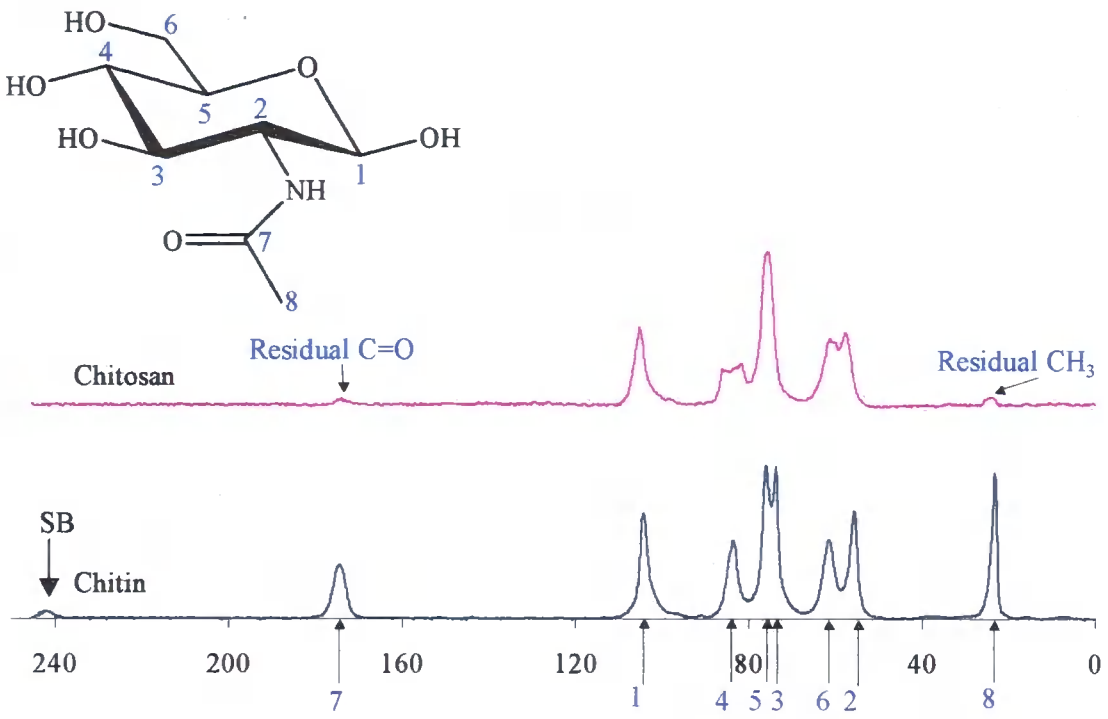


Figure 3.11 - ^{13}C CPMAS ssNMR spectra of commercial chitin and chitosan. SB indicates a spinning side-band.

Problems arise in using ^{13}C CPMAS ssNMR as a quantitative method to study DDA due to the use of CP to acquire the spectra. CP involves the transfer of magnetisation from the ^{1}H to the ^{13}C spins, this is achieved through heteronuclear dipolar interactions. The rate of build-up of the ^{13}C NMR signals varies, as it is dependent upon both the number of protons attached, or close, to a given carbon and on the distance between the ^{13}C and the ^{1}H nuclei. Molecular dynamics also affects CP, for example, fast rotation of methyl groups about the ternary axis partially averages out the ^{1}H-^{13}C dipolar interaction which tends to reduce the efficiency of CP.[172] As the intensities of the ^{13}C NMR resonances are influenced by the kinetics of the CP process, different contact times give rise to a modification of the relative intensities of the ^{13}C signals. The contact time is the time during which the r.f. fields are applied simultaneously to the ^{1}H and ^{13}C channels, this is described further in section 3.8.1. This means that carbons in different environments present maximum intensities when arrayed using different contact times. Therefore any quantitative study of the DDA must address the problem of whether or not the fraction of ^{13}C nuclei detected by NMR is representative of all the carbon in the sample. Duarte *et al.* carried out a

comprehensive relaxation study in order to obtain reliable values of DDA from ^{13}C CPMAS ssNMR.[173] The authors state the cross-polarisation relaxation times of chitin and chitosan are similar, indicating that equivalent carbons cross-polarise in the same length of time in samples with high and low DDA. They demonstrated that the methyl carbons require longer contact times to attain maximum cross-polarisation. The authors also studied the proton spin-lattice relaxation times and found them to be longer for more acetylated samples, indicating a lower molecular mobility. In order to quantitatively determine the DDA the contact time must be longer than the largest cross-polarisation relaxation time in the system so that all the carbon types are cross-polarised to an equal extent. However, the contact time must also be shorter than the proton spin-lattice relaxation times so that the ^{1}H magnetisation does not decay significantly during the contact period. The authors concluded these conditions are satisfied when using a contact time of 1 ms, affording spectra which are approximately quantitative for samples with varying DDA. However Heux *et al.* monitored the rise of the magnetisation with contact time and state that at a contact time of 1 ms the methyl and polysaccharide backbone magnetization reach a value of 88 % of the theoretical maximum magnetisation, whereas the carbonyl only reaches 84 %.[174] Therefore, the comparison of the integral of the methyl peak will produce more reliable results than the carbonyl peak

Another issue with the use of ^{13}C CPMAS ssNMR for the determination of the DDA of chitinous materials is the presence of impurities such as polysaccharides. In fungal sources, chitin is found in a matrix of interactions with polysaccharides, in particular β-glucans.[90, 175] The chemical shift of the sugar backbone carbons in these impurities usually coincides with those of chitinous materials, therefore, the presence of any polysaccharide impurities will distort the determination of the DDA. Therefore in order to obtain reliable determination of the DDA chitinous materials samples must be rigorously purified from polysaccharide impurities.

^{13}C CPMAS ssNMR analysis of a series of samples of commercial chitin deacetylated to varying extents was performed, as described in section 7.5. The spectra, displayed

in Figure 3.12, show the decrease in the intensities of the carbonyl and methyl peaks, which are clearly resolved, with increasing time of deacetylation.

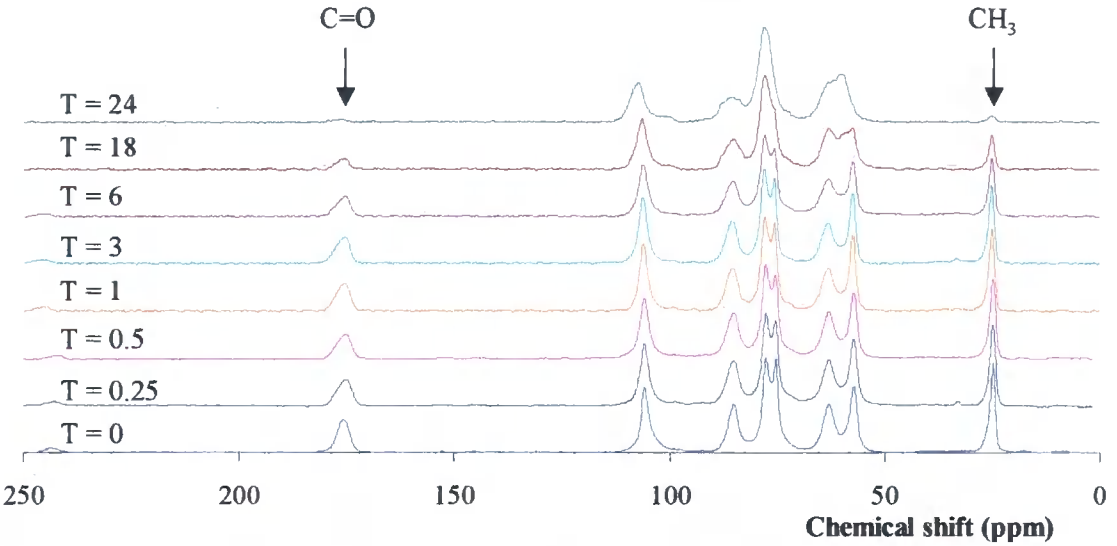


Figure 3.12 - ^{13}C CPMAS ssNMR spectra of commercial samples of chitin deacetylated in 40 % NaOH at 100 °C for varying lengths of time T (h). The carbonyl and methyl peaks are marked.

The DDA of the samples was calculated by comparing the intensity of both the carbonyl and methyl peaks against that of the sugar backbone carbons, which have chemical shifts between 50 and 110 ppm. These results are displayed in Table 3.5.

Deacetylation Time (h)	DDA (CH_3)	DDA (C=O)
T = 0	18	22
T = 0.25	17	19
T = 0.5	24	35
T = 1	21	26
T = 3	20	23
T = 6	36	43
T = 18	68	80
T = 24	93	95
Commercial chitosan	94	94

Table 3.5 - DDA of commercial samples of chitin deacetylated for differing lengths of time T, calculated from the relative intensities of the C=O and CH_3 peaks in the ^{13}C CPMAS ssNMR spectra.

As predicted from the work by Heux *et al.*[174], the DDA calculated from the integral of the carbonyl and methyl peaks are not in agreement, although they do follow the same general trend. We can hypothesise that this is due to the CP kinetics, the contact time is not optimal for the carbonyl carbon and therefore the peak has not reached maximum intensity, this results in overestimated DDA levels. Therefore, we shall consider the DDA calculated from the integral of the methyl peak. This value does seem unrealistically high for the commercial sample of chitin that has not undergone deacetylation.

3.6.4.4.1 ^{13}C CPMAS ssNMR determination of the DDA of fungal chitin

It has previously been mentioned that polysaccharide impurities will result in inaccurate determinations of the DDA of chitinous materials, this is a particularly issue when the samples are extracted from fungal sources. The major polysaccharide impurities in chitinous materials extracted from fungal sources are β-glucans, which are abundant in the fungal cell wall forming the major matricial component. It has been demonstrated that there is a covalent linkage between the carbonyl group of chitin and (1→3)-β-D-glucan.[90, 175] If these covalent linkages are not broken during the extraction procedure then glucans maybe present in the resulting chitin. Chitinous materials extracted from fungal sources by a standard chemical procedure, described in chapter 4, were analysed by elemental analysis, the resulting data indicated that they do contain impurities with low nitrogen contents. This is discussed further in section 3.2. If β-glucans do remain in the extracted materials then the ^{13}C CPMAS ssNMR analysis of the DDA will be complicated.

In order to address the issue of polysaccharide impurities in chitinous materials extracted from fungal sources a commercial source of chitin was mechanically mixed with a commercial source of β-glucan. This mixture studied by ^{13}C CPMAS ssNMR before and after the standard chemical extraction procedure described in section 7.4.3. The spectra are shown in Figure 3.13.

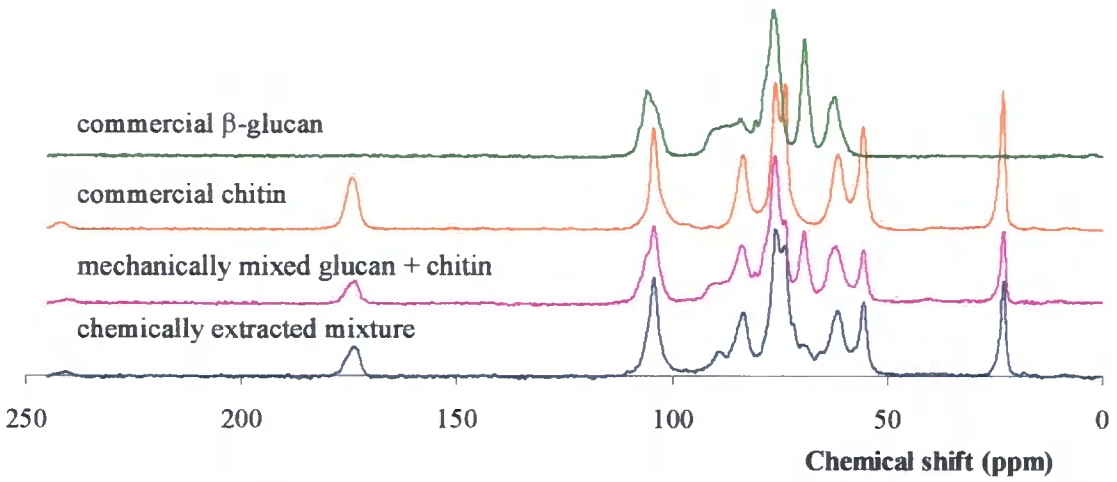


Figure 3.13 - ^{13}C CPMAS ssNMR spectra of a mechanical mixture of commercial chitin and β-glucan before and after chemical extraction. Spectra of the commercial chitin and β-glucan are shown for comparison.

Comparing the ^{13}C CPMAS ssNMR spectra of the commercial samples of chitin and β-glucan we can see that the resonances of the β-glucan polysaccharide fall in the same spectral region as the sugar backbone carbons of chitin. This validates the statement that β-glucan impurities will affect the determination of the DDA as the integral of these resonances is used as a reference. ^{13}C CPMAS ssNMR analysis of the mechanical mixture displays a general broadening of the peaks in this region, this is particularly apparent in the resonance of the anomeric carbon, additionally, the C3 and C5 peaks at approximately 74 and 75 ppm can no longer be resolved. An additional peak can also be seen at approximately 69 ppm and there is a notable large shoulder on the peak at approximately 86 ppm. The standard chemical extraction procedure is designed to removed polysaccharide impurities, however, the ^{13}C CPMAS ssNMR spectrum of the sample after extraction indicates that some β-glucan still remains. The shoulder on the peak at 86 ppm is still visible as is the additionally peak at approximately 69 ppm. This suggests that β-glucans are not removed by chemical extraction procedure, therefore ^{13}C CPMAS ssNMR analysis of chitinous materials extracted from fungal sources to determine the DDA will be inaccurate.

The ^{13}C CPMAS ssNMR spectrum of the chitinous material extracted from *P. chrysogenum*, as described in section 7.4.3, is shown in Figure 3.14. The spectra of commercial samples of chitin and β-glucan are shown for comparison. Elemental analysis of this extracted material suggests that it contains non-nitrogen containing impurities, as the carbon to nitrogen ratio is too high, see section 3.2. In comparing the sample extracted from the fungal source and the commercial sample of chitin we can see the similar spectral features, such as the general line broadening and the addition of a shoulder to the peak at approximately 86 ppm, as observed in the spectra of the mixed samples of commercial chitin and β-glucan. We can conclude from the ^{13}C CPMAS ssNMR spectra that a polysaccharide impurity, similar to β-glucan, is present in chitinous material extracted from *P. chrysogenum*.

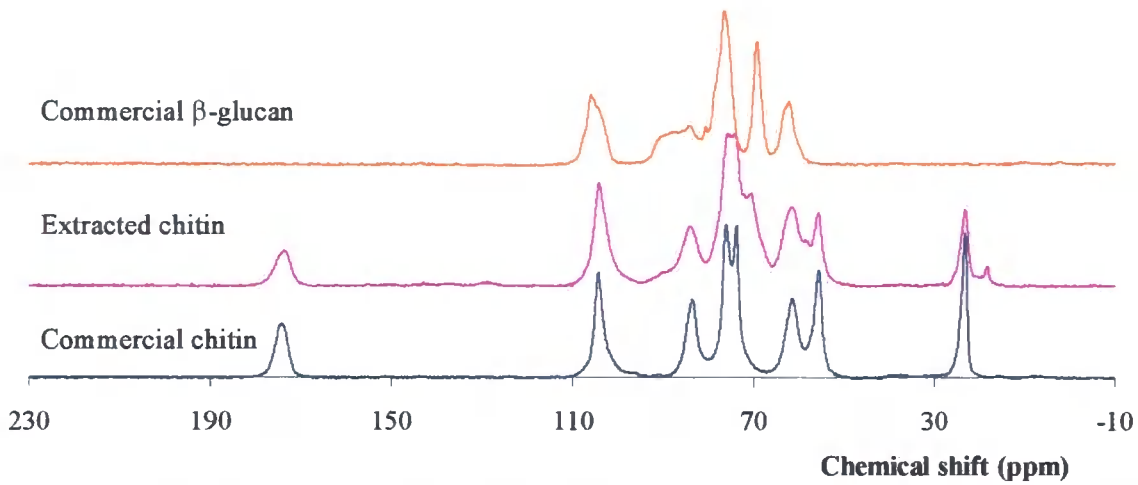


Figure 3.14 - ^{13}C CPMAS ssNMR spectra of chitinous material extracted from a fungal source, as described in sections 7.4.3 and 7.4.4, in comparison to commercial sources of chitin and β-glucan.

The use of ssNMR to determine the DDA of chitinous materials offers several advantages. The samples do not need to be extensively dried or derivatised in any way and the technique is applicable to a wide range of DDAs as the samples do not need to solubilised. However, we can conclude that ^{13}C CPMAS ssNMR is not a suitable method for the determination of the DDA of chitinous materials extracted from fungal sources as the extracts contain impurities that interfere with the analysis.

3.6.4.5 ^{15}N CPMAS ssNMR

^{15}N CPMAS ssNMR offers all the benefits of ^{13}C CPMAS ssNMR, described in section 3.6.4.4, in that the sample does not need to be solubilised, which increases the range of DDA which can be assayed, and an accurate weight is not needed, therefore the sample does not need to be extensively dried or purified. However, it has the additional advantage that there are relatively few non-equivalent nitrogen atoms present in biopolymers and the chemical shift differences are usually large. Therefore there are fewer overlapping resonances than in ^{13}C CPMAS ssNMR spectra, additionally the line-widths of the peaks are usually narrower. ^{15}N CPMAS ssNMR has been extensively applied to the investigation of biopolymers, especially the structure and conformation of DNA or peptides.[176, 177] It has also been applied to the structural analysis of polymeric materials such as nylon.[178] ^{15}N CPMAS ssNMR can provide information on chemical structure and bonding, reaction mechanisms, biosynthesis, nitrogen fixation, metal coordination and provide detail about active sites in biochemical systems.[177, 179] The natural abundance of ^{15}N nucleus is low in comparison to the ^{14}N nucleus, 0.36 % and 99.63 % respectively, however the ^{14}N nucleus is quadrupolar resulting in line-broadening over the order of several kHz hampering the interpretation of the spectra.[179]

Yu *et al.* first proposed the use of ^{15}N CPMAS ssNMR as a method for determining the DDA of chitinous materials in 1999, reporting that it is more reliable than ^{13}C ssNMR analysis.[180] Figure 3.15 displays ^{15}N CPMAS ssNMR spectra of commercial samples of chitin and chitosan (Sigma). The ^{15}N resonances of the acetylated and deacetylated nitrogen are at approximately – 257 and – 357 ppm respectively, this pronounced 100 ppm separation, combined with the absence of any other nitrogen peaks, increases the ease at which the ssNMR spectra can be analysed to determine the DDA.

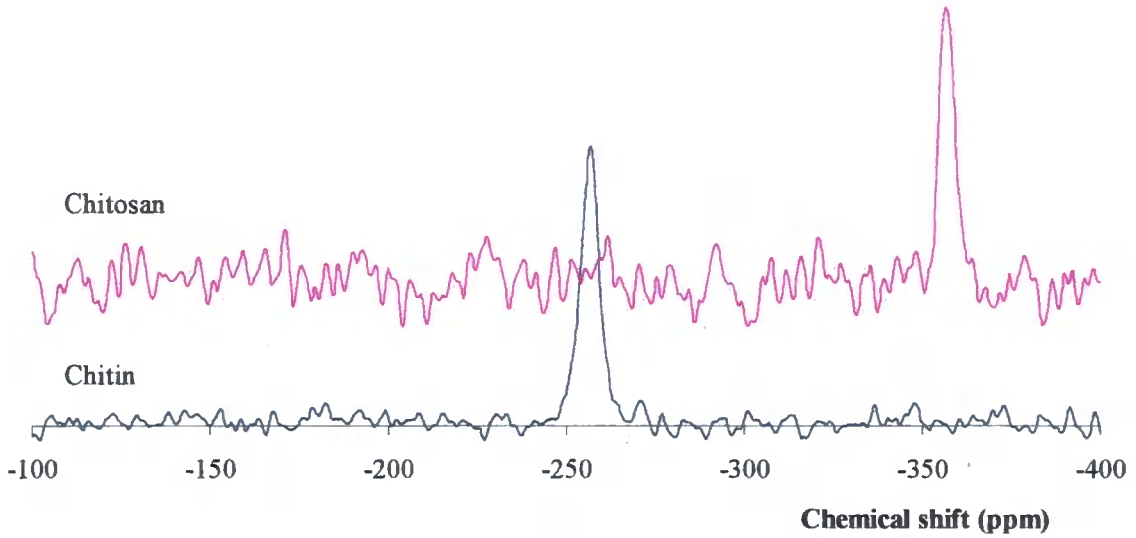


Figure 3.15 - ^{15}N CPMAS ssNMR spectra of commercial samples of chitin and chitosan (Sigma)

As described in section 3.6.4.4.1, chitin is frequently associated with polysaccharides such as β-glucans which complicate the analysis of the DDA by ^{13}C CPMAS ssNMR, however, these impurities do not contain nitrogen, therefore the ^{15}N CPMAS ssNMR spectra will be unaffected. Heux *et al.* compared the use of ^{13}C and ^{15}N CPMAS ssNMR to determine the DDA of a series of chitinous materials from *Aspergillus niger*. The authors studied the cross-polarisation kinetics and found that quantization by direct intergration of the amido and amino nitrogen peaks is reasonable.[174] They state that the limitation of ssNMR lies in the detection threshold being not higher than 5 %.

^{15}N CPMAS ssNMR appears to be the most accurate method of determining the DDA of chitinous materials derived from fungal sources. The literature indicates that it is applicable to a wide range of DDA, with an apparent detection limit of 5 % limiting the detection range of DDAs to between 5 and 95 %, and does not require the sample to be soluble or derivatised in any way. Additionally, ^{15}N CPMAS ssNMR assays both the amino and amine content of the sample in order to determine the DDA, as discussed previously, this produces more reliable results. Importantly, the presence of non-nitrogen containing impurities, such as β-glucans and adsorbed water, does not

affect the analysis. Therefore the samples do not need to be extensively purified or dried. However, ^{15}N NMR spectroscopy is hampered by low sensitivity, which results from very low ^{15}N natural abundance (0.37%), this means that long acquisition times are required in order to obtain reasonable spectra. Yu *et al.* stated that between 50,000 and 80,000 scans are required, this can take between 13 and 22 hours.[180]

We acquired ^{15}N CPMAS ssNMR spectra of a series of commercial samples of chitin which had been deacetylated in 40 % NaOH at 100 °C under nitrogen for varying lengths of time, see section 7.5. The spectra, shown in Figure 3.16, display the decrease in the intensity of the peak corresponding to the amido nitrogen of chitin with a concurrent increase in the intensity of the amino nitrogen of chitosan with increasing deacetylation time.

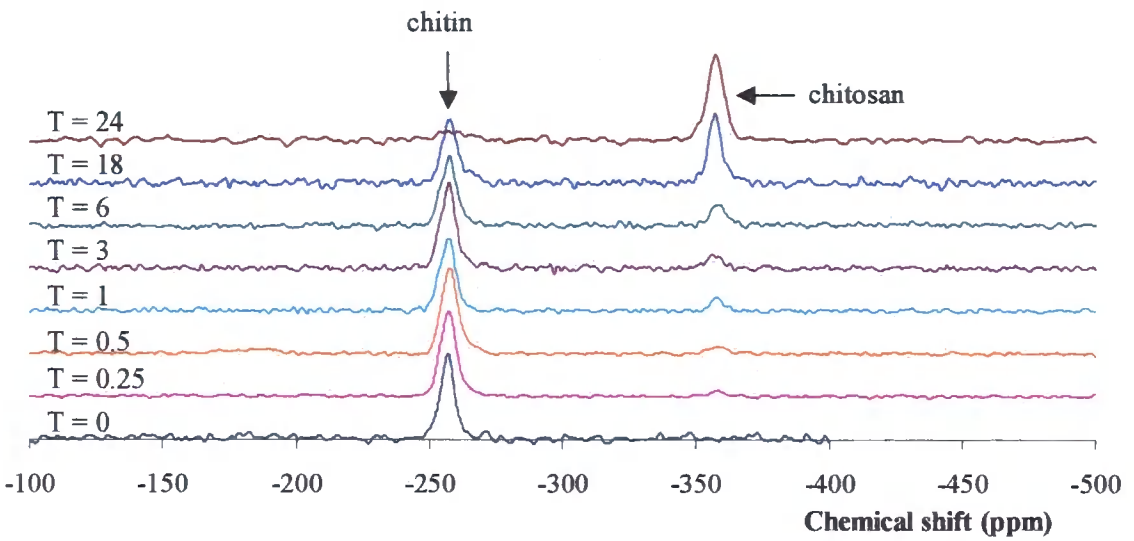


Figure 3.16 - ^{15}N CPMAS ssNMR spectra of commercial samples of chitin deacetylated for varying lengths of time T (h).

The relative integrals of the amino and amine peaks were used to calculate the DDA of the samples, see Table 3.6. These peaks are well resolved and free from interference in the spectra of deacetylated commercial samples of chitin, displayed in Figure 3.16.

Deacetylation Time (h)	DDA (%)
0	1.1
0.25	5.5
0.5	8.2
1	12
3	14
6	24
18	48
24	85

Table 3.6 - DDA of a series of commercial samples of chitin deacetylated for varying lengths of time. DDA determined by ^{15}N CPMAS ssNMR.

A ^{15}N CPMAS ssNMR spectrum of the chitinous material extracted from a fungal source was acquired in order to investigate the possibility of interference from remaining impurities. The elemental analysis of this sample indicates that the sample contains impurities, which the ^{13}C CPMAS ssNMR spectrum of this sample, shown in Figure 3.14, suggests are of polysaccharide nature. The ^{15}N CPMAS ssNMR spectrum of this sample is shown in comparison to a commercial sample of chitin in Figure 3.17. No additional peaks can be seen in the spectrum of the extracted chitin, indicating that no other nitrogen containing species are present. The peak corresponding to the amino nitrogen of chitin is broader than that of commercial chitin, however, this may be explained by a different conformational form of chitin and does not suggest the presence of impurities. This indicates that ^{15}N CPMAS ssNMR analysis of the DDA of chitinous materials is unaffected by the presence of polysaccharide impurities. Study of the levels of signal to noise seen in the ^{15}N CPMAS ssNMR spectra, acquired using upwards of 30,000 repetitions, of commercial chitin and chitinous samples extracted and purified from fungal biomass that contain natural abundance levels of ^{15}N suggests that a confidence limit of approximately $\pm$ 5 % can be employed when assessing the DDA.

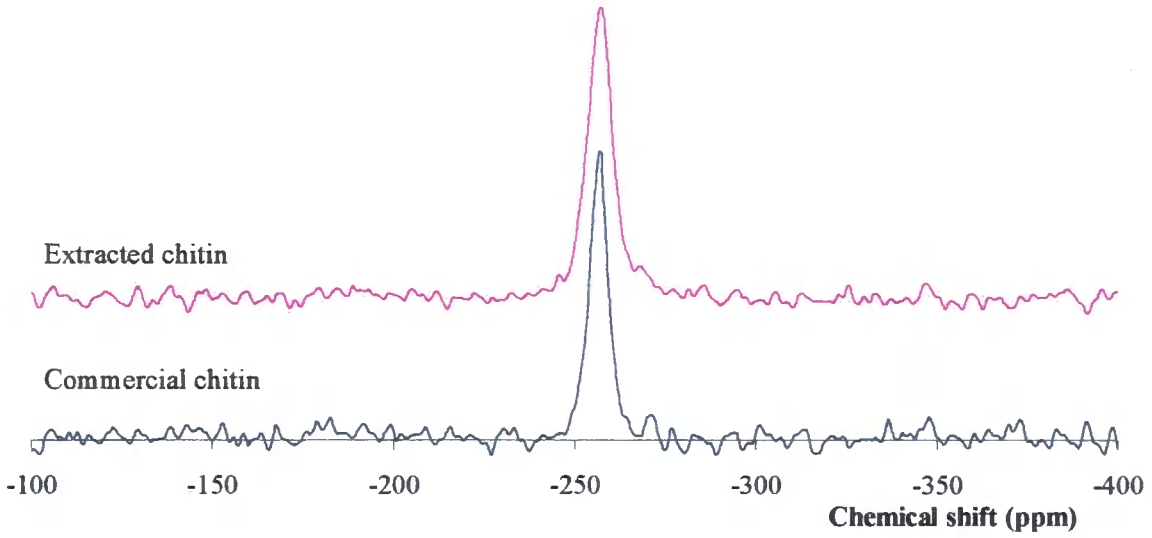


Figure 3.17 - ^{15}N CPMAS ssNMR spectrum of the chitinous extracts of *P. chrysogenum*. Extracted as described in sections 7.4.3 and 7.4.4.

^{15}N CPMAS ssNMR as a method of determining the DDA of chitinous materials appears to offer a great many advantages, as outlined in the previous section, however, it is limited by the poor sensitivity of the ^{15}N nucleus, which results from its low natural abundance, 0.36 %. This means that a high number of repetitions are required to obtain spectra of high enough quality to quantitatively assess the DDA, resulting in long acquisition times. The spectra in Figure 3.16 required 35,000 repetitions to acquire; this takes approximately 10 hours of spectrometer time leading to increased costs. In order to overcome this intrinsic problem, chitinous materials may be ^{15}N labelled. We investigated the ^{15}N labelling of fungal systems in order to produce labelled chitin that requires much shorter ^{15}N CPMAS ssNMR acquisition times and results in improved spectra, which have increased confidence levels, monitoring DDA below 5 %. This is discussed extensively in chapter 5.

3.6.5 Conclusion of the Determination of the DDA

Several methods of determining the DDA of chitinous materials proposed in the literature were investigated with the aim of identifying a fast and reliable method, which does not rely upon any standard of known DDA or calibration curve obtained by another technique and is applicable to the whole range of DDA. A series of commercial samples of chitin were deacetylated to varying extents by stirring in 40 % NaOH at 100 °C under nitrogen for varying lengths of time. The various methods of determination of the DDA were then applied to these samples. The calculated DDA of the commercial samples of chitin by all methods are shown in Table 3.7.

Time of deacetylation (h)	DDA (%) Calculated by				
	Elemental analysis	Dye adsorption	IR	^{13}C CPMAS ssNMR	^{15}N CPMAS ssNMR
0	5.0	3.0	6.3	18	1.1
0.25	-3.6	4.0	4.1	17	5.5
0.5	-2.7	4.0	12	24	8.2
1	8.0	3.8	18	21	12
3	9.6	2.9	23	20	14
6	18	2.1	35	36	24
18	49	2.3	60	68	48
24	81	3.3	82	93	85

Table 3.7 - DDA of a series of commercial samples of chitin deacetylated to varying extents, determined by several methods.

We can conclude that elemental analysis is not a suitable method of determining the DDA of samples as any organic impurities present affect the C to N ratio, altering the results. We demonstrated in section 3.6.4.4.1 that chitinous extracts from fungal sources contain polysaccharides, such as β-glucans, which will invalidate the elemental analysis determination of DDA.

We can also conclude that the dye adsorption method of determining the DDA of chitinous materials is not suitable. The values of DDA produced by this method do not vary significantly with increasing deacetylation time for the commercial sample of chitin or the samples extracted from fungal sources, see Table 3.7. We can hypothesise that this is due to the crystallinity of the samples, which render the dye sites inaccessible to the dye ions.

IR spectroscopy as a method for determining the DDA is discounted due to the hygroscopic nature of chitin. Adsorbed water produces absorption bands that interfere with the determination of the DDA. In addition there is much argument in the literature as to the choice of absorption ratios that are best suited to determining the DDA, the use of different ratios produces very different DDA results.

^{13}C CPMAS ssNMR is quoted in the literature as being the most reliable method of determination of DDA,[155, 168] however the presence of polysaccharide impurities in the sample results in inaccurate determination of the DDA. In section 3.6.4.4.1 we demonstrated that the chemical extraction procedure does not completely remove polysaccharide impurities, which will increase the intensities of the resonances in the region of chemical shift containing the sugar backbone carbons. Determination of the DDA by comparing the intensity of the carbonyl or methyl peaks to the integral of the peaks in this region will therefore result in inaccurately high DDA. As this study focuses on chitinous extracts of fungal sources we can conclude that ^{13}C CPMAS ssNMR is not a suitable method for determining the DDA.

Using the information gathered during literature searches and our own investigations, we concluded that ^{15}N CPMAS ssNMR analysis is the most suitable method of determining the DDA of chitinous materials extracted from fungal sources. ^{15}N CPMAS ssNMR offers all the benefits of ssNMR, in that the sample does not need to be solubilised, derivatised or extensively dried, and has the additional advantage that there are relatively few non-equivalent nitrogens present in biopolymers. The polysaccharide impurities present in chitin extracted from fungal systems do not contain nitrogen and, therefore, do not interfere with the analysis. Additionally the

amino and amide resonances of chitin and chitosan respectively have a 100 ppm separation, therefore the peaks are well resolved and free from interference. This method also considers both the concentration of amino and amine groups and is therefore more robust. The disadvantage of the use of ^{15}N CPMAS ssNMR to determine the DDA is the low natural abundance of ^{15}N, which results in long, costly, NMR acquisition times. However, the use of ^{15}N labelled samples may reduce the acquisition times, and hence the cost, of acquiring a ^{15}N CPMAS ssNMR considerably. This is discussed in chapter 5. Additionally the relatively low abundance of nitrogen containing compounds in the fungal cell, in comparison to carbon, may mean that the ^{15}N CPMAS ssNMR analysis of the whole fungal cell can be used to determine the DDA. This would remove the need for lengthy extraction and purification procedures, which is required for all other methods of analysis investigated. This is also investigated in chapter 5.

3.7 Determination of the Molecular Weight of Chitin and Chitosan

The characteristics of chitinous materials intended for biomedical applications must be definitively analysed in order to optimise the properties of the materials. To complete this study the molecular weight and polydispersity of the polymer should be determined. Gel permeation chromatography (GPC), is a widely used technique for the analysis of polymeric materials. GPC, or size exclusion chromatography (SEC), involves the separation of molecules according to their size in chromatographic columns filled with porous rigid gels, the pores are at least of the same size as the dimensions of the polymer molecules. A sample of dilute polymer solution is introduced into a solvent stream flowing through the columns and the polymer molecules diffuse into the internal pore structure of the gel to an extent depending on their size. Larger molecules can enter into only a small fraction of the pores, or are excluded completely, and therefore flow through the column faster. An elution curve is obtained by plotting the amount of polymeric solute, evidenced by an appropriate detector, against retention time of the different molecular sizes. A refractive index (RI) detector is commonly used to measure the change in the refractive index of the

eluent as the polymer concentration changes. This data can be used to calculate the concentration of all dissolved solutes at a given time and unlike many detection methods does not require the presence of a chromophore. In order to determine the molecular weight characteristics, this data can then be compared to the elution characteristics of standards of known molecular weight. The standards must therefore behave similarly in the given solvent system. Alternatively, a triple detector system that combines an RI detector coupled with a right-angle laser light scattering (RALS) detector and a viscometer can be employed to determine the molar mass characteristics without the use of standards. The Rayleigh equation relates the scattered laser light intensity (determined by LS) to the product of the polymers molecular weight, concentration (determined by RI) and an optical constant (the specific refractive index increment (dn/dc)). Therefore, providing the dn/dc value of the material in the particular solvent system is known the molar mass averages and molar mass distribution can be calculated. The dn/dc value is a measure of how much the refractive index of a solution varies for a given increment in concentration. The viscometer is needed to correct for the fact that scattered light is collected at 90 ° and not 0 ° as required by the Rayleigh equation.

In the following sections the literature precedents for the use of GPC to determine the molar mass characteristics of chitinous materials are discussed. As the chitinous materials studied in the following chapters generally have low DDAs, only the conditions for the analysis of chitin are discussed in detail. This discussion is followed by a report of the work we carried out to optimise the dissolution of chitin in the literature proposed solvent and the subsequent attempts of GPC analysis. This method of analysis and its poor reliability in our hands is then evaluated.

3.7.1 GPC Analysis of Chitosan

GPC analysis of chitosan has been extensively reported in the literature as this deacetylated chitinous polymer has increased solubility.[102, 181-183] Generally, chitosans with a DDA above 50 % are soluble in dilute aqueous acetic acid, however,

in this solvent chitosan acts as a cationic polyelectrolyte. This complicates GPC analysis as the hydrodynamic volume of polyelectrolytes is dependent upon the ionic strength and hence the concentration of the chitinous material and any other ionic species in solution. Additionally, conventional GPC gels carry a negative surface charge, so that the protonated chitosan chain would be expected to be strongly adsorbed onto the gel. For the separation process to operate correctly, it is necessary that there is no interaction between the gel and the polymer solute; therefore, any electrostatic effects must be eliminated. GPC analysis of chitosan has been reported in a 0.5 M acetic acid eluent as this eluent is expected to reduce ionisation of the carboxyl groups present on the support surface and therefore reduce adsorption of chitosan.[102] Sodium acetate is typically added to this eluent to generate enough ionic strength to overcome ion-exclusion effects.[183] However, fully deacetylated chitosan samples have been shown to extensively adsorb onto the columns when using this eluent. The use of 0.2 M ammonium acetate at pH 4.5 as the eluent is claimed to eliminate this adsorbtion.[181] In addition to considering electrostatic interactions between the polymer and the porous gel, the concentration dependent self-association of more highly acetylated chitosan to form aggregates must be taken into account when analysing by GPC.[181]

3.7.2 GPC Analysis of Chitin

The chitinous materials discussed in this study generally have DDAs below 50 % and are therefore known as chitin. Chitin is inherently insoluble in common solvents, including the ammonium acetate solution described for chitosan. This further complicates the analysis of the material by GPC. Deacetylation of chitin to increase its solubility may result in depolymerisation of the material, producing unreliable molecular weight results. The use of LiCl:*N,N*-dimethylacetamide (LiCl:DMAc) as a true, non-derivatising, solvent of polysaccharides such as chitin and cellulose has been proposed in the literature.[43, 184-187] The exact mechanism of the dissolution of chitin LiCl:DMAc is unknown, however, it is thought that LiCl:DMAc solutions dissolve chitin by disrupting the extensive hydrogen bonding network. The lithium

ion associates with the carbonyl oxygen of DMAc,[188] this weak complex then dissolves the polyelectrolyte formed by association between the chloride ions and the protons of the hydroxyl groups of the chitin chain.[189]

Solutions of 5 % (w/v) LiCl in DMAc are commonly reported in the literature for the dissolution of chitin, however, Poirier *et al.* proposed that the ability of the solvent system to solubilise chitin is dependent upon the concentration of LiCl in DMAc.[189] In order to maximise the potential of the solvent for the dissolution of chitin we investigated the effect of varying the concentrations of LiCl on the quantity of chitin dissolved. LiCl:DMAc solutions between 1 and 5 % (w/v) were prepared as described in section 7.2.7, care was taken to ensure that both constituents were thoroughly dried as moisture prevents the dissolution of LiCl. Known quantities of commercial chitin (Sigma) were then stirred in these solutions at room temperature for three days. After this time the solutions were centrifuged to remove the residual chitin and the supernatant was added to water to precipitate any dissolved chitin. The precipitate was washed with water and acetone and dried in a desiccator overnight. The yield of precipitated chitin, as a percentage of the original weight, from each solution is shown in Table 3.8. The precipitate was analysed by ^{13}C CPMAS ssNMR and elemental analysis to ensure that the chitin had not been derivatised in any way. The results indicate that higher concentrations of LiCl do increase the ability of the solvent system to dissolve chitin. Therefore, 5 % LiCl:DMAc solutions will be used in future studies as higher concentrations of salts present issues in the use of GPC. The use of 5 % LiCl:DMAc as the solvent and eluent for the analysis of chitin and cellulose by GPC has been reported in the literature.[189-192]

LiCl:DMAc (w/v)	Yield of precipitate (%)
1	16
3	24
5	28

Table 3.8 - Yield of chitin precipitated from LiCl:DMAc solutions of varying concentrations of LiCl.

It is important to note that only 28 % of the available chitin was dissolved in this solvent. As the solubility of polymers is molecular weight dependent it is likely that only the lower molecular weight chitin chains were dissolved, therefore, GPC analysis of these dissolved chitin chains may not be representative of the whole sample. Heating or refluxing insoluble samples is a frequent protocol to facilitate the subsequent dissolution, however, care must be taken to avoid chitin degradation. Potthast *et al.* demonstrated the degradation, and subsequent increase in solubility, of cellulosic material when they were heated in LiCl:DMAc solvent systems.[193] In order to investigate the effect of heating chitin on the degradation and dissolution of chitin in LiCl:DMAc, we stirred commercial samples of chitin in 5 % (w/v) LiCl:DMAc at 100 °C for varying lengths of time. After this time the resulting solutions were centrifuged to removed residual chitin, the supernatant was analysed by GPC, using 5 % (w/v) LiCl:DMAc as the eluent. The resulting refractive index (RI) traces are shown in Figure 3.18.

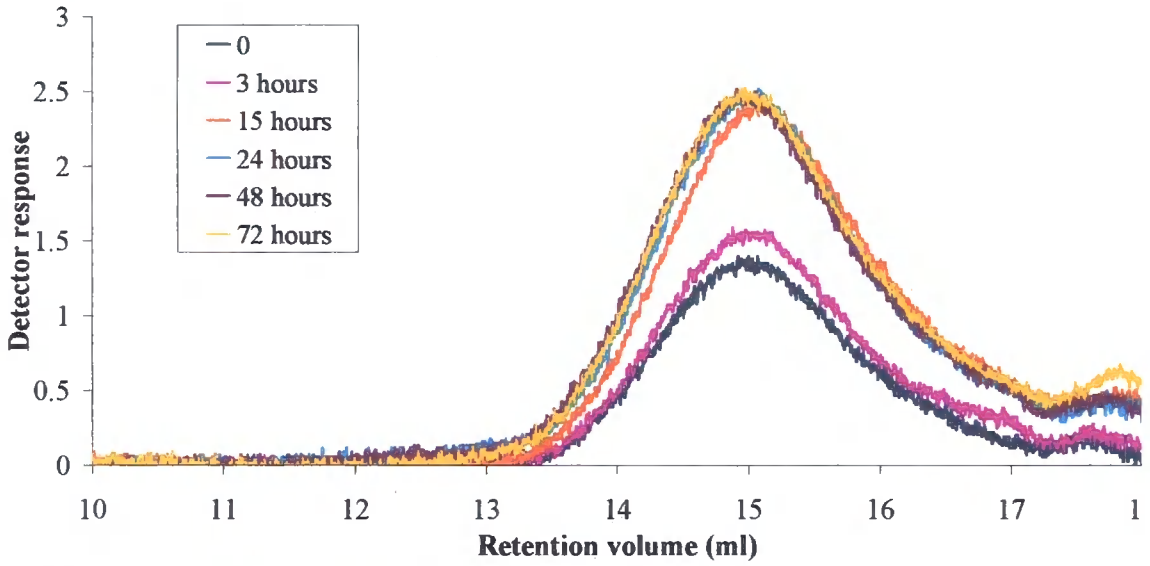


Figure 3.18 - Refractive index trace produced by the GPC analysis of commercial samples of chitin (Sigma) stirred in 5 % LiCl:DMAc at 100 °C for varying lengths of time.

The results suggest that heating in 5 % (w/v) LiCl:DMAc for longer than 3 hours increased the amount of chitin dissolved as the response curve has a greater intensity. Heating the samples for longer than 24 hours did not significantly increase the response seen by GPC. The retention volumes of all of the samples were similar, indicating that no significant degradation of the polymer had occurred upon heating.

Results from the light scattering traces, not shown, also displayed no change in retention volume upon heating of the samples for extended periods. Therefore, in future studies chitinous samples were stirred in 5 % (w/v) LiCl:DMAc at 100 °C for 24 hours prior to analysis by GPC.

In order to further maximise the concentration of chitin dissolved in 5 % LiCl:DMAc, a series of concentrations of commercial samples of chitin were stirred in 5 % LiCl:DMAc at 100 °C for 24 hours. The resulting solutions were centrifuged to remove residual chitin and the supernatants analysed by GPC. The RI traces produced are shown in Figure 3.19. At initial concentrations greater than 5 mg/ml, the RI traces are skewed towards higher retention volumes, indicating lower molecular weights. There are two possible explanations for this: firstly, if the solution is at the limit of solubility lower molecular weight polymers may be selectively solubilised skewing the results. Alternatively, at high concentrations and hence high viscosities, the polymers may shear reducing their molecular weight. In order to avoid these effects we avoided the use of concentrations above 5 mg/ml.

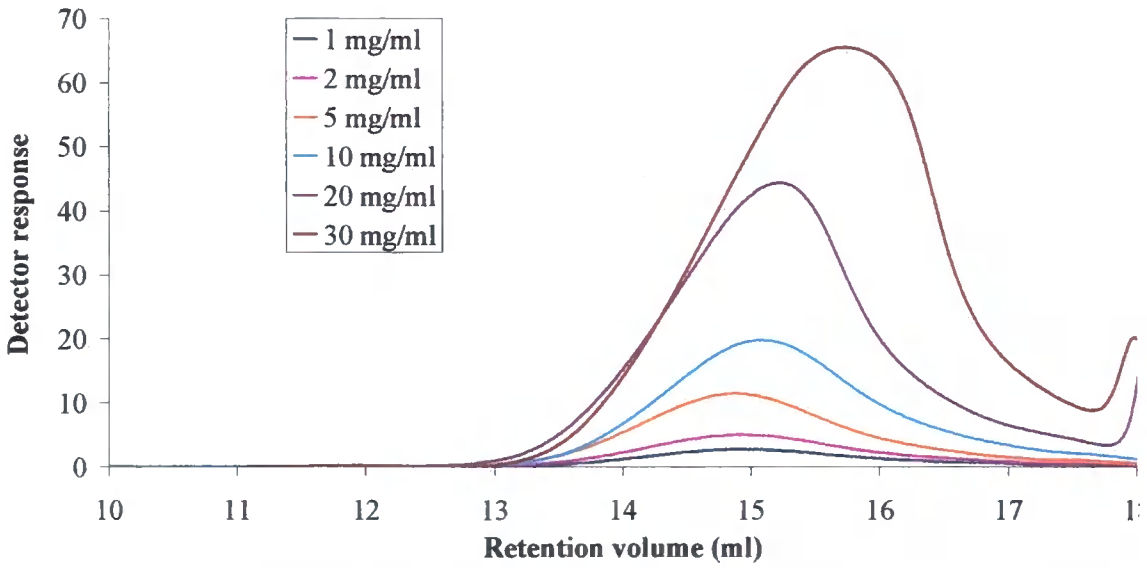


Figure 3.19 - Refractive index trace produced by the GPC analysis of difference concentrations of commercial chitin in 5 % LiCl:DMAc.

The increasing intensity of a large peak at a retention volume of approximately 10 ml in the light scattering traces, shown in Figure 3.20, of samples with an initial concentration above 5mg/ml indicates the presence of very high molecular weight materials that are not retarded by the column. This suggests that aggregation of the chitinous polymers has occurred. All of the LS traces present this high molecular weight peak, however, its intensity is far greater in solutions with an initial concentration of chitin of above 5 mg/ml.

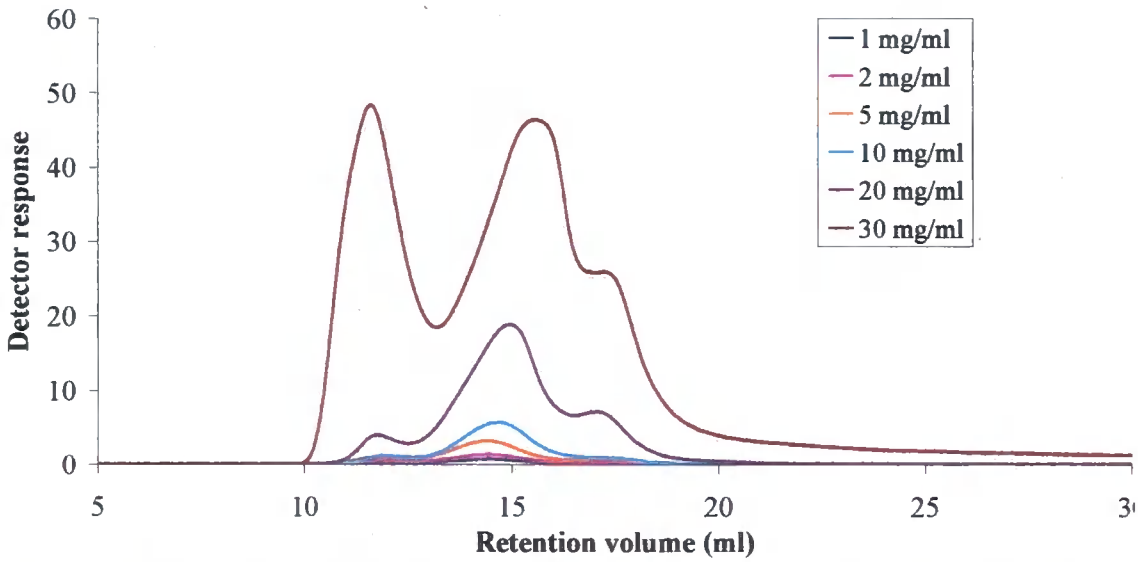


Figure 3.20 - Light scattering trace produced from the GPC analysis of varying concentrations of chitin in 5% LiCl:DMAc

Aggregation of cellulose in LiCl:DMAc solvent systems has been reported in the literature[194] and it has been suggested that the presence of adsorbed water increases the amount of aggregation seen.[186] Therefore, to reduce the possibility of aggregation initial concentrations of 5 mg/ml of chitin in 5 % LiCl:DMAc which has been extensively dried were used. A solution made to these specifications was prepared and analysed by GPC immediately and after standing for one week. The LS traces acquired (not shown), are identical indicating that no additional aggregation had taken place.

3.7.3 Conclusion of GPC Analysis of Chitin

The analysis of chitin by GPC using LiCl:DMAc as the solvent and eluent has been proposed in the literature. We have determined that the optimal solution conditions involve stirring 5 mg/ml of chitin in 5 % (w/v) LiCl:DMAc at 100 °C for 24 hours prior to analysis. In order to prepare the LiCl:DMAc solution, and to reduce the possibility of aggregation, LiCl and DMAc were extensively dried prior to use. As the dn/dc value of chitin in this solvent system was not known, evaluation of the molecular weight and polydispersity of the polymer solutions could only be achieved by comparison of the refractive index data to that of a standard of known molecular weight. The use of polysaccharide pullulan standards have been proposed in the literature.[191, 195] Pullulan consists of α-(1→4) and α-(1→6) linked glucose residues.

The RI and LS trace of a commercial sample of chitin (Sigma) acquired under these conditions is shown in Figure 3.21. The RI trace had a maximum at a retention volume of approximately 14 ml, however, the LS trace displayed an additional maximum at approximately 10 ml. RI results are concentration dependent, however, LS results are dependent upon the molecular weight of the polymer in solution. Therefore, a low concentration of a high molecular weight material presents as a large peak on a LS trace but may not be seen on a RI trace. The presence of this low elution volume peak on the LS trace indicates the presence of a very high molecular weight material, which is probably explained by the aggregation of chitin chains in solution, as described previously. Comparison to the Pullulan standards indicates that commercial chitin has a Mw of 6,700 Da and a polydispersity of 9.8. This molecular weight value is unreasonably low in comparison to literature values of chitin extracted from crab and shrimp sources which usually lie in the region of hundreds of kDa.[184, 190, 195] This reinforces our conclusion that the lower molecular weight chitin chains are preferentially solubilised.

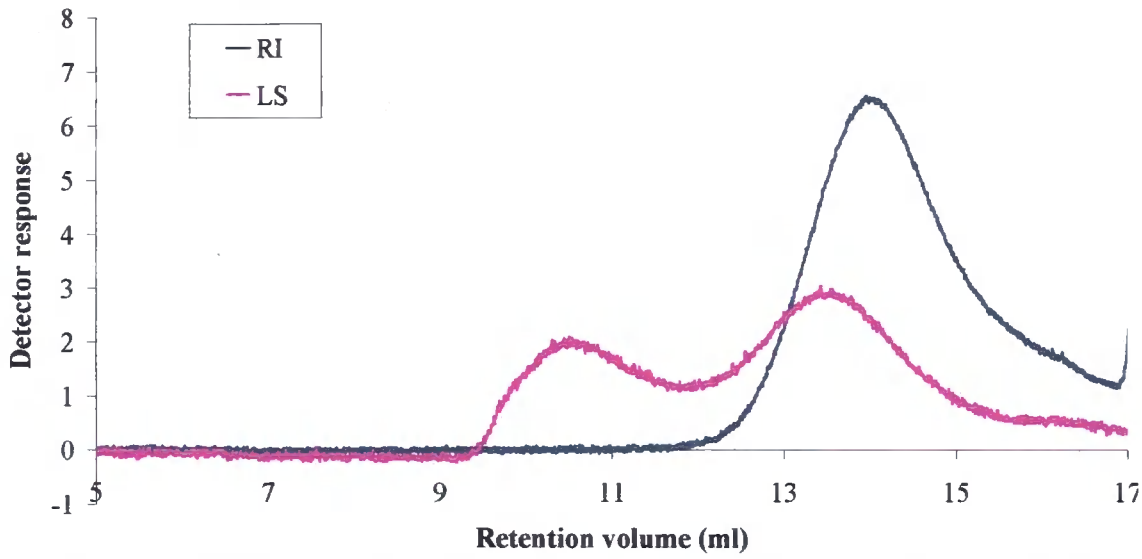


Figure 3.21 - Light scattering and refractive index trace produced from the GPC analysis of commercial chitin (Sigma) in 5% LiCl:DMAc

In our opinion, the use of GPC using a LiCl:DMAc solvent system to accurately determine the molar mass averages and molar mass distribution of chitinous materials present in fungal systems is limited. This is principally due to the poor solubility of chitin, even in the optimised conditions described above, the commercial samples of chitin were not fully solubilised. As solubility is dependent upon molecular weight, GPC analysis of the supernatant will therefore not be representative of the whole sample. The solubility of the chitinous polymers is also dependent upon the DDA of the sample. Chitosan polymers with a high DDA are insoluble in LiCl:DMAc solutions. As discussed in section 3.7, GPC analysis of chitosan has been reported in aqueous solutions, however, the results obtained in this way may not be comparable to those of chitin in the LiCl:DMAc system as the polymers behaviour differ in different solutions. Another possible introduction of error into the calculation is the comparison to pullulan standards required to quantitatively determine the molar mass averages. This requires the assumption that chitin and pullulan behave similarly in LiCl:DMAc, that is to say that at equal degrees of polymerisation the hydrodynamic volume of chitin and pullulan will be equal. This may not necessarily be the case as chitin is structurally more rigid than pullulan and the hydrodynamic volume of chitin may vary with DDA and distribution of acetylated groups in chitin.[195] Additionally, the presence of a peak at low retention volume in the light scattering

trace of the commercial samples indicates that aggregation of the chitin polymers takes place in solution, this will also affect the molecular weight data obtained. Finally, analysis of chitinous polymers extracted and purified from fungal sources is not necessarily representative of the molecular weight of the polymers *in vivo,* as the harsh chemical extraction procedures sometimes employed may degrade the polymer.

The study of chitin by GPC may be furthered by improving the solubility characteristics of chitin in LiCl:DMAc, or by the identification of another suitable solvent system. The dn/dc value of chitinous polymers in a known solvent system could also be calculated to improve the reliability of the results. However, this thesis of work has focused mainly on other areas of the analysis and extraction of chitinous materials therefore, these studies were not taken any further. Initial GPC studies of the chitinous materials extracted in the following chapters will be carried out, however, the reliability of the results attained is questionable. Therefore, comparative studies of the RI and LS traces will be used rather than the absolute values of molar mass averages and molar mass distributions.

3.8 Determination of the Polymorphic Form and Degree of Crystallinity of Chitinous Materials.

Chitin chains associate *via* a network of hydrogen bonds, the nature of which determines the polymorphic form of the material and its crystallinity, which in turn dictates the physiochemical properties. Variations in the conformation and structure of chitin have marked affects on the physical properties of the material, altering the degree of crystallinity of the sample, its solubility, reactivity and tensile strength.[196] In section 3.6 the variation in the physiochemical properties of chitinous materials with the DDA was discussed. This relationship is principally due to the change in the hydrogen-bonding network that occurs upon deacetylation. Therefore, when analysing chitinous materials it is important to consider conformation and structure. There are several possible methods available to do this. X-ray powder diffraction studies have been used to evaluate the crystal structure of chitin.[29] However, this

method of analysis is only suitable for crystalline materials and begins to fail when studying amorphous regions. IR spectroscopy can also provide information about the polymorphic form and degree of crystallinity, however, as discussed in section 3.6.4.1 this method of analysis has inherent disadvantages when studying chitin, due to chitin's hygroscopic nature. The physical structure of polymers influences the line positions and shapes in the ssNMR spectra, therefore, this offers the possibility to obtain information on the conformation and polymorphic form of the polymer.[34, 37, 197] The use of ssNMR as a powerful tool to investigate subtle molecular environments and local structure has been extensively employed in the study of biopolymers. For example, Hediger *et al.* used ssNMR techniques to study the effect of hydration on the mobility of polysaccharides in onion cell-wall material,[198] Newman *et al.* used ^{13}C CPMAS ssNMR to monitor cell wall changes in ripening kiwifruit.[199] Extensive research into insect sclerotized structures has also been carried out, utilising ssNMR.[200] This method, unlike diffraction techniques, is applicable to semi-crystalline, amorphous or heterogeneous samples and is therefore advantageous.

In the following chapters chitinous materials from several fungal sources, cultured under varying conditions, are analysed in the whole cell form and after extraction. In order to determine the structure and conformation of these materials they will be analysed by ^{13}C and ^{15}N CPMAS ssNMR and, where appropriate IR, spectroscopy. X-ray powder diffraction studies are commonly reported in the literature for the analysis of the polymorphic form and degree of crystallinity of chitinous materials and provide a reliable method of analysis. However, X-ray powder diffraction was not attempted in this body of work as it requires the chitinous material to be extracted and of a high degree of purity, therefore whole cell samples cannot be analysed in this way. Extraction procedures can feasibly alter the degree of crystallinity of chitin relative to its form *in vivo,* therefore, we required a universal method of analysis that can be performed on extracted chitinous materials and whole fungal cells. As this precludes X-ray diffraction studies the literature pertaining to it will not be extensively reviewed in the following sections, instead greater attention is paid to the use of CPMAS ssNMR techniques. The following sections aim to outline the features of the

spectra that will be analysed in order to gain more information on the polymorphic form and crystallinity of the chitinous materials. The experimental background to CPMAS ssNMR will be briefly reviewed. The application of CPMAS ssNMR to the elucidation of the structure and conformation of chitinous materials will then be discussed, in particular, the use of ssNMR to determine the polymorphic form of chitin. Where applicable IR spectroscopy techniques will also be discussed.

3.8.1 Solid-State NMR Basics

The introduction of new experimental techniques in the past 30 years has dramatically increased the power and applicability of ssNMR for the characterisation of the structure of polymers at a molecular level. As the physical structure of polymers influences the line positions and shapes in the ssNMR spectra, this offers the possibility to obtain information on conformations and polymorphic form of the polymer. Conventional ssNMR spectroscopy provides unresolved spectra with broad lines and a wide set of relaxation phenomena. However, the advent of three main experimental techniques has enabled the use of ssNMR for the analysis of polymers such as chitin. These techniques, which will be outlined in the following paragraphs, are: magic angle spinning (MAS) [201], dipolar decoupling and the cross polarisation technique[202]. The use of these three experimental procedures results a highly resolved spectra of magnetic 'diluted' spins.

Magic angle spinning is employed to average out dipolar interactions and chemical shift anisotropy to yield highly-resolved spectra, this involves spinning the sample at the so-called 'magic angle' of 54.74 °, with respect to the direction of the magnetic field. This is needed because the electronic environment of the nucleus is different in different directions in the molecule, resulting in chemical shift anisotropies that can range from 20 to 200 ppm, producing spectra with extremely broad lines. In liquids the rapid tumbling motions result in isotropic averages, this can be simulated in solids by spinning the sample at the 'magic angle' as the orientation dependent interactions are scaled by a factor of $\frac{1}{2}(3\cos^2\theta - 1)$. At a value of $\theta = 57.74$ this anisotropic part

of the interaction averages to zero and the chemical shift patterns collapse to single lines. Depending upon the ratio of the applied spinning rate to the chemical shift anisotropy, spinning sidebands can occur in the spectrum, which are separated by multiples of the rotation frequency. This is a particular problem for saturated carbons, which have large anisotropies.

The second technique to obtain highly resolved spectra is the dipolar decoupling technique. The strong magnetic dipole-dipole interactions between, for example, ^{13}C and ^{1}H spins lead to line-widths of up to 50kHz. These couplings can be removed by applying a decoupling field of sufficient power at the proton frequency during the acquisition of the carbon signal. However, dipolar decoupling is not always completely efficient. Tanner *et al.* observed asymmetric splitting of the carbonyl and C2 peaks in the ^{13}C CPMAS ssNMR spectrum of β-chitin and state that this splitting arises from ^{13}C-^{14}N dipolar coupling which occurs due to a failure in CPMAS to completely average the dipolar coupling.[37]

The third technique that is required is cross polarisation (CP), which is used to produce a detectable magnetisation of the 'rare' ^{13}C or ^{15}N spins by utilising the increased abundance and better relaxation properties of the ^{1}H spins. In a double resonance experiment a magnetisation transfer from the protons to the carbons or nitrogens to which they are coupled is achieved by simultaneous irradiation of two radio-frequency fields with frequencies ν_H and $\nu_{C/N}$ and field strengths that fulfil the Hartman-Hahn condition ($\gamma_H B_{1H} = \gamma_{C/N} B_{1C/N}$), where B is the field strength and γ the gyromagnetic ratio. The maximum signal enhancement that can be seen is given by the gyromagnetic ratios, carbon, for example, has a maximum signal enhancement of $\gamma_H / \gamma_C = 4$. An additional benefit of the CP technique is that the repetition rate of the process is determined by the much shorter longitudinal relaxation time of the protons (~1 s) and not by the carbon relaxation (10 to 1000 s). Therefore, the experiment time is shortened considerably. Quantitative analysis of CP ssNMR spectra must take into account the dependence of the carbon or nitrogen signal intensities upon the strength of the heteronuclear dipolar coupling, which is dependent upon the number of attached protons and the distance between the spins. Hence, the build up of the signals takes

place at different rates, depending on both the number of protons attached (or close to) a given carbon or nitrogen and on the distance between the $^{13}C/^{15}N$ nucleus and the ^{1}H nuclei. CP is also affected by molecular dynamics. For example, the fast rotation of methyl groups partially averages out the $^{1}H/^{13}C$ dipolar interaction, reducing the CP efficiency.

A general pulse sequence for a 'CPMAS ssNMR with dipolar decoupling' experiment is shown in Figure 3.22. First a ^{1}H 90° pulse is applied, which rotates the magnetisation from the z axis to the y axis. Once this has been achieved a pulse is applied to the y axis in order retain the magnetisation in the y axis, this is called the 'spin-lock' and prevents the decay of the magnetisation in the y axis by relaxation processes. A pulse is then applied on the X-channel (for example ^{13}C or ^{15}N) in such a way that the pulses in the ^{1}H and X channel fulfil the Hartman Hahn condition, ($\gamma_H B_{1H} = \gamma_{C/N} B_{1C/N}$). The time for which these two pulses are on together is known as the contact time, during this time the magnetisation is transferred from the ^{1}H spin to the $^{13}C/^{15}N$ spin. After this time the H-irradiation is extended to decouple the protons whilst X is observed on the other channel. A flip-back pulse is then applied, which is of the opposite phase to the first ^{1}H 90° pulse, this returns to the z direction any magnetisation that remains spin-locked at the end of the acquisition.

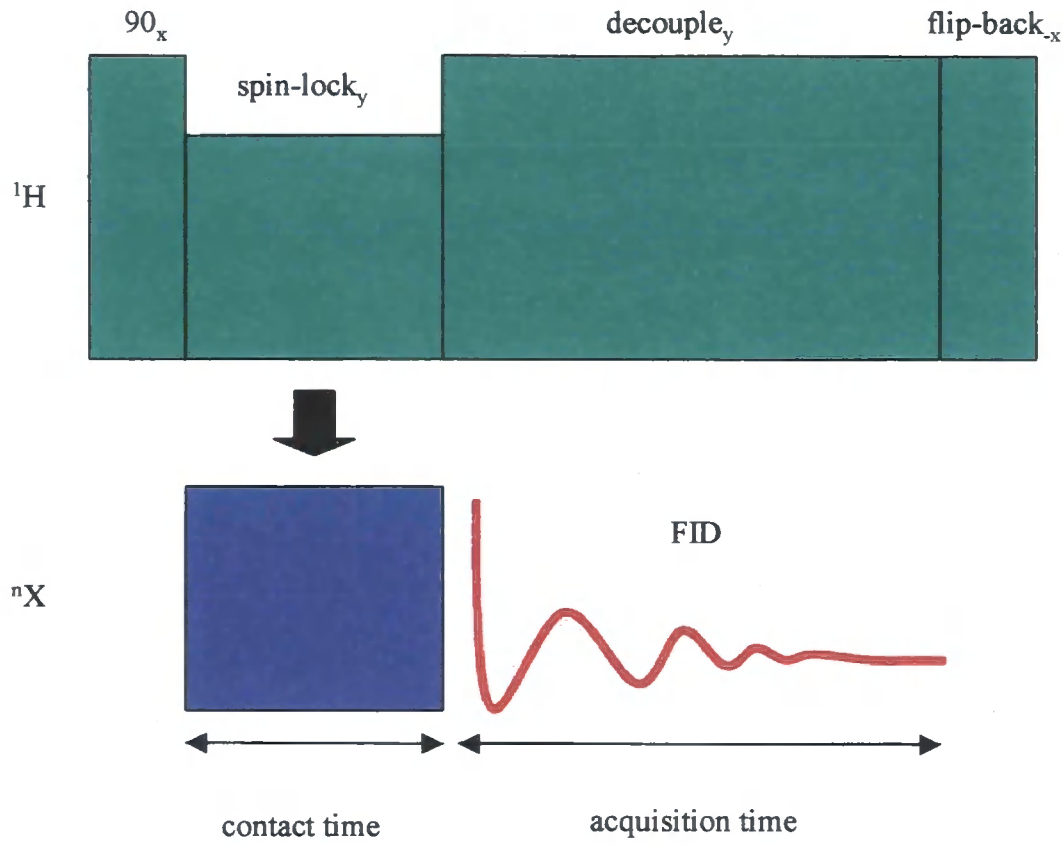


Figure 3.22 - Pulse sequence employed to obtain a ssNMR spectrum using the cross-polarisation technique.

There are several variables that must be optimised in order to obtain good CPMAS ssNMR spectra. The recycle delay, which is defined as the time between the end of the data acquisition from one FID and the start of the next period of radio-frequency excitation, is dependent upon the sample. This delay must be optimised as too long a delay results in wasted spectrometer time. However, if the delay is not long enough the sample may not have returned to its equilibrium state and therefore the maximum signal will not be seen. A sample returns to its equilibrium state at a rate of $1/T_1$, where T_1 is the spin lattice relaxation constant of ^1H. A recycle delay of approximately $5T_1$ is usually required to obtain full signal. This can be shortened by the use of a flip-back pulse, as described above. The contact time is another important variable that must be optimised in order to obtain maximum signal. The rise in observed signal varies according to the cross-polarisation rate, $1/T_{XH}$, which is dependent upon the strength of the coupling between X and H. The strength of this coupling is dependent upon the distance between the nuclei and any molecular motion.

Hence, the build up of the signal takes place at different rates, depending on both the number of attached protons and on the distance between the nuclei. Molecular dynamics also affects CP. For example, the fast rotation of methyl groups partially reduces the ^{1}H-^{13}C dipolar interaction, which in turn reduces the CP efficiency. As different groups will experience different T_{XH}, different contact times give rise to a modification of the relative intensities of the signals. At the same time as the signal build-up, the ^{1}H magnetisation available for CP is reduced through spin lattice relaxation, which causes an eventual decay in the CP signal. Therefore the contact time must be optimised to take these factors into account, it must be longer than the longest T_{XH} and much smaller than the spin lattice relaxation time.

3.8.2 Study of Chitin by ssNMR

The use ^{13}C CPMAS ssNMR to study the structure and conformation of chitin was first reported in the early 1980s and the ^{13}C chemical shifts of chitin/chitosan were assigned.[170, 171] The use of ^{13}C CPMAS ssNMR to study the conformations of chitin and chitosan and their behaviour under deacetylation and hydrolysis conditions has since been reported in the literature.[26, 30, 173, 203, 204] Saito *et al.* reported the use of ^{13}C CPMAS ssNMR to predict the conformation of chitin and chitosan in its solid state, the authors found that any distortion of the hydrogen bonding system may affect the chemical shift of the carbonyl carbon, shifting downfield with increased hydrogen bonding.[170] It has been demonstrated that the chemical shifts of the carbons adjacent to the glycosidic linkages in polysaccharides, C1 and C4 (see Figure 3.23), are very sensitive to the conformation of the glycosidic linkages and can therefore be used to evaluate conformational features.[205] Tanner *et al.* used ^{13}C CPMAS ssNMR to study chitin of varying polymorphic forms and degrees of hydration.[37] The authors demonstrated that the degree of crystallinity of a sample affects the line-widths. This is true of most polymers in CPMAS ssNMR, the more crystalline the material is, the more long-range order the polymer chains in the sample have, the narrower the line-widths will be. In an amorphous sample there is a continuum of orientations, which results in much broader peaks.[172] In studying β-

chitin with varying degrees of hydration, Tanner *et al.* proposed that hydrated samples of chitin have a higher degree of crystallinity and hypothesised that this is a result of associated water, which facilitates chain movement. As CPMAS experiments can result in loss of water due to spinning, this introduces variability in the line-widths produced. They also demonstrated that the ability of the polymer chains to pack in several different ways leads to the appearance of 'extra' peaks and broadening of the peaks in the ^{13}C CPMAS ssNMR spectrum. For example, the methyl peak may present a shoulder. In our ^{13}C CPMAS ssNMR analysis of commercial samples of chitin, shown in Figure 3.23, this shoulder is not seen, indicating a higher degree of order of the polymer chains. The spectra shown in Figure 3.23 will be referred to throughout the following sections in order to illustrate the carbon number strategy used and to identify resonance lines.

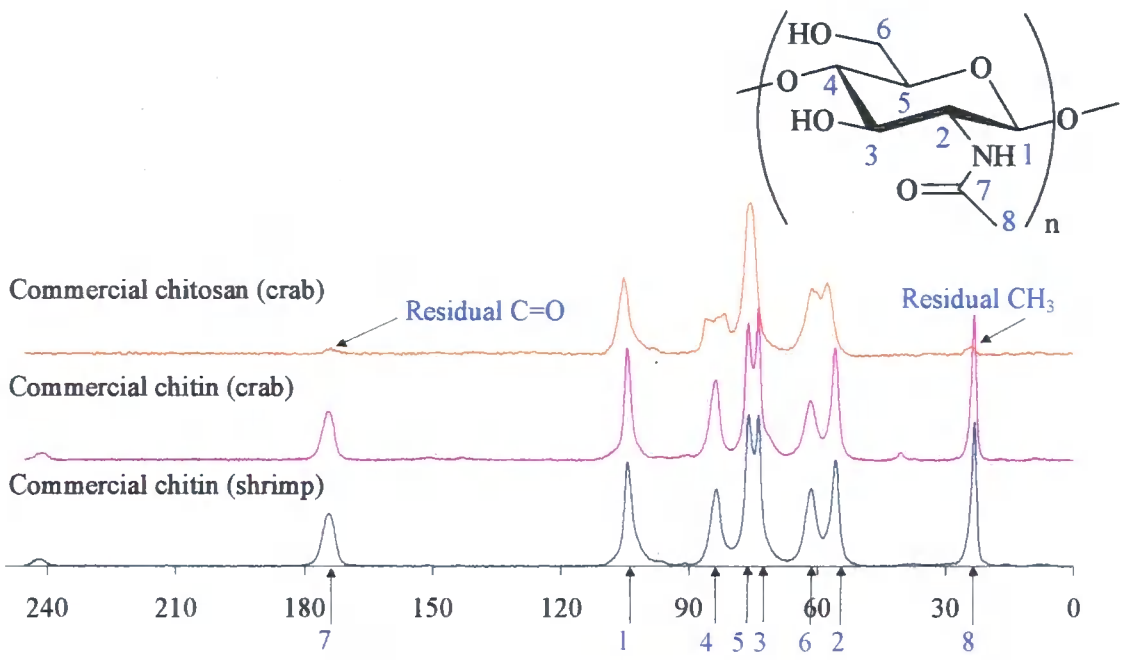


Figure 3.23 - ^{13}C CPMAS ssNMR of commercial sources of chitin and chitosan supplied by Sigma. The source of the chitinous material is shown in brackets.

3.8.2.1 Determination of the Polymorphic Form of Chitin

In nature chitin can assume several polymorphic forms, the most common of which are α and β-chitin.[30, 34, 37] As described in section 1.2, chitin chains associate by hydrogen bonding to form areas of highly ordered crystalline structure. In these areas the chitin chains form sheets that arrange in an anti-parallel, α-chitin, or parallel, β-chitin, manner. α-chitin has a more rigid network of hydrogen bonding which extends in all three directions, this results in a more crystalline and compacted structure. β-chitin has a less rigid framework and so a higher degree of solubility and an ability to swell.[30, 37] The difference in structure has marked affects on the physical properties of chitin, affecting its reactivity, for example its susceptibility to deacetylation.[34, 204]

Much work has been carried out in the literature into elucidating the 3D structure of chitin, with the aim of achieving a better understanding of its role in nature and ultimately the tuning of its physical properties to suit commercial applications.[30, 35, 37, 197, 205] The crystal structures of α and β-chitin have been proposed and reinforced by several authors on the basis of IR spectroscopy[27, 30, 35] and X-ray powder diffraction studies.[27, 29, 35, 206] X-ray diffraction studies showed that the polymorphic forms of chitin differ in the packing and polarities of adjacent chains in successive sheets, as described above.[27] IR studies established differences both in the hydrogen bonding network and the amide I vibrational mode.[29, 30] The generally accepted hydrogen bonding networks present in α- and β-chitin are described more fully in sections 1.3.1 and 1.3.2 and shown in Figure 1.4. The hydrogen bonding network of α-chitin is thought to extend in all three directions. It is proposed there are full intramolecular hydrogen bonds between the $C3_1$-OH and $C5_1$-O, and full intermolecular hydrogen bonds between $C2_1$-NH and $O{=}C_3$. In addition, there is a 50/50 statistical mixture of $O(6_1)H$ to $O{=}C_1$/ $O(6_2)H$ to $O(6_1)$ and $O(6_2)H$ to $O{=}C_2$/$O(6_1)H$ to $O(6_2)$ intra-/intermolecular pairs of hydrogen bonds, that is to say half of the CH_2OH groups form intermolecular hydrogen bonds with the OH-6 groups of the neighbouring chain and the other half form intramolecular with the C=O group

of the next residue along the same chain. Blackwell and Minke state that analysis of the IR spectrum of α-chitin supports this proposed hydrogen bonding structure as the amide I (C=O) band is split, see Figure 3.6. This is due to the two hydrogen-bonding environments in which the amide groups are found. All of the amide groups are involved in intermolecular hydrogen bonds and half are involved in intramolecular bonds.[29] Additionally, the authors report that the IR spectrum predicts that all of the hydroxyl groups form donor hydrogen bonds; which is accounted for in this model. The hydrogen bonding network of β-chitin is thought to contain hydrogen bonds in two directions only, forming intermolecular hydrogen bonds between the $C2_1$-NH and $O{=}C_3$ and between the $C6_1$-OH and $O{=}C_3$. Intramolecular bonds are also formed between the $C3_1$-OH and $C5_1$-O. This structure is confirmed by the presence of a single amide I (C=O) absorption band in the IR spectrum, as the amide groups are now all involved in the same type of hydrogen bonding.

Analysis of the α and β polymorphs of chitin by ^{13}C CPMAS ssNMR has been widely reported in the literature. It is generally reported that the line shape of α-chitin agrees with that of β-chitin with one exception in the ring carbon region (50 - 110 ppm). In the ^{13}CPMAS ssNMR spectrum of α-chitin all 8 peaks, representing the 8 carbons of the repeating unit, are well resolved, however, in β-chitin the C-3 and C-5 resonance lines, at 73 and 75 ppm respectively (see Figure 3.23), overlap to form a single resonance line at 74 ppm.[30, 35, 197, 204, 207] This is probably due to the different hydrogen bonding networks that are present in α and β-chitin. The lack of intrasheet hydrogen bonding between the C6 hydroxyl groups in β-chitin can be seen in the ^{13}C CPMAS ssNMR spectra as the loss of resolution between the C3 and C5 peaks. Kono investigated the structures of α and β-chitin using a series of 2D CPMAS ssNMR experiments, these through-bond ^{13}C-^{13}C and ^{1}H-^{13}C correlation spectra enabled the authors to precisely assign the ^{1}H and ^{13}C chemical shifts of α and β-chitin, with the exception of the OH and NH protons. The authors report that the most noticeable difference between α and β-chitin was observed in the chemical shift of protons attached to C6, α-chitin presenting two ^{1}H resonance lines and β-chitin only one.[197] These findings reinforce the conclusions drawn from X-ray diffraction and IR

spectroscopy investigations, that the C6 hydroxyl group is involved in intrasheet and intersheet hydrogen bonding interactions in α-chitin and intersheet hydrogen bonding alone in β-chitin. Kameda *et al.* studied the hydrogen bonding structure of α-chitin using ^{13}C CPMAS ssNMR, noting the appearance of shoulder peaks on the side of the main peak of the C=O resonance.[34] The authors stated that the peak decomposition indicates the presence of two peaks with similar line-widths which have a splitting width of over 200 Hz, and therefore can not be accounted for by ^{13}C-^{14}N dipolar coupling, which would result in a splitting width of 60 to 70 Hz. The splitting is also independent of the strength of the applied magnetic field. The authors concluded that this splitting does not arise from dipolar coupling and demonstrated that it can not be accounted for by crystalline and amorphous regions of the polymer. They concluded that the major cause of the isotropic shifts of the carbonyl carbon was a difference in hydrogen bonding and that the two distinct signals were derived from carbonyl carbons with two different types of hydrogen bonds. This is in agreement with the structure proposed by Blackwell *et al.*,[29] however, Kameda *et al.* state that the NMR data indicates that 60 % of the carbonyl groups contribute exclusively to intermolecular hydrogen bonding and 40 % of these groups contribute to inter and intramolecular hydrogen bonding. This is in disagreement with Blackwell and Minke who assumed a 1:1 ratio.[29]

Tanner *et al.* advise caution when assigning the polymorphic form of chitins using 1D CPMAS ssNMR experiments. The authors studied a variety of hydrated forms of β-chitin and concluded that the differences observed in the chemical shift of differing polymorphic forms of chitin are of the same order as those between the various hydrate forms of chitin.[37] Therefore, when analysing chitinous materials in this study, we made a tentative assignment of the polymorphic form of chitin using ^{13}C CPMAS ssNMR data, taking the level of hydration of the polymer into consideration. In addition we must also consider the DDA of the chitinous material, as the C3/C5 resolution is also lost on deacetylation chitin to chitosan, (Figure 3.23).

3.8.2.2 Study of Crystallinity

As discussed above chitin contains crystalline and amorphous regions. Chitin chains associate *via* hydrogen bonding to form areas of highly crystalline structure. The degree of crystallinity of the sample is dependent upon several factors, the polymorphic form of chitin, the DDA, the degree of hydration and the polymer chain length. The degree of crystallinity of the sample is influential in determining the physical properties of the material, for example its solubility and susceptibility to deacetylation and hydrolysis.[208] There are several methods of determining how crystalline a sample is, X-ray diffraction is commonly used, however, this method is limited as it can not be used to study materials with low levels of crystallinity. It is also reported in the literature that IR spectroscopy can be used to estimate the crystallinity of biopolymers such as chitin. Hatakeyama *et al.* suggested that the ratio of the intensities of the bands at 1379 and 2900 cm^{-1}, which represent the CH bending with some OH bending contributions and CH stretching bands respectively, can be used as an index of crystallinity for cellulose. [209] The CH stretching band acts as a reference band and the CH bending with OH contribution forms a probe band. The authors suggest that the relative intensity of bands observed for OH-stretching vibrations associated with hydrogen bonds indicates the degree of intermolecular hydrogen bonding, a change in the intensity of this band therefore reflects a conformational change in the material. Focher *et al.* reported that this probe band occurs at approximately 1380 cm^{-1} in chitin and can be used in a similar way to study the degree of order of the material.[210] However, the inherent disadvantages of IR analysis of chitinous materials make accurate identification of absorption bands and their intensities difficult. Chitin's hygroscopic nature compounds the difficulties seen in resolving individual absorbances in the IR spectra. This is discussed further in section 3.6.4.1. We analysed commercial sources of chitin and chitosan (Sigma) in this way and the results suggest absorption ratios of 2.5 and 0.97 respectively. These results must be treated with a degree of caution, however, this does indicate a decrease in order in going from chitin to chitosan, which can be explained by a reduction in the degree of hydrogen bonding.

Another method of assaying the degree of crystallinity of chitinous material is to compare the line-widths of the signals in CPMAS ssNMR spectra. Amorphous samples have an increased number of orientations of functional groups, which leads to slight variations in the chemical shift increasing the overall line-width of the peak. In a sample displaying long range order there are fewer possible orientations, therefore, the lines are narrower. This was demonstrated by Webster *et al.* when they used ^{13}C CPMAS ssNMR to monitor the conformational changes that occur during the deacetylation of chitin.[26] The authors reported that the removal of the bulky acetyl group results in an increase in the number of motional degrees of freedom, which increases the short-range disorder. This can be seen in a general broadening of the peaks in CPMAS ssNMR spectra.[26] Figure 3.23 shows the ^{13}C CPMAS ssNMR spectra of commercial samples of chitin and chitosan. Qualitatively we can see that the chitosan spectra displays greater line-widths than the chitin spectra. It has been reported in the literature that the proton spin lattice relation times (T_{1H}) for chitin are longer than the deacetylated chitosan. This indicates that in chitin, the protons relax in the rotating frame slower, suggesting that the molecular mobility of chitin is lower than in deacetylated chitin. T_{1H} values can therefore be used to monitor structural changes such as a decrease in hydrogen bonding.[173] The line-widths of ^{15}N CPMAS ssNMR can also be used to indicate the degree of crystallinity of a sample.[179]

3.8.2.3 Changes Seen Upon Depolymerisation

^{13}C CPMAS ssNMR has also been used to monitor the conformational or structural changes occurring during the deacetylation and hydrolysis reactions of chitin. It is reported that the sensitivity of the C1 and C4 signals to conformational changes about the glycosidic bond can be used to identify any depolymerisation of the sample.[37] Rajamohanan *et al.* studied the enzymatic hydrolysis of chitin by ^{13}C CPMAS ssNMR, observing that upon hydrolysis all of the sugar backbone carbon peaks experienced an upfield shift of varying magnitudes.[203] The C1 and C4 carbons, which are involved in the glycosidic linkage experienced the greatest shifts of

approximately 11 and 9 ppm respectively, compared to shifts of between 1 and 4 ppm of the C2,3,5,6 peaks. The authors state that the best way of monitoring the hydrolysis of chitin it to monitor the C1 chemical shift, which experienced the greatest shift, from approximately 103 ppm to 92 ppm. In partially hydrolysed chitin samples two C1 peaks were resolved and their relative intensities used to monitor the rate of formation of the monomer.[203] We will employ this technique to observe any possible hydrolysis of chitinous materials occurring during the extraction procedure or deacetylation process.

3.8.2.4 Conclusion - Monitoring the Structure and Conformation of Chitinous Materials Produced in this Study.

^{13}C and ^{15}N CPMAS ssNMR analysis of the chitinous materials produced in this study will be carried out in order to determine the presence of chitin/chitosan and any impurities and the DDA of the sample. In addition, the spectra will also be used to study any conformational differences in the samples. A tentative assignment of the polymorphic form of chitin, providing the DDA is known, will be made by considering the C3 and C5 resonances lines. If these lines can be clearly resolved it will be assumed that the chitinous material is in the α-chitin polymorphic form. The C1 and C4 resonances will also be analysed in order to determine if significant hydrolysis of the glycosidic bonds has taken place. A qualitative, comparative, study of the line-widths of the spectra, in comparison to those of a commercial sample of chitin, will also be used to reveal information about the degree of crystallinity of the samples, as a highly amorphous sample will present larger line-widths.

3.9 Whole Cell ssNMR Analysis

In the following chapters fungal species were cultured under varying conditions in an attempt to improve the accessibility of the chitinous materials for extraction, for example, in the presence of hydrolytic enzymes or additives known to induce

autolysis. In order to study the effect of these conditions on the structure and composition of the fungal cell and the environment in which chitin is found ^{13}C and ^{15}N CPMAS ssNMR analysis of the whole fungal cell was performed. The following section will outline how these experiments were carried out and highlight the salient points in the spectra that were monitored.

In addition to studying chitinous materials that have been extracted and purified from fungal material, ssNMR may be used to analyse the whole fungal cell. For example, we may use the ^{15}N CPMAS ssNMR spectra of the whole fungal cell to determine the DDA. This negates the need for a lengthy extraction procedure, which may have effected deacetylation or depolymerisation of the sample due to the harsh nature of the conditions required. This is discussed in chapter 4. Examination of the line-widths of the ^{15}N and ^{13}C CPMAS ssNMR spectra may also be used to indicate the native degree of crystallinity of the chitinous materials in the fungal cell wall, which may be altered upon extraction. ^{15}N CPMAS ssNMR may also be used to identify the presence of other nitrogen containing components in the cell. Similarly, ^{13}C CPMAS ssNMR of the whole fungal cell may be used to identify polysaccharides or lipids in the cell. Comparative studies of the CPMAS ssNMR spectra of whole fungal cells may be used to determine the effects of change in the culture conditions or growth media on the structure of the fungal cell.

3.9.1 Composition of the Fungal Cell

The composition and structure of the fungal cell is discussed in more detail in section 4.2, in particular the structure of the fungal cell wall as chitin forms its principal structural component. In brief, the basic cellular unit of fungi is described as a hypha, which is usually a tubular cell containing nuclei, mitochondria, ribosomes, golgi and membrane-bound vesicles within a plasma-membrane bound cytoplasm. The sub-cellular structures are supported and organized by micro-tubules and endoplasmic reticulum. The cell is surrounded by a rigid cell wall, which contains chitin. Fungal cell walls are usually composed of fibrillar polysaccharides embedded in a matrix of

amorphous components such as polysaccharides, lipids and proteins. The internal network of stress-bearing polysaccharides, usually comprised of chitin, serve as a scaffold for a dense external layer of glycoproteins. The protein composition of the cell wall depends upon environmental conditions and developmental stage. Proteins are usually comprise 3 to 20% of the fungal cell wall and are typically in the form of glycoproteins. Lipids form a minor component of the cell wall ranging from 1 to 10 %, the presence of sterols, sterol esters, triglycerides, free fatty acids and phospholipids has also been described. We would therefore expect to see characteristic polysaccharide, protein, glycoprotein and lipid peaks in the CPMAS ssNMR spectrum of a fungal cell.

3.9.2 Physical State of the Fungal Cell Wall

It is operationally preferable to sterilise fungal samples before analysing by ssNMR, however, this treatment may affect the fungal cell wall composition and structure. It is also probable that complete dehydration of the fungal cell will affect the structure of the fungal cell. Therefore, in order to ensure that the sterilisation process did not affect the ssNMR spectra produced, we analysed samples of fungal biomass after a series of sterilisation treatments. The P2 strain of *P. chrysogenum* was cultured under normal conditions for eight days, as described in section 7.3.2, After this time the resulting fungal biomass was collected by filtration and separated into four aliquots. One aliquot was sonicated for 40 minutes, one was autoclaved at 121 °C for 15 minutes and one sample was dried in an oven at 95 °C overnight. For comparison, a sample of 'live' fungal biomass was also studied. With the exception of the dried sample, all of the samples were filtered under vacuum and analysed whilst wet.

3.9.3 ^{13}C CPMAS ssNMR Analysis of the Whole Fungal Cell

The ^{13}CPMAS ssNMR spectra of the whole cell samples are shown in Figure 3.24. Comparison of the three spectra obtained from the 'wet' samples to that of the dried

sample demonstrates that drying results in a loss of resolution in the ^{13}C CPMAS ssNMR spectra. Additionally, the line-widths of the dried sample are much greater that those of 'wet' samples. This may be explained by a loss of mobility upon drying, which locks the cellular components into conformations, increasing the heterogeneous nature of the material. As discussed previously, Tanner *et al.* proposed that hydrated samples of chitin have a higher degree of crystallinity and hypothesised that this is a result of the associated water, which facilitates chain movement.[37] As CPMAS experiments can result in loss of water, due to spinning, this introduces the possibility of variability in the line-widths produced. Therefore if wet samples are to be used the spinning rate and acquisition time must be constant to minimise this effect. Comparison of the three 'wet' samples indicates that no significant change can be seen by ^{13}C CPMAS ssNMR when samples are sterilised as the line-widths, resolution and chemical shifts of the peaks do not vary. Therefore, all future fungal samples will be sterilised by autoclaving, as this is the simplest and most reproducible method of sterilisation, and analysed 'wet'.

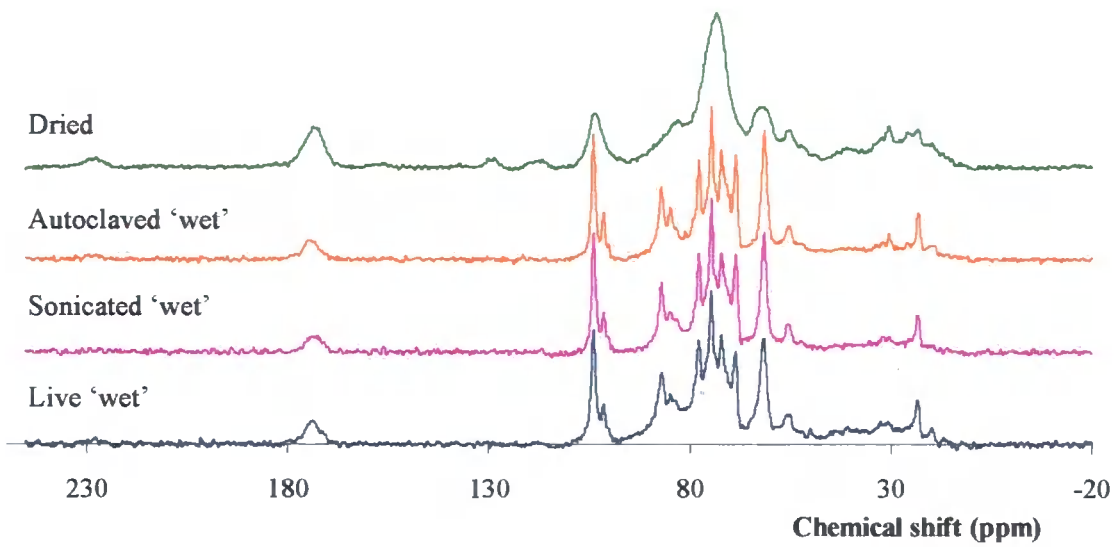


Figure 3.24 - ^{13}C CPMAS ssNMR spectra of whole cell samples of the P2 strain of *P. chrysogenum* analysed live and after sterilisation by sonication, autoclaving and drying.

The ^{13}C CPMAS ssNMR spectra of the whole fungal cell analysed 'wet' after sterilisation by autoclaving is shown in Figure 3.25 in comparison to the spectra of the

chitinous material extracted by the procedure described in section 7.4.6. The complex mixture of carbohydrates, proteins and lipids present in the fungal cell can be seen in the ^{13}C CPMAS ssNMR spectra. The presence of polysaccharides, other than chitin, is indicated by the additional peaks present in the chemical shift region of 50 to 120 ppm, in particular the additional peak at approximately 101 ppm which is characteristic of an anomeric carbon. There are notable additional peaks in the region of 0 to 50 ppm in the spectra of the whole fungal cell, which may be accounted for the presence of lipids or amino acid side chains.

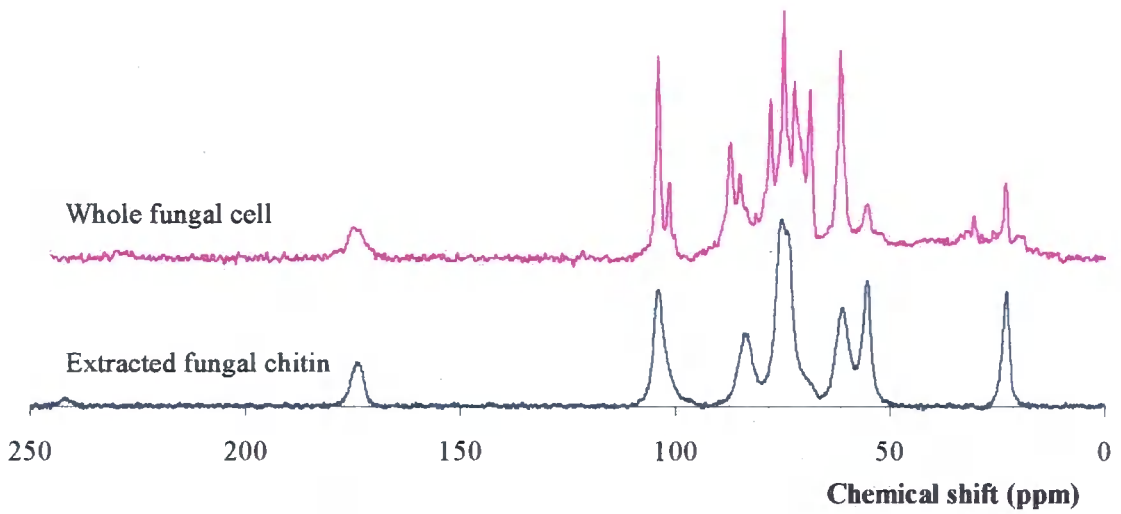


Figure 3.25 - ^{13}C CPMAS ssNMR spectra of the whole fungal cell analysed 'wet' after autoclaving and the chitinous material extracted as described in section 7.4.6.

In order to further investigate the effects of acquiring ssNMR of 'wet' samples, ^{13}C MAS ssNMR spectra of autoclaved samples acquired using cross-polarisation and direct-polarisation were compared, see Figure 3.26. As previously described ^{15}N and ^{13}C ssNMR spectra are normally acquired using the cross-polarisation technique, which involves the transfer of magnetisation from abundant ^{1}H to ^{13}C or ^{15}N. This technique is dependent upon the heteronuclear dipolar coupling interactions, which may be inefficient in highly mobile species. Ha *et al.* reported that in their studies of *Allium* cell walls highly mobile polysaccharides of hydrated cell walls were not visible in CPMAS ssNMR experiments using normal contact times.[211] Similarly Fenwick *et al.* discussed the effect of the mobility of polymers in tomatoes on the CP

kinetics.[212] The inability of ^{13}C CPMAS ssNMR experiments to visualise mobile components is explained by the decreasing rate of cross-polarisation that occurs with increasing mobility. Highly mobile species will have reduced spin-lattice relaxation times and the fast phase of cross-polarisation from covalently bonded protons will be eliminated by motion, therefore, transfer of the magnetisation to the $^{13}C/^{15}N$ is inefficient.[213, 214] Direct-polarisation experiments apply the pulse directly to the nucleus to be studied, as such they are independent of the heteronuclear dipolar interaction. Therefore, DP techniques reveal the more mobile constituents of the fungal cell, which are not seen in the CP experiment.

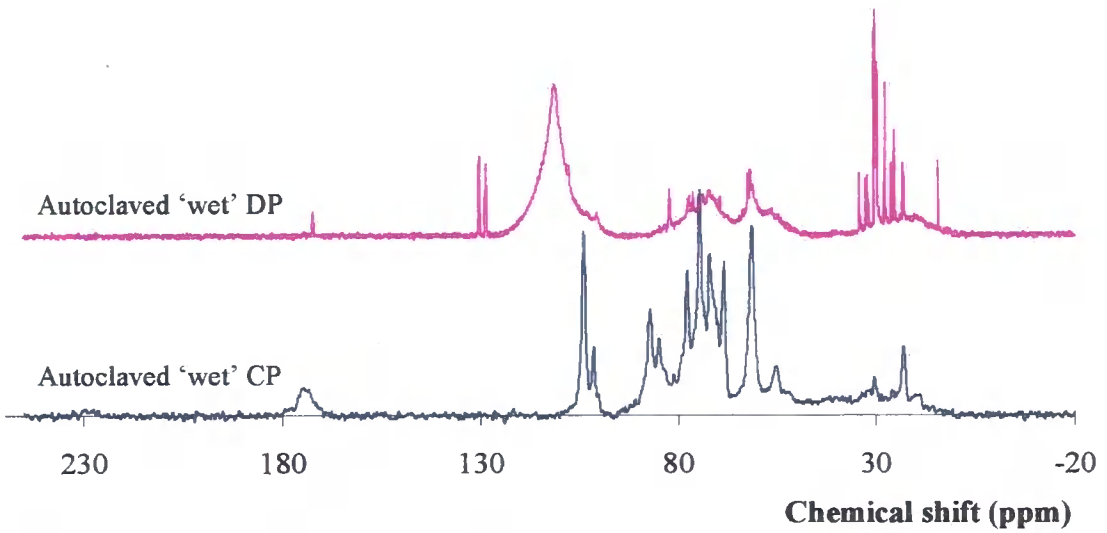


Figure 3.26 - ^{13}C MAS ssNMR spectra of autoclaved 'wet' whole cell samples of *P. chrysogenum* obtained using cross-polarisation (CP) and direct-polarisation (DP) techniques

Comparing the DP and CP spectra in Figure 3.26 we can see that the spectra vary greatly. The large, broad, peak at approximately 100 ppm in the DP spectrum is a result of the experimental equipment and can be ignored. The peaks seen in the chemical shift range of 0 to 40 ppm in the DP spectrum are typical of N-terminus CH_3 groups and aliphatic carbons, the DP spectrum also displays two distinctive alkenic peaks at 128 and 130 ppm and a carbonyl peak at 172 ppm. This spectrum is typical of that of a fatty acid and may be accounted for by mobile lipid components of the fungal cell. These peaks cannot be resolved in the CP spectrum. The characteristic polysaccharide peaks as well as the carbonyl peak of chitin seen in the CP spectrum

are not seen in the DP spectrum as these components form the more rigid components of the fungal cell. The DP spectrum is acquired with a limited recycle time, so that rigid components which have a T_1 significantly greater than the recycle time are not observed, but mobile components with short T_1 times are observed. As chitin forms the major structural component of the fungal cell wall and exists in a rigid network of hydrogen bonds, β-glucans and glycoproteins covalently attached to this chitinous matrix will have an imparted rigidity, these components will not be seen in a DP experiment. As we are interested in the environment and matrix of interactions in which chitin is found, as well as its structure and degree of crystallinity, we will employ CPMAS ssNMR for the study of the fungal whole cell in future studies.

3.9.4 ^{15}N CPMAS ssNMR Analysis of the Whole Fungal Cell

The ^{15}N CPMAS ssNMR spectra of the live and sterilised whole cell samples are shown in Figure 3.27. The spectra follow the same trend as the ^{13}C CPMAS ssNMR in that the three 'wet' samples exhibit much narrower line-widths than the dried sample. As discussed in section 3.9.3, this may be explained by a loss of mobility which occurs upon drying. In addition to an increase in line-widths, the spectrum of the dried sample displays additional peaks, which are not present in the 'wet' samples. The appearance of these peaks may be explained by consideration of the mobility of the cellular components. As discussed in section 3.9.3 the more mobile components of the fungal cell in hydrated samples will not be visualised by CPMAS ssNMR techniques. Drying the sample may reduce the mobility of these components, increasing the efficiency of the CP process. Dipolar dephasing experiments, which remove the signals from nitrogens directly bonded to a proton, were carried out and indicate that the peaks at approximately –295, –308, –332 and –358 ppm are protonated nitrogens whereas the peaks at approximately –209, –338 and –357 ppm are tertiary nitrogens. A contact time array was also carried out, varying the contact time used to acquire the spectra. As discussed in section 3.8.1, the contact time required is dependent upon the nature of the group being analysed. The contact time array indicated that the peaks form three distinct groups, indicating that there are three

different components to the cell, which may have different degrees of crystallinity or mobility. Analysing the peak at –258 ppm, which represents the amide nitrogen of chitin, more closely we can see that in the dried sample it is a composite of three different signals. This could suggest the presence of chitin in different conformations, or with different degrees of hydrogen bonding. Alternatively the peaks could indicate the presence of peptides or other amides, which have chemical shifts in the same region. The small differences in chemical shift and similar behaviour in the contact array and dipolar dephasing experiments indicate that these three signals all have a similar nature, suggesting the presence of chitin in different environments. Examination of the chemical shifts of the other peaks indicates the possible presence of amines, –330 to –360 ppm, nucleosides and nucleotides, –140 to –240 and –280 to –305 ppm and guanidinium nitrogens, –280 to –320 ppm.

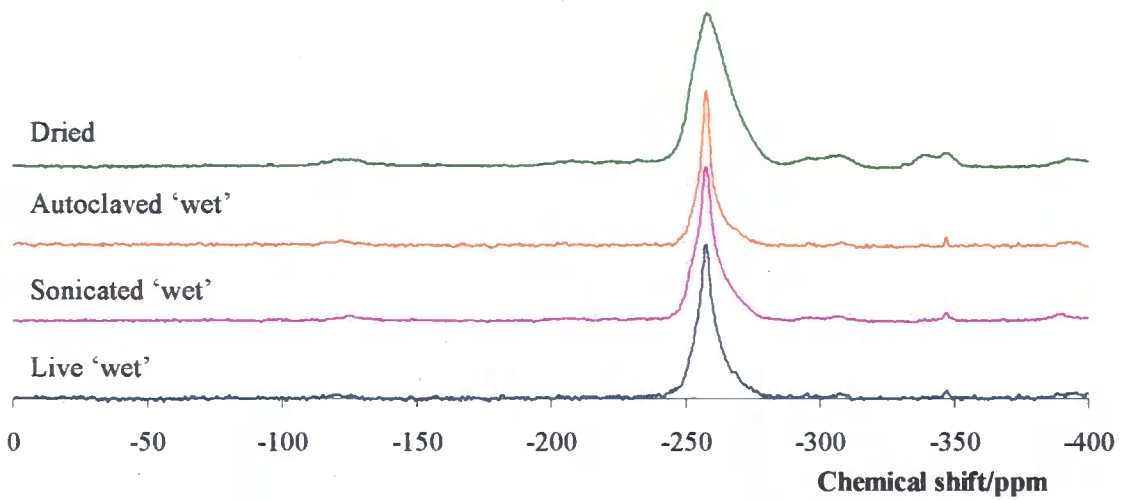


Figure 3.27 - ^{15}N CPMAS ssNMR spectra of whole cell samples of the P2 strain of *P. chrysogenum* analysed live and after sterilisation by sonication, autoclaving and drying.

In choosing to analyse samples of the whole fungal cell by ^{15}N CPMAS ssNMR in the 'wet' or dried state we must consider what we wish to observe. If the aim is to determine the DDA of the material or the degree of crystallinity of the chitinous components of the fungal cell wall then it is advantageous to study the whole cell 'wet' as the interference from other nitrogen containing components is avoided. We can also study the native crystallinity of the chitinous material in the fungal cell, which may be more reliable as the dried sample will give artificially low crystallinity

results. However, if we wish to study the other constituents of the fungal cell it is essential to dry the sample first in order to visualise these mobile components. This thesis focuses on the chitinous components of the fungal cell, therefore in subsequent studies samples were analysed 'wet'. Comparison of the spectra of the samples analysed 'wet' indicates that no change was seen when the samples were sterilised by sonication or autoclaving, therefore, in subsequent experiments all samples were autoclaved at 121 °C for 15 minutes before analysis as this is the simplest and most reproducible method of sterilisation.

The ^{15}N CPMAS ssNMR spectra of a whole cell sample that had been sterilised by autoclaving and analysed 'wet' is shown in comparison to the chitinous material extracted from the fungal biomass by the method described in section 7.4.6 in Figure 3.28. The major difference between the two spectra is the line-width of the chitin, amide, peak at – 258 ppm, which has decreased significantly upon extraction. This may indicate an increasing degree of crystallinity in the extracted sample. As discussed above, in the whole fungal cell the chitin amide peak in the ^{15}N CPMAS ssNMR spectrum is present as a composite of three peaks, this may be due to the presence of chitin in differing hydrogen bonding networks or the presence of another amide of peptone at a similar chemical shift. The narrowing of the line-width upon extraction may be explained by the removal of an impurity peak, or by chitin being present in a single conformation. During the purification process chitin is precipitated from cold HCl, it is probable than upon precipitation chitin will form its most stable polymorphic form, α-chitin, with a high degree of hydrogen-bonding. This could account for the increase in crystallinity.

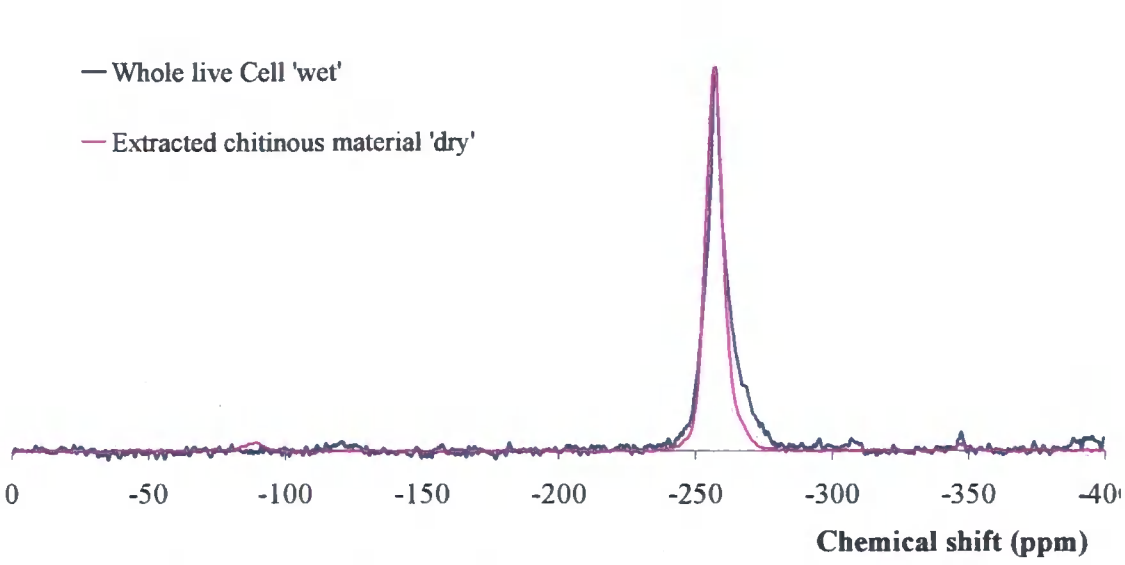


Figure 3.28 - ^{15}N CPMAS ssNMR of the 'wet' whole cell of *P. chrysogenum* and the chitinous material extracted from the fungal biomass by the procedure described in section 7.4.6.

3.10 Conclusion

Analysis of chitinous materials is hampered by their inherent insolubility and hygroscopic nature, which preclude the use of many standard analytical techniques. This has resulted in little literature agreement upon absolute methods of analysis of chitin and chitosan. In order to evaluate the effect of growth and extraction conditions on the chitinous materials produced in this study the aim of this chapter was to define a series of methods of analysis suitable for chitinous materials from fungal sources. These protocols aim to determine the DDA, the polymorphic form, degree of crystallinity and level of purity of the extracted chitinous materials.

The use of elemental analysis to determine the ratio of carbon to nitrogen content of chitinous samples was shown to be an indication of the level of purity of the sample, providing the DDA is known. However, in order to obtain a qualitative determination of the amount of chitin in a sample, it must be fully deacetylated and hydrolysed to form glucosamine, which can then be assayed by the colorimetric method of Tsuji *et al.*[131] This procedure was shown to be reliable for concentrations of glucosamine

between 0.01 and 10 mg/ml and produced consistently accurate results with commercial samples of chitin.

Evaluation of the available literature procedures for the determination of the DDA of chitinous materials highlights the extensive disagreement over absolute methods of analysis. In our hands the most reliable method of determination of the DDA of chitinous materials from fungal sources is ^{15}N CPMAS ssNMR as this technique does not require the sample to be derivatised, solubilised or extensively dried. Additionally, the presence of polysaccharide impurities, common in chitinous materials extracted from fungal sources, does not affect the analysis. Importantly, unlike most methods of determination of the DDA ^{15}N CPMAS ssNMR considers both the concentration of amine and amino groups and is applicable to a wide range of DDA. The low natural abundance of ^{15}N does result in long acquisition times for ^{15}N CPMAS ssNMR, however, this may be avoided by ^{15}N labelling the chitinous material. This is discussed further in chapter 5.

Methods of determining the polymorphic form and degree of crystallinity of chitinous samples were also investigated. X-ray diffraction studies were not attempted as these are time consuming and not applicable to poorly crystalline materials. IR spectroscopy has been proposed for the determination of the polymorphic form of chitin, however, the hygroscopic nature of chitin precludes this. ^{13}C CPMAS ssNMR provides a simple method of determining the polymorphic form of the chitinous material, providing the DDA is known, as the C3 and C5 resonance lines are well resolved in α-chitin but form a single peak in β-chitin. Additionally, the line-widths of the ^{15}N and ^{13}C CPMAS ssNMR spectra may be used to comparatively assess the degree of crystallinity of chitinous samples, as amorphous samples present broad lines due to the increased number of orientations of the functional groups. Analysis of the chemical shift of the anomeric carbon in ^{13}C CPMAS ssNMR may also be used to determine if extensive degradation has occurred as the chemical shift of the monomeric and polymeric unit are separated by approximately 10 ppm.

GPC analysis of chitin in 5 % LiCl:DMAc has been reported in the literature for the determination of the molar mass averages and molar mass distribution. However, we concluded that chitin could not be sufficiently solubilised in this solvent system. As the solubility of polymers is determined by the molecular weight, GPC analysis of the chitin that does dissolve will not be representative of the whole sample. Additionally, no universal solvent system for the GPC analysis of chitinous materials with a wide range of DDA has been identified. Therefore the molecular weight of chitin and chitosan can not be reliably compared.

$^{13}C/^{15}N$ CPMAS ssNMR analysis of the whole fungal cell was investigated. It was determined that autoclaving fungal samples did not affect chemical shifts, line-widths or resolution of the acquired spectra. However, drying the samples prior to analysis reduces the resolution seen in the ^{13}C CPMAS ssNMR spectra and results in broad peaks. Drying also resulted in the appearance of additional peaks in the ^{15}N CPMAS ssNMR. It was concluded that this was due to an increased level of mobility in hydrated samples, which reduces the efficiency of the CP process so more mobile components of the fungal cell wall are not seen in the spectra. This was verified by the comparison of CP and DP MAS ssNMR spectra. We concluded that ^{15}N and ^{13}C CPMAS ssNMR should be carried out on 'wet' autoclaved samples. The spectra may be used to evaluate the degree of crystallinity and DDA of chitinous materials in the fungal cell wall as the harsh chemical extraction procedures may affect the nature of the chitinous material. Additionally, these techniques may be used to monitor the environment in which chitin is found and the effect of changes in the nutrient compositions, such as the addition of hydrolytic enzymes and additives known to induce autolysis.

All chitinous materials extracted and purified in this study will be analysed by elemental analysis to determine the level of purity. In order to obtain a quantitative determination of the chitin content they will be hydrolysed and deacetylated to form glucosamine, which will be assayed by the colorimetric assay of Tsuji.[131] They will also be analysed by ^{15}N CPMAS ssNMR to determine the DDA, along with ^{13}C CPMAS ssNMR analysis, this will also be used to investigate the polymorphic form

and degree of crystallinity of the chitinous samples. GPC analysis of the materials in 5 % LiCl:DMAc will be performed to comparatively, qualitatively, assess the effect of extraction and deacetylation on the molecular weight. However, as discussed above the inherent issues with this method of analysis limits its reliability. Whole cell ^{15}N and ^{13}C CPMAS ssNMR analysis of fungal cultures will be performed to evaluate the native crystallinity and DDA of chitinous materials. These spectra were also used to evaluate any changes occurring upon variation of the nutrient conditions in the fungal cell that may affect the accessibility of chitin to extraction.

Chapter 4 - Extraction Procedures

4.1 Introduction

The main objective of this thesis was to investigate efficient procedures for the extraction of the chitinous material present in the residual biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* in the penicillin manufacturing industry. This material, which is currently a waste product, contains approximately 5 % (w/w) chitin in the cell wall. As described in Chapter 1, the current commercial source of chitinous materials are the waste products of the seafood processing industry. However, fermentable fungi represent a major alternative source of chitin which offers the benefits of a non-seasonal supply and requires a less severe extraction procedure resulting in a more homogenous product. However, the current extraction procedures, whilst cheap and resulting in reasonable overall recoveries, do result in degradation of chitin quality and are potentially polluting to the environment. There is therefore potentially great utility in developing new extraction methods for isolating chitin/chitosan from *Penicillium* biomass that avoid or minimise the harsh chemical treatments and produce a reliable and uniform chitin/chitosan product of high quality.

In order to assess a suitable procedure for the efficient extraction of pure chitinous material, with minimal degradation to the polymer chain, the source and matrix from which chitin or chitosan is to be extracted must be considered. Elucidation of the interactions in which chitin is involved and the identification of other components present in the source not only enable the design of the extraction procedure, but also the identification of any impurities in the final product. This thesis has focused upon the optimised extraction of chitin from the cell wall of fungal sources, therefore, in the following sections the role of chitin in fungi and the composition of the fungal cell wall are discussed. Traditional chemical extraction procedures and alternative enzymatic strategies are reviewed and the application of representative chemical extraction procedures to the biomass remaining from the large-scale fermentation of

Penicillium chrysogenum in the penicillin manufacturing industry is discussed. The characteristics, such as the level of purity and degree of deacetylation (DDA), of the resulting chitinous materials are established. These materials form a datum against which the materials extracted from all alternative extraction procedures are compared. As we were unable to extract chitin of a high level of purity from the dried biomass, the P2 strain of *P. chrysogenum* was cultured and the chemical and enzymatic extraction procedures were applied to the resultant, wet, fungal biomass. The culture conditions employed and the quality of the extracted materials are outlined. In order to assess the suitability of this source of chitin, the extracted materials were compared to those extracted from two other fungi that have been described as sources for chitin in the literature, *Aspergillus niger* and *Mucor rouxii*.

4.2 Fungal Cell Walls

Fungal cells depend upon an extracellular structure, the cell wall, to provide skeletal support whilst at the same time allowing interaction between the cell and the surrounding environment. The fungal cell wall is essential for maintaining the osmotic balance of the cell and for morphogenesis. The integrity of the structure is essential for the cell's survival in hostile environments, and fungal cells devoid of this wall, protoplasts, can not survive. The cell wall can also provide resistance to enzymatic attack and is responsible for the variety of shapes adopted by fungi, enabling them to adapt to their environment and the different functions required of them during their life span. Aside from rigidity, the cell wall is also required to convey information to the protoplast about the extracellular environment. It is the structure, which in response to environmental alterations, permits the cell to regulate its rate of growth and to respond to positive or negative tropic stimuli. Cell walls can also accumulate enzymes that digest impermeable compounds such as polysaccharides or proteins into smaller products that can penetrate the cell barrier in order to be further metabolized. Sugar moieties and proteins in the cell wall are also responsible for cell-to-cell recognition, which governs many interactions.

The fungal cell wall is a complex structure that has a species-specific composition, which may alter depending upon the phase of the cell cycle, environmental conditions and the developmental stage of the fungi.[215] Three main groups of polysaccharides form the cell wall, chitin accounts for approximately 2-10 % of the cell wall dry mass, β-(1→3) and (1→6) glucans form approximately 50-60 % of the cell wall dry mass and mannoproteins account for approximately 30-40 % of the cell wall dry mass.[216] The exact levels of each component is, to some extent, dependent upon the growth conditions, for example the nature of the nutrient source, pH, temperature and mode of cell cultivation.[113, 216] The general structure of the fungal cell wall is described in two layers, an inner layer of structural polysaccharides which are linked *via* β-(1→6)-glucans to an outer surface of densely packed mannoproteins which are extensively *O*- and *N*-glycosylated.[215, 217]

The mechanical strength of the wall is mainly due to the inner layer of the cell wall, which has been compared to that of man-made materials such as reinforced concrete.[208] Reinforced concrete gains its resistance to tension from the iron rods immersed in concrete, with the concrete providing resistance to compressions. In a similar way inner fungal cell walls are comprised of fibrillar polysaccharides embedded in a matrix of amorphous components such as polysaccharides, lipids and proteins. The fibrous polymers constitute the structural skeleton of the cell wall and the matricial gel-like polymers act as interconnecting molecules. The fibrillar structure arises from multiple sugar chains that associate to form microfibrils, which then in turn associate to form larger fibrils. This structure imparts rigidity on the cell wall, which is essential in order to protect the protoplast from the harmful extracellular environment. The fibrillar components are highly insoluble polysaccharides, predominantly chitin and β-glucan complexes.[217, 218] Chitin forms the principle structural component due to its inherent insolubility and high tensile strength. The presence of covalent linkages between β-glucan and chitin was first proposed by Sietsma *et al*;[90] the authors proposed that β-glucans were covalently linked to chitin *via* amino acids and this covalent linkage imparted the increased insolubility characteristics observed in β-glucans in fungal cell walls. Wessels *et al.* later provided evidence that the inter-polymer linkage in chitin-glucan

complexes involves a Schiff base link between free amino groups of deacetylated units in chitin and the reducing end of the glucan chains.[219] However, more recent studies suggest the presence of direct covalent linkages between chitin and highly branched β-(1→3)-glucan chains *via* a β-(1→4) linkage to the non-reducing end of β-(1→3)-glucan side chains.[216, 220, 221]

The outer layer of the cell wall consists of heavily glycosylated mannoproteins that emanate from the cell surface. These components are extremely important as they bear the most significant antigenic determinants of fungal cells and, as such, are involved in cell-cell recognition events. The outer layer also limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes such as cell-wall degrading enzymes. The carbohydrate side chains of the cell surface proteins contain multiple phosphodiester bridges, resulting in numerous negative charges at the cell surface at physiological pH. These side chains are responsible for the hydrophilic properties of the wall and may be involved in water retention and drought prevention. There are two main classes of cell wall proteins that are covalently coupled to cell wall polysaccharides, glycosyl phophotidylinositol (GPI)-anchored cell wall proteins are linked *via* their C-terminus to β-(1→3)-glucan through a connecting β-(1→6)-glucan moiety.[215] The second class of cell wall proteins are covalently O-linked directly through a serine or threonine residue to the reducing end of β-(1→3)-glucan.[216] A schematic of the proposed fungal cell wall structure is shown in Figure 4.29.

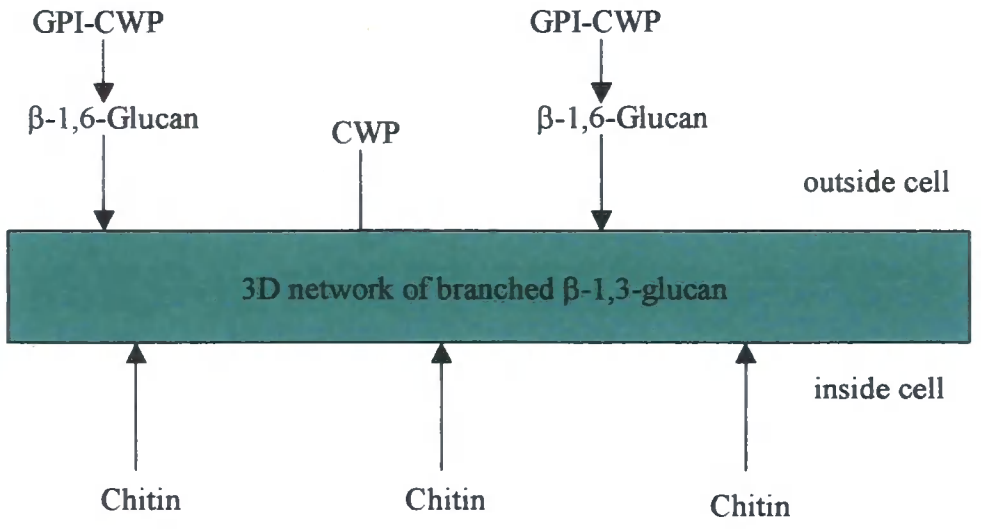


Figure 4.29 - Proposed molecular organisation of fungal cell wall. The arrows indicate that the terminal non-reducing ends of β-1,3-glucan side chains function as acceptor sites for β-1,6-glucan and chitin. The reducing end of β-1,3-glucan may be involved in linkages to cell wall proteins (CWP). GPI-anchored cell wall proteins (GPI-CWP) are tethered to the β-1,3-glucan network *via* β-1,6-glucan linkages.

Lipids form a minor component of the cell wall ranging from 1 to 10 %, the presence of sterols, sterol esters, triglycerides, free fatty acids and phospholipids has also been described, however, little is known of their roles in the cell wall. It is possible they play a role in protecting the walls from drying.[92] All other components are present in minor, variable, amounts. Certain fungal species possess pigments especially during the early developmental stages, most possess inorganic salts, typically phosphates, in small amounts.

4.3 Chitosan in Fungal Cell Walls

While it is probable that incompletely acetylated chitin exists in most fungi, the highly deacetylated form, chitosan, is less common. However, it is a characteristic component of the cell walls of Zygomycetes where its incorporation can exceed that of chitin.[9] The extraction of chitosan from the mycelia of various fungi has been reported in the literature, including *Mucor, Absifia, Rhizopus and Gongronella*.[101, 107, 222, 223] Chitosan is easily hydrated and the interchain hydrogen bonding is weaker than that of chitin indicating that it does not play a structural role and may

form the matricial component of the fungal cell wall. It has been hypothesised that chitosan protects chitin from hydrolytic attack by chitinases.[4] It also may accumulate different ionic substances through salt formation or fulfil the role that glucans perform in the matrix of the cell walls of other fungi. Nwe *et al.* proposed that chitosan is covalently linked to β-glucans *via* an α-(1→4) glucosidic bond as the chitosan/glucan complex can be cleaved by the heat-stable α-amylase.[94]

4.4 Extraction Procedures

It is generally accepted that chitin and chitosan do not appear in nature as discrete substances, but in fact occur in complexes with other substances. As described above chitin forms a structural component of fungal cell walls and is found in a matrix of interactions with other structural polysaccharides and mannoproteins. Although the exact nature of the linkages is contentious, it is generally accepted that chitin is covalently linked to a network of β-(1→3) and (1→6)-glucans, which are in turn covalently linked to a network of glycoproteins. Chitosan is present in only one fungal taxa, where it is also covalently linked to the β-glucan network. Therefore, in order to extract pure chitinous material this network of glycoproteins and β-glucans must be removed. In extracting chitinous materials care must be taken to ensure that the polymers are not degraded by either depolymerisation or deacetylatyion as the end use of the materials usually requires a high degree of homogeneity. The traditional chemical extraction procedures, described in the following section, are frequently harsh and may result in degradation of the materials, therefore, alternative enzymatic extraction procedures have been investigated. These procedures, along with deacetylation strategies to produce chitosan, are reviewed here.

4.4.1 Traditional Chemical Extraction Procedures

Various chemical extraction procedures have been reported in the literature and although no standard process has been developed, the procedures do follow a general

pattern. The design of the extraction procedure is dependent upon whether chitin or chitosan is to be extracted as these materials behave differently. For all fungal sources, and independent of chitin or chitosan extraction, the initial step is an alkali treatment. This deproteinisation step breaks down the cell wall removing the glycoproteins, outer-shell and matricial components of the cell. The residual 'alkali insoluble material' (AIM) contains the skeletal structure of the cell wall, principally chitinous materials and covalently linked β-glucans.[221] A wide range of reagents have been used for this deproteinisation step, however, NaOH is the preferred alkali in the literature, the concentration, temperature and duration of the treatment varying widely.[87] White *et al.* described the extraction of chitinous material from fungal sources in 1979, the authors used an initial alkali step involving the homogenisation of the biomass, by blending at high speed, followed by treatment with a mixture of 1 M NaOH and 95 % ethanol (1:2) at a ratio of 1:10 (w:v) of dry biomass to NaOH/EtOH in steam bath conditions for 90 minutes.[224] Subsequently Rane and Hoover reported an 'optimisation' of the alkali treatment, proposing that the most efficient method of separating the alkali insoluble fraction is to autoclave the homogenised cell in 1 M NaOH for 15 minutes at 121 °C with a 1:40 (w:v) ratio of dry cell mass to alkali solution.[224] This procedure has been employed by many authors, with variations in the concentration of NaOH, ratio of solid to alkali and reaction time.[101, 222, 223, 225] As harsh alkali conditions may result in the deacetylation of chitinous materials, see section 4.4.1.2, milder alkali treatments have also been proposed in the literature. Synowiecki *et al.* report efficient deproteinisation of *Mucor rouxii* in 2 % (w/v) NaOH solution at 90 °C for 2 hours at a ratio of 1:30 (w:v) of alkali solution to dry cell biomass.[103] This procedure has also been extensively utilised and reported with minor variations, in the literature.[102, 104, 107, 115] In order to obtain homogenous samples of chitin or chitosan that have not been extensively deacetylated or depolymerised, milder extraction conditions are preferred. Therefore, for the purposes of this study chemical extraction procedures based upon the milder conditions of Synowiecki *et al.* will be considered.

Once the AIM has been extracted from the fungal biomass the extraction procedure varies depending on whether chitin or chitosan is required. Native chitosan and chitin

in the AIM may be separated by an acid extraction step, which yields acid soluble chitosan and acid insoluble chitin.[103] The chitin fraction may then be purified to remove remaining β-glucans and, if required, subsequently deacetylated. Alternatively, the AIM may be treated with strong alkali at high temperatures to effect deacetylation of chitin, producing acid soluble chitosan, which may then be selectively extracted.[102, 115] The generalised extraction procedure for chitinous materials from fungal sources is shown schematically in Figure 4.30. For ease we will discuss the acid extraction step here and deacetylation strategies separately in section 4.4.1.2.

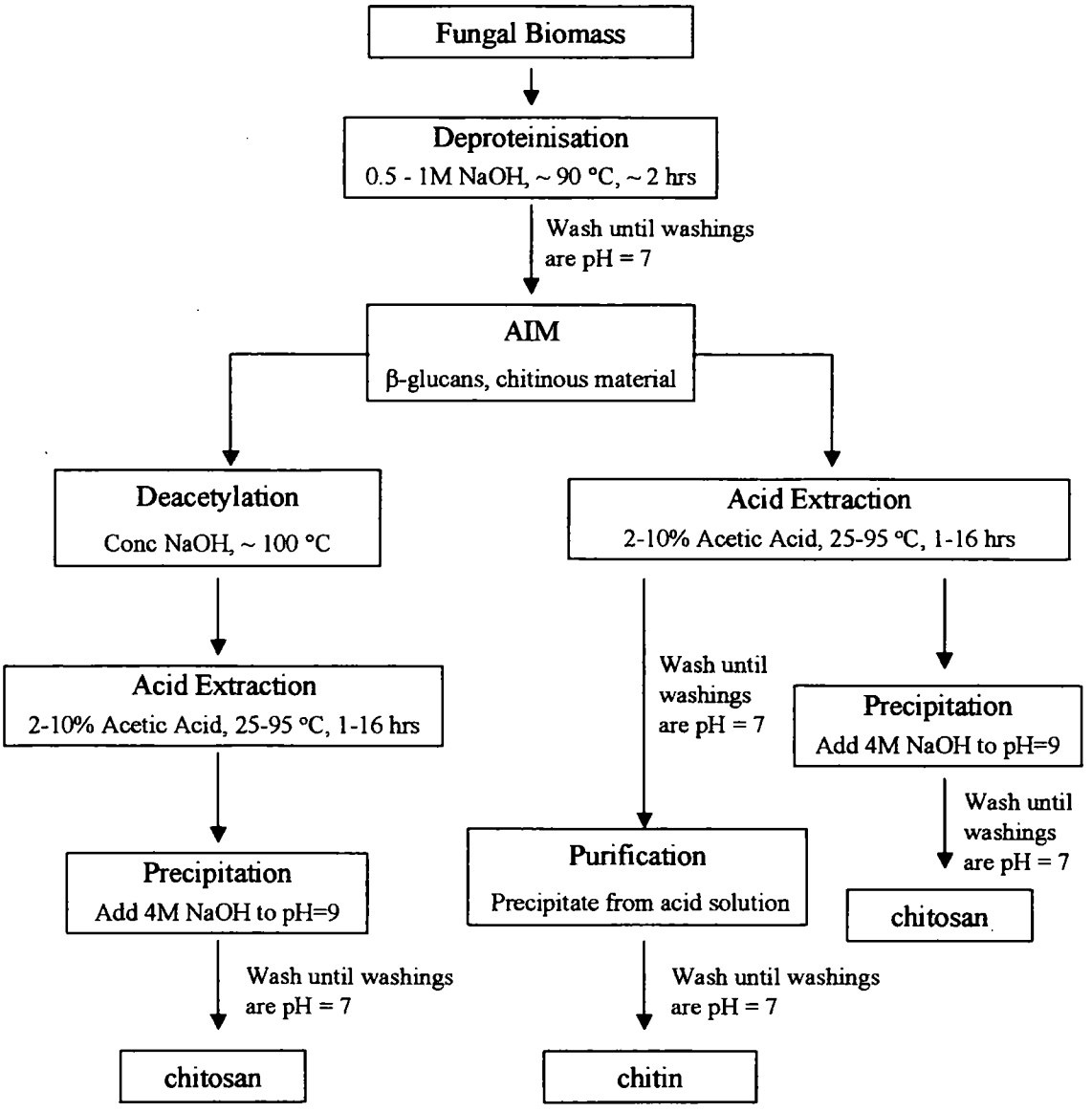


Figure 4.30 - Schematic of the general chemical extraction for chitin and chitosan from fungal sources. AIM - Alkali insoluble material

The acid extraction step involves the use of weak organic acids to solubilise chitosan, and other remaining polysaccharides that are not strongly bound to chitin, from the AIM. This results in an acid insoluble fraction containing chitin and an acid soluble fraction containing chitosan. Care must be taken to ensure that degradation of the chitinous polymer chains does not occur, as the glycosidic linkages are susceptible to acid hydrolysis. Therefore, the reaction conditions are a compromise between temperature, reaction time and acid concentration. The use of acetic acid is most frequently described in the literature at concentrations between 2 and 10 % (v/v) and at temperatures between 25 and 95 °C for between 1 and 16 hours.[102, 103, 222, 223, 225] Following this step the suspension is separated and the acid insoluble fraction washed with demineralised water, until the washings are pH = 7, and purified to produce chitin, as described in section 7.4.3. Chitosan can be precipitated from the acid solution by the addition of an alkali, such as NaOH, to raise the pH to 8.0-9.0.[222]

4.4.1.1 Preparation of 'Pure' Chitin

Acid extraction of the AIM material results in an insoluble fraction that should contain crude chitin, which is still covalently linked to β-glucans. Duarte *et al.* report that commercial samples of chitin can be purified by extended treatments with HCl and NaOH at high temperatures, however, this may result in depolymerisation and deacetylation of the chitinous material.[155] Austin proposes that the best method of purification is to dissolve and reprecipitate the chitinous material.[226] Chitin is described as being sparingly soluble in HCl, sulphuric acid, methanesulphonic acid and LiCl:DMAc solutions.[227] However, care must be taken to ensure that the acid labile glycosidic linkages are not broken in this procedure. Skujins *et al.* propose that dissolution of chitin in concentrated (37.5 %) HCl at 0 °C minimises this degradation. Chitin can then be precipitated by addition to a non-solvent, such as water or ethanol. Any purification procedure must be optimised to ensure that maximum dissolution of the chitinous material occurs with minimum degradation of the polymer chain. As the dissolution of polymers is dependent upon the molecular weight of the polymer it is

possible that the precipitated chitin will not be representative of the whole chitinous material in the fungal biomass.

An alternative method of producing chitin with an improved level of purity is to extract the chitinous material in a deacetylated form, with a DDA of 50 % or above, followed by the reacetylation to produce chitin. This would involve the use of a deacetylation step in the extraction procedure to produce acid soluble chitosan, which can be selectively solubilised and extracted with higher purity than the chitin in the AIM. Kurita *et al.* studied the acetylation of water-soluble chitosan using an acetic anhydride-pyridine system and an acetic acid-dicyclohexylcarbodiimide (DCC) system.[228] The authors described the formation of a highly swollen gel when aqueous chitin, with a high DDA, was added to pyridine and the subsequent acetylation by acetic anhydride. However, the acetylation process was not selective, requiring hydrolysis of the resulting ester groups. This was achieved using a weak base, $NaHCO_3$, with full deacetylation of the hydroxyl groups taking 141 hours. Highly acetylated chitin was produced, however, the process is lengthy therefore the authors attempted an alternative strategy of acetylating water soluble chitosan by treatment with acetic acid-DCC using a 20 fold excess of reagents in 60 % (v/v) aqueous DMF. This one step process selectively acetylated the amine groups producing highly acetylated chitin. Other methods of acetylation of chitosan have been reported in the literature, Hirano *et al.* reported the *N*-acetylation of chitosan in aqueous acetic acid-methanol mixtures using acetic anhydride, however, this process was also not selective resulting in the esterification of hydroxyl groups.[229] The one-step method of Kurita *et al.* appears to produce chitin of high purity with a low DDA, however, the procedure is lengthy and requires harsh alkali conditions to effect deacetylation. These harsh procedures could feasibly result in degradation of the polymer chain, resulting in a hetereogeneous end product.

4.4.1.2 Deacetylation Strategies

The increased interest in chitosan as a biomaterial and its improved solubilisation properties mean that chitinous materials are frequently extracted as chitosan. Therefore, if the native chitinous material has a low DDA, a deacetylation step is required in the extraction procedure. As previously discussed, the chitinous material in the AIM may be deacetylated directly and chitosan extracted as an acid soluble fraction. Alternatively, chitin may be extracted, purified and subsequently deacetylated.

In principle the acetamide groups of chitin may be hydrolysed under acidic or basic conditions, however, the use of acid hydrolysis is avoided due to the susceptibility of the glycosidic linkages to hydrolysis. Deacetylation under basic conditions is hampered by the trans arrangement of the C(2)-C(3) substituents, which increases the resistance of the C(2)-acetamido group to alkaline hydrolysis. This resistance means that severe treatments are required to bring about deacetylation, at the same time care must be taken to minimise the accompanying degradation of the polymer chain.[208] The extent of deacetylation and the severity of the reaction conditions required are to some degree dependent upon the source of the chitin.[230] Therefore, there is no one standard deacetylation procedure as the reaction conditions (time, temperature, concentration of alkali) must be optimised for each source of chitin in order to maximise the deacetylation whilst minimising the extent of degradation of the polymer chain.[204] Chitin in the β polymorphic form has been shown to be more susceptible to deacetylation than α-chitin, requiring milder reaction conditions and producing chitosan with a higher molecular weight and degree of deacetylation.[204] It is thought that this increased susceptibility is due to the lower degree of crystallinity of β-chitin compared to α-chitin. Lemarque *et al.* propose that the extent of the crystallinty of a chitinous sample is crucial in determining its deacetylation characteristics, as the acetamide groups of highly crystalline samples are inaccessible to the alkali.[231] The authors go on to postulate that deacetylation occurs in the

amorphous regions of chitinous materials, resulting in block copolymers, and full deacetylation requires a breakdown of the crystalline structure.[232]

Deacetylation is generally achieved by treatment with concentrated sodium or potassium hydroxide (40-50%) at temperatures of 100 °C or above, in heterogeneous conditions. Unfortunately, these harsh reaction conditions result in hydrolysis of the chitin chain, therefore a balance must be found between reaction time, temperature and alkali concentration in order to maximise the deacetylation and minimise the degradation of the polymer chain. Full deacetylation is difficult to achieve, as the crystallinity of the chitin structure means not all of the amine groups are accessible. The DDA may be increased by repeated solvation and reprecipitation of the partially deacetylation chitin, this multi-stage treatment may cause morphological changes rendering the remaining amide groups more accessible.[208, 231, 233] The extent of chain degradation can be decreased by excluding oxygen from the reaction or adding oxygen scavengers such as thiophenol. The reaction mechanism is not clearly established, but it is thought that oxygen acts as a catalyst in an oxido-reductive process by adding carbonyl groups at either the C_6 or C_3 positions which make the adjacent glycosidic bond very susceptible to β-alkoxy carbonyl elimination.[234] This can be reduced by adding $NaBH_4$ which will prevent oxidation of the hydroxyl groups or by removing oxygen from the reaction. Subjecting the reaction to freeze-pump-thaw cycles also increases the DDA and decreases the chain degradation as the process removes oxygen from the system whilst also opening the crystalline structure of the chitin chain to make it more permeable to the alkaline solution.[234]

4.5 Chemical extraction of Chitinous Materials from the Biomass Remaining from the Large-Scale Fermentation of *P. chrysogenum.*

The biomass remaining from the large scale fermentation of *Penicillium chrysogenum* for the production of Penicillin G was provided by Angel Biotechnology. This material is the solid resulting from the filtration of bulk cultures of *Penicillium chrysogenum,* the material has been dried at 113 °C and is a fine powder, it is

estimated to contain approximately 5-10 % chitin by weight. We initially employed literature, chemical, extraction procedures to this material in order to assess the quantity of chitinous material present. The characteristics of the materials extracted were then used as a standard against which alternative extraction procedures were compared to monitor improvements in yield or quality of the material extracted. As *Penicillium chrysogenum* is not part of the zygomycetes family, we can predict that it will not contain significant concentrations of chitosan.[9] Therefore the extraction procedures should either be tailored to the extraction of chitin, or the *in situ* deacetylation of chitin and the subsequent extraction of chitosan.

4.5.1 Chemical Extraction Procedure One [115]

Tianwei *et al.* have previously reported the chemical extraction of chitosan from *Penicillium chrysogenum*.[115] The authors employed a typical alkali deproteinisation step, followed by deacetylation in harsh alkaline conditions and acid extraction of the resulting chitosan. This procedure is described in section 7.4.2. We applied this extraction procedure to the dry biomass and recovered an off-white solid in 1.1 % yield (± 0.6 %, standard deviation of 4 repetitions). This material was analysed by ^{13}C CPMAS ssNMR, the resulting spectrum is shown in comparison to that of a commercial sample of chitosan in Figure 4.31. The ^{13}C CPMAS ssNMR spectrum indicates a low carbon content of the sample as 60,000 repetitions were required to acquire the spectra, which is of much poorer quality than that of commercial chitosan acquired in 2,000 repetitions.

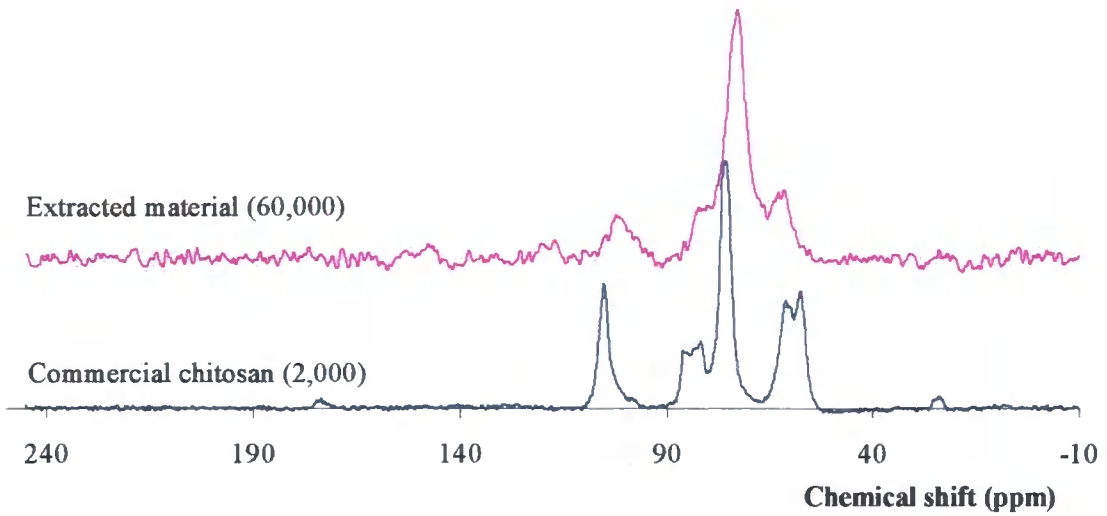


Figure 4.31 - ^{13}C CPMAS ssNMR spectra of commercial chitosan (Sigma) and a sample of material extracted by chemical extraction procedure 1.[115] Number of repetitions to acquire the spectra shown in brackets.

The low carbon content was verified by elemental analysis, which suggests that the solid contains only 2.19 % by weight of carbon and gave negligible nitrogen content. Further elemental analysis suggests the presence of 20.15 % and 15.21 % calcium and phosphorus respectively and ^{31}P CPMAS ssNMR analysis of the solid suggests the presence of an inorganic phosphate, see Figure 4.32.

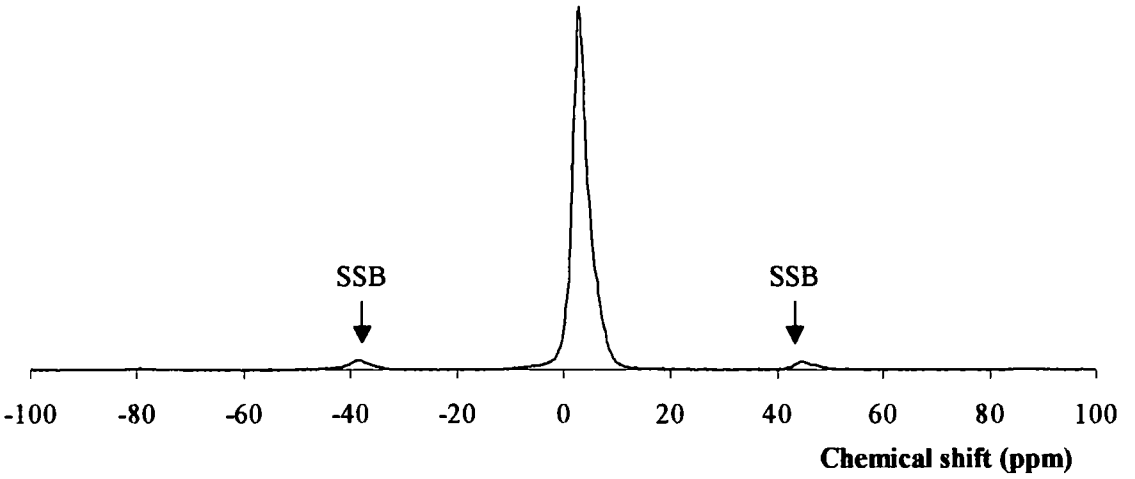


Figure 4.32 - ^{31}P CPMAS ssNMR spectra of solid produced by chemical extraction procedure one.[115] SSB = spinning side band

This extraction procedure produced a low yield of a solid, which ^{13}C CPMAS ssNMR suggest may contain chitosan in extremely low concentrations. However, elemental analysis indicates a much higher concentration of calcium and phosphorus in the sample and ^{31}P CPMAS ssNMR confirms the presence of an inorganic phosphate. We can therefore conclude that this extraction procedure does not produce chitosan when applied to the dried biomass in this study, as the sample obtained in 1 % yield predominantly contains an inorganic phosphate, such as calcium phosphate or calcium hydroxyapatite.

4.5.2 Chemical Extraction Procedure Two [103]

As the extraction procedure described in section 4.5.1 did not produce significant yields of chitinous material when applied to the dry biomass, we looked to employ a simpler extraction strategy that does not include a deacetylation step. Synowiecki *et al.* report a procedure for the extraction of native chitosan, without the use of a deacetylation step, and chitin in a crude form, complexed to β-glucans, from *Mucor rouxii*.[103] This procedure has been employed by many authors to extract chitinous materials from various fungal sources in the literature.[107, 235] The extraction

procedure involves an alkali deproteinisation step followed by an acid extraction to yield an insoluble residue containing crude chitin (solid A) and an acid soluble fraction containing chitosan (solid B). This procedure, which is described in detail in section 7.4.3, was applied to our dry biomass. For simplicity the two solids obtained will be discussed separately here.

4.5.2.1 Solid A – Chitin Containing Fraction

Solid A, containing crude chitin, was obtained in 15.9 % yield (± 1 %, standard deviation of 5 repetitions). This solid was analysed by elemental analysis and the glucosamine content was assayed in order to determine the content of chitinous material. As shown in Table 4.9, elemental analysis indicates a carbon to nitrogen ratio of 12.3 (± 0.8 %, standard deviation of 5 repetitions), an ideal sample of chitin with DDA of 0 % has a C:N of 6.86. The low nitrogen content of solid A suggests the presence of non-nitrogen containing impurities, which could be explained by the presence of polysaccharide impurities, such as β-glucans. As discussed above, chitin is found in a matrix of interactions with β-glucans in the fungal cell wall, these covalent interactions may not be destroyed in the extraction process. The glucosamine assay indicates that the sample contains 17 % by weight of glucosamine, suggesting that the sample is only 17 % chitinous.

^{13}C CPMAS ssNMR analysis was carried out to confirm the presence of chitin. The spectrum is shown in comparison to that of a commercial sample of chitin (Sigma) in Figure 4.33. The spectrum does indicate the presence of chitin as the distinctive carbonyl, methyl and sugar ring carbon peaks can be seen. Unlike the solid produced by chemical extraction method one, the spectra of the commercial chitin and the extracted sample were acquired using a similar number of repetitions, indicating that the sample contains a reasonable yield of chitin. However, the presence of impurities, which was also suggested by the low glucosamine content, can be seen in the ^{13}C CPMAS ssNMR spectrum. Notably, there is an additional peak in the region of the anomeric carbon at approximately 100 ppm, which suggests either the presence of

more than one polymorphic form of chitin, deacetylated chitin units, or other polysaccharide impurities. Consideration of the extraction procedure and the native state of chitin, combined with the general broadening of the peaks in the chemical shift range of sugar ring carbons, approximately 45 to 90 ppm, and the low glucosamine content suggested by the glucosamine assay leads us to the conclusion that solid A contains polysaccharide impurities. The inability of the extraction procedure to remove β-glucans is discussed further in section 3.6.4.4.1. The presence of these impurities will increase the amorphous nature of the sample, which accounts for the general line broadening seen in the ^{13}C CPMAS ssNMR spectrum. The spectrum also displays additional impurity peaks at 19.3, 27.7-42.6 (broad), 128.0, 136.8 and 154.3 ppm, which we can speculate arise from the presence of residual amino acids. Impurity peaks in the lower chemical shift range may be accounted for by aliphatic carbons of amino acids, those in the higher chemical shift range may represent aromatic carbons of amino acid side chains, phenoxy carbons of tyrosine or guanidino carbons of arginine.

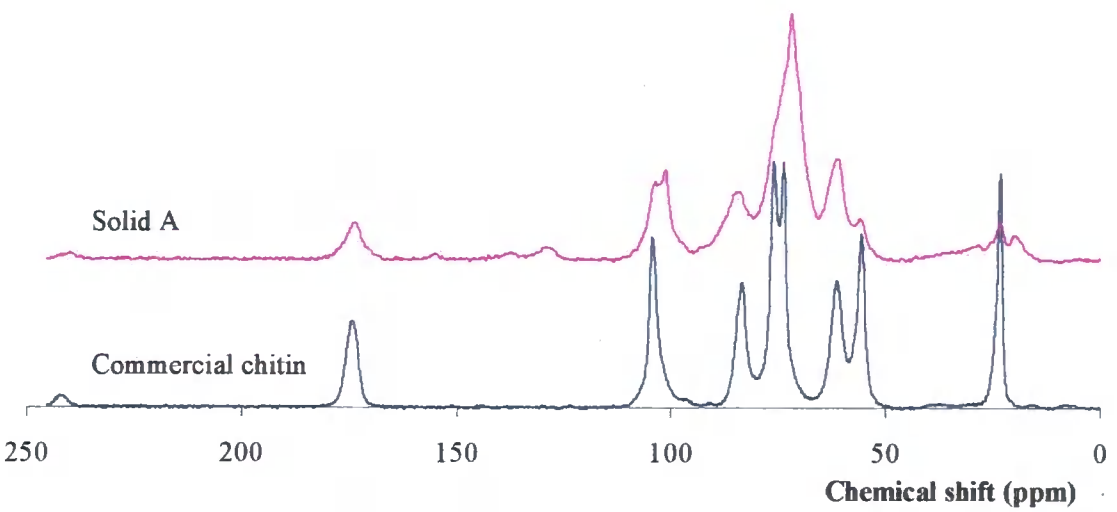


Figure 4.33 - ^{13}C CPMAS ssNMR spectra of 'solid A' extracted by chemical extraction procedure two,[103] shown in comparison to commercial chitin (Sigma).

^{15}N CPMAS ssNMR analysis was also performed on solid A, the spectrum is shown in comparison to that of a commercial sample of chitin (Sigma) in Figure 4.34. Again the coincident peak of the spectrum of solid A and that of commercial chitin indicates

the presence of chitin. The absence of a peak at approximately –350 ppm suggests that the chitinous material has a very low DDA. It is probable that the sample does contain deacetylated units, however the concentration of any such units is so low they can not be discerned from the baseline. Allowing for the confidence limits suggested in chapter 3, we can assume that the DDA of the sample is ≤ 5 %. The low purity of the sample, which results in an increasingly amorphous nature, is seen by the broad line-width of the peak when compared to that of commercial chitin.

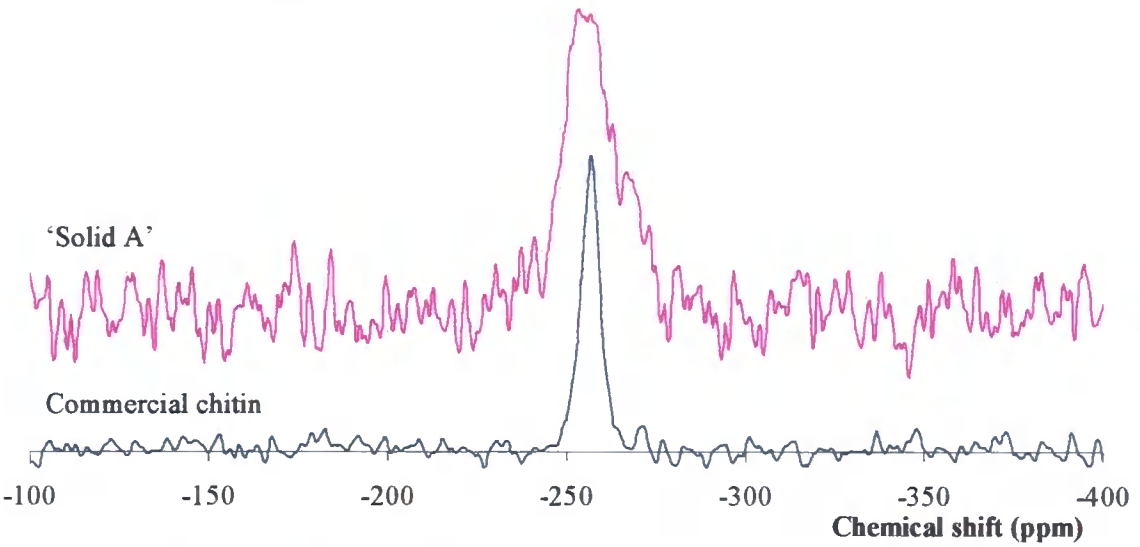


Figure 4.34 - ^{15}N CPMAS ssNMR spectra of commercial chitin (Sigma) and solid A extracted by chemical extraction procedure two.[103]

The characterisation of solid A is summarised in Table 4.9. The analysis suggests that chemical extraction procedure two produces approximately 16 % of a solid, solid A, which contains approximately 17 % chitinous material. ^{15}N CPMAS ssNMR analysis of this material indicates that it has a low DDA of ≤ 5 %. The ^{13}C CPMAS ssNMR spectrum indicates that the sample contains polysaccharide impurities such as β-glucans. As these impurities do not contain nitrogen, this accounts for the low nitrogen content as seen by elemental analysis.

Analysis	Result
Yield	15.9 ± 1.0 *
C:N**	12.3 ± 0.8 *
glucosamine content	17 %
DDA	≤ 5 %

Table 4.9 – Analysis of 'solid A', chitin containing fraction, of the dry biomass after subjection to the chemical extraction procedure two.[103] * errors given as standard deviations of 5 repetitions. ** C:N calculated by elemental analysis.

4.5.2.2 Solid B – Chitosan Containing Fraction

Solid B was extracted in 2.9 % yield (± 0.4 %, standard deviation from 5 repetitions) and elemental analysis was performed. The results suggest that solid B contains only 3.41 % carbon by weight (± 0.8 %, standard deviation from 5 repetitions) and does not contain nitrogen. This low carbon content is confirmed by the ^{13}C CPMAS ssNMR spectrum of the sample, shown in Figure 4.35, as the spectrum required 60,000 repetitions to acquire compared to 2,000 for the commercial sample and has a much poorer signal to noise ratio. The chemical shifts of the few peaks that are present are coincident with chitosan, however the low signal to noise ratio and lack of resolution of the peaks suggests that the concentration of chitosan extracted is extremely low.

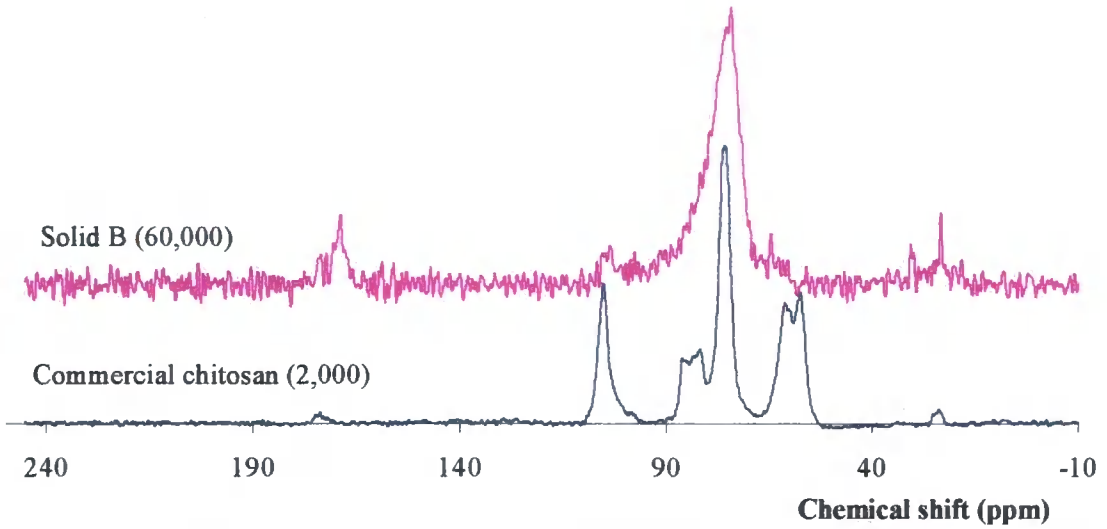


Figure 4.35 - ^{13}C CPMAS ssNMR spectra of solid B extracted by chemical extraction procedure two,[103] and commercial chitosan (Sigma) The number of repetitions required to obtain the spectra are shown in brackets.

Further elemental analysis indicates that the sample contains a high concentration of calcium and phosphorus, 23.1 and 12.0 % by weight respectively. The presence of an inorganic phosphate was confirmed by ^{31}P CPMAS ssNMR analysis, see Figure 4.36. We can therefore conclude that, as for chemical extraction procedure one, chitosan has not been extracted in a high yield and that solid B contains a high concentration of an inorganic phosphate such as calcium phosphate or calcium hydroxyapatite.

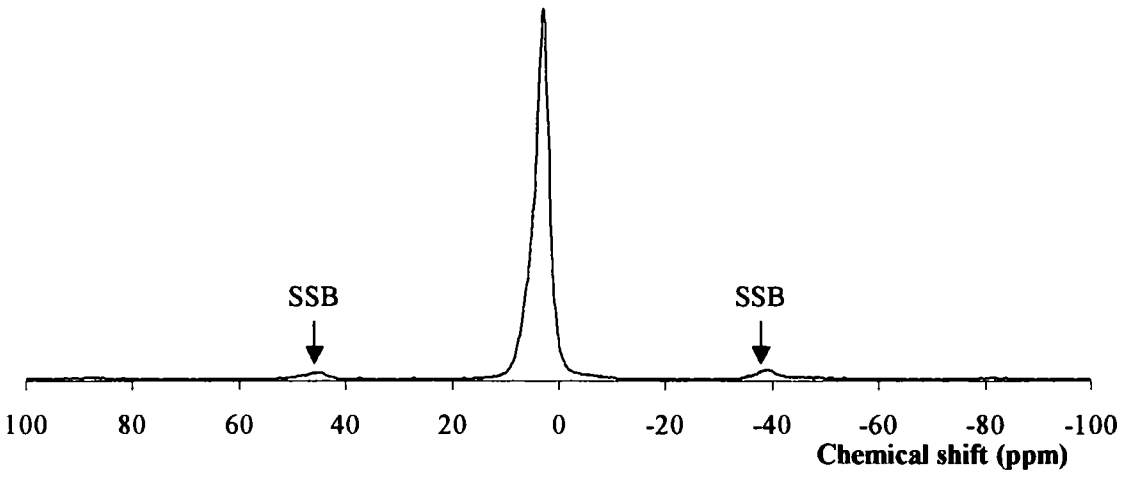


Figure 4.36 - ^{31}P CPMAS ssNMR spectrum of solid B extracted by chemical extraction procedure two. (SSB = spinning side band)

4.5.2.3 Modification of Chemical Extraction Procedure Two

The chemical extraction procedure described in 4.5.2 was modified by altering the concentration of NaOH in the deproteinisation step. It was thought that this may effect deacetylation of the chitinous material, which may increase the concentration of the acid soluble chitosan extracted, or it may increase the DDA of the acid insoluble chitin fraction increasing its accessibility to the extraction reagents. Concentrations of NaOH between 2 and 20 % were employed all other reaction conditions were maintained. It was found that the yields of solid A and B produced, displayed in Table 4.9, did not vary significantly when the concentration of NaOH in the deproteinisation step was increased.

[NaOH] (% (w/v))	Solid A		Yield solid B (%)
	Yield (%)	C:N *	
2	15.9	12.3	2.9
5	12.0	12.7	2.1
10	11.4	13.3	3.1
20	12.0	12.2	2.8

Table 4.10 - Yields of solid A and B produced by chemical extraction procedure one, modified to use varying concentrations of NaOH in the deproteinisation step. The C:N of solid A, given by elemental analysis, is also shown.

Study of the C:N of the solid A samples containing crude chitin, obtained by elemental analysis, suggests that the materials are of a similar level of purity as the values only vary by 1 %, which is within the error of the values obtained using the unmodified procedure. ^{13}C CPMAS ssNMR analysis was also performed, the resulting spectra are shown in comparison to a commercial source of chitin in Figure 4.37. No significant change in the chemical shifts of the chitinous peaks, the peak line-widths or the number and distribution of impurity peaks can be seen in the ^{13}C CPMAS ssNMR spectra upon alteration of the NaOH concentration.

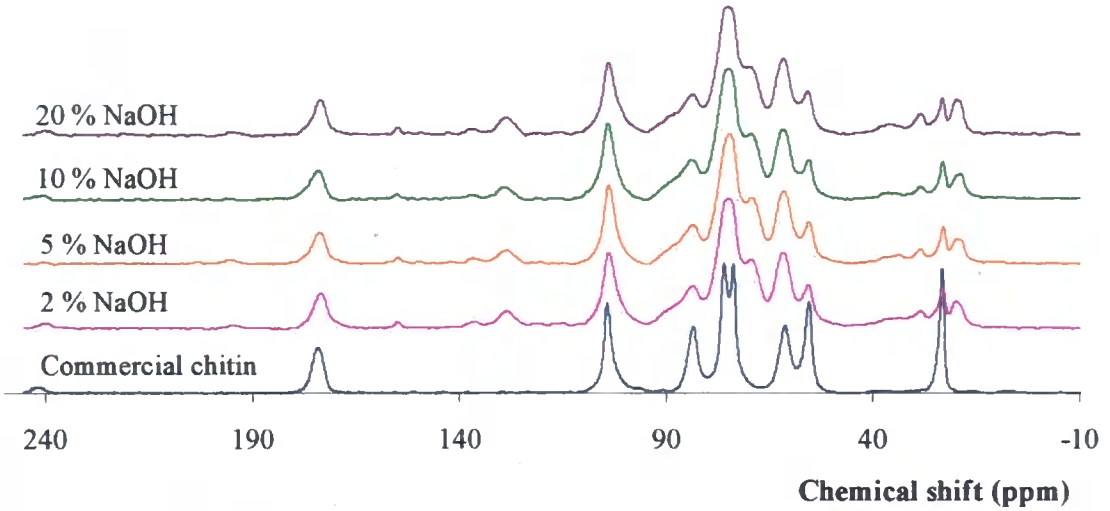


Figure 4.37 - ^{13}C CPMAS ssNMR spectra of commercial chitin and a series of samples of solid A, containing crude chitin, extracted by chemical extraction procedure two using varying concentrations of NaOH in the deproteinisation step.

Elemental analysis of the solid B fractions, which were supposed to contain the acid soluble chitosan material, are shown in Table 4.9. The results indicate that increasing the concentration of NaOH in the deproteinisation step did not result in an increased extraction of chitosan in the acid soluble fraction, as the carbon content of the samples does not increase significantly. The samples all contained a high concentration of calcium and phosphate, again indicating the presence of calcium phosphate or calcium hydroxyl apatite.

[NaOH] (% (w/v))	C	H	N	Ca	P
2	3.41	1.6	-	23.1	12.0
5	4.97	1.70	-	26.1	13.8
10	3.28	1.67	-	27.0	12.3
20	3.38	1.76	-	24.4	9.79

Table 4.4 - C,H,N, Ca and P content (% by weight) given by elemental analysis of samples of solid B extracted by chemical extraction procedure two, modified to use varying concentrations of NaOH in the deproteinisation step.

We can conclude that increasing the concentration of NaOH in the deproteinisation step did not effect significant deacetylation of the chitinous material as the yield of

chitinous material in the acid soluble fraction, solid B, was not improved. The yield and purity of the chitin containing fraction, solid A, was also unaffected by the change in NaOH concentration.

As discussed in section 3.7.3 the validity of GPC analysis of chitinous materials is limited due to their inherent insolubility, which means that only a small fraction of the sample is solubilised. As solubility is molecular weight dependent this means that the results are not representative of the whole sample. However, we may make tentative, qualitative, comparisons of samples. GPC analysis of the crude solid A samples extracted using the lowest and highest concentrations of NaOH was performed, the resulting refractive index traces are shown in Figure 4.38 in comparison to a commercial sample of chitin. The traces suggest that using a higher concentration of NaOH degrades the chitin polymer chain, as the sample has a higher retention volume, indicating a lower molecular weight. Therefore, as using higher concentrations of NaOH does not afford any advantages in the yield or purity of the chitinous material extracted and results in degradation of the polymer chain, the chemical extraction procedure was applied unmodified in subsequent extractions, using 2 % NaOH in the alkali extraction step.

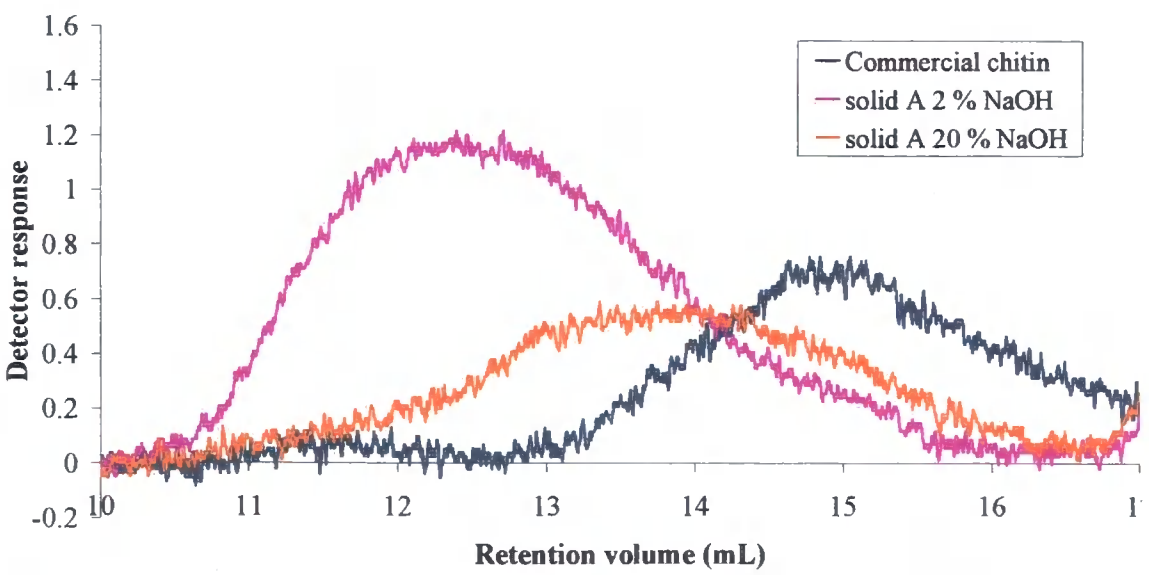


Figure 4.38 – Refractive index trace produced by GPC analysis of a commercial sample of chitin (Sigma) and two samples of solid A extracted by chemical extraction procedure two using varying concentrations of NaOH in the deproteinisation step. 5 % (w/v) LiCl:DMAc was used as the solvent and eluent.

4.5.2.4 'Purification' of Solid A

The acid insoluble fraction, solid A, remaining after the extraction of the dry biomass by chemical extraction procedure two, contains approximately 17 % chitin, as estimated by the glucosamine assay of Tsuji *et al.*[130] ^{13}C CPMAS ssNMR and elemental analysis indicates that this fraction also contains a high concentration of non-nitrogen containing polysaccharides, such as β-glucans which are known to be covalently linked to chitin in the fungal cell wall. In order to purify chitin from this material we used a modified version of the purification procedure of Skujins *et al*; see section 7.4.4.[236] This involved stirring the solid in concentrated HCl at 0 °C for six hours, after this time the suspension was separated by centrifugation and the supernatant poured into 50 % aqueous ethanol, which caused the precipitation a white solid. The acid insoluble residue and the precipitate were washed with deionised water until the washings became neutral and the residues dried.

The purification procedure produced a precipitate in 11.8 % yield (± 1 %, standard deviation of 5 repetitions) and resulted in 83.4 % (± 5 %, standard deviation of 5 repetitions) of an acid insoluble residue, with respect to the weight of the crude material. Elemental analysis suggests that the precipitate has a C:N of 9.7 (± 1 %, standard deviation of 5 repetitions), an ideal sample of chitin with 0 % DDA has a C:N of 6.86, this low nitrogen content indicates the sample still contains non-nitrogen containing impurities. However, this value is improved from that of the crude solid A, C:N = 12.3. The glucosamine assay suggests that 57 % of the sample is chitinous material; this has increased from 17 % in the crude solid A. ^{13}C CPMAS ssNMR analysis corroborates the indicated increase in chitin concentration of the sample, as the resolution of the spectrum, shown in Figure 4.39, is improved and the intensities of the C8 and C7 peaks are greatly increased relative to the sugar ring carbon peaks. The C3 and C5 peaks can now be resolved and the line-widths of the peaks have decreased. The impurity peaks in the chemical shift ranges of 0 to 40 and 110 to 160 ppm can no longer be seen. However, there are still broad shoulders on the C3 and C4 peaks, which indicates that there are still polysaccharide impurities present.

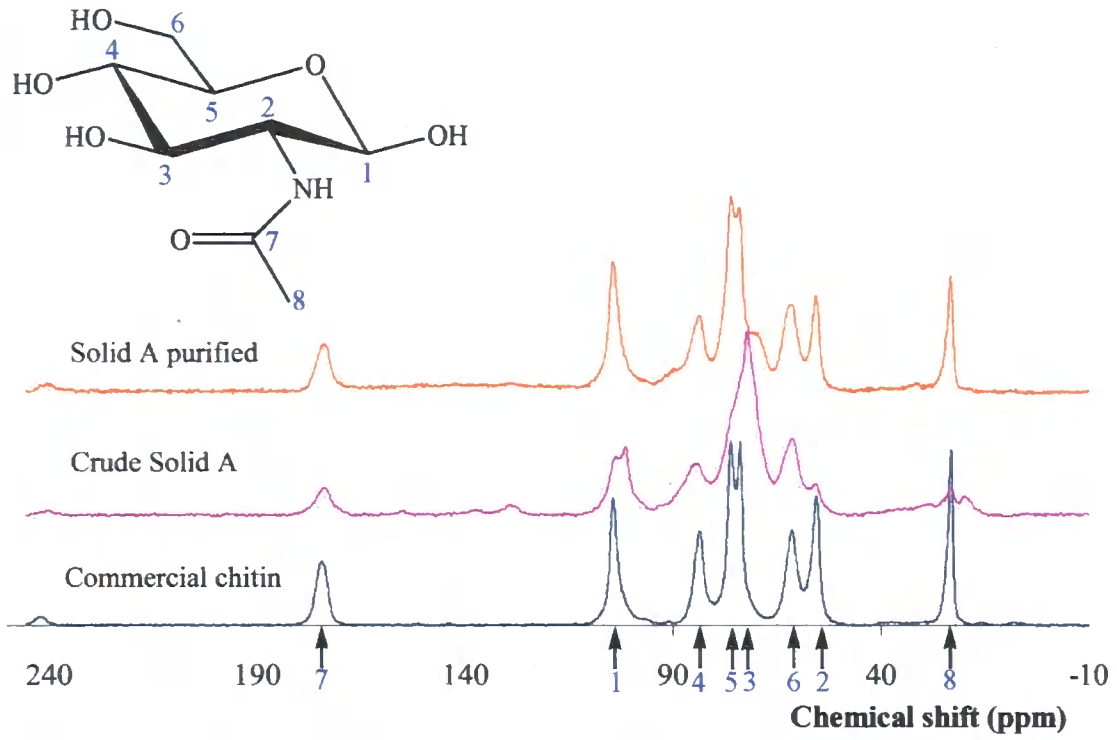


Figure 4.39 - ^{13}C CPMAS ssNMR spectra of commercial chitin (Sigma), crude solid A extracted by chemical extraction procedure two and solid A purified by precipitation from HCl.

^{15}N CPMAS ssNMR analysis was also performed, the spectrum of the purified sample, shown in Figure 4.40, displays the characteristic chitin peak. Upon purification we can see that the line-width of the ^{15}N CPMAS ssNMR spectrum has decreased in comparison to the crude solid and is now comparable with the commercial sample of chitin. This indicates a decrease in the amorphous nature of the sample as the concentration of impurities decreases. Again a peak cannot be seen at –350 ppm, indicating that the material has a low DDA ($\leq$ 5 %) and has not been deacetylated by the purification procedure.

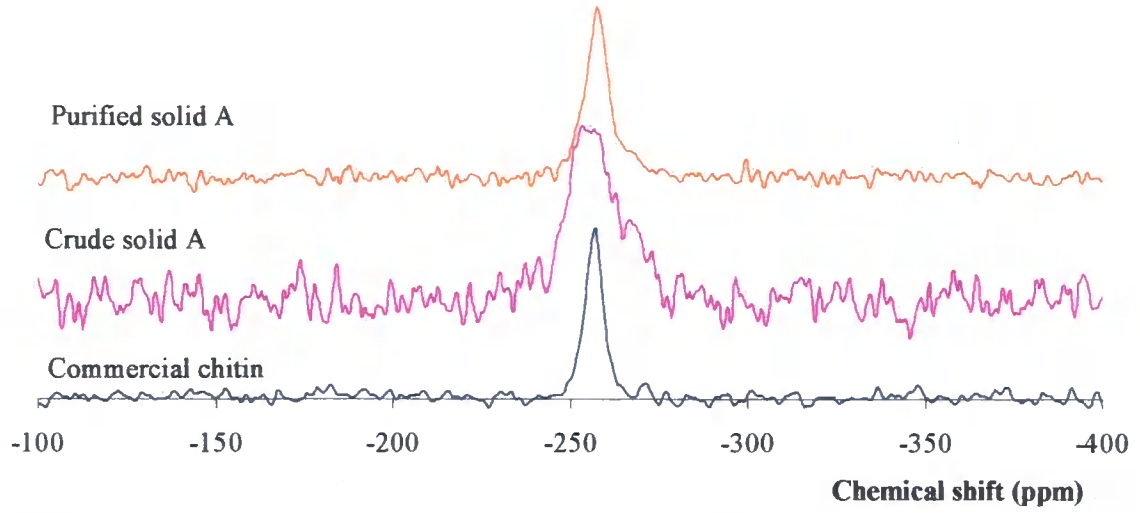


Figure 4.40 - ^{15}N CPMAS ssNMR spectra of commercial chitin (Sigma), the crude solid A extracted by chemical extraction procedure two and the purified solid A.

The characterisation of the purified solid A is summarised in Table 4.11, in comparison to the results obtained for the crude solid. The yield of the purified solid A has been calculated from the starting weight of the dry biomass before extraction. The results indicate that the purification procedure has removed approximately half of the impurities present as the purified sample contains 57 % glucosamine compared to 17 % of the crude material, as shown by the glucosamine assay. This is reflected in the decrease in the C:N ratio by elemental analysis. However, the polysaccharide impurities have not been completely removed, as 43 % of the sample is non-chitinous material.

Analysis	Crude solid A	Purified solid A
Yield	15.9 ± 1.0 *	1.88 ± 0.1 *#
C:N**	12.3 ± 0.8 *	9.7 ± 1.0 *
glucosamine content	17 %	57 %
DDA	≤ 5 %	≤ 5 %

Table 4.11 - Analysis of 'solid A', chitin containing fraction, of the dry biomass after subjection to the chemical extraction procedure two,[103] and purified 'solid A'. * errors given as standard deviations of 5 repetitions. ** C:N calculated by elemental analysis. # yield of purified solid A relative to starting weight of dry biomass.

The acid insoluble residue remaining from the purification of solid A was also analysed by ^{13}C CPMAS ssNMR. The spectrum, shown in Figure 4.41, indicates that not all of the chitinous material was solubilised in the concentrated HCl, as coincident peaks can be seen in the spectrum when it is compared to that of the commercial chitin. The spectrum also displays a number of peaks, of relatively high intensity compared to the chitinous peaks, which correspond to the impurity peaks seen in the spectrum of the crude solid A in the chemical shift ranges of 0 to 40 and 110 to 160 ppm. This indicates a high concentration of non-chitinous impurities present in the residue.

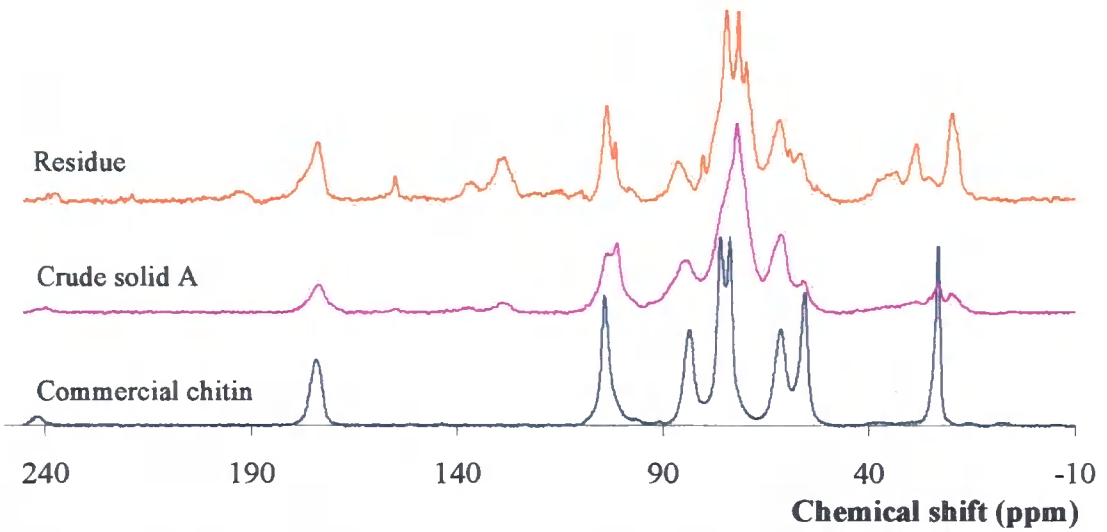


Figure 4.41 - ^{13}C CPMAS ssNMR spectra of commercial chitin (Sigma), the crude solid A from the chemical extraction procedure two and the acid insoluble residue remaining from the purification of the crude solid A.

4.5.2.5 GPC Analysis of Solid A

The refractive index trace obtained by the GPC analysis of the crude and purified solid A is shown in comparison to that of a commercial sample in Figure 4.42. The crude and purified samples both have retention volumes lower than that of commercial chitin, indicating that they have a higher molecular weight. As the purified sample has a higher retention volume that the crude extract we can assume that the molecular

weight has decreased upon purification, this may indicate that the polymer has been degraded by acid hydrolysis of the glycosidic linkages. Alternatively, as the solubility of polymers is dependent upon their molecular weight, it may be that the lower molecular weight chitin chains are selectively solubilised in the purification procedure. Additionally, impurities of high molecular weight may have been complexed to chitin, increasing the resulting molecular weight. The purification procedure may have removed these impurities and therefore resulted in a decrease in molecular weight. Interestingly the refractive index trace of the purified sample has a bimodal distribution, whereas the crude extract is unimodal, this does suggests that degradation of the polymer chain has taken place.

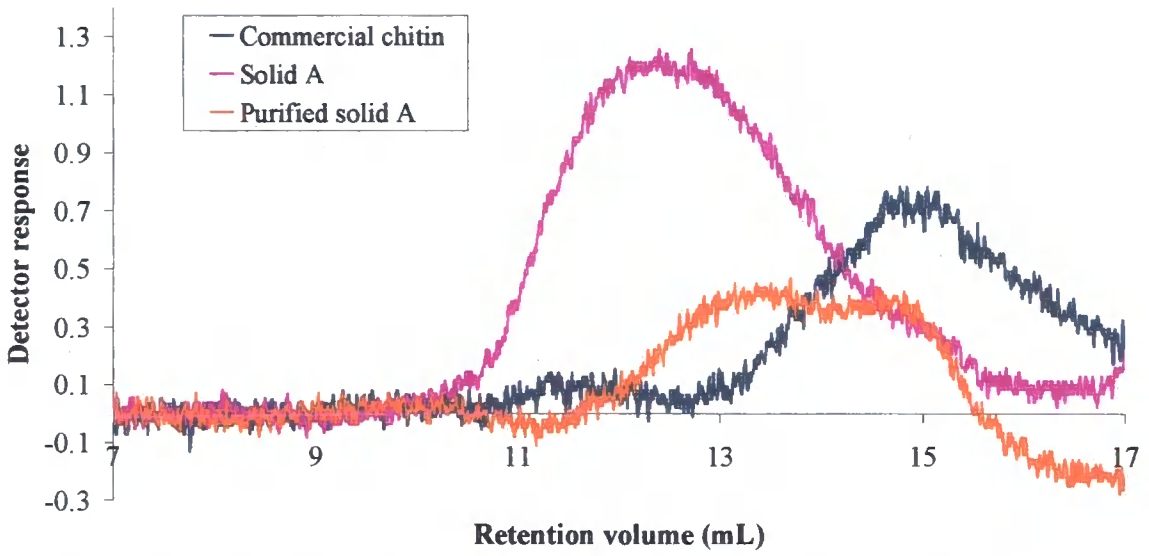


Figure 4.42 - Refractive index trace produced by the GPC analysis of commercial chitin (Sigma), the crude solid A and the purified solid A using 5 % (w/v) LiCl:DMAc as the solvent and eluent.

4.5.3 Conclusion – Standard Chemical Extraction Procedure

Evaluation of the literature procedures available for the extraction of chitinous material from fungal sources indicated that most chemical extraction procedures proceed in the same general route, with minor variations in reactants and concentrations. The main variation arises from the decision to extract native chitin, or to deacetylate and extract chitosan. We employed two literature extraction procedures

following each of these routes. Chemical extraction procedure one involved a step designed to deacetylated the chitinous material, followed by the acid extraction of chitosan.[115] However, we found that the low yielding solid produced had a very low carbon content. We concluded that the extract contains a high concentration of calcium phosphate, or calcium hydroxyapatite. Chemical extraction procedure two is designed to extract crude chitin as solid A and acid soluble chitosan, if it is present in the fungal material. Again we found that the acid soluble material contains mainly calcium and phosphorus and has a very low carbon content, however, the glucosamine assay suggested that solid A does contain approximately 17 % chitin, which was confirmed by ^{13}C and ^{15}N CPMAS ssNMR analysis. This crude material was purified by precipitation from concentrated HCl at 0 °C in to 50 % aqueous ethanol. The purified material contained approximately 57 % chitin and has a DDA below 5 %. The overall yield of solid A from the original dry biomass was 1.9 %. The characteristics of this material are summarised in Table 4.11. This material forms the datum against which material extracted by alternative procedures will be compared. The 'standard chemical extraction procedure', which was applied to other materials in the study is shown schematically in Figure 4.43. The results obtained using this procedure compare favourably with those reported in the literature for the extraction of chitin and chitosan. Tianwei *et al.*[115] and Wu *et al.*[104] report yields of crude chitin of 0.3 and 14 % yield respectively with glucosamine concentrations of approximately 45 % and DDA of between 80 and 8 %. The extraction of chitosan from a variety of fungal sources has been reported with yields of between 2 and 14 % with DDAs of between 70 and 90 % and glucosamine concentrations between 2 and 80 %.[101, 103, 225]

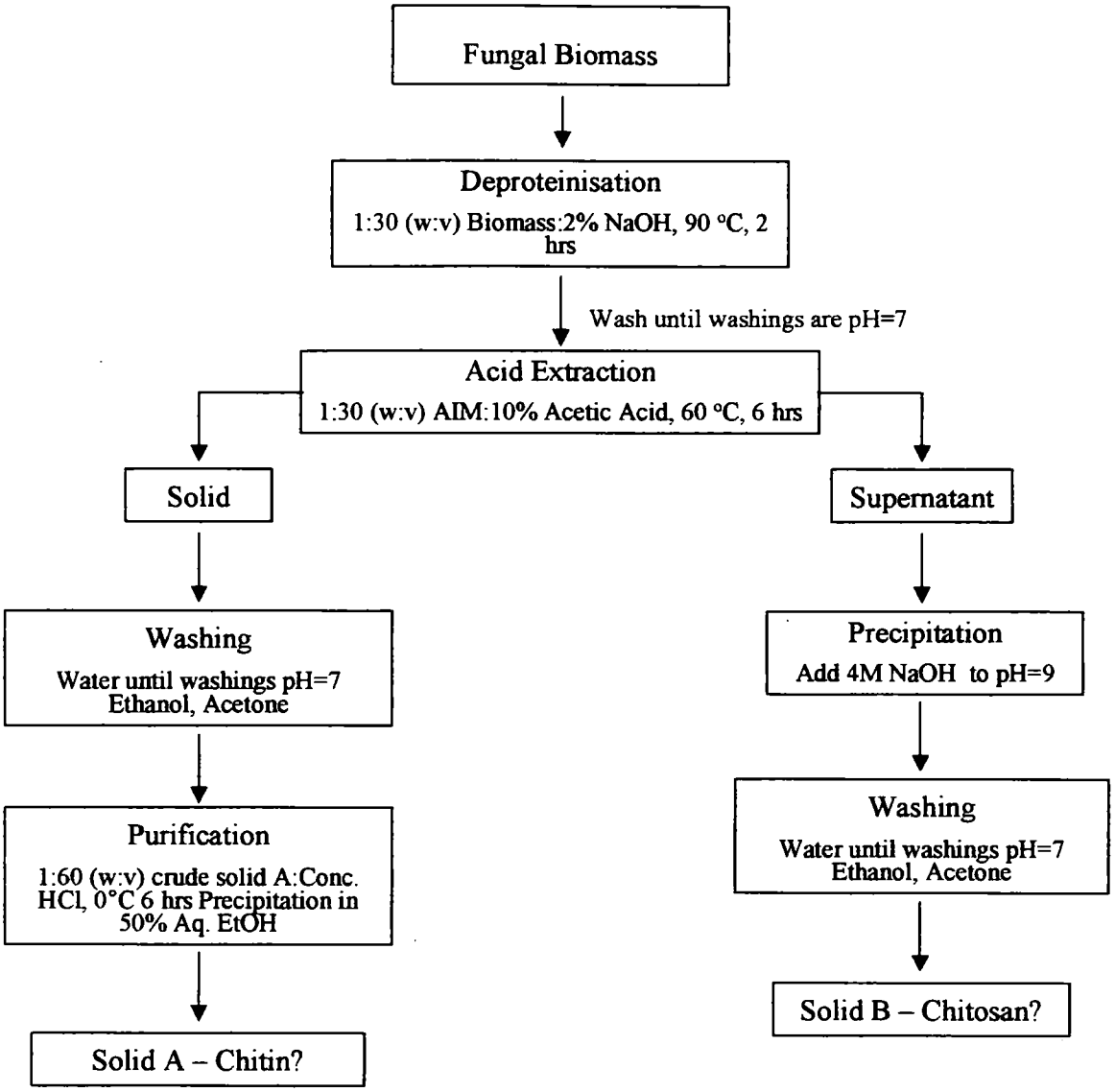


Figure 4.43 - Chemical extraction scheme

4.6 Alternative Extraction Procedures

Traditional chemical methods of extraction and deacetylation of chitin can result in unwanted deacetylation and degradation of the polymer chain; the product may therefore have a broad range of molecular weights and a heterogeneous deacetylation pattern. For many high-value biomedical applications, for which chitosan and chitin may have potential use, uniform materials with specific physical and chemical properties are required.[46] Chemical extraction procedures have been optimised to minimise the degradative effects, however, strong acids and alkalis are still

required.[230] Chemical treatments also result in waste disposal issues in industrial applications, as the waste stream requires neutralisation and detoxification to remove proteins and protein degradation products and high salt contents, which could lead to environmental pollution. There is therefore a great interest in developing extraction methods for isolating chitin and chitosan that avoid or minimise these harsh chemical treatments to produce a reliable and uniform product of high quality.

The most degradative procedure is the deacetylation step employed in the extraction of chitosan, as high concentrations of alkali are required at high temperatures. This can result in chitosan with an uncontrolled molecular weight and a heterogeneous deacetylation pattern, as the physiochemical properties and biological activity of chitosans are dependent upon these characteristics this can render the product unsuitable for biomedical applications.[4] Consequently a large volume of research in the literature has focused on the development of alternative, enzymatic, deacetylation procedures. The main commercial source of chitin and chitosan is from shrimp and crab processing waste produced by the seafood processing industry, extraction of chitin from these sources requires harsh deproteinisation and demineralistion procedures, therefore, there is also a large volume of work in the literature discussing enzymatic methods of extraction from these sources. The enzymatic extraction of chitin and chitosan from fungal sources has been studied to a lesser extent, however, the enzymatic deproteinisation techniques applied to crustacean sources may possibly be applied to fungal sources. In the following sections the literature procedures for enzymatic deproteinisation will be discussed along with a discussion of enzymatic extraction procedures for chitin and chitosan from fungal sources currently available in the literature. The use of chitin deacetylase will also be reviewed briefly, however, the lack of commercial availability of this enzyme meant that the use of this enzyme system was not explored in this thesis.

4.6.1 Alternative Deproteinisation and Demineralisation Strategies

The use of enzymatic deproteinisation of shrimp and crab shells has been widely reported in the literature.[237] Broussignac suggested the use of proteolytic enzymes such as pepsin, trypsin and papain for the deproteinisation of shrimp sources in 1968 and demonstrated that incubation with these enzymes results in the extraction of chitin with little change in DDA.[238] Yang *et al.* describe the production and purification of a neutral protease from *Bacillus subtilis* and reported that fermentation of untreated crab, shrimp and lobster shells with this microbe results in 88, 67 and 83 % protein removal.[239] Gagne *et al.* also describe the use of chymotrypsin and papain to facilitate the recovery of chitin from shrimp wastes, reporting that incubation with these proteolytic enzymes results in chitin containing 1.3 and 2.8 % residual protein respectively.[240] These results are comparable to the optimised chemical deproteinisation procedures, which generally result in residual protein levels between 0.5 and 2 % in extracted chitins.[88, 91] Teng et al. produced an interesting report on the investigation of the concurrent production of chitin from shrimp shells and fungi.[241] Fungal cell walls contain a rich source of enzymes of potential biotechnological interest including, hydrolases, synthases, oxidoreductases and transferases which have potential use in the extraction process.[242] The authors cultured a strain of fungi known to express proteases in the presence of shrimp shells, the release of protease by the fungi facilitated deproteinisation of the shrimp shell powder releasing hydrolysed proteins. These hydrolysed proteins then act as a nitrogen source for fungal growth, additionally the shrimp shells act as a mineral and trace element source resulting in demineralisation of the shrimp-shell powder.

Reports of the enzymatic extraction of chitin and chitosan from fungal sources are less abundant. Skujins *et al.* describe the use of $\beta(1\rightarrow3)$ glucanase and chitinase in combination to lyse fungal cell walls and report that $\beta(1\rightarrow3)$ glucanase can be employed to remove the glucan rich layer which shields the chitin-containing core of the cell wall.[236] Significant improvements in the harvesting of chitosan from fungal sources after a pre-treatment of the mycelia with a heat stable α-amylase has been

reported by Nwe *et al.*[94] The authors propose that the amylase reduces the crosslinking between the chitosan and glucan chains by hydrolysing α(1→6) glucosidic bonds resulting in chitosan of higher purity, however, a chemical deproteinisation procedure was still employed.[243] Gilmour *et al.* investigated the use of the plant protease actinidin to deproteinise Shitake mushrooms.[244] The authors found that the protease was successful in removing protein, however, it was not as efficient as the use of traditional hot NaOH treatments. To our knowledge, the only fully enzymatic extraction procedure described for fungal sources of chitinous material has been reported by Cai *et al.*[102] The authors describe the extraction of chitosan from the waste *Aspergillus niger* mycelium remaining from the citric acid production industry. Lysozyme and snailase (containing β-glucoronidase, sulfatase and β-D-mannosidase) enzymes were used to disrupt the cell wall, a neutral protease was then employed to deproteinate the sample followed by treatment with a chitin deacetylase to result in acid soluble chitosan. The authors compared the resulting chitosan with that extracted by a traditional chemical extraction procedure and found that the material was of similar purity and had a DDA equivalent to the chemical extraction product. However, it was of much higher molecular weight, which indicates that the enzymatic procedure is less degradative than the chemical procedure. Due to the similar nature of this work to the original outline of this project, the extraction procedure described was followed using the *Penicillium chrysogenum* mycelia studied in this thesis; this is discussed later in section 4.9.3.

4.6.2 Alternative Deacetylation Strategies

The traditional method of deacetylation involves the use of strongly alkaline conditions at high temperatures. This is environmentally unsuitable and produces a broad and heterogeneous range of products which have uncontrolled deacetylation patterns and lower degrees of polymerisation. Chitin deacetylases may offer an alternative, non-degradative and well-defined enzymatic process for chitosan production. The presence of a novel class of deacetylases which specifically catalyse the hydrolysis of the *N*-acetamido bonds of chitin has been demonstrated in several

fungal species which contain chitosan, for example *Mucor rouxii*,[99] *Colletotrichum lindemuthianum*[245] and *Saccharomyces cerevisae*,[246] It is suggested that chitin deacetylases catalyze the biosynthesis of chitosan in these species by deacetylating nascent chitin in the fungal cell wall. Tsigos *et al.* demonstrated that in *Mucor rouxii* chitin synthase and chitin deacetylase operate in tandem to produce chitosan as chitin deacetylase acts more efficiently upon nascent rather than microfibrilliar chitin.[247] The mode of action of the enzyme on water soluble, partially acetylated, chitosans, has been investigated by several authors who propose that a degree of polymerisation of 3 or more is required in the substrate.[248] The enzyme works by a multiple attack mechanism, by binding to the chitin chain to remove an acetyl group and processing down the chitin chain for at least 3 monomer units before disassociating and binding to another chitin chain.[249] This results in a more ordered deacetylation pattern than is produced using the traditional heterogeneous chemical deacetylation procedures.

The major disadvantage in the use of chitin deacetylases is the enzyme's reduced efficiencies when applied to highly crystalline substrates. The enzymes are only efficient when applied to water-soluble chitinous polymers, or oligosaccharides. Martinou *et al.* monitored the deacetylation by chitin deacetylase of highly acetylated crystalline chitin and amorphous chitin as well as two chitosan samples with DDAs of 72 and 58 %.[250] The authors found that chitin deacetylase did not efficiently deacetylate the crystalline and amorphous chitin substrates, which are not water-soluble, effecting only 0.5 and 9.5 % deacetylation respectively. Whereas the water-soluble chitosans achieved DDAs of 97-98 %, this was an improvement over chemical deacetylations which typically achieve DDAs of 85-93 %.[247] Similar results have been reported by other authors, Cai *et al.* describe a lower activity of chitin deacetylase on crystalline chitin substrates compared to amorphous materials.[110] It is proposed that this decrease in enzyme efficiency is due to the inaccessibility of the acetamide groups in highly crystalline chitin substrates. Attempts to increase the accessibility of these groups *via* pre-treatments have been made,[251] however, the conditions required to achieve this frequently result in degradation of the polymer chain.[252]

Chitin deacetylases offer the advantage of well-defined reactions resulting in chitosans with a homogenous deacetylation pattern, however at present their use is restricted to low molecular weight, or partially deacetylated, water-soluble chitin samples. It may be possible to use the chemical and enzymatic deacetylation techniques in a complementary manner to achieve full, or controlled, deacetylation of chitin with a defined deacetylation pattern and high molecular weight. However, to our knowledge, this has so far not been reported.

4.7 Enzymatic extraction of Chitinous Materials from the Biomass Remaining from the Large-Scale Fermentation of *P. chrysogenum*.

As described earlier, the current chemical procedures employed for the extraction of chitin and chitosan can result in heterogeneous products that are unsuitable for many high-value applications. Replacement of these procedures with controlled enzymatic protocols is not only environmentally appealing, but may also result in a more homogenous and well-defined product. To the best of our knowledge, to date, the total enzymatic extraction of chitinous material from fungal sources in high yield and purity has been described in the literature by only one group, who extracted the material in the deacetylated form.[102] If total enzymatic extraction cannot be achieved efficiently, it may be advantageous to use enzymatic and chemical extraction procedures in tandem. For example, hydrolytic enzymes may be used to reduce the interactions in the fungal cell wall matrix, releasing the majority of the lipids, proteins and glucans. The residual impurities may then be extracted by a chemical procedure. The enzymatic pre-treatment of the cell wall may increase the accessibility of the chitinous material thereby reducing the severity of the extraction conditions required, resulting in a chitinous product of high purity and well-defined characteristics. In order to explore the possibility of enzymatic extraction of chitin from the dry biomass remaining from the large-scale fermentation of *Penicillium chrysogenum*, we incubated the biomass with a series of hydrolytic enzymes designed to break down the fungal cell wall matrix. The biomass was subsequently subjected to the normal chemical extraction procedure, described in section 7.4.3, and the resulting products

were analysed to monitor any improvements in yield or quality. It was hoped that this would identify enzymes that could potentially be utilised in total enzymatic procedures. Following the literature precedent, described above, the effect of incubation with a neutral protease, an α-amylase and a β-glucanase was investigated. The enzymes were all commercially available from Sigma.

4.7.1 Incubation of Dry Biomass with Hydrolytic Enzymes

The dry biomass was stirred in 50 ml of buffered solution containing the hydrolytic enzymes at the required pH, see Table 4.12, for three days at 37 °C. The quantity of enzyme employed was estimated using the activities provided by the supplier (Sigma) and assuming that the fungal cells contain 50-60 % non-chitinous polysaccharides and 30-40 % proteins.

Enzyme	Buffer	pH	Mass of Enzyme	Activity of enzyme
Protease	50 mM potassium phosphate	7.5	1 g	0.3 units/mg
β-glucanase	100 mM potassium phosphate	6.5	20 mg	1 unit/mg
α-amylase	50 mM Tris-HCl	8.5	10 mg	0.3 units/mg

Table 4.12 - Buffer conditions used for the incubation of the dry biomass with 4 hydrolytic enzymes

After incubation the solid was collected by centrifugation and extracted by the chemical extraction procedure involving alkali deproteinisation and acid extraction as described in section 7.4.3, to produce an acid insoluble fraction, solid A, which should contain crude chitin, and an acid soluble fraction, solid B, which should contain chitosan. These crude materials were analysed and compared to the chemical standard. The resulting yields, glucosamine contents and ratios of carbon to nitrogen content, as indicated by elemental analysis, are shown in Table 4.13, the standard results obtained from chemical extraction of the dry biomass alone are shown for comparison.

Enzyme	Yield (%)		C/N*		Glucosamine content of Crude A (%)
	Solid A	Solid B	Solid A	Solid B	
Protease	15.5	2.8	11.4	-	15
β-glucanase	12.9	1.7	9.95	-	15
α-amylase	16.7	2.5	11.8	-	16
Chemical standard	15.9	2.9	12.3	-	17

Table 4.13 - Yields of solid A and B extracted from dry biomass by the standard chemical extraction procedure after incubation of the biomass with hydrolytic enzymes. * Ratio of carbon to nitrogen content indicated by elemental analysis

Incubation of the dry biomass with protease, β-glucanase and α-amylase did not significantly affect the yield of either the chitin or chitosan containing fractions extracted by the standard chemical extraction procedure. The ratio of carbon to nitrogen content of the crude chitin containing fraction extracted from the biomass which was incubated with β-glucanase is slightly reduced, suggesting an increase in the relative nitrogen concentration and hence a decrease in the concentration of non-nitrogen containing impurities, however, analysis of the glucosamine content of the sample indicates that it is of a similar level of purity. The carbon to nitrogen ratios of the samples extracted from biomass incubated with the protease and α-amylase are also slightly decreased, however, these values are almost within the ± 0.8 % error of the standard value. Analysis of the glucosamine concentration of the solids reinforces the conclusions drawn from elemental analysis data, that no significant change in the level of purity of the crude chitin containing fractions is seen upon incubation with hydrolytic enzymes. To better ascertain the nature of the solids ^{13}C CPMAS ssNMR analysis was performed, the resulting spectra are shown in Figure 4.44 in comparison to a commercial sample of chitin (Sigma) and the crude solid A extracted by the standard chemical procedure.

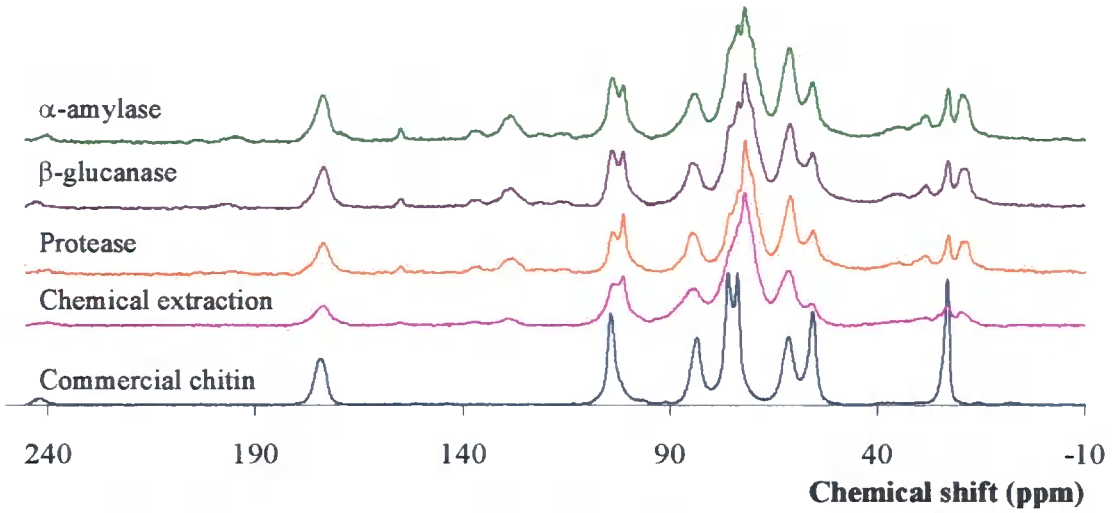


Figure 4.44 - ^{13}C CPMAS ssNMR spectra of solid A, chitin containing, samples extracted from the dry biomass by the chemical extraction procedure after incubation with a series of hydrolytic enzymes. The spectra of a commercial sample of chitin (Sigma) and a standard chemical extract are shown for comparison.

The ^{13}C CPMAS ssNMR spectra indicate that all of the samples contain chitin, however, impurity peaks are still present in each of the samples, the intensity, line-width and chemical shift of which remain largely unaltered. The anomeric carbon peak, at approximately 104 ppm, remains a composite of several peaks with a large line-width, suggesting the presence of non-chitinous polysaccharides, such as β-glucans. The peaks in the chemical shift region of sugar ring carbons also remain unresolved and broad. It was hoped that incubation with α-amylase and β-glucanase would remove these impurity polysaccharides, however, the ^{13}C CPMAS ssNMR spectra suggest that none of the enzymes investigated have achieved this. The remaining impurity peaks in the methylene envelope and in the chemical shift region of 110 to 160 ppm indicate that protease, as well as the other enzymes investigated, has been ineffective at removing the protein impurities.

Elemental analyses of the acid soluble fractions resulting from the chemical extraction procedure (solid B) indicate very low carbon contents and negligible nitrogen contents in all samples. Further analyses suggest that the samples contain high concentrations of calcium and phosphate, therefore we can conclude that chitosan has not been

extracted and the acid soluble fraction contains a calcium phosphate or calcium hydroxyl apatite.

We can conclude that incubation of the dry biomass with a neutral protease, α-amylase and β-glucanase did not significantly alter the yield of solid A, the chitin containing fraction, or solid B, the chitosan containing fraction. Elemental analysis of solid B suggests that, as in the case of the normal chemical extract, it is mainly comprised of calcium and phosphate. ^{13}C CPMAS ssNMR and elemental analysis of the crude chitin-containing fraction indicates the level of purity is not increased compared to the normal chemical extraction product.

It is possible that the quantities of enzymes incubated with the biomass were not sufficient to allow complete degradation of the associated cell wall component, as the quantities of enzymes added were estimated, using the activity details provided by the supplier (Sigma) and assumed concentrations of substrates in the cell wall. Therefore the experiment was repeated under the same conditions using twice the concentration of protease and β-glucanase, in place of α-amylase a control experiment was carried out incubating the biomass in a buffered solution at pH 7. After incubation the biomass was collected by centrifugation and subjected to the standard chemical extraction procedure, the crude chitin containing fraction, solid A, was also purified. The yields of solid A, purified solid A and solid B are shown in Table 4.14, the carbon to nitrogen ratio of the purified chitin, as indicated by elemental analysis, and glucosamine content are also shown.

Enzyme	Yield (%)			C/N*	Glucosamine
	Crude solid A	Purified solid A**	Solid B	Purified solid A	content of purified solid A (%)
Protease	10.0	3.1	2.65	17.0	40
β-glucanase	16.2	3.8	2.76	13.8	38
Control	12.0	3.48	2.65	12.0	55

Table 4.14 - Yield of solid A, purified solid A and solid B extracted from dry biomass by the normal chemical extraction procedure after incubation of the biomass with hydrolytic enzymes. * Ratio of carbon to nitrogen content indicated by elemental analysis. ** Yield of purified solid A given as percentage of starting dry biomass.

Elemental analyses of the acid soluble fractions, solid B, indicates that negligible levels of nitrogen are present in all of the acid soluble fractions, suggesting that chitosan has not been extracted in significant yield. Study of the extraction of solid A indicates that again incubation with protease and β-glucanase did not significantly affect the yield of crude or purified chitin extracted. The carbon to nitrogen values, indicated by elemental analysis of these samples, are largely unaffected by the enzymatic treatment. This is reflected in the results of the glucosamine assay of the purified materials, which suggest that samples incubated with hydrolytic enzymes do not have improved glucosamine concentrations. The ^{13}C CPMAS ssNMR spectra of the purified solid A samples are shown in comparison to a commercial sample of chitin in Figure 4.45. Again the spectra remain largely unaffected by incubation with the hydrolytic enzymes as the peak positions and line-widths are unaltered. The impurity peaks described previously, which indicate the presence of proteins and impurity polysaccharides, are present in the spectra.

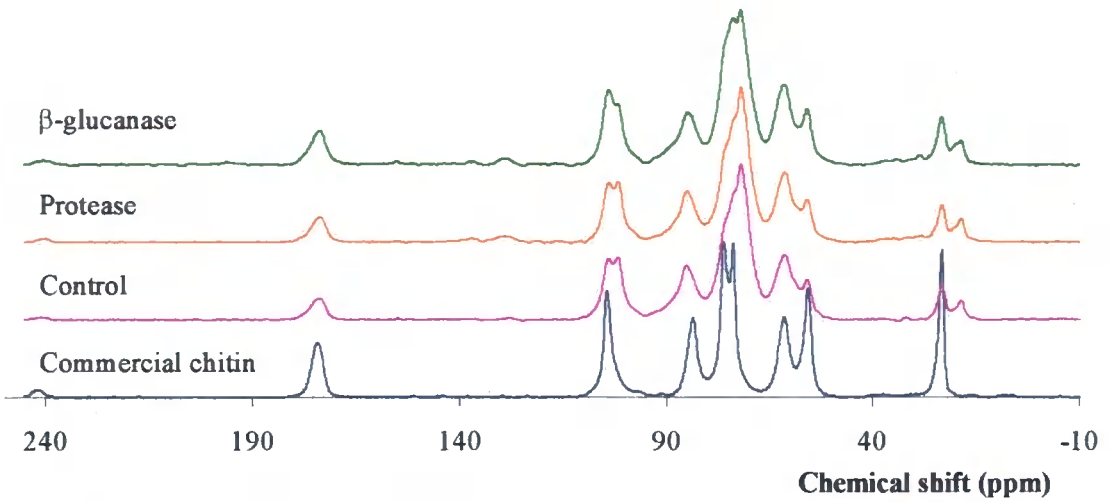


Figure 4.45 - ^{13}C CPMAS ssNMR spectra of purified chitin extracted from dry biomass that was incubated with protease or β-glucanase for 72 hours at 37 °C. The spectra of a purified control sample, incubated under the same conditions in buffer, and commercial chitin (Sigma) are also shown for comparison

We can conclude that incubation of the dry biomass with hydrolytic enzymes does not significantly alter the yield or purity of the chitinous material extracted. One possible explanation for this is that the components of the cell wall that we are aiming to degrade are too inaccessible for the enzymes to act efficiently. The biomass had been heat treated before it was delivered to us, which may have locked the cell wall components in their most stable conformations, resulting in a highly crystalline structure, which is impervious to enzyme action. We therefore hypothesised that treatment of the 'wet' fungal biomass may result in increased yields of chitin, of improved quality. We were unable to obtain the biomass remaining from the industrial fermentation of *Penicillium chrysogenum* that had not been heat-treated and, unfortunately, Angel Biotechnology were unable to provide the exact strain of *P. chrysogenum* used in their penicillin manufacturing process for commercial reasons. Therefore, under guidance from Angel Biotechnology, we acquired and cultured a strain of *P. chrysogenum* termed P2 that is also known to be a penicillin producer and which could be used as a suitable model system.

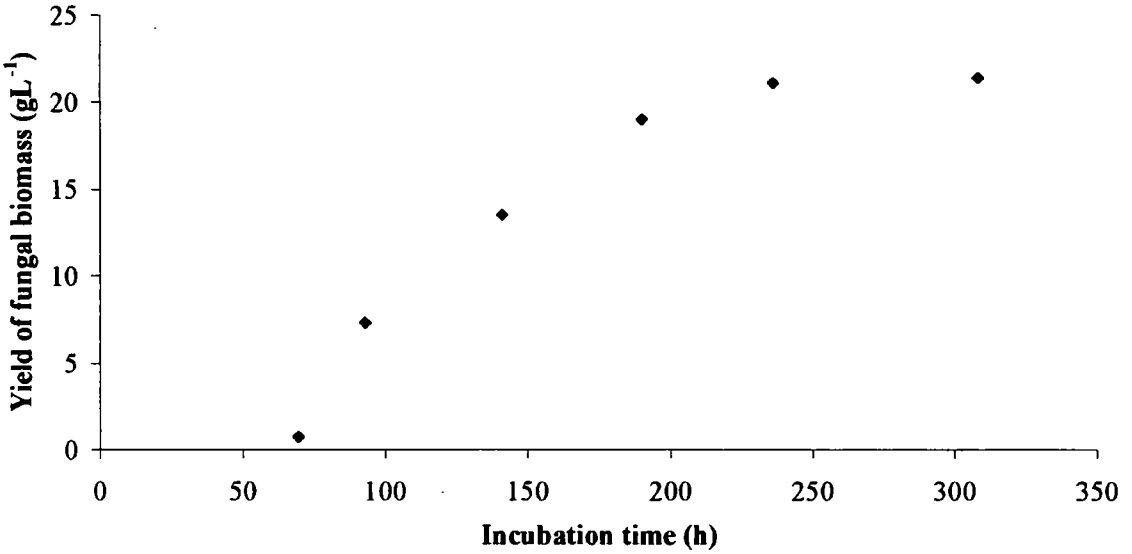
4.8 Extraction of Chitinous Materials from 'Wet' Fungal Material

In order to investigate the extraction of chitinous materials from 'wet' fungal biomass we acquired the P2 strain of *Penicillium chrysogenum*. As the growth rates of fungi vary, the growth curve must be determined in order to ensure that the maximum amount of fungal biomass is obtained. The growth curves of fungi generally display a rapid, 'exponential', growth stage, after which the growth slows and the fungi enter the 'stationary' phase. Therefore, in order to maximise the potential of chitin extraction from this species, its growth was monitored as a function of time. In studies of chitosan extraction from fungal sources reported in the literature it has been suggested that the maximum quantity of chitosan extracted from fungal biomass varies with the growth stage and does not necessarily coincide with the point at which maximum fungal biomass is achieved. Tan *et al.* demonstrated that the maximum quantity of extractable chitosan of several *zygomycetes* strains occurs during the 'late-exponential' growth stage, not in the stationary growth stage when the maximum amount of fungal biomass is seen.[101] The authors suggested that this was due to physiological changes in the fungal cell wall and the changing interactions in which chitosan is involved, as the concentration of free chitosan molecules decreased in the stationary growth phase due to anchoring to the cell wall by binding to chitin and other polysaccharides, rendering extraction more difficult. Chitin is likely to follow different extraction characteristics as it forms a more structural component of the fungal cell wall, therefore, in order to ensure that the maximum quantity of chitin was obtained the yield and purity of chitin extracted at several points on the growth curve was monitored.

4.8.1 Determination of the Incubation Time

As described in section 7.3.2, P2 was cultured in a complete medium, suggested by the supplier (LGC promochem), in 1 L shake flasks, containing 500 ml of medium, in a constant temperature, shaking, incubator at its optimum growth temperature, 24.5 °C, at 200 rpm. After 70 hours an appreciable quantity of biomass was seen,

therefore, flasks were stopped at varying time points between 70 and 308 hours. The biomass was collected by filtration, autoclaved and weighed wet. The resulting growth curve showing the yield of wet fungal biomass produced per litre of medium is shown in Graph 4.5. The amount of fungal biomass increased rapidly between 70 and 190 hours of incubation and continued to increase until 236 hours. After this time the growth slowed down and the fungus appeared to enter the 'stationary' phase.



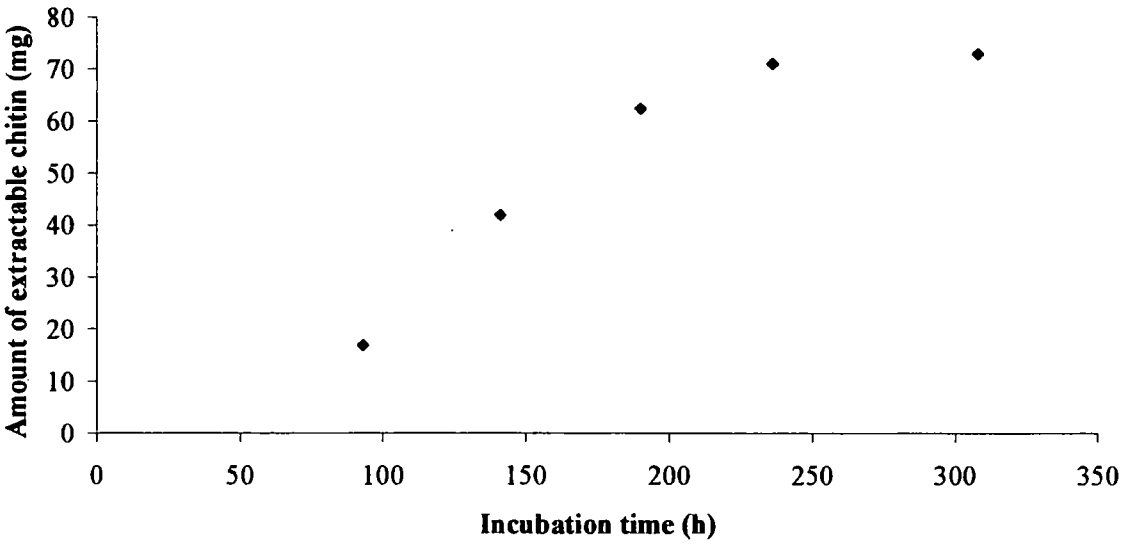
Graph 4.5 - Growth curve of the P2 strain of *Pencillium chrysogenum* cultured at 24.5 °C in a temperature controlled shaker at 200 rpm for varying lengths of time. Yields are shown as grams of wet weight of fungal biomass produced per litre of medium.

The fungal biomass collected was subjected to the standard chemical extraction procedure described in section 7.4.3, with a slight modification, the solids were collected and washed by filtration in place of centrifugation as this produced the same results and was more time efficient. The resulting samples of purified solid A were analysed by ^{13}C and ^{15}N CPMAS ssNMR, the spectra, not shown, indicate that the samples do contain chitin. The resulting yields of chitin containing samples are shown in Table 4.15. The glucosamine contents of each of the samples were assayed, as described in section 7.2.2, and used to determine the amount of extractable chitin present in the biomass.

Culture Time (h)	Yield of purified solid A (%)	Glucosamine content* (%)	Amount of extractable chitin ** (mg)
93	5.92	64	17
141	8.00	59	42
190	5.91	76	62
236	9.37	60	71
308	11.19	79	73

Table 4.15 - Yield of purified solid A, containing chitin, extracted by the standard chemical procedure from the fungal biomass produced by the incubation of P2 for varying lengths of time. * Glucosamine determination of the purified solid A was carried out as described in section 7.2.2. ** Amount of extractable chitin was calculated from the yield of purified solid A taking the glucosamine content of the sample into account.

As shown in Graph 4.6, the calculated amount of extractable chitin in the fungal biomass increases with incubation time and follows a similar trend to that seen in Graph 4.5, rising rapidly up to incubation times of approximately 200 hours. After 200 hours the increase in the amount of extractable chitin slows considerably. It was decided that 8 days, 192 hours, was sufficient incubation time to ensure that a reasonable yield of extractable chitin was observed as the fungus is in its 'late exponential' growth stage and increasing the incubation time further than this does not result in a significant increase in the yield of chitin extracted. Therefore all future cultures of the P2 strain of *Pencillium chrysogenum* were incubated at 24.5 °C for 8 days in a temperature controlled shaker at 200 rpm.



Graph 4.6 - The amount of extractable chitin present in fungal biomass produced by the incubation of P2 for varying lengths of time.

Our studies of the chitinous materials in fungal cell walls were hindered by the lack of a reliable and efficient method of studying the DDA of samples across a wide range of values, this is discussed at length in Chapter 3. We determined that the most reliable method of determining this characteristic is ^{15}N CPMAS ssNMR, as this method of analysis is not compromised by the presence of polysaccharide impurities and does not require solubilisation or derivatisation of the sample rendering it applicable to a wide range of DDAs. However, the analysis is limited by the low natural abundance of ^{15}N, which results in extremely long acquisition times to produce reasonable spectra. We therefore developed a simple, cost-effective, novel strategy for the ^{15}N labelling of chitinous materials in fungal cell walls that resulted in chitinous material in which upwards of 65 % of the monomer units contain a ^{15}N nuclide. This decreases the acquisition time required for ^{15}N CPMAS ssNMR significantly and increases the quality of the spectra obtained by two orders of magnitude allowing fast and reliable determination of the DDA of the chitinous material. Additionally, the confidence limits in determining the DDA are improved from ± 5 % to ± 0.5 %, allowing determination of DDAs between 0.5 and 99.5 %. This labelling method involves the addition of 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$ to the normal complete medium, this is described in full in Chapter 5. Therefore, from now onwards all fungal species will be cultured

in the complete medium containing 0.96 gL^{-1} of labelled additive as described in section 7.3.2.

4.8.2 Standard Chemical Extraction of 'Wet' Fungal Biomass

The P2 strain of *P. chrysogenum* was incubated at 24.5 °C in a temperature-controlled shaker, at 200 rpm, for eight days, as described in section 7.4.3. This produced fungal material with a spherical morphology, as shown in Figure E.83. The biomass was collected by filtration and weighed 'wet', 28 g (± 4 g from 8 repetitions) of 'wet' biomass were produced per litre of culture medium. Samples of this 'wet' biomass were dried at 95 °C until a constant weight was achieved; the biomass lost 82 % (± 1 % from 3 repetitions) of its mass, indicating that the samples, on average, contain 82 % water. ^{13}C and ^{15}N CPMAS ssNMR analysis was carried out on autoclaved samples of the whole cell, 'wet' biomass. The 'wet' biomass was then subjected to the standard chemical extraction procedure, as described in section 7.4.6. The extraction procedure produced an acid soluble fraction the elemental analysis of which indicates that it contains negligible concentrations of carbon and nitrogen. It was concluded, that as for the extractions from dry biomass, the acid soluble fractions is mainly comprised of calcium and phosphorus, in the form of a phosphate.

The acid insoluble fraction, solid A, was analysed by ^{13}C and ^{15}N CPMAS ssNMR in the crude and purified form, the ^{13}C spectra are shown along with the spectra of the whole 'wet' fungal cell prior to extraction and a commercial source of chitin (Sigma) in Figure 4.46. The presence of coincident peaks in the spectra of the whole cell and extracted samples with the spectrum of commercial chitin indicates that the samples do contain chitin. In the spectrum of the whole cell the presence of numerous peaks in the chemical shift region that is characteristic of anomeric carbons, ~100 ppm, suggests the presence of non-chitinous polysaccharides, such as β-glucans. This is reinforced by the presence of additional, non-chitinous, peaks between 50 and 90 ppm. The spectrum of the crude chitin containing extract, solid A, also indicates the presence of polysaccharide impurities as the peak representing the anomeric carbon is

broad and displays a shoulder at approximately 98 ppm. The peaks representing the sugar ring backbone carbons, with chemical shifts between 50 and 90 ppm, are also broad and unresolved. In particular a large shoulder can be seen on the peak at approximately 70 ppm, representing an impurity. Purification of this solid results in an overall decrease in the line-width of the spectra and a reduction in the intensity of the impurity shoulders at 98 ppm and 70 ppm, indicating that the chitinous material contains less impurity polysaccharides. This is reflected in the increased glucosamine concentration of the sample upon purification and the reduction in the ratio of carbon to nitrogen content, shown in Table 4.16. However, comparison of the spectra of the purified sample to that of commercial chitin indicates that polysaccharide impurities still remain.

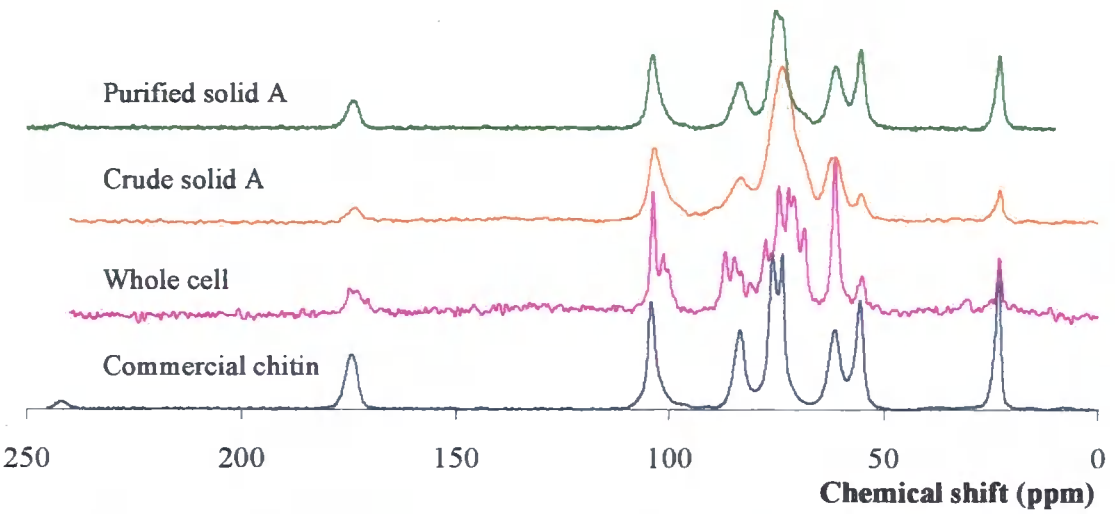


Figure 4.46 - ^{13}C CPMAS ssNMR spectra of the whole cell sample of *P. chrysogenum*, the crude chitin containing fraction, solid A, and the purified chitin fraction. The spectrum of a commercial sample of chitin (Sigma) is shown for comparison.

The peaks in the whole cell ^{13}C CPMAS ssNMR spectrum generally display narrower line-widths than the extracted samples, this probably due to an increased level of mobility within the sample as it is analysed 'wet', this is discussed more fully in section 3.9.3. The increased resolution that this affords may allow determination of the levels of the chitinous and non-chitinous, β-glucan, polysaccharides present in the whole fungal cell. This direct determination of the fungal cell wall composition *in vivo* has many potential applications, for example monitoring the effects of growth

conditions, nutritional supplements and anti-fungal agents in order to either maximise production of one cell wall component, chitin, or investigate potential fungicidal agents. The *in vivo* determination could negate the need for lengthy extraction procedures, which can degrade the cell wall components resulting in inaccurate indicators of cell wall composition. This is discussed further in the future work section of chapter 6.

The corresponding ^{15}N CPMAS ssNMR spectra of the materials are shown in Figure 4.47. No significant change in the chemical shift of the peaks representing the acetamide group of chitin and the amine group of chitosan are seen upon extraction or purification of the whole fungal cell material and no additional peaks are displayed in the spectrum. The line-width of the peak does increase slightly upon extraction and purification, this can be explained by a reduction in the mobility of the chitin chains upon drying of the sample as the whole cell material was analysed 'wet'. This is explained further in section 3.9.3. The small peak at approximately –357 ppm indicates that the chitinous material has a very low DDA of approximately 0.8 ± 0.5 %, this is constant in the ^{15}N CPMAS ssNMR spectra of the material at all stages of extraction. The pertinent portion of the ^{15}N CPMAS ssNMR spectrum of the purified solid A is expanded by a factor of ten to demonstrate this, this peak is seen in the spectra at all stages of extraction. The degree of similarity between the spectra reinforces the conclusion drawn in Chapter 5, that ^{15}N CPMAS ssNMR of ^{15}N labelled whole cell samples may be employed to analyse the DDA of chitinous materials without the need for lengthy extraction procedures.

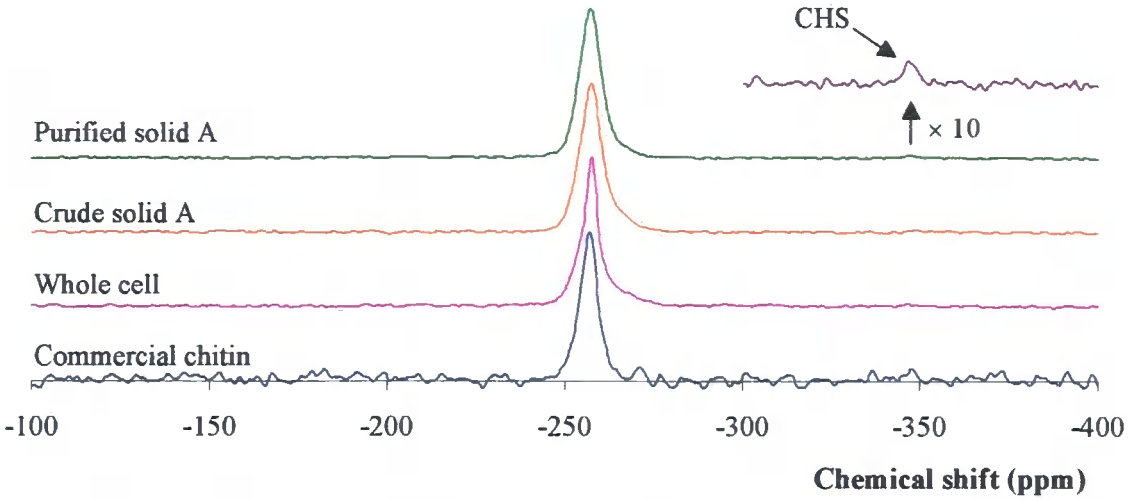


Figure 4.47 - ^{15}N CPMAS ssNMR spectra of the whole cell sample of *P. chrysogenum*, the crude chitin containing fraction, solid A, and the purified chitin fraction. The spectrum of a commercial sample of chitin (Sigma) is shown for comparison. The chemical shift range of purified solid A between –300 to – 400 ppm is expanded (× 10). CHS = amino peak of chitosan.

The yields and analyses of the crude and purified chitin containing fraction, solid A, are shown in Table 4.16. These results reinforce the conclusion drawn from the ^{13}C CPMAS ssNMR spectra, that purification of the crude solid A reduces the concentration of non-chitinous impurities as the glucosamine content increases from 33 % to 81 % and the carbon to nitrogen ratio decreases from 31.7 to 9.13. The very high ratio of carbon to nitrogen of the crude chitin material is not directly reflected in the analysis of the glucosamine content. Analysis of the crude chitinous material extracted from the dry biomass indicated a carbon to nitrogen ratio of 12.3, which is far lower than that of the crude extract from wet biomass indicating a higher degree of purity. This increased purity is seen in the glucosamine contents of the samples, however the difference does not appear to be as great, as glucosamine determination of the extract from dry biomass suggests the sample contains 17 % glucosamine compared to 33 % in the extract from wet biomass. There are two possible explanations for this variation; the glucosamine assay is dependent upon complete hydrolysis of the chitinous material, if the material is not sufficiently hydrolysed an inaccurately low concentration of glucosamine may be observed. If the extract from dry biomass is of a more crystalline nature than that of the wet biomass it may not be as efficiently hydrolysed. Alternatively, the impurities present in the extract from dry

biomass may contain a higher concentration of nitrogen, for example proteins. These nitrogen-containing impurities may be more efficiently removed in deproteinisation of wet biomass, resulting in a relatively higher concentration of non-nitrogen containing impurities and so a high ratio of carbon to nitrogen.

Analysis	Crude solid A	Purified solid A
Yield	31.8 ± 6 *	10.2 ± 1 *#
C:N**	31.7 ± 0.5 *	9.13 ± 1 *
Glucosamine content	33 %	81 %
DDA#	0.8 %	0.8 %

Table 4.16 – Analysis of the crude and purified chitin containing, solid A, fraction extracted by the standard chemical procedure from the wet fungal biomass produced upon incubation of *P. chrysogenum*. * errors given as standard deviations of 8 repetitions. ** C:N calculated by elemental analysis. # yield of purified solid A relative to starting weight of biomass assuming 82 % loss on drying. # ± 0.5 %

Taking the glucosamine concentration of the purified extract into account, the results suggest that the overall yield of chitin extracted from the 'wet' biomass is 8.29 %. The whole fungal cell material was subjected to the glucosamine assay, which indicated that it contains approximately 10 % chitinous material. Therefore we can conclude that the chemical extraction procedure results in the extraction of approximately 83 % of the chitinous material present. Analysis of the glucosamine content of the residue remaining from the purification procedure indicates that it contains approximately 4 % glucosamine, this may account for the remaining chitinous material not present in the purified chitin extract, as highly crystalline chitin may not be solubilised in the purification procedure.

4.8.3 GPC Analysis of Solid 'A' Extracted from 'Wet' Biomass

The refractive index trace obtained by the GPC analysis of the crude and purified solid A is shown in comparison to that of a commercial sample in Figure 4.48. The crude and purified samples both have retention volumes higher than that of commercial chitin, indicating that they have a lower molecular weight. As in the case of the

extracts from dry biomass, the purified sample has a higher retention volume than the crude extract; therefore, we can assume that the molecular weight has decreased upon purification due to degradation of the polymer chain. The whole cell sample has a multimodal distribution, which may be explained by the presence of more than one polysaccharide system such as chitin and β-glucans. Alternatively, this may be caused by the presence of chitinous polymers with varying degrees of polymerisation. Extraction of this material to give the crude solid A, containing chitin, results in a trace which has a similar multimodal pattern. If this behaviour can be explained by the presence of more than polysaccharide system, then this may indicate that the non-chitinous polysaccharides are still present. Purification of the material results in a unimodal trace, suggesting that either the majority of the impurities have been removed, or that degradation of the chitinous polymers has resulted in polymers with a unimodal distribution of molecular weights.

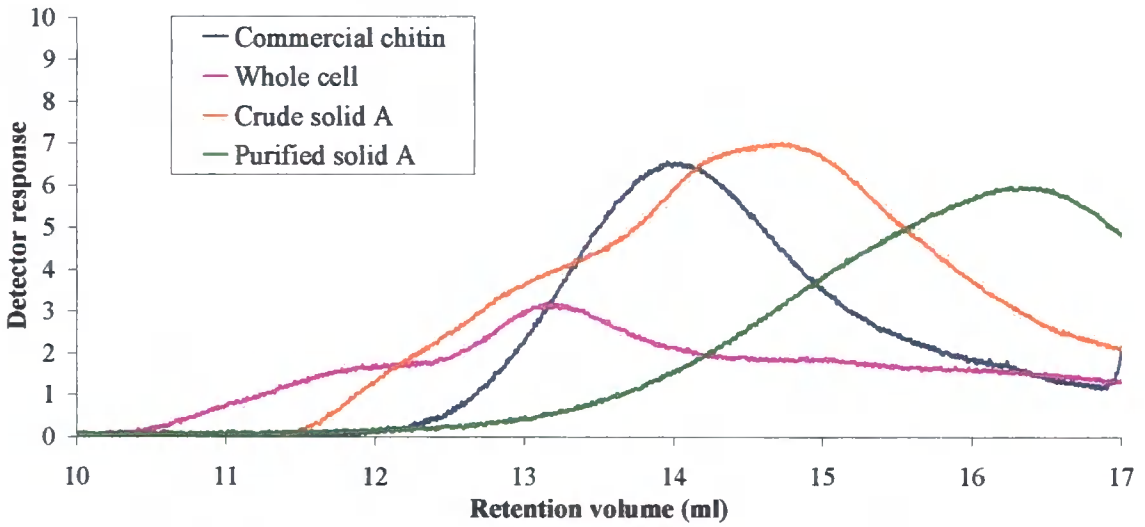


Figure 4.48 - Refractive index trace produced by the GPC analysis of commercial chitin (Sigma), the whole cell sample, the crude solid A and the purified solid A extracted from 'wet' biomass using 5 % (w/v) LiCl:DMAc as the solvent and eluent.

4.8.4 Comparison of Wet and Dry Biomass

The yields and analyses of the purified chitin extracted using the standard chemical extraction procedure, described in sections 7.4.3 and 7.4.6, from the dry biomass

provided by Angel Biotechnology and from wet fungal biomass produced by the incubation of *P. chrysogenum* are shown in Table 4.17.

Analysis	Dry Biomass	Wet Biomass
Yield	1.88 ± 0.1 (5) *#	10.2 ± 1 (8) *#
C:N**	9.7 ± 1 (5) *	9.13 ± 1 (8) *
Glucosamine content	57 %	81 %
DDA	≤ 5 %	0.8 ± 0.5 %

Table 4.17 - Analysis of the purified chitin containing, solid A, fraction extracted by the standard chemical procedure from the dry biomass provided by Angel Biotechnology and the wet fungal biomass produced upon incubation of *P. chrysogenum*. * errors given as standard deviations the number of repetitions are shown in brackets. ** C:N calculated by elemental analysis. # yield of purified solid A relative to starting weight of biomass, in the case of wet biomass assuming 82 % loss on drying.

The results suggest that the wet fungal biomass does provide a more efficient source of chitin as the yield and purity of the material is significantly higher. The glucosamine concentration, as indicated by the assay described in 7.2.2, of the sample extracted from wet biomass is 24 % higher than that from dry biomass, this is reflected in the ratio of carbon to nitrogen content of the samples, as indicated by elemental analysis, as the value of the extract from the dry biomass is lower than that from the 'wet'. Comparison of the ^{13}C CPMAS ssNMR spectra also indicates that the purified sample extracted from wet biomass contains fewer impurities than that from dry biomass, the spectra are shown in comparison to a spectrum of a commercial sample of chitin (Sigma) in Figure 4.49. The large shoulder peaks of the C4 (~83 ppm) and C3 (~73.6 ppm) peaks in the spectrum of the extract from dry biomass are markedly reduced in the spectrum of the extract from wet biomass. The impurity peaks present at approximately 30 ppm in the extract from dry biomass are also not present in the extract from 'wet' biomass.

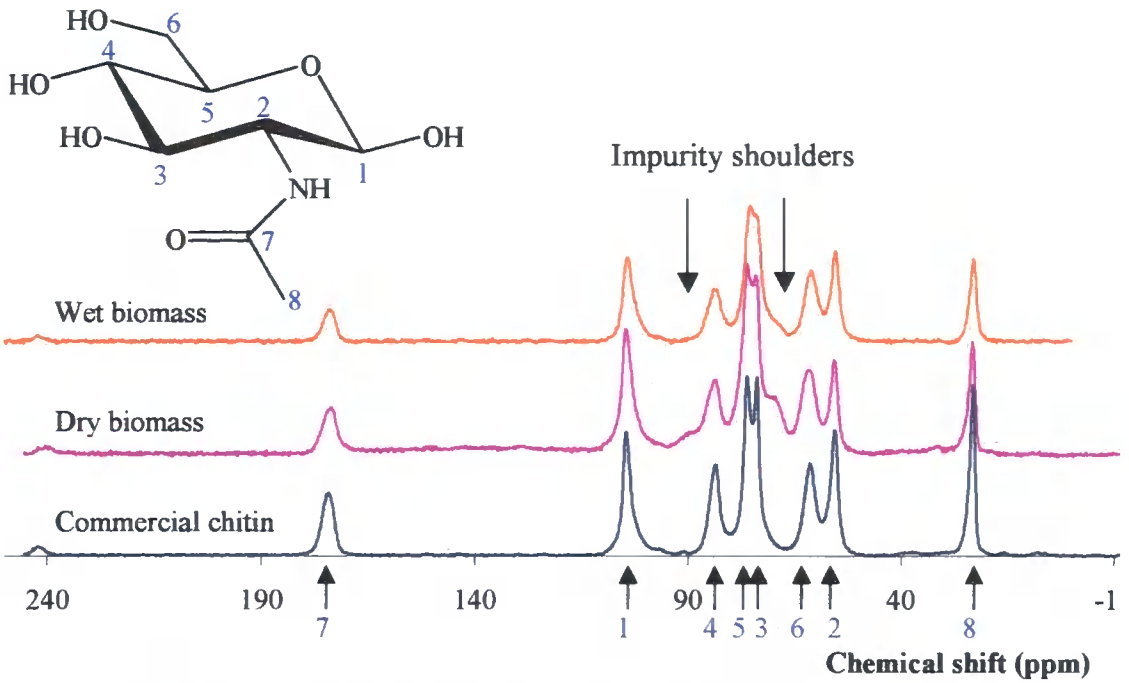


Figure 4.49 - ^{13}C CPMAS ssNMR spectra of purified chitin extracted from the dry biomass obtained from Angel Biotechnology and from wet biomass produced by the incubation of *P. chrysogenum*. The spectrum of a commercial sample of chitin (Sigma) is shown for comparison.

The ^{15}N CPMAS ssNMR spectrum of the extract from the dry biomass, shown in Figure 4.50, displays only one signal, the characteristic chitin peak at approximately –257 ppm. The low signal to noise ratio in the spectrum means that any signals with a relative intensity below 5 % cannot be resolved; therefore a DDA of ≤ 5 % is indicated. The ^{15}N CPMAS ssNMR spectrum of the extract from wet biomass demonstrates the major advantage of using wet biomass in these studies; namely, that the biomass can be cultured under ^{15}N labelling conditions, resulting in ^{15}N labelled chitinous material. The ^{15}N CPMAS ssNMR of the unlabelled samples extracted from dry biomass required 60,000 repetitions to acquire, however, the labelled sample required just 2,000. This reduces the acquisition time from over 16 hours to under one hour and produces a spectrum of higher quality allowing determination of the DDA of samples within a confidence limit of ± 0.5 %, compared to ± 5 % for unlabelled samples. The spectrum of the sample extracted from wet biomass displays a small peak at –348 ppm, the integration of which suggests that the sample has a DDA of 0.8 ± 0.5 %. The low intensity of this peak means that it is difficult to see in the spectrum

in this format, therefore, this peak is expanded in Figure 4.50. The ^{15}N labelling of fungal material and the associated benefits are discussed fully in Chapter 5.

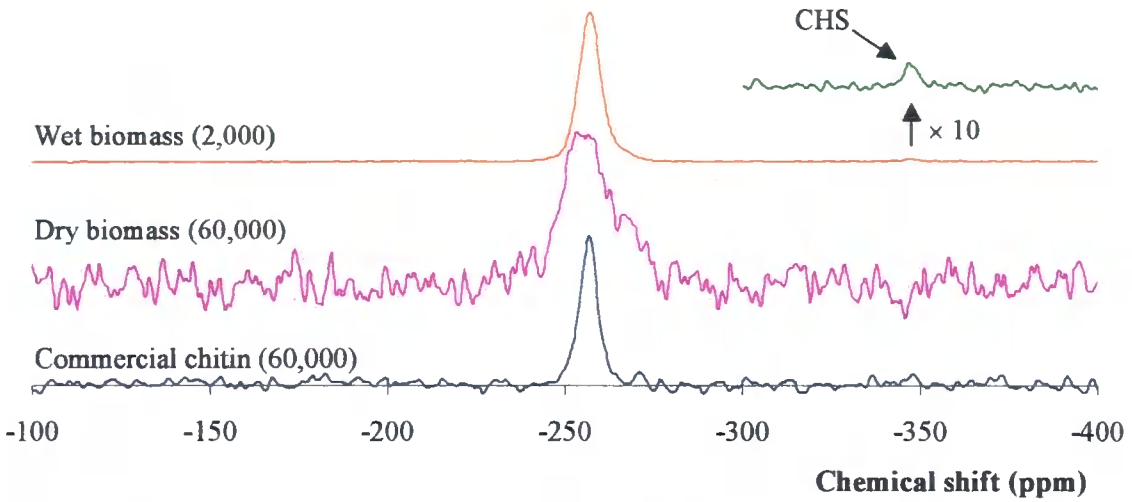


Figure 4.50 - ^{15}N CPMAS ssNMR spectra of purified chitin extracted from the dry biomass obtained from Angel Biotechnology and from wet biomass produced by the incubation of *P. chrysogenum*. The spectrum of a commercial source of chitin (Sigma) is shown for comparison. The number of repetitions required to acquire the spectra are shown in brackets. The chemical shift range from –300 to –400 ppm of the wet biomass spectrum is expanded by a factor of ten. CHS = the amino peak of chitosan which is expanded above.

The refractive index traces obtained by the GPC analysis of the purified solid A extracts, containing chitin, from the dry and 'wet' biomass are shown in comparison to that of a commercial sample in Figure 4.51. The trace of the extract from dry biomass indicates that the sample has a lower retention volume than that from 'wet' biomass, which indicates that it has a higher molecular weight. This may be explained by increased degradation of the chitinous polymers in the extract from 'wet' biomass, however, it is also possible that the different strains of *P. chrysogenum* possess chitinous systems with different degrees of polymerisation. The 'wet' biomass extract was produced from the culture of the P2 strain of *P. chrysogenum*, however, the dry biomass was a gift from Angel Biotechnology and due to Intellectual Property issues we were unable to ascertain from which strain of *P. chrysogenum* it was produced. The bimodal distribution seen in the extract from dry biomass was discussed earlier where it was proposed that this could indicate degradation of the polymer during the

extraction procedure. This is not seen in the extract from 'wet' biomass, which has a unimodal distribution, possibly suggesting a lower degree of degradation.

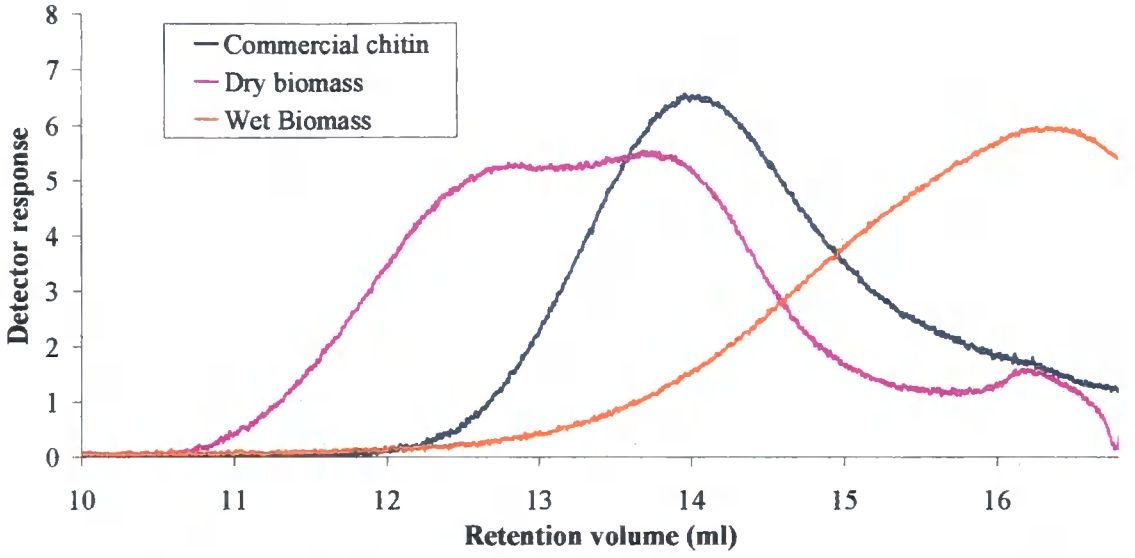


Figure 4.51 - Refractive index trace produced by the GPC analysis of commercial chitin (Sigma), the purified solid A extracts from 'wet' and dry biomass using 5 % (w/v) LiCl:DMAc as the solvent and eluent.

Comparison of the purified extracts produced upon application of the standard chemical extraction procedure to dry and wet biomass indicates that wet biomass produces a higher yield of chitinous material, which has a higher purity. Therefore, when applied to the waste materials of the production of penicillin, if it is possible to extract the biomass prior to heat treatment the yield and purity may be improved. For the purposes of this study, the culture of the fungi and use of 'wet' biomass is also advantageous as it allows the possibility of ^{15}N labelling the chitinous material, allowing fast, reliable, determination of the DDA without the need for lengthy extraction procedures.

4.9 Enzymatic Extraction of Chitinous Materials from Wet Biomass

The enzymatic extraction procedures discussed in relation to the dry biomass in section 4.7. were also applied to the 'wet' biomass produced upon incubation of

P. chrysogenum, as it was hypothesised that the constituents of the 'wet' fungal cell walls may be more available to enzymatic action due to a decrease in the level of crystallinity. The fungal material was also cultured in the presence of these hydrolytic enzymes in order to investigate their effect on nascent substrates, in the following sections these results will be discussed. In addition, the procedure proposed by Cai *et al.* for the total enzymatic extraction of chitinous materials from *A. niger*, which was discussed in section 4.6.1, is investigated and the solids obtained from extraction of *P. chrysogenum* and *A. niger* biomass in this way compared.

4.9.1 Incubation of Wet Biomass with Hydrolytic Enzymes

In order to investigate the difference in the susceptibility of wet and dry biomasses to enzymatic extraction procedures the wet fungal biomass produced by the incubation of the P2 strain of *P. chrysogenum* under standard, ^{15}N labelling conditions, was collected by filtration and then incubated with the hydrolytic enzymes described in section 4.7. The ratio of enzyme to biomass, the buffer conditions, incubation time and temperature were maintained at the same values as those employed in extraction of the dry biomass. A control experiment was also carried out by incubating the wet biomass in a buffered solution at pH 7.5 under the same conditions. After incubation the biomass from each experiment was collected by filtration and subjected to the standard chemical extraction procedure. This produced acid soluble fractions containing high concentrations of calcium and phosphorus and negligible concentrations of nitrogen and carbon, as seen by elemental analysis. The acid insoluble fractions were purified by the standard procedure, the yields and analyses are shown in Table 4.18.

	Yield of purified solid A (%)	C:N*	Glucosamine content of purified solid A (%)	DDA ** (%)
Control	9.6	19.8	64	≤ 0.5
Protease	8.6	15.7	53	≤ 0.5
β-glucansase	9.8	19.0	50	≤ 0.5
α-amylase	9.3	19.2	44	≤ 0.5

Table 4.18 - Analytical data of purified chitinous extracts from wet biomass incubated with hydrolytic enzymes prior to chemical extraction. *Indicated by elemental analysis. ** Indicated by ^{15}N CPMAS ssNMR.

The data indicate that, as with the extraction of dry biomass, incubation of the wet biomass with hydrolytic enzymes prior to chemical extraction does not significantly alter the yield or purity of the chitinous material extracted. Elemental analyses and assays of the glucosamine content both indicate that the levels of impurities of samples incubated with the enzymes are similar to those seen in the control. This can also be seen in the ^{13}C CPMAS ssNMR spectra of the samples, shown in Figure 4.52. The spectra of all of the samples display coincident peaks, which have similar line-widths and resolution. ^{15}N CPMAS ssNMR analysis of the samples was also carried out, these spectra (not shown) display the characteristic acetamide peak of chitin with unvarying chemical shifts and line-widths, indicating that the samples all have DDAs ≤ 0.5 % and are of a similar crystalline nature.

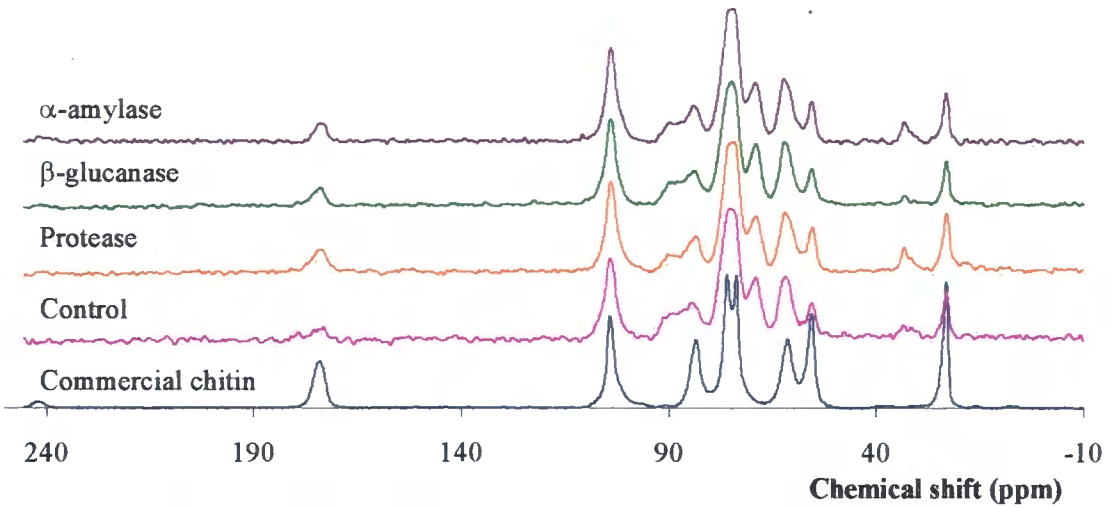


Figure 4.52 - ^{13}C CPMAS ssNMR spectra of chitin containing samples extracted by the standard chemical procedure from wet biomass that had been incubated with hydrolytic enzymes.

4.9.2 Culture of *P. chrysogenum* in the Presence of Hydrolytic Enzymes

The inability of the hydrolytic enzymes to reduce the interactions between chitin polymers and other cell wall components may be due to the highly crystalline nature of the chitin network, which prevents the enzymes from accessing the material. It was thought that the enzymes may be more efficient at hydrolysing nascent interactions in the substrate which have not yet formed the highly crystalline hydrogen bonding network. Therefore, the P2 strain of *P. chrysogenum* was cultured in the presence of the aforementioned hydrolytic enzymes. Solutions of the enzymes were added to the autoclaved standard culture medium, the pH of which was adjusted to the optimal pH of the enzymes by addition of 1 M KOH, by filter sterilisation. A control experiment, to which an equal volume of water was added, was also carried out. The medium was then inoculated with spores of P2 and cultured for 8 days under otherwise normal conditions, see section 7.3.2. After this time, the resulting biomass was collected by filtration and weighed 'wet'. The samples were then autoclaved and analysed by ^{13}C and ^{15}N CPMAS ssNMR. The yields of wet biomass produced, shown in Table 4.19,

were largely unaffected by the presence of the hydrolytic enzymes, the values all fall within the error limits of the standard culture (28 ± 4 gL^{-1}). The biomass was subjected to the standard chemical extraction procedure to obtain the crude chitin fraction, solid A. The acid soluble fraction, solid B, was again shown to contain low concentrations of carbon and nitrogen and high concentrations of calcium and phosphorus, indicating that deacetylated chitosan was not extracted in significant yield.

The ^{13}C CPMAS ssNMR spectra of the whole cell samples, analysed 'wet', are shown in Figure 4.53. Comparison of the spectrum of the control sample, cultured without the addition of an enzyme, to the spectra of the samples cultured in the presence of a protease and a β-glucanase, does not reveal any significant differences in the peak positions or line-widths. This indicates that no significant change in the composition of the fungal cell wall has occurred. The whole cell spectrum of the sample cultured in the presence of α-amylase does vary from that of the control sample in the chemical shift region of the anomeric carbons. In all of the spectra two distinct peaks can be seen in this region, at 101 and 104 ppm, with the intensity of the peak at 104 ppm, which coincides with the anomeric carbon peak of the commercial chitin spectrum, being greater. However, in the spectrum of the sample cultured with α-amylase the relative intensity of the peak at 101 ppm is much larger. This may infer a change in the fungal cell wall composition has occurred, increasing the relative concentration of the non-chitinous polysaccharides in this sample. Alternatively, this may imply that the interactions in which the cell wall components are involved may be altered, as the degree of mobility of the cell wall components affects the efficiency of the CP process required to obtain the spectrum. A more mobile component will not be visualised in a CPMAS ssNMR spectrum, therefore the change in relative intensities of these peaks may imply that the chitinous material has a greater degree of mobility, or that the non-chitinous polysaccharide has a lower degree of mobility in this sample.

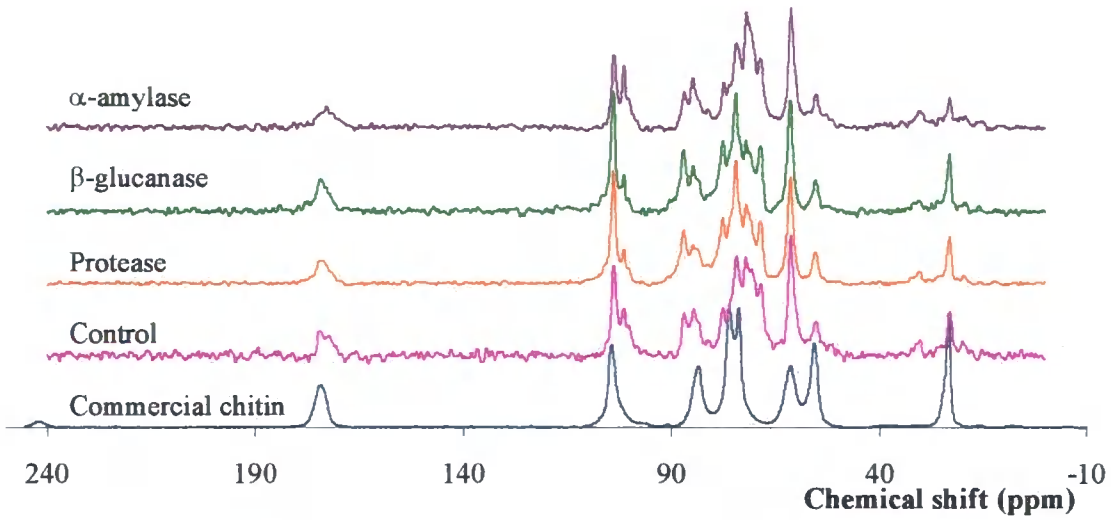


Figure 4.53 - ^{13}C CPMAS ssNMR spectra of the whole, 'wet', fungal biomass cultured in the presence of hydrolytic enzymes. The spectrum of a commercial source of chitin (Sigma) is shown for comparison.

^{15}N CPMAS ssNMR analysis of the samples reveals no significant change in chemical shift or line-width of the acetamide peak of the chitinous material. The spectra, shown in Figure 4.54, indicate that all of the samples have a low DDA. This is demonstrated by the expansion of the spectrum of the biomass culture in the presence of β-glucanase by a factor of ten in the chemical shift region of –300 to –400 ppm. A small peak may be present at approximately –350 ppm, however, this is within the confidence levels of the experiment, ± 0.5 %. Therefore, we can speculate that the samples probably have a non-zero DDA, which is below the limit of detection.

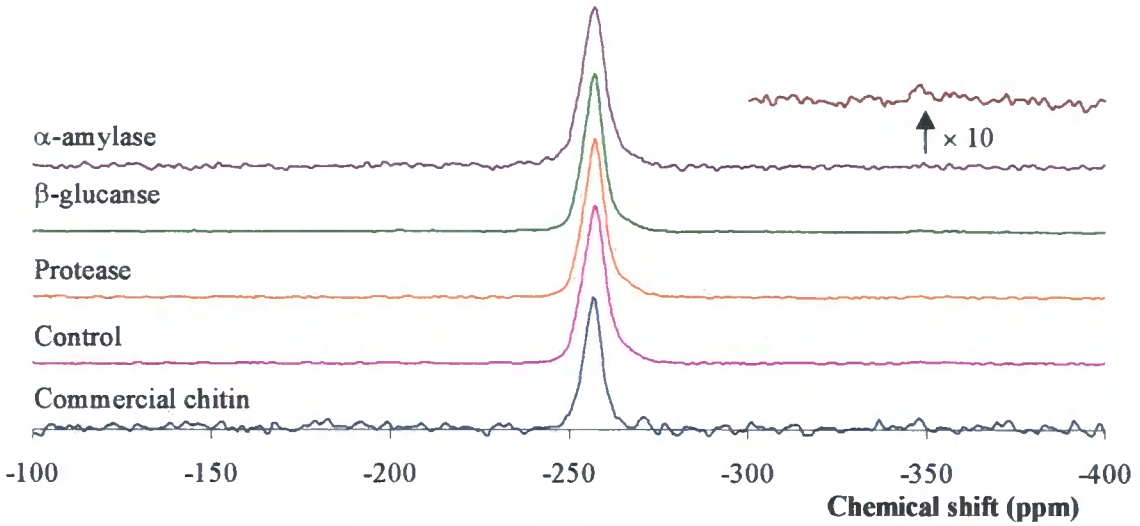


Figure 4.54 - ^{15}N CPMAS ssNMR spectra of whole 'wet' fungal biomass cultured in the presence of hydrolytic enzymes. The spectrum of commercial chitin (Sigma) is shown for comparison. The chemical shift range of –300 to-400 ppm of the β-glucanase spectrum is expanded by a factor of 10.

In order to investigate the effect of incubation with the hydrolytic enzymes on the extent of the interactions between the chitinous material and other cell wall components, the biomass was subjected to the standard chemical extraction procedure described in 7.4.6. The yields of the crude chitin containing fractions, solid A, and their glucosamine contents are shown in Table 4.19. This data suggests that the crude chitinous extracts are of similar levels of purity, as the yields and glucosamine content remain largely unaffected. ^{15}N CPMAS ssNMR analysis of the materials was performed, the spectra, not shown, all display the characteristic chitinous acetamide peak at approximately –257 ppm and no other peaks, indicating a very low DDA. The line-width of the peaks are again similar suggesting that the materials have similar levels of crystallinity.

	Yield biomass (gL^{-1})	Yield crude solid A (%)	Glucosamine content (%)	DDA* (%)
Control	30	36	35	≤ 0.5
Protease	31	32	36	≤ 0.5
β-glucanase	25	42	26	≤ 0.5
α-amylase	29	42	32	≤ 0.5

Table 4.19 - Yield of wet biomass produced from the incubation of P2 in the presence of hydrolytic enzymes. The yield and analysis of the crude chitin-containing fraction, solid A, are also shown. * DDA indicated by ^{15}N CPMAS ssNMR.

^{13}C CPMAS ssNMR analysis of the samples was also performed, the spectra are shown in Figure 4.55. Comparison of the spectra of the control and the sample incubated with protease indicates that little change in the cell wall composition of structure had occurred as a result of the protease treatment. The spectrum of the sample incubated with β-glucanase is largely unaltered from that of the control, with the exception of the impurity peak that occurs at approximately 68 ppm. In the spectrum of the control sample this appears as a shoulder on the chitinous peak at approximately 74 ppm, however in the spectrum of the β-glucanase sample this peak is has improved resolution. As this impurity peak can probably be accounted for by non-chitinous polysaccharides such as β-glucans, this change may indicate that the structure or nature of the chitin-β-glucan interaction has been altered. The spectrum of the sample incubated in the presence of α-amylase resembles that of the control sample with the exception of the impurity peak at 31 ppm, which has a greater relative intensity in comparison to the methyl peak of chitin at approximately 23 ppm. Again this may be explained by a change in the level of mobility of the polysaccharides, which would affect the efficiency of the magnetisation transfer in the cross-polarisation process.

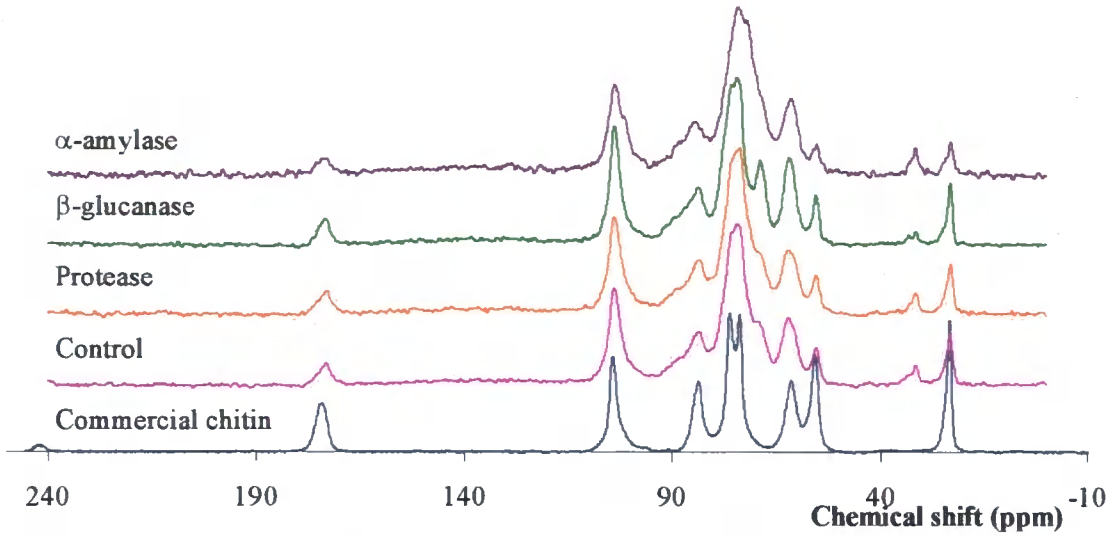


Figure 4.55 - ^{13}C CPMAS ssNMR spectra of crude chitin extracted by the standard chemical procedure from wet biomass which was cultured in the presence of the stated hydrolytic enzymes. The spectrum of a commercial sample of chitin (Sigma) is shown for comparison.

The ^{13}C CPMAS ssNMR spectra of the crude chitin containing samples extracted from biomass cultured in the presence of β-glucanase and α-amylase do indicate slight changes in comparison to the control sample, the nature of which has not been fully determined as the yield and purity of these samples is not improved in comparison to the control experiment this work has not been further examined at this point.

4.9.3 Enzymatic Extraction Procedure of Cai *et al.* [102]

In 2006, half way into our studies, Cai *et al.* reported the enzymatic preparation of chitosan from the waste *Aspergillus niger* mycelium of a citric acid production plant.[102] The authors objectives were not dissimilar to ours, in that they looked to establish a route for the extraction of chitinous material with defined characteristics, suitable for biomedical purposes, from a current waste source, in this case the mycelia produced by a citric acid production plant. They describe the use of three enzymatic systems to recover chitosan of a similar level of purity, structure and DDA as that produced by chemical extraction with a molecular weight three times that of the chemical extract. Snailase (containing a β-glucoronidase, a β-D-mannosidase and a

sulfatase) and lysozyme were first employed to disrupt the fungal cell wall. Incubation with a neutral protease, to deproteinate the sample, was then followed by treatment with a chitin deacetylase. The resulting chitosan was then recovered in an acetic acid extraction step. We wished to assess the applicability of this extraction procedure to *Penicillium* systems, however, unfortunately chitin deacetylase is not currently available commercially and therefore the procedure could not be fully carried out. Initial attempts at obtaining the enzyme from a known source, *Mucor rouxii*, were carried out, as described in section 6.5, however, this work was not optimised and remains as an area of future work. In the absence of a chitin deacetylase we carried out the cell disruption and deproteinisation procedures outlined by the authors, described in section 7.4.7.3, in order to compare the crude chitin extracted to that recovered using the chemical extraction method.

The extracted material was analysed by ^{13}C CPMAS ssNMR, the spectrum is shown in Figure 4.56 in comparison to that of crude chitin extracted by the standard chemical procedure and a commercial source of chitin (Sigma). The spectrum indicates that the sample does contain a polysaccharide, however the intensity of the carbonyl peak at approximately 173 ppm and the methyl peak at approximately 23 ppm are very low, indicating a low concentration of chitin.

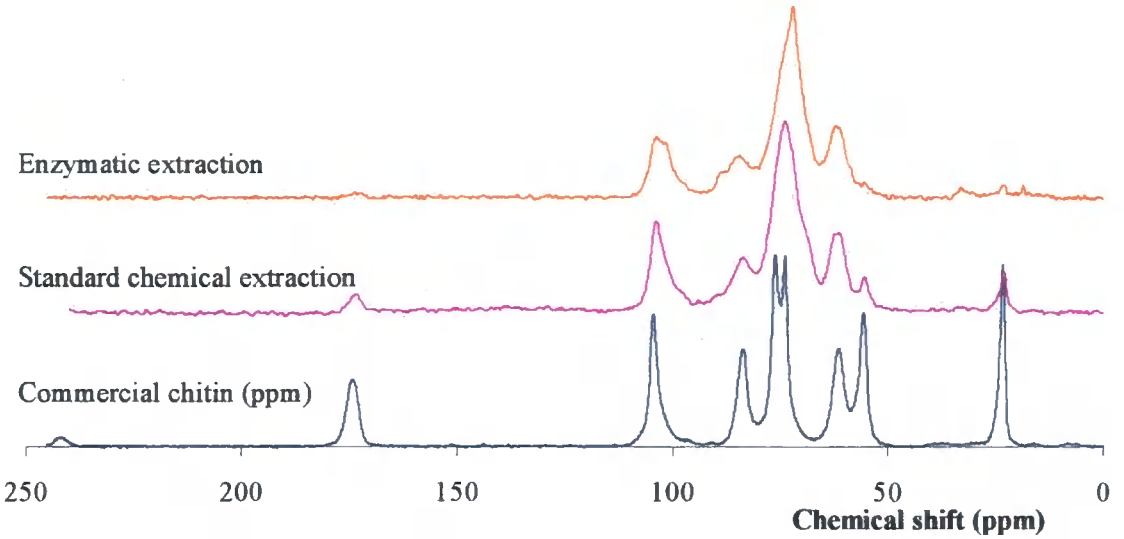


Figure 4.56 - ^{13}C CPMAS ssNMR spectra of crude chitin samples extracted from 'wet' *P. chrysogenum* by the standard chemical procedure and by the enzymatic procedure of Cai *et al.*[102]

Assay of the glucosamine content of the sample also indicates a low chitin concentration of 3.5 %, compared to 33 % in the crude chitin extracted by the standard chemical procedure, see Table 4.20. This conclusion is reinforced by elemental analysis, which indicates that the sample has very low nitrogen content. The data suggests that the sample does contain chitinous material, however the concentration of non-chitinous polysaccharides is far greater.

	Yield (%) *	C:N **	Glucosamine content (%)
Standard chemical extraction	31.8	31.7	33
Enzymatic extraction	33.1	59.6	3.5

Table 4.20 - Comparison of the yield and purity of crude chitin samples extracted from 'wet' *P. chrysogenum* biomass by the standard chemical procedure and the enzymatic procedure of Cai *et al.*[102] * Yield determined assuming 82 % loss on drying of original biomass. ** C:N indicated by elemental analysis.

We can therefore conclude that the enzymatic route of deproteinisation of fungal biomass resulting in crude chitin described by Cai *et al.*[102] is not as efficient as the standard chemical procedure currently employed. The procedures produced similar yields of crude chitin, however, elemental analysis and analysis of the glucosamine contents of the sample indicates that the purity of the crude chitin extracted by the chemical procedure is much greater than that produced by the enzymatic procedure. This can also be seen in the ^{13}C CPMAS ssNMR spectra of the materials. It is possible that the *in situ* deacetylation of the chitinous materials by chitin deacetylase is critical to the extraction of materials of high purity, removing the impurity polysaccharides present in the crude material. However, currently this enzyme is unavailable commercially, therefore this possibility cannot be explored until the extraction of chitin deacetylase has been optimised. Alternatively the low level of purity of the crude material may be due to the nature of the interactions of the fungal cell wall in this source, which renders the chitinous materials intractable. If the matrix of interactions in which chitin is found in *P. chrysogenum* render the material inaccessible to chemical or enzymatic extraction then this fungal species may not be a suitable source of chitin. It is possible that extraction from the mycelium of *Aspergillus niger*, used by the authors in their study, or *Mucor rouxii*, a commonly quoted source of chitinous materials, may yield better results.

4.9.3.1 Enzymatic Extraction of Crude Chitin from *A. niger*

Cai *et al.* do not describe the characteristics of the intermediate, un-deacetylated product in their study; therefore, we can not make a direct comparison between their results and ours. In order to explore the comparative extractability of our fungal source, *Aspergillus niger* was purchased and cultured in the same rich medium as *P. chrysogenum* under the same conditions, the resulting biomass was subjected to the enzymatic extraction procedure of Cai *et al.*[102] The resulting crude material was analysed by ^{13}C CPMAS ssNMR, the spectra are shown in Figure 4.57.

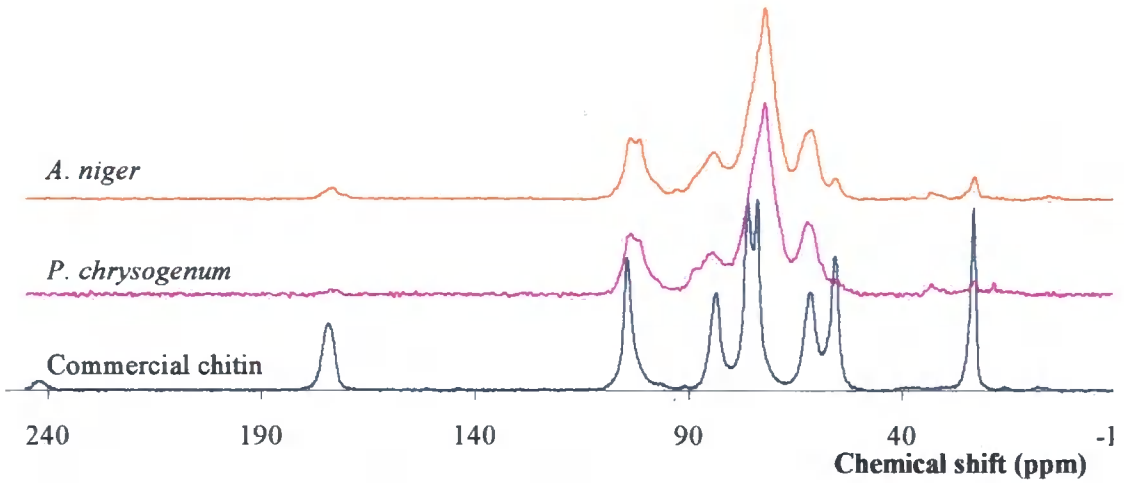


Figure 4.57 - ^{13}C CPMAS ssNMR spectrum of the crude materials obtained from the enzymatic extraction of 'wet' *A. niger* and *P. chrysogenum* biomass. A commercial source of chitin (Sigma) is shown for comparison.

Comparison of the ^{13}C CPMAS ssNMR spectra of the extracted materials to that of commercial chitin indicates that the samples do contain chitin in low yield, however, the presence of a large number of peaks, with large line-widths, in the chemical shift region of 45 to 90 ppm indicates that there is a high concentration of non-chitinous polysaccharide impurities present. The integral of the carbonyl and methyl peaks at 173 and 23 ppm respectively in the spectrum of the material extracted from *A. niger* are increased slightly in comparison to that of *P. chrysogenum*, which indicates that the sample contains a higher concentration of chitin. This is reflected in the glucosamine assay and elemental analysis of the materials, shown in Table 4.21.

	Yield (%) *	C:N **	Glucosamine content (%)
Aspergillus niger	35.1	42.7	15
P. chrysogenum	33.1	59.6	3.5

Table 4.21 - Yields and analysis of materials extracted from the 'wet' biomass of *A. niger* and *P. chrysogenum* by the enzymatic method of Cai *et al.*[102]* Yields calculated assuming 84 % and 82 % loss on drying of *A. niger* and *P. chrysogenum* respectively. ** C:N indicated by elemental analysis.

The results indicate that *A. niger* may provide a better source of chitin, which can be extracted in higher yield. However, without the enzymatic deacetylation step the extraction procedure described by Cai *et al.* does not produce crude chitin of improved yield or quality to that of the chemical extraction procedure. Further work in this area may be carried out in the future.

4.10 Comparison to Other Fungal Species

In order to evaluate the ease of extraction of chitin from *P. chrysogenum* in comparison to other fungal sources, the standard chemical extraction procedure was applied to two fungal species known to be producers of chitinous materials, the resulting materials were compared to the extracts of *P. chrysogenum*. *A. niger* and *Mucor rouxii* were employed for this study as the extraction of chitin has been described from both these sources in the literature.[103, 110, 223, 224] As *Mucor rouxii* is part of the *zygomycetes* it is also known to contain chitosan, which should be extracted as the acid soluble solid B.[103, 223] The fungi were cultured in the standard complete medium, containing 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$, in a temperature controlled shaker at 24 °C, 200 rpm, for eight days. After this time, the resulting biomass was collected by filtration and weighed 'wet'. Samples were dried at 95 °C until a constant weight was achieved to estimate the dry weight of the material. *A. niger* and *M. rouxii* were found to loose 84 and 79 % of their weight upon drying respectively. Samples were then autoclaved and the whole cell materials were analysed by ^{13}C and ^{15}N CPMAS ssNMR, whilst 'wet'. The biomass was subjected to the standard chemical extraction procedure.

The ^{13}C CPMAS ssNMR spectra of the whole cell samples are shown in Figure 4.58 in comparison to the spectrum of *P. chrysogenum* and a commercial source of chitin. The spectrum of the whole cell sample of *A. niger* does not vary greatly from that of *P. chrysogenum*. A similar patterns of peaks in the anomeric carbon region (approximately 100 ppm) and in the chemical shift region of sugar ring carbons (45 to

90 ppm) indicates that the two species possess similar polysaccharide compositions. The line-widths of the peaks are also similar indicating that the structure of the fungal cell walls has a similar level of crystallinity and degree of mobility. However, the spectrum of *M. rouxii* varies greatly from that of the other two fungal species. The poor resolution of the peaks and the broad line-widths suggest that the material has a lower degree of order, with the polysaccharide chains having a higher degree of mobility. There are also a greater number of peaks in the chemical shift region of 0 to 40 ppm, this may indicate a higher concentration of lipids or amino acid side chains. If this fungal material does possess a lower degree of order, and so crystallinity, this may increase the accessibility of the chitin chains resulting in a higher degree of purity upon extraction.

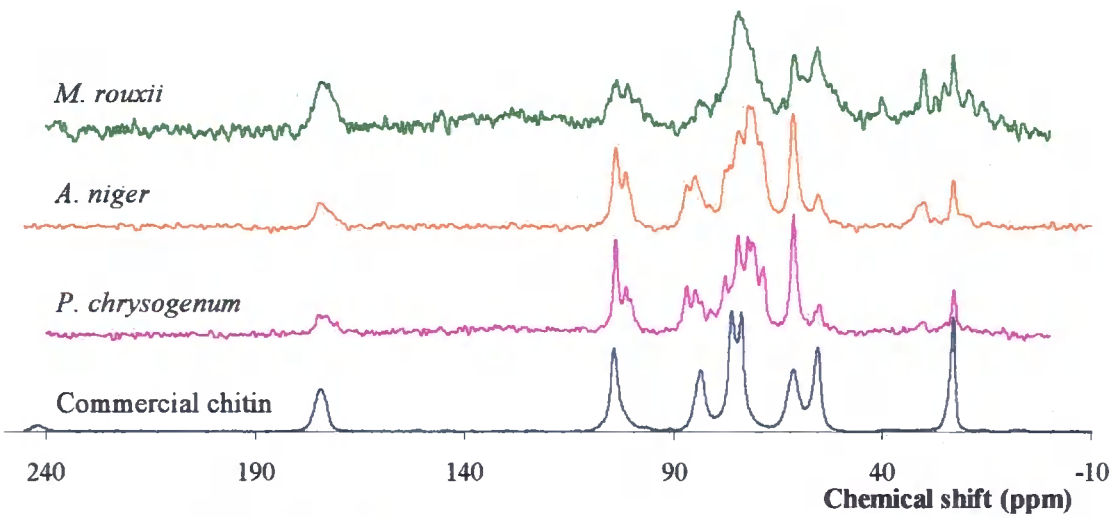


Figure 4.58 - ^{13}C CPMAS ssNMR spectra of the whole cell samples of *P. chrysogenum*, *A. niger* and *M. rouxii*. The spectrum of a commercial source of chitin (Sigma) is shown for comparison.

The corresponding ^{15}N CPMAS ssNMR spectra are shown in Figure 4.59. Again the spectra of *P. chrysogenum* and *A. niger* are very similar, displaying the characteristic acetamide peak of chitin with similar line-widths. The spectra indicate that *A. niger* contain chitinous material with a very low DDA, as the characteristic amino peak of chitosan is not seen, however the DDA of *P. chrysogenum* is estimated at 0.8 ± 0.5 %. Conversely, the spectrum of *M. rouxii* displays both amino and amido peaks indicating the presence of deacetylated chitosan. Study of the integrals of the two peaks suggests that the material has a DDA of 38 %, however, the literature indicates

that the chitinous material of *M. rouxii* exists as two discrete polymers of highly acetylated chitin and deacetylated chitosan, rather than as copolymers of acetylated and deacetylated units.[9] The decreased crystallinity of the sample suggested by ^{13}C CPMAS ssNMR is reflected in the acetamide peak of *Mucor rouxii*, which displays a greater line-width than that of the other two fungi. There is also a noticeable shoulder at –268 ppm, which may indicate the presence of more than one polymorphic form of chitin.

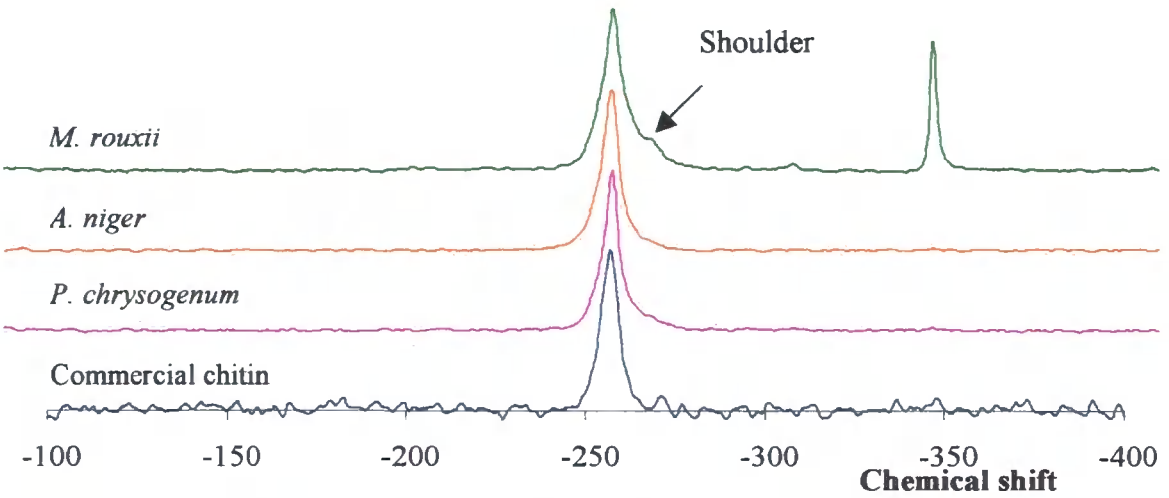


Figure 4.59 - ^{15}N CPMAS ssNMR spectra of the whole cell samples of *P. chrysogenum*, *A. niger* and *M. rouxii*. The spectrum of a commercial source of chitin (Sigma) is shown for comparison.

The wet biomass from each experiment was subjected to the standard chemical extraction and purification procedures described in section 7.4.6. The yields and analyses of the resulting chitin containing fractions, solid A, are shown in Table 4.22. The yield of chitin extracted from *A. niger* was similar to that from *P. chrysogenum*, however, elemental analysis and the glucosamine assay indicate that it was of a slightly higher purity. *Mucor rouxii* produced chitin of the highest level of purity, however, the yield was much lower. This may be because the chitinous material was also present as chitosan which is extracted as the acid soluble fraction solid B, this is discussed further later.

	Yield (%)	C:N *	Glucosamine content (%)	DDA **# (%)
P. chrysogenum	10.2	9.13	81	0.8
A. niger	11.6	8.90	87	≤ 0.5
M. rouxii	1.91	6.90	98	9.1

Table 4.22 - Yield and analysis of purified chitin extracted by the standard chemical procedure from three fungal sources. * C:N indicated by elemental analysis. * DDA indicated by ^{15}N CPMAS ssNMR. # DDAs ± 0.5 %

^{13}C CPMAS ssNMR analysis of the purified materials was carried out, the resulting spectra are shown in comparison to a commercial sample of chitin (Sigma) in Figure 4.60, the spectra indicate that all of the samples contain high concentrations of chitin. The spectra of the extracts from *P. chrysogenum* and *A. niger* again show a high degree of similarity. Both spectra display shoulders on the C4 and C3 peaks and a general broadening of the peaks in the chemical shift range of 50 to 90 ppm, indicating the presence of non-chitinous polysaccharide impurities. This is decreased in the spectrum of the extract from *M. rouxii*, which also shows greater resolution of the C3 and C5 peaks.

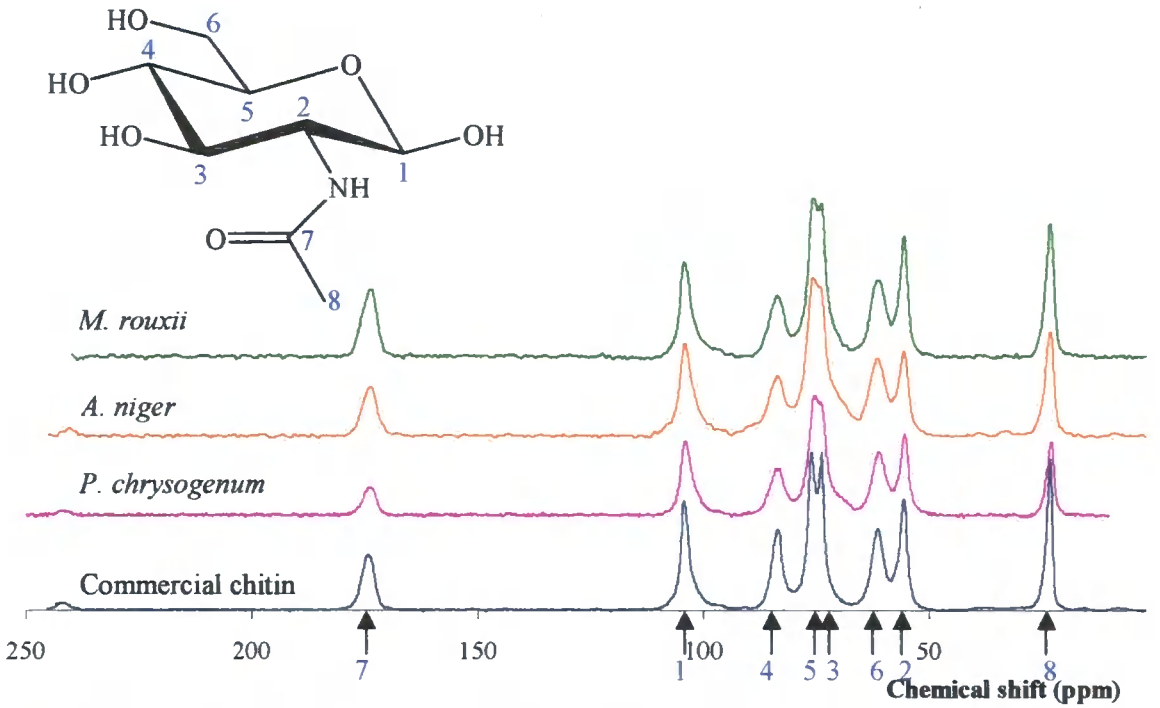


Figure 4.60 - ^{13}C CPMAS ssNMR spectra of purified chitin extracted by the standard chemical procedure from several fungal sources.

The corresponding ^{15}N CPMAS ssNMR spectra are shown in Figure 4.61. The spectra all display the characteristic acetamide peak of chitin at –257 ppm, which have similar line-widths indicating a similar level of purity in all of the samples. The spectrum of the extracts from *M. rouxii* and *P. chrysogenum* also display peaks at approximately –350 ppm, which indicates the presence of deacetylated chitin. In the case of *Mucor rouxii* is possible that this can be accounted for by the presence of chitosan chains, however, if we assume that this peaks represents deacetylated units in the chitin chain then integration of the spectra indicates that the material has a DDA of 9.1 ± 0.5 %.

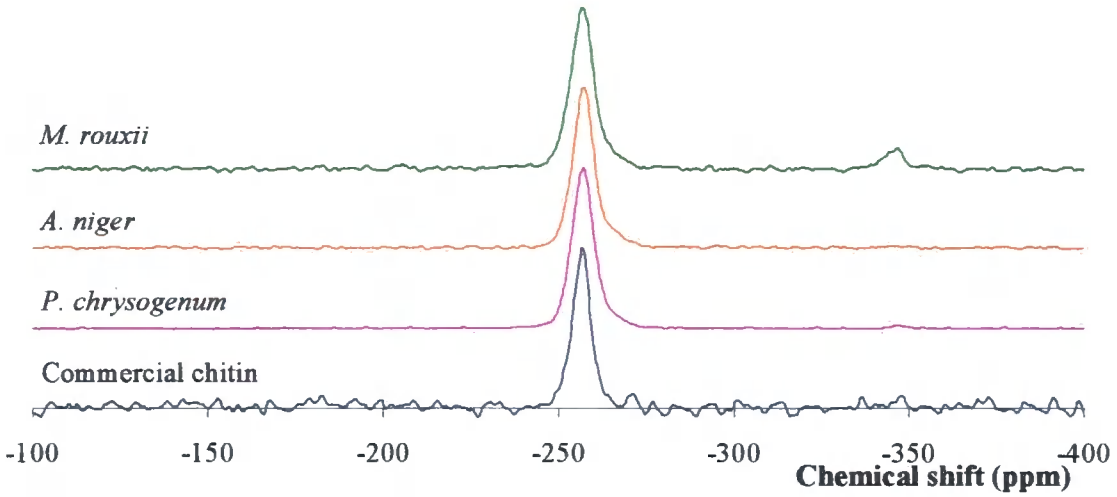


Figure 4.61 - ^{15}N CPMAS ssNMR spectra of purified chitin extracted by the standard chemical procedure from several fungal sources.

The refractive index trace obtained by the GPC analysis of the purified solid A, chitin, fractions extracted from the three fungal biomass are shown in comparison to that of a commercial sample in Figure 4.62. The traces indicate that the extracted chitinous material from *A. niger* has a higher molecular weight than those extracted from *M. rouxii* and *P. chrysogenum*. All of the traces display unimodal distributions, which, as discussed earlier, may indicate that they have not been significantly degraded during the extraction process.

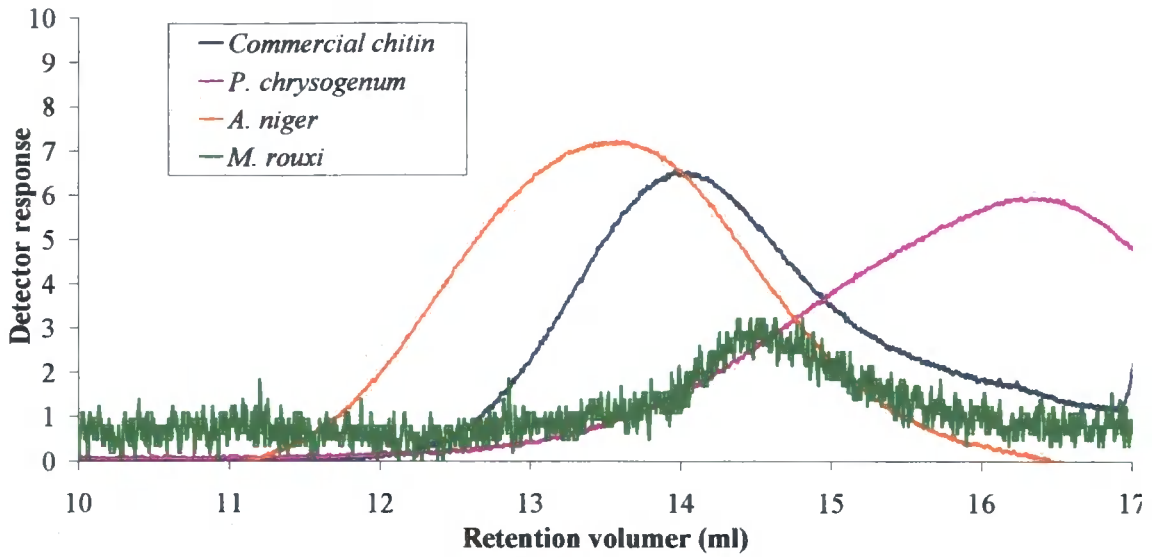


Figure 4.62 - Refractive index trace produced by the GPC analysis of commercial chitin (Sigma), the purified solid A extracts from 'wet' *P. chrysogenum, A. niger* and *M. rouxii* biomass using 5 % (w/v) LiCl:DMAc as the solvent and eluent.

As discussed previously, *M. rouxii* is known to contain chitosan, which will be extracted as the acid soluble fraction, solid B, in the standard chemical extraction procedure. Analysis of the acid soluble fractions resulting from the extraction of *P. chrysogenum* and *A. niger* indicates that they contain a very low concentration of carbon and negligible nitrogen and are comprised mainly of calcium and phosphorus. However, elemental analysis of the acid soluble fraction resulting from the extraction of *M. rouxii* indicates that the sample does contain carbon and nitrogen and has a carbon to nitrogen ratio of 6.77. Chitosan with a DDA of 100 % has a carbon to nitrogen ratio of 5.19, therefore the high carbon to nitrogen ratio of the extracted chitosan may indicate that the material contains impurities, this is reflected in the glucosamine assay of the material, which indicates that it contains 65 % glucosamine. However, some of the variation may be accounted for by the DDA of the sample as the carbon to nitrogen ratio varies with DDA, with a lower DDA giving a higher ratio, and the extracted chitosan is unlikely to have a DDA of 100 %. ^{13}C CPMAS ssNMR analysis of this material was carried out, the spectrum, not shown, indicates the presence of a chitinous material with a high DDA. This was confirmed by the ^{15}N CPMAS ssNMR spectrum of the solid, shown in Figure 4.63, which displays the characteristic acetamide peak of chitin at –257 ppm and the amino peak of chitosan at

−356 ppm. Integration of these peaks indicates a DDA of 65 ± 0.5 %. Chitosan was extracted from the 'wet' biomass produced by the incubation of *Mucor rouxii* in 14 % yield indicating that the majority of the chitinous material present in *M. rouxii* is in the more highly deacetylated form.

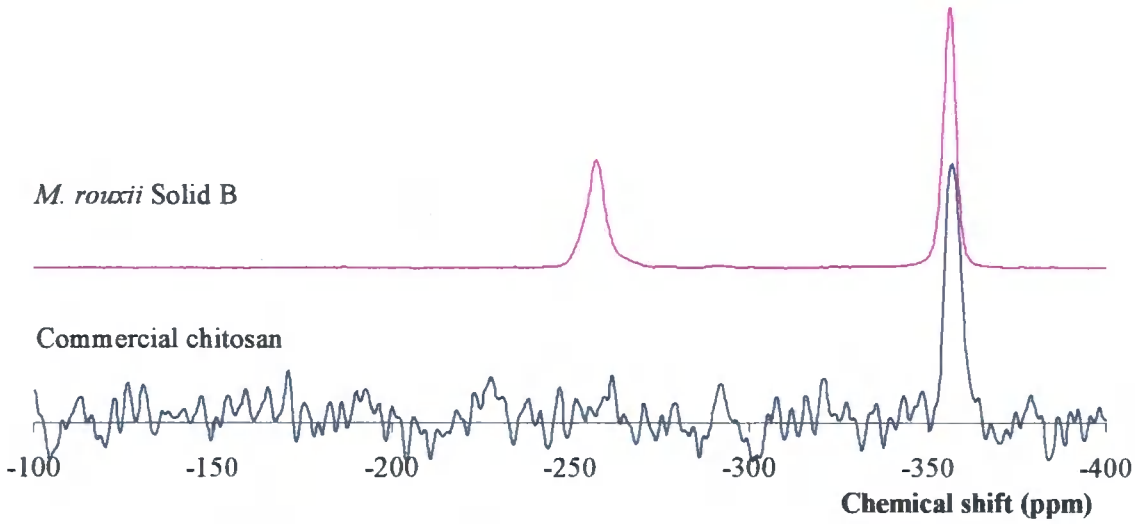


Figure 4.63 - ^{15}N CPMAS ssNMR spectrum of the acid soluble, solid B, fraction extracted from *Mucor rouxii* by the standard chemical extraction procedure.

Taking the yield of chitin and chitosan extracted into account *Mucor rouxii* appears to provide the highest yields of extractable material, which is of higher purity than that of *A. niger* or *P. chrysogenum*. The increased level of purity of chitin extracted from this source suggests that chitin is either involved in fewer matrix interactions in the fungal cell wall of *M. rouxii*, or that these interactions are more readily destroyed. Comparison of the ^{13}C and ^{15}N CPMAS ssNMR spectra of the whole cell sample also indicates that this source has a lower degree of order, which may render the chitinous material more accessible to enzymatic extraction. If the chitinous material is to be extracted as chitosan for use in biomedical applications, the increased concentration of chitosan in *M. rouxii* is preferable, and a possible increased accessibility may mean that the acetylated chitin is more amenable to *in situ* deacetylation reactions, increasing the yield of chitosan extracted. We can therefore conclude that of the three fungal sources examined *M. rouxii* appears to offer the best source of chitinous material.

4.11 Conclusion

A standard chemical procedure for the extraction of purified chitin from the dry biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* was defined and the yield, glucosamine content, ratio of carbon to nitrogen content and DDA of the resulting materials were assessed. It was concluded that the biomass does not contain a high concentration of chitosan because the acid soluble fraction from the extraction procedure was shown to contain high concentrations of calcium and phosphorus. The dry biomass was then incubated with a series of hydrolytic enzymes in order to decrease the series of interactions in which chitin is involved in the fungal cell wall, so when subjected to the standard chemical extraction procedure the chitinous material produced would be of a higher level of purity. However, it was found that incubation with these enzymes did not significantly affect the yield or quality of the chitinous material extracted. It was hypothesised that this can be explained by a high degree of crystallinity of the sample, which renders the chitinous network inaccessible to enzyme action. The heat treatment the biomass had undergone as part of the penicillin production procedure may have increased the level of crystallinity of the sample, decreasing the extractability of the sample. Therefore, in order to investigate the use of wet biomass, the P2 strain of *Penicillium chrysogenum* was acquired and the optimum culture conditions were examined. This biomass was analysed 'wet' by ^{13}C and ^{15}N CPMAS ssNMR and subjected to the standard chemical extraction procedure. The results indicate that extraction from this 'wet' source does produce chitin in greater yield and of a higher level of purity. However, incubation of this 'wet' biomass with hydrolytic enzymes again did not increase the yield or purity of the chitin extracted. It was thought that culture of the biomass in presence of these hydrolytic enzymes could increase their efficiency, as the nascent cell wall components may have a lower degree of crystallinity and therefore be more accessible to enzyme action. Culture in the presence of these enzymes did not significantly affect the yield of mycelia produced, ^{13}C CPMAS ssNMR analysis of the whole fungal cells indicated no significant change in the composition or structure of the fungal cell wall, with the exception of α-amylase which produced a slight change in the relative integrals of the chitinous and non-chitinous anomeric carbon

peaks. Chemical extraction of these materials again did not result in any significant change in yield or purity of the chitinous extracts.

Extraction under chemical and enzymatic conditions from both the wet and dry biomass did not result in a significant concentration of chitosan. The acid soluble fraction, solid B, was found to contain a high concentration of an inorganic phosphate, possibly calcium phosphate or calcium hydroxyl apatite. It was concluded that this does not indicate an inability to extract chitosan, rather that the chitinous material present in *P. chrysogenum* has a low DDA. ^{15}N CPMAS ssNMR analysis of all of the chitin fractions extracted indicate that the DDA is extremely low, ≤ 5 % in unlabelled samples and between 0.8 and ≤ 0.5 % in ^{15}N labelled samples, taking the confidence limits discussed in Chapter 3 into account.

During the course of the research a paper was published outlining an enzymatic procedure for the extraction of chitosan from the waste *Aspergillus niger* mycelium remaining from the production of citric acid.[102] Due to the similarities between this work and ours we employed this procedure, with the exception of the deacetylation step due to the lack of availability of chitin deacetylase, to the wet *Penicillium* biomass. This procedure produced chitin that was of much lower purity than that extracted by the standard chemical procedure. As the authors claimed to extract chitinous material of similar purity, and increased molecular weight, to chemical procedures it was hypothesised that the poor levels of purity seen were due either differences in the extractability of chitin from different fungal sources, or that the procedure is dependent upon *in situ* deacetylation resulting in chitosan which is more easily extracted. Therefore, *Aspergillus niger* was purchased and submitted to the same procedure, this was found to produce chitin with slightly increased levels of purity. In order to further investigate the effect of the fungal source upon the extractability of chitinous material, known chitin producers, *A. niger* and *Mucor rouxii* were obtained and subjected to the standard chemical extraction procedure. *A. niger* was found to produce chitin in similar yields and quality to *Penicillium chrysogenum*, however, *M. rouxii* was found to produce much improved yields of chitinous material of improved quality. It was proposed that this is due to a higher degree of mobility in

the fungal cell wall, which renders the chitinous material more accessible to the extraction procedure. The presence of native chitosan in the cell wall of *M. rouxii* may also be advantageous as there is a great commercial demand for chitosan of high, and controlled, molecular weight. Currently, commercial production of chitosan involves the deacetylation of chitin, which, as discussed previously, results in polymers of uncontrolled molecular weight and deacetylation pattern.

Therefore, in the production of chitinous materials from fungal sources we can conclude that *P. chrysogenum* may not provide the most suitable source when compared to other fungal systems. Standard chemical procedures do produce chitin in relatively high yield, however, the products still contain approximately 40 % non-chitinous material. Attempts at enzymatic extraction procedures proved unsuccessful at increasing the level of purity of this material. It is possible that the use of chitin deacetylase either in the treatment pre-culture biomass, or as an additive to the culture medium, to produce chitosan, which can be selectively solubilised and extracted with a higher level of purity, may improve the biomass' appeal as a source of chitin. However, at present we do not have access to these enzymes, therefore this remains as an area of future work. If this material is to be used as a source of chitin, extracting from 'wet' material, which has not been heat-treated, does appear to result in improved results.

Chapter 5 - ^{15}N Labelling of Chitinous Materials in Fungi

5.1 Introduction

The DDA of chitinous materials must be accurately determined as it governs the physiochemical properties of the polymer, such as solubility and tensile strength, however, to date there is no agreed literature procedure available to do this. Assessment of the available procedures and their associated drawbacks, discussed in section 3.6.5, indicates that ^{15}N CPMAS ssNMR provides the most reliable method for the determination of the DDA of the chitinous materials present in the fungal systems described within this project as this method is unaffected by the presence of polysaccharide impurities and covers a wide range of DDA values. However, ^{15}N NMR spectroscopy is hampered by low sensitivity, which results from a very low natural abundance of ^{15}N (0.37%), the small magnetogyric ratio of ^{15}N and its long spin-relaxation times. The long spin-relaxation times may be avoided by the use of ^{13}C cross polarisation magic angle spinning, (CPMAS) ssNMR methods as described in section 3.8.1. The issue of sensitivity may be overcome by ^{15}N labelling samples, therefore our aim was to develop a simple, cost-effective, and efficient method for ^{15}N labelling the chitinous materials in fungal systems to allow more accurate determination of the DDA. It was hoped that this would allow the study of the DDA of chitinous materials in fungal systems as a function of nutrition and growth stage. Culturing ^{15}N labelled fungal systems may also afford the possibility of real-time investigations into the relative abundance of chitinous materials in fungal cell walls; this may be used to monitor chitin biosynthesis at different stages in the growth cycle and to comparatively study the chitin content of different fungal systems quickly and accurately. NMR spectroscopy has been widely exploited to study the flow of metabolites in pathways *in vivo,* as it does not require the use of destructive chemical analysis or radioisotope labels. The literature provides many examples of the ^{15}N labelling of biological systems to provide information on reaction mechanisms, biosynthetic routes, nitrogen fixation, metal-coordination and the identification of active sites in biochemical systems.[253-256]

The following chapter will detail the two approaches taken to produce ^{15}N labelled chitinous materials in fungal systems. Literature examples of ^{15}N labelling of biological systems primarily make use of minimal media containing a single nitrogen source that can be supplied ^{15}N labelled,[256-260] therefore, we initially investigated this approach, as described in section 5.2. An alternative approach, investigated in section 5.5, identified a suitable ^{15}N labelled supplement that could be added to the rich medium that had previously been used to culture *Penicillium chrysogenum* in high yield, to produce ^{15}N labelled chitin. The most suitable culture conditions were then chosen. Following extraction and hydrolysis, the chitinous material produced under these conditions was then be analysed by LC-MS to determine the efficiency of ^{15}N labelling, section 5.6. ^{15}N CPMAS ssNMR analysis of the purified chitinous material was also performed, section 5.7. Taking these results into account, an optimum concentration of ^{15}N labelled additive was chosen which produces chitin with a degree of ^{15}N labelling high enough to ensure increased signal in ^{15}N CPMAS ssNMR, allowing quick and accurate determination of the DDA, without being cost-prohibitive. Additionally, in section 5.8 the labelling of other nitrogen containing components within the fungal cell was investigated. Finally, in section 5.9, we demonstrated that the chosen labelling methodology is not limited to the P2 strain of *Penicillium chrysogenum*.

5.2 Minimal Media Labelling Systems

The use of minimal media containing a sole nitrogen source to culture ^{15}N labelled fungal material was initially investigated. As nitrogen is present as a single species the concentration of ^{15}N nuclides relative to ^{14}N can be determined, therefore, assuming that ^{15}N and ^{14}N are assimilated at the same rate, the level of labelling of chitinous materials in the fungal cell wall can be determined. The assimilation of ammonium and nitrate ions into nitrogen metabolic routes to allow production of labelled amino acids in fungal species has been demonstrated in the literature.[257-261] Therefore media containing $(NH_4)_2SO_4$ or $NaNO_3$, which are commercially available ^{15}N labelled, as the sole nitrogen source were investigated. Minimal media

that contain $Na^{15}NO_3$ or $(^{15}NH_4)_2SO_4$ (see appendix) were chosen for this investigation, recipes for these media were available in the literature and have been shown to culture fungal species.[116, 262, 263]

The standard against which the media are compared is a rich medium, medium A, which is known to culture P2 effectively, see section 4.8. The nitrogen sources in this rich medium are varied and cannot be directly substituted for ^{15}N labelled analogues, as in the minimal media, therefore P2 was cultured unlabelled in this medium. The minimal media were prepared with 10 % of the nitrogen containing component, $NaNO_3$ or $(NH_4)_2SO_4$, in the ^{15}N form. Assuming that ^{14}N and ^{15}N are assimilated at the same rate, this should produce chitinous materials with a 10 % level of ^{15}N labelling.

The P2 strain of *Penicillium chrysogenum* was shown to grow on agar plates of all four of the media under consideration. P2 was then cultured in shake flasks under normal conditions, see section 7.3.2. The mycelia were then collected by filtration and weighed; the wet weights of mycelia are shown in Table 5.23.

Medium	Wet Weight Biomass Produced* (gL^{-1})
A – Blakeslee's Formula (Unlabelled)	25 ± 3 (20)
B - Bristol Minimal Medium ($Na^{15}NO_3$)	2.7 ± 0.4 (3)
C - Czapek Dox ($Na^{15}NO_3$)	0.9 ± 0.5 (3)
D - $(^{15}NH_4)_2SO_4$ Minimal Medium	9.0 ± 1.1 (7)

Table 5.23. Wet weight of biomass produced. * Errors given as standard deviations, number of replicates shown in brackets.

P2 grew to some extent in all media. However, medium C, containing $Na^{15}NO_3$, was immediately disregarded as the yield of mycelia produced was extremely low. Media B ($Na^{15}NO_3$) and D ($(^{15}NH_4)_2SO_4$) produced higher yields than C ($Na^{15}NO_3$), however the yields are notably lower than the yield from medium A (rich medium). The

mycelia produced in media A, B and D were autoclaved, washed with water and collected by filtration before drying in an oven at 90 °C overnight. ^{15}N CPMAS ssNMR analysis of the dried mycelia was performed, the spectra are shown in Figure 5.64.

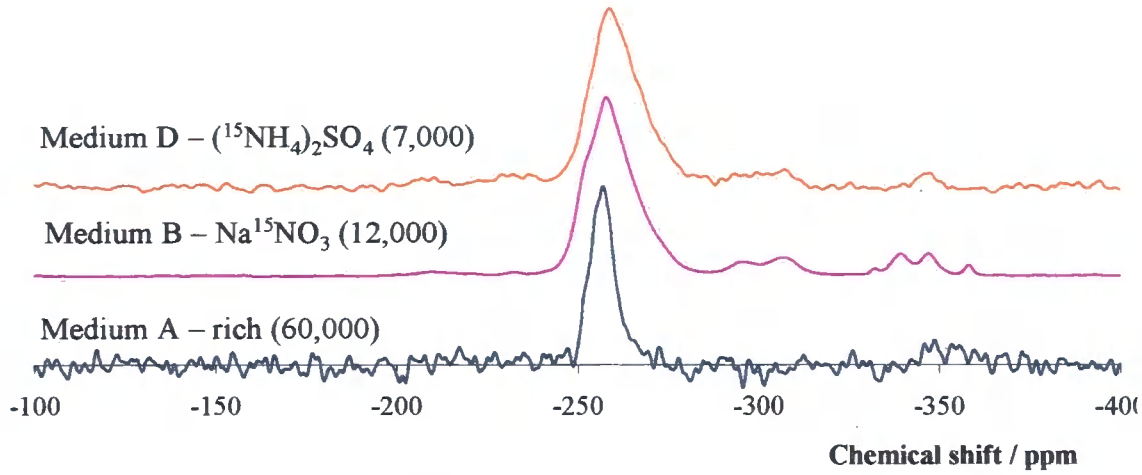


Figure 5.64 - Whole cell ^{15}N CPMAS ssNMR spectra of mycelia cultured in media A (rich), B ($Na^{15}NO_3$) and D ($(^{15}NH_4)_2SO_4$). The number of repetitions required to obtain the spectra are shown in brackets.

Mycelia cultured in media B ($Na^{15}NO_3$) and D ($(^{15}NH_4)_2SO_4$) required fewer repetitions to acquire than medium A (rich medium), which has only natural abundance levels of ^{15}N. Medium A (rich medium) required 60,000 repetitions equating to an acquisition time of approximately 15.5 hours. The acquisition times for Media B ($Na^{15}NO_3$) and D ($(^{15}NH_4)_2SO_4$) were 3.5 and 2 hours respectively, corresponding to 12,000 and 7,000 repetitions. The data intimates that despite the shorter acquisition times the spectra obtained from mycelia cultured under labelled conditions are of improved quality. Peaks in the region from –280 to –370 ppm, which can be explained by the presence of other nitrogen containing components in the fungal cell wall, this is discussed in chapter 3, are not discernable in the spectra produced from medium A (rich medium) however they can be resolved in the spectra of mycelia cultured in labelled conditions. We propose that this can be explained by an improvement in the amount of signal observed in the spectra of mycelia cultured in media B ($Na^{15}NO_3$) and D ($(^{15}NH_4)_2SO_4$) as a result of the increased level of ^{15}N

labelling. The improvement in signal to noise was not quantitatively established at this point, as these are preliminary experiments.

Further work in the area of minimal media utilised medium D ($(^{15}NH_4)_2SO_4$), as this produced the highest yields of fungal biomass of the minimal media tested, see Table 5.23. Following culture in this media under normal conditions, see section 7.3.2, the biomass was subjected to the standard chemical extraction procedure (see section 7.4.6) resulting in a solid, which ^{13}C CPMAS ssNMR indicates, contains chitin, see Figure 5.65. In comparison to the spectrum of a commercial sample of chitin (Sigma), there is a general broadening of the spectrum, this is particularly apparent at the peak representing the anomeric carbon at 100 ppm. This broadening may arise from impurities present in the sample, however, it may also result from the presence of differing polymorphic forms of chitin. This broadening of peaks has been observed in the spectra of chitin extracted from the mycelia cultured in the standard rich medium, this is further discussed in chapter 4, and is not peculiar to this labelled system.

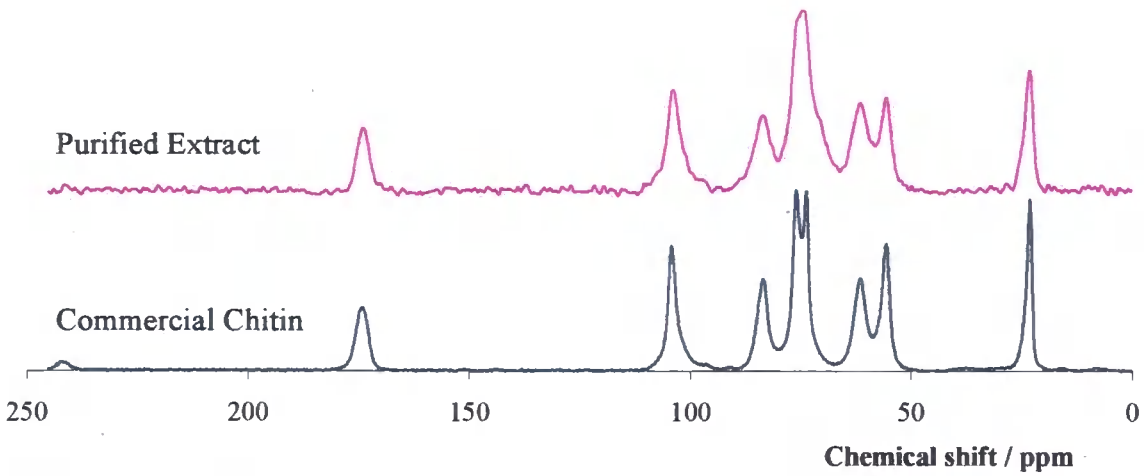


Figure 5.65 - ^{13}C CPMAS ssNMR spectra of purified chitinous material extracted from mycelia cultured in medium D ($(^{15}NH_4)_2SO_4$) and a commercial chitin sample.

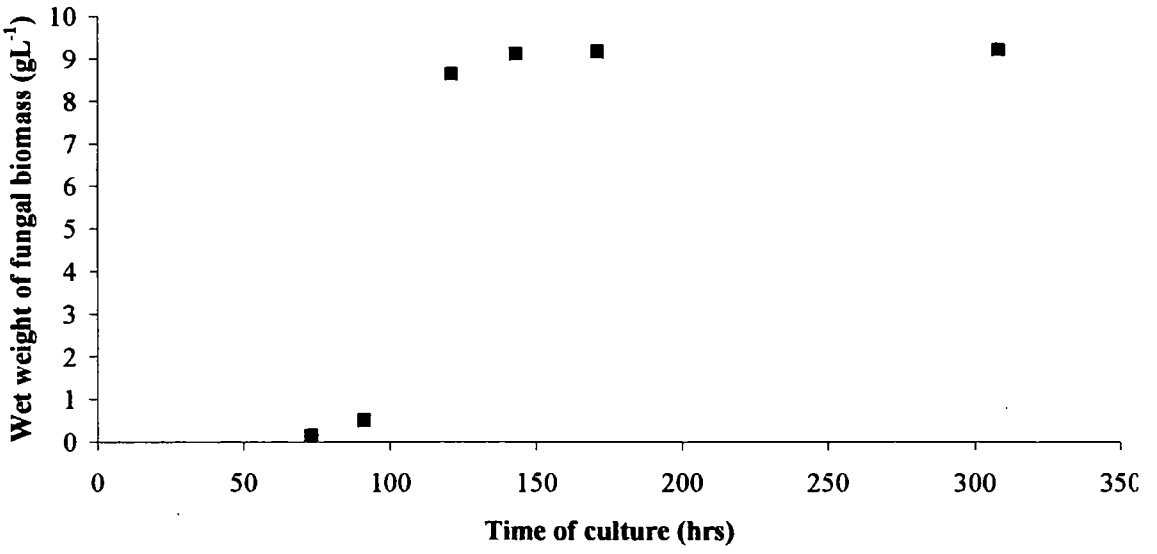
The yield of chitin extracted from mycelium cultured in medium D ($(^{15}NH_4)_2SO_4$) was 3.5 % compared to 5.6 % from medium A (rich medium), see Table 5.24. However, this decrease lies almost within the error of the yield of purified chitin from medium A

(rich medium) of ± 2 %. The C:N values indicate that the level of impurities in chitin extracted from mycelia cultured in the minimal media D were higher than that from the rich medium A, Table 5.24 shows an increase from 9.83 to 14.87. However, C:N ratios have been shown to fluctuate within these values, see section 4.8.2, therefore this is not the significant factor in disregarding this as a method of labelling mycelia. More importantly, the low yields of biomass produced in this minimal media mean that further experiments requiring extraction of the chitinous products would necessitate the use of large volumes of medium, as 1 L of medium D produces approximately 40 mg of chitin compared to approximately 250 mg when using the rich medium A. ^{15}N CPMAS ssNMR analysis requires at least 50 mg of sample. This was considered practically and economically unviable, as 1 L of medium D, which contains 10 % of the ammonium sulphate ^{15}N labelled, costs £ 49.70 (sigma) for the label alone.

	Medium A (rich)	Medium D ($(^{15}NH_4)_2SO_4$)
Wet weight of mycelia / gL^{-1}	25 ± 3 (20)	9.0 ± 1.1 (7)
Yield of purified extract (from estimated dry weight of mycelia) / %	5.6 ± 2 (8)	3.5
C:N	9.83 ± 3 (13)	14.87

Table 5.24 - Extracts from fungal biomass cultured in Media A (rich) and D ($(^{15}NH_4)_2SO_4$). Errors given as standard deviations, number of replicates shown in brackets.

In order to ensure that the maximum yield of biomass had been produced, *P.* chrysogenum was cultured in medium D for varying lengths of time, as described in 7.3.2. These results, displayed in Graph 5.7, show that no appreciable growth was seen for approximately 70 hours, after this time the system experienced a period of rapid growth, which began to plateau after 130 hours. As the cultures were all allowed to grow for 192 hours to achieve the yields quoted in Table 5.23 this data suggests that maximum yields were attained, culturing the media for longer periods would not result in higher yields.



Graph 5.7 – Wet weight of fungal biomass produce in medium D ($(^{15}NH_4)_2SO_4$) when cultured in shake flasks for varying periods of time.

We concluded that the use of minimal media containing a single nitrogen source, to effect ^{15}N labelling of the chitinous components of the fungal cell wall is not an economically efficient approach due to the low yields of biomass produced. We therefore explored an alternative strategy, discussed in section 5.5, utilising the rich medium A and a ^{15}N-labelled additive.

5.3 Targeted Labelling Strategy

As the use of minimal media containing a single nitrogen source to produce ^{15}N enriched samples resulted in poor yields of mycelia, section 5.2, an alternative approach was taken to ^{15}N label the chitinous material in the fungal cell wall. This focused on identifying a nutritional supplement to be added to the original rich medium, medium A, to produce ^{15}N labelled chitinous materials without a reduction in yield of fungal biomass. The biosynthesis of chitin is catalysed by the enzyme chitin synthase, this is discussed in section 1.6.3. As the numerous isozymes of chitin synthase all employ uridine diphosphate-*N*-acetyl-D-glucosamine (UDP-GlcNAc) as the substrate [3], the biosynthetic route of UDP-GlcNAc was investigated as a possible route for targeted labelling.

5.4 Biosynthetic Route of UDP-GlcNAc in Fungal Systems

In the 1950s Luis F. Leloir discovered the presence of sugar nucleotides, notably UDP-GlcNAc [264], and went on to establish that the general metabolic route for incorporation of sugar units into structural polysaccharides involves the prior activation of the sugar monomer by conversion into a sugar nucleoside, which then acts as a glycosyl donor.[265, 266] The sequences of biosynthetic reactions involved in this conversion are known as Leloir pathways. The sugar nucleotide precursor of the GlcNAc units, utilised by chitin synthases to synthesise chitin is UDP-GlcNAc, is formed from fructose-6-phosphate in a four-step version of the Leloir pathway. There are two variations of this pathway depending on whether the system involved is of eukaryote or prokaryote origin. In eukaryotes the pathway involves four successive steps each catalysed by a separate enzyme. These steps, summarised in Figure 5.66, are as follows: i, conversion of fructose-6-phosphate into glucosamine-6-phosphate; ii, acetylation of glucosamine-6-phosphate; iii, isomerisation of GlcNAc-1-phosphate; and iv, uridylation of GlcNAc-1-phosphate to give UDP-GlcNAc. This pathway is ubiquitous in fungal systems, producing the substrate for chitin synthase as well as the substrate for the formation of *N*-glycosylated mannoproteins and the glycosylphosphatidyllinositol (GPI) anchor of membrane bound proteins.[267] In some fungi UDP-GlcNAc can be additionally converted into UDP-GalNAc, to provide a building block for the galactosamine containing cell wall polysaccharide.[268]

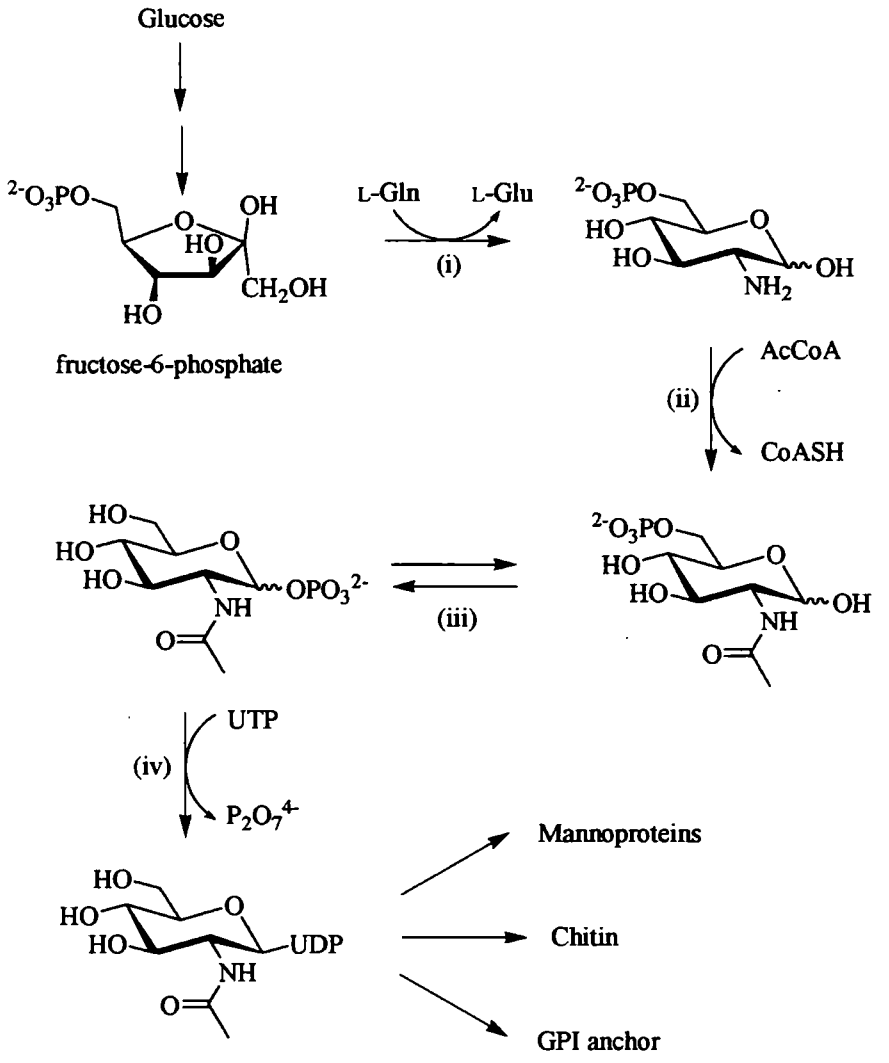


Figure 5.66. Leloir pathway for the multiple-enzyme system of biosynthesis of UDP-GlcNAc in fungi.[269] (UDP and UTP are uridine di/tri-phosphate respectively. AcCoA is Acetyl-Coenzyme A, CoASH is Coenzyme A.)

Nitrogen is incorporated into the nascent sugar nucleotides in the first step of the pathway, which is catalysed by an enzyme known by the trivial name of glucosamine-6-phosphate synthase, (the non-trivial name is L-glutamine:D-fructose-6-phosphate amido transferase). There are two distinguishable parts to this step, ammonia is first transferred from the amide group of L-Gln to Fru-6-P, this is followed by the isomerisation of fructosimine-6-P to form GlcN-6-P as shown in Figure 5.67. As the enzyme shows specificity for L-glutamine as an amino donor, this offers an opportunity for incorporation of a ^{15}N label.

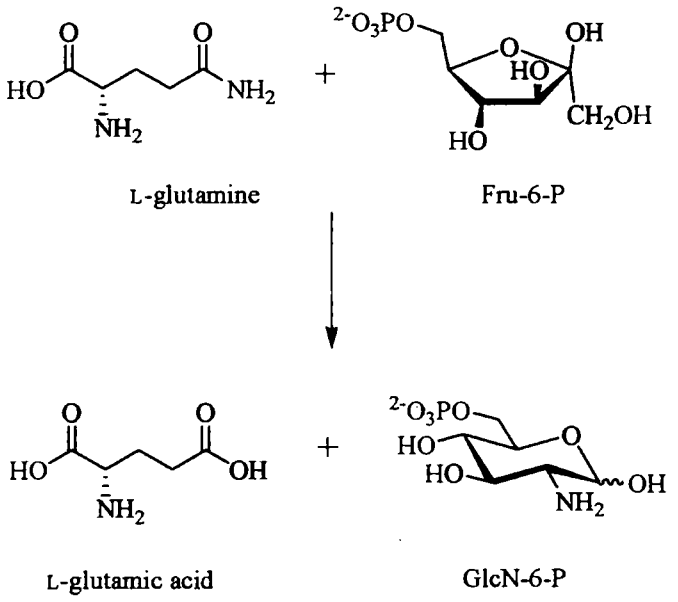


Figure 5.67 - Step (i) of the Leloir pathway in Figure 5.66. Conversion of fructose-6-phosphate (Fru-6-P) into glucosamine-6-phosphate (GlcN-6-P), catalysed by the enzyme glucosamine-6-phosphate synthase.

Addition of ^{15}N labelled L-Gln should theoretically result in ^{15}N labelled UDP-GlcNAc and therefore labelled chitin in the fungal cell wall, however this is currently cost prohibitive as commercially available doubly ^{15}N labelled L-Gln costs £559.00 for 250 mg (Sigma). As it is the amide nitrogen that is transferred to Fru-6-P, L-Gln labelled in the amide position should be sufficient to produce labelled UDP-GlcNAc, however this is also costly at £290.50 for 1 g. If we consider the biosynthetic route for L-Gln, shown in Figure 5.68, we can see that the amido nitrogen can be incorporated from any ammonium ion that is present. Therefore addition of a labelled ammonium ion to the culture media should result in the biosynthesis of labelled glutamine and hence labelled chitin.

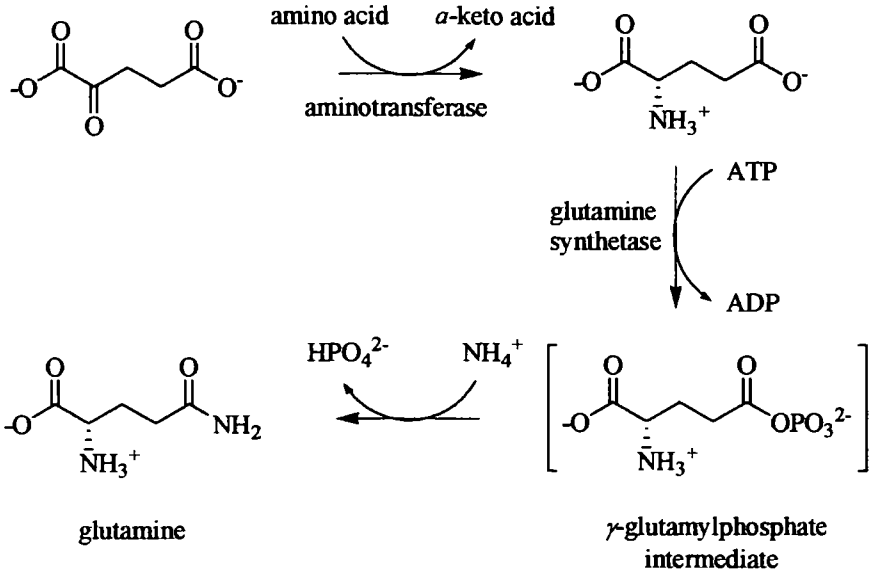


Figure 5.68 - Biosynthetic route of glutamine involving the transamination of α-ketoglutarate to form glutamate that subsequently undergoes amidation to form glutamine *via* an intermediate. These steps are catalysed by the aminotransferase and glutamine synthetase respectively.

The labelling of fungal systems *via* $^{15}NH_4^+$ to study nitrogen metabolism has been reported in the literature.[258-260, 270, 271] Johansen *et al.* studied the hyphal content and ^{15}N enrichment of free amino acids and fatty acids in the arbuscular mycorrhizal fungus *Glomus intraradices* following augmentation of the medium with ^{15}N labelled nitrate and ammonium ions.[261] In earlier studies of ^{15}N incorporation in soybeans it was shown that both the ammonium and nitrate nitrogens of NH_4NO_3 are incorporated into protein synthesis equally.[272] However, Johansen *et al.* found that, while both nitrate and ammonium ions were assimilated into free amino acids, the levels of enrichment were higher upon addition of $^{15}NH_4^+$ indicating that $^{15}NH_4^+$ is more easily assimilated, the total concentrations of free amino acids produced were the same for media containing each of the ions. As this work was carried out on fungal systems, these results reinforce the choice of ^{15}N labelled ammonium ions in this study. Martin has also demonstrated the assimilation of labelled ammonium ions into amino acids in fungal systems [257]. The author describes how the label is primarily incorporated into glutamine at the amide-N position, the amino-N being derived from the originally unlabelled glutamate, after extended periods glutamine becomes doubly labelled. It is the amido nitrogen that is transferred to Fru-6-P in the

biosynthesis of UDP-GlcNAc, therefore this evidence also supports the use of $^{15}NH_4^+$ to culture ^{15}N labelled chitinous materials in fungal systems.

5.5 Labelling *via* the Addition of $(^{15}NH_4)_2SO_4$ to a Rich Medium

We investigated the effect of the addition of a series of concentrations of $(^{15}NH_4)_2SO_4$ to the rich medium A on the mycelium produced. We wanted to investigate the concentration of $(^{15}NH_4)_2SO_4$ required to produce levels of labelling between that of natural abundance and 100 % ^{15}N, therefore we estimated the concentration required to effect 50 % labelling and used concentrations between 0.5 and 200 % of this value. In order to estimate the concentration of $(^{15}NH_4)_2SO_4$ that should be added to the medium to effect 50 % labelling, we determined the nitrogen content of the medium by elemental analysis. Malt extract and peptone contain 1.32 % and 16.54 % nitrogen by weight respectively, in 1 L of medium this equates to 0.4294 g of nitrogen. We therefore estimated that 1 L of media should be supplemented with 1.92 g of $(^{15}NH_4)_2SO_4$ to effect 50 % ^{15}N labelling, i.e. 50 % of the nitrogen present will be the ^{15}N isotope. This estimation assumes that the ^{15}N labelled ammonium ion will be assimilated at a comparable rate to other nitrogen sources available in the medium. A range of concentrations of $(^{15}NH_4)_2SO_4$ varying from 0.5 to 200 % of this value were added to medium A prior to sterilisation, shake flasks were inoculated with the P2 strain of *Penicillium chrysogenum* and cultured in the usual way for eight days, see section 7.3.2. The resulting fungal biomass was collected by filtration and weighed, it was then extracted by the normal chemical means, see section 7.4.6. The purified solid was analysed by elemental analysis and the glucosamine content was assayed, the results are shown in Table 5.25.

Concentration of $(^{15}NH_4)_2SO_4$ (gL^{-1})*	Yield of wet mycelium (gL^{-1})	Yield of purified chitin (%) **	C:N	Glucosamine determination (%)
0 (0 %)	24	5.6	8.8	64
0.0096 (0.5 %)	18	5.1	8.4	65
0.096 (4.8 %)	22	5.5	7.4	74
0.48 (20 %)	19	6.3	8.0	95
0.96 (33 %)	23	6.8	7.7	78
1.92 (50 %)	18	6.8	7.3	90
2.88 (60 %)	20	6.5	7.2	74
3.84 (67 %)	19	4.5	7.4	80

Table 5.25 – Yields and indicators of the level of purity of chitinous material extracted from fungal material cultured in medium A (rich) containing varying concentrations of $(^{15}NH_4)_2SO_4$. * Predicted level of labelling, above that of natural abundance, shown in brackets. ** Yield calculated from estimated dry weight of mycelium.

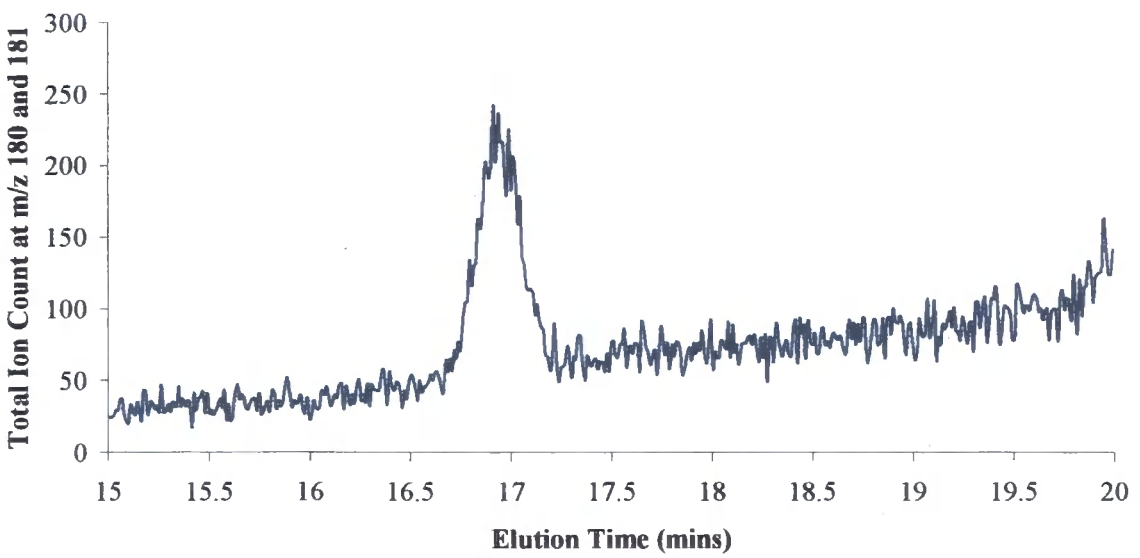
Altering the composition of the growth medium could feasibly alter the rate of growth of the fungi, however, addition of the label does not appear to inhibit or increase the growth of the fungal material as the yield of the wet weight of mycelia produced is largely unaffected. Additionally, the chitin content of the fungal cell wall is not significantly altered by the change in composition of growth medium, as the yields of purified chitin extracted from the mycelia do not vary with changing concentrations of $(^{15}NH_4)_2SO_4$. The carbon to nitrogen ratios, as given by elemental analysis, display no systematic change and do not vary greatly, indicating that the extracted chitin was of a similar level of purity. The results for the glucosamine determination do vary and there are two anomalously high results, however, this variation is within the error seen when analysing samples extracted from fungal material. These errors are discussed in section 3.5, where we have concluded that they most likely arise from incomplete hydrolysis of the samples.

The data indicates that the addition of $(^{15}NH_4)_2SO_4$ does not alter the growth of the fungal material, its chitin content or the ease of extraction of the chitinous material. This labelling methodology appears to be more suitable than using the minimal media

described in section 5.2 and was chosen for further investigation. The extent of ^{15}N labelling of the chitinous material present in the fungal cell wall was assayed in order to determine the concentration of additive that would provide the optimum level of labelling. For the purposes of this study the optimum level of labelling is defined as the level of ^{15}N labelling which increases the sensitivity in ^{15}N ssNMR experiments to such a level as to reduce the acquisition times to an acceptable level, without incurring too high a cost.

5.6 LC-MS Determination of the Degree of Labelling

The extent of ^{15}N labelling of the fungal biomass cultured in media containing varying concentrations of $(^{15}NH_4)_2SO_4$ was assayed by LC-MS analysis of the glucosamine monomer units that resulted from the purification and total hydrolysis of the chitinous materials in the fungal cell wall. Following hydrolysis the acidic solution was neutralised to give a final concentration of approximately 0.04mg/ml of glucosamine, see section 7.2.2. This was then further diluted to 0.004mg/ml with deionised water and analysed by LC-MS (see section 7.2.8).



Graph 5.8 - Chromatograph of hydrolysed chitinous material showing mass ions 180 and 181.

Graph 5.8 is a chromatograph of a hydrolysed sample of chitin, this is representative of all of the samples that were analysed. This chromatograph shows mass ions with an m/z of 180 and 181 as protonated glucosamine has a mass of 180, resulting in a parent ion peak with an m/z of 180, and the incorporation of ^{15}N would result in an increase in the intensity of the mass ion at m/z 181 in the isotopic distribution. If the high salt content, remaining from the hydrolysis and neutralisation of the sample, is allowed to pass into the mass spectrometer it would suppress the ionisation of the product in question and saturate the detector, therefore this must be diverted from the sample before it is analysed. Initial experiments demonstrated that glucosamine elutes at a retention time of approximately 16.9 minutes, therefore in subsequent experiments the first 15 minutes of the column eluent was diverted to waste and only a window of 15 to 20 minutes analysed by mass spectrometry. As salts elute at a shorter retention time than glucosamine, the column eluent in this window should not include a high concentration of salts. There are no other major peaks seen in this time frame.

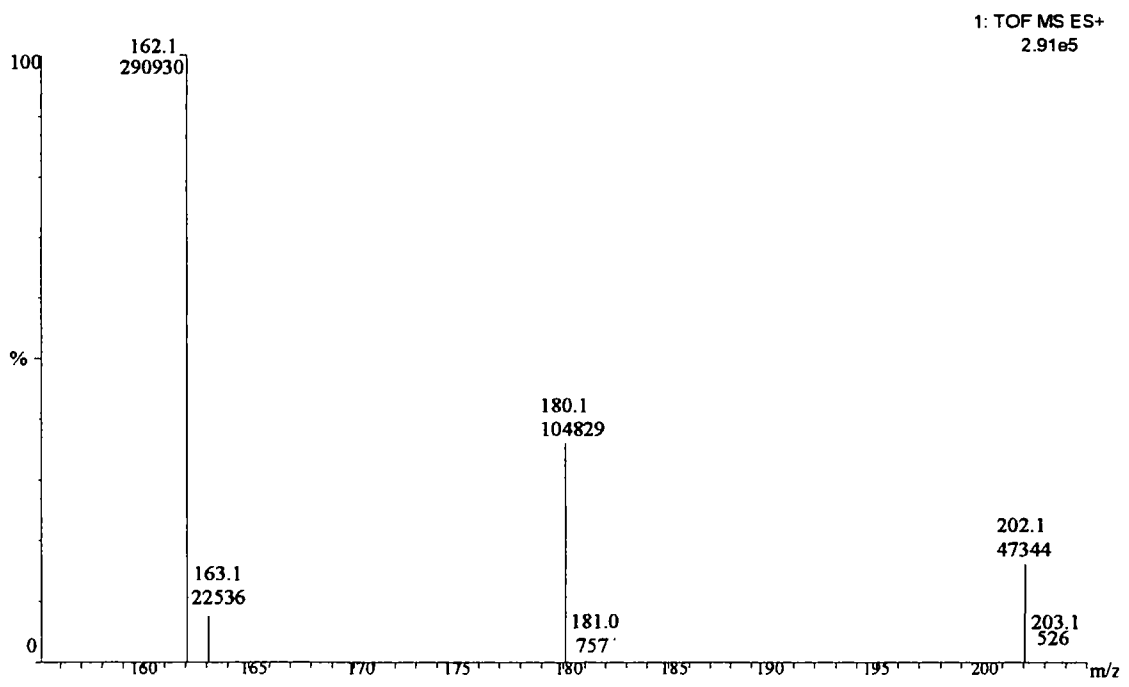


Figure 5.69 - Mass spectrum of a hydrolysed sample of chitin extracted from *P. chrysogenum*. Cultured in medium containing natural abundance levels of ^{15}N. (Background subtracted)

A single spectrum was constructed by summing the spectra obtained across the shoulder of the peak at approximately 16.9 minutes on the chromatogram. At high ion counts the detector will become saturated and will no longer produce a linear response, which will tend to give inaccurate ratios of isotopic abundances within the mass ion group. Therefore, in order to ensure that the detector had not reached saturation the shoulders of the peak before and after the maximum were independently summed and the resulting spectra compared to ensure agreement. Time intervals of similar lengths from either side of this peak were also summed and then subtracted from this spectrum, this removes the background mass ions that are present on the spectra and obscure the peaks of the mass ions in question. A representative spectrum of an unlabelled sample of hydrolysed chitin is shown in Figure 5.69. The major peak in this spectrum occurs at 162.1 m/z, this corresponds to the loss of water from a protonated glucosamine monomer. Due to the natural abundance of ^{13}C there is also a peak at 163.1 m/z, which is approximately 7 % of the height of the main mass peak. Peaks in the isotopic distribution around 180.1 account for the protonated molecular ion and those around 202.1 are the sodiated molecular ion.

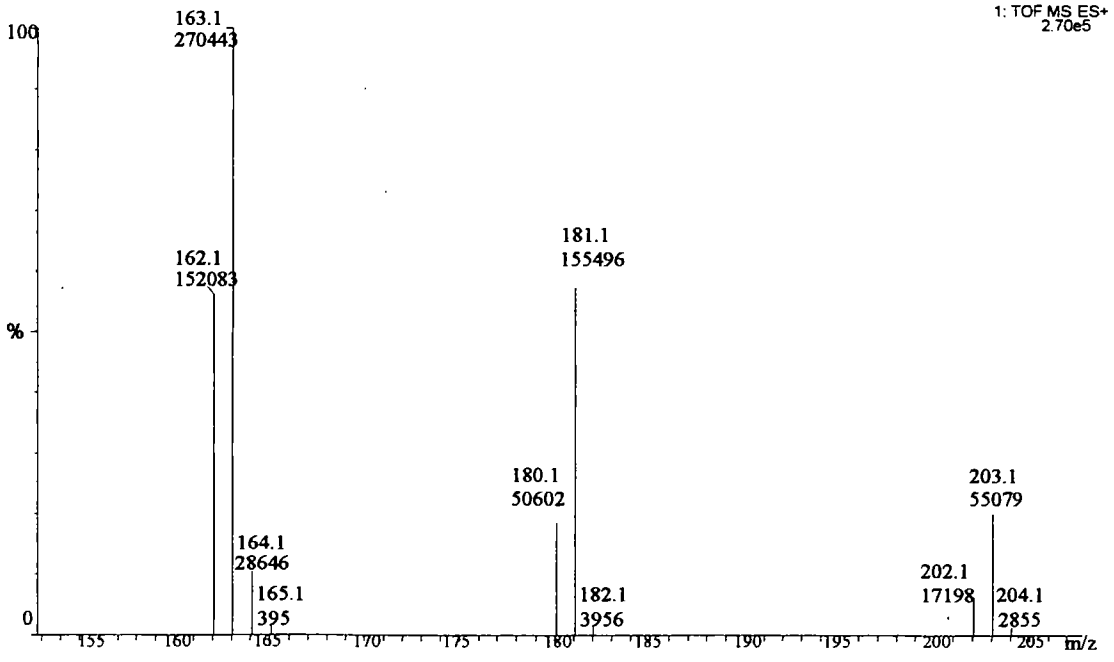


Figure 5.70 - Mass spectrum of a hydrolysed sample of ^{15}N labelled chitin extracted from *P. chrysogenum*. (Background subtracted)

A mass spectrum of a representative ^{15}N labelled sample of hydrolysed chitin, cultured and extracted as described in sections 7.3.2 and 7.4.6 respectively, is shown in Figure 5.70. The spectrum demonstrates that the sample has been ^{15}N labelled as the peaks at 163.1, 181.1 and 203.1, which represent ions containing a single ^{13}C or ^{15}N atom, are now of much greater intensity than the parents ions at 162.1, 180.1 and 202.1 respectively. Additionally peaks can be seen at 164.1, 182.1 and 204.1, representing ions containing both a ^{13}C and a ^{15}N atom. The relative intensities of the peaks in the isotopic distributions can be used to estimate the degree of ^{15}N labelling of the glucosamine units.

As the level of labelling will remain unchanged upon dehydration or sodiation the relative intensities of the peaks in the isotopic distributions should be the same for each parent ion. The isotopic distributions around the parent ions at 180.1, 162.1 and 202.1 do show agreement, see Table 5.26.

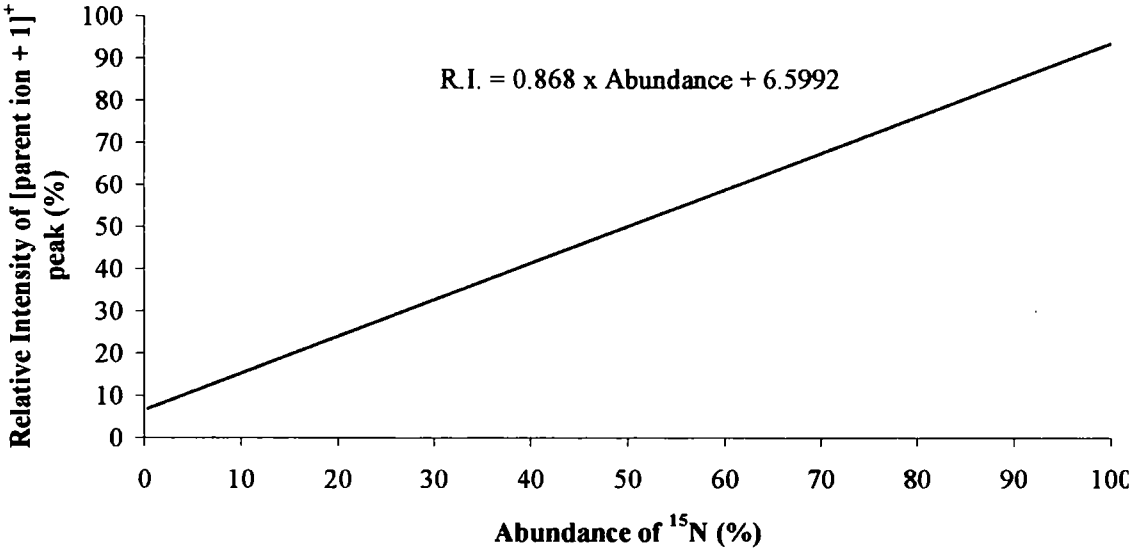
Mass Ion		Relative Intensity (In mass ion group) (%)
$[M+1-H_2O]^+$	162.1	23.7
	163.1	70.8
	164.1	5.55
$[M+1]^+$	180.1	23.4
	181.1	72.2
	182.1	4.36
$[M+Na]^+$	202.1	22.5
	203.1	71.3
	204.1	6.20

Table 5.26 - Relative Intensities of Mass Ion Peaks in Figure 5.70.

The relative intensities of corresponding mass ion peaks around the parent ions at 163.1, 180.1 and 202.1 agree to within 1.5 %, s.d. = 0.3. Therefore the degree of error inherent in this methodology for the quantification of isotopic distributions by mass

spectrometry shall be taken as 1.5 % for the purpose of these calculations. Additionally, for the purposes of this study the presence of ^{2}H, ^{17}O and ^{18}O are ignored, as the natural abundance of these isotopes is lower than the inherent error, 0.015, 0.037 and 0.204 % respectively. Similarly the possibility of two ^{13}C isotopes is ignored.

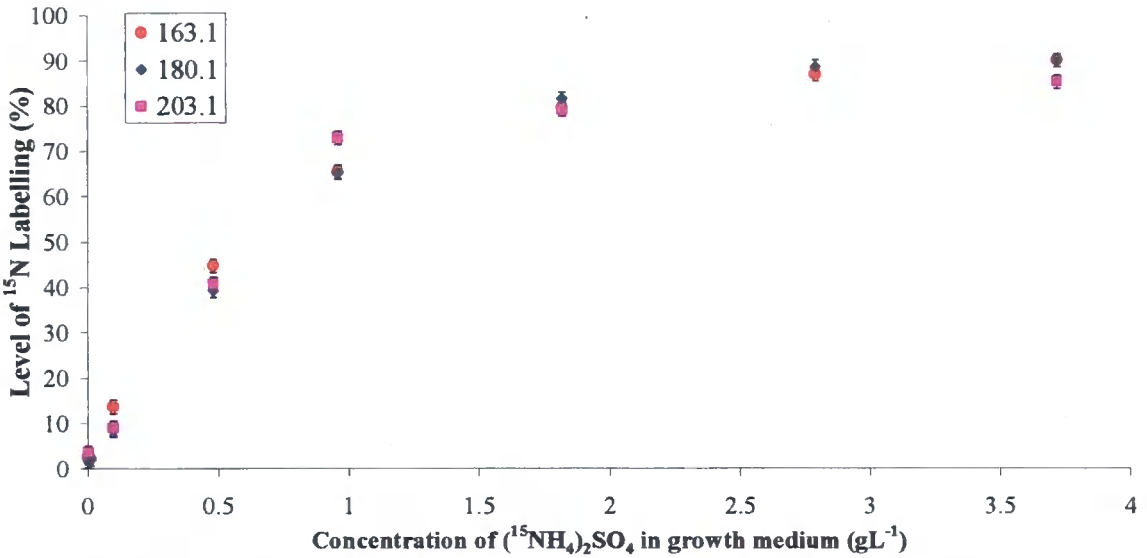
In order to quantify the ^{15}N isotope content a standard plot of the calculated theoretical relative intensity of a mass ion peak against predetermined levels of ^{15}N labelling was prepared. The mass ion peak representing the parent ion plus one mass ion was chosen, for example 163.1, 181.1 and 203.1, as it contains the majority of the ^{15}N isotopes present. This peak contains either all ^{12}C and one ^{15}N or one ^{13}C. The natural abundance of ^{13}C was assumed to be 1.1 %, therefore it was estimated 6.6 % of the ions contain ^{13}C, as there are 6 carbons. The natural abundance of ^{15}N was taken as 0.36 %. See Graph 5.9.



Graph 5.9 - Standard curve of the theoretical relative intensity of $[M+2]^{+}$ peak against abundance of ^{15}N. Assuming natural abundances of 1.1 and 0.36 % for ^{13}C and ^{15}N respectively.

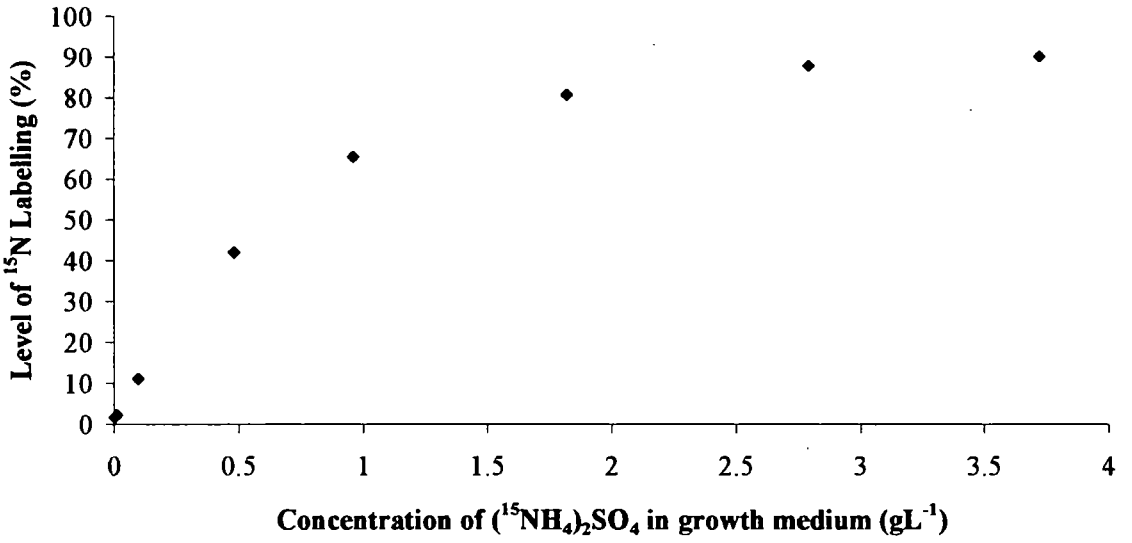
The relative intensities of the [parent ion +1]$^{+}$ peak in the isotopic distributions around the parent ions at 163.1, 180.1 and 202.1 were then calculated from the ion counts given in the mass spectra of samples that had been cultured in varying concentrations

of $(^{15}NH_4)_2SO_4$. These values, and the standard plot, were then used to calculate the level of ^{15}N labelling. Due to the low ion count of the parent ion at 202.1 it was not possible to visualise this peak in all of the samples.



Graph 5.10 - Level of ^{15}N labelling in samples cultured in media containing varying concentrations of $(^{15}NH_4)_2SO_4$. Calculated from experimental data of the relative intensity of the [parent ion + 1]$^+$ mass ion in isotopic distributions around 180.1 and 202.1.

The level of ^{15}N labelling in samples cultured in media containing a series of concentrations of $(^{15}NH_4)_2SO_4$ are compared when calculated using the relative intensities of the mass ion at m/z 163.1, 181.1 and 203.1 in Graph 5.10. The data suggests that the levels of labelling calculated from the three isotopic distributions follow a similar trend, however, the values calculated using the mass ion at m/z 203.1 do vary slightly from those calculated from the mass ions at m/z 163.1 and 180.1. This may be explained by the low intensity of the ion counts in the isotopic distribution around 202.1, which increases the error in the calculation of their relative intensities. The isotopic distribution about 202.1 was therefore discarded and the average relative intensity of the mass ion peaks at 163.1 and 181.1 used to calculate the level of ^{15}N labelling, this data is shown in Graph 5.11. The graph shows that the level of ^{15}N labelling increases with concentration of $(^{15}NH_4)_2SO_4$ rapidly up to a concentration of approximately 1 gL^{-1}, above which it begins to plateau.



Graph 5.11 - Level of ^{15}N labelling in samples cultured in media with varying concentrations of $(^{15}NH_4)_2SO_4$ calculated from the average relative intensity of the 163.1 and 181.1 mass ion peaks in the isotopic distribution about the mass ion at m/z 162.1 and 180.1 respectively.

The calculated values of ^{15}N labelling are shown in

Table 5.27, the sample extracted from fungal biomass cultured with no $(^{15}NH_4)_2SO_4$ supplement shows an estimated level of ^{15}N labelling of 1.69 %. Taking the error of 1.5 %, calculated previously, into account this value lies in the region of the natural abundance of ^{15}N, which is 0.36 %. As we simply wish to determine the optimum level of labelling, which will give optimal NMR characteristics whilst being economically viable, we do not need to accurately determine the level of labelling, therefore a degree of error in the calculations and the assumptions of no ^{2}H, ^{17}O and ^{18}O are acceptable. The level of ^{15}N labelling rises sharply up to 65 % at a concentration of approximately 1 gL^{-1} of $(^{15}NH_4)_2SO_4$, at concentrations above this the level of labelling begins to plateau to a maximum of approximately 90 % at a concentration of 3.88 gL^{-1}.

Concentration of $(^{15}NH4)_2SO_4$ in growth medium (gL^{-1})	Calculated level of ^{15}N labelling (± 1.6) (%)	Cost of label per Litre of medium (£)
0	1.6	0
0.0096	2.2	0.30
0.096	11	2.98
0.48	42	14.90
0.96	65	29.81
1.92	80	59.62
2.88	87	89.43
3.84	89	119.24

Table 5.27 - Level of ^{15}N labelling in samples cultured in varying concentrations of $(^{15}NH_4)_2SO_4$, calculated from the relative intensity of the mass ion at 181.1. Error of ± 1.5 %. Cost of label shown (Sigma)

LC-MS analysis of the purified and hydrolysed chitinous materials extracted from mycelia cultured in varying concentrations of $(^{15}NH_4)_2SO_4$ suggests that a concentration of 0.96 gL^{-1} results in 65 % of the glucosamine units containing the ^{15}N isotope. Increasing this concentration of additive does not significantly increase this level of labelling. In order to determine if this is sufficient to improve the sensitivity in ^{15}N CPMAS ssNMR studies, ^{15}N CPMAS ssNMR analysis of the purified chitin extracts described above was performed, prior to hydrolysis.

5.7 ^{15}N CPMAS ssNMR Analysis

^{15}N CPMAS ssNMR analysis of the purified extracts of the fungal biomass cultured in the varying concentrations of $(^{15}NH_4)_2SO_4$ was performed, the spectra are displayed in Figure 5.71. As described in section 3.6.4.5, the peak at approximately – 257 ppm represents the amido nitrogen of chitin, the amino nitrogen of chitosan occurs at approximately – 357 ppm. The spectra indicate the presence of chitin in all samples,

the absence of a peak at – 357 ppm, in all of the spectra, indicates that this chitinous material has a very low DDA. Increasing the concentration of $(^{15}NH_4)_2SO_4$ does not appear to change the chemical shift or line-width of the peak corresponding to chitin and no additional peaks are seen. This implies that the polymorphic form of chitin and DDA are unaffected by the addition of the label. Importantly the number of repetitions required in order to acquire the spectra decreased with increased concentration of additive in the growth medium, shown in brackets on Figure 5.71. This decreases the acquisition time required to obtain the spectra.

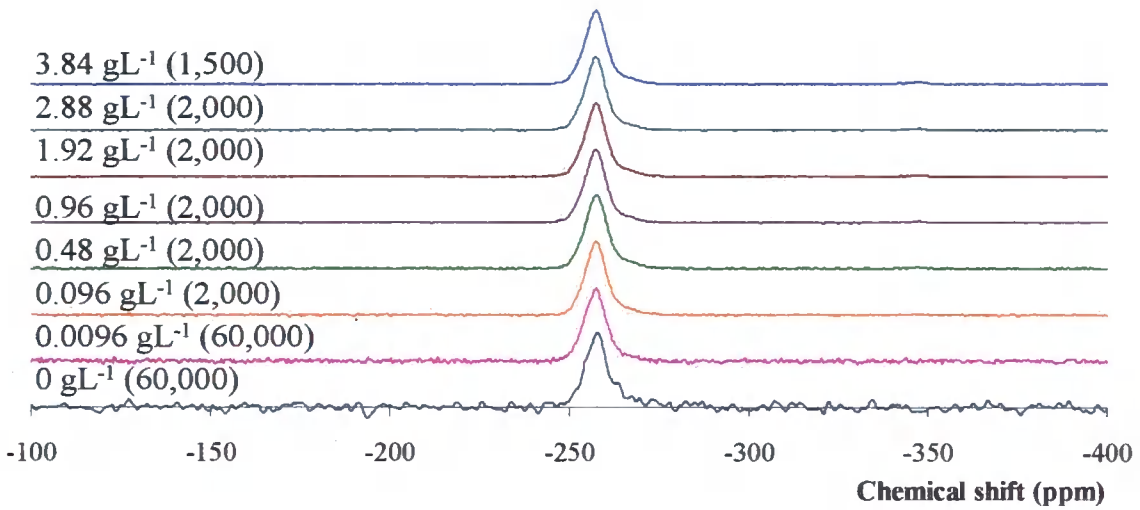
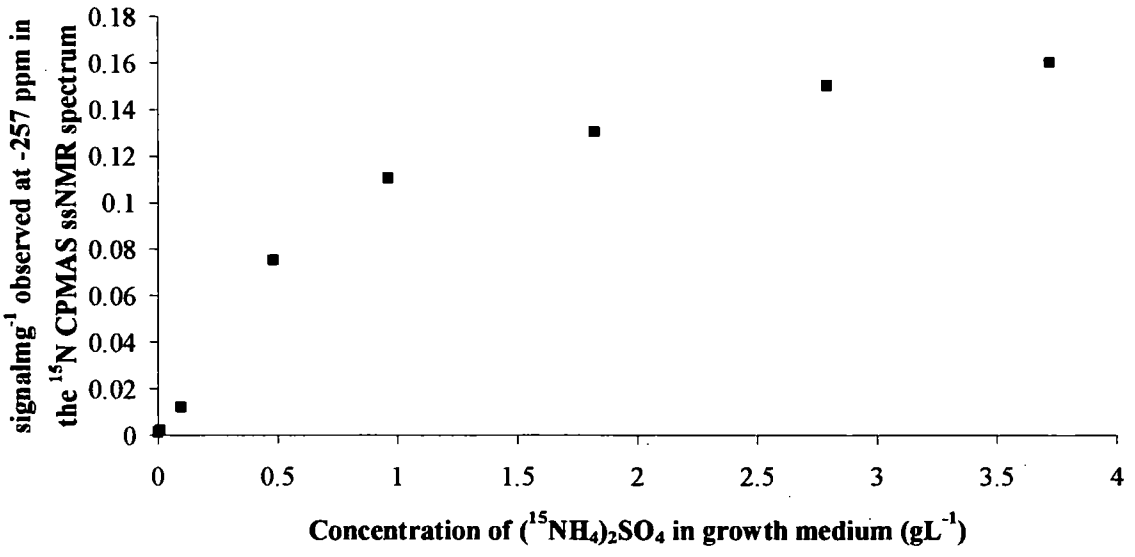


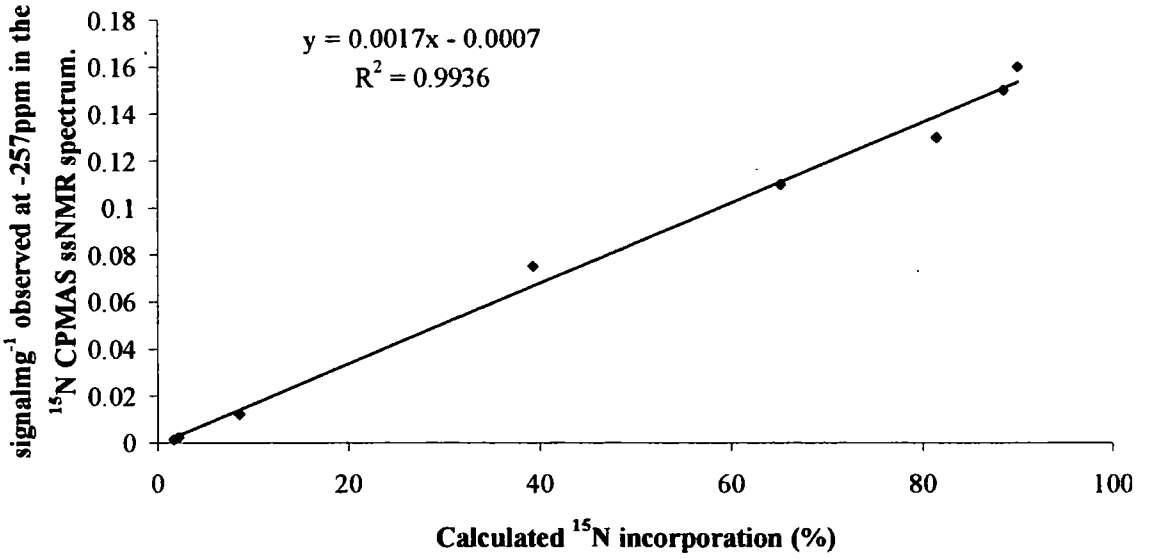
Figure 5.71 - ^{15}N CPMAS ssNMR spectra of chitin extracted from fungal biomass cultured in growth media containing varying concentrations of $(^{15}NH_4)_2SO_4$. The number of repetitions required to obtain the spectra are shown in brackets.

We can qualitatively conclude that the spectra obtained from materials extracted from biomass cultured with high concentrations of $(^{15}NH_4)_2SO_4$ are of better quality than that obtained from materials cultured in natural abundance conditions, despite the reduced acquisition times, as there appears to be less noise on the baseline of the spectra. To quantify this improvement in the ssNMR spectra of the labelled materials the amount of signal per unit mass of sample, adjusted to take the number of repetitions used to obtain the spectra into account, can be compared. This data is shown in Graph 5.12.



Graph 5.12 – Amount of signal seen at ~ -257 ppm, corresponding to chitin, per milligram of sample in the ^{15}N CPMAS ssNMR spectra of chitinous extracts of materials cultured in growth media of varying concentrations of $(^{15}NH_4)_2SO_4$.

The results follow a similar trend to that shown in Graph 5.11, the amount of signal seen per unit mass of sample increases rapidly up to a concentration of approximately 1 gL^{-1} of $(^{15}NH_4)_2SO_4$, above this value the amount of signal rises slowly and plateaus. This is to be expected, as the amount of signal should vary linearly with the amount of ^{15}N in the sample. Graph 5.13 demonstrates the linear relationship between the calculated level of ^{15}N labelling and the amount of signal per unit mass of sample observed.



Graph 5.13 - Amount of signal per milligram of sample seen at ~ –257 ppm (corresponding to chitin) in the ^{15}N CPMAS ssNMR spectra of samples against the calculated degree of ^{15}N labelling.

The ^{15}N CPMAS ssNMR data suggests that increasing the level of ^{15}N labelling of the samples improves the amount of signal observed and decreases the acquisition time required. The number of repetitions required to attain the spectra decreased from 60,000 to 2,000 for the materials extracted from growth media containing 0 and 0.48 gL^{-1}, at concentrations above this 2,000 repetitions were required. This decreases the acquisitions time from approximately 16 hours to under one hour. This data reinforces the conclusion drawn in section 5.6, that the optimal level of labelling is 0.96 gL^{-1}.

5.8 Labelling of Other Components in the Fungal Cell Wall

In order to ascertain the efficiency of the method of labelling we must consider how much of the ^{15}N label will be assimilated into the metabolic pathways of the fungi and how much will remain unused. Additionally what proportion of the ^{15}N label is taken up into the biosynthetic pathway of chitin and how much is assimilated by other nitrogen metabolic pathways.

In order to address this latter issue P2 was cultured in a modified version of Medium A, containing 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$ and varying concentrations of peptone. Elemental analysis indicates that peptone accounts for approximately half of the medium's nitrogen source; therefore decreasing the concentration of peptone should decrease the concentration of available ^{14}N, resulting in a larger uptake of $^{15}NH_4^+$ by the biosynthetic pathways of other nitrogen containing metabolites. If increased labelling of other metabolites occurs they may be visualised by ^{15}N CPMAS ssNMR analysis of the resulting fungal material. *P. chrysogenum* was cultured in media containing between 0 and 1 gL^{-1} of peptone under the usual conditions, see section 7.3.2.4. The wet mycelia were then collected by filtration and submitted for ^{15}N CPMAS ssNMR analysis. The yields of the wet weight of mycelium are shown in Table 5.28. The ssNMR spectra are shown in Figure 5.72. The spectra all display a peak at approximately -257 ppm corresponding to the amido nitrogen of chitin, the line-width and chemical shift of this peak remain unchanged with varying concentrations of peptone, indicating that the polymorphic form of chitin is unaltered. Notably the amount of signal observed at the chemical shift corresponding to chitin was not altered by the decrease in the concentration of peptone. This suggests that chitin biosynthesis preferentially uses the labelled additive as a nitrogen source

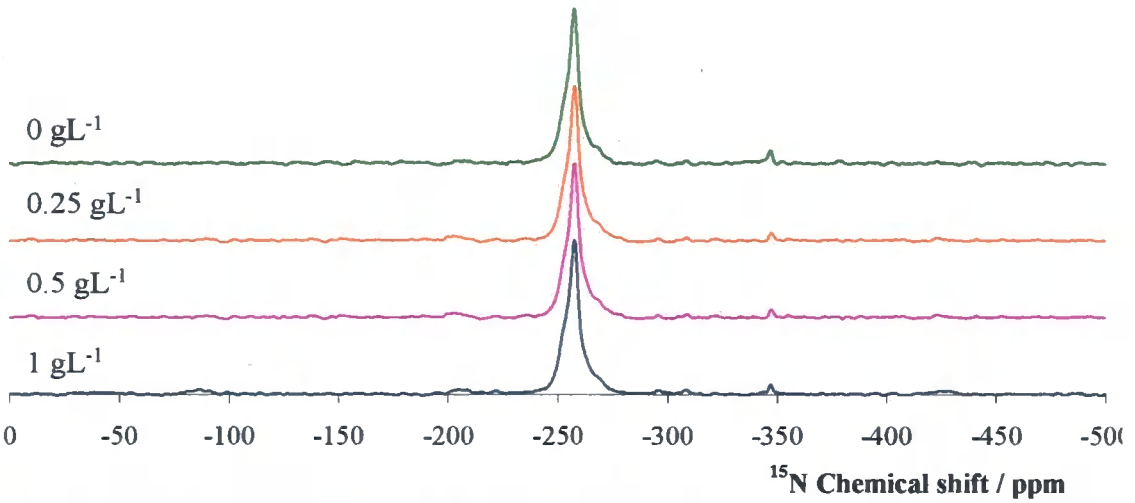


Figure 5.72 - ^{15}N CPMAS ssNMR spectra of sterilised 'whole cell' samples of P. chrysogenum cultured in modified medium A (rich) containing varying concentrations of peptone, concentration shown above each spectra. 2,000 repetitions were required to acquire the spectra.

Additional peaks, of low intensity, can be seen with chemical shifts between -260 and -350 ppm, and between -150 and -250 ppm in the spectra shown in Figure 5.72. As discussed in section 4.2, the fungal cell wall typically contains between 20 and 30 % glycoproteins, which are anchored to the plasma membrane by GPI anchors and can form covalent interactions with chitin and β-glucans. The additional peaks visible in the spectra may be accounted for by these glycoproteins, the intensities of which are typically lower than that of chitin, the assignment of these peaks is discussed in section 3.9.4. An increase in the ^{15}N content of the glycoproteins, resulting from the assimilation of $^{15}NH_4^+$ by their biosynthetic pathways, should increase the intensity of these peaks. However, varying the concentration of ^{14}N, by decreasing the concentration of peptone, in the media does not appear to have had a significant effect on the number or intensity of the additional peaks. This may indicate that the nitrogen containing metabolites do not assimilate the $^{15}NH_4^+$ label and the nitrogen sources available in the malt extract portion of the medium are preferentially metabolised. The yield of mycelia cultured in the medium containing no peptone was not significantly lower than that cultured in the normal media suggesting that the nutrient supply was not exhausted, see Table 5.28, therefore the labelled additive may not have been assimilated.

Concentration of Peptone (gL^{-1})	Wet weight of mycelia (gL^{-1})
1	31.8
0.5	30.1
0.25	25.8
0	23.6

Table 5.28 - Wet weight of mycelia produced in modified medium A (rich) containing varying concentrations of peptone.

Alternatively the nitrogen metabolic pathways may have assimilated the ^{15}N isotope but the peaks are at the limit of detection by NMR. Chitin accounts for less than 10 % of the fungal cell wall but has a small range of ^{15}N chemical shifts, therefore results in a resonance peak of high intensity. Although glycoproteins account for a larger proportion of the fungal cell wall they will display a number of chemical shifts, each of much lower intensity and at the limit of the detector. A comparison of the whole cell ^{15}N CPMAS ssNMR spectra of labelled and unlabelled mycelia is shown in Figure 5.73. The lower intensity peaks, corresponding to other nitrogen containing components of the fungal material, that are visible in the labelled sample are not visible in the unlabelled sample, they may be masked by the low signal:noise. Their appearance in the spectra of samples cultured in labelled conditions suggests that there are other ^{15}N containing species.

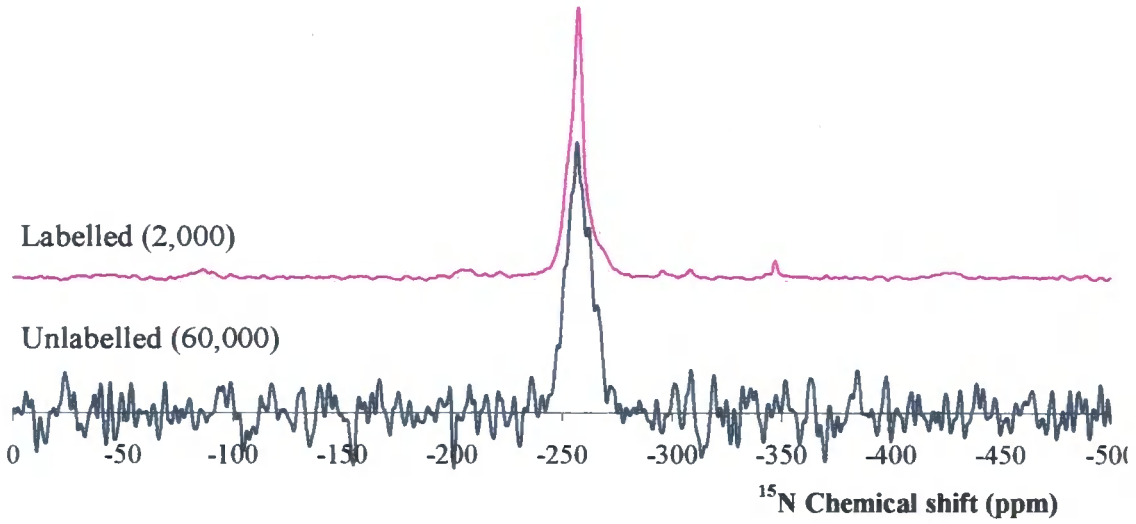


Figure 5.73 - ^{15}N CPMAS ssNMR spectra of wet mycelium cultured in labelled and unlabelled media. The number of repetitions required to obtain the spectra are shown in brackets.

These preliminary tests are inconclusive in determining the degree to which other nitrogen containing species are ^{15}N labelled within the cell wall. The selectivity of this method of labelling was not furthered investigated as the main aim of the study was to culture ^{15}N labelled chitinous materials.

In order to address the other question of efficiency, what proportion of the ammonium label is metabolised, the concentration of ammonium in the medium was determined at the start and end of the growth period by the modified Berthelot's method, as described in section 7.2.9.[273, 274] This assay has been shown in the literature to display minimal interference from amino acids and peptides.[275] The assay involves the reaction of ammonia with hypochlorite to form chloramines, which react with phenol to form the intermediate monochloroquinoimine. This intermediate then couples with a second phenolic molecule to for a blue indophenol chromophore. A standard plot was constructed with known $(NH4)_2SO_4$ concentrations (0 to 1 gL^{-1}) in medium A, see Graph E.17 in section 7.2.9. A linear relationship between concentration and absorption at 630 nm was observed in this region and best-fit analysis gave an equation of Abs = 8.80 x C + 0.264, with an R^2 value of 0.998, where Abs is the absorption at 630 nm and C is the concentration of ammonium (gL^{-1}).

Analysis of the standard medium A, with no addition of $(^{15}NH_4)_2SO_4$, indicated that following eight day's growth of *P. chrysogenum* under normal conditions, see section 7.3.2, the concentration of ammonium ions in the medium rises by 0.04 gL^{-1}. Taking this into account, assay of media containing 0.96 gL^{-1} $(^{15}NH_4)_2SO_4$ before inoculation with the P2 strain of *P. chrysogenum* and after culture for eight days under the normal conditions, see section 7.3.2, suggests that only 39 % of the ammonium remains in solution in the medium, ie. 61 % of the label has been incorporated into the fungal material. This data suggests that the labelling strategy is 61 % efficient, with 39 % of the labelled ammonium remaining in solution.

5.9 ^{15}N labelling of Other Fungal Species

In order to demonstrate that this method of labelling is not limited to the P2 strain of *Penicillium chrysogenum* two other fungal species, *Aspergillus niger* and *Mucor rouxii,* were cultured in the same medium, containing 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$, under the same conditions. The fungal material was then extracted and purified by the normal chemical means, see section 7.4.6, and analysed by ^{15}N CPMAS ssNMR. Samples were also hydrolysed and analysed by and LC-MS.

The data produced by LC-MS was analysed using the relative intensities of the peaks at 181.1 and the standard plot, Graph 5.9, as described in section 5.6. This analysis suggests that unlabelled samples of chitin extracted from *M. rouxii* and *A. niger* have calculated levels of ^{15}N labelling of 1.50 and 1.82 % respectively. Accounting for an error of ± 1.5 %, previously calculated in section 5.6, these values fall in the range of the natural abundance of ^{15}N isotopes. The data indicates that samples extracted from *M. rouxii* and *A. niger* cultured in the presence of 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$ have calculated levels of ^{15}N labelling of 72.9 and 69.6 % respectively, this compares to *P. chrysogenum* which contained 65.1 % ^{15}N under the same conditions.

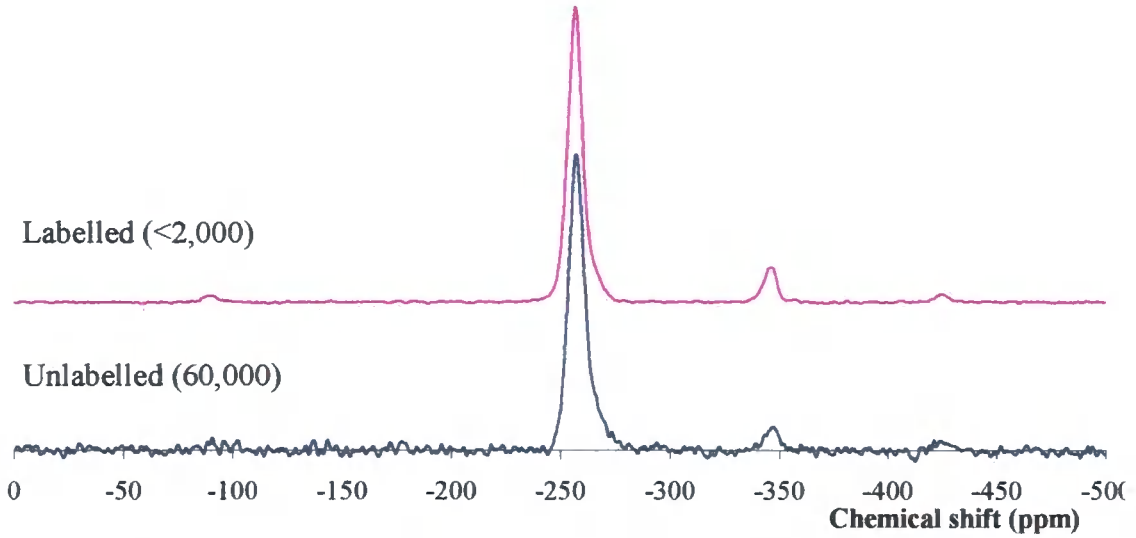


Figure 5.74 - ^{15}N CPMAS ssNMR of purified samples of chitin extracted from *M. rouxii* cultured in labelled (containing $(^{15}NH_4)_2SO_4$) and unlabelled media. The number of repetitions required to obtain the spectra are shown in brackets.

The ^{15}N CPMAS ssNMR spectra of purified samples of chitin extracted from *M. rouxii* cultured in labelled and unlabelled media are shown in Figure 5.74. The spectra both display a peak at approximately –257 ppm corresponding to the amido nitrogen of chitin. An additional peak at approximately –350 ppm can also be seen, this peak corresponds to chitosan, which is present as a matricial component of the fungal cell wall in *M. rouxii*, this is further discussed in section 4.10. LC-MS data indicates that 72.9 % of the nitrogen in chitinous materials extracted *M. rouxii* cultured in labelled media are present as ^{15}N isotope, this increase in the concentration of ^{15}N can be seen in the ^{15}N CPMAS ssNMR spectra as the number of repetitions required decreased from 60,000 to under 2,000 for the labelled sample. This reduces the acquisition time to under one hour. Qualitative assessment of the spectra indicates that the spectrum acquired from the sample cultured in labelled conditions is of higher quality, has an increased signal:noise, as the spinning side bands at approximately –90 and –220 ppm can be resolved from the noise on the baseline. The increase in quality of the spectra and the reduction in acquisition time implies that the sample is ^{15}N labelled. ^{15}N CPMAS ssNMR analysis of samples extracted from *A. niger* cultured in labelled conditions displayed similar results and a reduction of the acquisition time to under one hour, spectra not shown.

The data indicates that the labelling methodology is general to fungal systems producing chitin and is not specific to the P2 strain of *P. chrysogenum* as comparable levels of labelling were observed in two other fungal species.

5.10 Conclusion – Development of ^{15}N labelling Strategy

The work described in this chapter aimed to develop a simple, cost-effective, and efficient method to ^{15}N label the chitinous material in fungal systems, allowing more accurate determination of the DDA by ^{15}N CPMAS ssNMR. The use of minimal media containing discrete ^{15}N labelled nitrogen sources that are commercially available proved an inefficient method of culturing ^{15}N labelled fungal material for the extraction of chitin, as the yields of fungal biomass produced by these media were significantly lower than those produced by the complete medium A. As this strategy proved economically and practically unviable an alternative approach was investigated using the complete medium A, which is known to produce reasonable yields of *P. chrysogenum*. Study of the biosynthetic pathway of the chitin precursor, UDP-GlcNAc, suggested that the addition of $(^{15}NH_4)_2SO_4$ to the complete medium A would result in the production of ^{15}N labelled chitinous material in the fungal cell wall. Modification of the medium by the addition of $(^{15}NH_4)_2SO_4$ did not significantly affect the yield, or chitin content, of the fungal material produced. The degree of labelling of the chitinous material cultured in media containing a series of concentrations of this additive was monitored by LC-MS and the resulting improvement in the ^{15}N CPMAS ssNMR spectrums acquired observed. The results indicate that the optimal concentration of $(^{15}NH_4)_2SO_4$ in medium A is 0.96 gL^{-1}, at this concentration approximately 65 % of nitrogen in the chitinous material is present as the ^{15}N isotope. This increases the amount of signal observed in the ^{15}N CPMAS ssNMR spectrum by two orders of magnitude, decreasing the acquisition time from approximately 16 hours to under one hour. A further doubling of the concentration of $(^{15}NH_4)_2SO_4$ in the medium only effects a 15 % rise in the amount of ^{15}N labelling and hence a 15 % increase in the amount of signal seen by ^{15}N CPMAS ssNMR, as the

amount of signal observed by ^{15}N CPMAS ssNMR follows a linear relationship with the concentration of ^{15}N. This does not significantly increase the quality of the spectrum and does not reduce the acquisition time required to obtain the spectrum. As the cost of the medium is dominated by the cost of the label, doubling the concentration of label doubles the total cost of the experiment, but does not yield any significant advantage. We can therefore conclude that the optimal concentration of $(^{15}NH_4)_2SO_4$ in the growth medium is 0.96 gL^{-1}.

The labelling of chitinous materials has practical and economical ramifications; in practical terms an unlabelled sample requires 60,000 repetitions to acquire a spectrum, a sampled labelled at 65 % requires less than 2,000 repetitions. Only one 60,000 repetition run can be carried out per day, however, 10 to 12 short, 2000 repetition, runs may be carried out in one day. Additionally, even with 60,000 repetitions the quality of the spectrum of an unlabelled sample is not as good as that of a 2000 repetition run of a labelled sample. Therefore, practically, the analysis can be carried out more swiftly and to a higher standard when samples are ^{15}N labelled. Economically speaking, the EPSRC ssNMR service at Durham University charge a commercial client £135 for a short run, 2000 repetitions, however a longer run of 60000 repetitions would cost £370. The cost of labelled additive to achieve ~65 % labelled chitin, with a resultant reduction in the number of required repetitions to obtain a ssNMR spectrum giving a saving of £235, is approximately £30 per litre of medium, 1 L of medium produces approximately 0.25 g of labelled chitin, see Table 5.25.

The use of a labelled additive in medium A to produce ^{15}N labelled chitin is far more economically attractive than the use of minimal media, the costs of the label required to produce 1 g of 65 % labelled chitin is displayed in Table 5.29. The costs of the other ingredients of the media are insignificant in comparison to the label. If ^{15}N CPMAS ssNMR analysis is required it is more economically efficient to culture the fungal material in medium A containing 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$ than to use samples with natural abundance levels of ^{15}N due to the reduction in the cost of acquiring a ^{15}N CPMAS ssNMR spectrum for a sample that is labelled at 65 %. The total cost of

acquiring a ^{15}N CPMAS ssNMR spectrum labelled at this level, including the cost of producing the sample, is £141. A ^{15}N CPMAS ssNMR spectrum of an unlabelled sample will be of much poorer quality, as the amount of signal observed is decreased, see section 5.7, and will cost £370 to acquire.

Medium	Cost of ^{15}N label per Litre of medium for 65 % labelling (£)	Cost of ^{15}N label per gram of 65 % ^{15}N labelled chitin (£)	Total Cost (NMR time + sample)* (£)
A	No ^{15}N label	No ^{15}N label	370
A + 0.96 gl^{-1} $(^{15}NH_4)_2SO_4$	29.81	120	141
B	217.62	7996	534.80
D	141.28	1550	212.50

Table 5.29 - Comparison of the cost of three media required to achieve 65 % ^{15}N labelling of chitin. Costs shown per Litre of medium and as cost required to produced 1g of ^{15}N labelled chitin, assuming yield of chitin to be 5.6 % of dry weight of mycelia produced. *Total cost is defined as the cost of 50mg of sample, minimum requirement for ssNMR, plus the cost of acquiring a ^{15}N CPMAS ssNMR spectra. Costs of materials from Sigma. The cost of acquiring a ^{15}N CPMAS ssNMR of an unlabelled sample is shown for comparison.

Investigations into the efficiency of this method of labelling suggest that 39 % of the label remains unused. ^{15}N CPMAS ssNMR data suggests that other nitrogen containing species within the mycelia are ^{15}N labelled although further experiments are required to identify the extent of labelling and actual content of other nitrogen containing metabolites.

The aim of this chapter was to develop a simple, cost-effective, and efficient method for ^{15}N labelling the chitinous material in fungal systems to allow more accurate determination of the DDA. We have proposed a labelling strategy involving the addition of $(^{15}NH_4)_2SO_4$ to the complete medium A at a concentration of 0.96 gL^{-1} and demonstrated that this provides a general method for the culture of ^{15}N labelled chitin in fungal cell walls. Three fungal systems, *A. niger, M. rouxii and P. chrysogenum*, when cultured in this modified medium produce reasonable yields of chitinous material containing upwards of 65 % of the monomer units ^{15}N labelled. We

have demonstrated the practical and economic viability of this method. To our knowledge there is no literature precedent for ^{15}N labelling chitinous material in fungal systems.

5.11 Determination of the DDA of ^{15}N Labelled Chitinous Material by ^{15}N CPMAS ssNMR.

The intended application of the ^{15}N labelling strategy is the accurate determination of the DDA of chitinous materials from fungal sources. In section 3.6.5 we concluded that the ^{15}N CPMAS ssNMR is the most suitable method of analysis for determining the DDA of chitinous materials extracted from fungal sources as it does not require the samples to be solubilised, derivatised or extensively dried. Additionally, the presence of polysaccharide impurities common in chitinous materials extracted from fungal sources does not interfere with the determination. This assay is more reliable than other proposed literature procedures as it considers both the amine and amino group concentration, however, ^{15}N CPMAS ssNMR is hampered by the low natural abundance of ^{15}N, which results in long acquisition times. By ^{15}N labelling the chitinous material in the fungal cell wall we hoped to overcome this issue.

^{15}N CPMAS ssNMR spectra of chitin with a low DDA containing natural abundance ^{15}N and 65 % ^{15}N are shown in Figure 5.75. Both spectra display a peak at approximately –257 ppm, corresponding to the amido nitrogen of chitin, however in the unlabelled sample this is the only peak that can be resolved from the noise on the baseline. In the spectrum of the labelled sample the spinning side bands at approximately –90 and –420 ppm can be resolved and also, more importantly, a peak at approximately –350 ppm corresponding to deacetylated chitin. The relative intensities of the peaks at –257 and –350 ppm may be used to accurately determine the DDA, which in the case of the ^{15}N labelled samples indicates a DDA of 0.8 %. It is clear from Figure 5.75 that the DDA cannot be determined for chitinous materials with a low DDA using this method if they are not ^{15}N labelled. Comparison of the signal to noise ratios of these spectra indicates that unlabelled samples, acquired using

a minimum of 30,000 repetitions, have a confidence limit of approximately ± 5 %, that is to say DDAs below 5 % will not be determined. The spectra of chitinous samples ^{15}N labelled at 65 %, which are acquired using a minimum of 1,000 repetitions, have a confidence limit of ± 0.5 % allowing a much greater range of DDA values to be assayed.

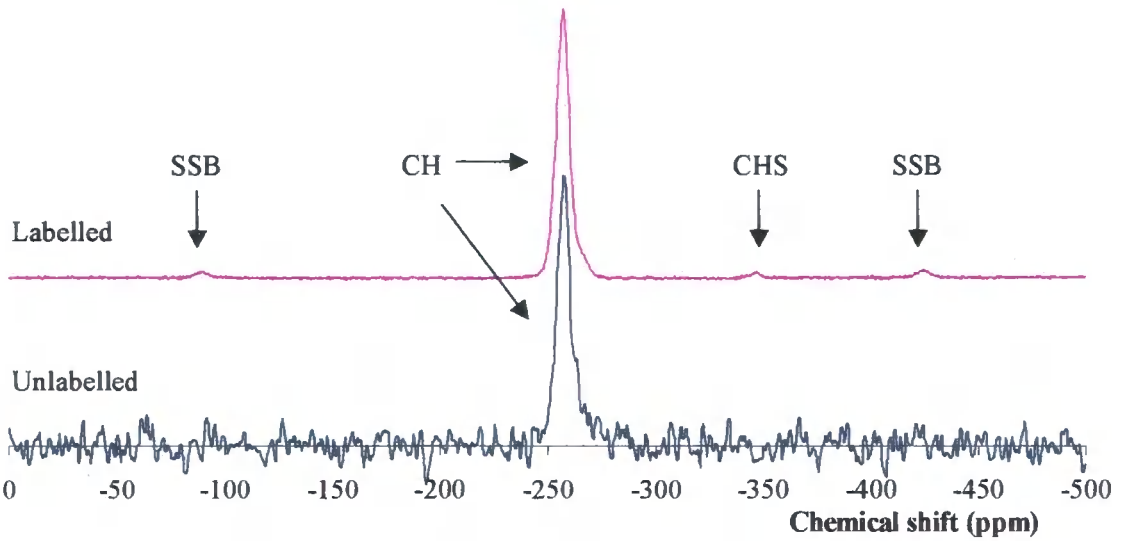
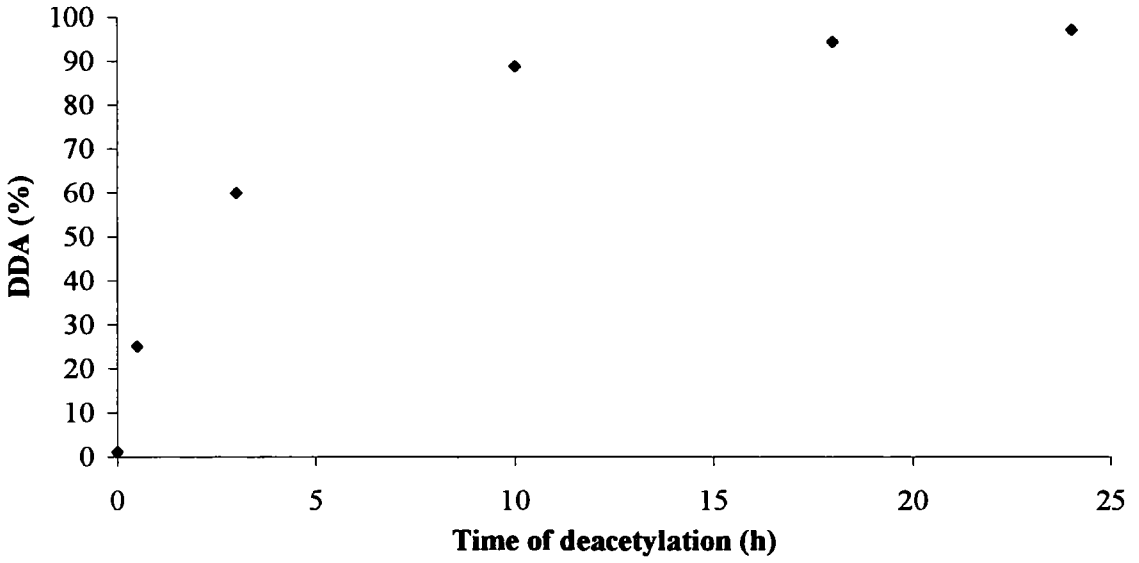


Figure 5.75 – ^{15}N CPMAS ssNMR of chitinous material extracted from fungal material cultured in labelled and unlabelled media. (SSB = spinning side bands, CH = chitin, CHS = chitosan.)

In order to prove the validity of this method of determination of the DDA a series of samples of chitin extracted from fungal biomass cultured under the ^{15}N labelling conditions outlined in section 5.5 were deacetylated in 40 % NaOH at 100 °C under nitrogen for varying lengths of time. The samples were then analysed by ^{15}N CPMAS ssNMR and the relative intensities of the amino and amine nitrogen peaks were used to determine the DDA of the samples. The results are shown in Graph 5.14.



Graph 5.14 - DDA of a series of samples of chitin extracted from P. chrysogenum, cultured in ^{15}N labelled conditions, deacetylated for varying lengths of time. DDA determined by ^{15}N CPMAS ssNMR

In order to reinforce our conclusion, that ^{15}N CPMAS ssNMR is the most suitable method of determining the DDA of chitinous materials extracted from fungal sources, the DDA of the samples was analysed by the alternate methods outlined in chapter 3. Elemental analysis, the dye adsorption method, FTIR spectroscopy and ^{13}C CPMAS ssNMR were all performed, the results are collated in Table 5.30 for comparison.

Time of deacetylation (h)	DDA (%)				
	Elemental analysis	Dye adsorption	IR	^{13}C CPMAS ssNMR	^{15}N CPMAS ssNMR
0	-57	1.2	17	40	1
0.5	23	4.1	58	53	25
6	78	3.0	95	94	87
18	84	3.4	96	98	94
24	78	3.2	91	99.9	97

Table 5.30 - DDA of a series of deacetylated sample of chitin extracted from fungal biomass cultured in ^{15}N labelled conditions, determined by different methods.

The elemental analysis method of determining the DDA relies upon an accurate ratio of carbon to nitrogen content of the sample. As described in section 4.5, chitinous materials extracted from fungal sources usually contain polysaccharide impurities. These non-nitrogen containing impurities will result in inflated carbon to nitrogen ratios and inaccurate determination of the DDA, this explains the improbable DDA values calculated by this method. The dye adsorption method requires that all of the dye sites, the primary amines, are accessible to the dye ions. The relatively unvarying DDA values acquired by this method reinforce the conclusion drawn in section 3.6.3.3, that the crystallinity of the samples prevents this. IR spectroscopy was discounted due to the hygroscopic nature of chitin, as the presence of adsorbed water interferes with the absorption bands required for analysis. ^{13}C CPMAS ssNMR as a method of determining the DDA produced unreasonably high values of DDA. This was predicted in section 3.6.4.4 and is thought to be due to the presence of polysaccharide impurities which have ^{13}C chemical shifts in the range of the sugar backbone carbons of chitin. ^{15}N CPMAS ssNMR analysis appears to give reasonable results for the DDA of the samples.

In order to more quantitatively compare the reliability of each method of determining the DDA we can consider the kinetics of the deacetylation reaction. The kinetics of the heterogeneous deacetylation of chitin in alkali conditions has been studied by several authors and is has been reported to be a pseudo first order reaction, if the concentration of alkali is in excess.[276-278] Therefore the rate of deacetylation can be written as:

$$-\frac{d[C]}{dt} = k[C] \times [NaOH] \approx k'[C]$$

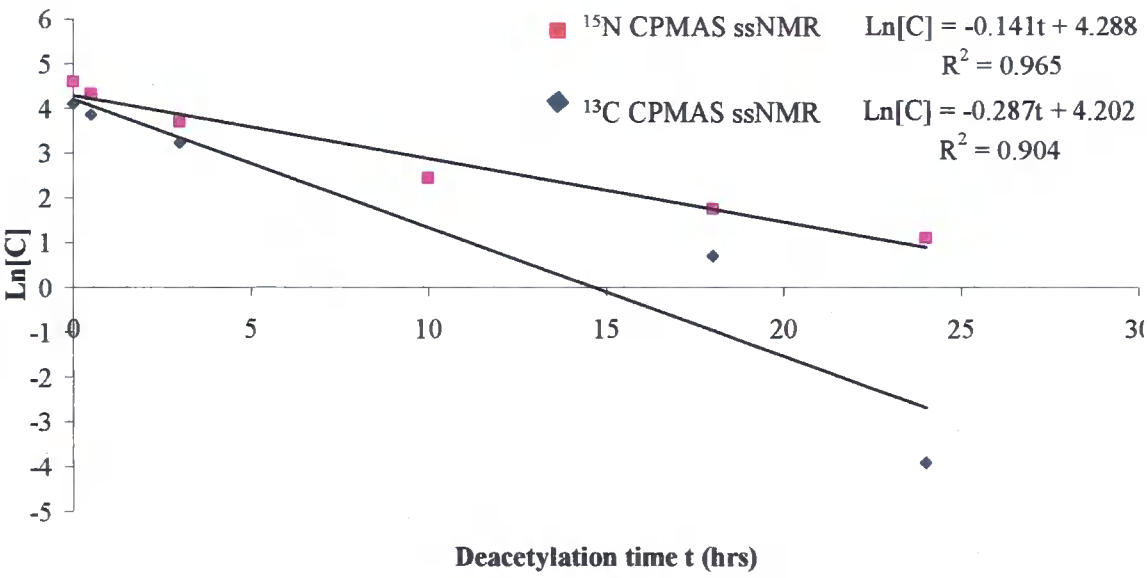
Where [C] is the concentration of *N*-acetyl-D-glucosamine residues in *N*-deacetylated chitin, k' is the pseudo first order rate constant and t is the deacetylation time. Therefore, the plot of Ln[C] against reaction time should produce a straight line, the slope of which gives the pseudo first rate constant, k'. Using the DDA values determined by each method, pseudo first order rate plots were constructed and the rate

constants calculated, these values along with the correlation coefficient, R^2, values are shown in Table 5.31.

Method of determination	Pseudo first order rate constant k' (min^{-1})	Correlation coefficient R^2
Elemental analysis	0.071	0.61
Dye adsorption	-0.0004	0.025
IR	0.086	0.50
^{13}C CPMAS ssNMR	0.29	0.90
^{15}N CPMAS ssNMR	0.14	0.96

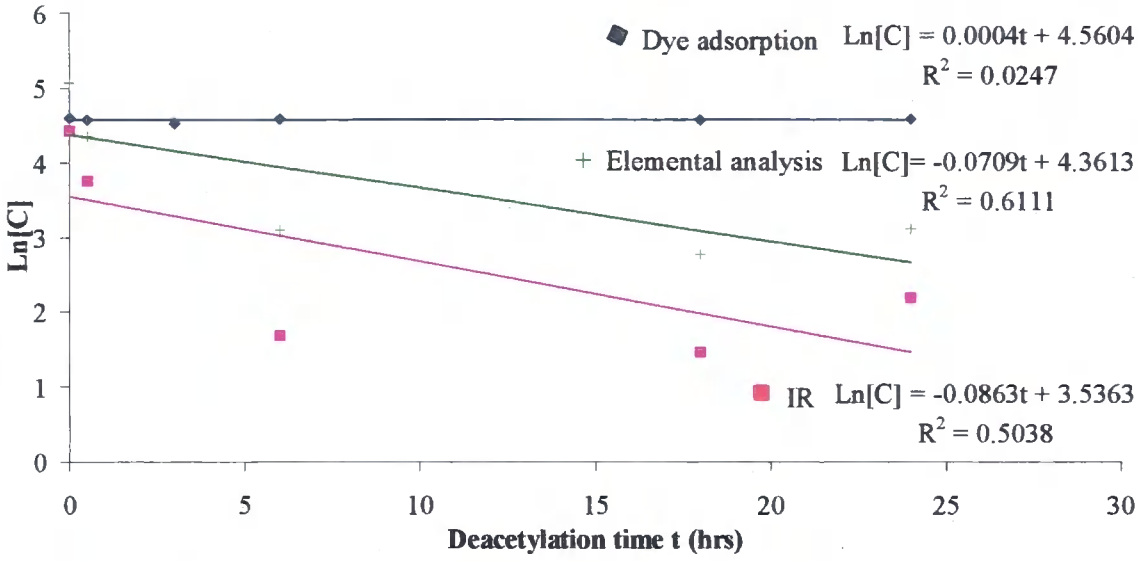
Table 5.31 - Pseudo first order rate constants, and correlation coefficients, for the deacetylation of chitin under alkali conditions as determined by differing methods of analysis.

Evaluation of the data shown in Table 5.31 indicates that the ssNMR methods of determining the DDA produce semi-logarithmic plots that have correlation coefficients above 0.90. This indicates that the deacetylation process does follow an approximate pseudo first order kinetics, these rate plots are shown in Graph 5.15.



Graph 5.15 - Pseudo first order rate plot of the deacetylation of chitin in alkali. Where [C], the concentration of *N*-acetyl-D-glucosamine, is determined by ^{15}N and ^{13}C CPMAS ssNMR

Using kinetic plots of the deacetylation reactions to assess the reliability of each of the methods of determining the DDA we can see that ssNMR is the only method that produces pseudo first order rate plots. The semi-logarithmic plots resulting from the DDA values obtained by elemental analysis, IR and the dye adsorption method are shown in Graph 5.16 for comparison, it can be seen that these methods of analysis do not produce straight-line plots. As the deacetylation behaviour of chitin under these conditions is known to be approximately pseudo first-order, this indicates that the DDA values obtained are not reliable. Comparison of the correlation coefficients of the ^{13}C and ^{15}N CPMAS ssNMR data indicates that ^{15}N CPMAS ssNMR provides more reliable results. This is reflected in the absolute values of DDA obtained, ^{13}C CPMAS ssNMR indicates that the untreated chitinous extract has a DDA of 40 %; the DDA of 1 % estimated by ^{15}N CPMAS ssNMR is more reasonable. As previously concluded, the high DDA values predicted by ^{13}C CPMAS ssNMR are probably due to the presence of polysaccharide impurities in the chitinous extracts. We can therefore conclude that ^{15}N CPMAS ssNMR analysis is the most reliable method of determining the DDA of ^{15}N labelled chitinous material extracted from fungal sources. Closer inspection of the rate plot produced from the ^{15}N CPMAS ssNMR data, shown in Graph 5.15, indicates that the results deviate from the straight-line plot, forming a slight curvature. This can be explained by consideration of the chitin source, the crystalline nature of which prevents the deacetylation of acetamide groups in highly crystalline regions. The deacetylation reaction will proceed quickly at the start as the available acetamide groups in amorphous regions are deacetylated, this rate will then decrease as the crystal structure of chitin sample must be destroyed in order to access the remaining acetamide groups. Therefore, the pseudo first order approximation is too simplistic, as it does not account for the variance in the accessibility of the acetamide groups. However, for the purpose of assessing the methods of determining the DDA of chitinous samples, we may use it as a rough approximation.



Graph 5.16 - Pseudo first order rate plot of the deacetylation of chitin in alkali. Where [C], the concentration of *N*-acetyl-D-glucosamine, is determined by the dye adsorption method, IR spectroscopy and elemental analysis.

The relatively low abundance of nitrogen containing compounds in the fungal cell wall, in comparison to carbon, presents the possibility of the use of ^{15}N CPMAS ssNMR analysis of the whole fungal cell to determine the DDA. This removes the need for the lengthy extraction and purification procedures, which are required for all other methods of analysis investigated. The ^{15}N CPMAS ssNMR spectrum of ^{15}N labelled raw fungal biomass, which has been sterilised by autoclaving and analysed whilst wet, is shown in Figure 5.76. Comparison to the ^{15}N CPMAS ssNMR spectrum of the extracted and purified chitin material from this fungal biomass demonstrates that there is little change upon extraction. The integration of the chitin and chitosan peaks indicate a DDA of 0.8 % in all spectra, therefore, the DDA may be determined from the whole cell material. The ^{15}N CPMAS ssNMR spectrum of a commercial sample of chitin is shown for comparison.

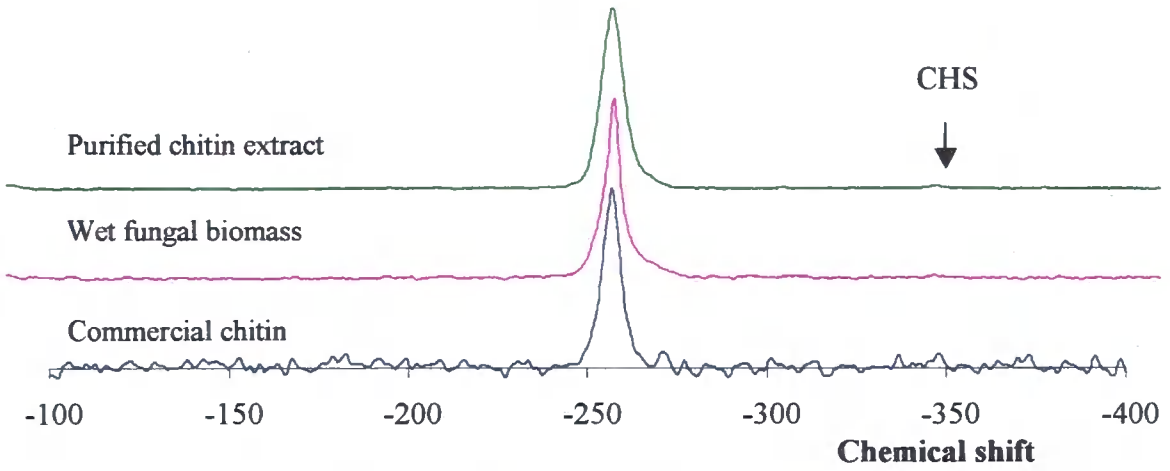


Figure 5.76 - ^{15}N CPMAS ssNMR spectra of the wet fungal biomass produced from the culture of the P2 strain of *P. chrysogenum* in ^{15}N labelled conditions and the purified chitin extract. The spectrum of commercial chitin is shown for reference. CHS = the amino peak of deacetylated units.

5.12 Conclusion

We have developed a simple, cost-effective, novel strategy for the ^{15}N labelling of chitinous materials in fungal cell walls that results in chitinous material containing upwards of 65 % of the monomer units ^{15}N labelled. This decreases the acquisition time required for ^{15}N CPMAS ssNMR significantly and increases the quality of the spectra obtained by two orders of magnitude allowing fast and reliable determination of the DDA of the chitinous material. We can conclude that ^{15}N CPMAS ssNMR is the optimal method of determining the DDA of chitinous extracts from fungal sources cultured in ^{15}N labelled conditions, as it is fast, reliable, economical and allows determination of DDAs between 0.5 and 99.5 %. This procedure is applicable to the whole range of DDA and does not require the sample to be solubilised, derivatised or extensively dried. It is also unaffected by the presence of polysaccharide impurities, common in fungal sources of chitin, and may be carried out on the whole fungal cell negating the need for lengthy extraction procedures. Comparison of the pseudo first order rate plots produced using the DDA data obtained by several different methods indicates that ^{15}N CPMAS ssNMR is the most accurate method of determination.

Additionally the relatively low abundance of nitrogen containing compounds in the fungal cell wall, in comparison to carbon, means the ^{15}N CPMAS ssNMR analysis of the whole fungal cell may be used to determine the DDA. This removes the need for lengthy extraction and purification procedures, which is required for all other methods of analysis investigated.

The novel labelling strategy developed here may allow more practical and accurate study of chitinous materials than was previously possible. For example, in addition to allowing more accurate determination of the DDA, this work may be extended to study the DDA of chitinous materials in fungal systems as a function of nutrition and growth stage. Additionally culturing ^{15}N labelled fungal systems may also afford the possibility of real-time investigations into the relative abundance of chitinous materials in fungal cell walls; this may be used to monitor chitin biosynthesis at different stages in the growth cycle and to comparatively study the chitin content of different fungal systems quickly and accurately.

Chapter 6 – Conclusions and Future Work

During the course of this thesis the extraction of chitinous materials from fungal sources by chemical and enzymatic procedures has been evaluated, with particular attention paid to the dry biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* in the penicillin manufacturing industry. Chitin and chitosan are high-value products that have a wide range of potential applications, however, currently the main commercial source of these biopolymers is the waste products of the seafood processing industry. This supply is seasonal and requires harsh extraction procedures, which result in a range of heterogeneous products. Fungal sources of chitinous materials, which offer a non-seasonal supply and do not require as severe extraction procedures, have so far been precluded due to the high costs of fermentation. However, the large-scale fermentation of *P. chrysogenum* for the production of penicillin produces vast quantities of waste fungal biomass containing chitin. It was hoped that a suitable extraction procedure could be evaluated for the production of high quality chitinous material, of known molecular weight and degree of deacetylation (DDA), from this waste material. Traditional chemical extraction procedures utilise harsh acid and alkali conditions, which can result in degradation of the chitin chain and heterogeneous deacetylation patterns. Therefore, the use of alternative enzymatic extraction procedures was explored.

In Chapter 4 the traditional chemical extraction procedures were evaluated and applied to the dry waste biomass, provided by Angel Biotechnology. The resulting chitinous materials were analysed and this material formed a datum against which alternative extraction procedures were compared. The extract was determined to contain 57 % glucosamine, indicating a high concentration of an impurity. An understanding of the nature of chitin in fungal cell walls and ^{13}C CPMAS ssNMR analysis of these materials suggested that this impurity is predominantly β-glucan. It was found that incubating the biomass with a series of hydrolytic enzymes, which were designed to remove the cell wall impurities, did not significantly improve the yield or quality of the materials produced upon subsequent chemical extraction. We hypothesised that this may be due to the highly crystalline nature of the sample, which renders the

material inaccessible to enzyme action. This material has been heat treated before we receive it, which may have increased this degree of crystallinity and therefore decreased the extractability of the chitinous material. As Angel Biotechnology were unable to provide the 'wet' biomass, in order to investigate this we acquired a strain of *P. chrysogenum* and subjected the biomass resulting from its incubation to the standard chemical extraction procedure. This did result in higher yields of chitin of improved purity, which may be explained by an increased level of extractability of the materials or may be due to a variance in the characteristics of the chitinous material in the different fungal strains employed. This 'wet' biomass was incubated with the hydrolytic enzymes and also cultured in media containing the same enzymes, however, neither of these experiments increased the level of purity of the chitinous materials obtained. Comparison to two other fungal sources, widely reported in the literature for the extraction of chitin and chitosan, indicated that the biomass remaining from the large-scale fermentation of *P. chrysogenum* might not provide the most efficient source of chitinous materials. *Mucor rouxii* was found to produce chitinous materials in much higher yields and of greater quality. In addition *Mucor rouxii* is a natural source of chitosan, which is of high-value in biomedical applications.

In order to study these chitinous extracts we first had to assign a set of analytical procedures in order to determine the key characteristics: the level of purity; the molecular weight; and the DDA. The literature procedures currently available, and their application to commercial sources of chitin and chitosan, are reviewed in Chapter 3. During the course of this research we were hindered by the lack of a reliable method of determining the DDA of chitinous materials. A plethora of procedures are reported in the literature, however, there is little agreement upon an absolute method. The majority of the available procedures require the sample to be solubilised, limiting the range of DDA values which can be assayed due to the insolubility of chitin. Alternatively they require the sample to be extensively dried, or of extremely high purity. Chitinous materials extracted from fungal sources generally are of low DDA, which precludes the use of methods that require solubilisation. They are also hygroscopic, which precludes FT-IR analysis, and they are found complexed to β-

glucans, which precludes determination of the DDA by ^{13}C CPMAS ssNMR. We therefore concluded that ^{15}N CPMAS ssNMR offers the most reliable method of determining the DDA. The use of this method has been reported in the literature, however, the low natural abundance of ^{15}N nuclides results in long and costly acquisition times. We therefore developed a simple, cost-effective, and efficient method to ^{15}N label the chitinous material in fungal systems, this is discussed in Chapter 5. We demonstrated that this directed labelling system, which is applicable to a number of fungal species, results in a more accurate determination of the DDA by ^{15}N CPMAS ssNMR and reduces the acquisition time of the spectra from sixteen hours to less than one hour. This procedure is applicable to the determination of a wide range of DDA (0.5 % to 99.5 %) and does not require the sample to be solubilised, derivatised or extensively dried. It is also unaffected by the presence of polysaccharide impurities, common in fungal sources of chitin, and may be carried out on the whole fungal cell negating the need for lengthy extraction procedures.

6.2 Future Work Potential

The CPMAS ssNMR procedures for whole cell analysis employed in this study, combined with the development of a novel and efficient ^{15}N labelling system opens up many interesting areas of possible research. The ability to accurately determine the DDA of fungal systems from the whole cell material may be extended to study the DDA of chitinous materials in fungal systems as a function of nutrition and growth stage without the need for lengthy extraction procedures, which in themselves may alter the DDA of the material.

In a wider view, the culturing of ^{15}N labelled fungal systems may also afford the possibility of real-time investigations into the relative abundance of chitinous materials in fungal cell walls by ^{15}N CPMAS ssNMR techniques. This may be used to monitor chitin biosynthesis at different stages in the growth cycle and to comparatively study the chitin content of different fungal systems quickly and accurately. Elucidation of the chitin content of the sample may then be transferred to

the ^{13}C CPMAS ssNMR spectrum of the sample in order to determine the concentration of non-chitinous materials in the fungal cell wall, in particular the polysaccharide contents that appears over a similar range of chemical shift values to chitin. Combining the ^{13}C and ^{15}N CPMAS ssNMR analysis of a whole cell sample may then allow fast, simple and dynamic study of fungal cell wall biogenesis. This presents the possibility of determining the effects of nutrition, feed additives and physical conditions such as agitation speed and temperature on the fungal mycelia produced.

There are two main areas in which this research could be beneficial. Firstly, this procedure could be employed to optimise the culture conditions of fungal species in order to produce maximum yields of chitin or chitosan of pre-determined DDA. Fungal strains may also be screened rapidly in order to determine the most suitable producers of chitin and chitosan. Additionally, this technique could find use in the development of anti-fungal agents. As chitinous materials are not present in the human body they present an obvious target for anti-fungal therapy, the development of this procedure could allow a fast and easy indicator of the effects of proposed anti-fungal agents on the fungal cell wall structure and composition.

To this end, preliminary experiments were undertaken during the course of this thesis. Unfortunately the time restraints of this project prevented the acquisition of any conclusive results, however, the results obtained so far are presented here.

6.3 Modification of the Compositions of the Fungal Cell Wall

Filamentous fungi are morphologically complex microorganisms that exhibit different structural forms throughout their life cycles. The basic structure of growth consists of a tubular filament known as hypha that originates from the germination of a single reproductive spore. As the hypha continues to grow, it frequently branches to form a mass of hyphal filaments referred to as mycelium. When grown in submerged culture, as the fungi in this study were, fungi can exhibit different morphological forms,

ranging from dispersed mycelial filaments to densely interwoven mycelial masses known as pellets. Due to the demands of cell growth and development, fungal cell walls are dynamic structures that are capable of changing continuously during growth and in response to changes in the environmental conditions. The morphology of the culture, and to some extent the cell wall composition, is affected by the genotype of the strain, the size of the inoculum and the chemical and physical culture conditions.[279] For example the frequency of hyphal branching has been shown to be dependent on the pH of the growth medium in continuous cultures of *P. chrysogenum*.[280] The nutrient composition can also affect the composition of the fungal cell wall, for example, Bulik *et al.* demonstrated that in the yeast *Saccharomyces cerevisiae* addition of glucosamine to the growth medium leads to a three- to fourfold increase in the cell wall chitin levels.[281] The composition of fungal cell walls can also vary drastically when the organism is subjected to stress, such as the presence of cell wall lytic enzymes, hypo-osmotic stress and heat stress. These conditions may result in the up-regulation of chitin synthase in order to maintain the cell-wall integrity, increasing chitin contents tenfold.[282] This was demonstrated in *Aspergillus niger* by Ram *et al.*, the authors proposed that cell wall stress in fungi may generally lead to activation of the chitin biosynthetic pathway based upon their observations of increased chitin deposition and increased transcription of the enzyme responsible for the first step in chitin synthesis.[283]

6.4 Monitoring Chitin Contents

High concentrations of dissolved carbon dioxide in the submerged cultures of *Pencillium chrysogenum* have been reported to result in stunted, swollen hyphae, increased branching, lower growth rates and lower penicillin productivity.[284] Edwards *et al.* hypothesised that this was due to an inability to control the plasticizing effect required during hyphal branching, which resulted in severe morphological changes.[285] Interestingly the authors reported that at influent carbon dioxide levels of 5 and 10 % chitin synthesis was up-regulated by approximately 100 and 200 % respectively. In the culture of filamentous fungi for the purpose of chitin extraction

this has obvious benefits. Unfortunately, due to the lack of suitable culture equipment we were unable to incubate submerged cultures at controlled carbon dioxide levels. As a preliminary experiment, agar-solidified plates of Blakeslee's formula medium (see appendix) were inoculated with *P. chrysogenum* and incubated in an oven at 24 °C in a 10 % carbon dioxide atmosphere. Control plates were also incubated under normal atmospheric conditions. After two weeks the resulting fungal biomass was collected, the low yield of fungal biomass produced by this method precludes the chemical extraction in order to determine the chitin content; therefore the samples were submitted for ^{15}N CPMAS ssNMR analysis. Methods for determining the chitin content of the sample directly from the ^{15}N CPMAS ssNMR spectra have not yet been fully developed, however, it was hoped that an increase in chitin content of the samples would result in an increase in the amount of signal seen per unit mass of sample in the NMR spectrum.

The resulting spectra, shown in Figure 6.77, are typical of whole cell samples described else where in this thesis; see section 3.9. Comparison of the spectra indicates that the chemical shifts and line-widths are unchanged upon incubation in elevated CO_2 atmospheres. The amount of signal seen per unit mass of sample also did not vary significantly between the control sample and the sample incubated in 10 % CO_2, 0.25 and 0.22 respectively.

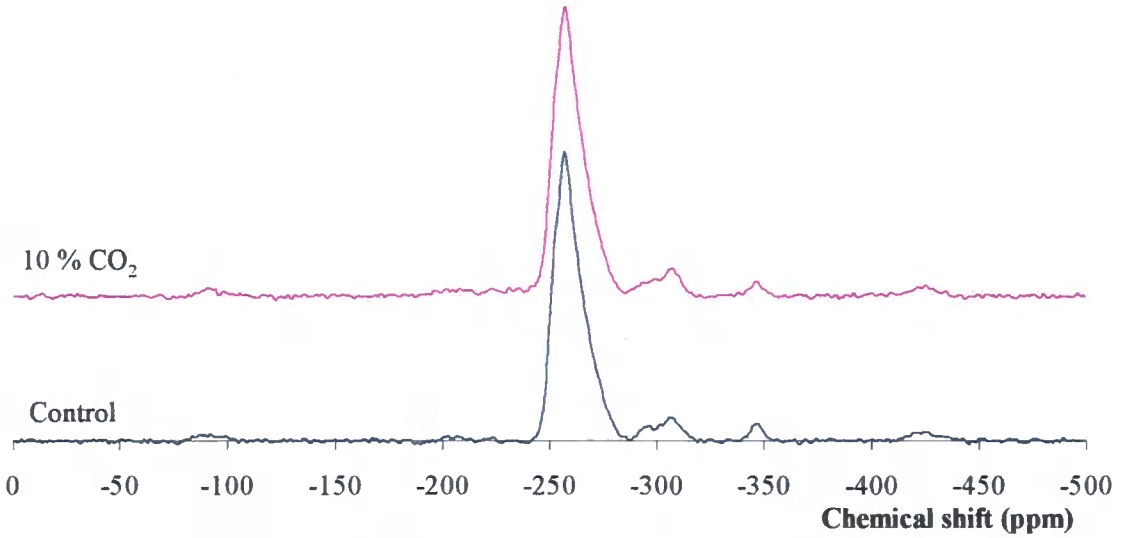


Figure 6.77 - ^{15}N CPMAS ssNMR of whole cell samples cultured on agar plates at 24 °C under normal atmospheric conditions and at carbon dioxide concentrations of 10 %.

Unfortunately, we could not discern any change in the chitin content of samples incubated in increased carbon dioxide conditions in this preliminary experiment. It is possible that submerged cultures incubated with increased concentrations of carbon dioxide would result in a variation in chitin content, which could be monitored by the ^{15}N CPMAS ssNMR method described. Unfortunately within the time constraints of this project we were unable to investigate this further and it remains an area of future work.

6.5 Monitoring Autolysis of Fungal Cell Walls

The process by which microbial cells cease growing and begin to break down by the action of their own enzymes is usually referred to as autolysis.[286] Autolysis can be induced by nutrient depletion, therefore, in nature this may be seen as a means of prolonged survival for filamentous fungi through recycling of nutrients within a culture.[280, 287] In the submerged cultivation of filamentous fungi for the production of antibiotics non-ideal process control can result in extensive autolysis in cultures. This can cause severe down stream processing problems as the broths will not filter easily, additionally this can result in a reduction biomass and the productive

capacity of the culture.[288, 289] Alternately, autolysis during the production of penicillins has been correlated with increased extracellular acylase activity, although this enzyme degrades the antibiotic its activity may be economically advantageous as the product, 6-aminopencillanic acid, is a precursor for semisynthetic antibiotic production.[290] Additionally, within the area of therapeutic antifungal agents we may wish to induce autolysis. There are several methods available to measure the extent of autolysis; biomass decline; NH_4^+ release; monitoring changes in the apparent viscosity of the culture; and by means of quantitative assessment of changes of micromorphology using a computerised image analysis system.[286, 291] Additionally, it may be possible to use $^{13}C/^{15}N$ CPMAS ssNMR techniques as a fast and reliable method of monitoring the appearance and effect of autolysis on fungal cultures.

White *et al.* describe the ability of high concentrations of the penicillin precursor phenylacetic acid (PAA) to induce autolysis in submerged batch cultures of *Penicillium chrysogenum*.[292] PAA is routinely added to penicillin production processes as a precursor to penicillin G, however, the addition must be rigorously monitored to ensure efficient conversion to product as at high concentrations PAA inhibits penicillin biosynthesis and is toxic.[293] The toxicity of high concentrations of PAA arises from the dissipation of the trans-membrane pH gradient, since the protonated, lipid soluble PAA will transport protons across the membrane. Concentrations of PAA between 0.1 and 1.0 gl^{-1} are described as optimum for penicillin production and White *et al.* demonstrated that within these levels the biomass and penicillin production and autolysis level were within the range of control processes. However, high concentrations (10.0 gl^{-1}) reduced biomass and penicillin production and were associated with increased cellular autolysis.

In order to investigate the possibility of monitoring autolysis by ^{13}C and ^{15}N CPMAS ssNMR spectroscopy, the conditions of culture of *P. chrysogenum* in above optimal concentrations of PAA described in the study of White *et al.* were employed. The P2 strain of *Penicillium chrysogenum* was cultured in Blakeslee's formula (see appendix) under normal culture conditions (see section 7.3.2) for 72 hours to allow significant

biomass to form, after this time PAA was added using a range of feeding profiles. White *et al.* designed PAA feeding profiles for batch processes that result in an extracellular PAA concentration in the culture fluid above that of the maximum recommended concentrations at a time when the process of autolysis was expected to be well underway. A variation of this feeding profile, adapted to account for the decrease in vessel size employed in our study, was employed. Additional feeding profiles containing concentrations of PAA below this maximum were also applied. PAA was fed continuously to the cultures, at the rates shown in Table 6.32, for five days, after this time the resulting biomass was collected by filtration and weighed 'wet', the results are shown in Table 6.32. The samples were then sterilised by autoclaving and the whole cell analysed 'wet' by ^{15}N and ^{13}C CPMAS ssNMR.

PAA addition rate (gh^{-1})	Yield of 'wet' biomass (gl^{-1})
0.055	2.57
0.028	4.53
0.0055	17.9
0.0028	19.5
0	22.4

Table 6.32 - Yield of 'wet' biomass produced upon incubation of *P. chrysogenum* under standard conditions, see section 7.3.2, with the addition of PAA at varying feed rates.

Addition of PAA at a rate above 0.0055 gh^{-1} resulted in a marked decrease in the yield of biomass. This is in agreement with the results reported from White *et al.* who describe the optimal addition rate of PAA to a 500 ml culture as 0.002 gh^{-1} and described the toxic effects, and resultant decrease in biomass production, of addition of PAA at rates above 0.02 gh^{-1}.[292] The ^{13}C CPMAS ssNMR spectra of the whole cell samples, analysed 'wet', are shown in Figure 6.78. Unfortunately no discernable differences can be seen in the spectra of samples cultured in higher concentrations of PAA. The chemical shifts, line-widths and intensities of the spectra display no significant variations. The agreement in chemical shift and intensity of the peaks seen in the chemical shift range of anomeric carbons in the spectra suggests that the cells contain similar compositions of polysaccharides. If autolysis of the fungal cell wall has occurred we would expect to see a change in the cell composition as hydrolytic

enzymes act upon the components, additionally, we would expect to see a change in the cell wall structure, which should manifest as a variation in the chemical shifts and line-widths of the peaks seen in the ^{13}C CPMAS ssNMR. The lack of variation seen in these spectra may suggest that autolysis has not occurred, however, this is in disagreement with the decreasing yields of biomass observed. Alternatively, this may be explained by the technique employed to acquire the spectra. As discussed in section 3.9.3. CPMAS ssNMR spectra only visualise the more rigid parts of the sample, therefore, if autolysis results in a greater degree of mobility in the components of the cell wall they may not be visualised. It may be that the CPMAS ssNMR spectra are only representing the intact cell walls that have not undergone autolysis. This could be verified by carrying DPMAS ssNMR experiments, which can be tailored to monitor the mobile fractions of samples only. Unfortunately, due to the time restraints of this project we were unable to carry this out.

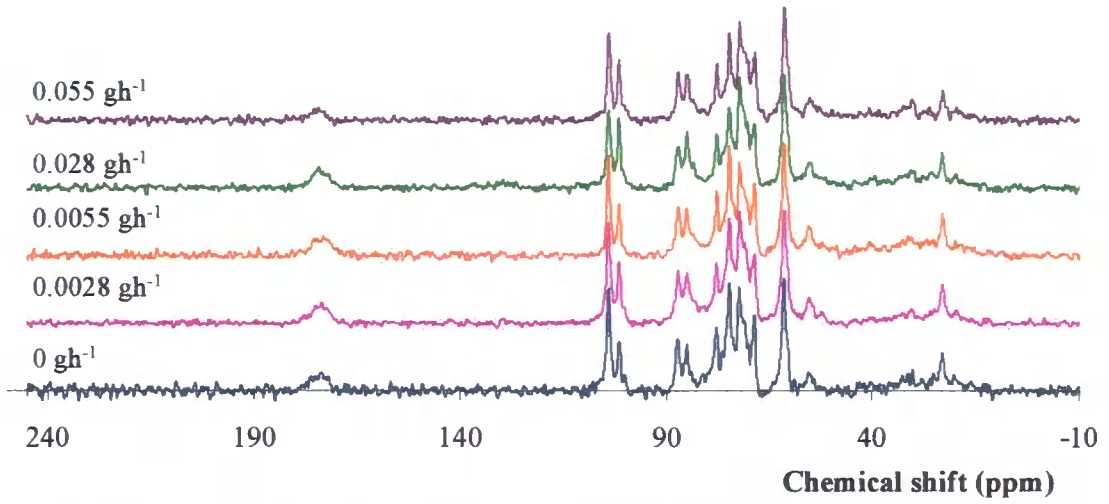


Figure 6.78 - ^{13}C CPMAS ssNMR spectra of whole 'wet' samples of biomass cultured with the addition of PAA at varying feeding rates

The corresponding ^{15}N CPMAS ssNMR spectra are shown in Figure 6.79. Again, no significant variation in the chemical shifts and line-widths can be seen and integration of the spectra indicates that the samples all have DDAs of the approximately 0.8 ± 0.5%.

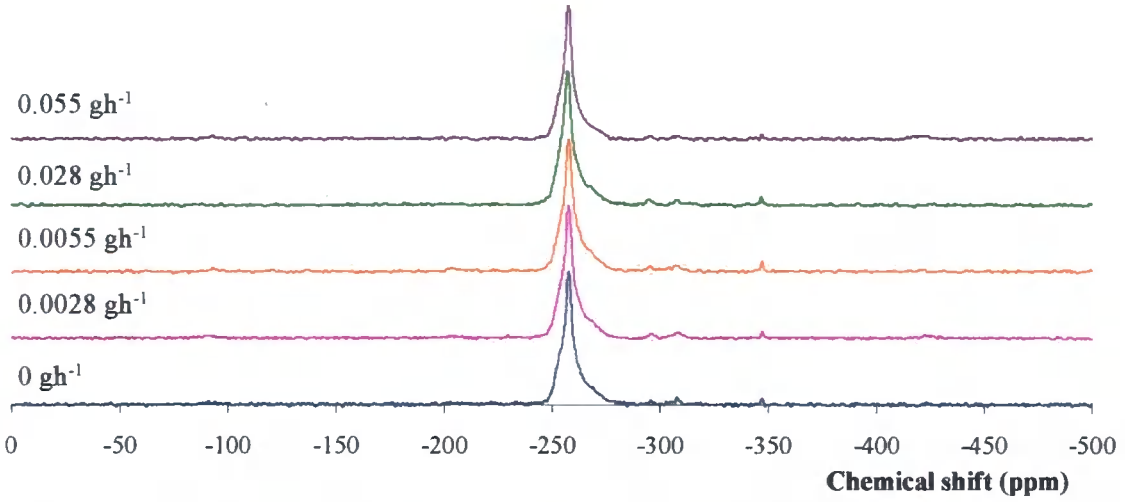


Figure 6.79 – ^{15}N CPMAS ssNMR spectra of whole 'wet' samples of biomass cultured with the addition of PAA at varying feeding rates

These initial experiments indicate that high concentrations of PAA do exert a toxic effect on *P. chrysogenum* as the yield of biomass produced is markedly decreased at addition rates of 0.028 gh^{-1} and above. Unfortunately we were unable observe variations in the ^{13}C and ^{15}N CPMAS ssNMR spectra of these samples, this may be explained by the procedure employed to obtain the spectra, however, we were unable to further investigate this. The importance of the regulation of autolysis in fungal cultures means that the development of a fast and efficient method of monitoring the changes in the fungal cell wall by CPMAS ssNMR could potentially be very beneficial.

6.6 Anti-Fungal Agents

Another potential application of the whole cell ssNMR studies of ^{15}N labelled material is in the development of anti-fungal agents. Invasive fungal infections, particularly in immuno-suppressed patients, have continued to increase in incidence in the past 20 years and are now significant causes of morbidity and mortalitiy.[294] There is therefore a great deal of interest in developing new antifungal agents, the optimal properties of which should include: inhibition of fungal cell wall biosynthesis;

fungicidal activity *in vitro* and *in vivo*; and a lack of toxicity to the patient.[295] Anti-fungal agents commonly act by disrupting membrane function by binding preferentially to ergosterol or inhibiting the biosynthesis of this fungal sterol.[296] As the fungal cell wall is one of the essential architectures for fungal growth and, as mammalian cells do not have such architectures, enzymes that synthesis, assemble, retain and remodel the fungal cell wall have been thought to be promising targets for antifungal agents.[297] In recent years the inhibition of 1,3-β-D-glucan synthesis has provided an increasingly popular target.[294, 298, 299] As chitin is absent from mammalian systems, it is also an ideal target for anti-fungal agents, either through inhibition of its biosynthesis,[300] or disruption of the assembly of chitin microfibrils.[301]

The development of the ^{13}C and ^{15}N CPMAS ssNMR techniques may provide a fast, simple and direct method of visualising the effect of anti-fungal drug candidates on the fungal cell wall. In order to investigate the possibility of this we carried out some preliminary investigations into inhibiting fungal cell wall biogenesis. A known chitin synthase inhibitor, Nikkomycin Z,[302] an antibiotic that inhibits eukaryotic protein synthesis, Cycloheximide,[303] and an inhibitor of *N*-acetylglucosamine transferesases, tunicamycin,[304] were added to cultures of *Penicillium chrysogenum* which had been incubated for 96 hours. PAA, which is claimed to induce autolysis of fungal cell walls at high concentrations,[292] was also investigated. The cultures were incubated in the presence of the inhibitors overnight, this is described in 7.6.3. In order to evaluate if the additives had a fungicidal effect on the cultures, each culture was used to inoculate agar-solidified plates of Blakeslee's formula medium (see appendix) under sterile conditions. The plates were incubated at room temperature for one week, after this time all of the plates showed growth consistent with that of *P. chrysogenum*, indicating that the cultures were still active. The biomass produced from the submerged culture in the presence of the additives was collected by filtration, weighed 'wet', and analysed by ^{13}C and ^{15}N CPMAS ssNMR. The 'wet' weights of biomass obtained did not vary significantly with the addition of any of these compounds. This biomass was subjected to the standard chemical extraction procedure, which again did not result in any significant variation in the yield of level

of purity of the chitinous material extracted, data not shown. The ^{13}C and ^{15}N CPMAS ssNMR spectra of the whole 'wet' samples also did not indicate any variation in the samples, the ^{13}C CPMAS ssNMR spectra are shown in Figure 6.80.

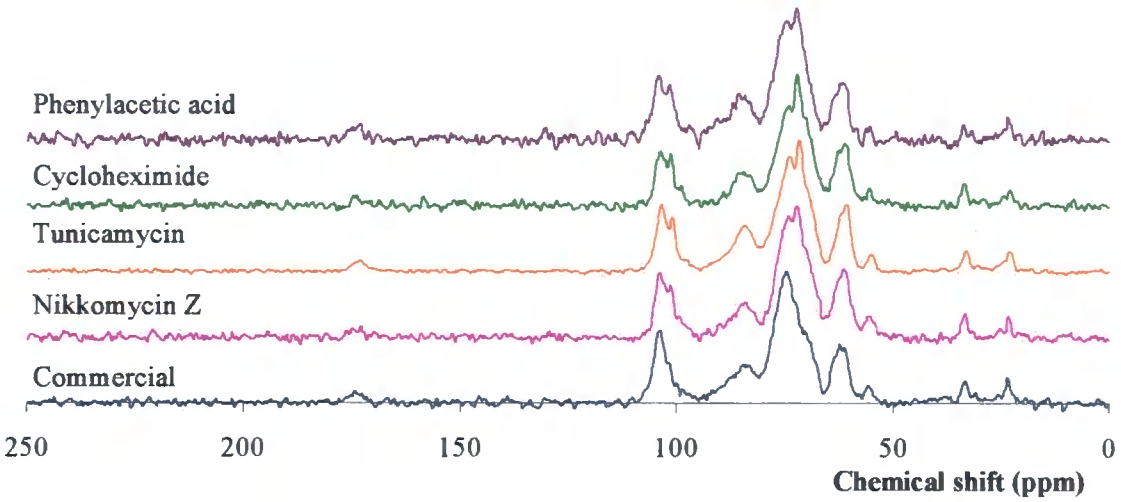


Figure 6.80 - ^{13}C CPMAS ssNMR spectra of whole 'wet' samples of *P. chrysogenum* cultured in the presence of various anti-fungal agents.

The inability of any of the additives to effect a change in the structure and composition of the fungal cell wall, as seen by CPMAS ssNMR, or the yield or purity on the chitinous material extracted may be explained by flaws in the experimental methods employed. The quantities of each of the compounds that were added were estimated using literature values of the IC_{50}, however, these values may differ between fungal species. Additionally, the cultures may not have been incubated in the presence of these additives for long enough for an effect to be seen. Therefore, these preliminary experiments may be repeated to take these factors into account.

6.5 Chitin Deacetylase

In our investigations into alternatives to the harsh chemical extraction procedures currently employed for the production of chitin and chitosan we were hindered by the lack of a commercial availability of chitin deacetylase, the enzyme which specifically

catalyse the hydrolysis of the *N*-acetamido bonds of chitin. Cai *et al.* described the use of chitin deacetylase in their total enzymatic extraction of chitosan from *Aspergillus niger* fungal biomass.[102] As discussed in section 4.9.3, replication of this extraction procedure with the omission of the deacetylation reaction resulted in the extraction of chitinous material of low purity. It was hypothesised at the time that the *in situ* deacetylation of the chitinous materials by chitin deacetylase may be critical to the extraction of materials of high purity, allowing easier removal of the impurity polysaccharides present in the crude material. Chitin deacetylases are commonly found in fungal species that are known to contain chitosan, for example *Mucor rouxii.*[99] We therefore postulated that incubation of the dry biomass with the lysate of a *Mucor rouxii* culture may effect deacetylation of the chitin present, which may result in the extraction of chitin with a higher DDA and increased purity, or the extraction of chitosan.

Mucor rouxii was cultured under standard conditions, see section, the resulting biomass was then ground in the presence of a glutamate buffer (25 mmol, pH 4.5) and acid washed sand. After dilution with the glutamate buffer the suspension was centrifuged (3,900 g, 5 min) and the supernatant used as a crude enzyme mixture. The dry *P. chrysogenum* biomass was incubated in this enzyme mixture at the optimum temperature of chitin deacetylase, 50 °C, for four days. A control, incubated in buffer alone, was also carried out. After this time the solids were subjected to the standard chemical extraction procedure described in section 7.4.6. This procedure should result in a crude chitin-containing fraction, solid A, and a chitosan containing fraction, solid B. The solids were analysed by ^{13}C CPMAS ssNMR and elemental analysis, unfortunately ^{15}N CPMAS ssNMR analysis was not performed as these experiments were carried out before the use of ^{15}N CPMAS ssNMR techniques were investigated. The resulting yields and carbon to nitrogen ratios are shown in Table 6.32.

	Yield of solid A (%)	C:N solid A *	Yield of solid B (%)	C:N solid B *
Control	9.8	11.7	2.6	-
Lysate	9.1	11.3	2.9	-

Table 6.33 - Yield and elemental analyses of solids extracted from the dry biomass by the standard chemical procedure, see section 7.5.3, after incubation with the lysate from *Mucor rouxii* and a control. * Indicated by elemental analysis

Incubation with the lysate of *Mucor rouxii* did not significantly alter the yield of solid A or solid B. Elemental analysis suggests that the level of purity of the solid A samples was also unaffected by incubation with the lysate. This is reflected in the ^{13}C CPMAS ssNMR spectra of the sample, shown in Figure 6.81. The chemical shifts, intensities and line-widths of the peaks seen do not vary significantly indicating that the material contains similar levels of polysaccharide impurities and that the structure and crystallinity of the material is unaltered. Although ^{13}C CPMAS ssNMR spectra can not be used to give a definitive determination of the DDA due to the presence of polysaccharide impurities, this is discussed in length in section 3.6.4.4, we can estimate from the integration of these spectra that the DDA of the sample incubated with the lysate of *M. rouxii* is similar to that of the control sample.

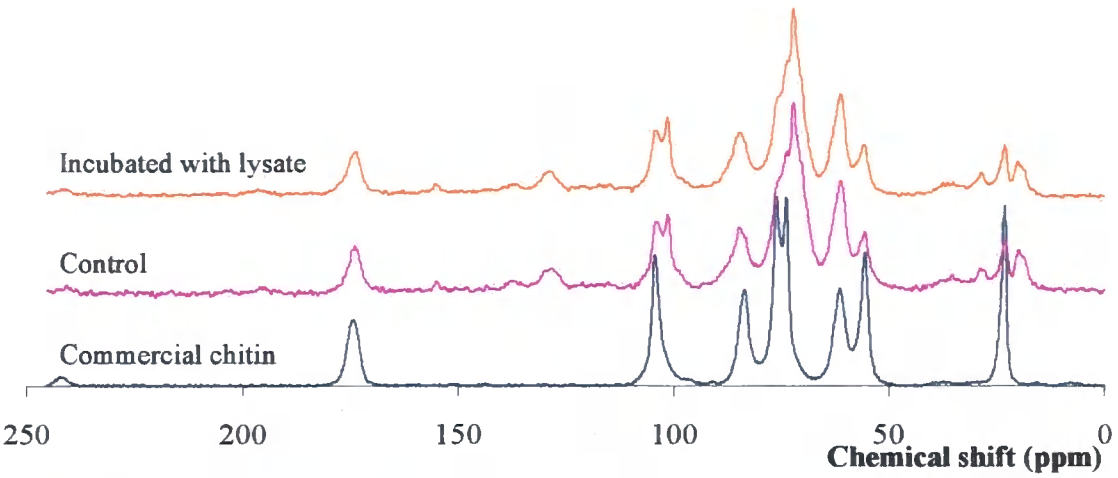


Figure 6.81 - ^{13}C CPMAS ssNMR of the crude solid A, chitin, extracted from dry biomass incubated in a buffered solution and in a buffered solution containing the lysate of *Mucor rouxii*. The spectrum of commercial chitin (Sigma) is shown for comparison.

Elemental analysis of the acid soluble fraction, solid B, indicated a very low carbon content and negligible nitrogen contents in the solids extracted from the control and the biomass incubated with the lysate. Further elemental analysis indicated that the solids contain high concentrations of calcium and phosphorus, therefore we can conclude that as in earlier experiments, see section 4.5.2.2, the acid soluble fraction extracted from the control and the sample incubated with lysate do not contain significant concentrations of chitosan and are mainly comprised of calcium phosphate or calcium hydroxyapatite.

We may conclude from these results that chitin deacetylase has not effected deacetylation of the chitinous material in the dry biomass. There are two possible explanations for this; as discussed previously chitin deacetylase is more efficient at catalysing the deacetylation of nascent chitin, which has not yet formed the rigid hydrogen-bonding network of interactions.[247] Therefore the substrate may not be suitable for enzymatic hydrolysis as the chitin chains are inaccessible to enzyme action. Alternatively, *Mucor rouxii* may not have been expressing the endogenous chitin deacetylase gene, due to the lack of substrate availability (chitin/glucosamine),

and therefore if this experiment were to be repeated additional feeding of one of these substrates would be utilised to potentially induce expression of chitin deacetylase.

6.6 Conclusion

It is hoped that the work that has been initiated in this thesis can be continued and developed to allow increased production of well-defined chitinous materials for use the wide ranging high-value applications outlined in chapter 1. As discussed above, there are a great many applications for which this science could be employed. This work may be utilised and developed to gain greater insight into the composition of the fungal cell wall and how the cell wall biogenesis can be controlled, either through inhibition or up-regulation of the biosynthesis of certain components, which has far ranging applications.

Chapter 7 - Experimental

During the course of this thesis a series of analytical techniques, chitin extraction procedures and fungal culture conditions were employed to study chitinous materials in fungal systems. Some of these methods are known literature procedures, and therefore will be briefly outlined with appropriate literature citations, whereas some have been developed for the needs of this project, so will be described in detail. Certain procedures have been utilised repetitively within this thesis with minor variations, therefore, these methods will be described once and any experiment containing a variation will only have that variation described. Where appropriate, results or representative results, from systems repeatedly extracted under the same protocols will be reported, however, the majority of the results will be found in the results and discussion chapters 3 to 6.

7.1 General

Reagents

All reagents were purchased from Sigma-Aldrich or Fluka and were used as supplied. The dry biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* was a gift from Angel Biotechnology. The P2 strain of *Penicillium chrysogenum* was supplied by LGC promochem UK (ATCC no. 48271). *Aspergillus niger* was supplied by the Fungal Genetics Stock Centre (FGCS), at the University of Missouri, Kansas City, USA, (FGCS no. A732, ATCC no. 9029). *Mucor rouxii* (DSM no. 1191, ATCC no. 24905) was supplied by DSMZ, Germany.

General equipment

Centrifugation was carried out using either a Beckman Coulter Avanti centrifuge (J-20 XP1) with a Beckman Coulter JLA-10.500 rotor, or a Beckman Coulter Allegra X-22R centrifuge. A Beckman Coulter DU350 LifeScience UV/Vis spectrophotometer was used in all spectrophotometric assays. A Forma Scientific incubated table-top

orbital shaker, model 420, was used in the culture of all fungal species, which were performed at a temperature of 24.5 °C. A Dixons Surgical Instruments LTD. Vario 19 portable autoclave and a Rodwell Scientific Instruments MP24 Control autoclave were used for sterilisation.

7.2 Analytical methods

7.2.1 Elemental Analysis

Elemental analyses were conducted on an Exeter analytical E-440 elemental analyser by Durham University Chemistry Department technical staff. Samples were analysed after standing for one week, open to the atmosphere, to ensure that the chitinous material had reached maximum weight due to water adsorption.

7.2.2 General Protocol for Glucosamine Determination[131]

Suspensions of the chitinous material to be analysed (1 mg/ml) were hydrolysed in 6 M HCl at 100 °C for 24 hrs. A 1ml portion of the resulting acidic solution was then added to a 25 ml volumetric flask using a Gilson P1000 pipette, followed by one drop of ethanolic phenolthalein solution. A 1 M solution of NaOH was then added dropwise until a pink colour persisted (~5 ml). A 1 % (w:v) solution of $KHSO_4$ was then added dropwise until the pink colour just disappeared (~0.5 ml) and the flask was made up to 25 ml with deionised water. A 1 ml portion of the resulting solution was added to 1 ml of 5 % (w:v) $KHSO_4$ and 1 ml of 5 % (w:v) $NaNO_2$. The mixture was shaken briefly and left to stand for 15 min. After this time 1 ml of 12.5 % (w:v) $NH_2SO_3.NH_4$ was added and the reaction mixture was shaken for 5 min, following this 1 ml of 0.5 % (w:v) 3-methyl-2-benzothiazolone hydrazone hydrochloride was added and the mixture was left to stand for 1 hr at room temperature. After this time 1 ml of 0.5 % (w:v) $FeCl_3$ was added and the mixture left to stand for 30 min at room temperature. The absorbance of the resulting solution was measured at 650 nm,

referenced against a blank prepared using 1 ml of deionised water in place of the analyte.

The assay was standardised using a series of solutions of commercial D-glucosamine hydrochloride. A 10 mg/ml solution of commercial D-glucosamine hydrochloride in 6 M HCl was prepared in a 200 ml volumetric flask. From this stock solution 7.5, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml solutions were prepared by serial dilutions of the stock solution and the assay was performed on each sample as described in section 7.2.2. The resulting solutions were diluted to 1 part analyte solution to 6 parts distilled water before the absorbance was measured. The resulting standard plot is shown in Graph 3.1.

7.2.2.1 Optimisation of the Glucosamine Assay

Study of the glucosamine deamination with respect to time

Solutions of commercial D-glucosamine hydrochloride in 6 M HCl were prepared (0.3 mg/ml). These solutions were subjected to the glucosamine assay described in section 7.2.2, however, the assay was modified by employing a series of deamination reaction times, after the addition of $NaNO_2$. Time periods of between 5 to 120 min were employed and the time at which the maximum absorbance at 650 nm occurs was determined. The results are discussed in section 3.5.

Study of the increase and decay of absorbance at 650 nm

Solutions of commercial D-glucosamine hydrochloride in 6 M HCl (0.3mg/ml) were prepared and subjected to a variation of the glucosamine assay described in above. Following the addition of $FeCl_3$ the absorbance of the solution at 650 nm was measured at regular time points over a 2 hour time period. The results are discussed in section 3.5.

7.2.2.2 Optimisation of Chitin Hydrolysis

Chitin from a commercial (Sigma) shrimp source (15 mg) was stirred in 6 M HCl (50 ml) at 100 °C. After 2, 8, 12, 24, 48, 72 and 192 hrs, 1 ml portions were removed and subjected to the glucosamine assay described in section 7.2.2. In addition, a sample of chitin extracted, as described in section 7.4.3, from a fungal source (*P. chrysogenum*), was stirred in 6 M HCl (50 ml) at 100 °C and assayed after 8, 24 and 48 hrs as described in section 7.2.2. The results are discussed in section 3.5.

7.2.3 Dye Adsorption Method of Determining the DDA of Chitinous Materials[148]

The chitinous material (40 mg) was added to 20 ml of a 5×10^{-4} M stock solution of C.I. Acid Orange 7 (4-(2-hydroxy-1-napthylazo)benzensulphonic acid, sodium salt), which was previously prepared by dissolving 175 mg of C.I. Acid Orange 7 in 1 L of 0.1 M acetic acid. A control containing no chitinous material was also prepared. The flasks were then sealed and heated to 60 °C, once this temperature had been reached the pressure was released and the flasks resealed and stirred at 60 °C overnight. After this time the solutions were filtered through glass wool whilst still hot, the resulting filtrates were sealed and allowed to cool to room temperature. The absorbances of each filtrate were then measured at 484 nm using 0.1 M acetic acid as a blank. The DDA of the material was calculated by the method of Roberts *et al.* as follows:[148]

$$EW = (w \times 22500)/(\Delta A \times f \times v)$$

Where:

EW = equivalent weight of the amine group

w = weight of chitinous material (g)

ΔA = Difference in the absorbance values of the reference and test solution

f = dilution factor (constant for all solutions)

v = volume of stock solution added to each sample

$$DDA\ (\%) = 100 - [(EW - 161) \times 100]/(EW + 42)$$

7.2.4 FT-IR Spectroscopy

Infrared spectra were recorded on solid samples using a PerkinElmer Spectrum100 FT-IR spectrometer a PIKE MIRacle® ATR accessory, which has a germanium crystal. Spectra were obtained between 700 and 4000 cm^{-1} at a resolution of 1 cm^{-1}. The data were analysed using the PerkinElmer software, Spectrum (Version 6.0.2.0025).

7.2.5 Solution-State ^{1}H NMR

Solution state NMR experiments were performed by Durham University solution-state NMR service. ^{1}H Nuclear Magnetic Resonance (NMR) spectra were obtained using a Varian 500 spectrometer at 499.827 MHz. All NMR samples were prepared in 20 % DCl in D_2O by stirring 20 mg of the sample in 1 ml of the NMR solvent for 6 hrs. After this time the suspensions were separated by centrifugation (3,900 g, 10 min) and the supernatants were analysed at 70 °C. The spectrum of an internal standard 3-(trimethylsilyl)-1-propane sulphonic acid in 20 % DCl in D_2O, acquired at 70 °C, was used to assign the chemical shift of the residual solvent peak (water) at 7.10 ppm. All subsequent spectra were indirectly referenced to 3-(trimethylsilyl)-1-propane sulphonic acid by setting the residual solvent peak (water) to 7.10 ppm. The results are discussed in 3.6.4.3.

7.2.6 Solid-State NMR

All solid-state NMR analyses were carried out by the Durham University solid-state NMR service. Solid state ^{13}C and ^{15}N NMR spectra were obtained using cross-polarisation and high-power proton decoupling techniques, either on a Varian Unity Inova 300 spectrometer at a ^{1}H operating frequency of 299.82 MHz with a 7.5mm Chemagnetics Magic Angle Spinning (MAS) probe, or on a Varian VNMRS 400 spectrometer at a ^{1}H operating frequency of 399.88 MHz with a 4.0 mm Chemagnetics

MAS probe. All samples were spun at the magic angle at a spinning speed of 4000-5000 Hz and the spectra were acquired at an ambient temperature of approximately 22 °C. ^{13}C CPMAS ssNMR spectra were acquired at either 75.398 MHz (Varian Unity Inova 300) or 100.562 MHz (Varian VNMRS 400). ^{15}N CPMAS ssNMR spectra were acquired at either 30.386 MHz (Varian Unity Inova 300) or 40.525 MHz (Varian VNMRS 400). A contact time of 1.00 ms and a recycle delay of 1.0 s were employed. ^{13}C CPMAS ssNMR spectra were acquired using between 2000 and 4000 repetitions. Natural abundance ^{15}N CPMAS ssNMR spectra were acquired using approximately 60,000 repetitions, approximately 2,000 repetitions were employed for the analysis of ^{15}N labelled samples. Carbon spectra were referenced indirectly to tetramethylsilane by setting the high-frequency signal from adamantine to 38.4 ppm. Nitrogen spectra were indirectly referenced to neat nitromethane by setting the nitrate signal from labelled solid ammonium nitrate to –5.1 ppm.

7.2.7 GPC

Preparation of LiCl:DMAc

Lithium chloride was dried at 100 °C under high vacuum and *N,N*-dimethylacetamide (DMAc) was dried over molecular sieves (4 Å) for four days prior to use. The required quantity of LiCl was then stirred in DMAc for 24 hrs to allow complete dissolution.

Preparation of chitinous materials in 5 % (w/v) LiCl:DMAc

The sample to be analysed was dried in a desiccator under vacuum, with NaOH as a desiccant, for two days prior to use. The sample was then stirred, open to air, in 5 % (w/v) LiCl:DMAc at an initial concentration of 5 mg/ml for 24 hrs at 100 °C. After this time the resulting suspension was centrifuged, (3,900 g, 5 min) and the supernatant was submitted for GPC analysis.

GPC analysis

GPC analysis was carried out by Dr. Lian Hutchings at Durham University. Gel permeation chromatography (GPC) analysis was undertaken using 5 % (w:v) LiCl:DMAc as the eluent. 100 µl of solution was injected at a flow rate of 0.50 $mlmin^{-1}$ by a Viscoteck SEC autochange module. A Polymer Labs PLGel Mixed C column (300 mm × 7.5 mm) column was used. A Viscoteck TriSEC Model 302 unit with triple detection (right angle laser scattering at 670 nm, differential refractometer and viscometer) was used. The materials were analysed by conventional calibration using Pullulan standards. Data analyses were undertaken using OmniSEC 4.0 software.

7.2.8 LC-MS Analysis of the Level of ^{15}N labelling

Preparation of samples

The chitinous samples discussed in section 5.5 were hydrolysed by the method described in the hydrolysis step of the glucosamine assay, see section 7.2.2, in order to allow LC-MS analysis and glucosamine assay of the resulting hydrolysis product. The sample to be analysed was stirred in 6 M HCl (1:1 w/v) at 100 °C for 24 hrs. After this time the solution was allowed to cool and a 1 ml portion was added to a 25 ml volumetric flask using a Gilson P1000 pipette, followed by one drop of ethanolic phenolpthalein solution. A solution of 1 M NaOH was then added dropwise until a pink colour persisted (~5 ml). A solution of 1 % $KHSO_4$ was then added dropwise until the pink colour just disappears (~0.5 ml) and the flask was made up to 25 ml with deionised water. This solution was then diluted by a factor of ten and submitted for LC-MS analysis.

LC-MS analysis

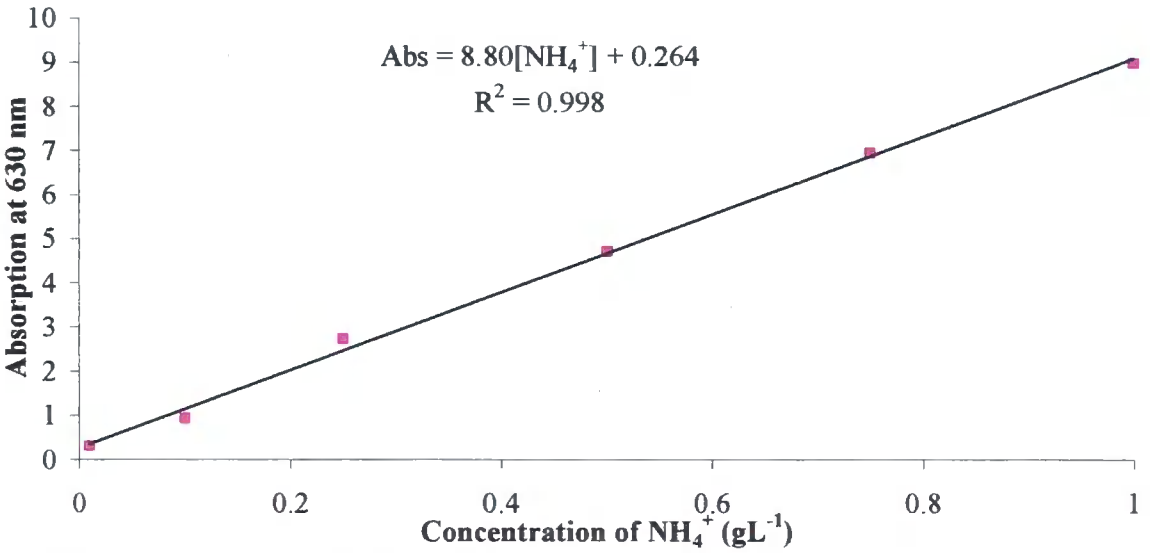
The glucosamine monomers produced by the acid hydrolysis of chitinous samples, as described above, were analysed by LC-MS (Durham University Protein Analysis Service). Samples (1 µl) were resolved on a TSKGel amide-80 column (5 µm, 100 mm × 2 mm), using a 20 min linear gradient of 100 % acetonitrile to 40 % acetonitrile in 0.1 % formic acid at a rate of 150 µl/min and the eluted materials were monitored by MS using a Waters Q-TOF Premier instrument operating in +ve electrospray mode (capillary 3.0 kV, sampling cone voltage 25.0 V, source temperature 100 °C, desolvation temperature 180 °C, sampling frequency 4 Hz, sampled in "V" mode, m/z 150 - 200). The instrument was externally calibrated using sodium formate and calibration was adjusted in real time using a leucine enkephalin lock spray (m/z 556.2771).

Ions corresponding to unlabelled (m/z 180.1) and ^{15}N-labelled (m/z 181.1) glucosamine ($[M+H]^+$) eluting at 16.9 min were quantified by integration of extracted ion chromatograms using Masslynx (V. 4.1) software. The relative intensity of the $[M+H]^+$ peak of ^{15}N labelled glucosamine was then used to calculate the quantity of ^{15}N present by comparison to a standard plot, shown in Graph 5.13, this was constructed using theoretical values, for the purposes of this study the presence of ^{2}H, ^{17}O, ^{18}O and the possibility of two ^{13}C isotopes were ignored. The results are discussed in section 5.6.

7.2.9 General Protocol for NH_4^+ assay [273] [274]

The solution to be assayed (240 µl) was added to phenol nitroprusside (400 µl) solution followed by 0.2 % sodium hypochlorite in alkaline solution (400 µl). The sample was then shaken and incubated at 37 °C for 15 min to allow the colour to develop. The absorbance of the resulting solution was read at 630 nm, referenced against a solution containing distilled water in place of the analyte. The results are discussed in section 5.9.

The assay was standardised using a series of solutions of ammonium sulfate. A 1 gL^{-1} solution of commercial ammonium sulphate in deionised water was prepared in a 200 ml volumetric flask. Solutions of 0.75, 0.5, 0.1 and 0.01 gL^{-1} were then prepared by successive dilutions of the stock solution and the assay was performed on all samples as described above. The resulting standard plot is shown in Graph E.17.



Graph E.17 - Standardisation of the NH_4^+ assay using known concentrations of ammonium sulfate

7.3 Culture and storage of fungi

7.3.1 Storage of Fungi

Penicillium chrysogenum, Aspergillus niger and *Mucor rouxii* were cultured on slopes of Blakeslee's formula containing 2 % agar, see appendix for medium recipe, for two weeks at ambient temperature to allow spore formation. The resulting cultures are shown in Figure E.82. After this time the spores were transferred to 2 ml cryogenic vials containing 50 % glycerol under sterile conditions, using a flame-sterilised inoculating loop under an open flame. These vials were flash frozen in liquid nitrogen and then stored at – 78 °C. Before use as an inoculum in submerged fungal culture,

each vial was used to inoculate agar-solidified plates of the Blakeslee's formula medium to ensure that no cross-contamination had occurred.

P. chrysogenum *A. niger* *M. rouxii*

Figure E.82 - Photos of *P. chrysogenum*, *A. niger* and *M. rouxii* cultured on slopes of Blakeslee's formula containing 2 % agar, see appendix, for two weeks

7.3.2 General Method for the Submerged Culture of Fungal Species

All fungi were cultured in either 250 ml shake flasks containing 100 ml of a liquid medium or 1 l shake flasks containing 500 ml of a liquid medium. The flasks were filled with distilled water and autoclaved at 121 °C for 15 min prior to use, the water was then discarded. Culture media were prepared using the recipes described in the appendix and autoclaved in the shake flasks at 121 °C for 15 min. The flasks were then allowed to cool to below 25 °C before inoculation under sterile conditions (using a flame-sterilised inoculating loop under an open flame). The inoculums were prepared and stored as described above. The inoculated flasks were incubated in a temperature controlled orbital shaker at 24.5 °C, 200 rpm, for 8 days. With the exception of the minimal media experiments, discussed below, the culture medium for all fungal species was Blakeslee's formula containing, unless stated, 0.96 gL^{-1} of $(^{15}NH_4)_2SO_4$ (see appendix). Initial investigations into the culture of ^{15}N labelled *P. chrysogenum* employed three alternative, minimal media. The recipes and methods for preparation are listed in the appendix. The results are discussed in section 4.8.

All fungal cultures displayed a similar spherical morphology, a photograph of an 8 day old culture of *P. chrysogenum* in Blakeslee's formula is shown in Figure E.83. After 8 days the biomass was collected by filtration and weighed 'wet' and samples of the 'wet' biomass were submitted for ^{13}C and ^{15}N CPMAS ssNMR. These spectra are discussed in sections 4.8.2 and 4.10. The submerged culture of *P. chrysogenum, A. niger* and *M. rouxii* in this way produced 28 ± 4 gL^{-1} (8 repetitions), 34 ± 6 gL^{-1} (3 repetitions) and 23 ± 5 (3 repetitions) gL^{-1} of fungal biomass respectively ('wet' weights). This biomass was dried in an oven at 95 °C until a constant weight was achieved; the biomass lost 82 ± 1 % (3 repetitions), 84 % and 79 % of its weight upon drying respectively.

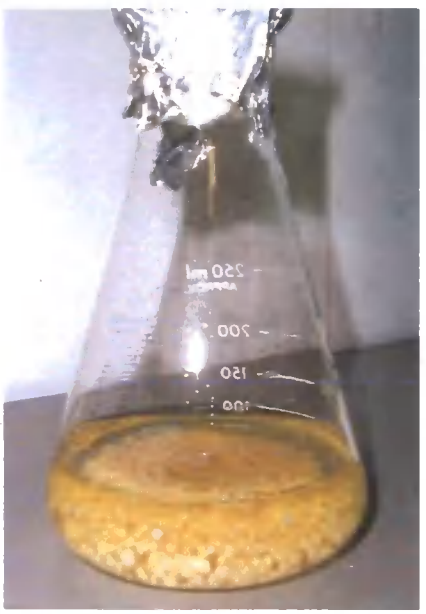


Figure E.83 - Photo of the fungal biomass resulting from the culture of *P. chrysogenum*.

7.3.2.1 Modifications of Culture Conditions – Growth Curves

In order to establish the time-dependent growth curve of *P. chrysogenum*, 6 × 1 L shake flasks, each containing 500 ml of medium, were inoculated and incubated under the conditions described in section 7.3.2 for varying lengths of time. Flasks were removed after 70, 93, 141, 190, 236 and 308 hrs, and were autoclaved at 121 °C for

30 min. The fungal biomass was then recovered by filtration and weighed 'wet'. With the exception of biomass produced during the 70 hr experiment, which was discarded due to the low yield. The biomass was then subjected to the extraction and purification processes described in 7.4.6. The resulting chitin containing material was analysed by ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay. The results are shown in Table E.34.

Culture Time (hrs)	Yield of biomass (gL^{-1})*	Yield of purified solid A (%)**	Glucosamine content*** (%)	C:N***
70	0.71	-	-	-
93	7.3	5.9	64	8.6
141	12	8.0	59	9.2
190	19	5.9	76	8.4
236	21	9.4	60	8.3
308	21	11.2	79	8.7

Table E.34 - Yield of biomass produced and yields and analysis of chitinous material extracted, from cultures incubated in Blakeslee's formula medium, containing varying concentrations of $(^{15}NH_4)_2SO_4$. * 'Wet' weight. ** Yield estimated assuming 82 % of weight on drying of 'wet' biomass. * Analysis of purified solid A.**

7.3.2.2 Modifications of Culture Conditions – Varying $[(^{15}NH_4)_2SO_4]$

A series of experiments was carried out to culture the P2 strain of *P. chrysogenum* under the conditions described in 7.3.2 in Blakeslee's formula medium (see appendix), modified to contain varying concentrations of $(^{15}NH_4)_2SO_4$, added prior to sterilisation. The resulting biomass was collected by filtration and the 'wet' weight was obtained before the biomass was subjected to the chemical extraction and purification procedures described in section 7.4.6. The solids obtained were analysed by elemental analysis, the glucosamine assay and ^{13}C and ^{15}N CPMAS ssNMR. The results are shown in Table E.35.

Concentration of $(^{15}NH_4)_2SO_4$ (gL^{-1})	Yield of wet Biomass (gL^{-1})*	Yield of purified Solid A (%) **	Glucosamine determination (%)***	C:N***
0 (0 %)	24	5.6	64	8.8
0.0096 (0.5 %)	18	5.1	65	8.4
0.096 (4.8 %)	22	5.5	74	7.4
0.48 (20 %)	19	6.3	95	8.0
0.96 (33 %)	23	6.8	78	7.7
1.92 (50 %)	18	6.8	90	7.3
2.88 (60 %)	20	6.5	74	7.2
3.84 (67 %)	19	4.5	80	7.4

Table E.35 – Yield of biomass produced and yields and analysis of chitinous material extracted, from cultures incubated in Blakeslee's formula medium, containing varying concentrations of $(^{15}NH_4)_2SO_4$. * 'Wet' weight. ** Yield estimated assuming 82 % of weight on drying of 'wet' biomass. * Analysis of purified solid A.**

7.3.2.3 Modifications of Culture Conditions – Hydrolytic Enzymes

Shake flasks (4 × 1 L), containing 500 ml of Blakeslee's formula were prepared as described in section 7.3.2. Prior to autoclaving the pH of three of the flasks was altered to 6.5, 7.5 and 8.5 by the addition of 1 M KOH. The flasks were then autoclaved at 121 °C for 15 min and allowed to cool. Either 10 ml of a 10 mg/ml solution of a neutral protease, or 10 ml of a 0.4 mg/ml solution of β-glucanase or 0.1 ml of α-amylase, or 10 ml of distilled water was then added to each of the flasks under sterile conditions by a process of filter sterilising through 0.2 μm Acrodisk® syringe filters (PALL corporation, USA). The flasks were then inoculated and cultured as described in section 7.3.2. The resulting biomass was collected by filtration and weighed 'wet', the yields are shown in Table E.36. The extraction of this biomass is described in section 7.4.6.

	Yield biomass (gL^{-1})
Control	30
Protease	31
β-glucanase	25
α-amylase	29

Table E.36 - Wet weights of biomass produced from the culture of the P2 strain of *P. chrysogenum* in Blakeslee's formula containing hydrolytic enzymes

7.3.2.4 Modifications of Culture Conditions – Variation [Peptone]

The P2 strain of *P. chrysogenum* was cultured under the conditions described in 7.3.2 in Blakeslee's formula medium which was modified to contain 1, 0.5, 0.25 and 0 g of peptone. The resulting biomass was collected by filtration and weighed 'wet', the resulting yields are shown in Table E.37. These results are discussed in section 5.9.

Concentration of Peptone (gL^{-1})	Wet weight of mycelia (gL^{-1})
1	32
0.5	30
0.25	26
0	24

Table E.37 - Wet weight of mycelia produced by the culture of *P. chrysogenum* in Blakeslee's formula medium containing varying concentrations of peptone.

7.4 Extraction procedures

7.4.1 Chemical Extraction of the Dry Biomass

Angel Biotechnology provided dried biomass remaining from the large-scale fermentation of *Penicillium chrysogenum* for the production of Penicillin G. This

material has been dried at 113 °C and contains residual penicillin at a level of < 50 ppb. This material was stored at 4 °C prior to use.

7.4.2 Chemical Extraction Procedure One – Chitosan Extraction[115]

The dry biomass (10 g) was stirred in 0.5 M NaOH (100 ml) at 70 °C for 5 hrs. After this time the suspension was allowed to cool and was separated by centrifugation (18,500 g, 10 min). The solid was then washed with deionised water (~5 × 100 ml) and separated by centrifugation (18,500 g, 10 min) until the supernatant became clear, colourless and pH 7.0-8.0.

The resulting solid was suspended in 30 % (w:v) NaOH (30 ml) and stirred at 130 °C for 1 hr. After this time the suspension was allowed to cool, separated by centrifugation (18,500 g, 10 min) and the solid washed with deionised water (~ 6 × 20 ml) until the pH of the supernatant became 9.0.

This solid was then stirred in 2 % (v:v) acetic acid (100 ml) at 100 °C for 5 hrs. The suspension was allowed to cool and was separated by centrifugation (18,500 g, 10 min). The acid insoluble fraction was discarded. The pH of the supernatant was adjusted to 8.0 by the addition of 2 M NaOH precipitating a solid that was collected by centrifugation (18,500 g, 10 min). The precipitate was washed with deionised water (2 × 20 ml) and then dissolved in 1% (v:v) acetic acid (40 ml). Addition of ethanol (200 ml) re-precipitated the solid, which was collected by centrifugation (18,500 g, 10 min) and dried in a desiccator under vacuum, with NaOH as the drying agent.

This produced an off-white solid with a yield of 1.1 % (± 0.6 % from 4 repetitions). ^{13}C CPMAS ssNMR and elemental analysis was performed. Characterisation: $C_6H_{11}O_4N$ requires: C 44.70, H 6.88, N 8.69 % C:N 5.14. Found: C 2.19, H 2.90, N 0, Ca 20.18, P 15.21 % C:N -; ^{13}C CPMAS ssNMR δ_{ppm}; **61.17** (C_2), **63.11** (C_6), **72.53** (C_5/C_3), **81.56** (C_4), **101.09** (impurity), **111.44** (impurity), **119.74** (impurity), **146.36** (impurity), **152.86** (impurity).

7.4.3 Chemical Extraction Procedure Two – Chitin and Chitosan Extraction – Standard Chemical Extraction Procedure[103]

The biomass was stirred in 2% (w:v) NaOH (1:30 w:v) at 90 °C for 2 hrs. The alkali insoluble fraction was then separated by centrifugation (18,500 g, 20 min) and washed with deionised water (~ 4 × 1:10 w:v) until the supernatant became clear, colourless and of pH 7.0. The resulting solid was stirred in 10 % (v:v) acetic acid (1:40 w:v) at 60 °C for 6 hrs. The suspension was then separated by centrifugation (18,500 g, 20 min) and the solid washed with deionised water (~6 × 1:10 w:v) until the supernatant was clear, colourless and of pH 7.0. The solid residue was then washed with ethanol (3 × 1:10 w:v) and acetone (2 × 1:10 w:v) to yield solid A ('Chitin'). The supernatant from the acid extraction was retained and the pH adjusted to 9.0 by addition of 4 M NaOH, the precipitated solid was washed with deionised water (~ 4 × 1:10 w:v), ethanol (1:10 w:v) and acetone (1:10 w:v), yielding solid B ('Chitosan'). Both solids were then milled and dried in a desiccator with dry NaOH as the drying agent.

7.4.3.1 Representative results of extraction of dry biomass

The acid insoluble fraction, solid A, produced an off white solid with a yield of 16 % (± 1 % standard deviation of 5 repetitions). ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 %, C:N 6.86 Found: C 47.31, H 6.65, N 3.86 %, C:N 12.3; Glucosamine assay - 17 % glucosamine content; ^{13}C CPMAS ssNMR δ_{ppm}; **19.89** (Impurity), **23.00** (C_8), **55.93** (C_2), **61.18** (C_6), **69.62**, **71.96** (C_5/C_3), **83.91** (C_4), **101.20** (impurity), **103.92** (C_1), **128.98** (impurity), **173.76** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; – **253.70** (NHCOR), 54100 repetitions to acquire, DDA ≤ 5 %.

The acid soluble fraction, solid B, produced a white solid with a yield of 2.9 % (± 0.4 %, standard deviation from 5 repetitions). ^{13}C and ^{31}P CPMAS ssNMR and

elemental analysis was performed. Characterisation: $C_6H_{11}O_4N$ requires: C 44.70, H 6.88, N 8.69 %, C:N 5.14 Found: C 3.41, H 1.66, N 0 %, C:N -. Ca 23.11, P 12.0 %; ^{31}P CPMAS ssNMR **2.80** (P*i*); ^{13}C CPMAS ssNMR δ_{ppm}; **23.25** (C_8) **29.95** (Impurity), **74.12** (C_3/C_5), **103.25** (C_1), **168.59** (C_7).

7.4.3.2 Modification of Extraction Procedure

The extraction procedure described in 7.4.3 was repeated using varying concentrations of NaOH in the initial step to acquire the alkali insoluble material (AIM). All other reaction conditions were maintained.

Concentration of NaOH (w/v)	Yield of Solid A	Yield of Solid B
2 %	15.9 %	2.9 %
5 %	12.0 %	2.1 %
10 %	11.4 %	3.1 %
20 %	12.0 %	1.8 %

Table E.38 - Yields of samples extracted using chemical extraction scheme 2 with varying concentrations of NaOH

All samples were analysed by solid-state ^{13}C CPMAS ssNMR and elemental analysis. Elemental analysis indicated that the samples of solid B have negligible nitrogen contents. These results are discussed in 4.5.2.3.

	C/N	C/N	Ca*	P*
Expected	6.86	5.14		
Concentration of NaOH	Solid A	Solid B		
2%	12.3	-	23.1	12.0
5%	12.7	-	26.1	13.8
10%	13.3	-	27.0	12.3
20%	12.2	-	24.4	9.79

Table E.39 – Ratio of carbon to nitrogen of samples extracted by procedure described in section 7.4.3 using varying concentrations of NaOH to extract the AIM. Carbon and nitrogen levels given by elemental analysis. * Ca and P % by weight of solid B samples.

7.4.4 Purification of Chitin

Solid A, extracted as described in section 7.4.3, was stirred in concentrated HCl (1:60 w:v) for 6 hrs at 0 °C. After this time the suspension was separated by centrifugation (18,500 g, 20 min) and the supernatant poured into 50 % aqueous ethanol (1:5 HCl:EtOH). The precipitated solid was collected by centrifugation (18,500 g, 20 min) and washed until neutrality (~ 6 × 1:100 w:v). The solid was then washed with acetone (1:10 w:v) and dried in a desiccator with NaOH as the drying agent. The HCl insoluble solid was also washed until neutrality (~ 8 × 1:100 w:v) and dried in a desiccator with NaOH as the drying agent.

7.4.4.1 Representative results of purification of extracted 'solid A' from dry biomass.

A white solid precipitate was produced in 11.8 % yield (± 1 %, standard deviation from 5 repetitions), ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 6.86. Found: C 47.11, H 6.65, N 4.83% C:N 9.7; Glucosamine assay - 57 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **23.00** (C_8), **55.54** (C_2),

60.79 (C_6), **73.70** (C_3), **74.00** (C_5), **83.42** (C_4), **104.11** (C_1), **173.86** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; **−257.63** (NHCOR), 60,000 repetitions to acquire, DDA ≤ 5 %.

The HCl insoluble residue was produced in 83.4 % yield (± 5 %, standard deviation from 8 repetitions). ^{13}C CPMAS ssNMR and elemental analysis were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 5.14. Found C 40.75, H 6.67, N 2.82 % C:N 14.5; ^{13}C CPMAS NMR δ_{ppm}; **19.72** (impurity), **28.34** (Possibly C_8) **33.20** (impurity), **55.61** (C_2), **61.47** (C_6), **69.62** (impurity), **71.47** (C_3), **74.48** (C_5), **80.33** (impurity), **86.33** (C_4), **101.58** (impurity), **103.72** (C_1), **128.39** (impurity), **136.55** (impurity), **155.11** (impurity), **173.66** (C_7).

7.4.5. Incubation of Dry Biomass with Hydrolytic Enzymes

The dry biomass (5 g) was incubated with a neutral protease, a β-glucanase and an α–amylase. In order to estimate the quantities of enzymes required, the dry biomass was assumed to contain 50-60 %, 2.5-3g, of non-chitinous polysaccharides (β-glucans) and 30-40 %, 1.5-2 g, of protein. The buffer conditions suggested by the supplier (Sigma) were employed, see Table E.40.

Enzyme	Source	Buffer	pH	Mass of Enzyme
Protease	*Aspergillus sojae*	50 mM potassium phosphate	7.5	1.0 g
β-glucanase	*Aspergillus niger*	100 mM potassium phosphate	6.5	20 mg
α-amylase	*Bacillus sp*	50 mM Tris-HCl	8.5	10 mg

Table E.40 - Source and buffer conditions of hydrolytic enzymes.

The biomass was stirred in the buffered solutions of the hydrolytic enzymes at 37 °C for 72 hrs. After this time the suspensions were separated by centrifugation, (18,500 g, 20 min) and subjected to the chemical extraction procedure described in section 7.4.3. The resulting solid A and solid B fractions were analysed by elemental

analysis, ^{13}C and ^{15}N CPMAS ssNMR and the glucosamine assay. The results are shown in Table E.41. These results are discussed in section 4.7.1.

Enzyme	Yield (%)		C/N*		Glucosamine content of Crude A (%)
	Solid A	Solid B	Solid A	Solid B	
Protease	15.5	2.8	11.4	-	15
β-glucanase	12.9	1.7	9.95	-	15
α-amylase	16.7	2.5	11.8	-	16
Chemical standard	15.9	2.9	12.3	-	17

Table E.41 - Yields of solid A and B extracted from dry biomass by the standard chemical extraction procedure after incubation of the biomass with hydrolytic enzymes. * Ratio of carbon to nitrogen content indicated by elemental analysis

The incubation was repeated using double the quantities of the neutral protease and β-glucanase under the same conditions, the α-amylase experiment was not repeated. A control reaction was also carried out, stirring the biomass in a buffered solution (100 mM potassium phosphate) at pH 7.0 under the same conditions. The resulting suspensions were then separated by centrifugation (18,500 g, 20 min) and subjected to the extraction procedure described in section 7.4.3 and the purification procedure described in 7.4.4. The resulting solids were analysed by elemental analysis, ^{13}C and ^{15}N CPMAS ssNMR and the glucosamine assay. The results are shown in Table E.42 and discussed in section 4.7.1.

Enzyme	Yield (%)			C/N*	Glucosamine content of purified solid A (%)
	Crude solid A	Purified solid A**	Solid B	Purified solid A	
Protease	10.0	3.1	2.65	17.0	40
β-glucanase	16.2	3.8	2.76	13.8	38
Control	12.0	3.48	2.65	12.0	55

Table E.42 - Yield of solid A, purified solid A and solid B extracted from dry biomass by the normal chemical extraction procedure after incubation of the biomass with hydrolytic enzymes. * Ratio of carbon to nitrogen content indicated by elemental analysis. ** Yield of purified solid A given as percentage of starting dry biomass.

7.4.6 Chemical Extraction of 'Wet' Biomass

The fungal biomass produced from the culture of either *P. chrysogenum, A. niger* or *M. rouxii,* as described in section 7.3.2, was subjected to the standard chemical extraction procedure described in section 7.4.3 with the minor modification that in place of centrifugation the AIM and crude solid A produced during the extraction procedure were collected and washed by filtration under vacuum. The purification process described in section 7.4.4 was carried out without modification.

7.4.6.1 Representative results of extraction of 'wet' *P. chrysogenum*

The acid insoluble fraction, solid A, produced an off white solid with a yield of 31.78 % (± 6 % standard deviation of 8 repetitions), assuming 82 % on drying of wet biomass. ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 %, C:N 6.86 Found: C 40.88, H 6.06, N 1.29 %, C:N 31.68; Glucosamine assay - 33 % glucosamine content; ^{13}C CPMAS ssNMR δ_{ppm}; **22.80** (C_8), **55.31** (C_2), **61.08** (C_6), **70** (impurity shoulder) **73.61** (C_5/C_3), **83.31** (C_4), **98** (impurity shoulder) **103.47** (C_1), **173.76** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; – **253.70** (NHCOR), 900 repetitions to acquire, DDA 0.8 %.

7.4.6.2 Representative results of purification of extracted 'solid A' from 'wet' *P. chrysogenum.*

A white solid precipitate was produced in 32.2 % yield, producing an overall yield of purified chitin from starting biomass, assuming 82 % loss on drying, of 10.24 % (± 1 %, standard deviation from 8 repetitions). ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 6.86. Found: C 42.90, H 6.59, N 4.70 % C:N 9.13; Glucosamine assay - 81 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **23.00** (C_8),

55.40 (C_2), **61.38** (C_6), **70** (impurity shoulder), **73.71** (C_3), **75.77** (C_5), **83.79** (C_4), **104.06** (C_1), **172.99** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; **−257.24** (NHCOR), 100 repetitions to acquire, DDA 0.8 %.

7.4.7 Enzymatic Treatment of 'Wet' *P. chrysogenum*

7.4.7.1 Incubation of 'Wet' Biomass with Hydrolytic Enzymes

Fungal biomass produced by the culture of *P. chrysogenum* as described in section 7.3.2 was incubated with a neutral protease, a β-glucanase and an α–amylase under the same conditions described for the dry biomass in section 7.4.5. A control experiment was carried out, incubating the biomass in 100 mM potassium phosphate at pH 7.0. After 72 hrs the biomass was collected by filtration and subjected to the standard chemical extraction and purification procedure described in sections 7.4.3 and 7.4.4. The resulting chitin containing fractions were analysed by elemental analysis, ^{13}C and ^{15}N CPMAS ssNMR and the glucosamine assay. The results are shown in Table E.43 and discussed in section 4.9.1.

	Yield of purified solid A (%)	C:N*	Glucosamine content of purified solid A (%)	DDA ** (%)
Control	9.6	19.8	64	≤ 0.5
Protease	8.6	15.7	53	≤ 0.5
β-glucansase	9.8	19.0	50	≤ 0.5
α-amylase	9.3	19.2	44	≤ 0.5

Table E.43 - Analytical data of purified chitinous extracts from wet biomass incubated with hydrolytic enzymes prior to chemical extraction. *Indicated by elemental analysis. ** Indicated by ^{15}N CPMAS ssNMR.

7.4.7.2 Culture of *P. chrysogenum* in the Presence of Hydrolytic Enzymes.

P. chrysogenum was cultured in the presence of the neutral protease, β-glucanase and α-amylase described in 7.4.5 by the method described in 7.3.2.3. After the normal culture period, 8 days, the fungal biomass was collected by filtration, autoclaved and weighed wet. Samples were analysed by ^{13}C and ^{15}N CPMAS ssNMR. The remaining biomass was subjected to the standard chemical extraction procedure, the resulting crude solids were analysed by ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay. The results are shown in Table E.44 and discussed in section 4.9.2.

	Yield biomass (gL^{-1})	Yield crude solid A (%)	Glucosamine content (%)	DDA* (%)
Control	30	36.2	35	≤ 0.5
Protease	31	32.4	36	≤ 0.5
β-glucanase	25	42.1	26	≤ 0.5
α-amylase	29	42.4	32	≤ 0.5

Table E.44 - Yield of wet biomass produced from the incubation of P2 in the presence of hydrolytic enzymes. The yield and analysis of the crude chitin-containing fraction, solid A, are also shown. * DDA indicated by ^{15}N CPMAS ssNMR.

7.4.7.3 Enzymatic Extraction Procedure of Cai *et al.*[102]

Fungal biomass produced from the culture of *P. chrysogenum* as described in 7.3.2 (44 g wet weight) was suspended in 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (600 ml) at pH 6.4. Lysozyme (1 g) and snailase (1g) were added and the suspension stirred continuously at 50 °C for 5 hrs. The solid was collected by centrifugation (18,500 g, 10 min) and washed with distilled water (400 ml). This resulted in 22 g (wet weight) of a solid that was suspended in Na_2HPO_4-NaH_2PO_4 buffer (1000 ml) at pH 7.0 and 1275 units (434 mg) of neutral protease was added, this suspension was stirred

continuously at 55 °C for 3 hrs. The solid was collected by centrifugation (18,500 g, 10 min) and washed with demineralised water (600 ml). This produced 15.2 g of solid (wet weight) which was stirred in 2 % (v:v) acetic acid (600 ml) at 30 °C for 16 hrs. After this time the solid was collected by centrifugation (18,500 g, 10 min) and washed until neutrality with distilled water (~5 × 300ml), followed by an ethanol (300 ml) and an acetone wash (300 ml).

This produced 2.6 g of an off-white solid in 33 % yield, assuming 82 % loss on drying of wet biomass. This solid was analysed ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 6.86. Found: C 42.34, H 6.29, N 0.71 % C:N 59.63; Glucosamine assay - 3.5 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **18.20** (impurity), **22.58** (C_8), **32.45** (impurity), **54.98** (C_2), **60.85** (C_6), **~70** (impurity shoulder), **73.47** (C_5/C_3), **83.72** (C_4), **88.15** (impurity), **96.85** (impurity), **101.20** (impurity), **103.48** (C_1), **172.75** (C_7).

7.4.7.3.1 Extraction of *A. niger* by the Enzymatic Procedure of Cai *et al.*[102]

The enzymatic procedure of Cai *et al.* described above was applied to the biomass resulting from the culture on *A. niger* under the conditions described in section 7.3.2. This produced an off-white solid in 35.10 % yield, assuming 84 % loss on drying of wet biomass. This solid was analysed ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and the glucosamine assay were performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 6.86. Found: C 41.40, H 6.15, N 0.97 % C:N 42.7; Glucosamine assay - 15 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **22.58** (C_8), **32.58** (impurity), **55.03** (C_2), **60.76** (C_6), **~70** (impurity shoulder), **71.64** (C_5/C_3), **83.11** (C_4), **~86** (impurity shoulder), **97.20** (impurity), **101.09** (impurity), **102.83** (C_1), **172.60** (C_7).

7.4.8 Chemical Extraction of *A. niger* and *M. rouxii*

Aspergillus niger and *Mucor rouxii* were cultured under the conditions described in section 7.3.2. The resulting biomasses were autoclaved, weighed wet and analysed by ^{13}C and ^{15}N CPMAS ssNMR. They were then subjected to the standard chemical extraction and purification procedure as described for wet *P. chrysogenum* biomass in section 7.4.6. The characterisation of the purified chitinous extracts are below:

Aspergillus niger

The extraction and purification produced a white solid in 11.6 % yield. ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and glucosamine assay was performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H 6.45, N 6.89 % C:N 6.86. Found: C 41.74, H 6.34, N 4.69 % C:N 8.90; Glucosamine assay - 87 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **22.96** (C_8), **55.32** (C_2), **61.05** (C_6), **68** (impurity shoulder), **73.88** (C_3), **75.14** (C_5), **82.91** (C_4), **~88** (impurity shoulder) **103.90** (C_1), **173.28** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; **−257.87** (NHCOR), 860 repetitions to acquire, DDA ≤ 0.5 %.

Mucor rouxii

The extraction produced an acetic acid soluble solid in 14.2 % yield. ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and glucosamine assay was performed. Characterisation: $C_6H_{11}O_4N$ requires: C 44.70, H 6.88, N 8.69 % C:N 5.14. Found: C 41.79, H 6.39, N 6.17 % C:N 6.77; Glucosamine assay - 65 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **22.22** (residual C_8), **58.00** (C_2), **60.53** (C_6), **75.01** (C_5/C_3), **82.68** (C_4), **105.33** (C_1), **174.5** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; **−356.83** (NH_2), **−258.00** (NHCOR), 1000 repetitions to acquire, DDA = 65 %

The extraction and purification of the acetic acid insoluble solid produced a white solid in 1.9 % yield. ^{13}C and ^{15}N CPMAS ssNMR, elemental analysis and glucosamine assay was performed. Characterisation: $C_8H_{13}O_5N$ requires: C 47.29, H

6.45, N 6.89 % C:N 6.86. Found: C 41.76, H 6.46, N 6.05 % C:N 6.90; Glucosamine assay - 98 % glucosamine content; ^{13}C CPMAS NMR δ_{ppm}; **23.02** (C_8), **55.43** (C_2), **61.30** (C_6), **73.74** (C_3), **75.50** (C_5), **83.53** (C_4), **104.18** (C_1), **173.50** (C_7); ^{15}N CPMAS ssNMR δ_{ppm}; **–257.87** (NHCOR), **–356.84** (NH_2), 1000 repetitions to acquire, DDA 9.1 %?

7.5 Deacetylation of Chitin

Samples of commercial chitin (Sigma) and the purified chitin extract from 'wet' *P. chrysogenum* biomass, extracted and purified as described in 7.4.6, were deacetylated under alkaline conditions. The chitin samples (30 mg) were stirred in 40 % (w:v) NaOH (40 ml) for between 0 and 24 hrs at 100 °C under a constant flow of nitrogen. After this time the suspensions were separated by centrifugation (3,900 g, 10 min) and washed with distilled water (~ 10 × 40 ml) until the washings became pH 7.0. The residues were then washed with acetone (40 ml) and dried in a desiccator under vacuum, with NaOH as the drying agent.

The resulting solids were analysed by elemental analysis, the dye adsorption method described in 7.2.3, ^{13}C CPMAS ssNMR, ^{15}N CPMAS ssNMR and FT-IR spectroscopy. The results are discussed in chapter 3.

7.6 Future Work

In the following sections the experimental techniques employed in the initial investigation experiments described in the future work area of this thesis, chapter 6, will be outlined.

7.6.1 CO_2

Agar-solidified plates of Blakeslee's formula medium (see appendix) were inoculated with the P2 strain of *P. chrysogenum* under sterile conditions, as described in section 7.3.1. Half of these plates were placed in a temperature-controlled growth chamber in which the gaseous environment is controlled to maintain a carbon dioxide level of 10 % and incubated at 24 °C for 2 weeks. The remaining plates were placed in a growth chamber under normal atmospheric conditions and incubated at 24 °C for 2 weeks. After this time the resulting mycelial biomass was removed from the agar plate surface and autoclaved prior to ^{13}C and ^{15}N CPMAS ssNMR analysis. The results are discussed in section 6.4.

7.6.2 Induction of Autolysis

A variation of the procedure described by White *et al.* was employed to culture *P. chrysogenum* in the presence of varying concentrations of phenylacetic acid (PAA).[292] The PAA stock solutions were prepared by producing potassium-phenylacetate by the addition of 5 M KOH solution, up to pH 10. The pH was then corrected to neutrality with 1 M HCl, leaving the dissolved protonated form of the precursor.

The P2 strain of *Pencillium chrysogenum* was used to inoculate 5 × 1 L shake flasks, containing 500 ml of medium, as described in 7.3.2. The flasks were incubated for 72 hrs under normal conditions, see section 7.3.2, after this time stock solutions of varying concentrations of PAA were added at a rate of 1.1 mlh^{-1} using a peristaltic pump. The PAA stock solution concentrations and resulting addition rates are shown in Table E.45.

PAA stock solution (gl^{-1})	PAA addition rate (gh^{-1})
50	0.055
25	0.028
5	0.0055
2	0.0028
0	0

Table E.45 - PAA feeding profiles for *P. chrysogenum*. Stock solutions added at a rate of 1.1 mlh^{-1}.

After 5 days the fungal biomass was recovered by filtration and weighed 'wet', the results are shown in Table E.46. The samples were then autoclaved and analysed by ^{13}C and ^{15}N CPMAS ssNMR. The results and spectra are discussed in section 6.5.

PAA addition rate (gh^{-1})	Yield of 'wet' biomass (gl^{-1})
0.055	2.6
0.028	4.5
0.0055	18
0.0028	20
0	22

Table E.46 - Yield of 'wet' biomass produced upon incubation of *P. chrysogenum* under standard conditions, see section 7.3.2, with the addition of PAA at varying feed rates.

7.6.3 Anti-Fungal Agents

P. chrysogenum (5 × 1 L shake flasks) was cultured under the standard conditions, described in 7.3.2, for four days. After this time a range of potential anti-fungal agents, see section 6.6, were added by a process of filter sterilising through 0.2 μm Acrodisk® syringe filters (PALL corporation, USA). These additives are described below:

- Nikkomycin Z (5 ml of a 1 $mgml^{-1}$ solution)
- Tunicamycin (5 ml of a 1 $mgml^{-1}$ solution at pH 10.5)
- Cycloheximide (1 ml of a 1.4 $mgml^{-1}$ solution)
- PAA – prepared by the addition of 5 M KOH solution, up to pH 10, to 0.4 g of PAA in distilled water. The pH was then corrected to neutrality with 1 M HCl.

The cultures were incubated under normal conditions, see section 7.3.2, in the presence of these additives overnight. After this time the cultures were utilised to inoculate agar-solidified plates of Blakeslee's formula medium (see appendix), as described in section 7.3.1, and these were incubated at room temperature for one week.

The biomass remaining from the submerged culture was collected by filtration, weighed 'wet' and analysed by ^{13}C and ^{15}N CPMAS ssNMR spectroscopy. The biomass was then subjected to the standard chemical extraction procedure, described in 7.4.6. The results are discussed in section 6.6.

7.6.4 Chitin Deacetylase

Mucor rouxii was cultured under the standard conditions, see section 7.3.2. The resulting biomass was collected by filtration (8.59 g wet weight) and ground in the presence of a glutamate buffer (20 ml, 25 mM, pH 4.5) and acid washed sand. After dilution with more glutamate buffer (10 ml) the suspension was centrifuged (3,900 g, 5 min) and the supernatant used as a crude enzyme mixture (40 ml). The protein content of this lysate was assayed using Bradford's reagent, using gamma globulin as a standard. The crude enzyme mixture was found to have a protein concentration of 0.594 mg/ml.

The dry biomass (5 g) was incubated in the crude enzyme mixture and in buffer solution alone at 37 °C for 96 hrs, after this time the solids were collected by centrifugation (18,500 g, 10min) and subjected to the standard chemical extraction

procedure, described 7.4.3. The resulting solids were analysed by elemental analysis and solid state ^{13}C CPMAS NMR. The results are shown in Table E.47 and discussed in section 6.6.

	Yield of solid A (%)	C:N solid A *	Yield of solid B (%)	C:N solid B *
Control	9.8	11.7	2.6	-
Lysate	9.1	11.3	2.9	-

Table E.47 - Yield and elemental analyses of solids extracted from the dry biomass by the standard chemical procedure, see section 7.4.3, after incubation with the lysate from *Mucor rouxii* and a control. * Indicated by elemental analysis

Appendix

Media.

Medium A - ATCC medium: 325 Malt Extract Agar (Blakeslee's Formula)

Malt Extract	20 g
Glucose	20 g
Peptone	1 g
Distilled Water	1 L
$(^{15}NH_4)_2SO_4$ (if required)	0.96 g
Agar (if required)	20 g

Add glucose prior to sterilisation, autoclave for 15 minutes at 121 °C

Medium B - Bristol Minimal Medium (BMM) also known as Aspergillus Minimal Media (AMM)[262]

$NaNO_3$	5.4 g
$Na^{15}NO_3$	0.6 g
KCl	0.52 g
KH_2PO_4	1.52 g
Trace Element Solution	1 ml
Distilled Water	1 L
Agar (if required)	20 g

Adjust to pH 6.5 with KOH, autoclave to sterilise for 15 minutes at 121 °C.

Supplements added after autoclaving, filter sterilised through 0.2 μm Acrodisk® syringe filters (PALL corporation, USA);

20 % $MgSO_4.7H_2O$.....2.5 ml
20 % Glucose.....50 ml
1 mg/ml $FeSO_4.7H_2O$.....1.0 ml

Trace Element Solution:

$ZnSO_4.7H_2O$.....8.8 g
$CuSO_4.5H_2O$.....0.4 g
$MnSO_4.4H_20$.....0.15 g
$Na_2B_4O_7.10H_2O$.....0.1 g
$(NH_4)_6Mo_7O_{24}.4H_2O$.....0.05 g
Distilled Water.....1 L

Autoclave to sterilise for 15 minutes at 121 °C.

Medium C - Modified Czapek Dox Medium (Oxoid www.oxoid.com)

$NaNO_3$.....1.8 g
$Na^{15}NO_3$.....0.2 g
KCl.....0.5 g
$C_3H_7MgO_6P.H_2O$.....6.0 g
K_2SO_4.....2.0 g
Sucrose.....30 g
Distilled Water.....1 L

Adjusted to pH to 6.8 with NaOH, autoclave 121 °C for 15 minutes.
Supplements added after autoclaving, filter sterilised through 0.2 μm Acrodisk® syringe filters (PALL corporation, USA);

$FeSO_4$ (10mg/ml).....1 ml

Medium D - $(NH_4)_2SO_4$ Minimal Medium [116]

$(NH_4)_2SO_4$	6.3 g
$(^{15}NH_4)_2SO_4$	0.7 g
KH_2PO_4	1.6 g
KCl	0.5 g
$MgSO_4$	0.1 g
$CaCl_2.2H_2O$	0.05 g
$CuSO_4.7H_2O$	0.005 g
$ZnSO_4.7H_2O$	0.02 g
$MnSO_4.7H_2O$	0.02 g
Glucose	6 g
Distilled Water	1 L

Adjusted to pH 6.5 with NaOH autoclave to sterilise for 15 minutes at 121 °C. Supplements added after autoclaving, filter sterilised through 0.2 μm Acrodisk® syringe filters (PALL corporation, USA);

$FeSO_4$ (10mg/ml)	4 ml

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