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Streptomycin Production from Chitin using *Streptomyces griseus*

by

Richard J. L. Meanwell

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy of Loughborough University

8th September, 2004

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Abstract

The production of streptomycin using *Streptomyces griseus* using two types of chitin as a substrate was studied using a variety of fermentation techniques. Commercial chitin was obtained (Sigma) and comprised chemically purified crab shell. Pre-fermented chitin was the solid product from the lactic acid fermentation of shrimp waste using *Lactobacillus paracasei* A3. Bioassay, HPLC and FTIR methods were developed during this project for the quantification of streptomycin both in liquid phase and adsorbed on solid chitin surfaces.

Shake flask experiments were carried out to determine basic production kinetics, as well as to establish if commercial and pre-fermented chitins produced different quantities of streptomycin. Shake flasks were also used to evaluate any effect of chitin concentration on streptomycin production. A range of submerged fermentations were undertaken in a standard 2 L bioreactor fitted with Rushton Turbines, at chitin concentrations from 0.4 %w/v to 10 %w/v, to study the effect on streptomycin yield. At concentrations of 5 %w/v and over, it was necessary to use an alternative, U-shaped agitator, as the Rushton Turbines did not provide adequate mixing. The U-shaped agitator was designed and produced as part of this project. The submerged fermenter was also used to determine if the re-use of chitin remaining post-fermentation was possible.

A solid state fermentation packed bed bioreactor was also developed, with a recycle loop for produced liquor. Four experiments evaluated the use of commercial and pre-fermented chitins, and different liquid media used for inoculation. In order to encompass the advantages of submerged and solid state fermentations, a vertical basket reactor was designed and manufactured, which used gentle fluidisation for the agitation of chitin particles contained inside the basket.
Shake flask experimentation showed that pre-fermented chitin produced approximately 3 times the streptomycin yield than that of commercial chitin. Both systems reached a maximum liquid phase yield after 8 days of fermentation. Maximum streptomycin yields were obtained at a chitin concentration of 10 %w/v.

The total streptomycin yields from submerged fermentation were fairly consistent over the range of chitin concentrations used. The amount of streptomycin adsorbed on the chitin surface, however, increased with increasing chitin concentration. Total streptomycin yields varied from 2 to 3.5 mg/L. The re-use of chitin remaining post-fermentation was found to be possible in two series of three experiments. In both cases (at approximately 7.5 %w/v and 10 %w/v chitin) the lag phase and time to reach maximum biomass concentration decreased. Particle size analysis and mathematical modelling suggest that this is due to increasing specific surface of chitin particles during the course of fermentation.

Both shake flask and submerged fermentation showed a bioassay inhibition peak in the troposphere, removable using 2 kDa membrane filters. Although it was not possible to determine the exact nature of the inhibiting component(s), streptomycin was eliminated through FTIR. A study of chitosan oligomers showed that short chain oligosaccharides inhibit *Bacillus subtilis* in a similar manner to streptomycin.

Solid state fermentation using a salts solution liquid medium, with intermittent aeration and recirculation proved to be the most effective, giving a streptomycin yield of 3.8 mg/L. The vertical basket reactor obtained higher streptomycin yields of 4.6 mg/L. Post-fermentation washing with pH 3 buffer was also successfully used in this fermenter for the *in-situ* extraction of streptomycin, before the addition of fresh sterile liquid medium and fermentation re-start.
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Acknowledgements

I would like to thank the following people:

Dr Gilbert Shama and Dr George Hall, my supervisors for this project. Both have provided me with invaluable advice over the last four years. I’d also like to thank Dr Shama particularly for all his help with the construction of this document.

Dave Smith and Andy Milne, for all their technical assistance in the laboratories.

Dr Ian Sutherland for guiding me in the use of FTIR, pointing me in the right direction for background work, and the repeated use of his analysis equipment during this project.

EPSRC, for their funding of the first three years of this project.

Eleni Karounou, for her tuition on GCMS.

Paul Izzard, for all the computer questions.

Kaddour Bouazza-Marouf and family, Sean Mitchell and family, and the Butler Court subwardens, particularly Graham and Jodie.

My parents, for all their support during the last 9 university years and more, and for the food parcels.
1 Introduction

The quantity of solid waste produced by the seafood industry, particularly shrimp processing, has been estimated at lying somewhere between 0.9 and 1.2 million metric tons per year worldwide (Gooday, 1990). This waste material is highly perishable and also highly polluting, and as environmental agencies worldwide tighten restrictions on disposal of wastes to sea, alternatives to disposal are likely to become increasingly important.

A significant proportion of this waste is chitin, in association with proteins and calcium carbonate. There are essentially two methods of purifying the chitin from shellfish waste. The first, and more traditional method, is extraction using strong acid and alkali, usually hydrochloric acid and sodium hydroxide (Cira et al., 2002). Large volumes of effluents are produced from this process and these in turn require safe disposal. The second method, pioneered in the Food and Bioprocessing Laboratory at Loughborough University, involves solid-state fermentation of minced waste using lactic acid bacteria (LAB) in a horizontal rotating bioreactor (Zakaria, 1998).

This LAB fermentation converts perishable shellfish waste into two products. The first, which for the purposes of this project will be considered a by-product, is a liquor, rich in protein, which has been found to have use both as a protein source in fish-feed and as an attractant. The second product is a chitin-rich sediment, which has had much of the protein and calcium carbonate removed and is referred throughout this work as ‘pre-fermented chitin’. This thesis is concerned with establishing whether chitin can be used as substrate for subsequent fermentation to yield useful antibiotics.

Because chitin is ubiquitous many organisms in nature are chitinolytic i.e. they possess the ability to break chitin down into oligosaccharides and monomers, usually for adsorption through the cell wall for use as a food source. Of particular
note are the streptomycetes, a genus belonging to the Order Actinomycetales and first described by Waksman in the 1940s. Streptomycetes are Gram-positive, aerobic, non-motile, spore-forming bacteria and many species are chitinolytic. Significantly, many of these species produce a wide variety of antibiotics. In particular, the species *Streptomyces griseus*, produces streptomycin. Streptomycin is an aminoglycoside antibiotic and is still in use as an effective agent in the fight against the causative agent of tuberculosis, *Mycobacterium tuberculosis*, and commercial strains of *S. griseus* are used for its large-scale production.

Chitin is known to absorb a wide variety of compounds, and indeed is used in water purification for just this property (Kumar, 2000). It seems possible that chitin could be fermented using solid state methods to produce streptomycin which could subsequently be recovered from unfermented chitin substrate. Solid state fermentation is the term used to denote the growth of microorganisms in the absence of free liquid. Most fermentation processes are carried under so-called ‘submerged fermentation’ conditions, but there are reports in the literature that claim that solid-state fermentation can produce higher yields of secondary metabolites, including those produced from streptomycetes (Hesseltine, 1972 and Barrios-Gonzalez and Mejia, 1996).

The extraction of a fermentation product at some stage during its production is known to enhance its rate of production. Such processes are referred to in the literature as *in-situ* extraction processes. Of particular relevance here is that extractions of fermentation products adsorbed onto solid substrates has been shown to be feasible (Salisbury, 1995 and Hernandez-Justiz et al., 1998).

In this project, the production of streptomycin by *S. griseus* from chitin will be investigated using a variety of fermentation methods. The objectives of this work are:

- To develop analytical techniques for the detection and quantification of streptomycin in both the liquid and solid phase.
- To investigate different fermentation techniques (shake flask, submerged and solid state) and their advantages for producing streptomycin from chitin.

- To identify methods for recovering streptomycin adsorbed onto solid chitin surface, and to establish whether in situ extraction is possible.

The remainder of this thesis comprises the following chapters:

Chapter 2: Literature Review. In this chapter the findings of the literature survey carried are presented. This includes information on chitin and chitinases, streptomycetes, and solid state fermentation.

Chapter 3: Streptomycin Analysis. This chapter details the techniques evaluated here to assay streptomycin. In addition to explaining the principles of detection behind the methods featured, their advantages and disadvantages are briefly discussed.

Chapter 4: Materials and Methods. The apparatus and experimental methods used for producing streptomycin from chitin are described in detail in this chapter.

Chapter 5: Fermentation Experiments for Streptomycin Production. The results from the various fermentation experiments carried out during this work are presented and discussed in this chapter.

Chapter 6: Conclusions and Suggestions for Further Work. The final conclusions for the project are presented here. Possible fruitful areas of future work are also identified.
2 Literature Review

2.1 Chitin

Chitin is a natural polymer and similar in structure to cellulose. Indeed, chitin is the second most abundantly biologically synthesized organic material after cellulose (Roberts, 1992). The most readily accessible source of chitin is the exoskeletons of crustaceans. Other sources of chitin also exist and include fungi, squid, krill, molluscs, insects, and certain types of algae.

Chitin itself is poly[β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose], the chemical structure of which can be seen below in Figure 2.1. Three polymeric forms have been identified, designated α, β, and γ. Figure 2.2 below shows the first two forms, showing the differences between antiparallel and parallel chitin chains. γ-chitin contains a mixture of both parallel and antiparallel chains. As with other polymers, the antiparallel chains of α-chitin provide greater rigidity and are found in e.g. cuticles, where as the more flexible β- and γ-chitin forms can be found in e.g. stomach linings.

![Chemical Structure of Chitin](image)

**Figure 2.1 Chemical Structure of Chitin (Tokura and Nishi, 1994)**
Chitin is difficult to dissolve and is only soluble in highly concentrated strong acids such as hydrochloric, sulphuric or phosphoric (Gooday, 1990), or more complex solvents, e.g. a lithium chloride and N,N-dimethylacetamide mixture (Austin, 1988). Chitin has currently relatively few applications, mainly being the adsorption of metals, dyes and proteins (Gooday, 1990). Chitin can be treated with fairly concentrated sodium hydroxide to remove the acetyl groups attached to the C2 amino group. Once this deacetylation has removed 50% or more of the acetyl groups, the polymer is referred to as chitosan, poly[β-(1→4)-2-amino-2-deoxy-D-glucopyranose] (see Figure 2.3). Chitosan becomes soluble at degrees of deacetylation of 60-70% and over in most dilute organic acids, typically formic and acetic acids. Industrially produced chitosans are typically 70-80% deacetylated.
Chitosan, in contrast to chitin being soluble and containing free NH$_2$ groups, has many applications, including those in the waste water treatment, medical, cosmetic and food industries (Gooday, 1990); however, these applications have been reviewed extensively elsewhere and are not specifically part of this work, and so will not be described further.

The main driving force in the marketplace for chitin derivatives comes from glucosamine, and its use as a dietary supplement in human nutrition. Sandford (2002) reports that the US market for this component was $288 million in 1999, whereas the next closest supplement (Coenzyme Q-10) was only $41 million. Approximately two-thirds of the worldwide market for glucosamine (7,500 metric tonnes) is in the US.
2.2 Streptomycetes

The generic name *Streptomyces* was first used in 1943 by Waksman to separate certain aerial mycelium-producing actinomycetes from the rest of the order Actinomycetales (Waksman, 1961).

The streptomycetes are contained within Family VII (Streptomycetaceae) of Order Actinomycetales (Buchanan and Gibbons, 1974), along with *Nocardia* (Family VI) and *Micromonospora* (Family VIII), among others. Streptomycetes are gram-positive, aerobic, vegetative hyphae and non-motile-spore producing bacteria, and produce a wide variety of secondary metabolites (Waksman, 1967), including antibiotics and fungicides. Streptomycetes do not form sporangia-like vesicles, and spores produced are not borne on verticillate sporophores (Waksman, 1961). Cell walls contain L-diaminopimelic acid and glycine, without large amount of arabinose (cell wall Type I). Most Streptomycetes are chitinolytic in nature (Gooday, 1994).

Waksman (1961) detailed 8 criteria to distinguish the streptomycetes from other actinomycetes:

1. A more or less branched, nonseptate, substrate or vegetative mycelium is produced.
2. Growth takes place either on the surface of agar or gelatin media or penetrates deep into the medium, forming a compact, often leathery mass, designated as a colony.
3. During growth in stationary liquid media, no turbidity is produced except on lysis; the masses of growth appear as clumps or compact masses.
4. The surface colony gradually becomes covered with an aerial mycelium, though this occasionally may not occur.
5. The aerial mycelium produces sporogenous hyphae or fruiting bodies, which are straight, or in the form of tufts, or curved, spiral-shaped, or verticillate.
6. The sporophores carry chains of single-celled spores (or conidia), which vary in shape from spherical to oblong or cylindrical, and also in surface appearance when viewed with the electron microscope.

7. The vegetative growth, the aerial mycelium, and the spores en masse frequently are coloured in a characteristic manner; the colour may also dissolve into the medium, producing a "soluble pigment".

8. The species are aerobic and mesophilic, non-acid-fast and gram-positive.

Since the genus was first described, many taxonomists have refined the characteristics of streptomyces but Waksman's work is quoted in Bergey's classifications (Buchanan and Gibbons, 1974). Waksman proposed separating species into groups, of which the *Griseus* group is one, and will be examined in more detail later in this chapter.

### 2.2.1 Ecology

Streptomycetes can be found in a variety of environments (Lechevalier, 1981), including marine environments (Cross, 1981), salt marshes (Hunter *et al.*, 1981) and the air (Lloyd, 1969). However, most streptomycetes are isolated from soils, in which their populations are usually present from $10^4$ to $10^7$ cfu/g of dry soil (Hirsch and McCann-McCormick, 1985).

Correlations have been found between the presence of streptomycetes and the organic matter and water content of the soil (Kutzner, 1981). As streptomycetes are obligate aerobes they prefer moist locations, but not those that are waterlogged (Williams *et al.*, 1972), however low moisture contents inhibit growth and can induce sporulation.

Most streptomycetes are mesophilic, and Kutzner (1981) classified them on the basis of the pH range over which they grow; acidophilic between 3.5 – 6.5, and neutrophilic between 5.0 – 9.0. Williams and Robinson (1981) developed a method of isolating *Streptomyces* species from soil using a solution of salts and chitin.
2.2.2 Structure and Composition

The structure of streptomycetes cell walls is typical of gram-positive bacteria, consisting of peptidoglycan cross-linked by L-alanine, L-glutamic acid and L-diaminopimelic acid (L-DAP). Nuclear material is located in the centre of cells, along the length of hyphae, in individual sections (Figure 2.4).

Vegetative hyphae and spore walls contain similar components, although the cell walls of spores are usually 1.5 – 2.0 times thicker than those of vegetative hyphae. In addition, the sheath formed by aerial hyphae prior to spore formation confers upon the spores a distinctive surface ornamentation (Hirsch and McCann-McCormick, 1985) lacking in vegetative hyphae, i.e. spines, hairs, warts or smoothness. The DNA content of spores is also higher than vegetative hyphae, each spore containing 1 – 2 copies of the genome. During germination, an additional nuclear region is found in the germ tube to that in the spore.

Figure 2.5 shows the differences in shape and thickness between vegetative and aerial hyphae, and the separation to form spores. Images (a) to (d) show the progression of conidia formation, and separation toward individual spores in aerial hyphae.
2.2.3 Physiology and Metabolism

Streptomyces are chemoheterotrophs, and do not require any special nutritional compounds, most being capable of growing on glucose and inorganic salts only (Hirsch and McCann-McCormick, 1985).

Many streptomyces use nitrates and/or ammonia; however, little information is available on the mechanism or regulation of nitrogen use (Hirsch and McCann-McCormick, 1985). Proteins are readily utilised by streptomyces, which elaborate proteolytic enzymes in order to do so (Nomoto et al., 1960). Alim and Ring (1976) showed in continuous culture experiments that the transport of amino acids correlates with growth rate.
Sanchez and Demain (2002) have shown that the metabolism of phosphates are intimately involved with the production of secondary metabolites, particularly in cases where aminoglycosidic antibiotics are produced. Biosynthesis intermediates are phosphorylated, and therefore phosphatases play a crucial role. Recent results (Sola-Landa et al., 2003) indicate that production of alkaline phosphatase (AP) occurs during the secondary metabolism of *Streptomyces lividans*, and that a two component phosphate receptor-transcriptional activator arrangement may serve as a general transduction system for secondary metabolism in other *Streptomyces* species. Biosynthesis of streptomycin by *S. griseus* involves three phosphate-cleavage steps, which are sensitive to phosphate concentration. The last enzymatic step is inhibited by inorganic phosphate (Mansouri et al., 1989).

### 2.2.4 Life Cycle

Figure 2.6 shows the life cycle of *Streptomyces*. Each main stage is examined in turn below, starting with a spore.
*Streptomyces* species use spores for distribution as well as a means of continuing their existence in adverse conditions. Spores can survive for several years at low moisture levels (Lapteva *et al.*, 1972). Moisture is an important factor in determining spore longevity and higher moisture levels may trigger the metabolism of endogenous spore components (Hirsch and McCann-McCormick, 1985). Spore submersion in water lowers heat resistance, however, spores are capable of recovery after heat treatment, although the mechanism behind this is uncertain (Kuimova, 1980).

Many different definitions of the phases that occur during spore germination are in current use (Ensign, 1978). For example, in their review, Hirsh and McCann-McCormick (1985) separate the process of germination into four discrete steps: activation, initiation, germination and outgrowth.

Secondary or reproductive hyphae, commonly called ‘aerial hyphae’ are covered in a hydrophobic sheath, and are produced from vegetative mycelia at the surface of the colony (Wildurmuth, 1970). These hyphae are segmented and form spores. This process of forming aerial mycelium and spores is collectively called sporulation.

Activation of spores can be induced by a number of factors. Some species can be activated by heat or chemical methods (Hirsch and Ensign, 1976; Grund and Ensign, 1982), or simply by shaking in submerged conditions (Kendrick and Ensign, 1983). The mechanism of initiation, however, is not known. Many species require metal ions such as calcium, magnesium or iron in order to commence germination. Current research (Gruzina *et al.*, 2003) is examining the effects of an auto-regulatory factor (A-factor) on germination. A-factor has been identified as 2S-isocapryloyl-3R-oxymethyl-8-butyrolactone by Pliner *et al* (1975).

RNA and protein production occurs rapidly during germination (Nagatsu and Matsuyama, 1970). DNA is produced as the outgrowth phase commences, i.e. the emergence of the germ tube. This germ tube extends and eventually forms branched mycelium, perhaps attaching itself to an available solid (e.g. agar).
It is not only nutritional factors that can induce sporulation: the A-factor (Khokhlov et al., 1973) produced by *S. griseus* is required for development. Also, recent work by Kawamoto *et al.* (1997) shows that the ssgA gene in a strain of *S. griseus* is responsible for the initiation / suppression of the sporulation process.

### 2.2.5 Chitinases of Streptomycetes

Chitinolytic bacteria are those which break down chitin by initially hydrolysing the (1→4)-β-glycosidic bond. This is carried out by a group of enzymes known as chitinases.

Chitinases can be split into two broad groups, *exo*-chitinases and *endo*-chitinases. Exo-chitinases split diacetylatedchitobiose units (essentially two *N*-acetylglucosamine units, sometimes called chitobiose) from the non-reducing end of a chitin chain by a hydrolysis cleaving of a glycosidic bond. Endo-chitinases split glycosidic bonds along a chitin chain at random, eventually producing diacetylatedchitobiose and some triacetylatedchitobiose. These short chains can then be split into monomers of *N*-acetylglucosamine by an enzyme defined as *N*-acetylglucosaminidase (Gooday, 1990). Either diacetylatedchitobiose or *N*-acetylglucosamine can be transported into cells and utilised.

An alternative pathway, found particularly in marine or estuarine environments is the deacetylation of chitin to chitosan using an enzyme called chitin deacetylase, and then broken down using chitosanases. These two pathways have been previously described (Gooday, 1990) and are summarised in Figure 2.7 below.
Much work has been done on the distribution of chitinolytic organisms in soils (many being streptomycetes, as well as *Pseudomonas* and *Bacillus* species (Gooday, 1990)), and their properties (Williams and Robertson, 1981), in addition to methods for measuring chitinolytic activity, such as enzyme activity evaluation (Fraendberg and Schnuerer, 1994), released sugar detection, and the use of fluorogenic compounds (McCreath and Gooday, 1992).

The enzymes that are able to hydrolyse glycosidic bonds are called glycoside hydrolases (or glycosidases) and are divided into more than 80 families (Henrissat and Davies, 1997). Families 18 and 19 contain over 800 enzymes, including the chitinases (Eijsink et al., 2002). Family 18 chitinases are the most wide-spread and best studied class of chitinolytic enzymes and much of the current knowledge has been obtained from the study of six chitinases produced by *Serratia marcescens*.

Figure 2.8 shows two chitinases (ChiA – upper and ChiB – lower) from *Serratia marcescens* in complex with chitin substrate, showing typical barrel-type structure. In the case of ChiB, the arrow in the lower diagram indicates a loop acting as a chitin-binding domain.
2.2.6 Streptomyces griseus

*S. griseus* was originally isolated by Waksman and Curtis in 1915 from soil and originally described as *Actinomyces griseus*. Krainsky claimed to have isolated the same organism in 1914; however, as there were reported differences between the two isolates (see below), and Krainsky’s organism was lost during World War I, it is Waksman’s isolates in 1915 and 1943 that remain the type strains (Waksman, 1967).

Whereas Krainsky’s organism produced a greenish-grey to dark grey aerial mycelium, with a green-yellow soluble pigment, Waksman and Curtis’ organism produced a water-green to yellow-green aerial mycelium, with no soluble pigment. Their colourations on potato were also different (grey compared to pale...
yellow). Whilst *S. griseus*, like other streptomycetes, can have colour variations on different artificial media, Krainsky’s initial isolate was described as being only weakly proteolytic, whereas Waksman (1961) described his *A. griseus* in 1919 as possessing a “very strong proteolytic power”. Baldacci *et al.* (1954) later suggested that Krainsky’s isolate was in fact *A. viridis*.

Two cultures were isolated in 1943 from the soil and the throat of a chicken (Schatz, Bugie and Waksman), which were found to be similar to *A. griseus* described by Waksman and Curtis in 1916, as part of a screening programme searching for antibiotics. Waksman and Henrici had, in the mean time, proposed that the sporulating actinomycetes be renamed as streptomycetes, and so the organism was named *Streptomyces griseus*, and has since been accepted as the type for streptomycin production.

Not all strains of *S. griseus* are capable of producing streptomycin. The majority were either non-producers or produced other antibiotics, e.g. cyclohexamide, grisein, streptocin, actinomycin and candicidin (Waksman, 1961). It was initially considered that the ability to produce streptomycin would be a strain characteristic, however in 1959 *S. griseus* was raised to the status of a series and the streptomycin producing strain to a species status, *Streptomyces griseus*, Waksman and Henrici. This particular organism is now classified as *S. griseus griseus*. Three synonyms of previous classifications exist: *A. globisporus*, *A. globisporus streptomycini* and *A. streptomycini*.

After reclassification and isolation of new species, Waksman divided the genus streptomycetes into a number of series, of which Griseus was one. It has four characteristic properties:

1. Sporophores straight, produced in tufts. Spores oval; surface smooth
2. Growth colourless to olive-buff. Aerial mycelium water-green to grass-green to grey.
3. Melanin-negative
4. Strong proteolytic activities. Produce a variety of antibiotics.
At least five distinct species have been identified within the Griseus series (Waksman, 1967). *S. griseus* itself is one, with strains capable of producing streptomycin as well as cyclohexamide, an anti-fungal agent. The following four species are also classified in the Griseus series, being very similar in many respects. Some strains of these organisms were originally classified as *S. griseus*.

*Streptomyces griseinus* strains produce grisein or grisein-like components, and are typically resistant to actinophage. Most strains differ in colouration from *S. griseus* in that although they often produce aerial mycelium with the typical greenish colour, they do not produce the yellow/green pigment in vegetative mycelium in a variety of media.

*Streptomyces coelicolor* comprises strains that produce candidicin, an antifungal agent, but no antibacterial components. The isolation of the first of these organisms was reported in by Mueller (1908) as *Streptothrix coelicolor*, and produced a soluble blue or brown pigment (depending on medium used), but no aerial mycelium unless serum, glycogen, dextrin or starch is added to the medium.

*Streptomyces californicus* strains produce viomycin, and this species is also classified as *S. griseus purpureus* (Burkholder *et al.*, 1955). Finally, *Streptomyces chrysomallus* contain actinomycin-producing strains.

These species identified, it must also be stated that there are strains that do not produce any antibiotic components at all, or produce others not listed here. It has been shown (Waksman, 1967) that streptomycin-producing strains are readily mutated; such mutants possess different colouration, antibiotic-producing capabilities, sensitivities to antibiotic, and may or may not produce aerial mycelium. Glasby (1992) lists 30 named antibiotics produced by *S. griseus* alone, not counting subspecies.

### 2.2.7 Antibiotic Production by the Genus Streptomycetes

It is estimated that the streptomycetes are responsible for producing 70% of the world’s antibiotics. Much is known about the secondary metabolism of
**Streptomyces** due to the variety of useful compounds that can be produced; however, much less is known about other aspects of their biology (Hirsch and McCann-McCormick, 1985).

In 1939 Selman Waksman and his research team began the first systematic search for fungal and bacterial antibiotics, only two being reported previously (penicillin by Fleming in 1929, and tyrothricin by Dubos in 1939). The first produced in his laboratory was actinomycin in 1940, from *Actinomycetes antibioticus*, which was highly inhibitory toward gram-positive bacteria but extremely toxic to humans. This was followed by streptothrycin, which was at first thought to be a promising antibiotic as it is less toxic than actinomycin. However, streptothrycin was later shown to exhibit delayed toxicity and this prevented it from being used clinically. In 1943, streptomycin was first isolated from *Streptomyces griseus*.

The 1943 strain of *S. griseus* was used for the large-scale production of streptomycin, found to be an effective agent in the fight against *Mycobacterium tuberculosis*, for which Waksman was awarded the Nobel Prize for Physiology and Medicine in 1952. Since then, a large number of antibiotics have been obtained and used from streptomycetes; Table 2.1 shows a selection of these, along with the organisms that synthesis them, and their uses.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Produced by</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aclarubicin (aclarinomycin A)</td>
<td><em>S. galileus</em></td>
<td>Used for treatment of acute Myeloid Leukaemia</td>
<td>Hiddeman and Mertelsman (1990)</td>
</tr>
<tr>
<td>Amphotericin</td>
<td><em>S. nodosus</em></td>
<td>Macrolide antifungal antibiotic</td>
<td>Herbrecht (1999)</td>
</tr>
<tr>
<td>Bleomycin</td>
<td><em>S. verticillus</em></td>
<td>Inhibits DNA metabolism, used as an antineoplastic, particularly for solid tumours</td>
<td>Caputo (1976)</td>
</tr>
<tr>
<td>Chromomycin</td>
<td><em>S. griseus</em></td>
<td>Glycosidic antibiotic, used as a fluorescent DNA stain and antineoplastic agent</td>
<td>Webster’s (2004)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td><em>S. erythreus</em></td>
<td>Effective against many Gram-positive and some Gram-negative bacteria</td>
<td>Icon Group (2004)</td>
</tr>
<tr>
<td>Tylosin</td>
<td><em>S. fradiae</em></td>
<td>Effective against many organisms in animals, but not humans</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Antibiotics produced by streptomycetes
2.3 Streptomycin

Streptomycin was the first antibiotic isolated through systematic searching that has a relatively low toxicity to humans. It is made up of 3 components: streptidine, streptose and N-methyl-L-glucosamine, linked together by glycosidic bonds (Figure 2.9), and is part of the aminoglycoside group of antibiotics.

Aminoglycosides are defined by the presence of a carbohydrate containing an aminosugar, linked via a glycosidic bond to an aglycone moiety, known as an “aminocyclitol”. This group of antibiotics includes amikacin, gentamicin, neomycin, netilmicin, tobramycin, and streptomycin, amongst others (Table 2.2). All Aminoglycosides are bactericidal and active against some Gram-positive and many Gram-negative organisms. Synthesis features little in their production; amikacin (a derivative of kanamycin) is the only clinically-used semi-synthetic product.
Streptomycin Production from chitin using *Streptomyces griseus*  Richard Meanwell

Aminoglycoside antibiotic manufacturers.

<table>
<thead>
<tr>
<th>Aminoglycoside antibiotic</th>
<th>Producing organism</th>
<th>Manufacturing(^{\text{a}}) companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmytomycin</td>
<td><em>Streptomyces rimosus</em></td>
<td>21</td>
</tr>
<tr>
<td>Fortimicins</td>
<td><em>Micromonospora pulchra</em></td>
<td>18</td>
</tr>
<tr>
<td>Genamicins</td>
<td><em>Micromonospora purpurea</em></td>
<td>5, 9, 32</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td><em>Streptomyces hygroscopicus</em></td>
<td>6, 19, 35</td>
</tr>
<tr>
<td>Kanamycins</td>
<td><em>Streptomyces kanamyceticus</em></td>
<td>2, 7, 8, 10, 21, 23, 28</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td><em>Streptomyces kasugensis</em></td>
<td>5, 15, 21, 31</td>
</tr>
<tr>
<td>Lividomycin</td>
<td><em>Streptomyces lividus</em></td>
<td>17, 29</td>
</tr>
<tr>
<td>Neomycins</td>
<td><em>Streptomyces fradiae, Streptomyces albogriseus</em></td>
<td>3, 4, 6, 8, 12, 20, 25, 26, 27, 29, 30, 53, 55, 56</td>
</tr>
<tr>
<td>Paromomycin</td>
<td><em>Streptomyces rimosus forma paromycinus</em></td>
<td>25</td>
</tr>
<tr>
<td>Ribostamycin</td>
<td><em>Streptomyces ribosidicus</em></td>
<td>21</td>
</tr>
<tr>
<td>Sagamicin</td>
<td><em>Micromonospora sagamiensis var. nonreducens</em></td>
<td>18</td>
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<tr>
<td>Sisomicin</td>
<td><em>Micromonospora anyosins</em></td>
<td>11, 32</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td><em>Streptomyces spectabilis, Streptomyces flavopersicus</em></td>
<td>1, 16, 26</td>
</tr>
<tr>
<td>Streptomycins</td>
<td><em>Streptomyces griseus</em></td>
<td>2, 3, 8, 13, 14, 21, 22, 24, 27, 34, 37</td>
</tr>
<tr>
<td>Tobramycin</td>
<td><em>Streptomyces tenebrarius</em></td>
<td>6, 19</td>
</tr>
<tr>
<td>Validation</td>
<td><em>Streptomyces hygroscopicus var. linearius</em></td>
<td>35</td>
</tr>
</tbody>
</table>

Table 2.2 Aminoglycoside antibiotics and their producers (Perlman, 1977)

Although active against many organisms, aminoglycosides are not as readily used as some antibiotics due to two main reasons. Firstly, they cannot be absorbed from the gut, and so must be injected. Secondly, they possess side effects, the most important being ototoxicity (affecting the ear) and nephrotoxicity (affecting the kidney), although they can also impair neuromuscular transmission and are not recommended during pregnancy.

The aminoglycosides, including streptomycin, operate by inhibiting protein synthesis, by binding to the 70S ribosome, which consists exclusively of protein and RNA. Ribosomes comprise two sub-units, designated 30S and 50S (Figure 2.10).
Streptomycin Production from chitin using *Streptomyces griseus*  

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**Figure 2.10** Simplified representation of the subunits of a prokaryotic ribosome and their cooperative interaction to form the 70S particle (Franklin and Snow, 1998).

This structural work has provided evidence that the RNA contained in these structures is responsible for both decoding and for peptide bond synthesis, with certain proteins being required to maintain the ribosome’s structure (Franklin and Snow, 1998). Streptomycin is bactericidal, but before cell death occurs there are visible effects on protein biosynthesis. The initiation of protein chains is inhibited, and the elongation of partially completed chains is slowed, although not stopped. The incorporation of certain peptide linkages are also inhibited, while misreading of the mRNA base is induced by stimulating other linkages.

The exact interaction between streptomycin and the 70S ribosome is, as yet, not entirely certain. However, based on its effects on the mRNA decoding and initiation of peptide chains, the S12 protein and 911-915 region of the 16S rRNA (decoding) are likely to be involved (Franklin and Snow, 1998).

It is thought that the induction of mRNA misreading allows the disruption of the cytoplasmic membrane and outer membrane of Gram-negative bacteria, making the cell "leaky", and allowing much more of the antibiotic to enter the cell, and halting protein synthesis altogether (Glazer and Nikaido, 1995).

Streptomycin is biologically active against gram-negative and gram-negative bacteria, and has been reported to have antialgal activity (Waksman and Lechevalier, 1962). Of note is its action against mycobacteria, particularly *Mycobacterium tuberculosis*, the organism responsible for tuberculosis or TB.
Streptomycin has been used to treat a number of infections including bacteremia, meningitis, tularemia, brucellosis and plague (Waksman, 1949). Streptomycin is also used in veterinary science to treat a variety of diseases in cattle, turkeys and chickens (Medina and Unruh, 1995).

Streptomycin is still used to treat TB today, in conjunction with several other drugs (e.g. isoniazid (INH), ethambutol, pyrazinamide, and aminosalicylic acid) in order to prevent resistant strains of *M. tuberculosis* being produced (Columbia Electronic Encyclopedia, 2004). In the last 20 years or so, streptomycin has been often replaced with rifampicin (a rifamycin), as it is capable of attacking bacteria in closed caseous lesions, and can be taken orally. Streptomycin is still used, however, in such cases where *M. tuberculosis* develops a resistance to rifampicin. Streptomycin is produced by a large number of companies, under a variety of brand names (Table 2.3). A number of high producing mutants are currently in use in industry and as early as 1967 Horner (1967) reported increases in production from 100-200 mg/litre to 10 g/litre.

Streptomycin production occurs during the idiophase of streptomyce growth, reaching a maximum concentration before reaching a plateau or declining. Figure 2.11 shows a typical fermentation pattern.
2.4 Solid State Fermentation

Solid State Fermentation (SSF) is referred to by many names, most of which ironically conform to the same acronym, e.g. Semi-Solid Fermentation, or Solid-Substrate Fermentation. SSF is not concerned with the metabolism of solid substrates in liquid media, even where the solids content is high. Neither does it mean that SSF is a condition where no water is present at all, as all biological activity ceases below about 12%.

Cannel and Moo-Young (1980) defined solid state fermentation as the growth of micro-organisms on solid materials without the presence of free liquid. While the presence of moisture is necessary in solid state fermentation, it exists in an absorbed or complexed form within the solid matrix.

The origins of solid state fermentation can be seen in written records before 1000 B.C. in one of the thirteen books of Confucius for the production of soy sauce, and was routinely used in food preparation as Buddhism flourished in China and other parts of the Orient. This practice continued in the home right up until the 18th Century, and continues industrially today. Other evidence has also placed SSF in the production of Roquefort cheese in the first century A.D (Hesseltine, 1972).
Perhaps the best example of solid state fermentation is the Koji Process, koji being an enzyme preparation produced from a mould (either Aspergillus oryzae or Aspergillus sojae). It is used as an intermediate in the production of soy sauce, sake and miso, of which literally billions of litres are produced per annum (Yamada, 1977).

2.4.1 Advantages and Disadvantages of Solid-State Fermentation

Much of the modern work on solid-state fermentation was pioneered by Hesseltine in the 1970's. His thoughts on the pros and cons of SSF still remain perhaps the most comprehensive list, expanded over the years, and briefly summarised below (Hesseltine, 1972, 1977a and 1977b).

Advantages:

a) Relatively simple media required, e.g. the solid substrate and enough water to soften the material.
b) Can be operated on either batch or continuous basis
c) Smaller equipment, due to lower water content. Complexity no greater than conventional fermentation equipment; may be less.
d) In fungal and natural flora fermentation, spores/cells can be used directly; no seed tanks required, so less capital cost, and one less step where contamination can occur
e) Reduced potential for bacterial contamination, as conditions are often adverse for establishing growth
f) Growth conditions can be closer to those in natural growth environment
g) Smaller quantity of solvent required for product extraction, therefore lower cost and smaller extraction equipment – lower capital cost
h) Fermentation mixture easy to aerate, due to interparticulate spacing
i) Yields are often much higher, and are reproducible
j) For animal feeds, solid product requires much less drying
k) Solids handling relatively simple
Disadvantages:

a) Only organisms not requiring free water can be used, i.e. fungi, some yeasts, and *Streptomyces*.  
b) Heating can be a problem for large scale operations; as there is no movement of liquid past the particles, it is more difficult for any heat generated to be removed.  
c) Monitoring and control can be more difficult, as many commercial sensor devices require the presence of liquid to operate. The solid substrate being used in the fermentation can also interfere with analysis, e.g. chitin interfering with use of glucosamine assay for biomass determination  
d) Solid substrate often requires treatment prior to fermentation  
e) Large scale shaking or rotation can be expensive  
f) Large number of spores required for fungal inoculation could cause problems  
g) Should sterile water addition be required during first few hours of fermentation, the risk of contamination is greater as the organism being used has had little time to establish itself.

### 2.4.2 Bioreactor Configurations for Solid State Fermentation

Cannel and Moo-Young (1980) have listed six broad categories of solid state fermentation systems:

a) Tray  
b) Windrow (see below)  
c) Tower  
d) Bed with recycled conditioned air  
e) Rotating Drum  
f) Stirred Tank

The first three reactor types are generally used in batch-mode, the latter three generally for continuous operation. Cannel and Moo-Young (1980) also further notes that the first two are likely to have a smaller capital cost (due to their
simplicity), however, tight process control is not possible. Reactor types c) to f) have higher capital costs, but better process control is afforded.

Key issues when deciding on a fermentation system are the required volume and whether agitation is necessary. Larger volume systems are cheaper to run in continuous mode, providing that aseptic conditions can be maintained (if required), and that the motion of the material through the system does not interfere with any agitation requirements.

Agitation requirements are not always as simple and provision must be made to ensure that sufficient oxygen is available for the fermentation, as well as for the removal of evolved carbon dioxide. This must be achieved without causing undue damage to the organism. *Streptomyces* are known to be relatively fragile organisms (Whitaker, 1992), and while some agitation will be necessary, both for aeration and to break down the aerial hyphae to a certain extent to aid in reproduction, excessive agitation will destroy the organism.

The use of tray reactors has been reported from as early as 1923 for the fermentation of koji using *Aspergillus oryzae* (Church, 1923). Koji fermentation is usually conducted in a chamber inside which are fixed horizontal trays containing the substrate. Heat generation is easier to control in such a reactor as the space between the trays allows excellent air flow. Figure 2.12 shows a typical automated koji process reported by Numokawa (1972).
Streptomycin Production from chitin using *Streptomycetes griseus*  

Richard Meanwell

Windrow reactors (Figure 2.13) are not strictly speaking reactors in the conventional sense, but rather rows of stacked waste material, usually around 6 feet high and 10 feet wide. Every few days, the row is pulled down and reconstructed in a space next to it, to provide aeration; specialist equipment is normally required to accomplish such a task. This method is usually used for composting.

Tower reactors are vertical vessels in which the solid material is agitated to promote mixing and help prevent localised excessive heating and build-up of
Streptomycin Production from chitin using *Streptomyces griseus* Richard MeanweIl

CO₂ (Figure 2.14). If the substrate is agitated, this is either provided by large internal paddles, or by rotating an internal cage or even the vessel itself. In other systems, the material is either simply located on shelves inside the tower, or transferred from shelf to shelf from the top to the bottom of the reactor (the latter normally used in a continuous system – Figure 2.15). A packed bed fermenter operates with a set-up similar to a typical packed bed unit operation. The solid substrate is supported on a grill or distribution plate, and air is passed through the column.

Tower reactors have been used for many applications, including the production of aflatoxin using *Aspergillus flavus* (Hesseltine, 1977a), and for the production of various secondary metabolites using streptomycetes, however little has been done with antibiotics specifically.

Berrocal et al. (2004) used *Streptomyces cyaneus* to ferment wheat straw in a typical packed column fermenter to produce laccase to pulp the substrate.

Ellaiah et al. (2004) carried out a study for neomycin production, using *Streptomyces marinensis* on wheat rawa. They found that the concentration of antibiotic reached a maximum after 10 days, and by using optimisation techniques, a yield could be obtained 1.85 times greater than that achieved using SmF.

In their review, Robinson et al. (2001) state that in theory, solid state fermentation could be used on an industrial scale for the production of antibiotics, in the same way that is has been advocated for other fermentation products, such as fuels and enzymes. Cephamycin C has been produced using *Streptomyces clavuligerus* by Kota and Sridhar (1998) on wheat rawa, obtaining a maximum yield of antibiotic after only 5 days.

Both Kota and Sridhar (1999) and Ellaiah (2004) showed that the optimum moisture content for solid state fermentation of their *Streptomyces* species was 80%.
A rotating drum reactor works on the same principle as a tower reactor with an internal cage. In most cases, however, the rotating drum reactor is in a horizontal...
orientation. Horizontal rotating bioreactors have been used for many purposes, including the bio-treatment of prawn and shrimp waste to produce chitin using lactic acid fermentation (Zakaria et al., 1998). Figure 2.16 shows this reactor arrangement.

![Figure 2.16 Horizontal Rotating Bioreactor for lactic acid fermentation (Zakaria et al., 1998).](image)

Key: 1: Outer QVF glass reactor shell. 2: Rotating 316L SS basket. 3: Central shaft for rotation. 4: Pulley system. 5: Variable speed motor.

2.4.3 Fermentation Conditions

Due to the inherent difficulties of measuring and controlling pH, temperature, water activity, biomass and other parameters, several methods have been investigated in order to measure, either directly or more often indirectly heat transfer (Gutierrez-Rojas et al., 1996), mass transfer (Rathbun and Shular, 1983), diffusion coefficients (Auria et al., 1992), water activity (Xavier and Karanth, 1992), and pH (Gaden, 2000). Of most important interest to this project, however, is the measurement of microbial biomass in a solid state fermentation system as a means of measuring growth for kinetic studies. This has further discussed in Section 3.2.7.
2.5 In-situ Extraction of Fermentation Products

Wu and Lin (2003) have shown that in-situ extraction of fermentation products is possible using solvent extraction for a two-phase system. Their research involved the extraction of Taxol (a secondary metabolite) from *Taxus chinensis* cells in a suspension culture using dibutyl phthalate on a shake flask scale. Wang *et al.* (2001) determined that such solvent extraction (along with addition of sucrose) could extend the production phase significantly.

Such extraction can also be used with antibiotics. Jermini and Demain (1989) used pH 3 0.1 M phosphate buffer to extract cephalosporins from wheat and barley after solid-state fermentation of *S. clavuligerus*, the time used to extract the antibiotic was 60 minutes in a three-stage extraction. Rettori and Duran (1998) have obtained purified violacein from the growth of *Chromobacterium violaceum* on cotton wool in modified Roux bottles. Following incubation, the antibiotic was eventually recovered by Soxhlet extraction using ethanol and purified through HPLC.

Fedeniuk and Shand’s (1998) review of antibiotic extraction from biomatrices highlights methods available for the recovery of aminoglycosides. Simple aqueous extraction using buffers allows recovery of approximately 50% in the presence of proteins. Removal of these proteins through acid precipitation or NaOH digestion pushed recoveries to 80% and 90% respectively (Salisbury, 1995).

Hernandez-Justiz *et al.* (1998) used a two-phase system to continuously strip water-soluble products (cephalexin – an antibiotic) using polyethylene glycol in an *E. coli* fermentation. The yield of cephalexin was increased from 55 % (using a mono-phasic system) to 90 %, mainly thought to be due to the removal of the antibiotic before rapid enzymatic hydrolysis could take place.
3 Streptomycin Analysis

3.1 Introduction

A number of techniques for determining the titre of streptomycin obtained during fermentation were evaluated during the course of this work. The concentration of the antibiotic had variously to be determined in aqueous solution but it was also essential to have estimates of the amounts that had adsorbed to the surface of solid chitin. The latter type of determination was initially undertaken by causing the streptomycin to become removed from the surface - i.e. into solution form - and then employing one of the techniques available developed specifically for solutions, e.g. bioassay. However, Fourier Transform Infra Red (FTIR) based methods were subsequently developed that obviated the need to remove adsorbed streptomycin from the surface of chitin particles and enabled direct estimates to be made.

Some of the techniques considered here, e.g. bioassay, were modifications of widely used methods, but others such as FTIR analysis, involved a considerable amount of development work. The development of these techniques is described in detail in this chapter. Also included is discussion of the applicability – and the limitations – of the methods in question.

Prior to the discussion of these methods, however, the procedures used throughout this project and the materials associated with them are first described, as well as the theory and background behind the different techniques used in this work.

3.2 Theory and Background

Several different methods have been investigated for the analysis of antibiotics, particularly in the last fifteen years, to provide a more rapid means of determining titre than standard bioassays. Methods having particular relevance included, liquid and high performance liquid chromatography, micelle
electrokinetic capillary chromatography, and gas chromatography. These three methods are discussed briefly below, with the addition of the standard bioassay, all of which analyse liquid samples.

Analysis of solids was examined using Fourier Transform Infra-Red (FTIR) spectroscopy, and discussed at the end of this section.

### 3.2.1 Liquid and High Performance Liquid Chromatography

Much work has been done in recent years on developing methods of detecting antibiotics using Liquid and High Performance Liquid Chromatography (LC & HPLC). HPLC offers a fast and reliable method of analysis, and while the chromatography hardware is, relative to operating a bioassay, expensive, many laboratories possess the necessary equipment due to its versatility.

Most of the methods revealed as part of this literature survey dealt with the detection of streptomycin or dihydrostreptomycin in serum, milk, or animal tissue. These methods are by their very nature specific to the particular systems used and would bear little relevance to the chitin-based fermentation system employed here. However, all these methods were considered or attempted as part of the development of the HPLC method currently being used (see Section 4), and will be briefly summarised here.

Two methods were investigated by Gerhardt and co-workers (1994a; 1994b), for both streptomycin and dihydrostreptomycin. However, in both cases, extraction of analytes using perchloric acid was required, in addition to post-column derivatisation using 1,2-naphthoquinone-4-sulphonic acid (which forms fluorescent products with guanidino groups in streptomycin) before being detected using fluorescence.

Another more general method has been more recently reported (Edder et al., 1999), for the analysis of streptomycin from a variety of sources, such as meat, milk, or honey. Ion-pair chromatography is again used, with the same post-column derivatisation found in Gerhardt et al.’s work. However, the sample
preparation before analysis is extremely involved, requiring approximately 15 stages.

Kubo et al. (1986) reported on an HPLC method for streptomycin determination. Although requiring less pre-treatment than the method of Gerhardt et al., the method remains involved. One advantage was that analysis was rapid with determinations taking less than 10 minutes.

A few methods using less expensive ultraviolet detection have also been used. Whall (1981) and Kurosawa et al. (1985) both utilised reverse-phase ion pair methods, with similar mobile phases (8:92 acetonitrile – sulphonate buffer), although pH of operation varies between pH 3 and 6. Both methods also used simple reverse phase C18 type columns, and detection wavelengths of 195nm. All these mobile phases and conditions were attempted here as part of the experimentation (Section 3.5).

Finally, excellent results were claimed by Abel et al. (1999) by combining HPLC with Nuclear Magnetic Resonance (NMR) Spectroscopy.

3.2.2 Micelle Electrokinetic Capillary Chromatography

Micelle Electrokinetic Capillary Chromatography (MECC) is a new method of analysis recently emerged in the last ten years. Essentially, a sample is placed into one of two reservoirs connected by a capillary and filled with detergent solution at a concentration above the critical micellar concentration (CMC). Above this concentration, the detergent forms micelles in the water, and analytes in the sample are absorbed onto and/or into these micelles.

A high potential difference is then applied to the two reservoirs as a driving force, and this causes the micelles to travel through the capillary. The capillary itself is passed through a UV detection system to pick up the analytes as they pass through the system. A diagram of such a system can be found in Figure 3.1.
Due to the novel nature of the method, little information is available on methods, particularly on specific antibiotics.

### 3.2.3 Gas Chromatography

Arrowood and co-workers (1991) produced a method of analysing for streptomycin contained in pharmaceutical preparations, by reacting the streptomycin with sodium hydroxide at 100°C and converting it to maltol. This approach is interesting as converting streptomycin to maltol could prove useful in HPLC analysis as a means of derivatisation if conflicting peaks are present.

### 3.2.4 Fourier Transform Infra-Red Analysis (FTIR)

The difficulties of *in-situ* analysis of secondary metabolites in solid state fermentation has been previously discussed (see Section 2.5). Although it will be shown that in chitin-based media fermentations with *S. griseus* HPLC provides a useful analytical technique when the concentration of streptomycin is greater
than 5 mg/l (see Section 3.5) these concentrations are only obtained with non-commercial strains near the end of the fermentation process.

FTIR spectroscopy provides a method of analysing the functional groups on the surface of a solid. It is known that FTIR has been previously used for determining the degree of de-acetylation of chitin (Shigemasa et al., 1996) and evaluating chemical derivatives through changes in surface functional groups, often through the prominent amide bond at ~1650cm\(^{-1}\) and NH\(_2\) group at >3000cm\(^{-1}\).

As the streptomycin molecule (see Figure 2.9) has a large number of NH\(_x\) groups it seems likely that however the molecule attaches to the chitin chain there is likely to be an increase in the number of NH\(_x\) groups on or near the chitin surface that could be detected by FTIR.

FTIR, therefore, represents a potential method of detecting streptomycin on the surface of chitin.

3.2.5 FTIR Theory

The energy of a molecule can be partitioned into four components: translational, rotational, vibrational and electronic (Colthup et al., 1975). Absorptions and emissions from each of these energies fall into different areas of the electromagnetic spectrum: electronic transitions, for example, absorb or emit throughout the ultraviolet and visible regions. Rotational energy provides absorption in the microwave and “far” (0.1 – 1 mm wavelength) infrared areas, and vibrational across the infrared zone.
3.2.5.1 Energies

Translational Energy

Translational energies occur when the molecule moves as a whole unit – individual atoms do not change position relative to each other. Such movement does not interact with the electromagnetic spectrum, and so this type of energy can be ignored for spectroscopy purposes.

Electronic Energy

Electronic energy involves three types of electrons (Kendall, 1966):

1. Those belonging to a single atom
2. Those shared between two adjacent atoms
3. Those shared between two or more atoms

Type 1 electrons, those found in the inner electron shells, do not significantly add to the molecular energy of a molecule and can be ignored. Types 2 and 3 are involved in transitions between electronic energy levels for rotational and vibrational energy levels under ultraviolet and visible radiation. As infrared is less energetic than ultraviolet and visible electromagnetic radiations, these contributions to the molecular energy are considered negligible.

Rotational Energy

The rotational energy of a molecule is entirely kinetic and is dependent on the molecule’s geometry and moments of inertia. Rotational energy is examined when analysing molecules in the vapour phase as free rotation is possible. However, inter-molecular forces in liquid and solid phase materials inhibit or prevent such movements (and thus absorptions) from taking place (Kendall, 1966).
As the other three energies associated with the energy of a molecule have been shown to be negligible in the infrared region, the energy of a molecule can be approximated to the contribution from vibrational energy.

**Vibrational Energy and Normal Modes of Vibration**

If a classical model of a molecule is taken, whereby the nuclei are represented as masses, each nuclei can be located in terms of three-dimensional co-ordinates. Each individual mass has three directions of movement possible, on x, y, and z axes, and so has three degrees of freedom of movement. Therefore, for a molecule of \(N\) atoms, there are \(3N\) degrees of freedom.

Three of these degrees of freedom occur when each atom of the molecule moves along the same path; as these merely change the location of the centre of mass of the molecule (by definition translational energy), they can be ignored as previously discussed. Another set of degrees of freedom are due to rotational energy; three for a three dimensional molecule, and two for a linear one (as any rotation around the molecular axis in a linear molecule is not due to any movement of atoms contained therein). Therefore, the number of degrees of freedom \(N_F\) for a molecule is either \(3N - 6\) or \(3N - 5\) for a three dimensional or linear molecule respectively. The degrees of freedom of a molecule due to vibrational energy calculated in this way are the *internal* degrees of freedom and are called the *normal modes of vibration* and can occur independently of each other.

3.2.5.2 *Energy Levels*

If a diatomic molecule is considered with a fixed centre of mass (ignoring translation), for a vibrating molecule containing atoms of different masses the two atoms must move different distances to maintain the centre of mass. If a dipole exists in the molecule, the dipole moment will change simultaneously with the distance of each atom from the centre of mass, creating an alternating electric field (van der Maas, 1972). Such a system can absorb energy provided at the
same frequency as the field oscillation, and as $E = h\nu$, the energy absorption is quantised.

The absence of a changing dipole moment would not produce an oscillating electric field and so no energy absorbance is possible, making it undetectable through the observance of IR-spectra.

3.2.5.3 Spectra and Wavenumber

Although a simple case of a diatomic molecule is taken, two facts hold for any vibration in any molecule; energy absorption is quantised for a particular vibration, and a vibrating molecule will only absorb infrared radiation if the vibration produces an oscillating electric field.

Figure 3.2 below shows a simple example of the vibrational modes of a triatomic linear molecule, such as CO$_2$. Table 3.1 following defines the vibrations.

![Vibrational modes of a triatomic linear molecule](Schimanouchi, 1972)

Figure 3.2 Vibrational modes of a triatomic linear molecule (Schimanouchi, 1972)
Vibration | Wavenumber (cm⁻¹) | Vibrational mode | Remarks |
---|---|---|---|
1. \( v_1 \) | 1285/1388 | Symmetric stretching vibration | C=O IR-inactive |
2. \( v_2 \) | 2349 | Asymmetric stretching vibration | C=O IR-active |
3 and 4. \( v_3 \) and \( v_4 \) | 667 | Deformation vibration (bending) | IR-active, two-fold degenerate |

Table 3.1 Normal modes of vibration of carbon dioxide (Schimanouchi, 1972)

Note that \( v_3 \) and \( v_4 \) are inherently the same, just rotated through an angle of 90°, and so will absorb infrared radiation at the same energy. Such vibrations are called degenerate vibrations and characterised by the degree of degeneracy; in this example, as there are two vibrations of identical frequency it is referred to as two-fold degenerate.

Figure 3.3 below shows a gas-phase IR-spectrum of \( \text{CO}_2 \) (containing a little \( \text{SO}_2 \)) and clearly shows the peaks attributing to the vibrations given in Table 3.1.

![Figure 3.3 Gas-phase IR-spectrum of a sample of \( \text{CO}_2 \) and \( \text{SO}_2 \) (Brisdon, 1998)](image-url)
The term "wavenumber" is widely used in IR spectroscopy and is defined as the number of waves in a 1cm long wave train, calculated by:

\[ \tilde{\nu} = \frac{v}{c} \text{ cm}^{-1} \] (3.1)

where \( \tilde{\nu} \) is wavenumber (cm\(^{-1}\)), \( v \) is frequency (s\(^{-1}\)) and \( c \) is the speed of light (cm/s).

3.2.5.4 *Types of vibration and Correlation Charts*

Once a spectrum for a sample has been obtained, correlation charts are used in order to analyse what the sample contains. An understanding of the different types of molecular vibration is essential for this.

Molecular vibrations can be split into two categories: stretching and bending. A stretching vibration is oscillating movement in line with the bond between two or three atoms, and can be symmetric or asymmetric depending on whether or not the oscillating motions are in or out of phase respectively.

There are four types of bending vibrations: deformation, wagging, rocking, and twisting vibrations. Deformation (sometimes called "scissors") bending occurs where the angle between atoms in the group concerned changes. Deformation vibrations can also be defined as skeletal in cases where the magnitude of the angle change is small, often due to the motions of other atoms.

Rocking vibrations involve no change of angle between atoms, only a rigid rotation of the concerned group about one atom. Wagging and twisting vibrations also involve no internal angle changes, but occur perpendicular to the molecular plane for wagging vibrations and around the bond connecting the concerned group to the rest of the molecule in twisting vibrations. Figure 3.4 shows these six types of vibration using a methylene group as an example.
Correlation charts are used in the analysis of spectra, and have been compiled from many practical analyses of known compounds. Entries are given for particular functional groups and their vibrations, and given a band where a peak or peaks corresponding to those groups can be found. Figure 3.5 shows an example part of a correlation chart.

Many procedures for analysing spectra start by splitting the base or “rocksalt” spectrum into two regions: the characteristic functional group region (4000 – 1333 cm⁻¹) and the fingerprint region (1333 – 667 cm⁻¹). The characteristic functional group region is examined first, starting with strongest absorptions,
with particular reference to the types of C-H vibrations (if any) to gain information on the molecular structures present. A similar procedure should then be carried out on the fingerprint region, and comparison made with existing spectra for suggested compounds present in the sample (Kendall, 1966).

3.2.5.5 IR and Fourier Transforms

Fourier transformation is a term combining two Fourier processes – synthesis and decomposition. Fourier decomposition is used in FTIR to take all the information on absorbed frequency magnitudes from the detector and split it into separate frequencies so that an IR spectra similar to that produced using scanning and a diffraction grating can be obtained. Figure 3.6 shows a simple graphical example of its use transforming a wave of three tuning forks combined into an amplitude vs. frequency plot.

![Graph](image_url)

**Figure 3.6** Use of Fourier decomposition to split a combined wave into individual components by frequency (Davis et al., 2001)
This tool allows all infra-red frequencies to be measured simultaneously rather than individually using a splitter (e.g. diffraction grating), a technique called multiplexing. Such a system allows a much faster analysis of a sample.

### 3.2.6 Sample techniques

Two sample techniques were used during the method development and analysis for this project: Attenuated Total Reflectance (ATR) and Diffuse Reflectance.

#### 3.2.6.1 Attenuated Total Reflectance

Also known as internal reflection spectroscopy (IRS), ATR is a non-destructive technique for analysing the surface of a material that is either too strongly absorbing or too thick for transmission spectroscopy (Guenzler and Gremlich, 2002). In this technique, the sample to be analysed is placed against the surface of an internal reflection element (IRE) (a material with a high refractive index – usually zinc selenide, silicon, germanium or diamond) where it interacts with the evanescent wave. The experimental configuration is shown in Figure 3.7.

![Figure 3.7 ATR setup.](image)

$n_1$ = refractive index of the internal reflection element; $n_2$ = refractive index of the sample ($n_2 < n_1$); $\theta$ = angle of incidence; $d_p$ = depth of penetration
ATR is regarded as a powerful analysis technique as the evanescent wave decays exponentially with distance from the IRE. Therefore, the depth of penetration of the evanescent wave is small (much less than a wavelength) and so the technique is generally independent of sample thickness. The spectra obtained from ATR are similar to those from transmission, however as the depth of penetration is dependent on wavelength, peaks at longer wavelengths are seen more intensively in ATR.

3.2.6.2 **Diffuse Reflectance**

Diffuse reflectance (also known as Kubelka-Munk reflection) is a technique used for the analysis of powders and solids with a rough surface. A small amount of the sample is usually combined with a powder that scatters radiation well (e.g. potassium bromide KBr), well mixed, and placed into a small sample holder. The radiation from the sample is scattered diffusely, and collected in wide angles through curved mirrors (see Figure 3.8). There are two fractions to this radiation: one fraction is reflected from the surface, the other penetrates the surface and is partially absorbed, and returns to the surface via internal scattering.

![Sample compartment accessories for measuring diffuse reflections (Schimanouchi, 1972)](image-url)
Rather than percentage transmission or absorbance, diffuse reflectance spectra are obtained as Kubelka-Munk (KM) ratio vs. frequency, the KM function being the ratio of moduli of radiation absorbed to radiation scattered. Spectra obtained this way are proportional to absorbance spectra, as the scattering modulus is constant.

3.2.7 Growth / Biomass Determination

As has already been discussed, determining the concentration of biomass in a solid state fermentation system is not straightforward as the presence of a solid substrate interferes with normal growth determination methods used in submerged fermentation. Methods developed for determining the growth of organisms in solid state fermentation are often indirect (Mallette, 1982).

Some of the various methods identified for growth and biomass determination are briefly discussed below.

3.2.7.1 Glucosamine

Arguably the most popular method currently is the use of glucosamine as an indirect measure of growth of solid state fermentations containing fungi. Several methods are available for determining the level of glucosamine in a sample (Zheng and Shetty, 1998; Tomaselli et al., 2001; Desgranges et al., 1991a). Glucosamine is of course a building block in both chitin and chitosan, either of which are often present in the cell wall of fungal species.

The assays detailed in the literature are generally similar. Each starts with homogenisation in order to break down the fungal cell wall and release the chitin contained within. A strong acid, either sulphuric or hydrochloric acid, followed by lengthy autoclaving, is used to break the chitin down into N-acetylglucosamine blocks. Some further treatment is often used, followed by colorimetry or spectrophotometry.
While this method seems to give excellent results for fungi, including some filamentous fungi, it is unsuited for use in a solid state fermentation system utilising chitin as a substrate; the solid substrate chitin will be hydrolysed in exactly the same way as that present in fungal cell walls.

Other methods have been developed for analysis of other components, such as total sugar and ergosterol; however, these are not present in this solid-state fermentation system.

3.2.7.2 I.R., Microscopy and Light Scattering Techniques

Infra-red analysis is used fairly extensively for the analysis of surface properties and the like. One report has been found that uses IR to analyse the amount of reflected light by a solid substrate at various wavelengths, and comparing those results to manual techniques such as those described in section 3.2.7.1 (Desgranges et al., 1991b). The results seem promising, however, as the methods in section 3.2.7.1 cannot be used, and given the expensive nature of the IR equipment, this method seems inappropriate for the solid state fermentations part of this project.

The techniques described for microscopy and light scattering also seem interesting. The light scattering technique uses a xenon lamp and computer equipment to analyse the scattered waves emitted from a beaker containing cells and suspended solids (Kennedy et al., 1992). The microscopy method uses a similar system in which the biomass is made to pass through a narrow orifice and monitored with the use of a microscope (Bittner et al., 1998). Both of these methods could potentially be used for direct online measurement of the biomass contained in the fermentation system, and indeed for a normal submerged fermentation system would be highly appropriate, particularly the latter microscope-based method as much of the equipment required is already available. However, it is unlikely that either of these methods would be suitable in solid state fermentations.
3.2.7.3 CO₂ Measurement

The indirect method of measuring the respiration of microorganisms by CO₂ analysis has been well documented (Desgranges et al., 1991b). Use of IR analysis, CO₂ probes, ion-selective electrodes, gas chromatography and mass spectroscopy are all methods that have been examined by Desgranges et al. (1991a & b), and all found to be reliable. The choice of method to use depends on whether on-line or off-line analysis is required.

3.2.7.4 Nucleic Acid/ATP Analysis

Also available are methods of assaying nucleic acids or ATP as an indicator of growth. One review on mycelial growth evaluation (Calam, 1982) proposes the estimation of either DNA or RNA as an indicator of growth.

Using ATP (Stouthamer, 1982) via the Luciferin/Luciferase reaction and fluorescence detection as a growth indicator is also becoming widely used.

3.3 Materials and Methods

3.3.1 Microorganisms

Bacillus subtilis

*Bacillus subtilis* (NCIMB 8054) suspensions were used for bioassaying streptomycin. A stock suspension was prepared for bioassays and it contained approximately 10⁶ spores/ml.

The *B. subtilis* suspensions were prepared according to the method of Gardner (1997). An Erlenmeyer flask containing 100 ml of Nutrient Broth (Oxoid Ltd., Basingstoke, Hants.) was inoculated with one loopful of *B. subtilis* from a Nutrient Agar slope, and incubated at 30 °C for 24 hours in a rotary incubator at 200 rpm.
Sporulation Agar (SA) plates (Harnulv and Snygg, 1972) were inoculated with 0.2 ml of the *B. subtilis* culture spread over the plates using a flamed glass spreader. The plates were incubated at 30 °C for 14 days, after which the *B. subtilis* spores produced were harvested by pipetting 5 ml of sterile distilled water onto the surface of the plate and scraping the surface with a flamed inoculation loop. The resulting suspension was pipetted into sterile Universal bottles and then immediately washed in order to remove small pieces of agar. The suspension was transferred to sterile centrifuge tubes and centrifuged at 8000 g for 20 minutes. The supernatant was poured off and 10 ml of sterile distilled water added, followed by re-suspension of the pellet by vigorous mixing using a vibrating mixer ("Whirlimixer", FB6500, Fisher Scientific Apparatus, Loughborough, Leics.) This procedure was repeated another two times.

The *B. subtilis* spore suspension was subjected to a ‘heat shock’ in order to inactivate any vegetative cells that may still have been present. Universal bottles containing the spore suspension were placed in a water bath at ambient temperature. The temperature of the water bath was then raised to 70 °C (typically an approximately 30 minute process) and kept at that temperature for 30 minutes. The Universals were then removed from the water bath and transferred to a refrigerator at 4 °C.

The streptomycin bioassay agar comprised:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef (Lab Lemco Powder)</td>
<td>1.5 g/l</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.0 g/l</td>
</tr>
<tr>
<td>Peptone</td>
<td>6.0 g/l</td>
</tr>
<tr>
<td>Agar (Bacteriological No. 1)</td>
<td>15.0 g/l</td>
</tr>
</tbody>
</table>

*Table 3.2 Bioassay Agar*
All materials used for this bioassay were obtained from Oxoid (Basingstoke, Hants.). Bacillus subtilis suspensions were stored at 4°C in 0.05M Tris-HCl buffer at pH 8.

### 3.3.2 Bioassay Plate Production

Bioassay plates used in this project consisted of two layers of medium (see Table 3.2 for composition). The base layer (20 ml) was allowed to set and dry first before adding the second (10 ml) layer, which was seeded with B. subtilis.

A syringe dispenser was then used to transfer both 10 and 20 ml quantities of molten medium into Universal bottles. The syringe system consisted of a 20 ml polypropylene syringe with a flat seal of the same material, with a 3-way switching valve on the base and two pieces of silicon tubing, one connected to each valve outlet. The Universals were filled by inserting one tube into a warm agar reservoir, and using the syringe to aspirate the agar into the syringe through the 3-way valve, followed by switching of the valve and expelling the agar from the syringe via the second tube. These Universals were then autoclaved at 15 psig for 15 minutes.

After autoclaving and cooling the Universals containing 20 ml of agar were poured into petri dishes. Those Universals containing 10 ml agar were rapidly cooled to 54°C, seeded with 20 µl B. subtilis spore suspension and overlayed onto the first layer of agar.

Four wells were then cut into each agar plate in a square arrangement, using the cutting equipment. This equipment consisted of a heat sterilised metal cylinder, approximately 9 mm in diameter and 100 mm in length, connected by flexible tubing to the side arm on a conical flask, used as a knockout pot for the agar removed. The conical flask was fitted with a bung through the centre of which a glass tube was fitted which extended approximately 30 mm below the level of the side arm. The top of the conical flask was connected to a vacuum pump.
The cutting tool was a stainless steel cylinder sharpened at one end and was pressed briefly to the base of each agar plate, removing an agar plug approximately 9 mm in diameter through both layers of agar (Figure 3.9).

Liquid samples (0.15 ml) were then added to each well then incubated at 30 °C for 48 hours to produce a zone of inhibition. A 1 μg/ml streptomycin standard was used on each plate for control purposes. In accordance with the recommendation given by AOAC (Association of Official Analytical Chemists), bioassay plates were made up on the day of use. It was subsequently found that plates could be prepared a day in advance and stored in a refrigerator without loss of experimental accuracy.

A calibration curve was produced to allow the zone diameter to be related to streptomycin concentration.
3.3.3 Recovery of Streptomycin from Solid Sediments

In order to determine the amounts of streptomycin adsorbed onto solid material in the fermentation medium, different pH buffers (see Table 3.3 below) were produced and used to wash the sediment from a streptomycete fermentation. A sample of separated (by centrifuge), dried sediment (0.2 g) was added to 2 ml of 50 mM strength buffer in a 10 ml conical flask and shaken together for 24 hours at room temperature. The resultant solutions were then centrifuged at 11,000 rpm for 20 minutes, and the supernatants filtered using a 20 kDa membrane. 0.15 ml of the filtrate was pipetted into wells cut into standard bioassay plates.

This method (using pH 3 buffer) was applied to obtain a filtrate as a means of determining the concentration of streptomycin adsorbed onto the chitin surface during normal experimentation.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Citric Acid &amp; Dibasic Phosphate Salt</td>
</tr>
<tr>
<td>4</td>
<td>Citric Acid &amp; Dibasic Phosphate Salt</td>
</tr>
<tr>
<td>5</td>
<td>Citric Acid &amp; Dibasic Phosphate Salt</td>
</tr>
<tr>
<td>6</td>
<td>Citric Acid &amp; Dibasic Phosphate Salt</td>
</tr>
<tr>
<td>7</td>
<td>Citric Acid &amp; Dibasic Phosphate Salt</td>
</tr>
<tr>
<td>8</td>
<td>Tris &amp; HCl Buffer</td>
</tr>
<tr>
<td>9</td>
<td>Tris &amp; HCl Buffer</td>
</tr>
<tr>
<td>10</td>
<td>Sodium Carbonate and Sodium Bicarbonate</td>
</tr>
</tbody>
</table>

Table 3.3 Buffers used for Solids Washing

3.3.4 Inhibition of *B. subtilis* by chitosan oligomers

In order to investigate whether some bioassay results could potentially be due to oligosaccharides resulting from the partial breakdown of chitin, an investigation to determine the inhibitory effects of chitosan oligomers was carried out using a logarithmic scale of concentrations. One control and seven chitosan solutions
were produced of the following concentrations: 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 %w/w, and were used in the standard inhibition bioassays described above. All solutions were produced in a pH 7.2 Tris/HCl buffer. The results can be found in Section 5.2.4.

3.3.5 Gas Chromatography

Gas Chromatography was used to determine CO₂ concentration in the exit air from fermentors. A Pye-Unicam 104 gas chromatograph (W. G. Pye & Co., Cambridge, UK) was used with a Thermal Conductivity Detector (TCD, or Katharometer). The column used for CO₂ analysis was glass, 6mm i.d., and 175 mm length and hand-packed with Molecular Sieve 5A, batch K7. The molecular sieve particles were 60-80 μm in diameter, and obtained from Phase Separations Ltd. (Deeside, UK).

Using a helium carrier gas at approximately 40 ml/min through the column and a current of 80 mA across the detector, a good response level to the range of CO₂ concentrations required was achieved. The separation between the nitrogen / oxygen (peaks were unresolved) and CO₂ peaks was sufficient to provide consistent readings across the required range.

An example GC Plot taken from a 20 ml sample of gas in the headspace of a typical fermentation is shown below (Figure 3.10). The plots obtained were then photocopied and enlarged twice (each time to 141%), and then the peaks cut from the sheet and weighed using a 4-figure balance with a precision of 0.1 mg. Weights were compared to a calibration line to determine actual CO₂ concentration, and the rate of CO₂ production for the micro-organism calculated from the flow rate of air through the fermenter (based on a dilution calculation).
3.3.6 High Performance Liquid Chromatography

Prior to analysis, samples were filtered using a Whatman GF 4.5 μm glass fibre pre-filter, followed by finer filtration using 0.4 μm exclusion size to avoid fouling of the HPLC. All samples were analysed using a Hewlett Packard (Bracknell, Berkshire, UK) HPLC 1100 system, equipped with an ultraviolet Diode Array Detector (DAD) set primarily at a wavelength of 192 nm and a bandwidth of 2 nm. The column was operated in reverse-phase at 30 °C, and consisted of a standard C18 column (Discovery ® C18, Model 504955. Supelco, Bellefonte, PA). Samples were passed through the system using a mobile phase consisting of a 10 mM orthophosphoric acid solution in deionised water (pH 2.6). The flowrate through the column was set at 0.5 ml/min, and the sample injection volume was 1 μl.

Streptomycin is the first peak to be eluted, and can be detected down to concentrations of 0.01 mg/l in relatively pure samples (e.g. aqueous solutions or buffers). However, poor resolution of streptomycin was achieved when fermentation broths were analysed. In order to improve resolution, a solid-phase and liquid-liquid extraction system was devised (see Figure 3.16a) and 3.16b) below). The liquid sample (10 ml) was pipetted into a separation funnel with 10 ml of hexane, shaken, and allowed to separate. The heavier aqueous fraction was poured out of the funnel, and then passed over a methanol-pre-treated C18 solid-phase extraction column. Methanol (Sigma-Aldrich) was used to elute any adsorbed compounds, and the resultant liquid sample was filtered in the same way described above.
Further experimentation showed that larger concentrations of streptomycin (5-6 mg/l) could be reliably detected using these conditions. However, due to the relatively poor resolution of the streptomycin peak, smaller concentrations are drawn into the leading edge of a second, larger peak and effectively disappear. Therefore, it was not possible to use this method to determine the streptomycin concentration using HPLC over the entire range of concentrations required.

3.3.7 Gas Chromatography / Mass Spectroscopy (GCMS)

A small number of investigative experiments were analysed using Gas Chromatography Mass Spectroscopy (GCMS), and the initial results were positive. A blank of di-chloromethane was analysed to give the first plot (left). A sample of broth was taken and liquid-liquid extraction carried out with di-chloromethane and then dosed with additional streptomycin (50 ppm) before analysis. These are the left and right plots respectively in Figure 3.11 below.

Figure 3.11 shows that the results are relatively simple in comparison to HPLC, and there is a clear indication that the peaks at 7.02, 16.74 and 20.13 minutes have been introduced in the second plot. Unfortunately, the software supplied with the equipment did not contain streptomycin in its library, so that these three peaks were either fragments of streptomycin or one of the peaks was streptomycin and the other peaks were compounds in the broth which were also present as impurities in the streptomycin product obtained from Sigma. One final possibility was that the three peaks featured were streptomycin A, streptomycin B, and dihydrostreptomycin. However, there were not sufficient time or resources to progress this GCMS technique further.
3.3.8 Fourier Transform Infra-Red (FTIR) Spectroscopy

Fourier decomposition is used in FTIR to take all the information on absorbed frequency magnitudes from an infra-red (IR) detector and split it into separate frequencies so that an IR spectrum can be obtained. Peaks shown in spectra correspond to energies released from a molecule during analysis, and correspond to different functional groups.

As an alternative to the solids washing technique previously described, the use of FTIR was investigated. The development of the method is discussed later (Section 3.7), however the processes behind each of the four methods used for the final experimentation are described here.

3.3.8.1 Fermentation Solids

In order to estimate the total amount of streptomycin produced during fermentation it was necessary to account for any streptomycin adsorbed to the surface of chitin and other solid components of the media employed. Solid was collected at the end of fermentation experiments and as much liquid as possible was removed through sedimentation and decanting (if necessary). The solid was
loosely washed to remove any material remaining from the fermentation liquor rather than adsorbed components, followed by sedimentation and decanting. The solid was finally dried at 45 – 55 °C overnight in a drying oven.

The resulting mass was ground using a pestle and mortar and sieved through a 54 μm sieve. An aliquot (0.2 mg) of this solid was weighed into a clean plastic bijou bottle, to which was added 400 mg of dried ground potassium bromide (grinding the KBr powder for 20 seconds in a coffee grinder produced particles with a diameter in the region of 20 μm) to yield a 0.5 %w/w sample concentration.

This mixture was then analysed using diffuse reflectance FTIR by slightly compacting the mixture into the sample dish and smoothing a flat surface using the flat end of a thin metal spatula.

3.3.8.2 Streptomycin Adsorption Isotherm

In order to determine if quantification of streptomycin adsorbed onto chitin was possible, and possibly the structure of such adsorption, an adsorption isotherm was undertaken using both pre-fermented and commercial chitin. HPLC was used to verify the results obtained as described below.

Four sets of one control (no streptomycin present) and eight concentrations (0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 25.0 and 50.0 mg/l streptomycin in pH 7 buffer) were set up in 50 ml conical flasks, two sets each for the pre-fermented material and commercial chitin. Aliquots (25 ml) of each concentration (including control) were pipetted into flasks and 0.75 g of material (fermented shrimp / commercial chitin) was weighed and added to make the four sets.

Each flask was covered with Parafilm to prevent moisture loss and agitated in a cool room (approximately 10°C) for 48 hours using a flask shaker.

After 48 hours the solids in each flask were allowed to settle, and a sample from the supernatant was taken and filtered using a Whatman GF 0.45 μm 45 mm diameter filter. The filtrate was analysed using the HPLC method described
above. The remaining mixture was then centrifuged at 8600g for 5 minutes at 10 °C and the supernatant poured off. The solid pellet was gently resuspended in ultra-pure water using a Whirlimixer to remove any surface liquid that could contain unadsorbed streptomycin, and re-centrifuged at the same conditions.

The supernatant was poured off, and the solid dried at 45 – 55 °C overnight in a drying oven. Samples for use in diffuse reflectance FTIR were produced following the same method as Section 3.3.8.1.

The HPLC samples were used as a means of determining the concentration of streptomycin remaining in each sample; this value was subtracted from the starting concentration to verify the results from FTIR. Both sets of results can be found in Section 3.7.6.

3.3.8.3 Water Gel

In the development of methods for assaying streptomycin, two methods were devised to produce a rapid screening method for putative streptomycin producers. The first used polyacrylamide crystals ("Water Gel", Homebase) dissolved at approximately 7.5 w/v% to produce a gel. The gel production was very rapid; the required weight of crystals was placed in a beaker, followed by adding water while stirring to produce an even mix.

A quantity of gel large enough to cover a 2 – 3 cm diameter circle (approximately 0.5 g) was placed in the middle of each agar plate used and left for 24 – 48 hours, sealed and under refrigerated conditions. After this period the water gel was removed from the agar plate (taking care not to remove any agar, although the gel and agar separated very easily) and placed into a foil boat. Each sample was dried for 24 hours at 45 – 55 °C in a drying oven.

The resultant solid gel crystals were ground in a large pestle and mortar, covered with cling film to prevent loss of material, followed by further grinding in a smaller pestle and mortar once a smaller particle size had been reached (<1 mm diameter approximately).
At this stage, the powder was treated in the same way as the fermentation chitin; sieving followed by dilution with KBr to 0.5 %w/w.

3.3.8.4 **Dried Agar**

The second assay technique involved drying the agar plates themselves rather than drying an adsorbent. Plates, with or without S. griseus growing on them, or with agar simply doped with streptomycin, were placed in a vacuum drying oven at approximately 0.5 bara and 75 deg C for 24 – 48 hours in order to remove the water from the agar and produce a thin film easily snapped when bent. This film was broken up into slightly smaller sections and ground in two stages using two different sized coffee grinders – a large followed by a smaller for two and four 15 second bursts respectively.

This grinding stage was followed by sieving to 54 μm and diluting in KBr to 0.5 %w/w, followed by preparation for diffuse reflectance in the same way as previously described.

3.3.8.5 **Direct Freeze-Drying of Fermentation Broths**

It was decided to test whether FTIR might provide a method of assaying streptomycin in liquid phase, as a potential alternative to bioassay.

By conducting a preliminary experiment, it was found that freeze-drying 50 ml of Yeast-Malt Extract doped with streptomycin could be used as a solid and prepared in the same way as the water gel (without the initial grinding stage with covered cling film roof).

Yeast-Malt Extract broths were doped with levels of streptomycin consistent with those found in the fermentations in this project (0.1 mg/l to 10 mg/l) and freeze-dried for 48 – 72 hours. The solids obtained were then ground and diluted with KBr and analysed using the method described in Section 3.3.8.3.
3.3.9 Analysis of FTIR spectra

After analysing obtained samples using FTIR, the resultant spectra required processing in order to obtain the concentration of streptomycin, as the method analyses surface functional groups rather than streptomycin itself. Therefore, two results analysis procedures were used, a manual method using the subtract function included with the OMNIC software package (to subtract a background spectrum from one obtained from a sample), and an automatic method using a QBASIC program.

3.3.9.1 Manual Subtraction Method

It is important that care be taken when subtracting one spectrum from another in order to find peak information, as it is very easy to misinterpret data. OMNIC uses a subtraction factor to multiply one spectrum's magnitude by when subtracting it from another, and so, for example, even if a spectrum was subtracted from itself, by choosing the wrong subtraction factor one could create peaks and data where non existed. Discussion of this issue can be found in Section 3.7.4.

3.3.9.2 Automated Results Processing

Whilst the manual technique described above gave good results, it was very time consuming when large numbers of samples were being processed. A few short programs were written to cover a large part of the processing required, using the y-axis ranges of the two spectra to calculate the subtraction factor for the two. The codes for all programs can be found in Appendix B.

The discussion for each of these analytical techniques described above now follows.
3.3.10 Extraction and Analysis of streptomycin adsorbed onto chitin surfaces

As already alluded to above, it was necessary throughout the course of this work to determine the amounts of streptomycin adsorbed on the surface of the chitin fraction of fermentation broths.

Figure 3.12 shows the results obtained when streptomycin-laden chitin was contacted with buffers of different pHs for 24 hours and the resulting solutions analysed by bioassay.

![Figure 3.12 Effect of pH on streptomycin removal from chitin](image)

The results show that streptomycin is most readily desorbed from chitin surfaces in the pH range 3.0 to 7.0. It was decided to employ the lowest pH (3.0) in subsequent analyses in desorbing the antibiotic from fermentation samples.

Jermini and Demain (1989) also used a pH of 3 to extract cephalosporins from wheat and barley after solid-state fermentation of *S. clavuligerus*, although the time used by these authors to extract the antibiotic was much shorter than that used here – 60 minutes compared with 24 hours. Jermini and Demain used a three stage extraction as opposed to the one used here, but the quantities recovered were of the same order of magnitude as those involved here. However,
they did not investigate whether in-situ extraction was possible (e.g. such as that by Hernandez-Justiz et al., 1998); in the case of this work, the use of chitin solid provides the necessary two-phase system. More recent work involving chitin highlights the importance of pH in the attachment of large molecules such as reactive dyes. In this case, Klimiuk et al. (2003) showed that decreasing the pH of a dye solution led to an increase in dye absorption to the chitin surface.

Salisbury (1995) showed streptomycin recoveries of 50% from biomatrices using aqueous buffer, rising to 80 – 90% with additional steps for protein removal. It was estimated from FTIR and HPLC results (see Section 3.7.6 below) that most (> 90%) of the streptomycin adsorbed to chitin could be removed using pH 3 buffer wash in this work. However, the chitin used had already undergone protein removal prior to use in streptomycete fermentations.

It seems likely that the functional groups on the dye molecule are anionic and take place in ion exchange at the chitin surface due to the protonation of amino groups (-NH$_2$ → -NH$_3^+$) (Longhinotti et al., 1998). Streptomycin, however, commonly takes a cationic form (e.g. Streptomycin sulphate) and so adsorption will more likely occur when the amino group is deprotonated or through interaction with the OH or NHCOCH$_3$ functional groups through hydrogen bond formation, van der Waals forces or ion-exchange (Longhinotti et al., 1998).

### 3.4 Bioassay

Figure 3.13 shows the calibration curve of the diameter of the zone of inhibition of the growth of *B. subtilis* against streptomycin concentration. The curve was divided into two sections for the purposes of converting inhibition zone diameters into streptomycin titres. In the exponential region of the curve (section 1) concentrations as low as 0.1 mg/l could be determined whilst the linear range (section 2) covered titres ranging from 1.2 to 12 mg/l.
Figure 3.13 Streptomycin bioassay calibration curve. (For Section 1, $y = 1.554 \ln(x) + 16.177$; for Section 2, $y = 1.1489x + 15.191$)

The method used here was based on that of the Association of Official Analytical Chemists "Official Methods of Analysis" 16th Edition (1996), which also employs Bacillus subtilis. The only substantial modification employed here was that spores of B. subtilis were prepared by the method described by Gardner (1997).

Some preliminary experimentation was undertaken to determine both the optimum concentration of spores and the volume that it was necessary to seed the agar with. The spore concentration had to be such that the zones of inhibition could clearly be delineated from the background confluent growth. It was also necessary to ensure that the volume of spore suspension used to seed the top layer of agar should not be so great as to cause significant cooling of the agar whilst not so small as to lead to inaccuracies in routinely dispensing the spore suspension. With these various constraints, bioassays were performed using 20 μL of a spore suspension of $5 \times 10^6$ cfu/ml.
The agar cutter for wells described in Materials and Methods enabled multiple samples to be efficiently processed without undue delay.

Bioassay is an established method of measuring the concentration of substances, including antibiotics, which inhibit microbial growth (Behnisch et al., 2001; Osada et al., 1997). Its disadvantages include the lengthy protocols required for preparing the plates and the delay in obtaining results. Perhaps more importantly here however, is the criticism that the response parameter at the heart of the assay – the zone of inhibition of the test organism – may be affected by compounds other than streptomycin. Caldwell et al. (2000) show evidence of this while examining bioassay techniques with three microorganisms, including B. cereus, which was inhibited by all five antibiotics tested above levels of 0.5 μg / ml.

Evidence of the existence of one or more compounds produced during fermentation that are almost certainly not streptomycin is presented in Chapter 5. For this reason, when the bioassay was employed on fermentation samples concentrations are quoted as ‘streptomycin equivalent’

3.5 HPLC
Detection of streptomycin by HPLC would circumvent some of the difficulties referred to above in analysing samples by bioassay when there is potentially more than one inhibitory compound present. However, other researchers’ techniques involve the use of ion-pair chromatography, a method unavailable to this project, and so a method was developed to analyse streptomycin concentration in liquid phase using a standard UV/Vis detector and C18 column system.

Figure 3.15 shows the chromatogram obtained when streptomycete fermentation broth (chromatogram shown in Figure 3.14) was artificially augmented with sufficient streptomycin to yield a concentration of 10 mg/L in excess of any of
the antibiotic that may originally have been present. The eluent used comprising a mixture of acetonitrile and water (8:92). Whilst some separation has been achieved better results were obtained using 10 mM orthophosphoric acids as eluent (Figure 3.15).

The concentration of streptomycin used was considerably greater than that detectable using the bioassay. Reducing the concentration to 10 mg/L still gives a measurable peak but now interference from an unknown compound is making precise determination difficult.

![Figure 3.14](image1.png)

**Figure 3.14** Analysis of fermentation liquor dosed with 10 mg/L streptomycin using an Acetonitrile / Water eluent mixture

![Figure 3.15](image2.png)

**Figure 3.15** HPLC Analysis of fermentation liquor (additionally dosed with 10 mg/l streptomycin) using 10 mM orthophosphoric acid

Greater sensitivity was achieved by a combination of liquid-liquid and solid-phase extraction techniques.
Figure 3.16a shows that extraction using hexane gave an improvement at the lower concentration of 10 mg/L. Solid-phase extraction (SPE) using a C18 column increased the area of the streptomycin peak relative to the remaining eluted compounds (Figure 3.16b).

Figure 3.16 Results obtained from a) liquid-liquid extraction with hexane, b) using C18 column solid-phase extraction

Using both of these techniques consecutively, adequate peak resolution could be obtained whilst analysing fermentation broths containing 5 mg/l (Figure 3.17). A calibration curve for this method was produced using streptomycin standards (Figure 3.18 shows a standard chromatogram, Figure 3.19 the calibration curve obtained). The calibration is linear over the range of streptomycin concentrations used in this project.

Figure 3.17 HPLC Analysis of Fermentation Broth containing approximately 5 mg/L streptomycin (using combined liquid-liquid extraction and solid-phase extraction)
The methods developed here to quantify streptomycin by HPLC gave a lower limit of detection of approximately 5mg/L, which made it too insensitive to use routinely with fermentation samples generated during the course of this work. It is worth mentioning here that industrial streptomycin titres are very much higher than those reported here (by possibly 3 orders of magnitude) and that consequently the methods developed here would be readily applicable.

However, HPLC was used in instances where streptomycin was extracted from broths into e.g. buffers as was the case when analysing for antibiotic absorbed on to the surface of solid chitin. In such instances the absence of compounds eluting in close proximity to streptomycin provided verification for the FTIR measurements discussed below.

![Figure 3.18 Solution of streptomycin (0.1 mg/l concentration) in pH 7 buffer](image)
3.6 Micelle Electrokinetic Capillary Chromatography (MECC)

The two main obstacles to using HPLC was firstly its insensitivity to the relatively low concentrations of streptomycin routinely encountered in this work and secondly interference from other compounds present in fermentation broths.

In MECC analytes are absorbed either onto or inside micelles that are transported along a capillary column under the influence of a potential difference (Figure 3.20). The micelles are naturally electrically charged and are formed by adding increasing amounts of detergents until a critical micelle concentration is reached. This method, which started attracting attention in the last decade, has several advantages over HPLC. The main advantage with chromatography using electroosmotic (e.g. MECC) over pressure (e.g. HPLC) methods of motility is that the latter operates under a laminar flow system whereas the analytes move through the MECC capillary in plug flow, resulting in better resolution of peaks.
The addition of micelles into this system provides another method of selection in addition to charge alone by the creation of a pseudo-stationary phase (Figure 3.20). Lucas et al. (2003) have used this technique for the analysis of glycopeptide antibiotics.

An attempt was made to assemble an experimental unit for using this technique for streptomycin but there were no protocols available in the literature. Initial attempts to use a UV detector (Milton Roy spectroMonitor 3100, LDC Analytical, Riviera Beach, FL.) proved abortive as the cell through which the capillary column had to pass was of metallic construction and this severely limited the potential difference that could be applied. Eventually an alternative cell was constructed from Acetal copolymer (RS UK), however the UV signals obtained were too low to detect changes in the composition of the suspending fluid from a solution of detergent (sodium dodecyl sulphate) to methanol. Increasing the UV energy available could only be achieved by designing a system for focussing the UV using lenses. This was a highly specialised task and was therefore abandoned.

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- detergent molecule with hydrophilic head, Hydrophobic tail
- solute molecule

Figure 3.20 Principle of MECC
3.7 Use of FTIR to detect adsorbed streptomycin

It was predicted that streptomycin would adsorb to the surface of chitin particles during fermentation and the work discussed above confirmed that streptomycin adsorption did occur. The experiments in question showed that streptomycin could efficiently be desorbed into buffers of low pH from commercially purified chitin. However, chitin recovered from streptomycete fermentations, or purified using lactic acid bacteria, might well respond differently if other compounds from the diverse mixtures existing in fermentation broths became adsorbed onto the surface of the chitin. Investigations were therefore conducted into methods of directly determining streptomycin on the surface of chitin particles.

3.7.1 Transmission and Attenuated Total Reflectance (ATR) using chitin flakes

ATR is a non-destructive technique for analysing the surface of a material that is either too strongly absorbing or too thick for transmission spectroscopy. In transmission mode, infrared is passed perpendicularly through the sample and the transmitted radiation is measured. Whereas in ATR, the sample to be analysed is placed against the surface of a material with a high refractive index. The latter is referred to as an ‘internal reflection element’ (IRE) and the materials used typically comprise zinc selenide, germanium, silicon, sapphire, and KRS-5 (an alloy of TlBr and TlI).

The first method of analysis attempted here involved the use of chitin flakes (4 – 10 mm diameter) (crab shell chitin, Sigma-Aldrich) using both transmission and ATR methods of operation. Unfortunately, the chitin flakes were too thick to allow infrared radiation to be transmitted through them, and so this method of analysis was no longer pursued. ATR was then employed with these flakes using a germanium internal reflection element (IRE) by clamping an individual flake onto the surface of the IRE. Although it was possible to obtain a signal by this method, the magnitude and reproducibility were very low as the surface of the chitin flake was too rough and uneven. The resulting poor contact with the IRE
produced intermittent and unreliable interaction, and so a poor analytical response.

To improve the contact between the sample and IRE surface the largest (20 – 35 mm diameter), flattest flakes of chitin available were selected. Compressing these flakes between two microscope slides using G-clamps for 48 hours further flattened them. The thickness of the flakes was found to be ~ 200 μm using electronic callipers.

This was still considered to be too thick for transmission FTIR, and so ATR was again tested using attachment to a germanium IRE. Although the signal was an improvement on the previous tests, it was still insufficient to conduct proper functional group analysis on the flake surface. Although other modifications could be attempted, such as using a AgCl (a malleable, IR transparent material) plate or poorly IR-adsorbing liquid used between the sample and IRE to increase the optical contact (Ishida, 1987), it was deemed that these techniques would not improve the analysis sufficiently.

3.7.2 Film Production

2,4 L-diaminopimelic acid (L-DAP) with 5 % w/v LiCl was used to create chitin solutions of concentrations between 0.1 % and 1 % w/w as described by Austin (1988). Following dissolution, chitin films were produced by both casting and drawing techniques. Film casting involved pouring chitin solution into a glass mould as described by Domszy and Roberts (1985) and Miya et al. (1980). The second method consisted of drawing thin films on glass microscopy slides using K-bars (stainless steel bars covered with a mesh having pockets of known depth – in this project, 100 μm) (Tokura and Nishi, 1995).

Both casting and drawing produced patchy films that fragmented easily when floated off the glass slides. This was attributed to the large amounts of LiCl necessary to bring about the dissolution of chitin. Attempts to use lower LiCl concentrations were made but proved unsuccessful.
3.7.3 Use of Diffuse Reflectance with ground chitin.

Due to the difficulties of using natural chitin flakes or producing chitin films it was decided to try the diffuse reflectance (DRIFT) or Kubelka-Munk reflection method using ground chitin. DRIFT differs from ATR in that rather than flat surfaces being pressed against a reflective surface, the material being analysed is finely powdered and mixed with KBr. Reflectance from the analyte is scattered, and collected using mirrors before being sent to the detector. This method has been reported by Domszy and Roberts (1985) to provide a clear spectrum and to enable the degree of deacetylation for a chitin sample to be determined.

Commercial and pre-fermented chitins were ground and sieved to obtain a particle size less than 56 μm. When analysed these samples produced discrete peaks at 0.5 %w/w in 20 μm KBr. The resulting spectrum (see Section 3.7.4) for commercial chitin is very similar to those already published elsewhere (Tokura and Nishi, 1995; Duarte et al., 2002; Domszy and Roberts, 1985).

3.7.4 Interpretation of FTIR Spectra

Correlation charts are an essential tool for analysing FTIR spectra. They detail ranges at which certain functional groups can be found based on experimental data for their energies of movement.

A set of correlation charts (Williams and Fleming, 1989) was used in this project to analyse FTIR spectra. The streptomycin molecule contains a large number of amine groups (Figure 3.21) it was assumed that even if some of them were involved in the attachment of the molecule to the surface, a portion at least would be available for detection by FTIR.
The region of the IR spectrum between 3300 and 3500 cm\(^{-1}\) can contain peaks relating to N-H stretching. However, O-H groups in various forms are also located across this range and with a number of such groups also present in the streptomycin molecule, it was decided to use other responses to amine groups.

N-H bending responses occur at approximately 1600 – 1650 cm\(^{-1}\) for primary amine groups. Compared with the area described above, O-H groups are not present in this range, but instead create a response between 1300 – 1490 cm\(^{-1}\).
Figure 3.22 FTIR Spectrum of commercial chitin sample with adsorbed streptomycin. Key:

A - Possible peaks due to N-H stretching / O-H stretching
B - C-H bond stretching
C - CO₂
D - Amine groups
E - Amide groups

Figure 3.22 shows the FTIR spectrum obtained from a sample of commercial chitin with adsorbed streptomycin. Although samples were thoroughly dried prior to analysis, and the sample chamber purged with dry nitrogen, small amounts of water vapour were still present in samples. This effect can be seen in two areas on most of the FTIR spectra detailed, between 3600 and 4000 cm⁻¹, and between 1500 and 1900 cm⁻¹, creating a “signal-noise” effect. Certain features can be identified, such as triple peak present at 2900 cm⁻¹, almost certainly due to C-H bond stretching, the triple peak nature being due to variations in the bond caused by the atoms adjoining (showing the presence of C-H groups, with a variety of adjacent molecules). The double peak evident at 2350 cm⁻¹ in most of the results obtained is due to CO₂ present in the analysis chamber.

The region below 1400 cm⁻¹ generally contains much diagnostic information and is commonly referred to as the ‘fingerprint’ region. Analysis of peaks in this region is more complicated due to the large number of peaks and their relative
proximity to one another. However, Figure 3.22 clearly shows a peak at 1640 cm$^{-1}$, indicative of the presence of amine groups on the surface of the sample. The peak adjacent to it, at approximately 1575 cm$^{-1}$ is possibly caused by secondary amides. The bands between 1000 and 1300 cm$^{-1}$ are likely to be C-O bending responses, as the other main responses in this region according to correlation charts are those with sulphur present (showing the presence of C-O groups in different arrangements). Finally, the peaks below this value are almost certainly due to C-H groups, those occurring below 800 cm$^{-1}$ likely to be due to 4 or 5 adjacent groups in an aromatic form.

Samples of pre-fermented and commercial chitin were allowed to adsorb streptomycin by gently shaking them in streptomycin solution for 48 hours at 10°C. The chitin was then processed as described above, and analysed alongside chitin samples that had been shaken in buffer (Citric Acid - Phosphate, pH 7.0). Using the OMNIC software, the spectra for samples in the buffer were subtracted from those for samples shaken in streptomycin solution. Examples of spectra for this case are shown in Figure 3.23a and Figure 3.23b. A prominent peak was present at a wavenumber of approximately 1650 cm$^{-1}$ for both pre-fermented and commercial chitin samples, consistent with an increase in the number of amine groups. It was determined that using the ratio of the sample and control spectra y-axis ranges (minimum y-axis value subtracted from maximum) provided a good approximation for the subtraction factor used in this stage of the processing, and removed the human error possible with this stage (e.g. using an incorrect factor and producing peaks where none may exist).

Once a subtraction spectrum had been obtained, it was further processed in two stages. Firstly, a water vapour spectrum such as that found in Figure 3.23c was taken at the start of each experiment, and this was subtracted from the result spectrum in an effort to remove some of the noise present in the spectrum – unfortunately, a proportion of which occurred at wavenumbers around 1650 cm$^{-1}$ (where the amine band used for analysis also resided). Secondly, if noise was still present the curve was smoothed using the OMNIC software to assist in the visual identification of local minima to determine the boundaries for integration underneath the peak.
Figure 3.23 FTIR analysis steps – (a) commercial chitin with adsorbed streptomycin spectra (b) commercial chitin from buffer solution spectra (c) water vapour
Figure 3.24 shows the final spectra obtained for commercial and pre-fermented chitin samples with streptomycin adsorbed onto the surface.

Differences are exhibited between the two samples, for example, two sharp, clearly defined peaks are shown in the pre-fermented spectrum at 1050 and 1150 cm⁻¹.

* The amine peak response was used as the determinant of streptomycin concentration.
Streptomycin Production from chitin using *Streptomyces griseus* Richard Meanwell

While other, lower magnitude peaks are present in both samples around this area. These additional peaks could be due to the presence of C-O bending movements, however it is also possible that these two peaks are resulting from –SO and –SO₂ groups. However, this lay outside the scope of the investigation.

3.7.5 **Investigation into possible adsorption mechanism.**

The FTIR data showing the removal of streptomycin from the surface of pre-fermented chitin (confirmed by bioassay) was re-examined in order to determine if any particular functional groups could be identified as taking place in the adsorption, to assist identification of any mechanisms.

Spectra shown in Figure 3.25 and Figure 3.26 comprise spectra of streptomycin-impregnated chitin before and after streptomycin has been recovered from the surface using pH 3 buffer. Another method of visualising the differences between these spectra is to examine the subtraction spectrum between these two (showing the differences between the initial and post-recovery spectra, including what has been removed), shown in Figure 3.27.

![Figure 3.25 Spectrum of pre-fermented chitin with adsorbed streptomycin](image)
Streptomycin Production from chitin using *Streptomyces griseus*  

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Figure 3.26 Spectrum of above sample after streptomycin has been recovered from surface  
* The amine peak response was used as the determinant of streptomycin concentration

Figure 3.27 Subtraction spectrum of removed from initial sample (i.e. this spectrum shows what has been removed).
The large peaks at 1630, 1379 and 3290 cm\(^{-1}\) (Figure 3.27) can most likely be assigned -NH\(_2\), -O-H, and a combination both groups respectively, both prominent in the streptomycin molecule. The peaks between 2800 – 3000 cm\(^{-1}\) are various CH\(_3\) and CH\(_2\) groups, and the two large peaks between 1000 – 1200 cm\(^{-1}\) are C-O groups. As shown above in Figure 3.21, these are common groups to streptomycin (as well as chitin itself).

The presence of a peak at 1540 cm\(^{-1}\) shows the difference in amide between the two samples. This could be due to the removal of protein (as the pre-fermented chitin still contained some protein material), or perhaps a change in the number of groups analysed from the chitin molecule itself. However, if the acetyl groups on chitin were involved in substitution with streptomycin it is likely there would be a larger number of amide groups shown after recovery, and thus either no peak or a negative peak at this area of the spectrum.

Further work would be required in order to determine the mechanism of streptomycin adsorption onto chitin.

### 3.7.6 Quantification of Adsorbed Streptomycin

In order to determine if quantification of streptomycin adsorbed at the surface of chitin was possible, an experiment to obtain the adsorption isotherms for commercial and pre-fermented chitins was carried out. Rather higher concentrations of streptomycin were used than were typically obtained by fermentation for both pure chitin and fermented shrimp waste, to ensure data could be verified using the existing HPLC technique.

HPLC was used to determine the change in streptomycin concentration in the residual liquid over the course of the adsorption, as the difference between the initial and final values was used to calculate the amount of streptomycin adsorbed per µg of chitin. The 1650cm\(^{-1}\) peaks resultant after subtraction were integrated. The results are shown in Figure 3.28.
Figure 3.28 Example adsorption isotherm for streptomycin on commercial chitin. The calculated values were determined by analysing the residual streptomycin in the liquid phase using HPLC, and using a mass balance.

Figure 3.28 shows that the FTIR data matches closely with the calibrated HPLC method. Note that in some cases data calculated from HPLC / mass balance is not available. In the case of samples at 50 mg/L liquid concentration, the quantity of streptomycin adsorbed was too low compared to the residual liquid concentration to obtain a reliable mass balance. Whereas at lower liquid concentrations, calculated data is not available due to the residual levels of streptomycin being too low to analyse using HPLC.

It is interesting to note that the pre-fermented waste is capable of adsorbing much more streptomycin than commercial chitin. This could be due to a number of factors including surface morphology, degree of acetylation, impurities with different functional groups, etc. However investigation of the exact mechanisms were not investigated further.
As the adsorption isotherm data previously discussed obtained using a combination of FTIR and HPLC techniques was Langmuir in shape, 1/q vs 1/c was plotted for both pure chitin and fermented waste material (q = quantity of streptomycin adsorbed, c = concentration of streptomycin in liquid phase); the data calculated from these plots (Figure 3.29) are shown in Table 3.4.

![Figure 3.29 Langmuir constant determination for commercial (left) and pre-fermented chitins (right)](image)

<table>
<thead>
<tr>
<th></th>
<th>Commercial</th>
<th>Pre-fermented</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.275</td>
<td>0.540</td>
</tr>
<tr>
<td>q&lt;sub&gt;max&lt;/sub&gt;</td>
<td>16.6</td>
<td>51.8</td>
</tr>
</tbody>
</table>

Table 3.4 Langmuir Isotherm Constants

Figure 3.29 shows that the Langmuir model adequately describes the experimental data obtained here. Klimiuk et al. (2003) used a double Langmuir model to account for the adsorption of dyes onto chitin. They postulated two adsorption mechanisms - ion exchange and physical adsorption. However, there was insufficient experimental data to establish whether a double Langmuir model was more appropriate.

Further validation of the FTIR results was obtained by comparing data obtained by this method with bioassay of streptomycin recovered from the surface. A
calibration curve was also produced by comparing FTIR results with those obtained by mass balance via bioassay of recovered adsorbed streptomycin. Figure 3.30 shows the calibration curve obtained. The relationship between streptomycin adsorbed and FTIR response is linear over the range of concentrations relevant to this work.

The level of response achieved from the FTIR equipment varied slightly for each batch of experiments, primarily as the equipment was used for a number of different projects using other techniques (ATR and transmission) and the apparatus used for holding samples changed each time. Therefore, each time a batch of samples was analysed using FTIR, chitin samples with a known amount of streptomycin adsorbed were also analysed. It was assumed that the calibration would remain linear, and the response from the standard used to correct the responses obtained to take into account variations between analysis batches.

![Figure 3.30 FTIR Streptomycin Calibration using Diffuse-Reflectance (DRIFT)](image-url)
These two methods (FTIR and Bioassay of recovered adsorbed streptomycin) were compared by analysing shake flask fermentations of several different microorganisms (see Section 4.5.1 for experimental method), including known producers and non-producers of streptomycin (Figure 3.31).

![Figure 3.31 Comparison of extraction and bioassay with FTIR quantification methods. S. griseus (8136) was the organism used throughout most of this project. S. purpureus is a known producer of vancomycin, but not streptomycin. S. griseus (8237) is also a known producer of streptomycin, as well as other components. Isolates A and B were putative streptomycetes isolated from compost samples.](image)

The results show that the two methods provide comparable data. However, FTIR was subsequently used as the method of choice as it was more reproducible than extraction followed by bioassay for streptomycin adsorbed onto chitin, although bioassays were used to verify the results obtained from each fermentation. Note that there is some variation between the samples (i.e. for S. griseus (8136) the bioassay result is higher than FTIR, whereas the opposite is true for the isolates).
For the strain 8237 case there may be other components produced that inhibit bioassay, but do not produce significant response at 1650 cm\(^{-1}\) in FTIR, and to a smaller extent also for 8136. However, the differences seen between all the samples except for \textit{S. griseus} (8237) are within the experimental error for bioassay.

### 3.8 FTIR-based assays

#### 3.8.1 Determination of liquid-phase Streptomycin concentration

The relative ease with which adsorbed streptomycin could be determined using FTIR prompted research into whether the technique could be used to analyse streptomycin in liquid samples. Figure 3.32 shows the result obtained when an aqueous mixture of yeast and malt extract (used to simulate a fermentation broth) was doped with streptomycin then freeze dried and analysed by DRIFT FTIR.

The FTIR traces that resulted were fairly simple to analyse, partly due to relatively low interference (some water vapour only) around 1650 cm\(^{-1}\) wavenumber and the reproducibility of the Yeast-Malt background spectra. Each subtracted peak was integrated and a calibration plotted (Figure 3.33).
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Figure 3.32 Example FTIR spectra from a Yeast-Malt Extract freeze-dried sample

Figure 3.33 Calibration Plot for concentration of streptomycin in Yeast-Malt Extract using DRIFT

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As the calibration shows, the method is accurate over the range where most of the concentrations from the fermentations in this work occur. Authentic yeast extract- malt extract fermentation broths in which \textit{S. griseus} had been cultured were then analysed by this method and the results shown in Table 3.5.

<table>
<thead>
<tr>
<th></th>
<th>FTIR Titre (mg/l)</th>
<th>Bioassay Titre (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask 1</td>
<td>2.10</td>
<td>1.85</td>
</tr>
<tr>
<td>Flask 2</td>
<td>2.30</td>
<td>2.40</td>
</tr>
<tr>
<td>Flask 3</td>
<td>1.70</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Table 3.5 Yeast-Malt Shake flask streptomycin production - FTIR and Bioassay

Both techniques provide similar results, Bioassay and FTIR providing results of $2.07 \pm 0.275 \text{ mg/L}$ and $2.03 \pm 0.3 \text{ mg/L}$ respectively.

3.8.2 Direct screening for streptomycin production using FTIR screening method

Using the experience gained in FTIR from developing the techniques described above, two methods were devised and tested in order to produce a direct and rapid screening method for streptomycin-producing microorganisms that could be applied to the agar plate on which the organisms were growing. The first used a proprietary polyacrylamide gel used to aid water retention in horticulture (‘Water Gel’, Gardman Ltd., Moulton, Lincs. UK), which was deposited on the surface of an agar plate upon which an organism had been growing for between 24-48 hours, and left there for 24 hours at 4°C. The gel was then harvested, dried, ground and analysed by the DRIFT method. In the second method the agar on which the organism had been growing was simply collected, freed of microbial growth and then processed in exactly the same manner.
Both methods were tried on yeast-malt agar plates that had been doped with various quantities of streptomycin in the range 1 – 100 mg/l. The results (Figure 3.34 shows the FTIR spectrum, Figure 3.35 the calibration curve) show that both methods can successfully be used for detecting streptomycin. The dried agar method yielded samples with higher streptomycin concentrations reflecting the fact that streptomycin adsorption by the polyacrylamide gel was not total.

Figure 3.34 10 mg/L of streptomycin detected using Dried Agar screening method

Figure 3.35 Streptomycin recovery by the dried agar (X) and Polyacrylamide Gel Absorption. A, B, and C are three individual sample sets.
The dried agar technique has also been used on plates on which antibiotic-producing *Streptomyces* were growing, with results obtainable in many cases (Figure 3.34). Some samples, after drying, were covered in very fine particles; such samples did not produce adequate FTIR spectra. It is suspected that these fine particles were generated by the fragmentation of aerial mycelium during the drying process.

The results obtained here show that both of the two screening methods could be used commercially to identify high streptomycin producers. This could increase the efficiency by which such strains might be identified.

Whilst this method can isolate those organisms that potentially produce streptomycin, the production of this particular antibiotic cannot be confirmed using this method - further work is needed.
3.9 Conclusions

The bioassay technique based on inhibition of *B. subtilis* proved a most reliable and sensitive technique and was successfully employed for quantifying streptomycin in fermentation broths and also adsorbed streptomycin recovered from the surface of sold chitin particles.

Attempts to use HPLC as an analytical method proved successful in so far as operating conditions were identified for separating the streptomycin peak from the complex constituents of fermentation broths, but the sensitivity of the method was insufficient for the levels of streptomycin being produced here by *S. griseus*. However, the method holds considerable promise for industrial applications where over-producing streptomycetes are employed.

The promises offered by MECC could not be realised owing to equipment limitations.

FTIR methods were successfully developed and made an important contribution to the analyses conducted in this work. The techniques developed were further refined to yield a method for rapid screening of potential streptomycin producers.
4 Materials & Methods

The experimental methods and apparatus used during this project are described in detail in this chapter, both for generation and analysis of results. Where a method has been developed (e.g. FTIR) the progress is discussed in the Streptomycin Analysis chapter; however, the final method used is detailed here. The microorganisms and materials used in this project are given first.

4.1 Microorganisms

Three species of microorganisms were routinely used in this project.

**Lactobacillus paracasei**

The lactic acid bacterium used for the partial purification of chitin was *Lactobacillus paracasei* A3, isolated from whole frozen prawns (from Thailand and India) in the Food and Bioprocessing Laboratory at Loughborough University and preserved in freeze-dried ampoules prepared by the NCIMB (National Collection of Industrial and Marine Bacteria), Aberdeen.

**Streptomyces griseus**

*Streptomyces griseus griseus* (hereafter abbreviated to *S. griseus*) (NCIMB 8136) was purchased in lyophilised form from the NCIMB. This particular microorganism was chosen as it is listed as being Waksman's original chitinolytic strain as well as being the type strain for *S. griseus* streptomycin producers.

4.2 Materials

**Chitin**

Purified chitin (chemically purified from crab shell) was obtained from Sigma Aldrich PLC (Poole, Dorset, UK) in the form of flakes (hereafter denoted as commercial chitin). Fermented chitin was obtained using scampi (*Nephrops*...
norvegicus) waste (heads and claws) obtained from Norfish Ltd., Sunderland, minced and fermented in a horizontal rotating bioreactor (Section 4.4).

Colloidal chitin was obtained according to the method laid out by Hsu and Lockwood (1975). Commercial chitin was ground using a Waring blender, and dissolved in concentrated HCl by stirring for 1 hour. A colloidal suspension was obtained by adding this mixture to 2 litres of water at approximately 5°C. The suspension was collected by first allowing it to sediment followed by removal of supernatant. The collected suspension was repeatedly washed with water in order to restore the pH to 3.5. Each wash was carried out by adding approximately 2.5 litres of water to the sedimented colloidal chitin suspension and stirring with a glass rod. The colloidal chitin was allowed to settle over 24 hours, and the supernatant poured off before measuring the pH and repeating the process. The colloidal chitin was washed approximately 8 times per preparation.

The resulting suspension was vacuum filtered using a 90 mm diameter Whatman GF filter (0.4 μm) (Fisher Scientific UK, Leics.) and the solid centrifuged at 8600 g for 25 minutes. Chitin recovery was not as high as that claimed in the literature (85%), the best results obtained in this work gave approximately 75% recovery.

Microbiological Media

Commercial MRS broth (Oxoid, Basingstoke, Hants.) was used for the growth and maintenance of the Lactobacillus paracasei A3. Several growth media were used for Streptomyces griseus, using pure chitin, colloidal chitin, and fermented chitin. However, each broth / agar used an identical salts solution (Table 4.1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂H₂PO₄</td>
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</tr>
<tr>
<td>KH₂PO₄</td>
<td>300 mg/l</td>
</tr>
<tr>
<td>MgSO₄</td>
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</tr>
<tr>
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</tr>
<tr>
<td>ZnSO₄</td>
<td>1.8 mg/l</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>1.6 mg/l</td>
</tr>
</tbody>
</table>

Table 4.1 Salt Solution Medium
4.3 General Methods

4.3.1 Isolation and Preliminary Identification of Streptomyces

Several samples were obtained from compost heaps located around the local area. Small aliquots (approximately 1 g) of these were taken and added to quarter-strength Ringer’s solution in sterile 10 ml test-tubes. Serial dilutions were carried out, and 0.25 ml samples of these were spread on agar plates containing Hsu and Lockwood’s isolation colloidal chitin agar, and incubated at 25°C and 30°C for 4-5 days.

Chitinolytic organisms could be clearly identified by the clear zone that developed as a result of utilisation of the chitin in the agar, and several of these appeared to be Streptomyces-like due to “fairy-ring” colony formation. Further confirmation was obtained by looking for the presence of fine abundant aerial mycelium under a microscope. Putative Streptomyces were isolated and purified by streaking on agar plates. The organisms were stored on sterile earth.

Sterile earth was prepared by drying earth at 60°C for 12 hours, followed by autoclaving at 121°C for 30 minutes in a foil sealed glass beaker. The earth was allowed to stand for 24 hours in the beaker (to allow any spores present to develop) before re-autoclaving under the same conditions. A third autoclaving followed another 24 hour rest period.

The sterile earth was then transferred in a laminar flow cupboard to sterile Universal bottles. Isolates were grown in 500 ml Erlenmeyer flasks containing 100 ml of Yeast – Malt agar in an orbital shaking incubator at 28°C for 5 days. A 2 ml aliquot of each culture was then added to separate sterile earth Universal bottles and these were stored at 4°C.

These organisms were subsequently used in evaluating FTIR antibiotic assay techniques (Section 3.7).
4.4 Lactic Acid Fermentation

4.4.1 Horizontal Rotating Bioreactor

The bioreactor is shown in Figure 4.1. It consisted of a QVF Glass shell, 295 mm long and 155 mm diameter, with a stainless steel plate sealed with PTFE gaskets and bolted at each end. One of the end plates contained ports for air exchange (via a Dreschel bottle), a pH probe, and taking of samples.

Inside the glass outer shell, a stainless steel basket was fitted, mounted on a central axial shaft supported by PTFE bearings to allow the basket to freely rotate. The basket itself was approximately 230 mm in length and 125 mm in diameter, and was manufactured from a cylindrical frame covered with a double layer of mesh, all stainless steel.

The axial shaft was connected to a fixed speed electric motor capable of delivering 70 Nm (Newton-meters) of torque (equivalent to 52 foot-pounds), giving the basket a speed of rotation of approximately 20 rpm. The rotation was controlled using an electronic timer so that the basket would rotate for 10 minutes every six hours; conditions shown in previous work (Zakaria, 1998) to have resulted in satisfactory operation.

Figure 4.1 Horizontal Rotating Bioreactor
Feed Preparation

The feed for the batch fermenter consisted of 750 g of scampi waste, 75 g of anhydrous glucose, and 75 ml of *Lactobacillus paracasei* A3 inoculum ($10^8$ cfu/ml).

The frozen scampi waste was minced with a 6 mm hole-diameter plate using an industrial mincer (Hobart E4522, Southgate, London), followed by slight thawing using a microwave oven (Deltaware III, Toshiba, setting 3, for approximately 5 minutes). As soon as this stage was complete, the minced waste, glucose and inoculum were all promptly charged into the bioreactor to prevent spoilage.

Inoculum Preparation

In order to prepare the inoculum, 48 hours before the reaction a sterile loop of *Lactobacillus paracasei* A3 was taken from MRS agar slope and transferred into 3 ml of sterile MRS broth in a Universal bottle, and incubated at 30 °C for 24 hours.

An aliquot (1.5 ml) of this culture was then transferred to 75ml of MRS broth in a 250ml conical flask and incubated at 30 °C for 24 hours. Previous work (Zakaria, 1997) has shown that this method produces an inoculum with an approximate cell count of $10^8$ cfu/ml.

4.4.2 Batch Fermentation

The batch fermentations were set-up in the manner described in the previous sections, with the pH being continually recorded by plotter. The fermenter was operated for 48 hours, or until the pH started to rise above a value of approximately pH 6 after having fallen to around pH 5-5.5; pH being an indicator of the commencement of spoilage.
The bioreactor itself was housed inside an incubator to provide a constant temperature environment. The temperature for all experiments as part of this project was $30 \, ^\circ C \pm 3 \, ^\circ C$.

Once the experiment had concluded, and the bioreactor was removed from the enclosure, the liquid product was poured from the bioreactor into a container. The basket was then removed, and the sediment was scraped from the inside of the basket. Both products were then freeze-dried (taking approximately 24 hours). The liquid fraction was used in other work on fish feed supplementation; the freeze-dried sediment constituted the source of ‘pre-fermented chitin’ used in the *Streptomyces* experimentation.

### 4.5 Streptomyces Fermentation

#### 4.5.1 Shake Flask Experiments

A preliminary shake flask scale experiment was carried out to evaluate three forms of chitin (commercial, colloidal and pre-fermented) for streptomycin production, as well as to provide an indication of the growth kinetics that could be expected in later experimentation.

Six 250 ml conical flasks containing 75 ml of one of three chitin broths were sterilised by autoclaving; two flasks each were made up of broths containing commercial, colloidal, and pre-fermented chitin (compositions in Appendix 1). At the start of the experiment, each flask was inoculated with 0.25 ml of a 72 hour old culture. The flasks were then incubated in an orbital shaker operating at 100 rpm rotation, at 25 $^\circ C$.

Samples (0.15 ml) were taken at 24-hourly intervals from each flask in a laminar flow cupboard using a sterile pipette, and analysed by bioassay.

Another experiment was carried out to determine the effect of chitin concentration on antibiotic production in shake flasks, as a precursor to performing the same experiment on bench fermenter scale. Three 250 ml shake...
flasks of each of the following chitin concentrations were produced: 0.4 %, 5 %, 10 %, 15 %, and 20 %w/v. Flasks were placed in an orbital shaker operating at 150rpm for 21 days, temperature controlled at 28 deg C.

At the end of the fermentation period, the flasks were removed and liquid samples taken for bioassay analysis. Solid samples were then taken and subjected to the solids washing technique previously described, using pH 3 buffer, to determine the quantity of streptomycin adsorbed onto the chitin surface.

4.5.2 Batch Streptomycete Fermentation

A stirred bioreactor was used for the Streptomycete fermentation and consisted of a 2-litre glass vessel with a stainless steel head plate and sealed with an O-ring and gasket (Figure 4.2 shows the experimental arrangement). Several connections were available on the head plate, and were used for the following:

**Heat Exchanger:** A small heat exchanger was present as a reactor internal, with two connections for inlet and outlet on the head plate. These were connected to a peristaltic pump supplying chilled water to the heat exchanger upon start-up to more rapidly cool the fermenter after removal from the autoclave. This was to ensure that the inoculum could be added as quickly as possible to the reactor and reduce the chance of contamination.

**Air inlet and outlet:** The agitator shaft had an air inlet, passing to the base of the shaft and air was forced out through four small holes slightly below the lower of two Rushton Turbines. In later experiments a separate air inlet line was used. This comprised a stainless steel tube (3/8" diameter) passing to the base of the bioreactor and bent at 90° so the outlet was directly beneath the agitator.

**Agitator shaft:** Two Rushton turbines were originally used to provide mixing and to break up inlet air bubbles, one fixed at 30 mm from the base and the other at 110 mm). An alternative agitator was later designed; this is dealt with in Section 4.5.4 below.

**Fluid transfer ports:** Three ports were connected to the top of the reactor. The first was an inoculum line used at the start of the experiment to connect a flask (via steri-connectors) to the reactor vessel. This line was then blocked off by
folding over the silicon tube and clamping both sides of the fold together. Another inlet was used for make-up water, connected via another steri-connector to a 500 ml bottle with a stainless steel screw cap. The sample acquisition port had a long, wide bore (approx. 8 mm i.d. – to prevent blocking) tube connected to a sample fitting for bijou bottles and cotton-wool filled glass filter. Samples were taken by pinching the air outlet, which increased the air pressure in the headspace and forced liquid out of the sample line.

**pH measurement and control:** For the first, material comparison experiments (those carried out with 0.4 %w/v chitin concentration, and using Yeast-Malt medium), a pH probe was also fitted to the reactor vessel, and the signal connected to a pH controller. Two small peristaltic pumps supplied acid and alkali (usually 1 M HCl and NaOH respectively) to the reactor vessel via 1/8” silicon tubing (narrow to decrease the fill time). For these experiments, the pH was controlled at pH 8.

**Thermocouple and heater:** Two ports were used for heating the reaction vessel, one for an internal thermocouple, and the second for the heating element. Both were left in-situ, and with their cables being disconnected and the sockets well-protected using cotton wool and aluminium foil during autoclaving.

### 4.5.3 Conduct of Fermentation Experiments

Experiments were typically carried out over a period of approximately 2½ - 3 weeks (depending on the results of CO₂ measurements and bioassay readings) whilst maintaining a constant temperature of 28 °C, an agitation of 150-200 rpm and either controlling or monitoring pH. Gas samples were taken from the air outlet stream at least once / twice per day, as the profile required (i.e. if the CO₂ level was currently varying significantly, more CO₂ readings were taken), and liquid samples for bioassay were taken once per day. The liquid samples were also used for contamination checks; two plates each of Yeast – Malt Extract (YM) and Potato Dextrose (PDA) Agars were streaked with a flamed wire loop and incubated at 25 °C for 48 hours. The plates were then checked for evidence of contamination.
4.5.4 Modified Agitator Design

Preliminary studies revealed that at high chitin concentrations, significant sedimentation occurred. In order to counteract this Rushton impellers were replaced by a specifically designed U-shaped paddle (Figure 4.3). The bottom of the paddle was flat and sat very close to the bioreactor base. The vertical sections were also close to the wall, and the section in-between was rounded to accommodate the shape of the inside and to prevent scraping.
Using this agitator combination, much better mixing was obtained without using baffles and at a much lower agitation rate – 55 rpm instead of 150 – 200 rpm.

4.5.5 Solid State Fermentations

In all 4 experiments the same arrangement was employed. The reactor resembled a packed bed and comprised a 300ml volume glass column with a silicone rubber head and base liquid take-off (Figure 4.4). An air inlet was attached just above the liquid outlet via a T-piece built into the glass column base. Air exited via a filter from the silicone rubber head. A liquid sparge was installed just below the bung, and was used for liquid inlet; the sparge inlet was fixed through the head bung and was connected via a strainer (to avoid sparge blockage) to a recirculation pump taking liquid from the base of the column.

The solid used in the column for fermentation was supported with a three-layer sandwich of mesh and foam. The base layer was a coarse mesh dish, and was
used to fit snugly into the base of the column to provide support for the other layers and solid. The second layer consisted of a much finer stainless steel mesh to prevent, as far as possible, fines and other solid particulates from passing through into the liquid recirculation and blocking the sparge or its filter. The top layer, a 10-15 mm thick foam section, was used to assist the middle layer in preventing particles from entering the liquid base take off; although the gaps in the foam were larger than the particles, the action of particles passing through part of the foam helped prevent other particles doing the same.

Inoculum Preparation

The inoculum was prepared by using three shake flasks containing 100 ml of Yeast-Malt Extract broth having been inoculated with a wire loop from an agar slope and shaken in an orbital shaker for 96 hours at 100 rpm and 28 °C. All flasks were checked for sterility using optical microscopy before use. Two flasks were used for the inoculum as 200 ml were required.
Fermenter Assembly and Charge

The fermenter was autoclaved in sections (due to size restrictions in the autoclave and charging mechanism), and assembled in a laminar flow cupboard after the chitin and inoculum were added.

The charge was prepared in a large beaker that the chitin had been autoclaved in by adding the inoculum flasks and stirring with a sterile glass rod. Preliminary experimentation showed that an inoculum of 200 ml was rapidly adsorbed onto the chitin leaving little free liquid.

The thick slurry mix was then added to the fermenter column as quickly as possible, and the fermenter sections closed. The column was removed from the laminar flow cupboard and filtered airflow was commenced immediately to reduce the risk of contamination. Silicone sealant was added around the join between bung and glass column to aid the seal, as well as around the top of the dreschel bottle used for water saturation of air before entering the column. Any liquid collected at the base of the column was recirculated through the column immediately to assist in good mixing of solid and inoculum throughout the column.

Constant aeration was continued until the CO₂ analysis rose significantly enough to show that the Streptomyces had established itself on the chitin, at which point the aeration was set on an electronic timer for 10 minutes each hour to reduce water loss from the column. Liquid was recirculated when it collected at the column base, usually three times per day.

4.5.6 Vertical Basket Bioreactor

A new unit was designed where the overall chitin concentration would be similar to that in slurry fermentation (above), and that would take the advantages of both submerged (mixing) and solid-state fermentations. Preliminary experimentation showed that re-use of chitin in the packed column reactor was experimentally
Streptomycin Production from chitin using *Streptomyces griseus*  
Richard Meanwell

straightforward as the liquid and solid phases were easily separated. It was decided to contain all the chitin in a vertical basket allowing easy separation of liquid and solid as in the packed column fermenter, while also allowing liquid to interact and assist with mixing in the reactor.

The basket consisted of a stainless steel frame with wire mesh fixed around it in a cylinder. The central support for the basket was hollow, and was also used for air inlet via small holes in the base of the basket’s lower central column (Figure 4.5 shows basket construction in more detail).

![Vertical Basket Construction](image)

Figure 4.5 Vertical Basket Construction

The air inlet flowrate was increased to a point where the chitin contained in the column became gently fluidised to provide good mixing between it and the bulk liquid. Two additional air lines were used to admit air into the vessel outside of the basket to mix the liquid in the vessel adequately; the ratio of these three
(most of the air pushed through the basket to achieve fluidisation) was controlled using small screw clamps to provide pressure drop.

The other connections via the head plate were for air outlet (via filter) and to allow liquid transfer after fermentation. Fermentations were carried out consecutively, without removing the basket from the reactor.

The fermenter itself was positioned inside a larger beaker containing water being continuously recirculated from a heated water bath to maintain a constant temperature inside the fermenter. Liquid samples were taken from the fermenter once per day, and analysed using bioassay.

### 4.6 Scanning Electron Microscopy

Some investigative work was done using SEM (Scanning Electron Microscopy). A Cambridge Stereoscan unit was used, with the scan resolution varying from <5 μm to 200 μm. Most of the images obtained were taken from directly above the surface, all except one image of the edge of a chitin flake that was taken from a 45° angle.

Initially three different samples were used, with different amounts of *S. griseus* attached to the surface. In all cases, chitin broths using large flakes of pure chitin were autoclaved and inoculated with a wire loop of cells from an existing culture. Two flasks were left to grow in an orbital shaker, another in an incubator at the same temperature (25 °C). Fresh, sterile chitin was added to the two shaken flasks after three days of growth, one of which was then left in the incubator rather than shaken. All flasks were left for one more day before samples of chitin were taken, dried, and then prepared for SEM by Mr. Frank Page, IPTME, at Loughborough University, using a standard gold setting technique (which essentially involved setting a chitin flake onto a disc, and then sputtering gold onto the surface under vacuum conditions). Additional samples were later taken from normal submerged fermentations, as well as solid-state fermentations, and following drying, prepared in the same way described above.
4.7 Effect of shear forces on mycelial size and inoculum size on pelleting.

Two methods of optical microscopy were used for two separate investigations. Firstly, the effect of agitation and chitin concentration on the size of mycelia; samples for this experiment were obtained by agitating two *S. griseus* cultures at 200 rpm for 4 hours, one of which contained 3 %w/v chitin. The second experiment that optical microscopy was used as an analytical technique was the effect of streptomycete inoculum size on pelleting in liquid media (Section 4.5.1). In this experiment pellets were generated by inoculating flasks of Yeast-Malt Extract broth with varying quantities of *S. griseus*. Both methods used the same image analysis techniques, only the method of obtaining the image varied.

For the mycelia-size investigation, a Leica ATC 2000 (Leica, New York, U.S.A.) optical microscope with a video capture device (JVC TK-C1381, Radio Spares 260-4654) was used, connected to a thermal printer output. The requested image was examined either through the eye-pieces, or on a monitor located as part of the system. Once the image field was positioned correctly, the sample number was superimposed and the image printed. This printout was then scanned at a resolution of 200 dpi under 24-bit colour conditions, and converted to an 8-bit greyscale bitmap for use in Scion Image, free software obtained from ScionCorp.

An optical microscope with a video capture camera was also used for the pelleting investigation; however, in this case, the camera output was taken straight to PC and the image captured as a TWAIN source. The saved image was then also converted to an 8-bit greyscale bitmap for use in Scion Image.

Each greyscale image (example shown in Figure 4.6) was then opened in the Scion Image application, inverted (i.e. subtracting each bitmap value from 255 to obtain a “negative” - Figure 4.7), and then converted to a two-tone black and white image using an auto-threshold function. This function takes a value between 0 and 255 and converts everything above that value to white, and everything below to black. The image can then be processed (Figure 4.8).
Figure 4.6 Example 8-bit Greyscale image

Figure 4.7 Inverted Image
Once a threshold image was obtained, a particle size analysis could be performed on the image. The number of pixels that made up each particle was measured using the software, and the results presented. In the case of the mycelial size investigation, the results were then normalised to obtain a ratio of sizes in the various cases, whereas for the pelleting experiments a scale was obtained (170 pixels to 1 mm) and a particle size distribution calculated.
5 Fermentation Experiments for Streptomycin Production

5.1 Introduction

In this chapter are presented the results of all fermentation experiments conducted using S. griseus. This includes preliminary shake flask studies, conventional submerged fermentations and solid substrate fermentations. Also included here are experiments in which chitin was 'recycled' following fermentation and re-used in subsequent fermentations. Finally, results are presented on in-situ extraction of streptomycin.

5.2 Shake Flask Studies

5.2.1 Introduction

Shake flask experiments permit the investigation of a number of important fermentation parameters with relative ease compared to the use of fully instrumented bioreactors. This was particularly important here where fermentation times of 20 days or more were typical. On the other hand, shake flask experiments have inherent limitations; it is not possible for example to monitor CO₂ evolution. Notwithstanding, it was necessary to gain an early indication of the time course of chitin fermentation and whether the streptomycin produced could be detected using the techniques described in the previous chapter. The first series of experiments described here was conducted to determine the inoculum levels necessary to avoid pellet formation. Following this, an account of investigations into the fermentation of three different types of chitin is given. These were commercially available chitin, colloidal chitin and finally pre-fermented chitin. In the last series of experiments the effect of increasing the concentration of chitin up to 20 % w/v on streptomycin titres was investigated.
5.2.2 Effect of inoculation concentration on pellet formation

Many filamentous organisms have a tendency to form pellets during cultivation under certain conditions, this is largely undesirable as growth in pellet form generally results in decreased antibiotic titres (Vandamme, 1984). It was necessary to ensure that for the strain of *S. griseus* used here pellet formation could be avoided.

Six 100ml Yeast-Malt flasks of different inoculum levels ranging from 0.05 to 10 ml of starter spore solution were incubated in a shaker at 100 rpm and 28 degrees C. The starter spore solution contained approximately $10^6$ cfu/ml. Samples were taken from each flask after 2 days growth and deposited onto glass slides. Ten electronic images were taken of each sample using a microscope fitted with a camera and attached to a PC.

The resulting images were then analysed using Scion Image, a freeware port of the NIH image analysis program provided for Exceed systems, to obtain the area of each particle. Program filters were applied to eliminate both very large and very small particles, and then the results were processed using a macro written in ExcelVB to remove data not corresponding to particles.

5.2.2.1 Results

Figure 5.1 shows the results obtained at each inoculum concentration. The particle size distributions are relatively narrow for high inoculum concentrations but become broader as the inoculum size decreases. Figure 5.2 shows the average particle size plotted against the inoculum concentration and clearly reveals that average pellet diameter decreases with the size of the inoculum.
Figure 5.1 Particle Size Distribution of Pellets of *S. griseus* grown in shake flasks at different inoculum levels.
Figure 5.2 Average Particle Size for different inoculum *S. griseus* spore concentrations
5.2.2.2 Discussion
Pelletting occurs when the formation of secondary hyphae is suppressed due to the absence of critical nutrients (mainly salts), Whitaker (1992). The findings reported here - that the pellet size produced was inversely proportional to the inoculum size - are corroborated by earlier work performed by Vecht-Lifshitz et al., (1990) for *S. tendae*. However, these workers claimed that the smallest pellet size obtained was 1 mm in diameter (average particle size) at $10^7 - 10^{10}$ spores / m$^3$ which is larger than that obtained here (see Figure 5.2), but as Whitaker (1992) showed characteristic pellet size varies between streptomycete species.

5.2.3 Streptomyces Fermentation

5.2.3.1 Introduction
Experiments were performed using three different types of chitin. These were the solid, primarily chitinous, material harvested after fermenting shellfish waste (*Nethrops norvegicus*, Norwegian prawns) with the lactic acid bacterium *Lactobacillus paracasei* for 48 hours. Also included was colloidal chitin prepared as described in sub-section 4.2, and finally commercially available chitin (Sigma Chemicals). The concentration of chitin employed in all of these experiments was 0.4 wt %. Samples were taken periodically over a period of 21 days and analysed for streptomycin production by bioassay based on *Bacillus subtilis* inhibition.

5.2.3.2 Results
Figure 5.3 shows streptomycin titres obtained during the time course of fermentation for all three types of chitin. The pre-fermented chitin clearly resulted in the highest titre. The other two types of chitin yielded lower titres of streptomycin. However, fermentation of commercial chitin commenced only after a lag period of 4 days duration.
Figure 5.3 Shake Flask scale kinetic study for streptomycin concentration
Colloidal chitin produced a slightly higher yield of streptomycin than commercial chitin and this may have been the result of the greater specific surface area possessed by the colloid which might have facilitated assimilation by *S. griseus*.

The concentration of streptomycin achieved reached a plateau, before gradually decreasing at the end of the fermentation. This suggests there is an optimum fermentation time for harvesting for maximum streptomycin production, and that fermentations should not be continued past this point as this would result in lower final yields.

However, most significantly of all, all three curves are bi-phasic in nature. The first streptomycin peak occurring approximately 3 days after the fermentation had commenced, and the second, approximately 5 days after the first.

### 5.2.3.3 Discussion

The higher streptomycin yield achieved using pre-fermented chitin was probably due to the presence of nutrients such as amino acid residues in the fermented waste, which stimulated growth of *S. griseus*.

The long lag phase experienced with commercial chitin was unusual but is difficult to account for in the absence of further data.

The detection of streptomycin titre so early in the fermentation of all three types of chitin was unexpected. The initial peaks in Figure 5.3 occurred in the tropophase, i.e. in the phase of growth in which primary metabolism – growth – was taking place. Antibiotics are secondary metabolites and are produced in the idiophase when the growth rate has virtually decreased to zero (Barrios-Gonzalez and Mejia, 1996). This effectively rules out the possibility that the first peaks in the curves could not have been due to streptomycin. In order to utilise the chitin, *S. griseus* must elaborate chitinases (Berger and Reynolds, 1958). These will
result in the generation of chitin oligomers that will accumulate in the growth medium.

Jermini and Demain (1989) carried out similar shake flask experiments using barley for the production of cephamycin (an antibiotic) by *Streptomyces clavuligerus*. Their results show that the maximum yield was reached between 7 and 9 days after the start of fermentation. No tropophase peak was in evidence, however they used a different bioassay technique based on a super-sensitive (to beta-lactams) strain of *Escherichia coli*.

The inhibitory effects of chitin and chitosan towards micro-organisms are well documented. Most of the literature focuses on growth inhibitory effects of these compounds as potential food additives, or for crop protection and textile use (Kumar, 2000; Lim and Hudson, 2002). Kendra et al. (1989) showed that the minimum size of chitosan oligomers for inhibition was 7-8 monomers, and that oligomers of 4-6 monomers in length did not cause inhibition. An extensive literature search revealed that there were no specific references to the inhibition of the strain of *B. subtilis* used here. However, work exists to show the effects of the deacetylation of chitin on the inhibition of *B. subtilis* (Omura, Y. et al., 2003).

### 5.2.4 Inhibition by Chitin Oligomers

In order to establish whether the first peaks revealed in Figure 5.3 might have been caused by chitin oligomers rather than by streptomycin, a trial was conducted to determine whether chitin oligomers could inhibit the strain of *B. subtilis* used in the bioassay. An uncharacterised commercial preparation of chitin oligomers was used. This was a gift of AT Chemicals (Zhengzhou, China).

#### 5.2.4.1 Results

The results are shown in Figure 5.4 and reveal clear dose-related inhibition. Figure 5.4 shows that the magnitude of the chitosan oligomers' inhibition is of the same order of magnitude as those found in shake flask fermentation, although
the levels are lower. This difference could be due to two reasons. Firstly, that the inhibition showed is not solely due to the chitin oligomers, which due to the number of secondary metabolites streptomycetes produce, is evidently possible. However, the response achieved in bioassay for the shake flask kinetic experiment is higher than those received later in submerged fermentation. This aspect will be discussed later in Section 5.3.9.

5.2.4.2 Discussion

The results presented above provide strong evidence, but not proof, that the first peaks shown in Figure 5.3 were not due to streptomycin but resulted because of the accumulation of chitin oligomers. The second peaks are more compatible with streptomycin production in that they correspond to the idiophase.

No attempts were made to determine the concentration of chitin oligosaccharides produced during fermentation as this would have involved a complicated analytical procedure (Schussler et al., 1996).

Definite proof of the identity of the substance(s) causing the early phase inhibition of *B. subtilis* could only have been obtained by employing chemical analysis of the fermentation broths at times corresponding to the peaks seen in Figure 5.3. HPLC methods were originally developed in order to help resolve this issue, but unfortunately, the methods developed were only sensitive to streptomycin concentrations found at the end of fermentations, and therefore HPLC was redundant in this case. The possibility of using streptomycinases to destroy any streptomycin present in fermentation broths was investigated as potentially offering a solution to the difficulties described above, however these enzymes were not commercially available and there was very little information in the literature as to how streptomycinases might be produced and isolated.
Figure 5.4 Effect of chitosan oligosaccharides on bioassay
As a result of the findings from the experiment described above it was decided to filter samples to remove long chain lengths of chitin oligomers that might interfere with the bioassay. Samples were filtered using 2 kDa membranes, which were capable of retaining oligomers of chain length greater than 10.

As it was clear that streptomycin was not the only component inhibiting *B. subtilis*, all the titres obtained from bioassay are denoted 'Streptomycin-Equivalent', i.e. the level of streptomycin equivalent to the inhibition obtained during analysis.

5.2.5 Effect of Chitin Concentration on Streptomycin Titres.

5.2.5.1 Introduction

The final series of shake flask experiments were conducted to investigate the effects of chitin concentrations ranging from 0.4 to 20 % w/v on streptomycin titre. Samples were taken for analysis at the end of fermentation 21 days after inoculation. Both the solid and liquid fractions were assayed. The liquid fraction was assayed directly by bioassay whereas the solid material was first suspended in citrate buffer pH 3 as described in Section 3.3.3. This allowed any adsorbed streptomycin to migrate into the liquid phase from where it could be assayed.

5.2.5.2 Results

The first column for each set of results in Figure 5.5 shows the total amount of streptomycin produced per litre of fermentation charge, whilst the other two columns indicate the amount of streptomycin in the liquid phase and adsorbed onto the solid respectively.
The lowest concentration fermentation produced low amounts of streptomycin on the solid phase, simply because there was less solid for adsorption to occur onto. However, there is a maximum concentration of streptomycin achievable on the solid phase (at around 10% w/v).

The streptomycin titre fell at chitin concentrations above this value.

5.2.5.3 Discussion

The apparent maximum in streptomycin titre is interesting. If the possibility of some inhibitory compound present in the commercial chitin can be ruled out, then one possible explanation is that increasing the chitin concentration above a critical level resulted in damage to the growing filaments as a result of abrasive forces. Alternatively, the increased viscosity at the higher chitin concentrations might have resulted in a diminution of oxygen transfer rates leading to oxygen
limitation. It is not inconceivable that both mechanisms may have operated alongside one another. Investigations into the mechanism of filament damage are considered below in section 5.3.3.

5.3 Submerged Fermentation (SmF)

5.3.1 Introduction

The shake flask experiments were primarily useful in revealing the presence of oligomers of chitin that could interfere with the true determination of streptomycin titres. These preliminary experiments also provided some information on the effects of chitin concentration on streptomycin titres. However, it was difficult to achieve good mixing in shake flasks for chitin slurries above a concentration of 5% and moreover shake flasks do not permit control of e.g. pH to be maintained or the measurement of important fermentation parameters such as CO₂ evolution. Instrumented bioreactors were therefore used to conduct further investigations using chitin at various concentrations that are described in this section. The use of high concentrations of chitin required modifications to be made to the bioreactor and these too are described here.

5.3.2 Preliminary experiments

5.3.2.1 Introduction

In this section three fermentation experiments are described. One was conducted using a simple yeast-malt extract medium (i.e. without chitin) in order to establish whether CO₂ production could serve as an indicator of biomass. This was necessary because in experiments in which solid chitin was present it was not possible to make direct determinations of cell dry weight. In the other two experiments commercial and pre-fermented chitin were used.
In each experiment, the pH was controlled automatically at pH 8 and the air flow rate maintained at approximately 150 ml/min. CO₂ measurements were taken initially every 6 hours for the first 7 days, and every 12 hours thereafter. Liquid fermentation samples were taken daily, and analysed using the standard bioassay. Bioassay results are presented for both filtered and unfiltered samples.

5.3.2.2 Results

Figure 5.6 shows the biomass concentration and exit gas CO₂ concentration over time obtained using the yeast-malt extract medium.

![Graph](image)

**Figure 5.6 CO₂ Measurement compared with Biomass Measurement**

CO₂ concentration shows a very similar trend to the biomass curve. In Figure 5.7 the CO₂ concentration and the filtered and unfiltered bioassay results are plotted against time. The bioassay results for the filtered and unfiltered samples are remarkably close to one another as would be expected for a medium devoid of chitin. Interestingly however small peaks with maxima at approximately 40 hours are revealed from the bioassay results.
Figure 5.7 Yeast-Malt Extract SmF
Results for commercial chitin are shown in Figure 5.8. Streptomycin titres are higher than for the yeast-malt medium. The unfiltered samples reveal the characteristic bi-phasic nature but filtration of liquid samples essentially eliminates the first peak. Still higher titres were obtained when pre-fermented chitin was employed (Figure 5.9). As for the commercial chitin sample the first peak in apparent titre is eliminated by filtration. However the CO₂ profile reveals an unexpected peak at 100 hours into the fermentation.

5.3.2.3 Discussion

Figure 5.6 shows that exit gas CO₂ concentration is a good indicator of growth as determined by cell dry mass and on this basis was used as an indicator of biomass production in subsequent fermentations. CO₂ evolution was used as an indicator of growth throughout this work. Desgranges et al. (1991b) compared CO₂ concentration with other indicators of growth such as glucosamine and ergosterol for Beauveria bassiana. They found that CO₂ analysis most closely matched biomass production.

Woodruff and Ruger's (1948) early work with streptomycin production showed similar growth trends to those shown here. The lag phase was slightly longer, approximately 4 – 5 days; however, they were using different, glucose based media, and no information is available on the levels of inoculum used.

The data in Figure 5.4, when compared to most of the tropophase peaks obtained during submerged fermentation shows that the response from the bioassay is of the same order of magnitude as that from the chitosan oligomers, making it possible that it is these small fractions that are responsible for the initial bioassay peak.

Filtering the fermentation samples through 2 kDa membranes seem from Figure 5.8 and Figure 5.9 to have resulted in the elimination of the factors causing interference in the bioassay. Most significantly this had no effect on the yeast-malt extract medium (Figure 5.7). However this last figure reveals a small peak in apparent streptomycin titre at about 40 hours. This was smaller (0.90 mg/l)
than that obtained in the chitin fermentations, which were approximately 3.2 – 4 mg/l. This obviously cannot be attributed to chitin oligomers as no chitin was present.

Streptomycin production also followed a similar trend to that shown by Woodruff and Ruger (1948), in that a peak was reached (in this case, after 7 – 8 days) followed by a slight decline leading to a steady-state. No additional peaks were found in the tropophase.

Although a different microorganism was used for the production of neomycin, useful comparisons can be made with work by Ellaiah et al. (2004). Growth kinetics were not determined, however the antibiotic production again followed a similar trend, reaching a maximum after 10 days followed by a slight decline.

Arguments were presented above as to why inhibition of the assay organism so early in the fermentation could not be due to the presence of a secondary metabolite and these arguments are valid here. The possible identity of the compounds resulting in inhibition remain unknown but it seems certain that they must be of low molecular weight as they are not retained by the membranes.

The CO₂ trace for the pre-fermented chitin revealed a second peak at approximately 100 hours (Figure 5.9). The fermented chitinous shrimp waste contained substantial amounts of calcium carbonate (Zakaria, 1998) this might have become exposed as chitinous material was broken down and could have acted as a source of additional CO₂. Certainly the amount of acid (approximately 80 ml of 0.1M HCl) added to maintain the pH at a value of 7.2 was greater than was used for the commercial chitin fermentation (approximately 5 ml). If the CO₂ curve is modified to take into account that this acid could react with available calcium carbonate (still contained in the shrimp waste) in equimolar quantities, Figure 5.10 shows the result.
Figure 5.8 Commercial Chitin SmF
Figure 5.9 Pre-Fermented Sediment SmF
A significant amount (75 ml) of 1 M HCl was added to the fermentation over the 24-hour period approximately either side of the 100-hour mark. If it is assumed that this acid liberates CO$_2$ from CaCO$_3$ present in the fermented chitin, evolved over the 24-hour period, the modified CO$_2$ production line is the result. The CO$_2$ trace adjusted in this way is shown in Figure 5.10 and shows a pattern more consistent with the other two fermentations (Figure 5.7 and Figure 5.8).

### 5.3.3 Agitation

#### 5.3.3.1 Introduction

Although the pre-fermented chitin resulted in higher streptomycin titres, it could not be conveniently produced and therefore commercial chitin was used as the source of chitin in subsequent fermentations. However, a further experiment with pre-fermented chitin was conducted using a packed bed reactor (see section 5.4)
In this section the effects of increasing the concentration of chitin on streptomycin titre was investigated.

5.3.3.2 Results

The results achieved with 3% chitin are shown in Figure 5.11. This reveals a higher streptomycin titre than the previous fermentations. The results for 5% are shown in Figure 5.12. An unusually long lag phase of almost 400 hours duration occurred. In addition to a rapid decrease in CO₂ evolution almost immediately after the start of the experiment, Figure 5.12 shows that the fermentation took over two weeks to achieve significant growth or streptomycin production. It was observed during fermentation that at this chitin concentration mixing was inefficient and that chitin was settling to the bottom of the bioreactor.

5.3.3.3 Discussion

The results obtained here suggest that for chitin concentrations above 3% difficulties were encountered. Two potential possibilities were considered.

Firstly, the increased viscosity of the chitin suspension resulted in poor mixing. The small diameter Rushton turbines supplied with the vessel for submerged fermentations are designed for efficient oxygen dispersion in media not containing insoluble solids. Secondly, the high rotational speed of the Rushton turbine required to achieve adequate mixing coupled with the increased chitin content of the medium may have resulted in damage to the growing *S. griseus* by some sort of attritional mechanism.
Figure 5.11 3 % w/v chitin concentration submerged fermentation
Figure 5.12 5% w/v chitin submerged fermentation
Moreover, on dismantling the bioreactor at the termination of experiments it was evident that some chitin had been trapped in-between the baffles and the reactor glass wall, and fairly heavy growth could be seen to have occurred on this material. This suggested that growth was occurring predominantly where shear forces were low.

5.3.4 Effect of Agitation and Presence of Chitin on Streptomycete length

5.3.4.1 Introduction

It was necessary to establish whether reduced streptomycin yields and long lag times seen in Figure 5.12 were the result of damage to the organism and not caused by other unidentified factors. In order to do this *S. griseus* was grown in a shake flask for 96 hours under standard conditions (see Methods Section). The biomass was gently filtered and dispensed into two vessels; one containing buffer at pH 7.2 and the other buffer and commercial chitin at a concentration of 0.4 % w/v. These were stirred for 30 mins at 200 rpm and the size of the mycelium determined by depositing samples onto a glass slide and obtaining digital images that were analysed using Scion Image Analysis (Frederick, Maryland, USA).

5.3.4.2 Results

A characteristic “hyphal length”, which was defined as the image area of a *Streptomycete* chain divided by the average *Streptomycete* filament thickness, was obtained using the image analysis software. These results were averaged over several samples (approximately 5 per case), and normalised to provide size ratios (Figure 5.13).
Figure 5.13 shows that agitation at 200 rpm – that employed in previous experiments - can result in a reduction in the size of filaments, this effect is more pronounced when chitin was added.

5.3.4.3 Discussion

These results suggest that at under the conditions employed in the fermentation with 5 % chitin (Figure 5.12) considerable damage was being done to streptomycete filaments in suspension and that the lower yields and delayed growth was probably the result of slow rate of growth occurring on chitin that had settled out of suspension and that received an inadequate supply of oxygen.

These results revealed the inadequacy of the existing agitator and highlighted a need for a design that maintained the chitin in suspension, dispersed oxygen adequately and did not damage the streptomycete filaments.
5.3.5 U-shaped Agitator design development

5.3.5.1 Introduction

As discussed above (Section 5.3.3.3), the original Rushton turbine arrangement provided insufficient mixing at chitin concentrations greater than 5 % w/v and caused damage to the growing streptomycete filaments.

It was decided to use an impeller configuration that comprised surfaces having a relatively large surface area and operating at a lower speed to maintain the chitin and biomass in suspension without causing high hydrodynamic and other shears to be generated. Moreover, the impeller had to feature a means of both scouring the bottom of the bioreactor to rapidly resuspend solids that settled there and preventing the aggregation of solids forming between the vessel wall and internals. These requirements are theoretically met by the U-shaped paddle (Figure 5.14a). The length of each vertical side section was set to be as long as possible without breaking the liquid surface and thereby generating foam.

5.3.5.2 Results And Discussion

Initial trials of the U-shaped agitator were made using suspensions of chitin in water with the agitator rotation speed reduced from 250-300 rpm to 100 rpm.

Although a substantial improvement on the Rushton turbine, mixing in the vicinity of the centre of the bioreactor was incomplete, and some material still collected around the impeller shaft. A second smaller U-shape agitator was added to the shaft above the first one but it had a tendency to foul the bioreactor internals (Figure 5.14b). Finally, the smaller U-shaped paddle was replaced by a Rushton turbine (Figure 4.3). This resulted in good overall mixing throughout the bioreactor at the low rotational speed of 55 rpm.
Figure 5.14 Agitator designs. a) U-shaped agitator, b) double U-shape

The U-shaped agitator was used for all subsequent experiments.
5.3.6 U-Shaped Agitator comparison with Rushton Turbine

5.3.6.1 Introduction

In order to test the new design of impeller under fermentation conditions an experiment was conducted at a chitin concentration previously investigated (5 \% w/v – Figure 5.15) in which the means of agitation were provided by Rushton impellers. Previous experiments had revealed that only very low quantities of alkali were necessary to maintain culture pH and this suggested that pH control was essentially redundant, consequently in these and subsequent fermentations pH control was dispensed with.

The first experiment carried out was to re-run the 5 \% w/v chitin concentration fermentation to determine if the agitator had made any improvement. Figure 5.15 shows that significant growth occurs much faster than with the previous agitation method, and therefore also produces increases in equivalent titres much more rapidly (after 140 hours instead of almost 400), although the levels of those titres are approximately the same.

As shown in Figure 5.15, the new agitator not only improved the rate of growth in the fermenter, but also reduced the duration of the lag phase. Figure 5.16a and Figure 5.16b show SEMs of solid material recovered at the termination of 5 \% fermentation. Figure 5.16a reveals that a network of vegetative mycelium, with significant production of aerial mycelium, covers the entire surface of the chitin particle. Figure 5.16b was taken at an angle of 45° to the chitin surface, and shows more clearly the aerial mycelium produced.
Figure 5.15 5% w/v Chitin SmF using U-shaped agitator
5.3.6.2 Discussion

The new impeller configuration was a substantial improvement over the previous one that employed Rushton turbines. In particular, no settling of solids or aggregation of solids was observed at a chitin concentration of 5% and it is suggested that the low rotational speed did not damage the growing streptomycete filaments.

A direct comparison between the CO₂ levels measured in the headspace throughout each fermentation (for biomass determination) is shown in Figure 5.17.

Culture pH remained surprisingly constant and justified the decision not to employ pH control. This is in contrast to Woodruff and Ruger (1948) where fermentation pH rose from 6 to 9 over the course of the fermentation. However, in that case, the medium used was glucose based, whereas the medium used in this project contained a weak phosphate buffer as well as chitin, which can itself act as a buffer.
5.3.7 Repeated Fermentation of Chitin

5.3.7.1 Introduction

With the problem caused by the settling out of suspension of high concentrations of chitin having been solved, it was decided to investigate whether at the end of a fermentation experiment the chitinous solids could be separated from the broth and adsorbed streptomycin could be recovered before re-using the solids in subsequent fermentations. The observation that streptomycin adsorbed to the surface of chitin suggested the possibility that the antibiotic might be extracted in-situ i.e. during the course of the fermentation. In order to test the feasibility of this approach a series of experiments was conducted in which at the end of the fermentation the solid chitinous material was separated from the liquid broth and the adsorbed streptomycin recovered as detailed in Section 3.3.10. Following extraction, the fermentation solids were sterilized and incorporated into a second fermentation experiment. This was repeated a further time.
These experiments were performed with chitin concentrations of 7.5 and 10 % w/v.

5.3.7.2 Results

Figure 5.18 – Figure 5.20 are for 7.5 % w/v and Figure 5.21 – Figure 5.23 are for 10 % w/v chitin. A similar trend is followed by each fermentation to those displayed previously.

Figure 5.24 compares CO2 profiles for all three fermentations at 7.5 % w/v. It is evident that each time the chitin is reused there is a reduction in the time required to achieve maximum growth in the bioreactor. Figure 5.25 and Figure 5.26 compares filtered SETs and shows that the titres are comparable between fermentations at the same chitin concentration, reaching similar maximum yields at similar times. These two figures also show that the streptomycin yields are generally slightly higher for the 7.5 % w/v experiments than those in the 10 % experiments.
Figure 5.18 7.5 % w/v chitin submerged fermentation
Figure 5.19 7.5 % w/v chitin submerged fermentation, second experiment
Figure 5.20 7.5 % w/v chitin submerged fermentation, third experiment
Figure 5.21 10 % w/v chitin submerged fermentation, first experiment
Figure 5.22 10 % w/v chitin submerged fermentation, second experiment
Figure 5.23 10 % w/v chitin submerged fermentation, third experiment
Streptomycin Production from chitin using *Streptomyces griseus*

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1.6

1.4

1.2

0

0.8

0.6

0.4

0.2

0

0 100 200 300 400 500 600

Time (hours)

CO₂ Respiration Rate (mmol/l)

--- 7.5% w/v First Expt
--- 7.5% w/v Second Expt
--- 7.5% w/v Third Expt

**Figure 5.24** Subsequent experiments using the same chitin charge

8

7

6

5

4

3

2

1

0

0 100 200 300 400 500 600

Time (hours)

Streptomycin Equivalent Titres for repeated fermentations at 7.5 % w/v approx. chitin

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Streptomycin Production from chitin using *Streptomyces griseus*  

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Figure 5.26 Streptomycin Equivalent Titres for repeated fermentations at 10 % w/v approx. chitin

Figure 5.27 Effect of repeated fermentation (at 7.5 % w/v approx) on the particle size distribution of chitin
Figure 5.27 shows the particle size distribution of solid material for each fermentation. From this data the Sauter Mean particle diameter was obtained. This decreased from 880 microns in the first fermentation to 280 microns at the end of the third fermentation. In addition the quantity of the “fines” i.e. particles below 120 μm in diameter, increases dramatically (a fraction of approximately 0.28 in the first fermentation, increasing to 0.55 in the third) resulting in a larger surface area to volume ratio for the solid in the fermentation. This might explain both the reduction in the lag phase and in the time taken for maximum growth to be attained.

5.3.8 Effect of Chitin Concentration on Streptomycin Yield

The streptomycin yields of all experiments were collated and analysed to discover any trends in the data, and parallels that could be drawn with the experiment carried out using shake flasks. Streptomycin titres from all of the 9 submerged fermentations conducted are summarised in Figure 5.28.
Streptomycin Production from chitin using *Streptomyces griseus* Richard Meanwell

**Figure 5.28** Effect of chitin concentration on streptomycin production in submerged fermentation

With the exception of the experiment conducted at 3 % w/v, total streptomycin yields (i.e. yield in the liquid + yield recovered from the chitin) do not appear to be significantly different. However, the solid streptomycin yield increases with chitin concentration.

The 3 % w/v chitin fermentation was anomalous for two reasons. First, although the change in the liquid yield is minimal, the increase in solid-phase production is significant – with error bars can be seen that there is a definite upward trend, which is expected with increasing solid concentration (up to a point). Secondly, all experiments carried out followed a similar trend with regard to shape of growth curve, transfer from tropo- to idio-phase, etc. except the 3 % experiment.

The submerged fermentation data follows the same trend as that found for the shake flasks (Figure 5.5).
Butler et al. (1996) carried out work using interactive continuous selection to achieve increased streptomycin yields produced by *S. griseus*. CO₂ production was in the same order of magnitude as the experiments conducted here (approximately 3.5 g/l), although the CO₂ level in the fermenter was controlled. Initial streptomycin production was of the same order also (approximately 15 mg/l), although this quickly increased during the selection process.

Of particular interest, however, are the trends seen in streptomycin production. All the strains, both original and mutated used in Butler et al.’s work, achieve greater streptomycin concentration with decreasing growth rate i.e. toward the idiophase. In addition, the yield of antibiotic follows the same trend as that found in this project; a delay period during the *S. griseus* growth phase, followed by a steady increase in streptomycin, finishing in nearly all cases with a gradual decline. Butler et al. make no mention of the decline phase.

Ellaiah et al. (2004) also saw a maximum yield of antibiotic production when using solid state experimentation – in their case, at an initial moisture content of 80%.

### 5.3.9 Inhibition Effect of Chitin Oligomers

By examining the various figures obtained from submerged fermentation experiments, two general trends are evident. Firstly, that there is an initial peak produced during the tropophase of each experiment, the concentration of which varies between 0.2 and 1 mg/L. Secondly, there is a difference between the filtered and unfiltered streptomycin equivalent titres obtained at the start of the idiophase. This difference rises from approximately 1.5 – 2 mg/L for 3 % and 5 % w/v chitin experiments to 3 – 4 mg/L for those between 7.5 % and 10 % w/v chitin.

Examination of Figure 5.4 shows that these latter results obtained during the idiophase are of a very similar magnitude to the inhibition obtained during bioassay for solutions of chitosan oligomers. While it is not possible that all of the chitin in the fermentation has been reduced to oligomers of chain lengths less
than 10, the particle size analysis results show that the mean particle size of the chitin is significantly reduced during fermentation, and that the amount of fine material also increases significantly. This leads to the hypothesis that chitin oligomers could be at least partially responsible for the increased inhibition seen in unfiltered streptomycin bioassay titres.

The lower response at the start of the fermentation could be due to a lower presence of finer particles that can inhibit the bioassay, and the reduction showing that these smaller chain length oligomers are being more rapidly hydrolysed by biosynthesised chitinases than the larger chitin particles.

The previous shake flask experiment showed a much higher response in this area than the submerged fermentation. This could be due to two reasons. Firstly, the kinetic experiment was the first carried out for the streptomycete fermentations, and the bioassay method was still under the final stages of refinement – therefore, it is possible that the responses are artificially high due to the nature of this process. However, it is also possible that the production of streptomycin or other inhibiting compounds was generally higher in shake flasks than submerged fermentation.

5.4 Solid State Fermentation

5.4.1 Introduction

The advantages of solid state fermentations were discussed at length in Chapter 2 but briefly these were that capital costs are lower as the volume of reaction is much less (less liquid), and the extraction costs are likely to also be lower, again as the product is much more concentrated due to the smaller volume of fermentation required. Relatively simple media are often required, and as the fermentation conditions are likely to be much closer to those in the environment from which the microorganism was isolated, there is reduced potential for bacterial contamination (due to the low water activity).
Three solid-state bioreactors were designed, fabricated and used over the course of this work. The first was a simple packed bed reactor (Figure 5.29a). The second featured a squat cylindrical mesh basket rotating about the central axis (Figure 5.29b) which was continuously irrigated with liquid medium. The third bioreactor was similar in outward appearance to the first, packed bed reactor described above, but was operated differently in that both aeration and liquid distribution were discontinuous.

5.4.2 Results and Discussion

Preliminary experiments carried out using the packed column reactor showed that *S. griseus* was able to grow weakly on the chitin under solid state conditions. This was evidenced by CO$_2$ profiles (not shown here). However, there were a number of disadvantages with this particular design, the main one being poor liquid distribution over the solids in the column. Growth of *S. griseus* appeared to be limited to a region at or near the top of the chitin packing.

Operation of the second design of solid substrate fermenter was initially encouraging; good liquid distribution over the solids was achieved. However, as the substrate became wet, it made turning the basket more difficult, and occasionally rotation stopped completely. The large liquid surface area at the base of the reactor also made evaporation a serious problem and it became apparent that the substrate was drying out. Moreover, particulates in the liquid phase tended to partially block the distributor ring only allowing liquid through one or two holes that resulted in poor liquid distribution. The third bioreactor was designed on the basis of the experience gained with the first two. To prevent blockage of the liquid distributor the liquid was filtered through a layer of foam situated at the base of the chitin packing. In addition, the liquid was collected at the base of the column (below the air inlet point to reduce evaporation) and recirculated only periodically to further reduce instances of blockage of the distributor. The experience of the first two reactors was that continuous aeration resulted in the drying out of the substrate and that it was difficult to exactly control the rate of liquid addition to compensate for evaporative losses. Drying
out of the chitin was minimised by only continuously aerating the column during
the growth phase of *S. griseus* (as determined by CO\textsubscript{2} concentration) and
thereafter only aerating for 10 minutes in each hour.

All the experiments detailed below were carried out on the third, the packed bed
reactor with recirculation.

**Figure 5.29** Solid-State Fermenters. a) Packed column, b) Vertical rotating basket reactor
The results of four solid state fermentation experiments are recorded here. Although all experiments were performed in the packed bed reactor described above there were differences in the operating procedures adopted. The first two were operated with the same parameters, whereas the third and fourth used the standard submerged fermentation salts solution as the re-circulating liquid. Also, in the first and second fermentations continuous aeration was employed. The third fermentation replaced the recirculation liquid used in the first two fermentations (a Yeast-Malt Extract broth) with the standard salt solution used in submerged fermentation. The fourth fermentation also used pre-fermented chitin in place of commercial chitin.

Figure 5.30 shows exit gas CO₂ concentrations and it is evident that there is slow growth in the early stages of the fermentation. The first fermentation follows a slow progress throughout, whereas in the second fermentation the aeration was switched over to timed after approximately 240 hours, to reduce drying in the column.

Whilst the first two fermentations are fairly similar in trend and values, the third and particularly fourth achieve growth earlier. The standard salts solution contains essential components for the growth of *Streptomyces* as well as acting as a mild buffer. The pre-fermented chitin resulted in still more rapid growth and this confirms the findings made with this substrate in submerged fermentation.

The results for the antibiotic yields from each experiment (both in the liquid and recovered from the solid fraction) are found below (Table 5.1). The yields are similar in the liquid for each fermentation. However, the yield recovered from the solids fraction is much higher for the experiment in which pre-fermented chitin was used. This was expected, as the isotherm data previously obtained showed that for the same liquid concentration the fermented waste adsorbed much more streptomycin than commercial chitin.
Figure 5.30 CO₂ Production in solid state fermentation experiments
Figure 5.31 shows the solid-state fermentation experimental data, along with a calculated total streptomycin yield (mg/l).

![Figure 5.31 Streptomycin yields from solid state fermentation](image)

<table>
<thead>
<tr>
<th>Fermentation Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid Streptomycin titre (mg/l)</strong></td>
<td>2.2</td>
<td>2.05</td>
<td>2.4</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Solid Streptomycin titre (mg/l)</strong></td>
<td>1.80</td>
<td>1.48</td>
<td>2.18</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Table 5.1 Solid State Fermentation - Streptomycin Production

The overall yields for the solid state fermentations are relatively similar to those for submerged fermentation.

Yang *et al.* (1994) have carried out solid-state fermentations with similar equipment and conditions using *S. rimosus* on sweet potato residue, using slightly more solid (25 – 32 %w/w, as opposed to 15 – 20 %w/w in this project). It was found that aeration significantly improved antibiotic production (over non-
aeration) for a static column. This corresponds to the results obtained except in this project an increased response was found by aerating intermittently. Analysis of internal temperatures of the packed column may have provided more information to determine if this intermittent aeration was keeping the temperature in the middle of the column to adequate levels.

5.5 Vertical Basket Bioreactor

5.5.1 S. griseus Fermentations

5.5.1.1 Introduction

The ability to recover adsorbed streptomycin from chitin solid prompted research into the possibility of conducting in-situ extraction of the antibiotic. A rotating vertical basket bioreactor was constructed and used for this purpose (Figure 4.5). The bioreactor was designed so that the chitin contained in the basket would be gently fluidised. This, it was hoped would allow good oxygen transfer whilst minimising damage to the biomass. The first fermentation was conducted for approximately 500 hours, after this time the liquid fraction was drained off, and the streptomycin was extracted with pH 3 buffer. Following this the bioreactor was refilled with fresh medium and the fermentation was allowed to continue for a further 500 hours.

5.5.1.2 Results

As shown in Figure 5.32 and Figure 5.33, the rate of growth is slightly faster than submerged fermentation experiments carried out at 10 % w/v, reaching maximum growth at approximately 200 hours rather than 275 hours for submerged fermentation. This is probably due to the improved mixing characteristics of and lower shear stress existing inside the basket. A comparison of the two experiments also highlights a point of interest (Figure 5.34).
Figure 5.32 Vertical Rotating Basket Bioreactor – First Experiment
Figure 5.33 Vertical Rotating Basket Bioreactor – Second Experiment
Moreover, the growth rate – as judged by the CO\(_2\) profile - is higher in the second experiment. This was observed in previous submerged fermentation experiments (Figure 5.24), and might be due either to residual nutrients remaining on the chitin surface by previous experiments, or a decrease in the particle size. Unfortunately, the experimental set-up in this case did not allow for solid samples to be taken and analysed.

5.5.1.3 Discussion

The vertical basket bioreactor was successfully operated and permitted the extraction of streptomycin from the chitin.

The CO\(_2\) profiles display a steeper increase with time than was previously observed for submerged fermentation (Figure 5.21 shows those of similar concentration) results, and much faster than those for solid-state fermentation (although in this case the concentrations used of chitin were higher - see Figure
5.30). This indicates that a higher rate of growth of *S. griseus* was achieved in the vertical basket bioreactor.

Examination of the filtered streptomycin equivalent titre for the second experiment (Figure 5.33) also shows a slight peak before the change from tropophase to idiophase. However, in this case the peak is almost certainly due to the presence of small amounts of residual streptomycin present on the chitin surface after washing that has leached into the fermentation broth.

Jermini and Demain (1989) also used low pH buffer to extract cephalosporins from wheat and barley after solid-state fermentation by *S. clavuligerus*. However, they employed a different method of contacting the cereal grains with the buffer which comprised three extractions using 20 g of material in 60 ml of 0.1 M pH 3 phosphate buffer, agitated in a rotary shaker at 175 rpm for 60 minutes at 25 °C. This method was found to extract 93 % of the cephalosporin produced.

5.5.2 Fermentation Modelling / Prediction – Growth Kinetics

Attempts were made to model / predict both the biomass production and streptomycin production in submerged fermentations using *S. griseus*.

Three models were initially screened for the growth of *S. griseus* in submerged chitin-based fermentation: an exponential growth function, a second order reaction, and a cubic function. These were compared to an example fermentation (the first of three fermentations carried out at 7.5 %w/v chitin).
Chitinase production is stimulated in *S. griseus* fermentations that contain chitin. Chitinases (either exo- or endo-chitinases or both) split chitin chains into N-acetylglucosamine monomers, which can be absorbed through the *S. griseus* cell wall and used as nutrients. A standard second order reaction was derived from two assumptions: that the rate of chitinase production is proportional to the amount of biomass, and that the rate of biomass production is proportional to the amount of chitinase in the fermentation.

Marshall and Alexander (1960) produced a cubic model for the growth of dispersed mycelia in batch culture where substrates are in excess (Equation 5.1):

\[ M^3 = kt + M_0^3 \]  

(5.1)

where \( M \) and \( M_0 \) are amounts of biomass at time \( t \) and 0 respectively, and \( k \) is a constant.
The second order and cubic growth models both provided good estimations of biomass production in the submerged fermentations. It was decided when initial biomass concentrations (the concentration of biomass at the start of the experiment – from inoculation) were taken into account that the cubic function provided a better (lower difference between estimated and experimental values) across the range, particularly at higher values.

Pirt (1966) detailed the constant $k$ for pelleted growth, based on a growth rate and a spherical morphology. Of greater interest to this project, however, was the effect of subsequent runs on the chitin particle size distribution and growth kinetics. It was decided to modify the constant used in the cubic model to include the particle size data obtained, rather than assume uniform spheres as in Pirt’s pellet size function, to see if the model held for these subsequent fermentations.

The Sauter Mean Diameter (SMD, or $d_{3,2}$) was used for two reasons: ease of use in equations (one variable, rather than a summation) and that as previously discussed it is thought that the key reason for increased growth rates in subsequent fermentations is due to the increased surface area : volume ratio of the solid substrate. The constant $k$ was therefore modified as follows:

$$ k = \frac{\mu}{d_{3,2}} \quad (5.2) $$

where $\mu$ is a constant (similar to that used by Pirt (1966) – a specific growth rate – Pirt’s model is shown below (Equation 5.3), where $\mu$ is the specific growth rate, $w$ is the shell thickness, and $n$ the number of particles with density $\rho$ and radius $r$). Figure 5.36 shows the result of using this model on three subsequent fermentations where the particle size distribution was analysed, and shows that use of the SMD in this way provides a useful modification of the constant in Marshall and Alexander’s model to estimate the effect of particle size to distinguish fermentations.
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\[ k = \left( \frac{4}{3} \pi \rho n \right)^{\frac{1}{3}} \mu \nu \]  

(5.3)

The three fermentations taken are three fermentations carried out using the same chitin, at approximately 7.5 %w/v chitin. As the chitin is re-used in the subsequent experiments, the SMD decreases, which is taken into account in the constant \( k \) from Equation 5.2. As Figure 5.36 shows, even though the particle size distribution changes over the course of three experiments, the model still holds well for the fermentations once this is taken into account.

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**Figure 5.36** Three slurry fermentations at 7.5 %w/v chitin concentration (First Experiment: top right, Second: top left, Third: bottom).
5.6 Conclusions

Shake flask experiments were used to validate analytical methods previously described for determining streptomycin and to provide information about the length of time necessary to achieve maximum streptomycin yields. These experiments revealed that pre-fermented chitin produced approximately 3 times more streptomycin than commercial chitin. In addition it was established that the liquid-phase yield of streptomycin was at the maximum value after 8 days of fermentation. Shake flasks also provided a convenient way of establishing what the effect of chitin concentration on streptomycin titre were. Streptomycin yields ranging from 1.0 to 5.3 mg/l were achieved when S. griseus was grown in shake flasks. Maximum streptomycin concentration was achieved at a chitin concentration of 10 %w/v.

Successful fermentations were carried out using bioreactor equipped with Rushton turbines at low chitin concentrations. However, at concentrations above 5 % difficulties were encountered when the chitin was found to be settling out of suspension and very low streptomycin yields were obtained (< less than 1 mg/litre). Evidence was provided to show that low yields could result from damage to the Streptomycese filaments caused by high agitation rates in the presence of chitin. A U-shaped impeller was designed and fabricated and this enabled fermentations to be conducted at high chitin concentrations. Exit gas CO₂ measurements were shown to correspond to cell dry weight measurements in media not containing chitin and was used as a measure of cell growth throughout the work described here. Apparent streptomycin production early in the fermentation (during idiophase) could be explained by the presence of chitin oligomers that had an inhibitory effect on the bioassay organism (B. subtilis) similar to that of streptomycin. FTIR studies described in the previous chapter were able to confirm that the initial peak was not streptomycin. However, it was discovered that interference to the bioassay results were largely eliminated by filtering samples prior to assay through 2 kDa membrane filters. The levels of streptomycin produced under submerged fermentation conditions (2 to 3.5 mg/L) were of a similar order to those achieved using shake flasks.
Experiments were conducted to show that at the end of a fermentation streptomycin could be recovered from the solid material remaining by extraction into pH 3 buffer and, most significantly that the chitin could be re-used in subsequent fermentations. Under these conditions it was observed that the lag phase was shorter and the time taken for CO₂ measurements to reach their maximum was also reduced. Particle size analysis of solid samples from submerged fermentation showed that the Sauter Mean Diameter of the chitin used decreased during fermentation.

It was possible to model the growth curve for submerged fermentations using a modified version of Marshall and Alexander (1960)'s cubic model and Pirt (1966)'s constant to include the effect that subsequent fermentations had on the Sauter Mean Diameter.

Three configurations of solid substrate bioreactors were evaluated and a simple packed column with intermittent aeration and recirculation proved to be the most effective giving streptomycin yields of 3.8 mg/l.

The benefits of the packed column reactor were combined with those from submerged fermentation by developing a vertical basket reactor. This permitted ready extraction of the streptomycin adsorbed to the surface of chitin and continued fermentation without having to dismantle the fermenter vessel. Using this configuration streptomycin yields of 4.6 mg/L were achieved.

The issue of early streptomycin production was finally resolved after FTIR techniques had been developed for these fermentations. Samples of both solid chitin and freeze-dried liquid (from yeast-malt based fermentations) were taken at the time period specified by bioassay, and the spectra examined for any indications of streptomycin production. This is described in more detail in the previous chapter.
6 Conclusions and Suggestions for Future Work

6.1 Conclusions

The main conclusions arising from this work are listed below.

- A bioassay technique based on *B. subtilis* inhibition was used successfully for the detection and quantification of streptomycin in the liquid phase. This technique was also used to yield estimates of the amounts of streptomycin bound to the surface of chitin by first extracting the antibiotic using buffer at pH 3.

- A diffuse-reflectance method was successfully developed for the direct analysis of streptomycin on the surface of chitin using FTIR. This method was also modified to enable the rapid identification of putative streptomycin producers.

- An HPLC method of streptomycin analysis was developed. The method was considerably less involved than the standard HPLC method, however, it proved to be too insensitive to quantify the concentrations of streptomycin produced with the strain of *S. griseus* used here.

- Shake flask experiments were used to identify the conditions for streptomycin production from different forms of chitin using *S. griseus*.

- Submerged fermentation experiments were conducted in a conventional stirred bioreactor and showed that streptomycin was produced from commercial chitin at concentrations up to 5% wt.

- Submerged fermentation experiments revealed that at chitin concentrations above 5% w/v long lag phases and low streptomycin yields were obtained. It was suggested that at high solids concentrations damage to bacterial hyphae...
occurred and that this served to limit both the growth rate and entry into the idiophase. This was confirmed by tests in which the characteristic length of bacterial hyphae in the presence of a high concentration of chitin under agitated conditions was shown to be significantly smaller than controls.

- A novel agitator was designed to enable fermentation of chitin concentrations above 5 % wt. to be conducted in submerged fermentation. The agitator comprised a U-shaped element with a single Rushton impeller and was operated at a speed of 55 rpm.

- Experiments were conducted to show that the chitin remaining after submerged fermentation could be re-used in subsequent fermentations. Particle size analysis showed that the Sauter Mean Diameter of the chitin was decreased by the fermentation process. In subsequent fermentations the lag phase and the time taken to reach maximum growth were shorter.

- One or more compounds showing inhibition against Bacillus subtilis were identified in fermentation broths early in the tropophase during S. griseus fermentations. Secondary metabolism does not occur during the tropophase and the observed inhibition was therefore not due to streptomycin or to any related aminoglycoside antibiotics. This was confirmed by FTIR analysis. Filtering the fermentation broths with a 2 kDa membrane largely eliminated the source of inhibition in the tropophase but not in the idiophase and it is suggested that the compounds comprise oligomers of chitin. Evidence of the inhibitory effects of chitin oligomers on the strain of B. subtilis used here was obtained and reinforces this conjecture.

- An existing mathematical growth model was modified to incorporate the Sauter Mean Diameter of chitin particles and was used to describe the growth of S. griseus in submerged fermentation.

- The pre-fermented chitin material showed faster growth of S. griseus and greater secondary metabolite production than commercial chitins. One
possibility for this is the presence of additional phosphates on the chitin surface, which have been shown to have an effect on the rate of production of secondary metabolites.

- Experiments using a packed column bioreactor showed that production of streptomycin using S. griseus from chitin was possible under solid state conditions. It was found that the most effective operating regime for this column was intermittent circulation and aeration.

- A number of different configurations of solid state bioreactors were evaluated. The best results were achieved using a simple packed column that was operated with intermittent aeration and liquid circulation.

- A novel vertical basket reactor was designed to incorporate the benefits of both submerged and solid state fermentation. Using this arrangement it was possible to interrupt the fermentation to extract adsorbed streptomycin produced and then resume fermentation.

6.2 Suggestions for further work

Much work was conducted here on methods of assaying streptomycin. The most promising results were obtained using FTIR for the estimation of streptomycin adsorbed onto the surface of chitin. Suggestions were made earlier that this method might form the basis of a screening assay for streptomycin producers and it would be interesting to compare an FTIR-based method with the techniques currently employed in industry. Indeed, there is scope for developing FTIR methods for the rapid detection of organisms able to produce other antibiotics, particularly those that are difficult to assay by conventional methods.

Micelle Electrokinetic Capillary Chromatography was briefly investigated here as a method for assaying streptomycin. Although no headway was made with this particular technique, its potential advantages are so great as to merit further investigation in relation to streptomycin.
The ability of chitin to both serve as a substrate for *S. griseus* and absorb streptomycin is an unusual finding. It would be interesting to establish whether other antibiotics produced by the streptomycetes might bind with greater affinity to chitin and whether this might serve as a method of extraction.

The strain of *S. griseus* used here, although the type strain, was not a particularly good streptomycin producer. The highest yields of the antibiotic are produced by industrial strains that are not readily available to academic researchers. However, without data obtained using these strains it would be difficult to assess the potential of the vertical basket bioreactor developed here. One possible way forward might be to approach existing commercial producers and propose collaborative research.

The existing design of the vertical basket bioreactor only permitted the sampling of the liquid phase for the generation of kinetic data for streptomycin production. Some method of sampling the solid material inside the basket during operation is required. With such a method, the optimum times for terminating the fermentation phase and initiating extraction could be determined with greater certainty.

Experiments conducted here showed that it was possible to produce streptomycin at the laboratory scale using a simple packed bed bioreactor. As discussed above, a method of removing solid samples for analysis would have added greatly to the data obtained here. Whether such systems could ever be used industrially remains an uncertainty. There are obvious scale-up problems to be encountered and overcome but the system constitutes a ‘low-tech’ method of producing antibiotics that might be profitably exploited in developing countries.

Experiments should be carried out to identify the compounds causing interference to the streptomycin bioassay in the tropophase. It seems likely that they are oligomers of chitin and this could be confirmed by chromatographic analyses in future studies. If the compounds were found to be chitin oligomers use could be made of their antibiotic properties in a number of different areas. As
chitin is recognised as safe for food use there might be a number of potential applications related to foods. Oligomers could be produced by using mutant strains of streptomycetes that did not produce antibiotics.

Operating conditions were developed for isolating streptomycin from complex fermentation media in HPLC. However, the method was not sensitive enough to quantify streptomycin concentrations of those typically produced by *S. griseus* during this work. Further work would need to be carried out, either by refining solid-phase extraction and liquid-liquid extraction techniques or using a different detection system (e.g. ion-pair chromatography) in order to increase the sensitivity of this method.

Final identification of the inhibitor(s) has not been possible, and could be an area for further work.

Two areas for further work on this system have been identified. Firstly, to develop a method of solid sampling during experimentation, for the quantification of adsorbed streptomycin – something not possible during this project. Secondly, the logical development of this system would be to include an extraction technique whilst the fermentation is being carried out, to continuously remove streptomycin. Whether this would allow continuous production of streptomycin is thought worthy of investigation.

As the column was small in size it is believed that there were not any localised areas of excessive temperature, however if this method were to be scaled up, it is thought that this could be an issue. Further experimentation incorporating temperature sensors into the reactor body would identify any localisation issues.
7 References


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Caputo, A. (1976) *Biological Basis of Clinical Effect of Bleomycin (Contributions to Primatology)*, S. Karger AG, Switzerland.


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Appendix A - Lactic Acid Fermentation

Several lactic acid fermentations were carried out in order to produce sufficient fermented waste for *Streptomyces* based experimentation. The pH profile of each fermentation run was plotted (Figure A.1)

![Figure A.1 pH profiles of Lactic Acid Fermentation Runs](image)

The pH profiles of the lactic acid fermentation runs show that the experiments carried out were reproducible. Investigative work carried out part of another project (Baynes-Clarke and Taylor, 2001) showed that the chitin chain molecular weights were of the same order of magnitude as each other (and generally higher than chitin products produced by traditional chemical treatment methods).

The pH trend of the experiments shows the initial pH at approximately 8, dropping after a short delay period to between 4.5 and 5.5 over a 24-hour period. This period of rapid pH decrease is followed by a gentle rise in pH over the next 24-period until the fermentation is halted.

As the horizontal rotating bioreactor is operated with an air vent preventing significant carbon dioxide build-up the *Lactobacillus paracasei A3* (obligatory
heterofermentative lactic acid bacteria) used follows the heterolactic fermentation pentose phosphate pathway, where lactic acid is produced in a ratio of 1:1 stoichiometrically with glucose consumed; the rapid pH decrease shows that in this phase of the fermentation the glucose added as part of the fermentation charge is being converted to lactic acid. If it is assumed that the rate of lactic acid production is approximately proportional to the number of microorganisms present in the fermentation, by examining the (negative) gradient of the pH profile an approximate growth curve can be obtained. This gives the expected result, with a delay period initially followed by rapid growth and a stationary phase before the rate of cell lysis overtakes that of cell growth.

There are two possible explanations have been identified for the slight troughs that occur at the start of the fermentation runs. The amount of liquid present at the start of each fermentation is low, and the first possibility is simply that even though the pH probe is situated as close to the base of the fermentation as possible, there is insufficient liquid present to obtain accurate readings. The second possibility is that as more liquid is produced at the start of the fermentation without significant lactic acid production there is a dilution effect, pushing the pH toward neutral. As this trough is similarly evident in each fermentation, the second explanation of a dilution effect is considered the more likely.

The pH profile shown has been reproduced by other researchers (Shirai et al., 2001; Zakaria et al., 1998; Passos et al., 1994) although the pH analysis in the first stated case was done by taking samples and so the number of points shown is much lower. Zakaria et al. (1998) developed the fermenter on which the work in this project was based. However, she allowed the fermentations to continue beyond 48 hours and hence they show a much more rapid rise in pH due to the buffering capacity of solubilised calcium.

The fermented waste produced, once freeze dried and ground, was used throughout the project, including the fermentations using *Streptomyces*. 
Appendix B – FTIR Analysis Program Code

DECLARE SUB ftirbatch (bchin$, bchbg$, bchop$, area!)  
DECLARE SUB csvtest (f$)
CLS

' *****************************************  
' * Dimension required variables *  
' *****************************************

aa = 3500  
DIM FILEVAL!(aa, 2)  
DIM BGVAL!(aa, 2)  
DIM OUTVAL(aa, 2)  
DIM PEAK(aa, 2)  
DIM AREAVAL(100)

' *****************************************  
' * Program front end *  
' *****************************************

PRINT "**************************************************************************"  
PRINT "* FTIR Spectra Analysis *"  
PRINT "**************************************************************************"  
PRINT PRINT "Please choose whether you would like to:"  
PRINT "1. Run the program as normal"  
PRINT "2. Run a batch file series"  
INPUT "Selection: ", a  
IF a = 2 THEN GOTO batch

Normal:  

INPUT "Please enter sample filename (return for default)? ", filename$  
IF filename$ = "" THEN filename$ = "P:\a6.csv"  
CALL csvtest(filename$)  
INPUT "Please enter background filename (return for default)? ", bgname$
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```plaintext
IF bgname$ = "" THEN bgname$ = "P:\ac.csv"
CALL csvtest(bgname$)
INPUT "Please enter output filename (return for default)? ", output$
IF output$ = "" THEN output$ = "P:\default.csv"
CALL csvtest(output$)

C = 0
YMAX1 = -99999
YMIN1 = 99999
YMAX2 = -99999
YMIN2 = 99999

' **************************
' * Access sample input file *
' **************************

PRINT "Opening sample file "; filename$
OPEN filename$ FOR INPUT AS #1
FOR X = 1 TO 3476
    FOR Y = 1 TO 2
        INPUT #1, RANDER
        FILEVAL!(X, Y) = RANDER
        NEXT Y
    IF FILEVAL!(X, 2) > YMAX1 THEN YMAX1 = FILEVAL!(X, 2)
    IF FILEVAL!(X, 2) < YMIN1 THEN YMIN1 = FILEVAL!(X, 2)
NEXT X
'    PRINT YMIN1, YMAX1
CLOSE #1

' **************************
' * Access background file *
' **************************

PRINT "Opening background file "; bgname$
OPEN bgname$ FOR INPUT AS #2
FOR X = 1 TO 3476
    FOR Y = 1 TO 2
        INPUT #2, RANDER
        BGVAL!(X, Y) = RANDER
```
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NEXT Y
    IF BGVAL!(X, 2) > YMAX2 THEN YMAX2 = BGVAL!(X, 2)
    IF BGVAL!(X, 2) < YMIN2 THEN YMIN2 = BGVAL!(X, 2)
NEXT X
'   PRINT YMIN2, YMAX2
CLOSE #2

' **************************  
' * Calculate output data *  
' **************************

DY1 = YMAX1 - YMIN1
DY2 = YMAX2 - YMIN2
YR = DY1 / DY2
'PRINT "Ratio used for subtraction: ", YR

PRINT
PRINT "Sample file: YMIN "; YMIN1
PRINT " YMAX "; YMAX1
PRINT "Background file: YMIN "; YMIN2
PRINT " YMAX "; YMAX2
PRINT "Subtraction Ratio YR: "; YR
PRINT "************************************"

PRINT "Creating output file "; outputS$  
comma$ = ","
OPEN outputS$ FOR OUTPUT AS #3
FOR X = 1 TO 3476  
    OUTVAL(X, 1) = FILEVAL(X, 1)
    OUTVAL(X, 2) = FILEVAL(X, 2) - YR * BGVAL(X, 2)
    PRINT #3, OUTVAL(X, 1); comma$; OUTVAL(X, 2)
'   PRINT #3, OUTVAL(X, 2)
NEXT X
CLOSE #3

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```
' *******************************
' * Peak Integration routine *
' *******************************

PRINT
PRINT
INPUT "Carry out integration between known limits? ", Y$
IF Y$ = "" THEN Y$ = "y"
IF Y$ = "Y" THEN Y$ = "y"
IF Y$ <> "y" THEN GOTO Question
PRINT
INPUT "Enter lower integration x-limit: ", xlow
INPUT "Enter higher integration x-limit: ", xhigh
PRINT
PRINT

FOR X = 1 TO 3475
C = 0
D = 0
X1 = OUTVAL(X, 1)
X2 = OUTVAL(X + 1, 1)
IF xlow >= X1 THEN C = C + 1
IF xlow <= X2 THEN C = C + 1
IF C = 2 THEN xmin = X
IF C = 2 THEN PRINT "Lower limit found..."
IF xhigh >= X1 THEN D = D + 1
IF xhigh <= X2 THEN D = D + 1
IF D = 2 THEN xmax = X
IF D = 2 THEN PRINT "Upper limit found..."
NEXT X
Y1 = OUTVAL(xmin, 2)
Y2 = OUTVAL(xmax, 2)
M = (Y2 - Y1) / (xhigh - xlow)

' DO PEAK INTEGRATION

SUMY = 0
count = 0
FOR X = xmin TO xmax
Y = OUTVAL(X, 2)
PEAK(X, 1) = OUTVAL(X, 1)
```
YB = M * (PEAK(X, 1) - xlow) + Y1
PEAK(X, 2) = Y - YB
SUMY = SUMY + PEAK(X, 2)
count = count + 1

NEXT X

SUMY = SUMY * 1000
PRINT
PRINT "***********************************************************
* Produce output screen *
***********************************************************
PRINT "Total peak area: "; SUMY; " mRU"
PRINT "Total points used: "; count
PRINT "***********************************************************

Question:
PRINT
INPUT "Run another fileset? ", a$
IF a$ = "Y" THEN a$ = "y"
IF a$ <> "y" THEN END ELSE GOTO Normal

batch:
PRINT "Batch File Operation."
INPUT "Enter batch filename: "; batch$
CALL csvtest(batch$)

OPEN batch$ FOR INPUT AS #5
   INPUT #5, batchname$

   INPUT #5, X
   INPUT #5, BLANK$
   PRINT batchname$, X
   DIM BATCHIN$(X)
   DIM BATCHBG$(X)
   DIM BATCHOP$(X)
   FOR Y = 1 TO X
      INPUT #5, BATCHIN$(Y)
      INPUT #5, BATCHBG$(Y)
      INPUT #5, BATCHOP$(Y)
PRINT BATCHIN$(Y), BATCHBG$(Y), BATCHOP$(Y)
NEXT Y
Z = X
CLOSE #5

INPUT "Press Return to continue.", g$

FOR Y = 1 TO Z

bchin$ = BATCHIN$(Y)
CALL csvtest(bchin$)
bchbg$ = BATCHBG$(Y)
CALL csvtest(bchbg$)
bchop$ = BATCHOP$(Y)
CALL csvtest(bchop$)

area! = 0
CALL ftirbatch(bchin$, bchbg$, bchop$, area!)
PRINT "Area": Y; ": "; area!
AREAVAL(Y) = area!
NEXT Y

PRINT "Batch Process Complete."

SUB csvtest (f$)
    r$ = RIGHT$(f$, 4)
    IF r$ <> ".csv" THEN f$ = f$ + ".csv"
    ll$ = LEFT$(f$, 2)
    l2$ = RIGHT$(ll$, 1)
    IF l2$ <> ":" THEN f$ = "R:\" + f$
END SUB

SUB ftirbatch (bchin$, bchbg$, bchop$, area!)

aa = 3500
DIM FILEVAL!(aa, 2)
DIM BGVAL!(aa, 2)
DIM OUTVAL(aa, 2)
DIM PEAK(aa, 2)
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\[ C = 0 \]
\[ YMAX1 = -99999 \]
\[ YMIN1 = 99999 \]
\[ YMAX2 = -99999 \]
\[ YMIN2 = 99999 \]

OPEN bchin$ FOR INPUT AS #1
FOR X = 1 TO 3476
  FOR Y = 1 TO 2
    INPUT #1, RANDEr
    FILEVAL! (X, Y) = RANDEr
  NEXT Y
  IF FILEVAL! (X, 2) > YMAX1 THEN YMAX1 = FILEVAL! (X, 2)
  IF FILEVAL! (X, 2) < YMIN1 THEN YMIN1 = FILEVAL! (X, 2)
NEXT X
CLOSE #1

OPEN bchbg$ FOR INPUT AS #2
FOR X = 1 TO 3476
  FOR Y = 1 TO 2
    INPUT #2, RANDEr
    BGVAL! (X, Y) = RANDEr
  NEXT Y
  IF BGVAL! (X, 2) > YMAX2 THEN YMAX2 = BGVAL! (X, 2)
  IF BGVAL! (X, 2) < YMIN2 THEN YMIN2 = BGVAL! (X, 2)
NEXT X
CLOSE #2

DY1 = YMAX1 - YMIN1
DY2 = YMAX2 - YMIN2
YR = DY1 / DY2
comma$ = ","
OPEN bchop$ FOR OUTPUT AS #3
FOR X = 1 TO 3476
  OUTVAL(X, 1) = FILEVAL(X, 1)
  OUTVAL(X, 2) = FILEVAL(X, 2) - YR * BGVAL(X, 2)
  PRINT #3, OUTVAL(X, 1); comma$; OUTVAL(X, 2)
NEXT X
CLOSE #3

xlow = 1582
xhigh = 1732

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FOR X = 1 TO 3475
C = 0
D = 0
X1 = OUTVAL(X, 1)
X2 = OUTVAL(X + 1, 1)
IF xlow >= X1 THEN C = C + 1
IF xlow <= X2 THEN C = C + 1
IF C = 2 THEN xmin = X
' IF C = 2 THEN PRINT "Lower limit found..."
IF xhigh >= X1 THEN D = D + 1
IF xhigh <= X2 THEN D = D + 1
IF D = 2 THEN xmax = X
' IF D = 2 THEN PRINT "Upper limit found..."
NEXT X
Y1 = OUTVAL(xmin, 2)
Y2 = OUTVAL(xmax, 2)
M = (Y2 - Y1) / (xhigh - xlow)

' DO PEAK INTEGRATION

SUMY = 0
count = 0
FOR X = xmin TO xmax
Y = OUTVAL(X, 2)
PEAK(X, 1) = OUTVAL(X, 1)
YB = M * (PEAK(X, 1) - xlow) + Y1
PEAK(X, 2) = Y - YB
SUMY = SUMY + PEAK(X, 2)
count = count + 1
NEXT X
SUMY = SUMY * 1000
PRINT
PRINT "***********************************"
PRINT "Total peak area: ", SUMY, " mRU"
PRINT "Total points used: ", count
PRINT "***********************************"
PRINT
area! = SUMY

END SUB