In Situ Recovery of Secondary Metabolites Using Adsorption Resins

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Philosophy

at Massey University, Palmerston North,
New Zealand

Jason L J Ryan

2006

Abstract

Almost without exception a two to three fold increase in microbial secondary metabolite concentration was measured when adsorption resins were added *in-situ* during a submerged liquid fermentation. Anguidine was produced at a final concentration of 440 mg/L after five days in a shake flask that contained adsorption resin, compared to 300 mg/L without resin. Rapamcyin was produced at a final concentration of 87 mg/L after six days in a shake flask that had resin present, compared to 28 mg/L without resin. Ansamitocin P3 was produced at a final concentration of 24 mg/L after six days in a shake flask with resin, compared to 9.75 mg/L without resin. The increase in secondary metabolite concentration confirmed that the resins used provided a positive influence on secondary metabolite production. Adsorption resins for shake flask studies were selected based on their ability to achieve maximum adsorption of specific secondary metabolites in various fermentation systems.

A library of adsorbed concentrations was collected for the three secondary metabolites studied. The lipophilicty of the metabolite, calculated by several software packages, was compared to the polarity of the adsorption resin to generate a relationship. By using the preceding set of data it is possible to select adsorption resins that improved the produced concentrations of the target organic secondary metabolites.

The fermentation media compositions tested appeared to have no effect on the final product concentration when adsorption resins were added *in situ* during the fermentations.

Based on the lipohilictiy of the secondary metabolite and the polarity of the resins, it is possible to select a resin that achieves a high adsorption concentration of the target organic secondary metabolite.

Acknowledgments

This thesis was undertaken with assistance from Industrial Research Limited.

It gives me great pleasure to acknowledge the many people who have assisted me throughout this work. I would like to thank my supervisors Professor Yusuf Chisti and Sarah Reader for their assistance and guidance over the course of this project.

I would like to thank Randy Greasham for providing the impetus upon which I started this work. Without his encouragement and support I would not have achieved this work.

For their support and insight over the length of this work and more I would like to thank my colleagues Max Kennedy, Tony Woolhouse, Sarah Underwood, Simon Hinkley and Yin Zhu.

Figure

Tables

Equations

Nomenclature

Å	Angstroms
k_La	Overall gas-liquid volumetric mass transfer coefficient (h^{-1})
LogP	Logarithmic ratio of the concentrations of the solute in the solvent
π	*Pi*

1 Introduction

1.1 Motivation

It has become obvious in recent years that the use of adsorption resins in submerged fermentations has become more prevalent in fermentation science as a means of removing high value secondary metabolites. The benefits that adsorption resins convey into this system are an increase in metabolite stability, a reduction in feedback inhibition and an increase in extraction efficiency. Adsorption resin selection is usually based upon non-systematic considerations that are, in turn based upon the fermentation 'macro-system'. This process is therefore *ad hoc*, time consuming and costly to operate to cover all resin types that are commercially available.

The research investigated the screening of a range of adsorption resins against three secondary metabolites to identify their maximum adsorption from a simple aqueous solution with the express purpose of applying those results to a fermentation system. Fermentations were conducted under normal conditions and aliquots of resins were added by addition of a small, but representative selection of adsorption resins, daily, based on their anticipated ability to adsorb secondary metabolites. A guideline was generated based upon the results for possible use with other secondary metabolite production systems.

1.2 Objectives

The overall aims of this research were to understand the interaction between adsorption resins and secondary metabolites, to optimise conditions for using the resins in submerged cultures and to generate a methodology to screen secondary metabolites against resin systems for implementation in a fermentation system. The specific objectives were:

a. to screen a range of commercially available adsorption resins for the capture of anguidine, rapamycin and ansamitocin P3 to identify the maximum adsorption for each resin and compound;
b. to propose and identify a set of guidelines for the use of adsorption resins in submerged cultures for particular secondary metabolites;
c. to characterise the fermentation system of *F. sambucinum* in the presence of adsorption resins to optimise the conditions for maximum secondary metabolite production based on conclusion from point b;
d. to confirm the resin selection methodology for rapamycin from *Streptomyces hygroscopicus* and for ansamitocin P3 from *Actinosynnema pretiosum*.

1.3 Approach

The approach taken in this study was to screen a selected range of commercially available adsorption resins for their capacity to adsorb anguidine and two other secondary metabolites, rapamycin and ansamitocin P3 and confirm the recovery of each compound from each resin. Analyses of the extent of secondary metabolite adsorption were used to select two resins for further study.

Fermentations were conducted to identify the effect of adding adsorption resins and to identify the optimum combination of addition time and medium interactions. A range of resin addition times was chosen to span the critical stages in the fermentation; namely, growth and secondary metabolite production. The secondary metabolite accretion, pH and packed cell volume were assayed to generate a correlation between secondary metabolite expression and resin addition time.

A model for implementation of adsorption resins in submerged culture for secondary metabolite production was developed and tested. The model predictions were tested against *S. hygroscopicus* for the production of rapamycin and *A. pretiosum* for the production of ansamitocin P3.

1.4 Overview

Chapters 2 reviews the subjects of fermentation technology and adsorption resins selection, respectively, providing the foundation upon which the subsequent discussion is based. Specifically the chapter reviews the broad subjects of fermentation technology to provide information on submerged fermentations systems, fermentation medium and secondary metabolite production. Additionally the chapter also reviews the current use of adsorption resins in fermentation systems, the different types of adsorption resins and their mechanism of action. Finally, a method for comparing the lipophilicty of organic compounds is discussed. The experimental methods employed in this study are detailed in Chapter 3. Chapter 4 presents the experimental results and discussions characterising the development of a defined medium for *F. sambucinum*, the fermentation for the production of anguidine and the screening of a number of resins for the binding of anguidine, rapamycin and ansamitocin P3. A model was generated and a system for resin screening was tested against rapamycin production by *S. hygroscopicus* and *A. preitosum* for the production of ansamitocin P3. The conclusions for each stage are summarised in Chapter 5.

2 Literature Review

2.1 Fermentation technology

2.1.1 Aspects of submerged culture

There are three distinct phases of growth of micro-organisms in submerged culture fermentations. These are lag, exponential (log, linear and slowing) and stationary, Figure 1. The autolytic, or death phase, is not usually classed as a growth phase. The exponential phase is sometimes referred to as the tropophase and the stationary phase is sometimes referred to as the idiophase. The period of time before the exponential growth is called the lag phase, the length of this phase is dependant upon the micro-organism, cell concentration, conditions and growth medium (Pelczar and Reid, 1972).

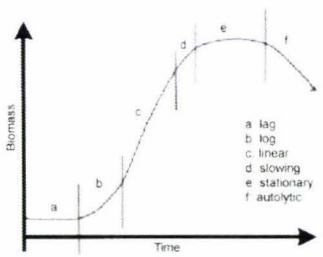

Figure 1 Growth phases of micro-organisms in submerged culture (Pelczar and Reid, 1972)

Pelczar and Reid (1972) report that submerged cultures are widely used to produce many secondary metabolites due to their ability to allow filamentous fungi and bacteria to produce freely suspended mycelia and pellets, essential for secondary metabolite production.

Submerged culture fermentations require a stirred nutrient medium and, in the case of aerobes, oxygen to be present.

2.1.1.1 Micro-organisms

Fungi and bacteria are commonly used in submerged fermentations to produce a wide range of primary and secondary metabolites. Many other

plant, insect and mammalian cell lines are also used to produce secondary metabolites, but these systems are not as effective as bacteria and fungi for their range and production. The main focus of fermentation is to enhance the production of the product of choice, whether it is primary metabolite, secondary metabolite and/or biomass. Therefore optimisation of fermentation is critical to ensure the appropriate growth or production phase is extended. The morphology of the micro-organism during the growth phase is dependant upon the strain, culture initiation method, growth medium and the hydrodynamic regime (Casas Lopez *et al.* 2005; Pelczar and Reid, 1972).

In submerged cultures, the morphology of filamentous bacteria and fungi in the growth phase varies between a network of freely dispersed mycelia to tightly packed, discrete pellets. In the case of a secondary metabolite lovastatin, produced by *Aspergillus terreus*, tightly packed pellets significantly increased its production compared to free mycelia (Casas Lopez *et al.* 2005).

A high oxygen requirement is important in most bacterial and fungal fermentations for biomass production and metabolite formation (Monnet *et al.* 1988; Kalakoutskii and Agre 1976; Casas Lopez *et al.* 2005). Rapidly growing filamentous fungi increase the viscosity of submerged cultures thereby impeding the oxygen transfer from the sparged air into the culture and concomitantly impeding the production of secondary metabolites (Casas Lopez *et al.* 2005). Additionally, to support microbial growth and metabolite production in bacteria and fungi, a media containing at least a nitrogen and carbon source is required (Zhang *et al.* 2002).

2.1.1.2 Types of bioreactors

Bioreactors for submerged microbial growth can be identified as shake flasks, stirred tanks, airlift, tower or immobilized beds (McNeil and Harvey 1990; Pelczar and Reid, 1972).

The most common type of bioreactor for bacterial and fungal fermentations is the stirred tank due to its relative high oxygen transfer rate (OTR), *in situ* sterilisation capability and established scale up techniques, Figure 2 (Casas Lopez *et al.* 2005; McNeil and Harvey 1990; Atkinson and Mavituna 1983).

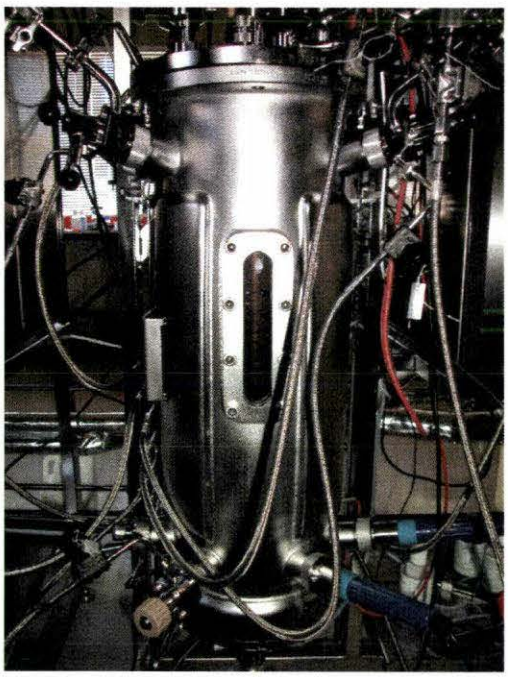

Figure 2 18L working volume stirred tank bioreactor

Oxygen transfer is an important aspect of fermentation during the growth phase, with oxygen demand decreasing during stationary phase (Atkinson and Mavituna 1983). Oxygen transfer in the aqueous phase is described by Equation 1 (McNeil and Harvey 1990).

Equation 1 Oxygen transfer rate

$N_a = k_L a\ (C_g^* - C)$

Where N_a is the oxygen uptake rate (mmol litre^{-1}h^{-1}), $k_L a$ is the mass transfer coefficient (h^{-1}), a is the interfacial area for mass transfer (m^{-1}), C_g is the saturation level for oxygen in the liquid (mmol litre^{-1}) and C is the actual dissolved level (mmol litre^{-1}) (McNeil and Harvey 1990). Casas Lopez *et al.* (2005) reported that experiment conducted on $k_L a$ values with *Aspergillus terreus* indicated that the value increased with an increase in agitation, but varied little during the growth of the organism.

Stirred tank bioreactors are able to vary the oxygen transfer rate through agitation and airflow manipulations to ensure the conditions of the bioreactor are conducive to optimal growth and metabolite production. In the presence of micro-organisms with complex morphology, such as filamentous bacteria and fungi, the OTR requirement is usually higher than

that required for simple bacteria and as such requires additional oxygen input via agitation, airflow, baffles and impellor design (Atkinson and Mavituna 1983; Chen *et al.* 1999). In stirred bioreactors agitation has a direct impact on the culture morphology, especially in relation to filamentous fungi and bacteria. Casas Lopez *et al.* (2005) reported that agitation speeds (u_t) of ~2.0 m s^{-1} damaged *Aspergillus terreus* mycelia pellets, reducing them in size and lowering the overall production of the secondary metabolite, lovastatin (Casas Lopez *et al.* 2005).

Shake flasks are routinely used for small scale fermentations and are used in inoculum preparation for larger bioreactors. Shake flasks vary greatly in size and form with the standard Erlenmeyer flasks, baffled shake flasks and Fernbach flasks amongst the most common type used. Oxygen transfer is limited within the shake flask and is one of the main constraints when using this system for microbial fermentations, as indicated in Table 1. (McNeil and Harvey 1990)

Table 1 Typical values of oxygen transfer coefficient (K_La) value (h^{-1}) in various systems

Fermenter	K_La value (h^{-1}) [the smaller the value the lower the oxygen transfer]
Test-tube	20
Flat-bed bottle	50
Erlenmeyer	500
Baffled shake flask	1200
~10L stirred bioreactor	3000-4000

(McNeil and Harvey 1990)

2.1.1.3 Operating modes for submerged bioreactors

A variety of techniques has been developed for the growth of micro-organisms in submerged culture. Batch and batch-fed have been used as a method for the cultivation of micro-organisms and metabolites since antiquity, with continuous culture techniques evolving as technology for

monitoring and control has evolved. All fermentation systems require an inoculation culture to be prepared from a spore or vegetative cell bank directly or via a streak plate or slant. Due to the large volumes of liquid medium in industrial fermentation production systems, the preparation of at least a two stage inoculum is required to increase the biomass for transfer to a larger vessel (Miller and Litsky 1976; McNeil and Harvey 1990).

Batch fermentation systems are used throughout the fermentation industry with food products and secondary metabolite production ideally suited to this technology. Batch fermentation systems require all nutrients for growth and production to be added to the bioreactor during initial setup and result in the micro-organism utilising all the nutrients until the fermentation is complete or harvested (Casas Lopez *et al.* 2005; McNeil and Harvey 1990).

Fed-batch fermentation is a fermentation which is supplied with fresh medium containing specific nutrients during any stage of the fermentation. The nutrient addition occurs in response to specific events occurring within the fermentation system. For example, a precursor to a metabolite may be added during the metabolite production phase to enhance final concentration of the metabolite. This system allows greater control over the growth and production phase in attempts to increase the final production concentration of relevant metabolites above that of a batch fermentation system (Miller and Litsky 1976; McNeil and Harvey 1990).

Continuous fermentation systems have been used successfully over the last 50 years. The continuous fermentation system acts by prolonging the growth phase of a micro-organism in culture by feeding in fresh nutrients and removing spent medium and cells. The system then maintains the nutrient, organisms and metabolites produced at constant concentrations, known as a steady state (McNeil and Harvey 1990).

2.1.2 Fermentation medium development

Fermentation medium utilised by the micro-organism is incorporated into biomass and/or used for metabolite biosynthesis. When medium is used for biomass production only, the biomass formed can be estimated by stoichiometry. If however the micro-organism utilises the medium substrate

for metabolite production as well as for biomass, efficiency of metabolite formation determines the concentration of biomass and metabolite. The nutrient supplied to the micro-organism by the medium has a significant impact on the concentration of metabolite, both directly through metabolism and indirectly by influencing the specific metabolite production rate of the biomass. (Atkinson and Mavituna 1983) The specific production rate of secondary metabolites can be influenced by catabolite regulation. Aharonwitz and Demain (1978) reported that cephalosporin production by *S. clavuligerus* is regulated by catabolic control. Specific production (measured as $gL^{-1}hr^{-1}$) of cephalosporin was reduced when glycerol and maltose carbon sources were used, but increased when starch was used as a carbon source. The results show that rapidly growing cultures negatively affect secondary metabolite production, suggesting that fermentation medium selection is critical for optimal metabolite production (Aharonwitz and Demain 1978).

2.1.2.1 Complex medium

Fermentation medium is a critical component of industrial fermentation as it directly affects the productivity (measured as $gL^{-1}hr^{-1}$) and operations, such as sterilisation. The medium also affects the shear sensitivity of cells, nutrient feeding requirements and broth viscosity. The operation of the submerged fermentation system is directly affected by the medium components impacting upon the sterilisation method, metabolite production and growth profiles (Zhang *et al.* 2003).

Initial development medium uses components that are known, to assist in the growth of the micro-organism and metabolite production. Payne and Hagler (1983) observed that extracts from Brazilian peanuts that are known to contain aflatoxins produced from *Aspergillus parasiticus* induced, aflatoxin production in submerged culture. Bacon *et al.* (1996) identified that *Fusarium* sp. produced fusaric acid in an agricultural mixed corn feed and successfully substituted corn feed with corn meal to produce fusaric acid in submerged culture.

Complex carbon and nitrogen sources, such as yeast extract and peptones, are commonly used as they are inexpensive but provide a wide range of

nutrients required for growth and metabolite production (Zhang *et al.* 2003; Degeest and De Vuyst 1999). Degeest and De Vuyst (1999) identified that by modifying the carbon/nitrogen ratio in a complex medium, different exopolysacchrides could be produced from *Streptococcus thermophilus*. The trichothecene family of mycotoxins were initially identified in stored grains and cereals. By using yeast extract as a medium component, cultures were propagated for isolation studies (Wannemacher and Weiner 1997).

Yeast extract is produced from baker's or brewer's yeast through autolysis at around 50°C and is a rich source of various amino acids, peptides, water-soluble vitamins, trace elements and carbohydrates (Zakriskie *et al.* 1980; Zhang *et al.* 2003). However, due to the poorly controlled supply of starting material and downstream processing the biomass and growth rates obtained on yeast extract can vary by as much as 50% between different lots (Potvin *et al.* 1997).

Zhang *et al.* (2003) and Potvin *et al.* (1997) recommend that testing of complex medium component be conducted by testing a representative sample of each raw material batch to compare its performance based on a number of pre determined parameters. This testing is used to minimise the variability of fermentations because of batch to batch variations in components of the medium. The substitution methodology can be used to also substitute a number of key carbon, nitrogen and trace minerals following standard statistical substitution models. Secondary metabolite production improvement and consistency are possible by screening a selection of common complex media components that are known to influence micro-organism growth or metabolite production (Zhang *et al.* 2003; Potvin *et al.* 1997).

2.1.2.2 Defined medium

Chemically defined media are used in the area of industrial microbiology to enhance metabolite production through directed biosynthesis, improved process control, fermentation consistency and enhanced product recovery (Zhang *et al.* 1996; Kenneth *et al.* 1977; Zhang *et al.* 2003; Majumdar and Kutzner 1961; Basak and Majumdar 1973). Other benefits for the use of defined medium proposed by Zhang *et al.* (1996) are that foaming and

viscosity are reduced and this improves the ease and economy of product recovery. Chemically defined media for the production of antibiotics have been used successfully for a wide range of *Streptomyces* species. The use of defined medium for secondary metabolite production allows consistency of microbial growth and metabolite production between batches, an essential requirement for large scale industrial antibiotic production (Zhang *et al.* 2003; Zhang *et al.* 1996; Majumdar and Kutzner 1962; Basak and Majumdar 1973; Dulaney 1948; Shirato and Motoyama 1966; Darken *et al* 1960; Williams and Katz 1977; Shirato and Nagatsu 1965; Perlman and O'Brien 1956).

These media, also called synthetic media, consist of fully known cocktail of carbon, nitrogen and trace metal components. Hajjaj *et al.* (2001) propose that the use of specifically defined carbon and nitrogen plays a critical role as the source of precursors and cofactors for the synthesis of secondary metabolites and that stringent starvation conditions promulgated by selective medium nutrients promote secondary metabolite production. Draken *et al.* (1960) reported that addition of chloride ions in a synthetic medium in the presence of *Streptomyces aureofaciens* produces the compound chlortetracycline whereas the same medium without chloride produces tetracycline.

The use of defined carbon sources allows specific metabolic activities to be influenced during the fermentation and conversely may exert complex regulations on gene expression and enzyme activities for antibiotic synthesis (Willams and Katz, 1977; Hajjaj *et al.* 2001). Williams and Katz (1977) show that the use of D-fructose in a synthetic medium for the production of actinomycin D by *Streptomyces parvulus* decreased the rate of biomass production and ensured that both carbon and energy were available during the antibiotic production.

In a defined medium the nitrogen source may either be a combination of organic (amino acids, amines, etc) or inorganic (nitrates, ammonia, nitrite, etc). For most filamentous fungi nitrogen sources, such as ammonium nitrate, sodium nitrate, and urea can be used for biomass formation and metabolite production (Hajja *et al.* 2001). Filamentous bacteria, however, utilise organic nitrogen sources more effectively than inorganic medium

nutrients, for example *Streptomyces griseus* was found to not utilise nitrates as the sole nitrogen source (William and Katz 1977; Zhang *et al.* 1996; Dulaney 1948). William and Katz (1977) observed that organic acids, such as glutamic acid, histidine and proline produced three times more actinomycin D than inorganic nitrates, nitrites and ammonium salts. Payne and Halger (1983) identified that the addition of amino acids, specifically asparagine and proline, to synthetic media stimulated biomass growth, respiration and secondary metabolite production in some *Streptomyces* spp. and *Aspergillus* spp.

The basal trace metal solution used for most chemically defined media for *Streptomyces* spp. contains magnesium sulphate, calcium chloride, cobalt chloride, ferric sulphate, zinc sulphate and di-potassium orthophosphate (Williams and Katz 1977; Basak and Majumdar 1973; Majumdar and Kutzner 1962; Zhang *et al.* 1996; Hajjaj *et al.* 2001). Williams and Katz (1977) reported that low concentrations of the secondary metabolite, actinomycin D, are observed when a chemically defined media lacks iron, magnesium and sometimes zinc ions. Knowledge of the metabolic pathways required for secondary and primary metabolite production allows defined medium to be optimised for secondary metabolite production (Williams and Katz 1977).

2.1.3 Overview of metabolite production

2.1.3.1 Primary metabolites or metabolism

Primary metabolites are involved in enzyme-mediated catabolic, amphibolic and anabolic pathways that provide energy and biosynthetic intermediates that can be converted into essential macromolecules (Martin and Demain 1980). Metabolites that are formed during cell multiplication, like proteins, carbohydrates, nucleic acids and lipids are classed as primary metabolites. Maggon *et al.* (1977) identify that at the onset of secondary metabolism the enzymes that biosynthesise primary metabolites are either blocked or inhibited. In fungal fermentations, a depletion of nitrogen or phosphorous results in decline of growth and primary metabolite accumulation, leading to

the initiation of secondary metabolite production. The pathways used for primary metabolite production, such as the polyketide route, the terpenoid route and the amino acid utilization route are all pathways used by secondary metabolite biosynthesis. (Maggon *et al.* 1977)

2.1.3.2 Secondary metabolites

Guarro *et al.* (1999) classifies secondary metabolites as, 'compounds neither essential for growth nor key intermediates of the organism's basic metabolism but presumably playing some other role...'. Secondary metabolites are most commonly produced by bacteria from the Actinomycetales group (commonly called Actinomycetes) and by most fungi. These micro-organisms exhibit filamentous growth and complex morphology (Guarro *et al.* 1999; Martin and Demain 1980; Calvo *et al.* 2002). Secondary metabolites can be classified into three broad categories, antibiotics (helpful for humans), pigments and toxins (harmful to humans) (Maggon *et al.* 1977; Calvo *et al.* 2002). The first antibiotic identified was actinomycin from a filamentous bacterium *Streptomyces* species in 1940, with a successive range of similar antibiotics identified in the following decades (Baltz 2005).

Many secondary metabolites with complex chemical structures are produced via the polyketide pathway (Hajjaj *et al.* 2001; Maggon *et al.* 1977). The chemical structures of secondary metabolites include a range of peculiar and rare structures such as β-lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes and large rings of macrolides (Guarro *et al.* 1999; Martin and Demain 1980).

Secondary metabolite production uniquely occurs during the idiophone phase and thus secondary metabolites have also been termed "idiolites". However, the distinction between trophophase and idiophase is not clearly defined with filamentous micro-organisms as sometimes the cell density continues to increase during the trophophase, so the timing of secondary metabolite production should not be used exclusively to define if a compound is a secondary metabolite (Martin and Demain 1980).

Williams and Katz (1977) reported that under synthetic medium conditions, secondary metabolite production in filamentous bacteria and fungi does not follow the typical trophophase-idiophase production pattern. Secondary metabolite production occurs earlier in the fermentation profiles with defined medium than complex medium. The change to the secondary metabolite production curve can be attributed to the modifications to the biomass production and consequently the repression of primary metabolite pathways (Williams and Katz 1977).

Maggon *et al.* (1977) state that biosynthesis of secondary metabolites occurs in batch fermentations when specific nutrient concentrations, mostly nitrogen and phosphorous, are depleted. Secondary metabolite production has not been successfully conducted in continuous fermentation as these systems are ideally designed for primary metabolite and biomass production (Maggon *et al.* 1977).

Martin and Demain (1980) observed that micro-organisms mainly produce antibiotics when the specific growth rate decreases below a certain level. The secondary metabolite producing micro-organism avoids self inhibition effects by two mechanisms:

1. Modification of the antibiotic by enzymes produced by the micro-organism;
2. Decreased inward permeability to the antibiotic after it has been excreted.

Growth of *Streptomyces antibioticus* is reduced by 50% by the addition of 4 µg/mL of actinomycin, although the strain can produce 120 µg/mL (Demain and Martin 1980). Additionally, Casas Lopez *et al.* (2004) reported that lovastatin inhibited and delayed production of its own biosynthesis, but did not interfere with biomass production.

Calvo *et al.* (2002) identifies that production of secondary metabolites by fungi is usually associated with the sporulation process. This process is a result either directly or a combination of the following activities:

1. Metabolites that induce sporulation
2. Pigment that is required for sporulation

3. Toxic metabolites that are secreted by growing colonies at the time of sporulation.

2.1.4 *Fusarium sambucinum*

Fusarium sambucinum is a type A heterothallic ascomycete fungus and facultative anaerobe that produces sesquiterpene epoxides called trichothecenes (Desjardins *et al.* 1993; Ueno *et al.* 1980). The first of the trichothecene family of compounds was identified from *Trichothecium roseum* (later renamed *Fusarium)* in 1959 (Eaker and Wadstrom 1980). The most common type A trichothecenes produced by *F. sambucinum* are T-2, HT-2, neosolaniol and anguidine. (Ueno *et al.* 1975)

2.1.4.1 Growth conditions

The conditions, for anguidine production, referenced in the literature vary markedly along with the final production concentrations (Richardson *et al.* 1987). The most common growth media used are complex media, based on potato dextrose. A low pH inhibits fungal growth and anguidine production (Ueno *et al.* 1980). Ueno (1980), states that the production of mycotoxins depends upon the substrate, the temperature of cultivation and the duration of incubation time.

Shaking the culture in the absence of light greatly improves the concentration of anguidine, but there is no evidence that the product is photo-labile (Altomare *et al.* 1995). Some trichothecene toxins have been observed to be unstable in an aqueous environment, due to their relative insolubility in water. The addition of a hydrophobic resin may increase stability by providing a matrix to bind to (Hagler *et al.* 1981).

2.1.4.2 Trichothecene biosynthetic pathway

The trichothecene family of sesquiterpenoid metabolites is produced by a number of fungal genera, including *Fusarium, Myrothecium, Phomopsis, Stachybotrys, Trichoderma* and *Tricothecium* (Bennett and Klich 2003). Desjardins *et al.* (1993) identified that the biosynthesis of trichothecenes is

preceded by an ordered sequence of esterifications to form the precursor trichodiene (Figure 3) and then a series of oxygenations to form the final trichothecene end product, Figure 4.

Acetyl coenzyme A, β-hydroxy-β-methyl-glutaryl coenzyme A (HMGCoA) and mevalonate are three of the main cofactors required for the esterification in anguidine production. Sub-inhibitory levels of sorbic acid (an intermediate of acetyl coenzyme A) stimulated anguidine production. The HMGCoA precursor additions (isovaleric acid, ethyl isovalerate and L-leucine) did not stimulate anguidine production whereas isovaleric acid inhibited production (Altomare *et al.* 1995).

The oxygenation enzymes are unstable, however oxygen isotope incorporation studies have identified that pyran, epoxide and hydroxide oxygenations are all catalyzed by molecular oxygen (Desjardins *et al.* 1993). Desjardins *et al.* (1987) showed that the addition of a cytochrome P-450 monooxygenase inhibitor blocked trichothecene production leading to accumulation of the biosynthetic precursor trichodiene.

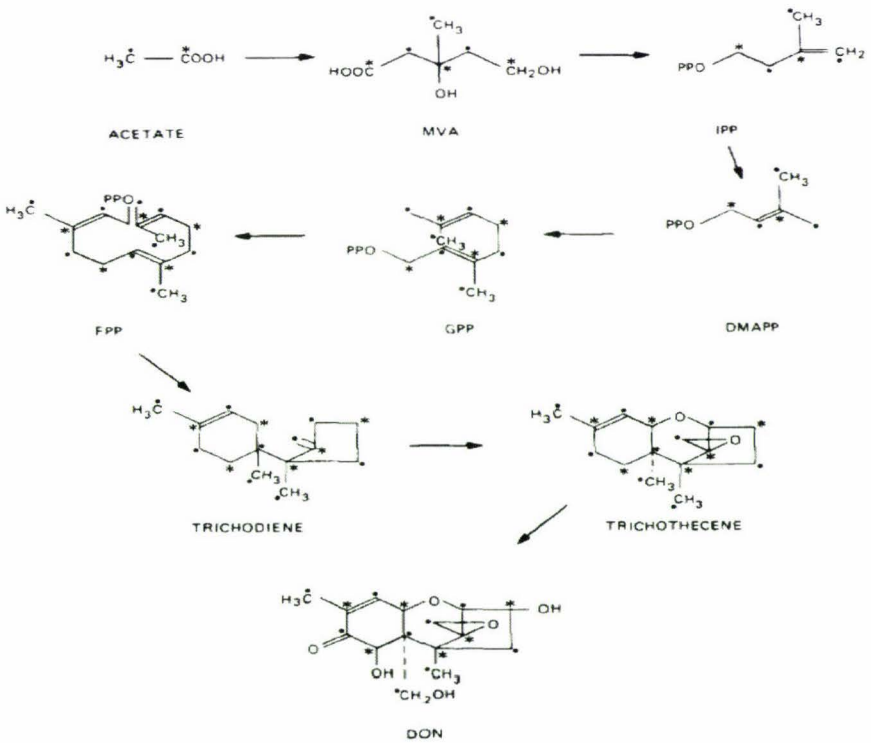

Figure 3 Proposed biosynthesis of trichothecenes (Blackwell *et al.* 1985)

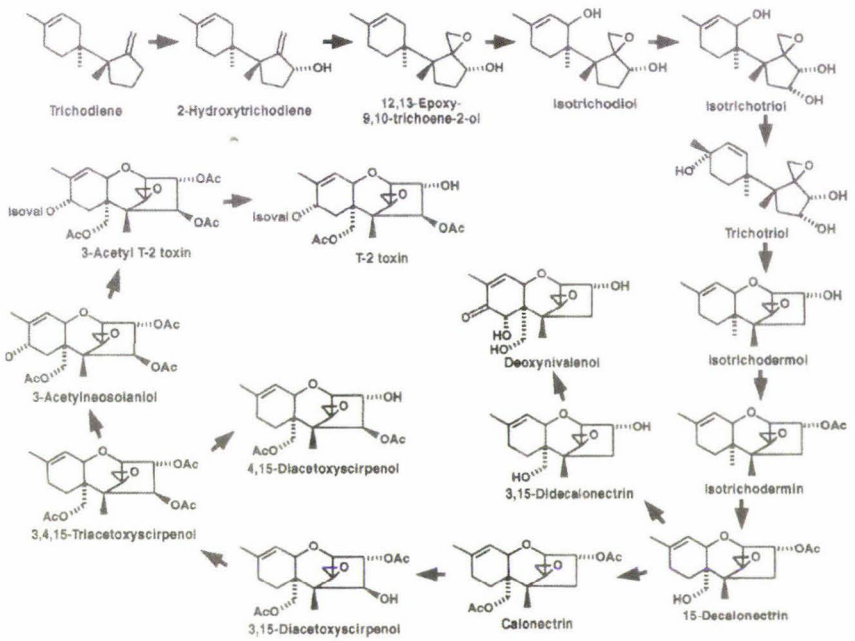

Figure 4 Trichothecene biosynthesis from tricodiene (Desjardins *et al.* 1993)

2.1.4.3 Anguidine

Anguidine ($C_{19}H_{26}O_7$) is a slightly hydrophobic compound containing two acetate side chains, a pyranyl and epoxide ether and a free hydroxyl group (Figure 5). The LogP (section 2.3.1) of anguidine is 0.81 (XLogP computational analysis). The high toxicity of anguidine (LD_{50} 625 µg/kg rat i.p.) and its variable production in solid-state and submerged culture fermentation has hindered its study to date (Ueno *et al.* 1972).

Anguidine is a colourless, crystalline, optically active compound, soluble in alcohol, acetone, ethyl acetate and chloroform (Uneo 1980). Trichothecenes inhibit eukaryotic protein synthesis and have a direct effect on human health (Bennett and Klich 2003; Desjardins *et al.* 1993).

Uneo (1980) describes anguidine as stable solid but, under mild alkaline conditions, the esters are readily saponified to give various hydrolysis products. Under acidic conditions, the C12-C13 epoxide ring is opened by hydrolysis. The presence of C9-C10 double bond confers a pseudo-chain conformation to the six membered ring (A) typical of substituted

cyclohexenes. The central nucleus consists of a fused six membered pyram ring (B) bridged across C2 and C5 by an ethano linkage (C) (Uneo 1980).

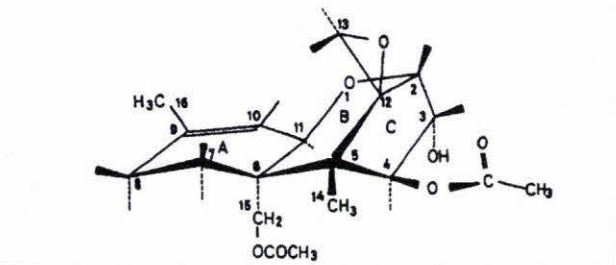

Figure 5 Conformation of anguidine (Uneo, 1980).

2.1.5 *Streptomyces hygroscopicus*

S. hygroscopicus, from the Actinomyces group, are gram-positive, obligate aerobic bacteria. They form filamentous or densely aggregated mycelia that coalesce into pellet-like particles. Actinomyces, in general are highly variable in phenotype and susceptible to changing conditions. Additionally the resulting conditions from dense pelleting cause the rheological conditions of the fermentation to change from newtonian to non-newtionian, thereby decreasing the oxygen transfer rate into the pellet. (Chen *et al*, 1999)

2.1.5.1 Growth conditions

Rapamycin is produced by a complex medium usually containing glycerol, peptone, yeast extract, sodium chloride, lysine and phosphate salts. Trace salts are also added at low concentrations. The micro-organism is grown in aerated, stirred, submerged culture at temperatures between 25 and 28 °C and a controlled pH of between 6.2 and 6.8. Additional carbon and lysine feeding is sometimes used during the tropophase to enhance rapamycin production by providing biosynthetic precursors. Improved rapamycin production has also been observed when the dissolved oxygen is controlled above 30% air saturation (Chen *et al.* 1999).

Kojima and colleagues (1995) have reported a defined medium for rapamycin production. The complex carbon and nitrogen medium

components were replaced with fructose, mannose, lysine and ammonium sulphate. The pH of the fermentation decreased significantly with the addition of ammonia sulphate, requiring a higher concentration of MES buffer (Kojima *et al.* 1995; Lee *et al.* 1997).

2.1.5.2 Rapamycin biosynthetic pathway

Rapamycin (Figure 6) is a 31-membered nitrogen-containing macrocyclic triene polyketide with potent immunosuppressive properties which was first identified in a soil sample from Easter Island in 1975. (Vezina *et al.* 1975; Khaw 1998; Ritacco *et al.* 2005)

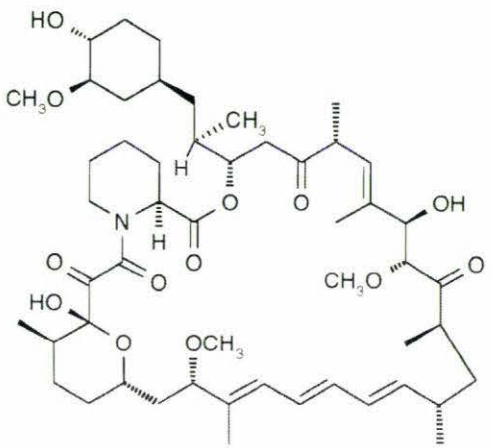

Figure 6 Structure of rapamycin (Ritacco *et al.* 2005)

The compound is known for immunosuppresive effects, in humans, and its antifungal properties (Chen *et al.* 1999). Rapamycin is synthesised by a mixed polyketide synthase and peptide synthase enzymatic complex. The biosynthesises occurs using the precursors, acetate and propionate, which are assembled in a 'head-to-tail' fashion to form the macrolide ring. Additionally, the three methoxy carbons are derived from methionine, the substituted cyclohexane moiety is derived from shikimic acid and the heterocyclic ring is derived from lysine via pipecolic acid (Paiva *et al.* 1993; Ritacco *et al.* 2005).

The enzyme lysine cyclodeaminase catalyses the cyclization and conversion of *L*-lysine to *L*-pipecolate, which is then incorporated into rapamycin prior to the final closure of the macrocyclic ring. A pipecolate-incorporating

enzyme is thought to be responsible for the catalysis of the *L*-pipecolate incorporation and the ring closure. Other studies have shown that *L*-proline can be substituted for L-pipecolate, indicating that the pipecolate-incorporating enzyme allows some substitution (Ritacco *et al.* 2005).

The starter unit for rapamycin biosynthesis was identified as shikimic acid, by incorporation studies using radio labelled shikimic acid. However, addition of shikimic acid to the fermentation medium did not increase the production of rapamycin (Paiva *et al.* 1993).

2.1.6 *Actinosynnema pretiosum*

The strain *Actinosynnema pretiosum* is an aerobic, motile nocardioform actinomycetes that forms branched mycelia and pellets in submerged culture fermentation. Originally identified as a *Norcadio* sp. by Tandia *et al.* (1980), the organism was reclassified as *Actinosynnema pretiosum* by Hatano *et al.* (1983). The strain *Actinosynnema pretiosum* subsp. *auranticum* (ATCC 31565) is an ethidium bromide mutant, isolated from Japan (Tanida *et al.* 1980; Hatano *et al.* 1983; Hasegawa *et al.* 1983).

2.1.6.1 Growth strategies

Actinosynnema pretiosum is grown in submerged culture fermentations using a complex medium containing yeast extract, malt extract and glucose at a pH of 7.0. The micro-organism grown on a solid medium of yeast extract malt agar (YMA) produced colonies with yellow vegetative growth. The cell morphology during growth in submerged culture involves formation of branched mycelia that break into motile elements with peritrichous flagella in late stationary phase (Hasegawa *et al.* 1983).

Actinosynnema pretiosum produces a range of ansamitocins that are varied at the C-3 position, to primarily produce the moieties of either propionyl (p-2), isobutyl (p-3) or isovaleryl (p-4), Figure 7. The selective accumulation of the specific moiety can be enhanced by the addition alcohols, aldehydes and fatty acids that contain the same number of carbon atoms as those of the acyl moieties required. The relative production of ansamitocin P-3 can be

increased by 151% with the addition of isobutyl alcohol, in addition to the standard media for submerged fermentation. (Hatano *et al.* 1984)

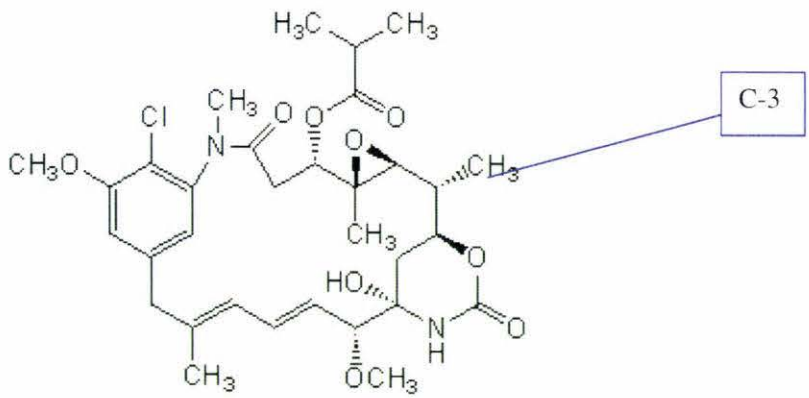

Figure 7 Structure of ansamitocin P3 (Yu *et al.* 2002)

2.1.6.2 Ansamitocin P-3 biosynthetic pathway

Ansamitocin P-3 is synthesised by a seven step chain extension facilitated by a type I modular polyketide synthase (PKS), Figure 8 (Carroll *et al.* 2002; Cassady *et al.* 2004).

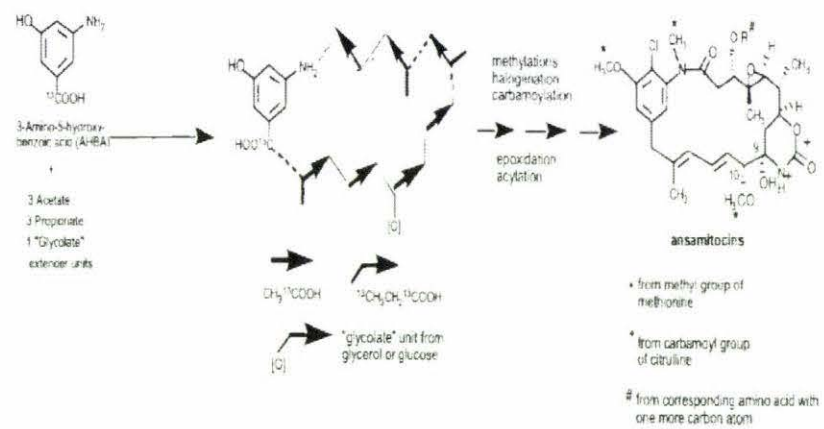

Figure 8 Ansamitocin biosynthesis deduced by Cassady *et al.* (2004) by addition of labelled precursors

The initial building block for biosynthesis is 3-amino-5-hydroxybenzoic acid (AHBA), derived from UDP-glucose, via kanosamine to AHBA by enzymatic steps that introduce nitrogen, phosphate and structural modifications. Feeding of ^{13}C-labelled AHBA to a *A. pretiosum* submerged

culture fermentation specifically enriched at C-15 and resulted in a 30% increase in production of ansamitocins.

The remainder of the marocycle backbone is built up by seven chain extensions which incorporate three acetates, three propionates and a glycolate unit. The glycolate unit is an oxygenated, two carbon unit derived from either glucose or glycerol. Finally, the acyl moiety that attaches to C-3 is derived from their corresponding amino acids with one or more carbon atom, through transamination and oxidative decarboxylation or via the corresponding alcohol or aldehyde through oxidation. During fermentation the addition of valine, isobutyrate, isobutyraldehyde or isobutanol results in the selective accumulation of ansamitocin P3, at the expense of ansamitocin P2 and P4 (Cassady *et al.* 2004).

2.2 Adsorption resins

2.2.1 Resins: chemical and physical properties

Two broad categories of neutral adsorption resins are used in the pharmaceutical industry for the capture of metabolites. The first is the macroporous, hydrophobic types, representatives of which have different surface areas and pore sizes. Of the hydrophobic resins used, the most common is the Amberlite 'XAD' range of resins produced by Rohm and Haas. (Douila *et al.* 2001; Rohm and Haas 2003; Rohm and Haas 2005; Rohm and Haas 2006) The second category is the macroporous, hydrophilic types, representatives of which have different surface areas and pore sizes (Lee *et al.* 2003; Mitsubishi Chemical Corporation. 2001).

Table 2 Sample of resin types used in this work

Resin	Pore size (Å)	Total Surface Area (m^2/g)	Dipole Moment (deybe)	Matrix	Adsorption Type
XAD2	90	330	0.4	Polystyrene	Hydrophobic
XAD16	100	825	0.4	Polystyrene	Hydrophobic
XAD1180	400	450	0.5	Polystyrene	Hydrophobic
SP207	105	630	0.3	Brominated polystyrene	Hydrophobic
HP2MG	200	500	0.8	Polymethacrylic	Moderately Hydrophilic
XAD761	600	200	0.4	Phenol-formaldehyde polycondensate	Hydrophilic
L285	25	800	unknown	Styrene divinylbenzene	Hydrophilic

(Rohm and Haas, 2003; Rohm and Haas, 2005; Rohm and Haas, 2006; Mitsubishi Chemical, 2001 ; Dowex, 2006)

2.2.1.1 Hydrophobic resins - XAD2/XAD16/XAD1180/SP207

The three hydrophobic XAD resins (XAD2/XAD16/XAD1180) are all variably cross linked polystyrenes (Figure 9), with varying pore sizes and surface areas (Table 2). The hydrophobic XAD resins are used for adsorption of hydrophobic substances from aqueous systems and polar solvents (Rohm and Haas 2003).

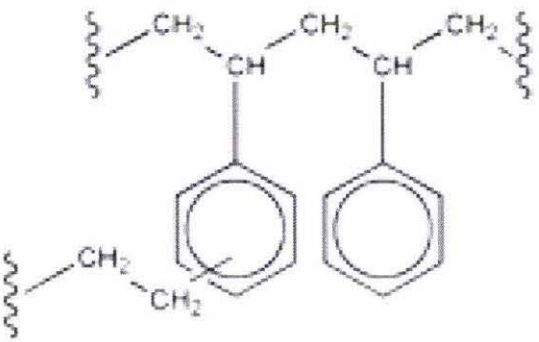

Figure 9 XAD family chemical structure (Rohm and Haas 2003)

Sepabead SP207 (Figure 10), with respect to the XAD series (Table 2) is a (co-*p*-bromosytrene)-DVB resin with enhanced hydrophobicity, supplied by Mitsubishi Chemical Corporation (Table 2). SP207 has a higher density (1.18 g/cm^3) than the standard polysytrene XAD resins (~1.02 g/cm^3). (Mitsubishi Chemical Corporation, 2001)

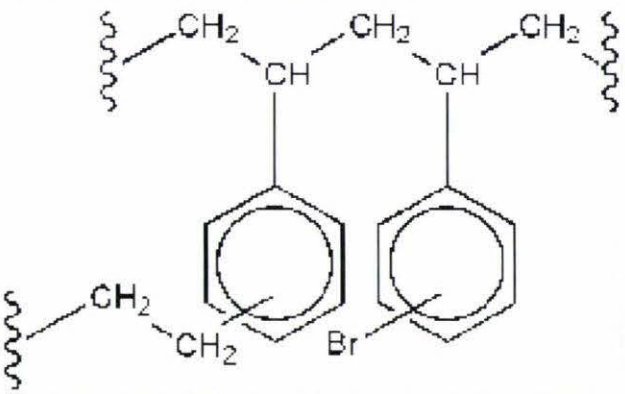

Figure 10 SP207 chemical structure (Mitsubishi Chemical Corporation, 2001)

2.2.1.2 Hydrophilic resins – HP2MG/XAD761/L285

Diaion HP2MG (Figure 11), produced by Mitsubishi Chemical Corporation, is a polymethacrylate ester that is considered an intermediate hydrophilic resin and is used to adsorb hydrophilic organic compounds from polar solvents. The resin is based upon variably cross linked methacrylate ester copolymer, and has relatively hydrophilic characteristics. The polarity of the resin was not available from the manufacturer, therefore the polarity of the structurally similar cross linked methacrylate ester copolymer, XAD7,

Amberlite resin was used for this study (Mitsubishi Chemical Corporation, 2001; Rohm and Haas 2003).

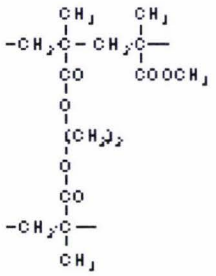

Figure 11 HP2MG chemical structure (Mitsubishi Chemical Corporation, 2001)

XAD761 has a matrix based upon a phenol-formaldehyde polycondensate in which the phenolic hydroxyl groups account for the hydrophilic activity. The maximum operating temperature for XAD761 is 80°C, making it unsuitable for industrial applications where high temperature sterilisation is required. High molecular weight polar, organic substances are absorbed well onto XAD761 (Rohm and Haas, 2006).

The final hydrophilic resin tested was L 285, a hydrophilic functionalized polyaromatic adsorbent which is used for adsorption of very hydrophobic proteins and decolourisation. This resin is used for waste and food industry applications, little information was available from the manufacturer on the resin characteristics (Dowex, 2006).

2.2.2 Adsorption resin use in submerged culture fermentations

Resin addition to fermentation systems has been used to increase the final concentrations of secondary and primary metabolites that are inhibitory to fermentation or their total synthesis. The actual mechanisms for the increase have not been investigated in detail, but it has been suggested that the metabolite is sequestered onto the resin and is thereby stabilised through its removal from the aqueous environment. Additionally it has been suggested that the sequestration of the metabolite onto the resin facilitates its removal from the aqueous phase, thereby reducing any feedback

inhibition that may occur (Warr, *et al.* 1996; Kusunose and Wang, 2004; Marshall *et al.* 1990).

Zhaoyi *et al.* (1997) confirm that hydrophobic/hydrophilic interactions between the resin and metabolite, increase adsorption of the complementary hydrophobic/hydrophilic compound primarily; pore size and surface area are secondary considerations or effects that influence the quantity of metabolite that may be adsorbed per unit mass of the resin.

Jarvis *et al.* (1990) observed that the timing of the resin addition over of the course of fermentation had a major impact on the increase of macrocyclic trichothecenes in *Mycothecium verrucaria* CL-72. By varying the day of resin addition, the levels of production of verrucarin A and J (moderately hydrophilic macrocyclic trichothecenes) could be increased. The earlier addition was better than later addition. Also, depending upon the hydrophilicity of the macrocyclic trichothecenes, the production differed depending upon the hydrophilic resin used. However, these results are not universal and differing cultures may produce different results, *Mycothecium verrucaria* (ATCC 36872) produces less verrucarin A when a hydrophilic resin is used, while precursor concentrations are increased. The results indicated that, in general, higher production occurred when a hydrophilic resin was used with a hydrophilic compound, with the alterative also true for hydrophobic compounds and their complimentary hydrophobic resins (Jarvis *et al.* 1990).

Rachev *et al.* (1997) indicated that production pathways may be altered by the addition of resins to remove precursors, based on hydrophobicity and its properties, to modify the end product of biosynthesis.

Marshall *et al.* (1990) investigated the effect of resin addition during the production of rubradirin. Three XAD resins (XAD2, XAD7 and XAD16) and two HP resins (HP20 and HP21) were added at various times during the fermentation and at varying resin concentrations. Experiments conducted indicated that the two resins, XAD16 (60 g/L) and HP21 (60 g/L), significantly increased rubradirin production when added after 20 hours (Marshall *et al.* 1990).

The use of XAD2 in the production of paulomycin, produced by *Streptomyces paulus* was investigated and resulted in an approximate eight-

fold increase. The improved production concentrations were attributed to adsorption of the paulomycin onto the resin, removing end product inhibition and secondly removing the product from the solution, preventing further modifications by the micro-organism to paulomenol (Marsall *et al.* 1987).

Two adsorption resins (XAD16 and HP 20) were used to improve the production of the secondary metabolite teicoplanin, a large polar molecule produced by *Actinoplanes teicomyceticus*. The addition of HP20 and to a lesser extent XAD16, at the start of the fermentation improved the production of teicoplanin four fold. The improvement in production was attributed to sequestering the toxic end product (removing feedback inhibition) and removing the teicoplanin from the solution and preventing its degradation. Recovery efficiency was also improved by 15% through the reduction in the number of steps required for purification (Lee *et al.* 2003).

The adsorption of a range of amino acids was tested against XAD2 and XAD4. The results indicated that adsorption of the amino acid onto the resin increased as the hydrophobicity of the amino acid increased. Conversely as the hydrophilicity of the amino acid increased the adsorption onto the XAD2 and XAD4 resin decreased. Overall adsorption was greater with XAD4 than with XAD2, due to the greater porosity and surface area of the former (Doulia *et al.* 2001).

Zhaoyi *et al.* (1997) investigated the adsorbent actions of four XAD adsorbent resins on the primary metabolite, napthalene and its derivatives. A correlation was observed that indicated an increase in the surface area of the adsorbent increased the adsorption capacity of the resin. However one adsorbent resin, XAD8, did not follow the surface area assumption. Analysis of napthalene and XAD8 indicated that both compounds are relatively hydrophobic, suggesting that the hydrophobic interactions were related to its increased adsorption capacity. The suggested causative actions of adsorption depend upon:

1 The surface area and hydrophobicity/hydrophilicity of the resin.

2 The solubility, availability and hydrophobicity/hydrophilicity of the target compound (Zhaoyi *et al.* 1997).

2.2.3 Mechanisms of action

The mechanism of adsorption of amino acid on hydrophobic resins was investigated by Doulia *et al.* (2001). By measuring the relative adsorption of eight amino acids a mechanism based on terminal groups and orientation was suggested. The adsorption of amino acids onto XAD resins decreased as the hydrocarbon chain length of the R-group decreased or as the hydrophilic part of the amino acid increased. Of the amino acids tested *D,L*-tryptophan was absorbed at a higher concentration than the other amino acids. The orientation of the adsorption was with the hydrocarbon chain on the surface of the adsorbent particles and the hydrophile directed towards the solution, Figure 12 (Doulia *et al.* 2001).

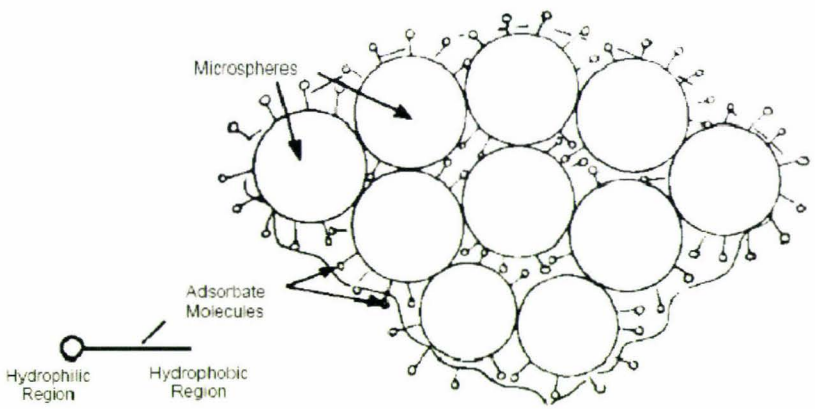

Figure 12 Adsorption onto hydrophobic resin (Sigma 1996)

As the hydrocarbon chain decreased in size the adsorption strength also decreased. However, the addition of an aromatic group had an effect on hydrophobicity of about three and one half methylene groups. Adsorption comparisons between tyrosine and phenylalinine, amino acids with the same hydrocarbon chain lengths but differing terminal groups, showed a lower adsorption of tyrosine due to the polar terminal hydrophilic (OH) group. Doulia *et al.* (2001) concluded that strong negative adsorption from aqueous

solutions on hydrophobic adsorbents could be attributed to the presence of a hydrophilic group and its position on the molecule (Doulia *et al.* 2001).
Lee *et al.* (2003) suggest that teicoplanin was absorbed onto some resins due to its ionic properties primarily and its polarity secondarily.

2.2.4 Specific adsorption resin applications

The *in situ* application of adsorption resins in submerged fermentations is not widespread through the industry; however several researchers have successfully used these systems to improve secondary metabolite production. Selection of adsorption resin in fermentation application is approached through empirical screening system of resins. Comparisons of resins with control fermentations (no resins) are used to determine the optimal adoption resin and its time of addition. A systematic approach for *a priori* prediction of adsorption performance based on properties of the resin and metabolite of interest does not yet exist (Warr *et al.* 1996; Marshall *et al.* 1990; Gerth *et al.* 1994).

2.2.4.1 Anguidine resin applications

Extensive biosynthetic and resin free fermentation studies have been undertaken by Richardson *et al.* (1987) and Altomare *et al.* (1987, 1995). However a search of the published literature did not indicate any fermentation systems that used adsorption resins, in situ, to assist in the production of anguidine.
The macrocycle trichothecenes verrucarin J and roridin A, produced by *Mycothecium verrucaria* in submerged culture, have been investigated with a range of hydrophobic and hydrophilic adsorption resins. Verrucarin J is a moderately non-polar compound, while roridin A is a moderately polar compound. Jarvis *et al.* (1990) identified that the moderately non-polar absorption resins were ineffective at adsorbing roridin A. Alternatively the more polar resins XAD7, XAD8 and CG161, were effective at absorbing more than 80% of the total trichothecenes produced from the fermentation broth. Relative adsorption of the three resins indicated that XAD8 was less

efficient, due to its smaller surface area. Upon addition of the resins XAD7 and CG161 to the fermentation the final concentration of verrucarin was increased by 75-100%, with CG161 increasing the concentration more effectively than XAD7. This increase may be due to the improved quality under which CG161 is produced, leading to a more uniform matrix and surface area (Jarvis *et al.* 1990; Rohm and Haas 2002).

2.2.4.2 Rapamycin resin applications

There has been no published work on the use of adsorption resins for the production of rapamycin in a submerged fermentation system.

2.2.4.3 Ansamitocin P3 resin applications

There has been no published work on the use of adsorption resins for the production of ansamitocin P3 in a submerged fermentation system.

2.3 Metabolite descriptions and interactions

2.3.1 Lipophilicity

Lipophilicity (Log *P*) is used to identify the molecular hydrophobicity of a compound for drug design and interaction modelling and is used exclusively for Quantitative Structure-Property Relationship (QSPR) studies. The Log *P* of a compound is the logarithm of the partition coefficient in *n*-octanol / water and this value can be calculated either experimentally or by various computational methods. The higher the value of Log *P* the more hydrophobic is the compound. This value allows the compound's hydrophobicity to be compared using a standard technique. There are at least nine computer models that are currently being used by researchers to calculate theoretical Log *P* values. Four programs, XLOGP, LogKow, CLOGP, IA LogP and CSLogP have been identified as predicting within 0.5% of the experimental value (Medic-Saric *et al.* 2004; Hansch and Fujita 1963).

The Log *P* values used in this thesis were obtained using Simplified Molecular Input Line Entry System (SMILES) entries for the three secondary metabolites into Molinspiration version 2 (Molinspiration Property Calculation Service, Medic-Saric *et al.* 2004). Alternatively, the structure can be inputted directly into the program to calculate the Log *P* values (Medic-Saric *et al.* 2004).

2.3.2 Hydrophobic/hydrophilic interactions

The mechanism by which neutral resins adsorb secondary metabolites is by hydrophobic interactions. Due to the aqueous (hydrophilic) environment of a submerged fermentation, the hydrophobic components of the resin and secondary metabolite group together by hydrophobic interactions. The driving force of the interaction is the entropy of water and the lowering of the energy state between the hydrophobic components. Recovery of metabolites from resins requires a non-polar solvent to be used (Stryer, 1981).

3 Materials and Methods

3.1 Model system for cultivation studies

3.1.1 Organism

3.1.1.1 *Fusarium sambucinum*

This organism was obtained from American Type Culture Collection (ATCC) and is designated as ATCC 64043 in their culture collection. Stock cultures were stored in 1mL aliquots in 10% glycerol solutions and held at -80°C. The medium used for propagation is described in section 3.1.2.1.

3.1.1.2 *Streptomyces hygroscopicus*

This organism was obtained by mutation of the wild type and is designated IRL362B. Stock cultures were stored in 1mL aliquots in 10% glycerol solutions and held at -80°C. The medium used for propagation is described in section 3.1.2.2.

3.1.1.3 *Actinosynnema pretiosum*

This organism was obtained from ATCC and is designated ATCC 31565. Stock cultures were stored in 1mL aliquots in 10% glycerol solutions and held at -80°. The medium used for propagation is described in section 3.1.2.3.

3.1.2 Cultivation medium

3.1.2.1 Cultivation medium for *F. sambucinum*

The seed media (GHY, Appendix 1) consists of glucose 10 g, Hy soy 1 g and yeast extract 1 g in 1L of reverse osmosis (RO) water and adjusted to pH 5.4 before being autoclaved at 121°C for 20 minutes (Ueno *et al.* 1975). The control medium used for the initial production of anguidine and growth studies for *F. sambucinum* was obtained from the literature (Ueno *et al.*

1975). The defined medium (DMP1 medium, Appendix 1) was developed by IRL-Biopharm. Erlenmeyer flasks (250mL) containing 50mL of the medium per flask were stoppered with Bioshield[tm] (0.2 μm pore size biological paper) and autoclaved for 20 minutes at 121°C.

Glucose was obtained from Roquette (Lestrem, France), Hy Soy was obtained from Kerry Bioscience (Hoffman Estates, IL, USA), yeast extract was obtained from Oxoid (Basingstoke, Hampshire, UK), SAG471 antifoam was obtained from GE Advance (Wilton, CT, USA) and all other medium components were obtained from BDH (Poole, Dorset, UK).

3.1.2.2 Cultivation medium for *S. hygroscopicus*

The production and seed media for *S. hygroscopicus* were developed by IRL Biopharm. The seed media (TJ media, Appendix 1) consists of tomato juice 200 g and calcium carbonate 3 g in 1L of RO water and adjusted to pH 6.8 with 1 M sodium hydroxide before being autoclaved at 121°C for 20 minutes.

The production media (SYLGG, Appendix 1) consists of Hy Soy 10 g, yeast extract 6.5 g, L-Lysine 6.5g, di-potassium orthophosphate 1.14 g, potassium di-hydrogen orthophosphate 0.7 g, sodium chloride 5 g, glycerol 30 g, iron sulphate 0.01 g and SAG antifoam 0.1 mL in 1 L of RO water and adjusted to pH 6 using 4 M sulphuric acid. Erlenmeyer flasks (250mL) containing 50mL of the medium per flask were stopped with Bioshield[tm] (0.2 μm pore size biological paper) and autoclaved for 20 minutes at 121°C.

All other media components were sourced as previously specified in section 3.1.2.1.

3.1.2.3 Cultivation medium for *A. pretiosum*

The production and seed media for *A. pretiosum* were obtained from Tandia *et al.* (1980). The seed media (Appendix 1) consisted of dextrose monohydrate 20 g, soluble starch 30 g, soybean flour 10 g, solulys steep liquor 10g, sodium chloride 3g and calcium carbonate 5g in 1L of RO water autoclaved at 121°C for 20 minutes.

The production media (Appendix 1) consists of Avon malt 70 g, proflo (powder) 10 g, soy flour 1 g, sodium acetate 0.1 g, ammonium sulphate 0.5

g, di-potassium hydrogen orthophosphate 0.6 g, potassium di-hydrogen orthophosphate 0.4 g, calcium chloride 5 g, SAG471 antifoam 0.1 mL in 1 litre of RO water. Erlenmeyer flasks (250mL) containing 50mL of the medium per flask were stopped with Bioshield^{tm} (0.2 µm pore size biological paper) and autoclaved for 20 minutes at 121°C.

Avon malt was obtained from Penfords (Taranaki, NZ), solulys steep liquor was obtained from Roquette (Lestrem, France), proflo was obtained from Trader's Protein (Memphis, TN) and soyflour was obtained from ADM (Decatur, IL). All other media components were sourced as previously specified in section 3.1.2.1.

3.1.3 Adsorption resins

A range of commercially available polymer resins were obtained to conduct binding studies with. XAD2, XAD16, XAD1180 and XAD761 were obtained from Rohm and Haas (Philadelphia, PA, USA). Diaion HP 2MG and Sepabeads SP207 were obtained from Mitsubishi (Minato-ku, Tokyo, Japan). Dowex Optipore L-285 was obtained from Dow chemicals (Midlands, MI, USA).

The adsorption resins were selected for their ease of supply, differing pore sizes, surface areas and modes of actions.

The adsorption resins were solvent washed with each solvent being removed through vacuum draining in a Buckner funnel before the addition of the next solvent. The quantity of solvent used is related directly to the number of volume of resin required. The solvent order for washing was 3 volumes of water, 2 volumes of ethanol, 1 volume of methanol, 1 volume of *iso*-propanol and finally 5 volumes of water. Resin washing is required to remove residual chemicals left over from the manufacture of the resins. From each adsorption resin type a specific amount of wet resin (0.15 mL) was added to 2 mL of purified water and then autoclaved at 121°C for 30 min. The resin slurry was added aseptically to the shake flask as needed.

3.2 Protocol for cultivation studies

3.2.1 *F. sambucinum* cultivation

Inoculum preparation

The inoculum for the shake flask system was prepared using the following protocol to 3.1.2.1. The number of shake flasks required for the inoculum varied depending upon the volume needed. A number of 2000mL unbaffeled shake flasks were prepared and charged with 250mL of GHY seed medium. The shake flask opening was covered with Bioshield and cheesecloth. The flasks were sterilised at 121°C for 20 minutes in an autoclave. The shake flasks were inoculated with 0.1mL of the spore stock and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 26mm throw for 3 days for the primary seed. The secondary seed shake flasks were inoculated with 4% (v/v) of the primary seed and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 26mm throw for 1 days.

50mL of DMP1 (Appendix 1) production medium was inoculated with 2 mL (4 % v/v) of the inoculum seed in a 250 mL Erlenmeyer flask. The shake flask was incubated at 28°C and 200 rpm on a rotary shaker incubator with a 26mm throw for 5 days.

3.2.1 *S. hygroscopicus* cultivation

Inoculum preparation

The inoculum for the shake flask system was prepared using the following protocol to 3.1.2.3. The number of shake flasks required for the inoculum varied depending upon the transfer volume. A number of 2000mL unbaffeled shake flasks were prepared and charged with 250mL of RAPA seed medium (Appendix 1). The shake flask opening was covered with Bioshield and cheesecloth. The flasks were sterilised at 121°C for 20

minutes in an autoclave. The shake flasks were inoculated with 0.1mL of the spore stock and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 26mm throw for 3 days. The secondary seed shake flasks were inoculated with 4% (v/v) of the primary seed and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 26mm throw for 1 days.

50mL of SYLGG (Appendix 1) production medium was inoculated with 3 mL (6 % v/v) of the inoculum seed in a 250 mL Erlenmeyer flask. The shake flask was incubated at 28°C and 200 rpm on a rotary shaker incubator with a 26mm throw for 7 days.

3.2.1 *A. pretiosum* cultivation

Inoculum preparation

The inoculum for the shake flask system was prepared using the following protocol to 3.1.2.3. The number of shake flasks required for the inoculum varied depending upon the transfer volume. A number of 2000mL unbaffeled shake flasks were prepared and charged with 250mL of AP3 seed medium. The shake flask opening was covered with Bioshield and cheesecloth. The flasks were sterilised at 121°C for 20 minutes in an autoclave. The shake flasks were inoculated with 0.1mL of the spore stock and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 5cm throw for 3 days.

The secondary seed shake flasks were inoculated with 4% (v/v) of the primary seed and incubated at 28°C and agitated at 200rpm on a rotary shaker incubator (Multitron 2, Infors, Bottmingen, Switzerland) with a 26mm throw for 1 days.

50mL of ansamitocin P3 production medium (Appendix 1) was inoculated with 3 mL (6 % v/v) of the inoculum seed in a 250 mL Erlenmeyer flask. The shake flask was incubated at 28°C and 200 rpm on a rotary shaker incubator with a 26mm throw for 8 days.

3.3 Measurements

3.3.1 Anguidine concentration

The analysis of anguidine was conducted on a Waters High Performance Liquid Chromatography (HPLC) using an Agilnet 1100 series DAD wavelength detector set at 205nm. The gradient analysis conditions were 30-90% by volume acetonitrile/water gradient over a 30 minute period at a flow rate of 1 mL/min on a Zorbax XDB-C8 150 x 4.6mm column with a C8 AJO-4290 guard column at room temperature. Standard curves for anguidine were generated by injecting a known amount of pure anguidine (>98% by HPLC) produced by IRLBiopharm.

The fermentation broth was sampled at specific time periods by removing 3 mL of broth and adding 3 mL of ethyl acetate, vortexed (Glas-Col, VB4, IN, USA) mixed on high (2000 rpm) for 10 minutes then centrifuging (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. A sample of 1 mL was removed, evaporated at room temperature under nitrogen and redissolved in 100% acetonitrile, and analysed by HPLC with 10 µl injections using the method described above.

3.3.2 Rapamycin concentration

The analysis of rapamycin was conducted on a Waters High Performance Liquid Chromatography (HPLC) using an Agilnet 1100 series DAD wavelength detector set at 280nm. The isocratic analysis conditions were 80/20% by volume methanol/water over a 20 minute period at a flow rate of 1 mL/min on a Phenomenex Hypersil C8 250 x 4.6mm column with a C8 AJO-4290 guard column at room temperature. Standard curves for rapamycin were generated by injecting a know amount of pure rapamycin (>98% by HPLC) produced by IRLBiopharm.

The fermentation broth was sampled at specific time periods by removing 3mL of broth and adding 3mL of acetone, homogenized at 1000rpm for 1 minute (Ultra-Turrex T-25, IKA, Staufen, Germany) then centrifuging (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. A sample of 1 mL was removed, and analysed by HPLC with 10 µl injections using the method described above.

3.3.3 Ansamitocin P3 concentration

The analysis of ansamitocin P3 was conducted on a Waters High Performance Liquid Chromatography (HPLC) using an Agilnet 1100 series DAD wavelength detector set at 252nm. The isocratic analysis conditions were 55/45% by volume acetonitrile/water over a 10 minute period at a flow rate of 1 mL/min on a Phenomenex Luna C8 150 x 4.6mm column with a C8 AJO-4290 guard column at room temperature. Standard curves for ansamitocin P3 were generated by injecting a known amount of pure ansamitocin P3 (95%) supplied by Sigma (Sydney, Australia).
The fermentation broth was sampled at specific time periods by removing 3mL of broth and adding 3mL of ethanol, vortexed (Glas-Col, VB4, IN, USA) mixed on high (2000 rpm) for 10 minutes then centrifuged (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. A sample of 1 mL of the supernatant was removed, and analysed by HPLC with 10 µl injections using the method described above.

3.3.4 Cell mass and broth chemical composition

Cell biomass was determined by gravimetric analysis (2576 g for 5 minutes). Biomass was represented as a percentage of cell biomass to supernatant, packed cell volume (PCV %).

3.4 Experimental protocol

As stated in the Introduction, section 1.2, the specific objectives of this work are to:

1. Screen a range of commercially available adsorption resins against anguidine to identify resins with a high adsorption affinity and maximum adsorption for anguidine (Section 3.4.1);
2. Propose and identify a set of selection criteria for use of adsorption resins in submerged cultures for secondary metabolite production (Section 3.4.2);
3. Characterise the fermentation system of *Fusarium sambucinum* in the presence of adsorption resins to identify the optimal conditions for maximum anguidine production based on results from point 2 (Section 4.1.1);
4. Confirm the resin selection methodology in *Streptomyces hygroscopicus* fermentation for the production of rapamycin and *Actinomyces preitosum* fermentation for the production of ansamitocin P3 (Section 4.1.2 – Section 4.1.3).

3.4.1 Adsorption of secondary metabolites using adsorption resins in aqueous phase

To improve the production and stability of the selected metabolites (anguidine, rapamycin, ansamitocin P3) the approach used was to experimentally identify the maximum adsorption capacity for each metabolite on the various resins in an aqueous phase. This data would then be used to identify significant trends to describe the interactions between the metabolites and the various resins. The results would then be used to attempt to predict the optimal resin to be added for increased metabolite production in shake flask fermentation. A series of common adsorption resins were selected based on their pore volume, surface area and polarity, Table 3.

Table 3 Adsorption resin surface areas and pore sizes.

Resin	Pore Volume (mL/g)	Surface Area (m^2/g)	Dipole Moment (Debye)
XAD2	0.65	330	0.4
XAD16	0.8	825	0.4
HP2MG	1.2	500	0.8
XAD761	1	200	1.6
SP207	1.82	630	0.3
XAD1180	1.68	500	0.5
L285	unknown	800	unknown

(Excerpt from Table 2)

3.4.1.1 Experiment 1

27.5 mL of a fermentation medium DMP1 (Appendix 1) was dispensed into a 50 mL Falcon tube. A stock solution of 95% pure anguidine was prepared in DMSO to a concentration of 7.6 mg/mL. 2.5 mL of the anguidine concentrate solution was added to the bulk fermentation media of 27.5 mL, to yield a spiked anguidine/fermentation broth containing a concentration of 633 µg/mL of anguidine.

The seven resins tested were prepared, section 3.1.3, and suspended into a solution of RO water to a ratio of 1mL resin to 0.5 mL of RO water. A volume of 0.15 mL (50 mL of resin per litre of broth) of each adsorption resin/water mixture was added to an individual 10 mL extraction tube, with one tube receiving no resin, but 0.15mL of RO water to act as the control. A 3 mL sample of spiked anguidine/fermentation media was added to each 10 mL extraction tube containing either resin or the RO water control and was agitated for 1 hour on a micro-vortex at room temperature and then centrifuged (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. The supernatants were decanted into separate tubes.

The resins and supernatants were then analysed independently for anguidine concentration using the method in section 3.3.1.

3.4.1.2 Experiment 2

27.5 mL of a fermentation medium SLYGG (appendix 1) was dispensed into a 50 mL falcon tubes. A stock solution of 95% pure rapamycin was prepared in DMSO to a concentration of 5.1 mg/mL. 2.5 mL of the rapamycin concentrate solution was added to the bulk fermentation media of 27.5 mL, to yield a spiked rapamycin/fermentation broth containing a concentration of 425 µg/mL of rapamycin.

The seven resins tested were prepared, section 3.1.3, and suspended into a solution of RO water to a ratio of 1mL resin to 0.5 mL of RO water. A volume of 0.15 mL (50 mL of resin per litre of broth) of each adsorption resin/water mixture was added to an individual 10 mL extraction tube, with one tube receiving no resin, but 0.15mL of RO water to act as the control. A 3 mL sample of spiked rapamycin/fermentation media was added to each 10 mL extraction tube and was agitated for 1 hour on a micro-vortex at room temperature and then centrifuge (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. The supernatants were decanted into separate tubes.

The resins and supernatants were then analysed independently for rapamycin concentration using the method in section 3.3.2.

3.4.1.3 Experiment 3

27.5 mL of ansamitocin P3 production medium (Appendix 1) was dispensed into a 50 mL Falcon tubes. A concentrated solution of 95% pure ansamitocin P3 was prepared in DMSO to a concentration of 4.6 mg/mL. 2.5 mL of the ansamitocin P3 concentrate solution was added to the bulk fermentation media of 27.5 mL, to yield a spiked ansamitocin P3/fermentation broth containing a concentration of 383 µg/mL of ansamitocin P3.

The seven resins tested were prepared, section 3.1.3, and suspended into a solution of RO water to a ratio of 1mL resin to 0.5 mL of RO water. A volume of 0.15 mL (50 mL of resin per litre of broth) of each adsorption resin/water mixture was added to an individual 10 mL extraction tube, with one tube receiving no resin, but 0.15mL of RO water to act as the control.

A 3 mL sample of spiked ansamitocin P3/fermentation media was added to each 10 mL extraction tube and was agitated for 1 hour on a micro-vortex at room temperature and then centrifuge (Biofuge pico, Heraus, Buckinghamshire, UK) at 2576 g for 5 minutes. The supernatants were decanted into separate tubes.

The resins and supernatants were then analysed independently for ansamitocin P3 concentration using the method in section 3.3.3.

3.4.2 Addition of adsorption resin to fermentation system to enhance final concentration of metabolite

3.4.2.1 Experiment 4

Results of Experiment 1 (section 3.4.1.1) indicated that the resin SP207 had the highest adsorption capacity for purified anguidine (12.5 mg anguidine per mL of resin) and the resin XAD1180 had the second highest adsorption capacity (i.e. 11.3 mg anguidine per mL of resin). These two resins were used to conduct an in situ study on anguidine production in actual fermentation in the presence of the resins.

XAD1180 and SP207 resins were added to individual shake flasks (in duplicate) on day 0, 1, 2, 3 and 4 of the 5 day fermentation, with an additional 2 shake flasks containing no resin. At day 5 all shake flasks were assayed for anguidine and biomass concentrations.

The 50 mL working volume (200 mL total volume) primary seed medium shake flasks were inoculated with 0.1 mL of spore suspension per shake flask. The shake flasks were incubated at 28°C, 200 rpm (26 mm throw) for 3 days.

The 200 mL working volume (2000 mL total volume) secondary seed medium shake flask was inoculated with 8 mL of the primary seed medium and incubated at 28°C, 200 rpm (26 mm throw)for 1 day.

A total of 24 production shake flasks with a 50 mL working volume (200mL total volume) were inoculated with 2mL of the secondary seed medium and incubated at 28°C and 200 rpm (26 mm throw). The resins tested in the production flasks as specified earlier.

To a set of two shake flasks, two sterile 2.5 mL aliquots of SP207 were added daily on day 0, 1, 2, 3 and 4. The resin additions were also conducted for XAD1180 in the same manner as SP207. All shake flasks were harvested on day 5 (two duplicate resin additions for days 0, 1, 2, 3 and 4 for both XAD1180 and SP207 plus a control of two shake flasks containing no resin) and were analysed for anguidine concentration using the method in Section 3.3.1.

3.4.2.2 Experiment 5

This experiment was analogous to experiment 4 (section 3.4.2.1), except that purified rapamycin was used as the selection metabolite instead of anguidine. The resin HP2MG was identified as having the highest adsorption capacity for rapamycin (4.6 mg rapamycin per mL of resin) and XAD1180 was identified as having the fourth highest adsorption capacity (2.5 mg rapamycin per mL of resin). These two resins were used to conduct an in situ study of rapamycin production in actual fermentation in the presence of the resins.

HP2MG and XAD1180 resins were added to individual shake flasks (in duplicate) on day 0, 1, 2, 3, 4, 5 and 6 of a 7 day fermentation, with an additional 2 shake flasks containing no resin. At day 7 all shake flasks were assayed for rapamycin and biomass concentrations

The 50 mL working volume (200 mL total volume) primary seed medium shake flask were inoculated with 0.1 mL of spore suspension per shake flask. The shakes flasks were incubated at 28°C, 200 rpm (26 mm throw) for 3 days.

The 200 mL working volume (2000 mL total volume) secondary seed medium shake flask was inoculated with 8 mL of the primary seed medium and incubated at 28°C, 200 rpm (26 mm throw) for 1 day.

A total of 28 production shake flasks with a 50 mL working volume (200mL total volume) were inoculated with 2mL of the secondary seed medium and incubated at 28°C and 200 rpm (26 mm throw). The resins tested in the production flasks as specified earlier.

To a set of two shake flasks, two sterile 2.5 mL aliquots of HP2MG were added daily on day 0, 1, 2, 3, 4, 5 and 6. The resin additions were also conducted for XAD1180 in the same manner as HP2MG. All shake flasks were harvested on day 7 (two duplicate resin additions for days 0, 1, 2, 3, 4, 5 and 6 for both XAD1180 and HP2MG plus a control of two shake flasks containing no resin) and were analysed for rapamycin concentration using the method in Section 3.3.2.

3.4.2.3 Experiment 6

This experiment was analogous to experiment 4 (section 3.4.2.1), except that purified ansamitocin P3 was used as the selection metabolite instead of anguidine. HP2MG was identified as having the highest adsorption capacity for ansamitocin P3 (6.8 mg ansamitocin P3 per mL of resin) and XAD16 the fourth highest adsorption capacity (5.1 mg ansamitocin P3 per mL of resin). These two resins were used to conduct an in situ study on the effects of ansamitocin P3 production in the presence of the resins.

HP2MG and XAD16 resin were added to individual shake flasks (in duplicate) on day 0, 1, 2, 3, 4, 5, and day 6 of the 7 day fermentation, with an additional 2 shake flasks containing no resin. At day 7 all shake flasks were assayed for ansamitocin P3 and biomass concentrations.

The 50 mL working volume (200 mL total volume) primary seed medium shake flask were inoculated with 0.1 mL of spore suspension per shake flask. The shakes flasks were incubated at 28°C, 200 rpm (26 mm throw) for 3 days.

The 200 mL working volume (2000 mL total volume) secondary seed medium shake flask was inoculated with 8 mL of the primary seed medium and incubated at 28°C, 200 rpm (26 mm throw)for 1 day.

A total of 30 production shake flasks with a 50 mL working volume (200mL total volume) were inoculated with 3 mL of the secondary seed medium and incubated at 28°C and 200 rpm (26 mm throw). The resins tested in the production flasks as specified earlier.

To a set of two shake flasks, two sterile 2.5 mL aliquots of XAD16 were added daily on day 0, 1, 2, 3, 4, 5, and 6. The resin additions were also

conducted for HP2MG in the same manner as XAD16. Two additional shake flasks were All shake flasks were harvested on day 7 (two duplicate resin additions for days 0, 1, 2, 3, 4, 5, and 6 for both HP2MG and XAD16 plus a two resin free control shake flasks) and were analysed for anguidine concentration using the method in section 3.3.3.

4 Results and Discussion

4.1 Adsorption of secondary metabolites using adsorption resins in aqueous phase

4.1.1 Experiment 1

Seven resins were used, as described in Section 3.4.2.1, to identify the maximum adsorption of anguidine. Each resin is a complex matrix of cross-linked aromatic polymers, styrene divinylbenzene matrixes, phenol-formaldehyde polycondensate or polymethacrylates, depending upon the resin type. A varied amount of adsorption was observed across the range of resins tested, Figure 13.

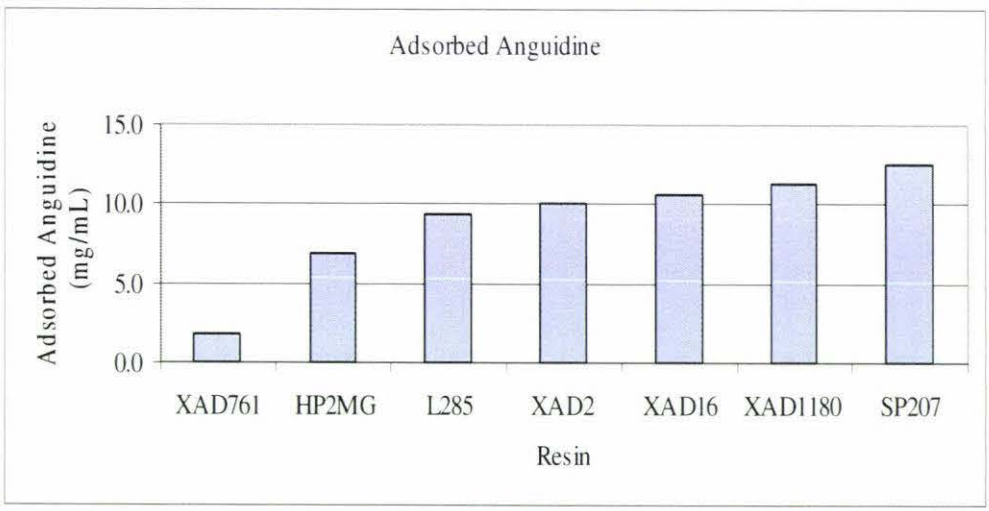

Figure 13 Adsorption of anguidine for various resins as maximum adsorption per mL of resin

The XAD and SP hydrophobic resins adsorbed the highest concentration of anguidine, the intermediate hydrophobic resins (i.e. HP2MG, L285) absorbed anguidine less effectively and the hydrophilic resin (i.e. XAD761) poorly absorbed anguidine. Doulia *et al.* (2001), Lee *et al,* (2003),and Zhaoyi *et al.* (1997) have observed this correlation for a range of

hydrophobic compounds. As the compounds become less hydrophobic the optimal resins for improved absorption were decreasingly hydrophobic.
The internal and external surface areas for each resin were calculated where possible, a pore volume for L285 was not supplied and therefore the internal surface area could not be estimated. A plot of the total surface area per mL of resin versus anguidine adsorption per mL of resin indicated that as the surface area increased the maximum adsorption increased, though L285 adsorption per unit area was significantly lower than the other resins tested, Figure 14.

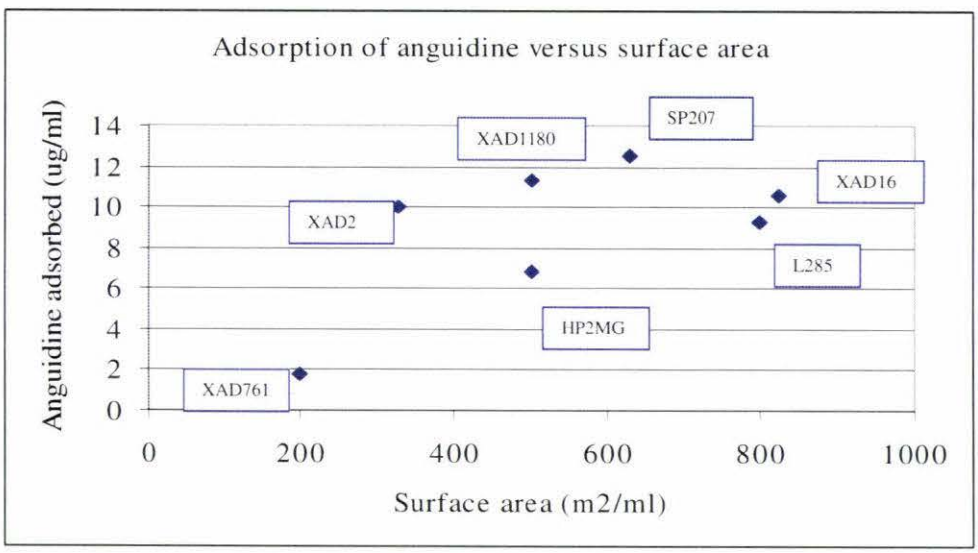

Figure 14 Adsorption resin comparison for adsorbing of anguidine and total surface area per mL of resin

XAD761 is a hydrophilic adsorption resin with the smallest surface area of the resins tested. The maximum adsorption of XAD761 was also the lowest of the resins tested; it is unknown, however, whether this is as a result of the resins mode of action or surface area. HP2MG was the resin with the second lowest maximum adsorption. This resin is a moderately hydrophobic resin with a moderately high surface area compared to the other resins tested. The amount of anguidine absorbed by SP207 (a super hydrophobic resin) is significantly more than the other XAD resins and also

has the greatest surface area. Of the other hydrophobic XAD adsorption resins tested, XAD2, XAD1180 and XAD16, the maximum adsorption did not vary with an increase to the surface area.

The results indicated that while a high surface area is required for a greater maximum adsorption, the adsorption resins mode of action has a significant impact of the adsorption of anguidine.

The diameter of the pore for each resin tested was plotted against the amount of anguidine adsorbed, Figure 15.

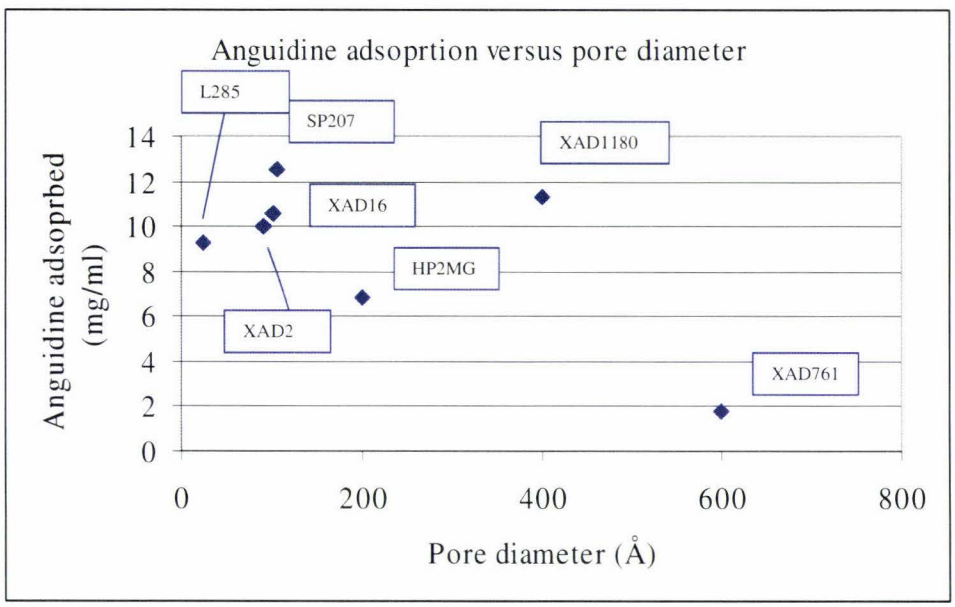

Figure 15 Adsorption of anguidine compared to pore size

There was no correlation between the size of the pore and the amount of anguidine adsorbed, confirming the analysis obtained from the surface area plot. A small surface area was generally found to reduce anguidine adsorption, though the resin HP2MG absorbed a lower concentration. The data suggests that the surface area and pore size are clearly not the only factors that increase adsorption on polymeric resins. For anguidine the most likely mechanism for improved adsorption appears to be hydrophobic interactions.

4.1.2 Experiment 2

Seven resins were used, as described in Section 3.4.2.2, to identify the maximum adsorption of rapamycin, Figure 16.

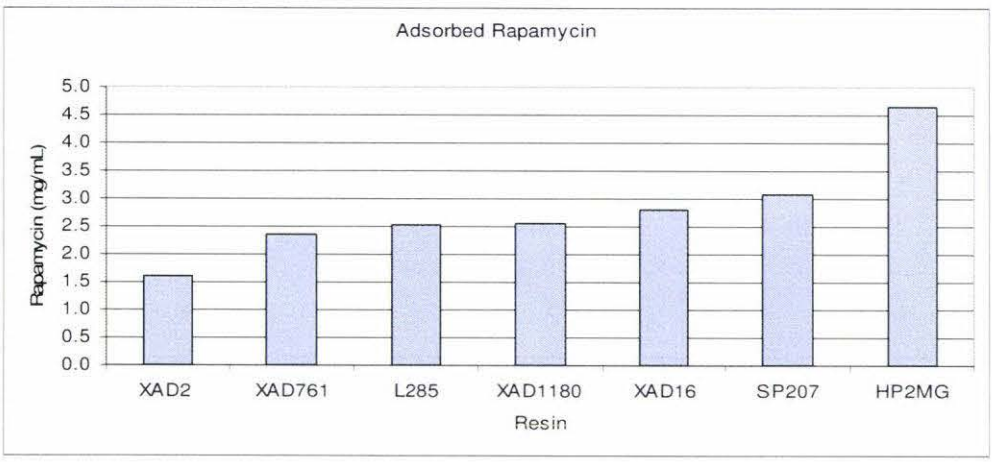

Figure 16 Adsorption of rapamycin per resin type as maximum adsorption per mL of resin

HP2MG absorbed significantly more rapamycin than any other resin. The other resins absorbed approximately similar amount of rapamycin, but XAD2 absorbed the least amount.

A plot of the total surface area per mL of each resin and maximum rapamycin absorbed, indicated that adsorption increased as the specific surface area increased somewhat, Figure 17.

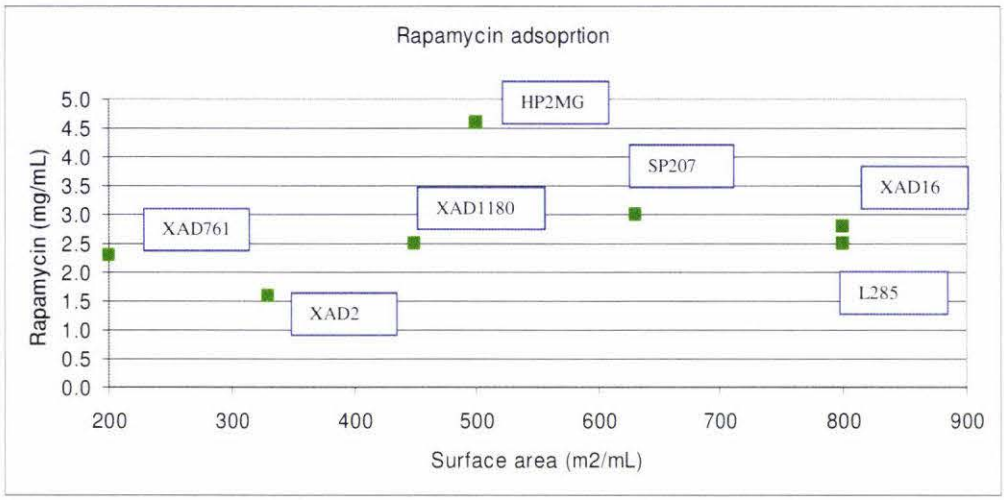

Figure 17 Adsorption resin comparison of absorbed rapamycin and surface area

Of the hydrophobic resins tested, XAD16, XAD1180 and SP207 absorbed similar amounts of rapamycin per ml of resin added. Compared with the other hydrophobic resins XAD2 absorbed approximately half the amount of rapamycin, but also had more than half the specific adsorption area compared with the structurally equivalent resin XAD16. The amount of rapamycin absorbed by HP2MG was significantly more than the other XAD resins of similar surface areas. Rapamycin is the largest of the secondary metabolites tested and correspondingly a lower amount of it is absorbed onto the resins compared with adsorption of anguidine on the same resins.

The maximum adsorption of rapamycin onto the tested resins was not directly influenced by the surface area. HP2MG, a moderately hydrophilic resin, absorbed the highest concentration of rapamycin, with SP207, a hydrophobic resin, adsorbing the second highest concentration. The data suggests that the type of interaction occurring between the resin and the compound is critical for determining the extent of adsorption.

4.1.3 Experiment 3

Seven resins were used, as described in Section 3.4.2.3, to identify the maximum adsorption of ansamitocin P3, Figure 18.

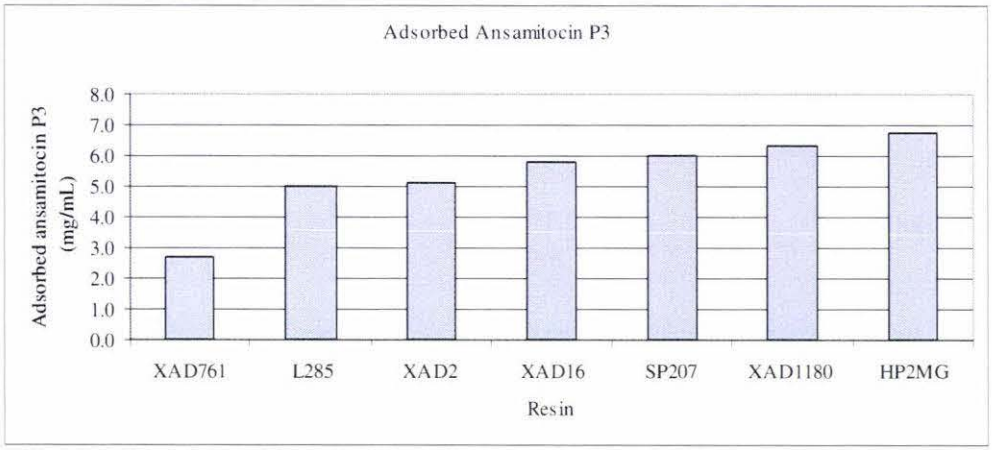

Figure 18 Adsorption of ansamitocin P3 (AP3) per resin type as maximum adsorption per mL of resin

The maximum adsorption of ansamitocin P3 onto all of the resins tested, except XAD761, was similar. XAD761 absorbed approximately a third of the maximum ansamitocin P3 compared to HP2MG.

A plot of the total surface area per mL of resin and the maximum ansamitocin P3 adsorbed per mL indicated that as the surface area increased the amount of ansamitocin P3 absorbed increased slightly, Figure 19.

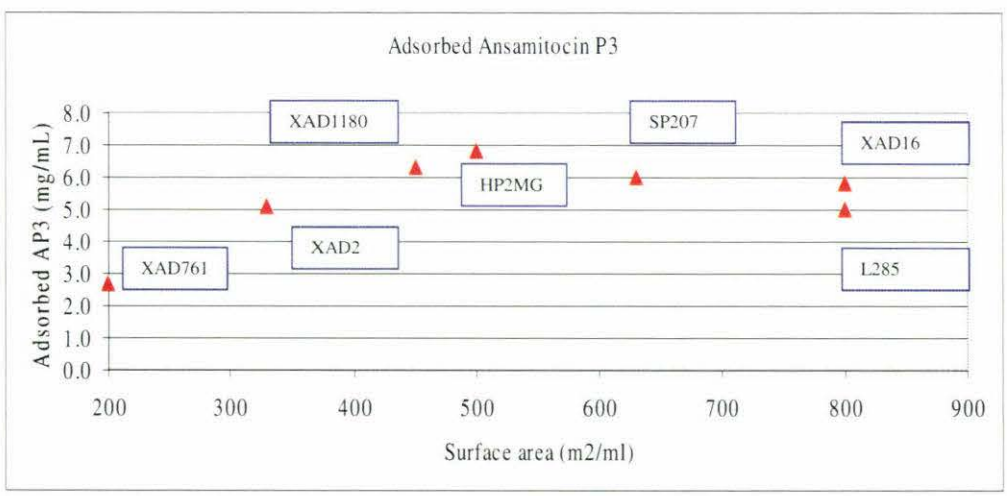

Figure 19 Adsorption resin comparison of absorbed ansamitocin P3 and surface area

As the surface area increased from 200 to 500 m^2/mL the maximum adsorption increased. However, above 500 m^2/mL there was no noticeable change in the adsorption of ansamitocin P3. The results indicate that the

adsorption of ansamitocin P3 is not exclusively influenced by surface area, though a low surface area is not optimal.

However, as seen with anguidine and rapamycin, an increase in surface area does not automatically equate to an increase in adsorption capacity.

4.1.4 Discussion of adsorption data

Analysis of adsorption data for anguidine, rapamycin and ansamitocin P3 indicated that while a large surface area was beneficial, there was no direct correlation between surface area and an increased metabolite adsorption that could be used to assist in a model that predicts an optimal metabolite-resin system, Figure 20.

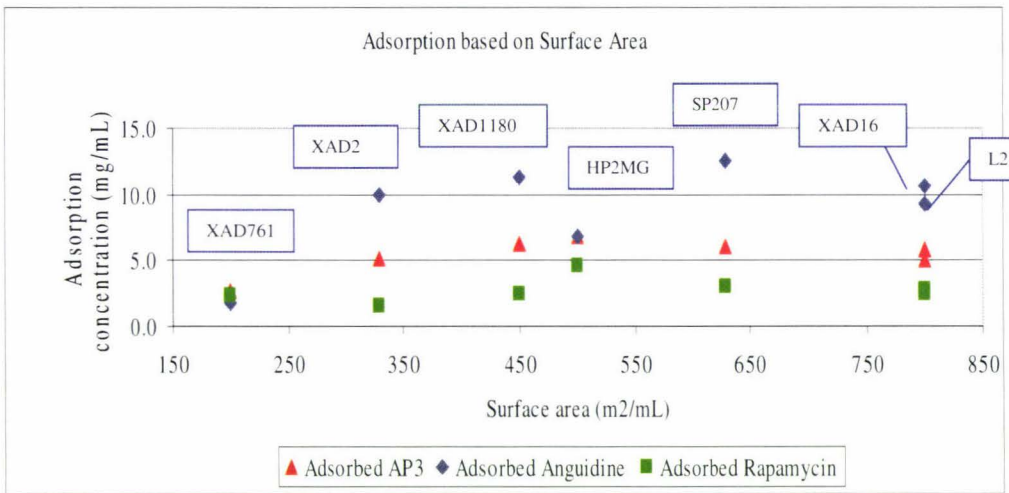

Figure 20 Maximum adsorption of three secondary metabolites on resins with differing surface areas

The maximum adsorption of the secondary metabolites analysed were not universally influenced by surface area. For example, HP2MG absorbed the highest concentration of ansamitocin P3, with XAD1180 adsorbing the second highest concentration. HP2MG is classed as a moderately hydrophilic resin with a surface area of 500 m^2/mL, whilst XAD1180 is identified as a hydrophobic resin with a surface area of 450 m^2/mL. The results are similar to those obtained with rapamycin. Anguidine however, appears to adsorb in a linear fashion with surface area, with the exception of the resin HP2MG, that adsorbs considerably less for a resin with a large

surface area. The data suggests that there are complex interactions that determine the extent of adsorption of ansamitocin P3 on the resin.

While the relationship between the surface area and the maximum adsorption is critical for optimal resin selection, a number of anomalies are observed, specifically in regards to the hydrophilic resin, which cannot be explained by the metabolite size and resin surface areas. As the two resins in question are different to the others tested through their mechanism of bonding, the critical component to a predictability model for adsorption resin selection requires this to be taken into account.

4.1.5 Maximum adsorption analysis using dipole moment

The discussion in the previous section indicated that the nature of interactions (hydrophobic and hydrophilic) between the resin and the metabolite may be the principal factor controlling the extent of adsorption.

Based on the expectations that the dipole moment, a descriptor of the relative hydrophobicity of a resin, is a critical parameter for adsorption each compound per unit area of resin was plotted against resin polarity, Figure 21. The dipole moment of each resin was obtained from the manufacturer, with the exception of HP2MG and SP207, where a value could only be inferred from a structurally similar resin. The manufacturer for L285 was unable to provide a dipole moment for the resin.

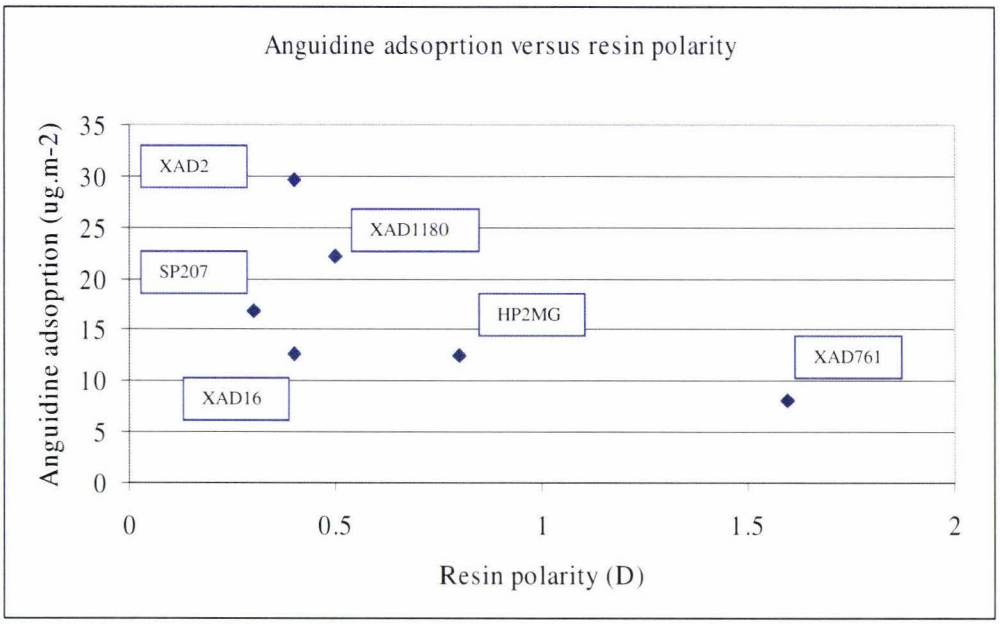

Figure 21 Maximum adsorption of anguidine based on resin polarity

Figure 21 suggests that for the adsorption of anguidine there is a correlation between the polarity of the resin and the amount adsorbed per unit area. The adsorption of anguidine decreased as the polarity of the resin increased. Within resins that had the same structural matrix and therefore dipole moment, (i.e. XAD2 and XAD16), the resin with a lower surface area per mL of resin, XAD2, adsorbed more anguidine.

The maximum adsorption of rapamycin was plotted in a like manner against the polarity of the resins used, Figure 22.

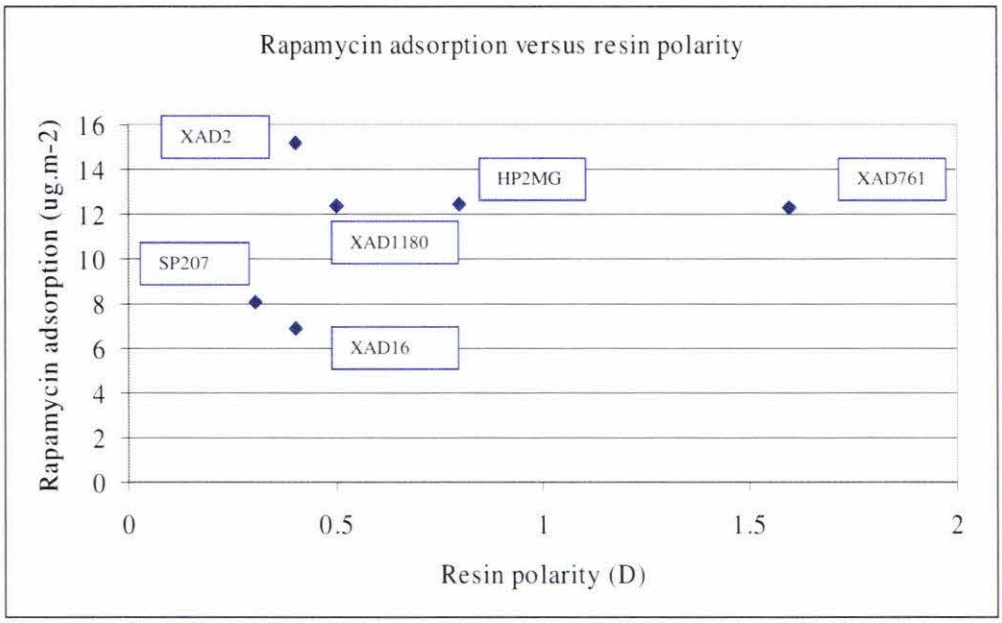

Figure 22 Maximum adsorption of rapamycin based on resin polarity

As the polarity of the resin increased, the adsorption of rapamycin onto the resin increased. As observed with anguidine, the similarly structural resins (XAD2 and XAD16) adsorbed significantly differing amount of the metabolite, with the lower surface area per mL resin (XAD2) adsorbing more rapamycin. The moderately hydrophilic resin HP2MG and the hydrophilic resin XAD761 absorbed the high amount of rapamycin compared to the other strongly hydrophobic resin SP207.

The maximum adsorption of ansamitocin P3 was also plotted against the polarity of the resins used, Figure 23.

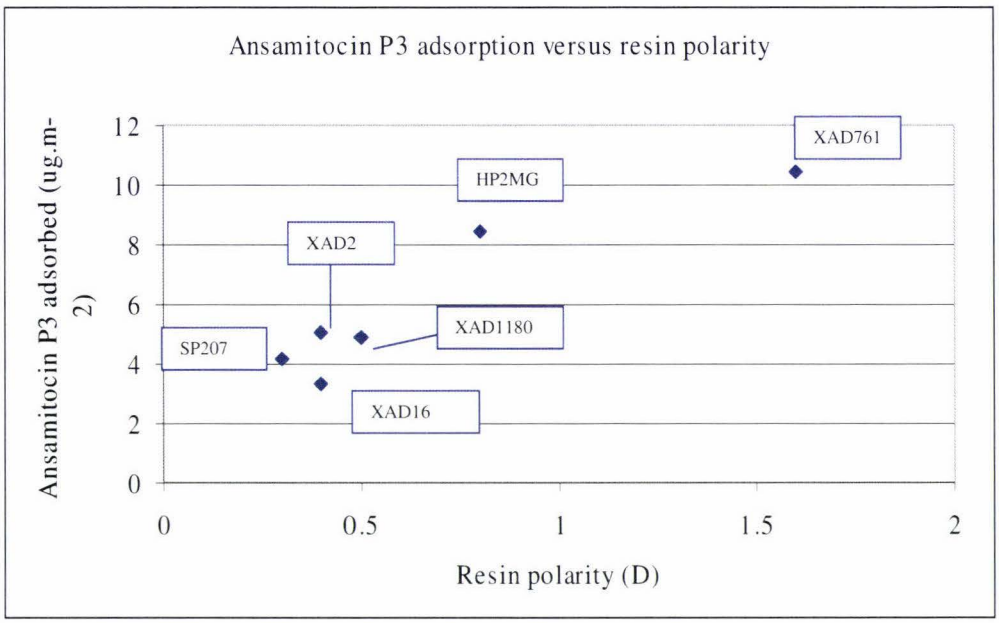

Figure 23 Maximum adsorption of ansamitocin P3 based on resin polarity

A general correlation was observed, similar to that recorded for rapamcyin. As the polarity of the resin increased the amount of ansamitocin P3 absorbed onto the resin increased. As with rapamycin, a similar relationship was observed with HP2MG and XAD761 absorbing the largest amount of anamitocin P3. XAD761 can not be sterilised and as such has limited value in the pharmaceutical industry.

A plot of the maximum adsorption per area of resin of the three secondary metabolites versus the polarity of the adsorption resin on the same graph provides an overview of the adsorption data, Figure 24.

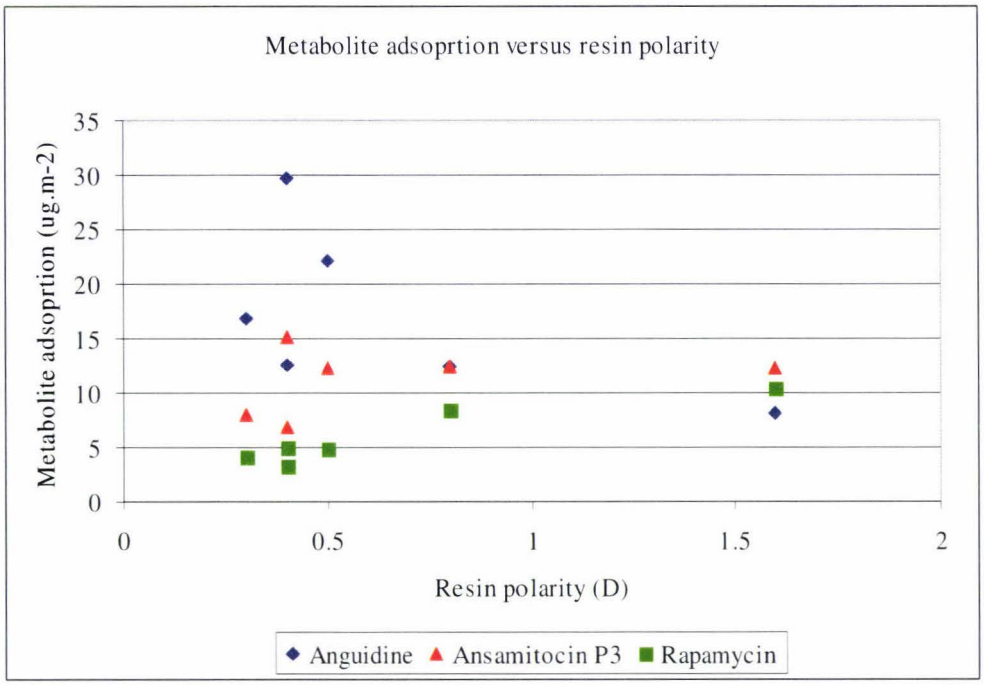

Figure 24 Maximum adsorption per unit area for hydrophobic secondary metabolites based on the polarity of adsorption resins

Figure 24 suggest that the nature of the adsorbed metabolite also influences the adsorption process, an expanded slope of the lines in Figure 24 increase as follows; anguidine > ansamitocin P3 > rapamycin.

The equation for each adsorption curve versus resin polarity was calculated, Table 4. The results suggest that the correlation for each curve is variable and that additional metabolites should be investigated to improve the data set.

Table 4 Gradient and intercept for rapamycin, ansamitocin P3 and anguidine versus adsorption resin polarity

Compound	Gradient	Intercept	R^2
Rapamcyin	5.2	2.5	0.3842
Ansamitocin P3	1.68	9.9	0.086
Anguidine	-9.9	23	0.8674

A method to describe the properties of organic secondary metabolites that is currently used in QSAR studies is based upon lipophilicity. The

lipophilicity of a molecule allows researchers to predict the relative hydrophobic or hydrophilic bonding that occurs between the compound and the resin.

The calculated Log *P* value of anguidine, rapamycin and ansamitocin P3 using various computational methods indicate that there are variations that depend upon the program used, Figure 25 - Figure 27).

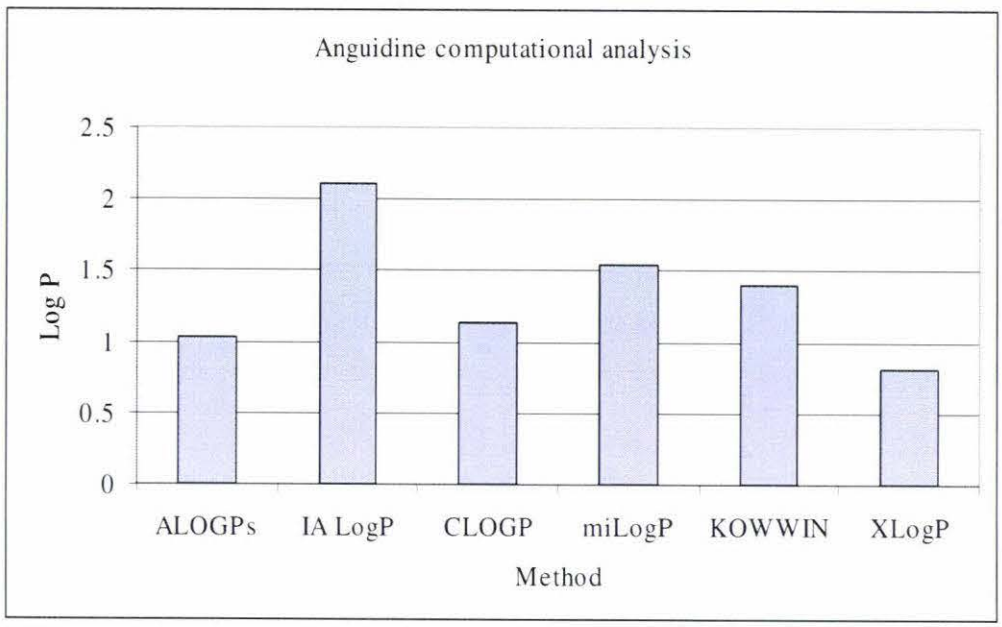

Figure 25 Log *P* for anguidine evaluated using 6 computational programs

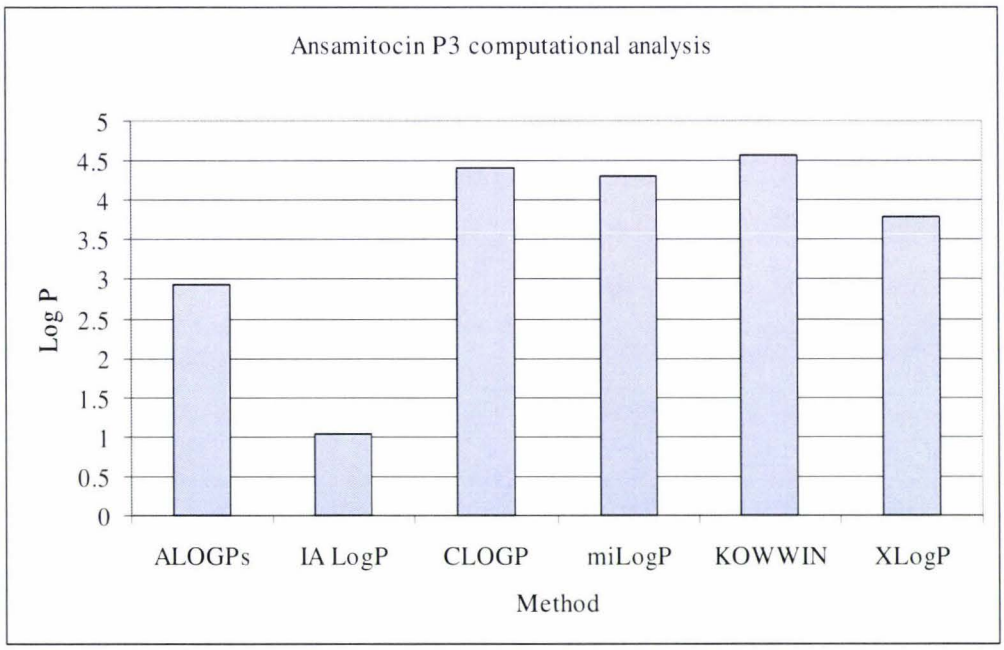

Figure 26 Log *P* for rapamcyin evaluated using 6 computational programs

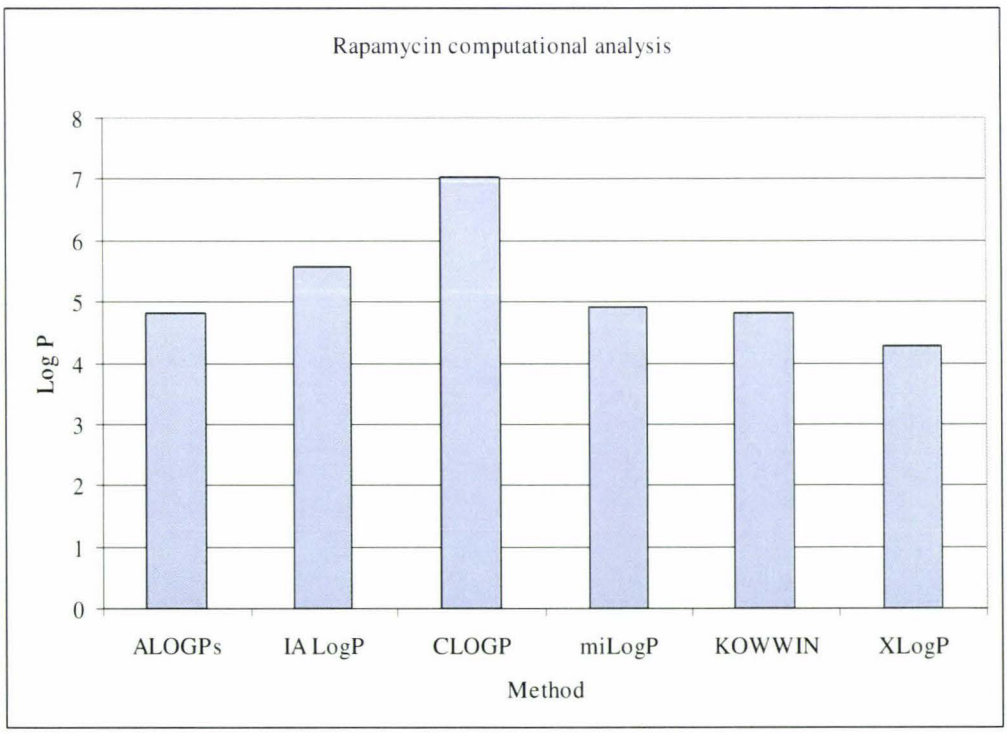

Figure 27 Log *P* for ansamitocin P3 evaluated using 6 computational programs

The average log *P* values with corresponding standard deviations were 1.33 ± 0.45 for anguidine, 3.5 ± 1.34 for ansamitocin P3 and 5.23 ± 0.97 for rapamycin.

Average Log *P* rates of the three secondary metabolites plotted against the gradient (Table 4) are shown in, Figure 28.

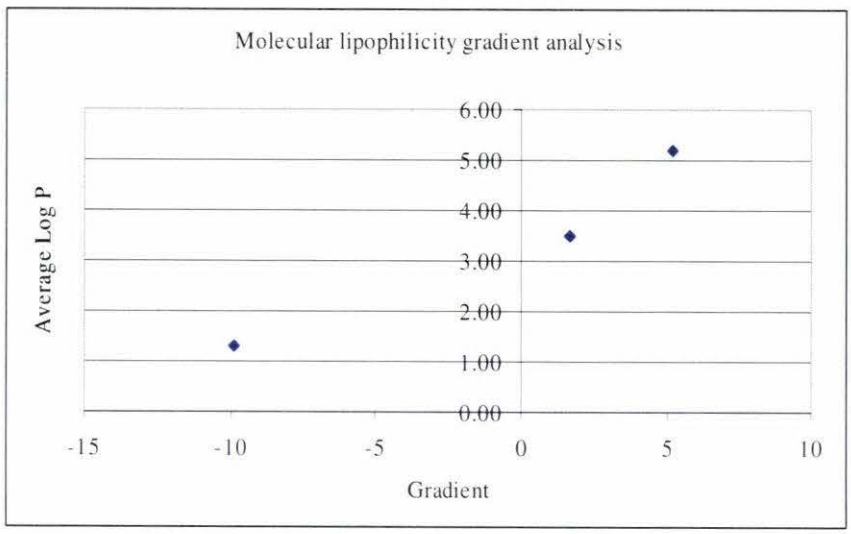

Figure 28 Average Log *P* resin gradient from Table 4

The plot indicates that average Log *P* adsorption of anguidine, rapamycin and ansamitocin P3 can be correlated with the gradient of plots in Figure 24. The data in Figure 28 were fitted by regression to the following linear equation.

Equation 2 LogP equation for the lipophilicity gradient

$y = 0.2396x + 3.5602$

Where x is the lipophilicity gradient. The R^2 value for Equation 2 was 0.9446.

A comparison of the lipophilicity of the three secondary metabolites to the three adsorption intercepts (Table 4) indicated that there was also a correlation between their average Log *P* values and lipophilicity intercept, Figure 29.

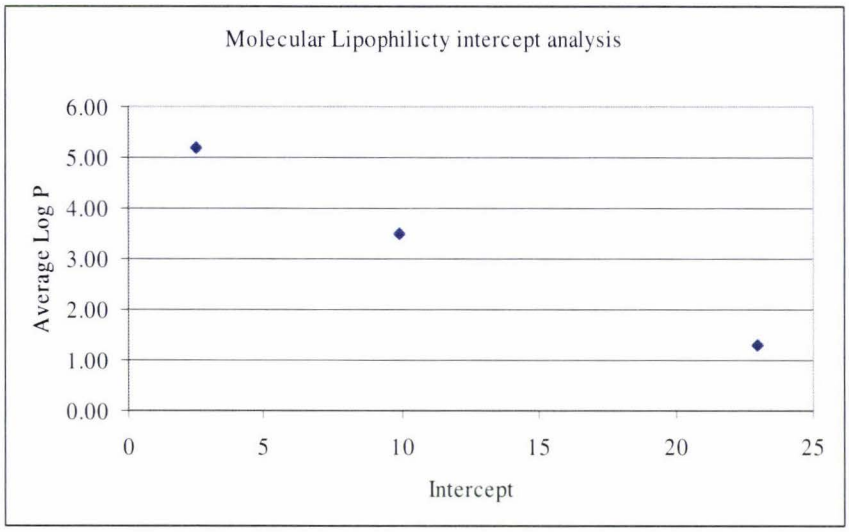

Figure 29 Average Log *P* versus lipophilicity intercept (Table 4)

The data in Figure 29 was correlated with the following linear equation.

Equation 3 LogP equation for the lipophilicity intercept

$y = -0.1877x + 5.5478$

Where x is the lipophilicity intercept (Table 4). The R^2 value for Equation 3 was 0.9927.

A linear relationship is obtained when the adsorption of the three secondary metabolites onto a neutral resin is related to the lipophilicity of the compounds. Based on the results from Table 4, further investigations of other similar metabolites are required to confirm the suggested relationship.

4.2 Addition of adsorption resin to fermentation system to enhance final concentration of metabolite

4.2.1 Experiment 4

Two resins, XAD1180 and SP207 resin were identified for their high adsorption of anguidine and added to a set of shake flasks each day for four days as described in Section 3.4.2.1. All shake flasks were sampled on day five and analysed for anguidine production, Figure 30.

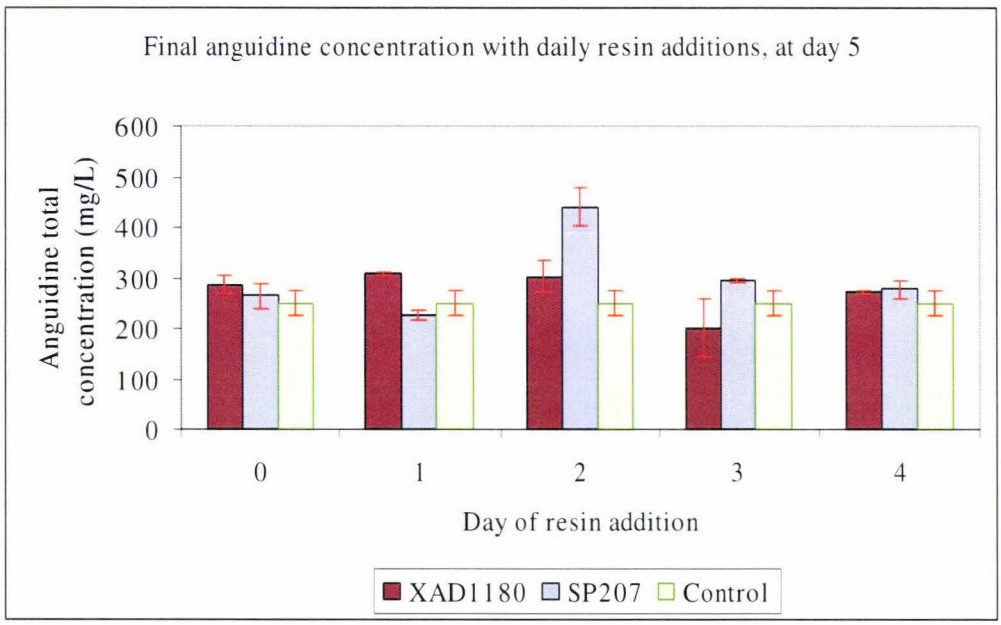

Figure 30 Anguidine production at day five with XAD1180 and SP207 resin addition on day 0, 1, 2, 3 and 4

Previous experiments showed that the start of anguidine production occurs at day one and ends at day four. From the results obtained, shake flasks that had SP207 added to them at day two, produced more anguidine at day 5 when compared to the control. In comparison when XAD1180 was added to shake flasks under the same conditions, the increase in anguidine concentration was lower than SP207, but higher than the control.

Of all the resins tested, SP207 absorbed the highest concentration (12.5 mg/mL) of anguidine, with XAD1180 having the second highest concentration (11.3 mg/mL). The resin additions were in excess of the production of anguidine (2.5 mL SP207 resin can adsorb 31 mg anguidine and 2.5 mL XAD1180 can adsorb 28.25 mg) as the maximum production that obtained was 22 mg of anguidine (obtained from the day two resin addition sample). The adsorption of all anguidine onto the resin was confirmed by there being no anguidine being present in the extraction from the supernatant.

The anguidine fermentation production media consists of a range of salts and glucose and does not contain any hydrophobic components that could be absorbed onto the hydrophobic resins. However, optimal resin addition

was at day two, with resin additions at day zero and one having a minimal impact on production, compared to the control. The reason for the decrease in production when resin is added to the fermentation on day zero and one, compared to resin addition at day two, is not obvious. No additional metabolites were detected by HPLC (operated under conditions specified in Section 3.3.1.) on any of the days resin was added, compared to the control fermentation.

4.2.2 Experiment 5

Two resins, XAD1180 and HP2MG were identified for their high adsorption of rapamycin and were added to a set of shake flasks each day for 7 days, as described in Section 3.4.2.2. All the shake flasks were then sampled on day 7 and analysed for rapamycin production, Figure 31.

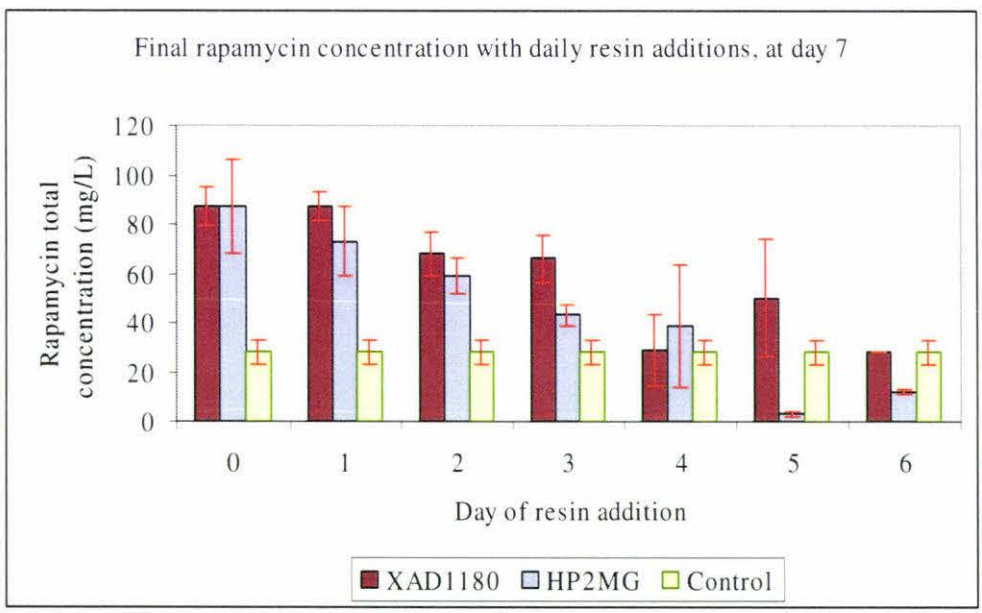

Figure 31 Rapamycin production on day 7 based on day of resin added on day 0, 1, 2, 3, 4, 5 and 6

The concentration of rapamycin at day six was significantly higher when either resin was added at day zero. Addition of resin later in the fermentation progressively decreased the day seven the concentration of rapamycin decreased from ~85 mg/L to a final concentration of ~30 mg/L.

Both resin, XAD1180 and HP2MG, appeared to be equally effective for improving the concentration of rapamycin produced. The maximum adsorption of rapamycin onto HP2MG was 4.5 mg/mL and for XAD1180 was 2.5 mg/mL. The maximum rapamycin produced per shake flask was 4.25 mg, well within the capacity for both resins to adsorb. The adsorption of all rapamycin onto the resin was confirmed as no rapamycin could be extraction from the supernatant.

The medium used for rapamycin production contains a range of salts, glycerol, HySoy™ (a hydrolysed soy peptone component), additional amounts of the polar amino acid *L*-lysine and yeast extract. HySoy™ is an enzymatically hydrolysed soy peptone that provides essential and non-essential amino acids in the form of peptides (Sigma-Aldrich). Yeast extract is an autolysate powder of *Saccharomyces cerevisiae* that provides vitamin B complex and additional free amino acids. From the medium components described, the moderately hydrophobic resins would only absorb the hydrophobic amino acids from the peptone and yeast extract. *L*-lysine improves the production of rapamycin via its use as a precursor. As it is a polar amino acid, absorption onto the hydrophobic resins would be minimal.

The high rapamycin concentration attained when the resin was added on day zero of to the fermentation, would suggest that no essential component of the media are being adsorbed onto the resin. A feedback inhibition mechanism may be limiting the production of rapamycin, as early addition of the resin would most effectively remove this feedback loop. This can potentially explain why an early addition of the resin is more effective in enhancing the final amount produced. Also, rapamycin is known to have a poor stability in an aqueous environment, the adsorption of rapamycin onto a hydrophobic surface maybe contributing to its stabilisation.

4.2.3 Experiment 6

Two resins, XAD1180 and SP207 were identified for their high adsorption of ansamitocin P3 and were added to a set of shake flasks on different days,

as described in section 3.4.2.3. All the shake flasks were then sampled on day seven and analysed for ansamitocin P3 production, Figure 32.

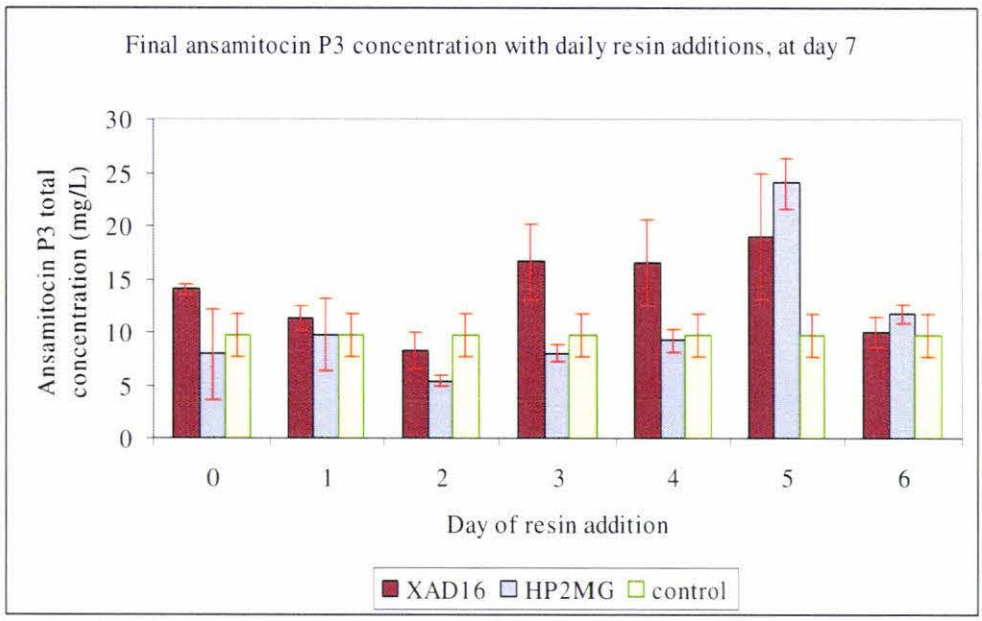

Figure 32 Ansamitocin P3 production based on day of resin addition

The concentration of ansamitocin P3 at day seven was significantly higher when HP2MG was added on day five. When XAD16 resin was added to the fermentation an increase in ansamitocin P3 concentration was observed when the resin was added at either day zero, three, four or five.

The highest concentrations of ansamitocin P3 occurred when HP2MG was added at day five. However, when XAD16 was added to the fermentation at day three and four the concentration of ansamitocin P3 was double that achieved with HP2MG. Iso-butyl alcohol (IBA) is added to the fermentation at day zero and is used to increase the percentage of ansamitocin P3 produced by providing a precursor (IBA) for biosynthesis.

The final concentration produced is considerably lower (91 mg/L) than previously reported using a different producing organism (Tandia *et al.* 1981). An increase in ansamitocin P3 production (500 mg/L) has also been reported through the use of strain improvement via mutation (Chung *et al.* 2003). The poor concentration, compared to other published data, is due to the fact our procedure is an un-optimised process that is currently undergoing process development. Additional seed stage experiments

conducted at IRL-BioPharm subsequent to this work has increased the final titre of ansamitocin P3 to 55 mg/L, without resin additions (results not shown). IBA is again used as precursor for ansamitocin P3 production, without it the co-metabolites ansamitocin P2 and P4 are produced at greater concentrations. An HPLC analysis of sample from the daily resin additions indicated that no other co-metabolites where produced at a measurable concentration. IBA was not identified by HPLC as being present on the resin at any stage of the fermentation.

4.3 Effect of resins on fermentations

The effects of resin addition during the fermentation for the production of rapamycin, ansamitocin P3 and anguidine were all significantly different. Resin addition at day zero increased the concentration of rapamycin compared to a later resin addition. In contrast, resin addition for ansamitocin P3 yielded an increase in the final concentration when added at the end of the fermentation. Lastly an increase in anguidine concentration was observed when resin was added mid way through the fermentation.

There appears to be no clear correlation as to the optimal day for resin addition. The differences are explained by the different ways metabolites can affect their own production and differences in stability of metabolites in aqueous media. The main mechanisms suggested by researchers for the increase in a compound's concentration when resin is added during the fermentation, are an increase in product stability and the removal of feedback inhibition when the compound is absorbed onto the resin. Most secondary metabolites in an aqueous environment are unstable and degrade as the fermentation progresses. Resin addition appears to be optimal before or during the exponential growth phase or at the start of secondary metabolite production, depending on fermentation.

Experiments undertaken at IRL-BioPharm confirmed that anguidine is a non-cell associated secondary metabolite (results not reported). As a result the metabolite is readily available in the aqueous environment for adsorption. In contrast experiments indicated that rapamycin is

approximately 70% cellular associated, suggesting that a majority of the metabolite cannot be absorbed onto the resin unless cells are disrupted (results not shown). As rapamycin is unstable in an aqueous environment, the presence of adsorption resins for stability earlier in the fermentation, during the exponential phase, allows adsorption onto a stabilising matrix when the metabolite is released from the cell (Carraway and Hidalgo, 2004). Ansamitocin P3 is approximately 50% cellular associated, a noticeable increase in the final concentration was observed when resins were present in the fermentation broth towards the end of the production phase.

The effects of resin addition on the production of secondary metabolites vary between each fermentation system and metabolite produced. The more complex and hydrophobic the secondary metabolite, the less stable the compound appears to be in an aqueous environment.

The cause for the decrease in metabolite production when resin was added to the shake flask is unknown. For the fermentations systems tested, addition of resin at the start of secondary metabolite production is optimal.

5 Conclusion

5.1 Adsorption resin screening

Based on the lipohilicity of the secondary metabolite, it appears to be possible to select a resin with the highest adsorption concentration based on the metabolites' properties and resin polarity. Hydrophobic resins adsorbed hydrophobic secondary metabolites used in this work with a greater specificity than did hydrophilic resins. As the total surface area of the resin increased, there was a corresponding increase in maximum adsorption per unit volume of the resin.

5.2 Anguidine production

A two-fold increase in anguidine concentration was observed when SP207 resin was added to shake flasks at day two, after four days of growth. A concentration of 440 mg/L of anguidine was produced in the presence of SP207 resin. A concentration of 300 mg/L of anguidine was produced in the presence of XAD1180 resin. The difference in anguidine produced between the two resins increased as the specific adsorption capacity of the resin increased.

5.3 Rapamycin production

A three-fold increase in rapamycin production was observed when either XAD1180 or HP2MG resin was added to shake flasks at day zero, after six days of growth. A concentration of 87 mg/L of rapamycin was produced in the presence of XAD1180 resin. A concentration of 87 mg/L of rapamycin was produced in the presence of HP2MG resin. No difference on production was observed between the two resins tested.

5.4 Ansamitocin P3 production

A two-fold increase in ansamitocin P3 production was observed when either XAD16 or HP2MG resin was added at day five, after six days of growth. A concentration of 24 mg/L of ansamitocin P3 was produced in the presence of HP2MG resin. A concentration of 19 mg/L of ansamitocin P3 was produced in the presence of XAD16 resin. The difference in ansamitocin P3 produced between the two resins increased as the specific adsorption capacity of the resin increased.

5.5 Selection of adsorption resin

An increase in metabolite production in the presence of adsorption resins was observed as the total adsorption of the metabolite measured increased. The differences between the resins used were less pronounced as the percentage of the metabolite that was cellular associated increased. The results of measured adsorption capacity suggest that the higher the adsorption capacity of the resin, the greater the production when added during the fermentation.

As the target compound increased in lipophilicity, the amount that could be adsorbed on to any given resin decreased. The hydrophobic resins tested adsorbed the metabolites similar levels per unit of area in surface area. The hydrophilic resins tested, however, adsorbed the compounds to varying degrees.

6 Appendix

Media Components

Anguidine

Table 5 Anguidine seed medium (GHY)

Component	g/L
Glucose	10
HySoy	1
Yeast extract	1

Table 6 Anguidine production medium (DMP1)

Component	g/L
Glucose	50
KH_2PO_4	2
$MgSO_4.7H_2O$	0.3
$NaNO_3$	10
$FeSO_4.\ 7H_2O$	0.005
$ZnSO_4.\ 7H_2O$	0.005
$CaCl_2$	0.2

Rapamycin

Table 7 seed medium (TJ)

Component	g/L
Tomato juice	200
$CaCO_3$	3
Adjust to pH6.8 using 1M NaOH	

Table 8 production medium (SYLGG)

Component	g/L

Hy Soy	10
Yeast extract	6.5
L-lysine	6.5
K_2HPO_4	1.14
KH_2PO_4	0.7
NaCl	5
Glycerol	30
$FeSO_4.7H_2O$	0.01
SAG471	0.1
Adjust to pH6.0 using 4M H_2SO_4.	

Ansamitocin P3

Table 9 Ansamitocin P3 seed medium

Component	g/L
Dextrose monohydrate	20
Soluble Starch	30
Soybean flour	10
Solulys Steep Liquor	10
Sodium Chloride	3
Calcium carbonate	5

Table 10 Ansamitocin P3 production medium

Component	g/L
Avon Malt	70
Proflo (Powder)	10
Soy four	1
Sodium acetate	0.1
(NH4)2SO4	0.5
K2HPO4	0.6
KH2PO4	0.4
CaCO3	5
SAG471 antifoam	0.1

7 References

1. Aharonowitz Y and Demain A. Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus*. *Antimicrobial Agents and Chemotherapy*. 1978. 14(2):159-164.
2. Altomare C, Logrieco A, Bottalico A, Mulé G, Moretti A and Evidente A. Production of type A trichothecenes and enniatin B by *Fusarium sambucinum* Fuckel sensu lato. *Mycopathologia*. 1987. 129(3):177-181.
3. Altomare C, Ritieni A, Perrone G, Fogliano V, Mannina L and Logrieco A. Production of neosolaniol by *Fusarium tumidum*. *Mycopathologia*. 1995. 130(3):179-84.
4. Atkinson B and Mavituna F. **Biochemical Engineering and Biotechnology Handbook**. Macmillan Publishers, Surrey. 1983.
5. Bacon C, Porter J, Norred W and Leslie J. Production of fusaric acid by *Fusarium* species. *Applied and Environmental Microbiology*. 1996. 62(11):4039-4043.
6. Baltz R. Antibiotic discovery from Actinomycetes: Will a renaissance follow the decline and fall? *SIM News*. 2005. 55(5):186-196.
7. Basak K and Majumdar S K. Ultilization of carbon and nitrogen sources by *Streptomyces kanamycesticus* for kanamycin production. *Antimicrobial Agents and Chemotherapy*. 1973. 4(1):6-10.
8. Bennett J and Klich M. Mycotoxins. *Clinical Microbiological Reviews*. 2003. 16(3)497-516.
9. Blackwell B, Miller J and Greenhalgh R. ^{12}C NMR study of biosynthesis of toxins by *Fusarium graminearum*. *Journal of Biological Chemistry*. 1985. 260(7):4243-4247.
10. Cassady J, Chan K, Floss H and Leistner E. Recent developments in the maytansinoid antitumor agents. *Chemical and Pharmaceutical Bulletin*. 2004. 52(1):1-26.

11. Carroll B, Moss S, Bai L, Kato Y, Toelzer S, Yu T and Floss H. Identification of a set of genes involved in the formation of the substrate for the incorporation of the unusual 'glycolate' chain extension unit in ansamitocin biosynthesis. *Journal of the American Chemical Society*. 2002. 124:4176-4177.

12. Carroway H and Hidalgo M. New targets for therapy in breast cancer: Mammalian target of rapamycin (mTOR) antagonists. *Breast Cancer Research*. 2004. 6(5): 219–224.

13. Casas Lopez J, Sanchez Perez J, Fernandez Sevilla J, Rodriguez Porcel E and Chisti Y. Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. *Journal of Biotechnology*. 2005. 116:61-77.

14. Casas Lopez J, Rodriguez Porcel E, Vilches Ferron M, Sanchez Perez J, Fernandez Sevilla J and Chisti Y. Lovastatin inhibits its own synthesis in *Aspergillus terreus*. *Journal of Industrial Microbiology and Biotechnology*. 2004. 31:48-50.

15. Calvo A, Wilson R, Bok J and Keller N. Relationship between secondary metabolites and fungal development. *Microbiology and Molecular Biology Reviews*. 2002. 66(3):447-459.

16. Chen Y, Krol J, Sterkin V, Fan W, Yan X, Huanh W, Cino J and Julien C. New process control strategy used in rapamycin fermentation. *Process Biochemistry*. 1999. 34:383-389.

17. Cheng Y, Fang A and Demain A. Effects of amino acids on rapamycin biosynthesis by *Streptomyces hygroscopicus*. *Journal of Industrial Microbiology*. 1995. 43:1096-1098.

18. Cheng Y, Hauck L and Demain A. Phosphate, ammonium, magnesium and iron nutrition of *Streptomyces hygroscopicus* with respect to rapamycin biosynthesis. *Journal of Industrial Microbiology*. 1995. 14:424-427.

19. Cheng Y, Huang J, Qiang H, Lin W and Demian A. Mutagenesis of the rapamycin producer *Streptomyces hygroscopicus* FC904. *Journal of Antibiotics*. 2001. 54:967-972.

20. Chung J and Byng G. U.S. Patent No. 20030157694. Washington D.C. U.S. Patent and Trademark Office. 2003.

21. Cullen D, Smalley E and Caldwell R. New process for T-2 toxin production. *Applied and Environmental Microbiology*. 1982. 44(2):371-375.

22. Darken M, Berenson H, Shirk R and Sjolander N. Production of tetracycline by *Streptomyces aureofaciens* in synthetic medium. *Applied Microbiology*. 1960. 8(1):46-51.

23. Degeest B and De Vuyst L. Indication that the nitrogen source influences both amount and size of exopolysaccharides Produced by *Streptococcus thermophilus* LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. *Applied and Environmental Microbiology*. 1999. 65(7):2863-2870.

24. Desjardins A, Hohn T and McCormick S. Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics and significance. *Microbiological Reviews*. 1993. 57(3):595-604.

25. Desjardins A, Plattner R and Beremand M. Ancymidol blocks trichothecene biosynthesis and leads to accumulation of trichodiene in *Fusarium sporotrichioides* and *Gibberalla pulicaris*. *Applied and Environmental Microbiology*. 1987. 53(8):1860-1865.

26. Doulia D, Rigas F and Gimouhopoulos C. Removal of amino acids from water by adsorption on polystyrene resins. *Journal of Chemical Technology and Biotechnology*. 2001. 76:83-89.

27. Dowex Optipore. Product information sheet L-285. Form No 177-01731-1105. 2006.

28. Dulaney E. Observations on *Streptomyces* griseus II. Nitrogen sources for growth and streptomycin production. *Journal of Bacteriology*. 1948. 56(3):305-313.

29. Eaker D and Wadstrom T. **Natural Toxins: Toxicological Evaluation of Trichothecene Mycotoxins**. Pergamon Press, Potts Point. 1980.

30. Gerth K, Bedorf N, Irschik H, Holfe G and Reichenbach H. The soraphens: A family of novel antifungal compounds from *Sorangium cellulosum*. I. Soraphen $A_{1\alpha}$: fermentation, isolation, biological properties. *Journal of Antibiotics*. 1994. 47:23-31.

31. Guarro J, Gene J and Stchigel A. Development in fungal taxonomy. *Clinical Microbiology Reviews*. 1999. 12(3):454-500.

32. Hajjaj H, Niederberger P and Duboc P. Lovastatin biosynthesis by *Asperillus terreus* in a chemically defined medium. *Applied and Environmental Microbiology*. 2001. 67(6):2596-2602.

33. Hansch C and Fujita T. Analysis. A method for the correlation of biological activity and chemical structure. *Journal of American Chemical Society*. 1963. 86:1616-1626.

34. Hasegawa T, Tanida S, Hatano K, Higashide E and Masahiko Y. Motile Actinomycetes: *Actinosynnema pretiosum* subsp. *Pretiousm* sp. nov., subsp. nov. and *Actinosynnema pretiosum* subsp. *auranticum* subsp. nov. *International Journal of Systematic Bacteriology*. 1983. 33(2):341-320.

35. Hatano K, Higashide E, Akiyama S and Yoneda M. Selective accumulation of ansamitocins P-2, P-3 and P-4 and biosynthetic origins of their acyl moieties. *Agriculture and Biological Chemistry*. 1984. 48(7):1721-1729.

36. Hohn T and Beremand M. Regulation of tricodiene synthase in *Fusarium sporotrichiodes* and *Gibberella pulicaris* (*Fusraium sambucinum*). *Applied and Environmental Microbiology*. 1989. 55(6):1500-1503.

37. Ingraham J, Maaloe O and Neidhart F. **Growth of the Bacterial Cell.** Sinauer Associates, Sunderland. 1983.

38. Jackson M and Bennett G. Production of fumonisin B by *Fusarium moniliforme* NRRL 13616 in submerged culture. *Applied and Environmental Microbiology*. 1990. 56(8):2296-2298.

39. Jackson M and Bothast R. Carbon concentration and carbon-to-nitrogen ratio influence submerged-culture condition by the potential herbicide *Colletotrichum truncatum* NRRL 13737. *Applied and Environmental Microbiology*. 1990 56(11):3435-3438.

40. Jackson M, Slininger P and Bothast R. Effects of zinc, iron, cobalt and manganese on *Fusarium moniliforme* NRRL 13616 growth and fusarium C biosynthesis in submerged cultures. *Applied and Environmental Microbiology*. 1989. 55(3):649-955.

41. Jarvis B, Armstrong C and Zeng M. Use of resins for trichothecene production in liquid cultures. *Journal of Antibiotics*. 1990 43(11):1502-1504.

42. Kalakoutskii L and Agre N. Comparitive aspects of development and differentiation in actionmycetes. *Bacteriological Reviews*. 1976. 40(2):469-524.

43. Khaw L, Bohm G, Metcalfe S, Staunton J and Leadlay P. Mutational biosynthesis of novel rapamycins by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. *Journal of Bacteriology*. 1988. 180(4):809-814.

44. Kojima I, Cheng Y, Mohan V and Demain A. Carbon source nutririon of rapamycin biosynthesis in *Streptomyces hygroscopicus*. *Journal of Industrial Microbiology*. 1995. 14:436-439.

45. Lee M, Kojima I and Demain A. Effect of nitrogen source of biosynthesis of rapamycin by *Streptomyces hygroscopicus*. *Journal of Industrial Microbiology and Biotechnology*. 1997. 19:83-86

46. Lee J, Park H, Park D, Lee H, Kim Y and Kim C. Improved production of teicoplanin using adsorbent resin in fermentations. *Letters in Applied Microbiology*. 2003. 37:196-200.

47. Lu C, Bai L and Shen Y. A novel amide N-glycoside of ansamitocins from *Actinosynnema pretiosum*. *Journal of Antibiotics*. 2004. 57(5):348-350.

48. Madry N, Zocher R, Kleinkauf H. Enniatin production by *Fusarium oxysporum* in chemically defined medium. *European Journal of Applied Microbiology and Biotechnology*. 1983. 17:75-79.

49. Maggon K, Gupta S and Venkitasubramanian T. Biosynthesis of aflatoxins. *Bacteriological Reviews*. 1977. 41(4):822-855.

50. Martin J and Demain A. Control of antibiotic synthesis. *Microbiological Reviews*. 1980. 44(2):220-251.

51. Marshall V, McWethy S, Sirotti J and Cialdella J. The effect of neutral resins on the fermentation production of rubradirin. *Journal of Industrial Microbiology*. 1990. 5:283-288.

52. Marshall V, McWethy S, Visser J, Cialdella J and Laborde A. Current fermentation technology for the production of antibiotics from actinomycetes: the example of paulomycin. *Developments in Industrial Microbiology*. 1987. 28(2):105-113.

53. McNeil B and Harvey L M. **Fermentation: a Practical Approach**. Oxford University press, Oxford. 1990.

54. Mitsubishi Chemical Corporation. Types and Properties of Synthetic Adsorbents, 2001.

55. Medic-Saric M, Mornar A and Jasprica I. Lipophilicity study of aalicylamide. *Acta Pharm*. 2004. 54:91-101.

56. Miller B and Litsky W. **Industrial Microbiology**. McGraw-Hill Book Company, New York. 1976.

57. Minas W, Bailey J and Duetz W. Streptomyces in micro-cultures: Growth, production of secondary metabolites and storage and retrieval in the 96-well format. *Antonie van Leeuwenhoek*. 2000. 78:297-305.

58. Molinspiration version 2. http://www.molinspiration.com

59. Monnet D, Vidal D and Creach O. Influence of metabolic and physical factors on production of diacetoxyscripenol by *Fusarium sambucinum* kuckel. *Applied and Environmental Microbiology*. 1988. 54(9):2167-2169.

60. O'Cleirigh C, Casey J, Walsh P and O'Shea D. Morphological engineering of *Streptmoyces hygroscopicus* var. *geldanus*:

regulation of pellet morphology through manipulation of broth viscosity. *Journal of Antibiotics.* 2005. 68:305-310.

61. Paiva N, Demain A and Roberts M. The immediate precursor of the nitrogen-containing ring of rapamycin is free pipecolic acid. *Enzyme and Microbiological Technology.* 1993. 15:581-584.

62. Paiva N, Roberts M and Demain A. The cyclohexane moiety of rapamycin in derived from shikimic acid in *Streptomyces hygroscopicus. Journal of Industrial Microbiology.* 1993. 12:423-428.

63. Payne G and Hagler W. Effects of specific amino acids on growth and aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus* in defined medium. *Applied and Environmental Microbiology.* 1983. 46(4):805-812.

64. Pelczar M and Reid R. **Microbiology.** McGraw-Hill, Sydney. 1972.

65. Perlman D and O'Brien E. Utilization of carbohydrates by strains of *Streptomyces griseus. Journal of Bacteriology.* 1956. 72(2):214-218.

66. Potvin J, Fonchy E, Conway J, Champagne C P. An automatic turbidimetric method to screen yeast extracts as fermentation nutrient ingredients. *Journal of Microbiological Methods.* 1997. 29:153-160

67. Rachev R, Gancheva V, Bojkova S, Christov C, Zafirova T. 1997. Gibberellin biosynthesis by *Fusarium moniliforme* in the presence of hydrophobic resin amberlite XAD-2. *Bulgarian Journal of Plant Physiology.* 23:23–32.

68. Richardson KE, Hamilton PB. Preparation of 4,15-diacetoxyscirpenol from cultures of *Fusarium sambucinum* NRRL 13495. *Applied Environmental Microbiology.* 1987. 53(2):460-462.

69. Ritacco F, Graziani E, Summers M, Zabriskie T, Yu K, Bernan V, Carter G and Greenstien M. Production of novel rapamycin analogs by precursor-directed biosynthesis. *Applied and Environmental Microbiology.* 2005. 71(4):1971-1976.

70. Rohm and Haas Company. Amberlite XAD761. PDS 0103A. 2006.

71. Rohm and Haas Company. Amberlite XAD2. PDS 0140A. 2003.

72. Rohm and Haas Company. Amberlite XAD16. PDS 0170A. 2003.

73. Rohm and Haas Company. Amberlite XAD1180. PDS 0205A. 2005.

74. Rohm and Haas Company. Amberchrom CG161. IE-687EDS. 2002

75. Sehgal S, Baker H and Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *Journal of Antibiotics.* 1975. 28(10):727-732.

76. Shoun H and Tanimoto T. Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrate reduction. *Journal of Biological Chemistry.* 1991. 266(17):11078-11082.

77. Shirato S and Nagatsu C. Fermentation studies with *Streptomyces griseus* I. Carbohydrate sources for the production of protease and streptomycin. *Applied Microbiology.* 1965. 13(5):669-672.

78. Shirato S and Motoyama H. Fermentation studies with *Streptomyces griseus* II. Synthetic medium for the production of Streptomycin. *Applied Microbiology.* 1966 14(5):706-710.

79. Sigma-Aldrich Company. Ready-to-Use, Sterile, Endotoxin-Free Adsorbent Cartridges for Purifying Biological Preparations. Bulletin 871B. 1996.

80. Stryer L. **Biochemistry**. W H Freeman and Company, San Francisco. 1981.

81. Tanida S, Hasegawa T, Hatano K, Higashide E and Yoneda M. Asamitocins, maytansinoid antitumor antibiotics producing organism, fermentation and antimicrobial activities. *Journal of Antibiotics.* 1980. 33(2):192-198.

82. Tanida S, Izawa M and Hasegawa T. Ansamitocin analogs from a mutant strain of *Nocardia*. I. Isolation of the mutant, fermentation and antimicrobial properties. *Journal of Antibiotics*. 1980. 34:489-495.

83. Tetko I, Gasteiger J, Todeschini R, Mauri A, Livingstone D, Ertl P, Palyulin V, Radchenko E, Zefirov N, Makarenko A, Tanchuk V and Prokopenko V. Virtual computational chemistry laboratory - design and description. *Journal of Computational Aided Molecular Discovery*. 2005. 19:453-63.

84. Ueno Y. Trichothecene mycotoxins: Mycology, chemistry and toxiciology. *Advances in Food and Nutrition Research*. 1980. 3:301-353.

85. Ueno Y, Sawano M and Ishii K. Production of trichothecene mycotoxins by *Fusarium* species in shake culture. *Applied Microbiology*. 1975. 30(1):4-9.

86. Vasavada A, Hsieh D. Effects of metals on 3-acetyldeoxynivalenol production by *Fusarium graminearum* R2118 in submerged cultures. *Applied and Environmental Microbiology*. 1988. 54(4):1063-1065.

87. Vezina C, Kudelski A and Sehgal S. Rapamycin (AY-22,989), A new antifungal antibiotic. *Journal of Antibiotics*. 1975. 28(10):721-726.

88. Wannemacher E and Wiener S. **Medical Aspects of Chemical and Biological Warfare.** Chapter 34, Trichothecene Mycotoxins. TMM Publications. Washington. 1997.

89. Warr G, Veitch J, Walsh A, Hesler G, Pirnik D, Leet J, Lin P, Medina I, McBrien K, Forenza S, Clark J and Lam K. BMS-182123, a fungal metabolite that inhibits the production of TNF-α by macrophages and monocytes. *Journal of Antibiotics*. 1996. 49(3):234-240.

90. Wiebe M, Robson G, Shuster J and Trinci A. pH regulation of recombinant glucoamylase production in *Fusarium venenatum* JeRS 325, a transformant with *Fusarium oxysporum* alkaline

(trypsin-like) protease promoter. *Biotechnology and Bioengineering*. 1999. 64(3):368-372.

91. Williams W and Katz E. Development of a chemically defined medium for the synthesis of actinomycin D by *Streptomyces parvulus*. *Antimicrobial Agents and Chemotherapy*. 1977. 11(2):281-290.

92. Zajtchuk R. **Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare, US Army.** Office of the Surgeon General. Washington DC. 1997

93. Zakriskie D, Armiger W, Phillips D, Albano P. Traders' guide to fermentation medium formulation. **Traders Protein**. 1980.

94. Zhang J, Marcin C, Shifflet M, Salmon P, Brix T, Greasham R, Buckland B, Chartrain M. Development of a defined medium fermentation process for physostigmine production by *Streptomyces griseofuscus*. *Applied Microbiology and Biotechnology*. 1996. 44:568-575.

95. Zhang J, Reddy J, Buckland B and Greasham R. Towards consistent and productive complex medium for industrial fermentations: Studies on yeast extract for a recombinant yeast fermentation process. *Biotechnology and Bioengineering*. 2003. 82(6):640-652.

96. Zhao Y, Abraham M and Zissmos A. Fast calculation of van der Waals volume as a sum of atomic and bond contributions and its application to drug compounds. *Journal of Organic Chemistry*. 2003. 68:7368-7373.

97. Zhaoyi Z, Quanxing Z, Changlong W and Liansheng W. Adsorption of napthalene derivatives on different macroporous polymeric adsorbents. *Chemosphere*. 1997. 35(10):2269-2276.

98. Zhou Z, Takaya N, Nakamure A, Yamaguchi M, Takeo K, Shoun H. Ammonia fermentation, A novel anoxic metabolism of nitrate by fungi. *Journal of Biological Chemistry*. 2002. 277(3):1892-1896.