Applicability of adsorbent resins for the recovery of geldanamycin from *Streptomyces hygroscopicus* var. *geldanus* fermentation broths

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I hereby certify that this material, which I now submit for the assessment of the programme of study leading to the award of Ph.D. (Biotechnology) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

Applicability of adsorbent resins for the recovery of geldanamycin from* Streptomyces hygroscopicus *var.* geldanus *fermentation broths

Adsorbent resins are gaining increased application in recovery bioprocesses, thus it was decided to assess their applicability for the recovery of geldanamycin, an antibiotic produced *Streptomyces hygroscopicus* var. *geldanus*, in both a Downstream Processing (DSP) and In-Situ Product Recovery (ISPR) context.

Antibiotic production was initially assessed using the conventional disk diffusion assay. This was inefficient for large sample sets, therefore a microtiter plate-based bioassay was developed. This assay was an improvement on the disk diffusion assay, it was high throughput, allowed quantitative assessment of sample bioactivity, but quantification of geldanamycin in fermentation samples, was not possible. To achieve this, a High Performance Liquid Chromatography (HPLC) method was developed. During method development, significant difficulties, including column fouling, low sample throughput and poor geldanamycin solubility and had to be addressed. Once these issues were resolved, the HPLC method could be used to treat large sample sets, with minimal column damage, and was therefore employed for analysis of all geldanamycin containing samples.

Product recovery is key in bioprocesses, and it was found that the resins assessed had capacity and affinity for geldanamycin adsorption when applied in a DSP context. They were robust to temperature and pH changes and facilitated the generation of product streams of high product purity and concentration. Addition of solvent increase the selectivity of adsorption from fermentation broths, by approximately 5-fold. Two resins, Amberlite XAD-1600 and Diaion HP-20 were selected for further examination in an ISPR context based on their performance in DSP studies.

Applied in an ISPR context, it was found that the resins were capable of adsorbing compounds other than geldanamycin and their inclusion impacted on the growth rate of the organism. An approximate 3-fold increase in production could be achieved depending on resin concentration and addition time. In summary adsorbent resins are suitable for recovery of geldanamycin from fermentation broth and their correct application can increase product yields.

List of Figures

List of Tables

Chapter 1. Introduction

1.1 Biotechnology and Bioprocessing

The European Federation of Biotechnology proposed a definition of biotechnology in 1981 as 'The integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological (industrial) application of the abilities of microorganisms, cultured tissues and parts thereof' (Lilly, 1997). From this, it is clear to see that biotechnology encompasses an array of methodologies and techniques which can be considered bioprocesses. The two terms 'biotechnology' and 'bioprocessing' are interlinked, and should be considered as involving a wide variety of distinct subject areas (Trevan *et al.*, 1987, Rehm and Reed, 1985), and for the remainder of this document, the use of either term can be considered as meaning the other.

Biotechnology and bioprocessing can be considered disciplines of great antiquity, with such examples as silage production, traditional foods, wastewater treatment and alcohol production through brewing, spanning centuries (Smith, 1981, Brown *et al.*, 1987, Scragg, 1991). Traditional, and modern, biotechnological processes have contributed greatly to the quantity and quality of our food, medicines, environment and our personal health and vitality (Bailey, 1995). The spectrum of bioprocesses, by extension, incorporates the production of fermented foods and chemicals such as antibiotics, enzymes, ethanol, vinegar, citric acid and vitamin B_{12}, cell cultivation, wastewater treatment and a number activities, such as energy production, oil recovery, and nitrogen fixation (Rehm and Reed, 1985).

Even though there are a vast range of compounds which fall under the classification of bioproducts, one can argue that there are three major techniques, or process disciplines, which can be routinely and repeatedly

employed in biotechnological methodologies. These include enzyme catalysis, cell-based systems, and tissue or whole-organism systems.

In general, enzymes are considered as proteins which catalyse reactions in a very specific manner. These biocatalysis reactions (meaning catalysed by biological material), use the enzyme to perform actions which would usually be associated with chemical processes. The specificity of the reactions means the application of enzymes in biological processes is limited to single enzymes for single, or closely related, processes. There is a great deal of interest in extending enzyme use in food processing, chemical production, analytical and diagnostic systems and in the treatment of diseases. Over 2000 enzymes have been identified, and a few hundred have commercial applications, such as those used for genetic manipulations, like restriction endonucleases, ligases and editing enzymes (Towalski and Rothman, 1995).

Where enzymes function through catalysis of specific reactions, the microbial cell performs a series of such reactions. They can, by virtue of this, become the basis of more complex bioprocesses than enzymatic processes. The cell can essentially act as a complete biomanufacturing plant. The umbrella of microbial cell bioprocesses incorporates scientific activities ranging from relatively modern applications for the production of recombinant human hormones and microbial insecticides, to the more traditional mineral leaching and to bioremediation of toxic wastes (Glazer and Nikaido, 1995).

The use of microbial cells for waste water treatment include so-called 'green', or environmentally friendly bioprocesses, such as bioremediation and biodegradation. Many human activities have resulted in negative impacts on the environment, especially the contamination of water, the atmosphere and soil (Zhong, 2004). Bioremediation and biodegradation involve the application of microorganisms to remove toxins from the environment and are steadily becoming the technologies of choice for the remediation of many contaminated environments (Crawford and Crawford, 1996), and their application has expanded enormously (Alexander, 1994).

Microbial cells are, however, more frequently applied in production fermentations. These types of processes involve the controlled culturing of specific cells, in specially designed media, for the production of desirable products. The major products produced from such microbial cell systems include fermented juices and liquors, cheese, cell biomass, enzymes, vitamins, vaccines and antibiotics. During the last two decades there has been a large increase in the range of commercial products, especially secondary metabolites and recombinant proteins, and an associated increase in fermenter and facility design to improve performance (Buckland and Lilly, 1993), with antibiotic production being one of the most significant microbial cell-based processes.

Of the many thousands of microbial species relatively few are exploited in production processes. The principal microorganisms involved are all chemo—organotrophs, and derive their carbon and energy supply from the metabolism of organic compounds. Of the Gram-positive organisms, aerobic, endospore-forming bacteria of the genus *Bacillus*, some coryneform bacteria and the filamentous bacteria, particularly of the genus *Streptomyces*, are well represented. The Gram-negative organisms include acetic acid bacteria and xanthomonads, with various yeast and fungi also prevalent, and the relevant characteristics of all microorganisms involved in these processes are that they should be non-pathogenic, easy to maintain and cheap to culture (Brown *et al.*, 1987).

Microbial cells are not the only cell type utilised in fermentation processes. Animal and plant culturing is also employed for the production of beneficial compounds. Animal cell bioprocesses involve an interplay between medicine, biology and engineering. Modern industrial animal cell culture began in the 1950's, with the use of animal cells for the development of vaccines (Schügerl, 2000a). An important example of the types of cells used in animal cell culture are the hybridomas, which have infinite life spans and are the most frequently employed cell type in animal cell culture (Kelley *et al.*, 1993). An excellent description of these cells, and their use for the production of monoclonal antibodies is detailed by Primrose (1987),

who described how hybridomas are created from the fusing of myeloma (a type of tumour) cells with antibody producing spleen lymphocytes, and can be grown indefinitely while continuing to secrete antibody. All the antibodies produced by culture of any particular hybridoma will be identical and are termed monoclonal, i.e. they are all derived from a single clone of lymphocytes. These monoclonal antibodies can be purified easily and find application in many different areas, from diagnostic kits to cancer therapy and protein purification.

Plant cell-based bioprocessing and culture dates back to the beginning of the last century, and since the 1930's a great deal of progress has been achieved. The concept of culturing plant cells includes the culture of plant organs, tissue, cells, protoplasts, embryos and plantlets, the main aspects of which are the production of secondary metabolites, microproagation and the study of plant cell genetics, physiology, biochemistry and pathology (Zhong, 2001). Plants are valuable sources of numerous metabolites, including pharmaceuticals, agrochemicals, flavours, colours, biopesticides, food additives and biologically active compounds, with more than 100,000 plant secondary metabolites having been identified (Zhong and Yue, 2005, Zhong, 2002).

Although bioprocesses have existed for millennia, since the 1970's, there has been somewhat of a biological revolution (Trevan *et al.*, 1987). The breakthrough into what is termed 'new biotechnology' came about with the advances in the knowledge of the genetic make-up of organisms, and was based on a combination of cell culture and recombinant DNA technology, (Vasil, 1990). True gene technology succeeded after the first gene transfer into *Escherichia coli* in 1973, and since then, gene transfer in microbial, animal and plant cells has become a well established technology (Fiecher, 2000). The ability to manipulate the genetic makeup of living organisms has made it possible to enhance the ability of an organism to produce a particular chemical product, to prevent it from producing a product and to enable an organism to produce an entirely new product (Mc Gloughlin and Burke, 2000). Genetic manipulation of microbial cells is most prevalent in bioprocessing, however, it is foreseen that the genetic modification of

animals could be of significant benefit in future bioprocessing applications (Mc Gloughlin and Burke, 2000, Stranzinger and Went, 1996).

It is one thing for the laboratory scientist to clone a novel gene, discover a new antibiotic or invent an enzyme catalysed process, but it is quite another to transfer that knowledge to the scale of operation required to make useful products in significant quantities (Trevan *et al.*, 1987). The two key aspects of any bioprocess can be summarised as compound production and recovery of the product of interest. Brauer, (1985) wrote the following with respect to production and product recovery, with focus on a successful bioprocess.

> 'The problems addressed are not only related to microbial mass conversion in bioreactors, but also to upstream and downstream processes.....microbial mass conversion in bioreactors is a function of the laws describing the transport momentum, heat and mass as well as bioreaction kinetics.....in upstream and downstream processes, transport phenomena play the decisive role. Especially in downstream processes, designed for separation of desired products of microbial mass conversion from undesired ones, transport phenomena occur, in many cases, under extreme conditions and this is primarily due to the small concentration of these products in the fluid and the low density difference between the microorganism and the fluid'.

Biotechnological and bioprocess applications encompass a range of technologies and an even more diverse range of products. These techniques employed can be traditional or at the cutting edge of scientific research, but either way, the benefits which they afford mankind are unmistakable. It is important to note however, that even though bioprocessing techniques and their products are encountered on a daily basis, there is considerable effort required to establish a suitable process for production of a desired compound. The work performed in this pursuit centres on development of appropriate production and recovery methodologies.

1.1.1 Fermentation

The process of fermentation is one of the most important facets of cell-based culture systems. In its strictest sense, fermentation refers to the metabolic breakdown of nutrient molecules, such as glucose, and historically, refers to the anaerobic consumption of sugars, by yeast, for the production of alcohol. In modern bioprocesses however, this definition is incomplete, and more accurately, may be considered to refer to the bulk growth of microorganisms on a growth medium with no distinction being made between aerobic and anaerobic metabolism. The cells involved in fermentation processes vary from animal and plant cells to microbial cells, and the nutrients consumed and products produced are similarly diverse. The core elements of importance in any fermentation include the fermentation reactor design and the mode of fermentation employed.

Bioprocess technology encompasses all the basic and applied sciences as well as the engineering required to fully exploit living systems and bring their products to market and typically, in commercial production, this begins at the fermentation reactor (Asenjo and Merchuk, 1995). The fermentation reactor, fermenter, or bioreactor, is the vessel in which the fermentation is carried out. In the fermenter, an organism is cultivated in a controlled manner to produce more of the organism or a product, or in some specialised cases, to carry out specific reactions (Scragg, 1991), and is an enclosed system, comprising of the vessel and its seal, the head-plate, which allows sterile conditions to be maintained. In liquid cultures, these are frequently cylindrical vessels, fabricated from glass, up to a volume of three to five litres, and fabricated from stainless steel above this volume. Commonly, the fermentation process incorporates additional devices to confer control upon the system and includes methods which allow control of the significant fermentation parameters, such as bulk liquid motion, mass transfer, aeration, temperature, pH and fermentation volume.

Adequate bulk liquid mixing is important in fermentation processes to ensure adequate agitation and mixing of the fermentation system, which facilitates dispersion of biomass, nutrients and oxygen. A suitably agitated system is characterised by a high homogeneity and potential for heat and mass transfer (Solomons, 1980). In order to achieve these goals, agitators are employed. These are located internally and may comprise of a stainless steel shaft running down, from the head-plate, into the fermentation liquid. Attached along this shaft, or at its end, are impellers. Impellers are stainless steel implements which come in a variety of forms, including disc, vaned disc, open disc, variable pitch, paddle, anchor, gate anchor, marine propeller and helical crew (Solomons, 1980, Doran, 1995). The impeller-type employed is dependent on, among other things, the fermentation process being performed, the organism involved and the viscosity of the system (Doran, 1995).

In order to increase turbulence, baffles can be included in the system. These are vertical strips of stainless steel, mounted against the walls of the fermenter, protruding into the culture liquid. Baffles are placed equidistant from each other in the fermenter and serve to reduce vortexing and swirling of the culture fluid, which would otherwise prevent sufficient bulk liquid motion (Doran, 1995).

In fermentations where oxygen is required, air is usually introduced into the system via a sparger. Air enters into the system, after being filter sterilised, in the form of bubbles. The sparger is usually located beneath the impeller, to facilitate bubble break-up and increase the potential for gas dispersion and mass transfer. Airlift bioreactors can also be used. These are fermentation vessels which do not employ mechanical agitation, instead, the introduction of air bubbles, through sparging devices, acts as an aeration aid and to provide bulk liquid flow to the fermentation system (Doran, 1995).

Temperature maintenance is important in fermentation systems because the producing cells may have an optimum temperature at which they proliferate and produce. The product of the fermentation may also exhibit

sensitivity to temperature extremes, and therefore without proper temperature control and regulation, both the producing cells and the product may be adversely affected. In circumstances where the optimum temperature for cell growth and viability is not the same for product stability, a trade-off in the temperature at which the fermentation is performed may be required. For temperature control, the fermenter can be jacketed, whereby an external space or jacket is fabricated into the design of the vessel walls or incorporated as a coil internally. Such devices allow the introduction of heated steam or cooling water to maintain or alter the fermentation temperature.

Feed and bleed lines may also be included as part of the fermenter. These lines allow the addition of feed streams into the system and removal of culture fluid out of the system. Feed lines are most frequently employed for addition of nutrients or pH regulators. In fermentation processes, the organism metabolises nutrient sources to product. If a nutrient becomes limited it may impact on the health of the organism or may induce a shift in the organisms' metabolism. These events can be prevented, or encouraged in the case of nutrient limited cultures, for antibiotic production, through the addition of substrates into the fermentation system.

pH control is another important consideration in fermentation processes. Frequently the organisms employed in the fermentation will have a pH range over which they can survive, and an optimal pH at which their growth will be encouraged. The organism may be capable of the production of acids, or other metabolic products, which change the fermentation broth pH and impact on the organism. Addition of liquids to alter pH is therefore necessary to maintain the fermentation under optimal conditions.

Wang *et al.*, (2005) highlighted the importance of selecting and applying the appropriate fermentation system, and the potential benefits of employing batch, fed-batch or continuous fermentations for specific production purposes. Although these are the most commonly employed

fermentation configurations, there are a number of others in existence, and the selection of the appropriate one is dependent on the product being produced and the organism used.

A batch fermentation is a system whereby apart from oxygen in aerobic processes, all the required constituents from the initiation of the fermentation and product recovery takes place when the process is complete (Doran, 1995). In batch fermentation systems, the organism proliferates and produces metabolites until one or more factors exert an influence on the system. The main influencing factors include substrate limitation and end product inhibition (Crueger and Crueger, 1982). Substrate limitation involves either one or more substrates being exhausted and thus preventing further growth or production (Doran, 1995). End product inhibition involves the build up of product or toxic by-product in the system (Glazer and Nikaido, 1995). The batch fermentation is a simple process, but the use of methods which allow feeding of compounds into the fermentation system may lead to higher yield and to greater process control and reproducibility (Küenzi, 1978).

Fed-batch processes are applied to overcome some of the problems associated with batch fermentation including catabolite repression of secondary metabolite production (Crueger and Crueger, 1982). Fed-batch systems essentially comprise of two 'separate' operations; batch and feeding. The batch operation favours biomass growth and the feeding operation is ideal for controlled production. The feeding of the substrate involves continuous or intermittent addition of nutrients without the removal of culture fluid (Doran, 1995), thus there is an increase in fermentation volume and addition of nutrients will ultimately be limited by the volumetric confines of the vessel.

A milestone in the development of bioprocessing was reached with the perfection of the continuous fermentation process. These were first used for the production of food and feed from yeast and bacteria (single cell protein) (Crueger and Crueger, 1982). Continuous fermentations are open systems and are aimed at prolonging growth and increasing product yield

via the maintenance of substrate and product concentrations at optimum levels. High productivity is achieved by feeding continuously and removing product (Schügerl, 2000b). In this manner, substrate inhibition is reduced and the fermentation working volume is maintained. Ideally in continuous fermentations the rate of conversion of substrate to biomass and products should balance the output rate, maintaining steady state in the fermentation (Smith, 1981).

The fermentation processes and the conditions employed are dictated by the producing organism and the product generated. Frequently considerable effort may be exerted in order to develop an optimised fermentation processes. Once this is achieved however, the onus for a successful bioprocess is transferred to the recovery of the product.

1.1.2 DownStream Processing (DSP) of Fermentation Products

The yield of a bioprocess is not only dictated by the production process employed, but also the recovery methods. Fermentations are characterised by relatively low product concentrations and by the complex nature of the fermentation medium utilised. Separation and purification of the products generated from fermentation processes can have a great impact on the complexity of the overall production process, are laborious, and dominate overall process economics (Wang and Sobnosky, 1985, Gordon *et al.*, 1990).

DownStream Processing (DSP) is a name given to any treatment of the fermentation broth post-fermentation to recover or purify the product (Doran, 1995). Downstream processing operations have long been seen as the bottleneck for many production processes (DePalma, 2005b). However, downstream operations also represent some of the greatest opportunities for creating value during biomanufacturing through their optimisation (DePalma, 2005a). DownStream Process operations follow a flow, from treatment of a crude broth to recovery of a purified product, and commonly involve three main processes; primary separation, secondary

separation, and product polishing.

Primary separations involve a stream separation of the fermentation broth, employing techniques to separate the biomass (solid stream) from the fermentation liquor (liquid phase). In DSP operations solid-liquid separation techniques, such as centrifugation, filtration and sedimentation are commonly the first major operation in the recovery of bioproducts (Krijgsman *et al.*, 1993). Once this is achieved removal of non-desired compounds, or isolation of the desired product, is performed (van Erkel *et al.*, 2004). Even though these processes fulfil a relatively simple role in the overall recovery process, the loss of product associated with them can be significant.

An important consideration, when examining the recovery of any compound of interest, is its location. When the fermentation is completed the product may be found in the cytosol, in the periplasmic space or in the extracellular medium, depending on the secreting ability of the organism (Hedman, 1984). If the product is secreted into the production environment, the liquid phase is of predominant importance and the biomass may be discarded. If the product is associated with the biomass, being either intracellular or intraorganelle associated, discarding the solid stream would mean discarding the product (Zhukovsk *et al.*, 1973).

In some cases, the product may be present in both the liquid and solid phases of the fermentation broth. In this scenario, one must establish the benefits of treating both phases as a source of product, with relation to the contribution of each, on the total amount of product recovered. The separation method or technique employed in the primary separation may also be a source of product loss.

Secondary separation techniques are those which facilitate concentration and recovery of maximal amounts of the product of interest in as pure a form as possible. In liquid cultures, concentration of the product often entails removing or reducing aqueous volume of the fermentation feed.

The methods employed included membrane separation techniques, volatilisation, extraction and chromatography.

When membrane techniques are used to concentrate the product feed, techniques such as microfiltration, ultrafiltration or nanofiltration are the most common. These techniques have become firmly established as technologies which ensure purity, safety and efficacy of modern biopharmaceuticals (Christy and Vermant, 2002). Li *et al.*, (2004) and Tessier *et al.*, (2005) outlined the use of such techniques to improve separation, purification and extraction of bioproducts. These techniques separate fermentation broth feeds via the use of membranes and maintenance of a transmembrane pressure as the driving force for separation. The pore size of the membranes dictates the retention capacities of the filtration process, and such techniques have wide application in clarification of fermentation broths, biomass recovery and in particular protein recovery.

If the product is volatile, it may be recovered via evaporation. Once the product has a lower boiling temperature than the water which is the prime constituent of the fermentation broth, then evaporation and subsequent distillation can be used for product recovery. In some cases, the product may be removed via sparging (Pankow *et al.*, 1993). In this process the compound of interest is evaporated from the liquid culture using airflow. The volatile products are then recovered, in a concentrated form, through processes such as distillation and condensation.

Extracting the compound of interest is another means of concentrating the product. Techniques including liquid-mediated extractions, using appropriate solvents (Brunner, 1985, Roffler *et al.*, 1987, Bruce and Daugulis, 1991, Daugulis *et al.*, 1991) and solid-mediated extractions using chromatographic materials are commonly employed (Crueger and Crueger, 1982, Stoffels *et al.*, 1993, Brocklebank and Kalyanpur, 1993, Ramos *et al.*, 2004).

Solvent extraction involves contacting the fermentation liquor with a suitable solvent for which the target molecule has an affinity. These solvents are generally immiscible with the fermentation liquor in order to facilitate subsequent separation, and back extraction of the product of interest into a new aqueous phase, if required. There are a number of problems associated with solvent extraction, including the generation of solvent waste which requires either costly disposal, or development of processes to facilitate recycling. Solvents may form emulsions which are difficult to treat, and from which, recovery of product is difficult, and recovery efficiencies are therefore reduced (Doig *et al.*, 1999, Doig *et al.*, 1998).

Chromatography is a process of selective adsorption of the target molecule from a solvent onto an adsorbent (Strube *et al.*, 2002). Chromatography-mediated recovery processes are of major significance in industrial production processes. Two commonly applied methodologies are hydrophobic interaction-mediated recovery and ion exchange-mediated recovery. The development of adsorbent resins, chemically synthesised or conferred with desired properties like a biospecificity or affinity (Wang and Schultz, 1981), hydrophobic interaction capabilities (Kwon *et al.*, 1998) or ion-exchange capabilities (Bartels *et al.*, 1958) has facilitated the development of a number of novel recovery strategies.

Hydrophobic interaction chromatography is based on hydrophobic attraction and is a technique for the purification and separation of biomolecules based on differences in their surface hydrophobicity, particularly applied to protein recovery (Bywater and Marsden, 1983). Molecules exposing hydrophobic areas on their surface may be separated due to their interaction with a non-polar ligand (Sofer and Hagel, 1997). The materials employed in such separations usually contain-polar functional groups (such as butyl, octyl or phenyl) attached to a hydrophobic polymer matrix.

Where hydrophobic interaction chromatography relies on the hydrophobic properties of the compounds being targeted, ion-exchange

chromatography relies on the charge of the compound. If the compound to be recovered is a positively charged compound, cationic chromatographic materials are used, and if the compound is negatively charged, anionic chromatographic materials are used. Thus recovery processes employing ion-exchange chromatography techniques rely on the interactions of charged functional groups with ionic functional groups of opposite charge on the adsorbent surface (Ghosh *et al.*, 1997).

Biospecific adsorbents use very complex, characteristic attributes to bring about adsorption. This can take the form of antibody or enzymatic-like complexes, and are thus among the most specific adsorption processes. Pyrzynska and Wierzbicki, (2005) detailed the use of modified selective adsorbent resins for the recovery of vanadium. The resins were functionalised with porphyrin ligands to increase pre-concentration of the vanadium species.

Advances in genetic modification techniques mean it is now possible to genetically engineer an organism to produce a product with a specific recognition site to aid its subsequent recovery. Common examples of this include incorporation of a poly amino acid tag, such as repeated units of histidine or arginine, onto the product. These can then be recognised and adsorbed by the chromatography material used, which makes subsequent recovery of the product easier (Levin *et al.*, 2005, Sontag and Cattini, 2003).

It is important to note however, that irrespective of the mode of separation employed (affinity, hydrophobic or ionic-based) such events are often very complex, and the recovery or adsorption of a product may be attributable to a combination of such interactions, despite one being dominant.

Product polishing is the final purification stage of the DSP process. The bioprocessing techniques involved in final product polishing are primarily focused on obtaining the product in an appropriate form for its subsequent use, and in pharmaceutical biotechnology it is considered the most laborious and exacting part of the whole downstream process (Jungbaur

and Janson, 1993). The most commonly applied techniques include high selectivity chromatography (Jungbaur and Janson, 1993), drying (Tijsterman, 1993) and crystallisation (Schügerl, 2000b). Some bioproducts are more stable in solid form, thus as a result, if they can be recovered in crystalline form, their longevity and storage potential will be increased. In order to bring about crystallisation there must be a high concentration of pure product in the recovered stream. Strube *et al.*, (2002) outlined the considerations one must take into account when selecting the optimal refining strategy for a product and also suggested that a combination of unit operations may be advantageous.

In summary, with respect to the general procedures involved in DSP recovery of biocompounds, the ideal recovery process should facilitate the removal of maximal amounts of product in as short a time as possible since bioproducts can exhibit sensitivities to the production environment and other external factors including temperature, light, pH, etc (Wang and Sobnosky, 1985). In DownStream Processing, a number of unit operations may be required to return the product in a suitable form (Strube *et al.*, 2002). The greater the number of processes or unit operations involved in the recovery of product, the greater the potential for product loss, and this is one of the reasons *In-Situ* Product Recovery processes were developed.

1.1.3 Integration of DownStream Processing into Fermentation – Use of *In-Situ* Product Recovery (ISPR)

As previously mentioned, the downstream recovery of products from a fermentation feed usually involves numerous clarification steps including centrifugation, filtration, extraction etc. Xu *et al.,* (2005) suggested that there is significant potential for product losses and contamination during such steps and suggested that a process which could combine separation functions with recovery and purification of the product would be extremely beneficial. Such processes are known as integrated processes and can be applied for the recovery of a range of bioproducts including alcohols,

organic acids, antibiotics and proteins.

In-Situ Product Recovery (ISPR), often referred to as extractive or integrated fermentation, involves actions taken for the immediate separation of product from its producing cell (Freeman *et al.*, 1993) or the production environment. Continuous recovery of product may improve productivity by limiting the exposure of the product to a potentially destructive environment (Schügerl, 2000b). ISPR is implemented to improve yield and productivity via minimisation of product inhibition, product losses due to degradation or evaporation, and reduction of the number of subsequent DownStream Processing steps (Dukler and Freeman, 1998, Freeman *et al.*, 1993).

For a successful ISPR protocol to be established, the integrated bioprocess set-up should consist of a bioreactor and a downstream unit, coupled with a means that guarantees the fast removal of the products (Bluemke and Schrade, 2001). The most applicable technique is dictated by the process conditions and limitations, thus a particular ISPR method may not be suitable for the recovery of a wide range of bioproducts. There are five main techniques which can be employed in ISPR methodologies for the recovery of product from its production environment, and they are: 1) Evaporation, 2) Extraction, 3) Permeation, 4) Immobilization and 5) Precipitation (Stark and von Stockar, 2003).

1) Evaporation is used for the recovery of volatile compounds such as alcohols and some of the techniques employed include vacuum fermentation, flash fermentation, gas stripping and pervaporation (Roffler *et al.*, 1984, Freeman *et al.*, 1993).

Vacuum evaporation involves maintaining the fermenter under vacuum so that the product evaporates at the normal temperature at which the fermentation is run. In flash evaporation procedures, the fermenter is kept at atmospheric pressures, and the broth is removed to an evaporation chamber where the product can be boiled off (Roffler *et al.*, 1984). Daugulis *et al.*, (1991) explained how this flash evaporation method could

be adapted, whereby the product could be extracted Into a solvent, which in turn underwent the flash evaporation process with associated condensing and recycling of the extracting solvent. Gas stripping uses a sparger to sparge gas bubbles into a fermenter, which removes the volatile compounds from the medium upon rupturing (Ezeji *et al.*, 2005). Finally, pervaporation is essentially an integrated system involving permeation and evaporation of the product. Pervaporation distinguishes itself from other membrane process, since on the feed side there are liquid mixtures, but on the permeate side the product is removed as a vapour. The driving force for the permeation is the high pressure difference across the membrane (Huang *et al.*, 2006).

2) Extraction is primarily used for the recovery of compounds from liquid cultures, where they are in solution and present in low concentration. Extraction methodologies essentially involve removing product from one stream to another. The techniques generally employed are liquid-liquid extraction, aqueous two phase systems and solid-liquid extractions, although the latter can be included as an immobilisation technique and will be covered as such in later sections.

Liquid-liquid extraction involves removal of the product from the production medium into an appropriate organic solvent. The use of organic solvents to extract products continuously from fermentation broths is a mature technology, and can be used for a variety of biocompounds (Brocklebank and Kalyanpur, 1993). The general technique of liquid-liquid extraction involves contacting the broth with the solvent, either in the fermenting vessel or in an external extracting vessel, at which point the products dissolve into the solvent and can be recovered later through processes such as back-extraction or distillation (Roffler *et al.*, 1984).

Extraction with organic solvents is a major technique in bioprocessing, however organic solvents may be unsuitable for recovery of sensitive biomolecules (Doran, 1995), or the solvent itself may be toxic to the producing cell or environment (Stark and von Stockar, 2003). In such cases aqueous two phase systems gain significance. Aqueous two phase

systems (ATPS) are employed to overcome the problems associated with organic liquid use in extractive fermentations. These systems generally employ polymers (e.g. polyethylene glycol) to establish a second liquid phase in the fermentation, which allows the product to distribute between the two phases (Roffler *et al.*, 1984). In ATPS the cells may be considered to be immobilised 'on' one of the phases and the required product is made partition into the other phase by proper manipulation of the system (Banik *et al.*, 2003). This manipulation of the system can include alteration of parameters such as polymer concentration, salt concentration, system pH and phase volume ratio (Benavides and Rito-Palomares, 2004).

Frequently, a combination of extraction and evaporation can be used to increase product yields and recoveries. Roffler *et al.*, (1988) detailed the *In-Situ* extractive fermentation of acetone and butanol using oleyl alcohol as the extractant and employed a steam stripper and condenser to recover product and recycle the extractant.

3) Permeation brings about separation of the compound of interest from the production environment through the use of permeable or semi-permeable barriers or membranes. In membrane fermentations, a feed composed of two or more components is separated using a semi-permeable barrier, the membrane, into a permeate (the fraction of the feed that passes through the membrane) and a retentate (the fraction of the feed retained by the membrane). This barrier can be made of a solid material or a fluid (gas or liquid) (Fernandes *et al.*, 2003) and the product can be recovered into the extractant on the other side of the membrane.

Fernandes *et al.,* (2003) suggested that mass transfer in the porous supports generally used in membrane bioreactors is a diffusion-controlled process, often becoming the rate-limiting step, however, this can be overcome or reduced by the use of membrane modules. Where membrane modules are used it is essential to maintain a concentration driving force by continually removing product on the downstream side, via use of an appropriate extractant (Freeman et al., 1993).

Membrane bioreactors can serve as a complete fermentation and recovery device. The unique character of membrane bioreactors, compared with other simultaneous bioreaction and bioproduct separation processes, is that neither extractant nor the membrane, as a kind of mass separation agent, will mix with the product stream, thus simplifying the further separation and purification process (Cen and Tsao, 1993). Xu *et al.,* (2005) used a novel integrated membrane chromatography device to improve protein separation. The unit consisted of a hollow fibre filtration unit, with packing of chromatographic resin beads on the shell side of the unit. It combined filtration and chromatography and reduced the process steps involved in protein recovery, limiting the potential for degradation of product between steps.

Dialysis membranes have also been employed in ISPR, in the form of electrodialysis. Electrodialysis is applied predominantly for the recovery of acids. van Erkel *et al.*, (2004) noted that electrodialysis was applicable for recovery of dissociated acids, for separation of amino acids and for conversion of dissociated acid into the corresponding acid. Zelić *et al.,* (2004) used an novel electrodialysis ISPR process to prevent product inhibition during the production of pyruvate. The technique involved applying a charge across an ion permeable membrane, allowing the passage of ions from one solution to another. The entire ISPR approach comprised of a fermentation with protein separation and cell retention and recycling by ultrafiltration, product recovery by electrodialysis, sterilisation by microfiltration and subsequent recycling of pyruvate-reduced fermentation permeate. This approach allowed additional product concentration in a separate liquid phase and reduced water handling over the continuous and repeated fed-batch systems which were also examined.

A novel variation of this technique was detailed by Stark *et al.,* (2003) for the extraction of 2-Phenylethanol from fermentations of *Saccharomyces cerevisiae*, where a solvent, dibutylsebacate, was required for the extraction process. The yeast used in the production process could not tolerate the extracting solvent, thus a novel method was developed to

allow ISPR of the 2-Phenylethanol. This took the form of microcapsules comprised of an outer layer of alginate acting as the permeable barrier, which prevented contact between the inner core extraction liquid and the producing organism but allowed permeation and extraction of the product across the barrier and into the dibutylsebacate. A similar method was detailed by Serp *et al.,* (2002). Wyss *et al.,* (2004) also used a similar methodology for herbicide and pesticide extraction from water. These types of extraction processes were termed perstraction, elucidating to the fact that they involved an agglomeration of permeation and extraction techniques.

4) Immobilisation involves removal of the product from the production liquid onto a solid phase. Product removal by immobilisation has been demonstrated for a large variety of products (Freeman *et al.*, 1993), and can serve to address some of the limitations associated with solvent-based *In-Situ* extractions for product recovery such as solvent toxicity.

There are a multitude of adsorbents in existence which have a huge variety of applications. Adsorbents commonly applied range from activated carbon, sand, charcoal and alumina (Dutta *et al.*, 1999, Muhammad *et al.*, 1998, Goyne *et al.*, 2005, Arias *et al.*, 1979) to adsorbent resins and biomass (Grezegorczyk and Carta, 1996, Lee *et al.*, 2003, Veit *et al.*, 2002, Aksu and Tunç, 2005). The application of adsorbents is not confined to ISPR application or for recovery of specific compounds. They have been used in many applications ranging from the adsorption of coloured compounds (Kim *et al.*, 1999, Gökmen and Serpen, 2002), proteins (Hamilton *et al.*, 2000), pesticides (Kyriakopoulos *et al.*, 2005), acids (Otero *et al.*, 2004), phenols (Ku and Lee, 2000) mutagenic organics (Daignault *et al.*, 1988), pollutants (Pyrzynska and Wierzbicki, 2005) and have even been applied to induce gene expression (Ermolaeva *et al.*, 2004).

5) Precipitation is perhaps the least frequently employed technique in ISPR processes, since only certain cases exist in which it can be achieved. Precipitation may involve product crystallisation and

precipitation events to occur, where a charged product can be precipitated by a counter-ion during fermentation (Stark and von Stockar, 2003). van Erkel *et al.*, (2004) described the benefits of *In-Situ* crystallisation of carboxylic acid, whereby changing pH to bring about crystallisation not only enhanced productivity, but also resulted in the production of a raw product which required less subsequent purification treatments.

A novel variation of this idea was detailed as a method of ISPR, demonstrated by Wei *et al.,* (2003) and Yang *et al.,* (2004). In their process, the ISPR of Cefaclor was mediated by the formation of insoluble complexes of product and extractant and recovery and product yield was facilitated and increased in this manner.

It is clear from discussing the use of general ISPR techniques, that an efficient ISPR process may take advantage of more than one of these techniques to bring about product recovery. It should be emphasised that while ISPR is considered mostly for the improvement of existing processes, in some cases, where product-cell interference is intensive, ISPR may be found to be essential in carrying out the process (Freeman *et al.*, 1993). In the case where the producing organism has a minimum inhibitory concentration for its own product, Gastaldo *et al.*, (1996) found that inclusion of adsorbent resins in fermentations of *Actinoplanes* sp. A8924, resulted in the removal of the product, kirromycin, which inhibited *Actinoplanes* growth.

Alternatively, the product could be susceptible to further treatments or degradation in the production environment e.g. hydrolysis. Ahmed *et al.*, (2001) applied ISPR techniques to increase the yield of kinetically-controlled biocatalytic reactions in which competing reactions lead to product degradation. The product could also impart detrimental effects on the production environment as a whole. If the product increases the viscosity of the environment, this may lower oxygen transfer and reduce production or growth potential of the organism (Solomons, 1980). These are just some of the reasons why ISPR has gained significance, and how the correctly selected methodology can benefit fermentation success.

1.2 Antibiotics

The term 'natural product' is commonly reserved for those organic compounds of natural origin that are unique to one organism, or common to a small number of closely related organisms (Mann, 1987). Antibiotics are important examples of natural products which exhibit an ability to stop microbial growth (bacteriostatic activity) or to kill microbes completely (bactericidal activity), through interruption and interaction with specific cellular components and disordering cell metabolism (Hammond and Lambert, 1978). Zähner (1978) suggested that antibiotics could also be defined as products of secondary metabolism with an incidental action in minimal concentration on growth processes.

Louis Pasteur made the first recorded observation of antibiotics, the inhibition of one organism by the products of another, when in 1877, he and Joubert demonstrated that anthrax bacilli were killed when the culture became contaminated by certain other bacteria (Hammond and Lambert, 1978). The discovery in 1929, by the Scottish biologist Alexander Fleming that *Penicillium notatum*, a common mould, could produce a compound able to selectively inactivate a wide range of bacteria without unduly influencing the host (Smith, 1981) gave an indication that antibiotics derived from natural sources could be exploited for the benefit of mankind.

During World War II, the demand for chemotherapeutic agents to treat wound infections lead to the development of a production process for penicillin and the beginning of the era of antibiotic research (Crueger and Crueger, 1982). Some time later, Professor L. P. Garrod, a leading bacteriologist, reflected back on Flemings discovery and commented that no other such casual observation has had such momentous consequences (Calam, 1987). Antibiotic production continues to be one of the most important areas of microbiology today. Intensive screening programs in all countries continue to increase the number of described antibiotics; 513 were known in 1963, 4076 in 1974, and it is estimated that

up until 1982, between 100 and 200 new compounds were discovered annually, with screening procedures continuing to occur to this day (Crueger and Crueger, 1982, Watve *et al.*, 2001). Since the discovery of the first antibiotics and penicillin, more than 6000 natural microbial compounds have been described, all of which display antibiotic activity (VanDamme, 1983).

Before the discovery of antibiotics *Staphylococcus aureus* was fatal in 80 percent of infected wounds, however, after the first prescriptions of antibiotics in the 1930's, bacterial infection as a cause of death plummeted and between 1944 and 1972 life expectancy increased by eight years, largely attributed to the impact of antibiotics (Walsh and McManus, 1999).

Antibiotics have not only been employed in medicine, but also in a huge range of industries from food to agriculture. The successful use of antibiotics has greatly expanded the fermentation industry, and has resulted in the research, development and production of antibiotics now representing a multibillion dollar industry worldwide (Ōmura, 1986). In terms of monetary value, apart from the traditional products of cheese and alcoholic beverages, antibiotics are currently the most important products of microbial biotechnology, with the worldwide antibiotic production estimated at a value of $16 billion in 1995 (Glazer and Nikaido, 1995), and continuing to the present day.

There is, however, a note of caution. The widespread misuse of antibiotics is believed to pose a significant danger to the future health of the modern world. The increase in emergence of antibiotic resistant strains in a number of microorganism species (Crueger and Crueger, 1982, Smith, 1981, Russell and Chopra, 1990, Walsh, 2003), has meant that antibiotic use must now be tightly regulated and controlled, and there is therefore, a continued need for research and development into new antibiotics.

1.2.1 Classification of Antibiotics

Antibiotics currently available can be classified in four major ways. Classification can be based upon the microbial origin of the antibiotic, its mode of action, its target organisms or its chemical structure.

Antibiotics are predominantly produced by microorganisms, thus classification of antibiotics on the basis of their origin usually means identifying the microorganism which produced them as either bacterial, fungi or actinomycete. These are the major microbial classes of antibiotic producers, with the latter two being the most prevalent (Hammond and Lambert, 1978). By extension, the physiological diversity among these microorganisms can be illustrated by the variety of the antibiotics they produce (Ensign, 1981).

Classification of antibiotics based on their mode of action is more complex. There are four main modes of action of antibiotics which can be used for their classification. Antibiotics can exert their influence via prevention of cell wall synthesis, DNA replication and repair, protein biosynthesis and nucleic acid synthesis (Hammond and Lambert, 1978, Walsh, 2003).

Antibiotics which act through prevention of cell wall biosynthesis include the β-lactams and glycopeptides. In general these types of antibiotics exert their effect during one of the steps in bacterial cell wall assembly, and usually inhibit enzymes or sequester substrates involved in peptidoglycan (the major unit of the cell wall structure) assembly and cross-linking. Antibiotics which block DNA replication and repair include the fluoroquinolones. These antibiotics act by inhibition of enzymes essential in processes of DNA replication and repair. They interact with and inhibit enzymes such as topoisomerases and gyrases which are essential for cell viability and result in increased cleavage of DNA (Walsh, 2003).

Antibiotics that block bacterial protein biosynthesis include the aminoglycosides, tetracyclines and the macrolides. These antibiotics exert

their actions by blockade of one or more of the protein biosynthetic steps that occur on the 30s and 50s subunits of the bacterial ribsome. The final major mode of antibiotic activity used for classification is prevention of nucleic acid synthesis and is displayed by antibiotics including the sulphonamides and trimethoprim (Walsh, 2003). The growth and division of cells depends upon, amongst other factors, DNA and RNA synthesis. Antibiotics classified as nucleic acid synthesis inhibitors do so by either interruption of nucleotide metabolism, interruption of DNA template formation or direct inhibition of enzymatic processes essential to nucleic acid synthesis (Russell and Chopra, 1990).

Classification may also be based on the range of target organisms against which the antibiotic is efficacious. Three classes exist: broad spectrum, meaning the antibiotic has activity against a large group of organisms, medium spectrum, meaning the antibiotic has activity against a medium sized group of organisms, or narrow spectrum, meaning the antibiotic is relatively specific, and exerts its activity on only a small group of organisms.

Classification of antibiotics can be aided by the fact that they show wide varieties of chemical structures encompassing aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines. Although antibiotic chemical structures may be varied, the pool of primary metabolites from which they are derived is small, thus their chemical diversity is a result of variations in metabolic pathways and processes (Turner, 1973, Okami and Hotta, 1988).

1.2.2 Sources of Antibiotics

Antibiotics belong to a group of substances referred to as secondary metabolites (Calam, 1987). Secondary metabolism is non-essential for growth and reproduction of the producing organism and the secondary metabolites are formed by a limited number of organisms, and encoded by

dispensable genes, which are highly regulated and usually organised into clusters (Martín *et al.*, 2005). Secondary metabolism is a characteristic of lower forms of life, such as microorganisms, and secondary metabolites can accumulate in substantial quantities and be excreted into the environment in which the producing organism is growing (Hammond and Lambert, 1978).

The taxonomic distribution of antibiotic-producing organisms is restricted to relatively few groups. All antibiotics of bacterial origin used in medicine are produced by the genus *Bacillus.* The Fungi are a more important group, with antibiotics of chemotherapeutic use being derived from the genera *Aspergillus* and *Penicillum* (a group of filamentous, spore-forming moulds), including penicillins, cephalosporins and fusidic acid. The actinomycetes, and in particular the genera *Streptomyces,* are easily the most important antibiotic producing group, synthesising a wide range of antibiotics, from chloramphenicol to streptomycin (Hammond and Lambert, 1978).

1.2.2.1 Fungi

The fungi comprise of a polyphyletic group of eukaryotic organisms which are united by a number of common characteristics of nutrition and morphology. Currently, some 65,000 species of fungi are accepted and new species are being described at a rate of 1500 per year, however only a few of these have been exploited for industrial use (Williams and Kirk, 1988). The determination of the number of individual fungi is impossible, given that in a gram of soil, one could expect to find in excess of 100,000 fungal spores (Cooke, 1980). Fungi are typically filamentous microorganisms, capable of spore forming, whose growth patterns involve production of hyphae (the individual filaments), which grow from their tips and branch out to form a hyphal mass known as a mycelium (Deacon, 1984).

Fungi can cause immense economic losses. Their harmful activities as saprotrophs include damage to timber, fuel, food and manufacturing goods. As parasites they cause heavy crop losses and diseases of man and domestic animals. However, the beneficial activities of fungi are also of great significance. They have long been exploited as food, in food processing, in brewing, and in modern fermentation processes and contribute to the production of valuable products such as vitamins, enzymes and most importantly, antibiotics (Carlile and Watkinson, 1994). Cultivation of fungi can be achieved in surface, shaken, stirred aerated and continuous culture, and the method employed and materials utilised are dependent on the product being produced and the producing fungi (Turner, 1971).

Two of the most important types of antibiotics are however produced by fungi, the penicillins and the cephalosporins (Carlile and Watkinson, 1994). Penicillins and cephalosporins belong chemically to a group of antibiotics known as β-lactams. Industrial production of penicillin and cephalosporin was achieved using *Penicillium chrysogenum* and *Acremonium chrysogenum* respectively, and importantly from a synthesis and production point of view, all naturally occurring penicillins and cephalosporins are synthesised from the same three amino acid precursors, L-α-aminoadipic acid (L-α-AAA), L-cysteine and L-valine (Brakhage *et al.*, 2005).

Penicillins are the most important compounds to have been isolated from fungi, and even to this day, penicillin represents one of the worlds major biotechnology products (Turner, 1971, Li *et al.*, 2005). Penicillin was first produced for clinical use at Oxford, in 1940, by a surface culture of the fungi *Penicillium notatum* (Carlile and Watkinson, 1994). Now however, *Penicillium chrysogenum* is the most commonly employed organism for the production of penicillin (El-Sabbagh *et al.*, 2005).

The continued successful application of penicillins can be attributed to structural alterations of the penicillin molecule 6-amino penicillanic acid (6-APA) for the generation of semi-synthetic antibiotics. This molecule

consists of two amino acids, cysteine and valine, to which various acyl side-chains may be attached and therefore forming 'new' penicillins, with altered antibiotic activity (Deacon, 1984).

In 1945 *Cephalosporium acremonium* was shown to inhibit bacterial growth, and after detailed studies were carried out, the antibiotic cephalosporin was isolated (Carlile and Watkinson, 1994). One of the cephalosporin antibiotics, cephalosporin C, is produced industrially and is similar to 6-APA in that it can be altered, to yield a range of new, semi-synthetic antibiotics of industrial and medical significance, via the addition of different sub-chains (Carlile and Watkinson, 1994, Schmidt, 2002, Araujo *et al.*, 1996).

1.2.2.2 Actinomycetes

A classification of any microbial order is a temporary and man-made arrangement in which similar individuals, sharing certain common features, are grouped together as taxonomic units at different levels in the taxonomic hierarchy (Cross and Goodfellow, 1973). Traditionally there had been much confusion about the taxonomic classification of Actinomycetes, with bacteriologists considering them bacteria and mycologists considering them fungi. The general consensus is now that Actinomycetes are more accurately classified as bacteria (Gottleib, 1973).

Actinomycetes are Gram positive, soil dwelling bacteria, ubiquitous in nature. Reproduction is usually asexual, though sexual processes have been shown to occur by genetic analysis. In the nonhyphal forms, asexual reproduction is by fragmentation or perhaps even by the usual fission of single cells. Where stable hyphae are produced, vegetative reproduction is by well formed spores resembling fungal arthropores, bourne either free or in sporangia. The free spores are usually in the form of sporophores and may consist of one, two or many spore chains arising from primary hyphae, which may either be straight, looped or spiral (Gottleib, 1973).

Actinomycetes play both detrimental and beneficial roles in nature. They are opportunistic pathogens, can cause diseases of animals and humans and plants. They can and cause problems in sewage treatment plants, and have been known to cause spoilage of plant and hydrocarbon-based products such as hay, straw, cereal grains, seeds, rubber and plastic. However, far outweighing any negative attributes the actinomycetes may have, is their beneficial activities in biodegradation and above all, antibiotic production (Piret and Demain, 1988). Although the first useful antibiotic, penicillin, came from a fungus, *Penicillium notatum*, most of the antibiotics in clinical use come form the Actinomycetes, especially *Streptomyces* (Carlile and Watkinson, 1994). Today, 60% of the known antibiotics are produced by actinomycetes (Ōmura, 1986) and include almost all known structural classes of commercially important antibiotics (Ōmura, 1986).

Streptomyces are the source genus for the majority of the secondary metabolites produced by the actinomycetes (Bushell, 1988). Of the antibiotics produced by actinomycetes, 90% originate from *Streptomyces* (Ōmura, 1986), and as a result it is the species which exhibits the greatest variety of antibiotic production (Crueger and Crueger, 1982). Since the potential of *Streptomyces* as source organisms for efficacious biocompounds was first identified over fifty years ago, they have been the subject of intensive investigation (Dietz, 1986) and the majority of antibiotics discovered have been isolated from them (Thompson *et al.*, 2002). Crandall and Hamill, (1986) have catalogued ten major structural types of antibiotics produced by the genus.

The number of antimicrobial compounds reported from the species increased almost exponentially for about two decades until the 1970s, at which point there has been a decline until the present day (Watve *et al.*, 2001). Within the antibiotic industry, there are thousands of scientists whose careers are dedicated to developing a better understanding of the growth and secondary metabolism of *Streptomyces* on a large scale. With the exception of yeast fermentations for food and beverages, there is more fermentation tank capacity dedicated to *Streptomyces* fermentations than any other class of microorganism (Bader, 1986).

Identification of microorganisms capable of producing antibiotics is achieved through screening programmes. These programmes involve taking samples from the environment, such as soil samples, plating them and recovering sample colonies. These colonies are then grown further on slopes and examined for antibiotic production (Calam, 1987, Hammond and Lambert, 1978). Figure 1.1 outlines the typical *Streptomyces* life cycle on solid culture, which is similar to the growth and differentiation of fungi (Ōmura, 1986).

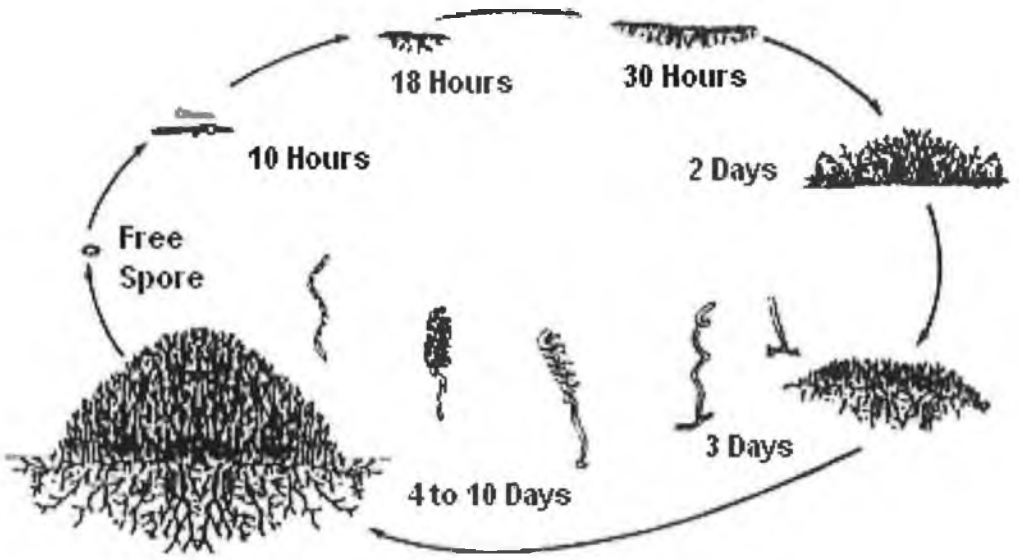


Figure 1.1: Schematic of the *Streptomyces* life-cycle (van Wezel, 2002)

1.2.3 Production of Antibiotics

It is generally agreed, that the production of antibiotics by microorganisms, is done so to gain some growth advantage in their natural environment (Gottleib, 1973, Turner, 1971). Competition among microorganisms for limited nutrients is commonplace, and it is as a result of these stresses, or during certain stages of growth of the microorganism, that antibiotic production occurs (Mann, 1987). When microorganisms enter the stationary phase of growth and face competition for space and nutrients

the genes responsible for the timing and level of antibiotic production are expressed and the antibiotics formed are used to regulate the growth of, or perhaps more accurately wage 'chemical war' on, their neighbours (Walsh, 2003). Antibiotic production therefore provides the organism with a survival advantage from an evolutionary point of view (Bader, 1986, Demain and Fang, 2000).

In nature, the generation of antibiotics is tightly regulated. Since antibiotics are frequently associated with limitation of nutrient supply (Roubos *et al.*, 2002), and are believed to be non-essential to the producing organism, antibiotic production represents a drain of resources, to produce a product which is frequently inhibitory to future cellular growth (Mann, 1987). Metabolic control systems are in place to ensure easily metabolised nutrients are used first and that growth has priority over less urgent activities such as secondary metabolite production (Vining and Chatterjee, 1982).

These control systems therefore, need an external 'triggering force', in order to initiate production. In the natural environment, this is supplied by the previously mentioned conditions of nutrient limitation, environmental stress, and competition for growth. In antibiotic fermentation processes, it is necessary to simulate this competition (through environmental control) in order to facilitate over-production of the antibiotic.

Initial investigations into antibiotic production took place in solid cultures, termed surface or static cultures (Kristiansen and Bu'Lock, 1980). Static cultures were then employed to produce the first microbial-derived compounds and antibiotics, since this static culture was suitable for growth of the most prolific producers of antibiotics, fungi and actinomycetes, which exhibit minimal nutrient requirements (Glazer and Nikaido, 1995). However, advances in bioprocessing procedures and a lack of reproducibility of product formation on static cultures (Bushell, 1988), resulted in movement of antibiotic fermentations from solid culture to liquid culture, termed submerged culture.

Submerged cultures take place in suitable fermentation vessels, which serve to provide the culture organism with its physical environment (Solomons, 1980), and take advantage of the wide variety of instrumentation available in the bioprocessing industries to provide tight control over the fermentation process. As a result of this, antibiotic fermentation now predominantly takes place in liquid cultures and under conditions of aeration and agitation (Küenzi, 1978). The media used for the liquid culture of antibiotics can be comprised of ill-defined, cost effective, complex materials and the fermentations can take place at the preferred temperatures of 24 - 28°C, and at a pH in range of 5.6 - 7.0 (but can be performed in some cases, as low as pH 5), which suit the producing organism (Calam, 1987).

An important point of note regarding the production of antibiotics is that static cultures are characterised by low product yields. The most significant difference between static and liquid cultures is that in static cultures, all the different stages of differentiation of the organism under investigation are present in the colony at any particular time. Where sequential formation of different products occurs within liquid cultures during the culturing processes, one could reasonably expect, in solid cultures, that all of those products would be present, somewhere in the colony, with their spatial distribution reflecting their temporal separation, in the equivalent liquid culture (Bushell, 1988). This variability in existence in the production profile in solid culture contributes to the limited application of solid cultures for large scale antibiotic fermentations.

1.2.3.1 Fermentation

Almost all antibiotics of commercial importance are manufactured by large scale aerobic fermentation, involving stirred tank reactors (Gupta *et al.*, 1997, Carlile and Watkinson, 1994). Antibiotic production has historically been a batch process-based industry, where cells, having been developed through successive rounds of inoculum development, are inoculated into the medium. The organism continues to grow and consumes nutrients until

depletion of some nutrient causes a reduction in cell growth and an associated production of antibiotic (Bader, 1986). Feed-type fermentations are also significant in antibiotic production processes, with their main advantage being that many antibiotic producing organisms are less productive in the presence of excess carbon source. This is especially the case if the compounds are rapidly degradable carbon sources such as glucose (Glazer and Nikaido, 1995).

Limiting nutrient supply, or supplying nutrients which can only be metabolised at a slow rate, mimics the natural growth environment of the antibiotic producing organism, and results in a successful antibiotic fermentation. Improvements in fermentation yields of antibiotics are often brought about by running the process in two stages. The first is submerged culture of the organism with sufficient aeration and generous nutrient supply to attain near-maximal cell density in a short period of time. In the second stage, when the culture reaches the stationary phase or stops growing, and antibiotic production begins, the concentration of key nutrients, such as carbon, nitrogen and phosphate, must be controlled carefully (Glazer and Nikaido, 1995). This is best achieved in sequential batch fermentations or using feed-type fermentations.

It is important to recognise that the development of antibiotic fermentation processes requires a triangular interaction between organism improvement, development of media and optimisation of process conditions (Bader, 1986). Antibiotic synthesis often requires dozens of enzymes and complex metabolic pathways. It may therefore, be important to understand the physiology of the producing organism in order to maximise the fermentative production of antibiotic (Glazer and Nikaido, 1995).

Antibiotic fermentations differ from fermentations used for the production of biomass, primary metabolites or other products in four main aspects, which are important from a process engineering perspective, and must be considered when developing an antibiotic fermentation process. These

are: (i) growth and production, (ii) morphological change and impacts, (iii) growth rates, (iv) production media (Küenzi, 1978):

(i) In antibiotic fermentations the organism generally must grow to a sufficient state before production occurs. Dykstra and Wang, (1990) detailed this phenomenon in their assessment of the production of the antibiotic cyclochexamide. They found that in batch fermentations, the onset of the stationary phase was marked by the beginning of significant antibiotic production and a substantial downturn in the rate of protein synthesis, while the end of the production phase was marked by the depletion of glucose in the fermentation medium.

From a processing point of view, this may be regulated by the use of either feeding mechanisms, such as fed-batch systems, or by having single unit operations, whereby a single fermentation serves to deliver maximum growth of cells utilising the optimal growth media and conditions. Once this is achieved, the medium is drained and replaced by fresh medium, designed for optimal production, or the cells are harvested and re-inoculated into fresh medium. However, there is considerable contamination risk from the second scenario, thus fed-batch systems would generally be favoured. An important point of note with regards to growth and production in antibiotic fermentations is that optimal nutrition for growth is not necessarily the same for production (Schrader and Blevins, 2001, Glazebrook *et al.*, 1992, Kojima *et al.*, 1995).

(ii) Understanding the impacts of morphology on antibiotic fermentations provides a better understanding of the complexities that exist over those fermentations not involved in antibiotic production. The most frequently applied organisms for antibiotic production are filamentous microorganisms such as *Streptomyces* and fungi, both of which are known to undergo pronounced morphological changes during culture (Küenzi, 1978). This can impact on the production and bioprocessing parameters employed, and greatly influence culture development in such systems (Papagianni, 2004, Prosser and Tough, 1991).

Submerged growth of filamentous organisms in liquid culture results in free movement of the microbial particles in the liquid medium, due to agitation, and maximises the potential of branched growth, by enabling the organism to develop in three dimensions (O'Cleirigh, 2005). Two of the distinct morphological states which can exist in antibiotic fermentations involving filamentous organisms are dispersed filamentous and pelleted growth, and the occurrence of these can be influenced by both the organisms and the fermentation conditions.

Dispersed filamentous growth is essentially the simplest morphological form, comprising of relatively uniform, short, branched hyphae, and is the closest approximation to unicellular behaviour possible for filamentous organisms. Dispersed filamentous growth is usually brought about in environments of high shear, since this confers a high level of disruption and dispersion of biomass in the system. The benefit of dispersed filamentous growth is that there is a greater proximity between organism and liquid medium, thus mass transfer can be maximised (Prosser and Tough, 1991), however such systems frequently suffer from high viscosities.

The filamentous nature of *Streptomyces* growth tends to be the primary contributing factor to the creation of these highly viscous, non-Newtonian, fermentation broths, which are characterised by a sensitivity to shear (Bader, 1986). However, the problems associated with this may be compounded, if the organism can also produce compounds capable of affecting the production environment rheology.

Pellet formation, agglomeration of mycelial masses, can occur in agitated systems due to collision of the biomass particles and resultant adhesion and intertwining of growth (Lu *et al.*, 1998). The pellets can range in form, from loosely packed 'fluffy' pellets to tightly packed, compact pellets (Papagianni, 2004), and the form which is produced, can influence the production of the system through mass transfer and viscosity effects. The growth of filamentous organisms in pellets is preferable to that of dispersed mycelia, from a bioprocessing point of view, as it reduces the

tendency of the microorganism to grow on fermenter walls, around impellers, and to foul gas distribution apparatus. This therefore improves operating conditions (Papagianni, 2004), and also reduces broth viscosity (Sinha *et al.*, 2001), which insures more efficient power consumption for mass transfer processes within the system (O'Cleirigh, 2005).

To summarise the morphological characteristics of filamentous organisms in submerged culture one should note that three major characteristics can be observed; complex morphology, by growing in different forms; complex rheology, producing non-viscous Newtonian cultures or viscous non-Newtonian cultures depending on morphology medium; and growth on surfaces, as a compact mass in the fermenter, below the liquid line, adhered to baffles, probes etc, or as surface growth, on fermenter head-plate and walls above the liquid level (Solomons, 1980).

(iii) Growth rates of the filamentous organisms used in antibiotic fermentations can have a number of influences on the success of the fermentation process. Firstly, a slow growth rate means that there is an increased potential for the growth environment to become contaminated by microorganisms with faster doubling times and growth rates. Also, these slower growth rates result in longer fermentation times, thus any errors incurred early in the fermentation process may only become visible or exert their effects, late on in the fermentation (Küenzi, 1978). The net result of this is that antibiotic fermentations generally take longer than other types of fermentations, and require careful monitoring and control to prevent costly losses of fermentation viability.

(iv) The medium employed in any fermentation process is a primary influencing factor on the success of the process and provides energy and essential nutrient sources for the organism to metabolise (Corbett, 1980). The basic requirements of filamentous microorganisms for antibiotic production in submerged culture include water, molecular oxygen, an energy source, organic carbon, nitrogen other than in a molecular form and a variety of other elements (O'Cleirigh, 2005). There are approximately thirteen elements essential to growth, five required in large

quantities (macronutrients: including carbon, hydrogen and oxygen), the remaining eight required in small amounts (micronutrients: including potassium, iron and zinc) (O'Cleirigh, 2005, Papagianni, 2004). Filamentous organisms depend heavily on the presence of the requisite nutritional compounds at optimal concentrations to ensure maximal growth and product formation (O'Cleirigh, 2005).

The nutrient requirements of antibiotic producing organisms such as *Streptomyces* are generally less chemically defined than those of other microorganisms (Glazer and Nikaido, 1995). It is common practice, with antibiotic fermentations, to use complex materials as nutrient sources, and two commonly employed products are corn-steep liquor and cotton seed oil. These materials are at least partially soluble and can be broken down by enzymes, produced by the cells, at a slow and steady rate which matches the slow growth of the organisms associated with antibiotic production (Calam, 1987). Although the most common substrates for antibiotic fermentations are starch, oils and various types of simple or cost effective sugar sources such as beet molasses, many fermentation media contain small amounts of glucose, which provide the culture with a rapidly utilisable carbon source (Bader, 1986).

The above considerations highlight the differences between antibiotic fermentation processes and those aimed at the production of other bioproducts. The primary goal of antibiotic fermentations is production of maximal amounts of product, but achieving this is not always a simple process. A better insight into the manipulation and regulation of secondary metabolite fermentations has generated new ideas on how to force cultures into over-production (Stephanopoulos *et al.*, 1998). Even so, there is considerable potential for reduction of product yields as a result of fermentation processes.

In antibiotic fermentations, the production and accumulation of the product can inhibit further production and cell growth. Three main means by which antibiotic yields may be diminished during the fermentation process are product feedback inhibition, product utilisation and degradation and

environmental impacting factors.

Product feedback inhibition is a common event in antibiotic fermentations (Wang *et al.*, 1989) and can reduce antibiotic yields. Feedback inhibition occurs when a product of a pathway controls the rate of its own synthesis through inhibition of an earlier step in the pathway (Horton *et al.*, 2006). These feedback mechanisms are in place to control reaction rates to production, via the use of enzymatic conversions (Calam, 1987). In the natural environment, once sufficient amounts of antibiotic have been produced and excreted into the surroundings of the producing organism to confer a growth advantage, production is ceased, and wastage of valuable, limited nutrients, is prevented.

In industrial antibiotic production processes, this natural 'safety mechanism' can limit product yield once a threshold level (set by the nature of the organism) is met. If yields are to be increased past this level, it is necessary to prevent this threshold from being reached, by removing the product as it is formed, or to change the threshold level, by genetically modifying the producing organism to facilitate over production. Tone et al., (1968) found during their studies of salicylic acid fermentations, that the fermentation was limited by product inhibition and that if the inhibitory product was removed from the culture, the fermentation could proceed further.

In some antibiotic fermentations, the product may be degraded or utilised by the mechanisms of the organism or by catabolic processes in the production system. Dykstra and Wang (1990) noted that cycloheximide, produced in *Streptomyces griseus* fermentations, was degraded during the fermentation process, and that there was evidence that both chemical and enzymatic mechanisms were responsible. They also suggested that maintenance of low broth concentrations of cycloheximide could alleviate this degradation. Product utilisation occurs when the organism degrades its own product, and usually as a result of a primary nutrient becoming limited, and is therefore performed to maintain cellular viability or metabolic processes (Roubos *et al.*, 2002, Junker *et al.*, 2001, Turner,

1971).

The production environment itself can also influence growth and production in antibiotic fermentations. This can occur not only by exposure to detrimental elements associated with the production process, such as shear forces (Tamura *et al.*, 1997) and temperature (Wei *et al.*, 2003), but can also be as a result of the product or producing organism imparting some negative influence.

The productivity of mycelial fermentations may be governed by the limitation of the mass transfer of oxygen or other compounds (Garcia-Ochoa and Gomez, 1998, Garcia-Ochoa *et al.*, 2000). This is due to the high non-Newtonian viscosities associated with the filamentous structure of mycelial cells (Gbewonyo and Wang, 1981) and the general fermentation of filamentous antibiotic producing organisms (Aiba and Okabe, 1976). The reduction in the overall product yields which results,, is attributed to limitation of the growth and production capabilities of the organism, under such fermentation conditions.

1.2.4 DownStream Processing of Antibiotics

In antibiotic fermentations, recovery of the product has a critical bearing on the success of the process. Since the product is frequently in a low concentration in the fermentation broth, its recovery can require extensive purification procedures (Crueger and Crueger, 1982). Antibiotics are characterised by structural duversity and frequently display sensitivities to environment or fermentation treatments such as temperature, pH and further processing or degradation (Aksu and Tunç, 2005, Bersanetti *et al.*, 2005, Wang and Sobnosky, 1985, Roubos *et al.*, 2002). As a result, the successful DownStream recovery process should be expected to recover maximal amounts of product in an efficient time interval and take advantage of the structural properties of the antibiotics of interest.

Antibiotic fermentation media provide the necessary nutrients for suitable

growth and product formation, but can often cause bioprocessing problems such as increased viscosity, as a result of the complex substrates from which they are frequently comprised. On completion of the antibiotic fermentation, the culture is passed for filtration, at which point filter aid or coagulating materials may be added to aid the filtration process. Filtration is usually achieved by rotary vacuum filtration, which may employ filter-aid to limit clogging. The antibiotic is recovered post filtration by extraction with suitable materials. The ease and efficiency of the filtration and recovery process therefore depends on the quality of the fermentation (Calam, 1987), which is, in turn, affected by the raw materials employed. It is therefore clear that, if crude raw materials are employed in the antibiotic fermentation, the filtration process may become less efficient.

In general, the recovery of antibiotics requires the same generic DownStream Processing unit operations as the recovery of the majority of bioproducts, such as stream harvesting and undissolved nutrient removal. Although there are a variety of means by which antibiotics can be recovered from fermentation broths, including membrane techniques (Alves *et al.*, 2002), among the most significant and frequently applied methodologies are liquid extraction and adsorption (Soto *et al.*, 2005).

Liquid extraction for the recovery of antibiotics generally involves the use of a suitable solvent into which the antibiotic will be preferentially recovered. The extraction process serves to enrich the product in the solvent stream, which is then back extracted into an aqueous phase for further treatment. The performance of a solvent is sometimes described in terms of its distribution co-efficient, which is a measure of the solvent's capacity for the product, and is defined as the ratio of the product concentration in the solvent to that in the aqueous culture medium, at equilibrium (Bruce and Daugulis, 1991). The advantage of solvent extraction is that it can be accomplished quickly. Penicillin for example, is subjected to two steps of solvent extraction and is transferred back to the aqueous phase in a 90 second period, and such rapid extraction procedures are especially desired if the antibiotic is potentially unstable (Crueger and Crueger, 1982).

The apparatus / techniques commonly employed in solvent extractions of antibiotics include centrifugal mixer / settler countercurrent contactors and disc stack extractors. Recovery yields of in excess of 95% are reported to have been achieved in one to four contacting steps (Brocklebank and Kalyanpur, 1993). Brunner (1985) described the development of a new extractor, which used two counter-current extracting decanters to extract penicillin and erythromycin, with the added benefit of being able to deal with whole cell broth. This resulted in significant increases in product recovery yields, since antibiotic associated with the biomass was also recovered.

There are considerable problems associated with the safe handling and use of solvents in antibiotic recovery applications, which relate to their toxic and flammable nature and phase separation limitations (Hollmann *et al.*, 1995). As a result, alternate means of antibiotic recovery have been investigated and employed (Cull *et al.*, 2000). There has been considerable interest shown in the development and application of recovery strategies involving the use of aqueous two phase systems and liquid membrane techniques.

Bora *et al.*, (2005) examined the downstream processing and separation of cephalosporin antibiotics, and determined that aqueous two-phase systems showed good prospects as separation techniques. Aqueous two-phase systems provide an alternative, and efficient approach, by facilitating partitioning between two liquid phases (Banik *et al.*, 2003), with a reduction in the hazards associated with organic solvent use. Soto *et al.*, (2005) substituted the use of organic solvents in a solvent extraction process with room temperature ionic liquids in an aqueous two phase extraction in their processes for antibiotic recovery. They found that the aqueous two phase process performed suitably and was a desirable substitute for the use of organic solvent extractions. Yang *et al.*, (1994) detailed how an aqueous two-phase system could not only be applied for the extraction of cephalosporin C from whole broth, but that the difficult separation of cephalosporin C and disacetyl cephalosporin C could also

possibly be achieved via this system.

Preferential transport in adsorptive membranes can be used to selectively remove biochemicals directly from fermentation broth (Agrawal and Burns, 1997). In antibiotic recovery processes, these membranes need not necessarily be comprised of the porous supports commonly employed in membrane separations, they can also be in the form of liquid membranes. Extraction using liquid membranes has been studied since the 1980's and is one of the most advantageous techniques of separation at present (Cascaval *et al.*, 2001). Extraction by liquid membranes is a separation method for recovery and concentration of antibiotics from their dilute aqueous solutions (Boyadzhiev *et al.*, 2003).

In liquid membrane separations, an intermediate, immiscible liquid, plays the role of a membrane, separating the feed and stripping solutions of the antibiotic (Kawasaki *et al.*, 1996). These liquid membranes may be unsupported, whereby the solvent or carrier layer is achieved via emulsification, or supported, whereby the solvent is included in a hydrophobic porous polymer matrix (Cascaval *et al.*, 2001, Sahoo and Dutta, 2002). Lee *et al.*,(1994) found that the use of a supported liquid membrane system, employing Amberlite LA-2 dissolved in 1-decanol, showed promising results for the selective separation and recovery of Penicillin G.

The main advantages of using liquid membranes, over conventional liquid-liquid extraction methods is that the quantity of solvent used is reduced because it is continually regenerated. In liquid membrane systems, there is potential for partitioning of product, against its concentration gradient, as long as the pH gradient is maintained (Cascaval *et al.*, 2001). A novel downstream application of liquid membranes was detailed by Barenschee *et al.*, (1992). In this process, extraction of penicillin was coupled with conversion into new penicillin derivatives via penicillin-G-amidase, immobilised in the liquid membrane carrier.

Recovery and purification of bioproducts, including antibiotics, from their

crude sources involve various steps including precipitation, centrifugation, extraction, membrane filtration and sorption (Ramos *et al.*, 2004). Güzeltunç and Ülgen, (2001) suggested that incorporation of a chromatographic technique early in this sequence of purification steps will lead to higher product yield. Chromatography-mediated recovery processes have long been associated with the recovery of proteins, but applications in antibiotic recovery has been the focus of much interest (Lee *et al.*, 2003, Ribeiro and Ribeiro, 2003), even though solvent-mediated recovery strategies were more traditionally employed. Aiba and Okabe, (1976) suggested that ion-exchange adsorbents could be employed in place of solvent exchange reactors in their configuration of an optimised antibiotic recovery process.

Of particular significance in chromatography-mediated antibiotic recovery processes is the use of adsorbent resins. In 1965 Rohm and Haas commercialised the first synthetic organic macroporous or macroreticular adsorbents, the so called Amberllte XAD resins (Voser, 1982). In a document by Voser, (1982) an extensive technical bulletin describing the resins was detailed. This document stated that the adsorbent resins were characterised by a selection of surface polarities, surface properties, and sorption behaviour. The bulletin went on to suggest that it is not possible to predict accurately just what materials will be adsorbed well by a given adsorbent. This technical bulletin, though focused on the Amberlite series of adsorbents, holds true for the majority of synthetic adsorbents, and adsorbent resins in general.

Dutta *et al.*, (1999) stated that a significant amount of commercially produced cephalosporin is isolated using polymeric adsorbents, and found that adsorbent resins could be used for the recovery of a range of β-lactam antibiotics. Adsorbent resins can also be used to recover antibiotics from a range of feeds. Robberson *et al.*, (2006) demonstrated that adsorbent resins could successfully recover antibiotics from water feeds, which is a positive result with regards to the antibiotic recovery from liquid fermentations, since the majority of antibiotics produced must be recovered from aqueous-based fermentation broths. Dutta and Dutta,

(2006) therefore suggested that, in view of the fact that the high concentrating factor of adsorption can satisfy the requirements of high recoveries and a large volume reduction, adsorption can be considered as an efficient separation method for the recovery of antibiotics from very dilute sources.

Adsorbent resins have a broad application range, can be applied in novel reactor configurations (Hicketier and Buchholz, 2002) and modified to enhance or confer application (Jung *et al.*, 2001, Wang and Sobnosky, 1985). Modification of adsorbent resins involves engineering the resins to comprise of moieties which will only adhere, adsorb or interact with other specific compounds. It is even possible to develop mixed mode adsorbents. An example would be adsorbents prepared using chemistries containing hydrophobic and ionic groups (Hamilton *et al.*, 2000). It is therefore important to note, that antibiotic recovery by adsorbent resins may not be attributed to a single means of chemical separation but instead, an interrelationship between the possible modes of actions may exist. This point was also considered to by Voser, (1982).

In summary, adsorbent resins can be successfully employed for the recovery of antibiotics in a solid-liquid contacting method, as an alternative to liquid-liquid recovery of antibiotics, and via the maintenance of appropriate contacting conditions, the success and selectivity of the adsorption processes can be increased (Barboza *et al.*, 2003, Barboza *et al.*, 2001, Chaubal *et al.*, 1995).

Combinational processes for antibiotic recovery also exist. These involve novel techniques which combine extraction and permeation and are occasionally termed perstraction systems. An example of one such system was detailed by Wyss *et al.*, (2005). In their assessments Penicillin G, the most common raw material for the production of β-lactam antibiotics, and the use of liquid-core capsules for product recovery, was examined. These capsules were composed of a dibutyl sebacetate solvent core, encapsulated in a crosslinked alginate / polyacrylamide membrane. The product permeated through the membrane and was extracted into the

solvent. A further novelty of this process was that it was possible to immobilise the enzyme penicillin acylase onto the surface of the capsule and therefore increase the operational stability of the enzyme during the process.

A similarly novel extraction process was described by Lye and Stuckey, (2000) and Lye and Stuckey, (2001). The processes detailed involved the application of colloidal liquid aphrons (CLAs) in the recovery of erythromycin. These CLAs are micron-sized solvent droplets surrounded by a thin aqueous film which is stabilised by a mixture of non-ionic and ionic surfactants. It was also found that CLA use was an attractive alternative to conventional liquid-liquid extraction for the recovery of erythromycin and that their use allowed an extremely rapid recovery process, due to the large interfacial area for mass transfer available.

1.2.5 *In-Situ* Product Recovery of Antibiotics

Previously, it was highlighted that *In-Situ* Product Recovery techniques are applied to increase the production of biotechnological processes by removal of the product from the vicinity of the biocatalyst as soon as it is formed. Employing ISPR helps to overcome toxic effects of the product which are noted in antibiotic fermentations, minimise product degradation and reduce subsequent DownStream Processing requirements (Stark and von Stockar, 2003, Freeman *et al.*, 1993, Martín *et al.*, 2005). Considering *In-Situ* recovery of antibiotics, Schügerl (2000b) suggested that the most important *In-Situ* recovery methods for antibiotics are solvent extraction, crystallisation and adsorption.

Schügerl (2000b) suggested that, although solvent extraction is a commonly applied method for the recovery of antibiotics from fermentation broths, there are relatively few examples of its use *In-Situ*. The main reason *In-Situ* solvent extraction application is restricted, is due to many organic solvents being toxic to microbes (Stark and von Stockar, 2003), which therefore limits the solvents which can be used in extractive

fermentations (Roffler *et al.*, 1984). Inclusion of a solvent, which is immiscible with the culture fluid, into the fermentation environment may also result in formation of emulsions. Phase separation of, and back-extraction from, these emulsions is difficult and constitutes a potential source of product loss (Tessier *et al.*, 2005, Li *et al.*, 2004, Stark and von Stockar, 2003).

The organic solvent used in *In-Situ* separation processes, especially when viable cells are participating, has to be biocompatible, have a favourable distribution coefficient for the product, have a low price and established commercial production, low viscosity emulsion tendency and mutual solubility, and have a high chemical stability (Cen and Tsao, 1993). Although *In-Situ* solvent extraction has been detailed for product recovery from plant cell cultures (Choi *et al.*, 2001), not many solvents satisfy these requirements, and therefore alternate *In-Situ* extraction strategies for recovery of antibiotics are frequently investigated.

The application of aqueous two-phase systems was previously outlined with relation to downstream recovery of antibiotics. Paquet *et al.*, (1994) examined the partitioning of pristinamycins, produced by *Streptomyces pritinaespiralis*, in aqueous two-phase systems and found that even in the presence of cells, recovery of the antibiotics from the fermentation broth could be achieved. The cells were confined to the bottom phase and the pristinamycins partitioned in the top phase. Paquet *et al.*, (1994) believed that this represented a first step towards the development of antibiotic production by extractive fermentation using aqueous two-phase systems.

Production of subtillin by *Bacillus subtillus* ATTC 6633 has been studied in an aqueous two-phase system composed of 20% polyethylene glycol 6000 and 5.5% potassium phosphate. Although the amount of subtillin produced in the two-phase system was 60% of the single-phase fermentation, a maximum of 13.1 U/ml subtillin could be recovered from the top phase after 10 h fermentation, compared to 8.2 U/ml produced in minimal salts medium. This was because subtillin mainly partitioned in favour of the top phase, in contrast to cells which partitioned in favour of

the bottom phase (Sinha *et al.*, 2000, Kuboi *et al.*, 1994).

Crystallisation *In-Situ*, although feasible, is even less commonly applied than *In-Situ* extraction of antibiotics using solvents. *In-Situ* crystallisation usually takes the form of a precipitation event in the fermentation, like that of tetracycline production by *S. aureofaciens*. *In-Situ* crystallisation of the tetracycline results from the accumulation of product at the bottom of the fermenter during the process (Schügerl, 2000b).

One of the most significant methodologies for the *In-Situ* recovery of antibiotics examined has been the application of adsorbent resins. Kim *et al.,* (1999) described the potential advantages of solid sorbents over organic solvents as extractants, and believed that there was a lower risk of toxicity when using polymeric materials as sorbents over solvents.

Adsorbents are generally applied in *In-Situ* systems to limit the impact of some environmental or physiological conditions, and it is believed the primary mechanism by which adsorbents return increased product yield and recovery, is through rapid removal of the product from the fermentation environment, and the associated benefits of such (Wang *et al.*, 1989). Freeman *et al.*, (1993) concurred with this finding and gave evidence that increases in both antibiotic yield and productivity could be achieved using adsorption-based ISPR techniques.

Examining the production profile of antibiotic fermentations can give an indication of how production may be limited. It is frequently the case that identifying the fermentation parameter responsible for limitation of production can highlight the benefits of applying adsorbent resins for *In-Situ* antibiotic recovery.

Lee *et al.*, (2003) outlined the production of teicoplanin, an antibiotic produced by *Actinoplanes teicomyceticus*. They found that *Actinoplanes teicomyceticus* was sensitive towards its own antibiotic in fermentation. They investigated the addition of adsorbent resins into the fermentation as an *In-Situ* product recovery processes and found that the toxic effect on

growth was eliminated, feedback repression of teicoplanin was reduced and the overall recovery process was shortened. Wang, (1983) noted a similar result. They found that final concentrations of cyclohexamide, whose production in fermentation had been found to be feedback regulated, and its synthesis rate, could be increased by adding adsorbent resins directly to the fermentation.

Gastaldo *et al.*, (1996) hypothesised that the two main factors which may influence yields of kirromycin fermentations were inhibition of the producing organism through antibiotic production and loss of antibiotic through degradation, and therefore postulated that the benefits they observed from the use of adsorbent resins in an *In-Situ* recovery process could be attributed to sequestering of the toxic end-product and removal of the antibiotic from the potentially degradative fermentation environment. These theories were shared by Marshall *et al.*, (1990), who found that using adsorbent resins to recover rubradirin *In-Situ*, resulted in enhanced production.

The benefits of applying these adsorbents in *In-Situ* antibiotic production and recovery processes have been made clear. The adsorbents applied are usually solid, porous adsorbents with extremely large surface areas. They can be applied in a variety of contexts, they can be added directly into the fermenter, placed in a separate vessel with circulation of the fermentation broth or incorporated in dialysis membranes (Roffler *et al.*, 1984, Wang, 1983).

1.3 Summary

Biotechnology and Bioprocessing are scientific disciplines which employ the combined knowledge of an array of other disciplines, from biology to engineering, to control the mechanisms of selected organisms, to produce valuable compounds, or carry out desired operations. The techniques associated with these disciplines have their applications far reaching into history. One of the first examples was believed to be the development of brewing by the Sumerians, about 6000 BC (Scragg, 1991). Biotechnology and Bioprocessing methods have developed with time. Their applications have resulted in the production of numerous compounds of significance to the modern world, such as interferon and antibiotics. These processes employ a variety of organisms, from microbes to plants, to produce the desired compounds, and to facilitate the successful production process, considerable effort is expended on development and optimisation of the production and recovery process.

The two most important facets of any biotechnology process are the production and recovery methods employed. The most commonly employed method to produce compounds of interest is through fermentation. The choice of the fermentation process which is employed, however, is dictated by the organism to be cultured and the product formed. The growth requirement of the organism must be met, thus agitation, nutrients, heat, aeration, etc, must be supplied by the fermentation system. The fermentation system employed must be capable of addressing any sensitivities of, or problems caused by, the product. If, for example, the product is pH sensitive the fermentation must be able to maintain pH in a suitable range, or if the product is toxic to the producing cell, the fermentation process must provide an ability to remove the product. All these issues are addressed by the selection of the appropriate fermentation technique, and the subsequent optimisation of operational parameters.

The recovery process is the second major consideration in biotechnological processes. Similar to the production method employed,

which must suit the producing organism, the recovery method must suit the product generated, in order for maximal amounts of product to be recovered. In general, it can be argued that there are two main means by which a product can be recovered, and depend on the time at which recovery occurs during the production process. These are downstream recovery, whereby the product is recovered when the production process has been completed, or *In-Situ* product recovery, whereby the product is recovered continuously, during the production process, as it is being produced.

In general, production processes are characterised by low product yields, thus the product frequently requires recovery form a dilute environment. As a result, downstream processing techniques are generally aimed at concentrating and purifying the product once its production has ceased. The general techniques involved include identification of the location of the product in the fermentation system, and the subsequent removal of the contaminating stream. Once primary separation of the product feed has been achieved, a number of processing steps are carried out, from chromatography and extraction, to evaporation and crystallisation, in order to concentrate and purify the product for further use or sale. It is during these recovery steps that product yields can be reduced, through degradation for example, thus the downstream recovery process employed must take into account the susceptibilities of the compound, and be developed to minimise product losses during recovery.

In-Situ product recovery involves techniques employed to reduce the potential losses in product recovery which can be frequently encountered during downstream processing of product feeds, degradation and further metabolism of product. In conjunction with limitation of product loss, the advantage of *In-Situ* product recovery techniques is that by recovering the product of interest as it is formed, the unit operations required for total recovery of product are usually reduced over those required in downstream processing applications. In some instances the product can negatively impact on the production system. For example the product may be toxic to the producing cell, or may negatively impact on the rheological

properties of the fermentation, through increasing the viscosity, which would lower the oxygen mass transfer potential of the system. In such instances, the use of *In-Situ* product recovery techniques may not only be advantageous for product recovery, but may be essential for a successful production process.

As outlined in this Chapter, the number of compounds which are produced through biotechnological and bioprocess applications is extensive, and although antibiotics are not the only products of these processes, they are among the most important. Antibiotics have application in an array of fields, from medicine to forestry, industry to the farm. Despite an increase in the chemical synthesis of antibiotics, a considerable amount are still derived from microbial sources, and it is believed continued research and screening procedures will unearth even more compounds of interest (Watve *et al.*, 2001, Overbye and Barrett, 2005, Thompson *et al.*, 2002). As with any bioprocess, the production methodology is of key importance, and the vast majority of antibiotics are produced through fermentation. The antibiotic fermentation can be complex, and the production scientist must deliberate on the optimal organism, fermentation type, media constituents, fermentation parameters, requirements of the organism and how each of these may impact on the product, both in-fermentation, and duing recovery.

Leading from optimal production of antibiotics, the importance of the recovery process becomes obvious. Like many bioproducts, antibiotics can be recovered post-fermentation, via downstream processing techniques. Antibiotics are generally excreted into the culture fluid during fermentation, and will be present in the liquid phase. Recovery of the compound would require removal of biomass and fermentation particulate, and the subsequent retrieval of the antibiotic in a purer, more concentrated form. Downstream antibiotic recovery therefore frequently employs techniques of extraction to achieve this. The two main extraction techniques are liquid-liquid and solid-liquid.

In liquid-liquid extractions, the antibiotics are usually recovered into a

suitable organic solvent, for which the antibiotic has an affinity. These solvents are generally immiscible with the culture liquid, and the antibiotic partitions preferentially into the solvent. Once present in the solvent phase, the stream can be treated further, if necessary, and the antibiotic recovered in a more concentrated, purer form, than what it was present in in the fermentation culture liquid.

Solid-liquid extractions of antibiotics usually involve some sort of chromatographic step. A solid adsorbent, with an affinity for the antibiotic, or some chemical structure which is part of the make up of the antibiotic, is brought in contact with the antibiotic and adsorption takes place. The antibiotic can then be eluted from the material, yielding a purified product stream. The development of polymeric adsorbent resins, with specific chemistries, has given rise to a number of interesting applications. These resins can be applied in a batch recovery or arranged in column modes, and can be modified to increase the selectivity of the recovery process. Such adsorbents reduce the handling of toxic solvents commonly used in liquid-liquid antibiotic recovery processes, and are applicable in a wide range of modes.

The *In-Situ* product recovery of antibiotics is an interesting area, since it allows the issue of product stability, commonly encountered with antibiotic production processes, to be addressed. Antibiotics can subjected to detrimental influences in fermentation. They may be susceptible to fermentation conditions such as temperature and pH, and exposure to oxygen and light during processing. Thus, any method which could hasten their recovery, minimise the number of steps required to do so, and limit their exposure to detrimental elements, would be beneficial to the production process. *In-Situ* antibiotic recovery processes are methods employed to achieve these goals.

There are relatively few *In-Situ* recovery methods which can be applied for the recovery of antibiotics. Solvent mediated liquid-liquid extractions have a limited applicability because the solvents used are usually toxic to the producing organism. *In-Situ* crystallisation is limited because there are few

antibiotics which undergo crystallisation in-fermentation. A number of novel techniques, such as membrane-assisted extractions, aqueous two-phase systems and perstraction methods have been developed, which show some very interesting application potential for the recovery of antibiotics *In-Situ*.

The use of adsorbent resins however, has been shown to possess particular potential in these types of applications. These resins are generally less toxic than solvents and can thus be incorporated into the fermentation environment. They can also be arranged in different configurations, such as in external loops, to aid the recovery process and minimise treatment steps. The resins can selectively adsorb the compound of interest and allow its subsequent concentration and purification, with minimal negative impact on the production system. An added benefit of application of these resins is that, in some *In-Situ* applications, the yield of product has been seen to increase over the normal fermentation. This is believed to occur as a result of prevention of product degradation and perhaps stimulation of metabolic paths as a result of the adsorption, and therefore regulation, of other fermentation media constituents and metabolic intermediates.

Chapter 2. Aims and Objectives

2.1 Geldanamycin and its' significance

Geldanamycin (Figure 2.1), a yellow antibiotic produced by *Streptomyces hygroscopicus* var. *geldanus* (Sasaki *et al.*, 1970), is a benzoquinone ansamycin produced as a secondary metabolite, which exhibits sensitivities to temperature, light, oxidation, acid and base (DeBoer *et al.*, 1970). Geldanamycin is a broad spectrum antibiotic which exhibits activity against Gram positive and Gram negative bacteria, protozoa and fungi (DeBoer *et al.*, 1970), and in nature geldanamycin is produced to serve the organism in situations of competition or protection.

Apart from its antimicrobial applications, geldanamycin gains clinical significance due to its anticancer properties. Interest in such benzoquinone ansamycins increased greatly upon the discovery of the broad antiviral and antitumour properties of geldanamycin (Rascher *et al.*, 2003, He *et al.*, 2006). Geldanamycin displays an anticancer activity through its interaction with the Heat Shock Protein (Hsp) 90 family of molecular chaperone proteins, binding them in a stable and pharmacologically specific manner (Whitesell *et al.*, 1994). Although geldanamycin is not a clinically employed antibiotic, a number of its analogues, in particular 17-allaylamino-17-demethoxygeldanamycin (17-AAG), are presently under evaluation (Hwang *et al.*, 2006).

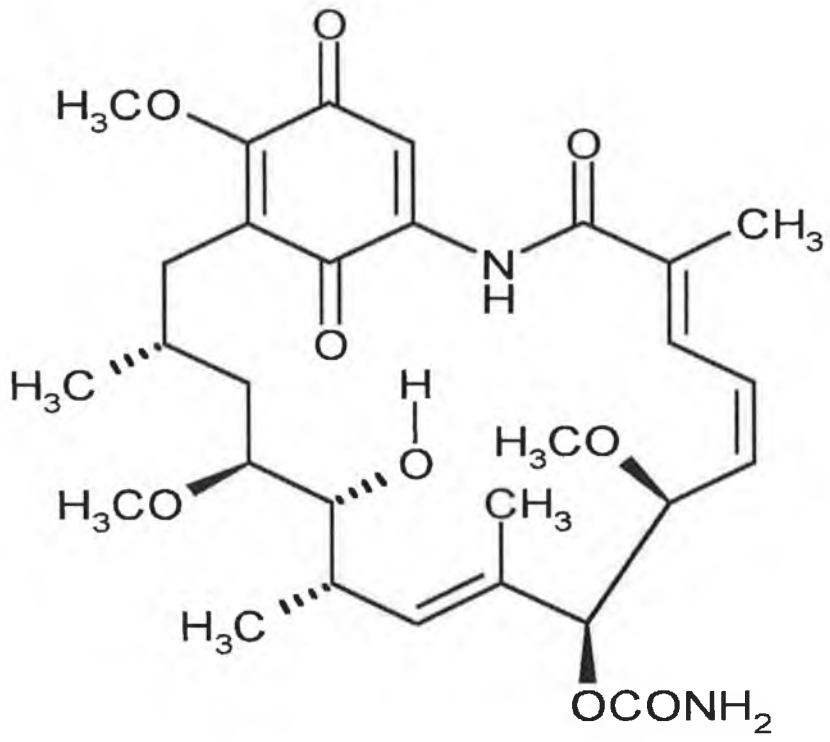


Figure 2.1: Structural diagram of the antibiotic geldanamycin

Hsp90 is a ubiquitous protein, present in the cytosol of both eukaryotic and prokaryotic cells, and is one of the most abundant cellular proteins (Neckers *et al.*, 1999). Hsp90 is over expressed in many malignancies, possibly as a result of stress that is induced by the mutation and aberrant expression of oncoproteins (Hwang *et al.*, 2006). Therefore, through its association with the activation of proteins involved in cell-cycle regulation, signal transduction, and steroid hormone response, it is an attractive target for antitumour drug development (Roe *et al.*, 1999). Hsps play the role of 'molecular chaperone', binding and stabilising proteins, aiding their assembly and transport across membranes. It is the N-terminal domain of Hsp90 which binds ATP and drives the chaperone activity of the protein, and therefore binding of ligands to this site, results in Hsp90 inhibition and the development of therapeutic opportunities (Barril *et al.*, 2005). Geldanamycin binds with a high affinity to the ATP binding pocket of

Hsp90 resulting in cancer-causing proteins being left malformed and readily degradable by the cells own mechanisms (Roe et al., 1999).

The significance of *Streptomyces* and their role as antibiotic producing organism can not be over-stressed. *Streptomyces* are the most widely studied and well known genus of the Actinomycete family. They are soil dwelling organisms, ubiquitous in nature, play a role as natural decomposers and are the largest antibiotic producing genus of microorganism known at present (Watve *et al.*, 2001). The organism which is the focus of the research outlined in this document is *Streptomyces hygroscopicus* var. *geldanus*, which was originally isolated from a Kalamazoo soil (DeBoer *et al.*, 1970). As with many *Streptomyces*, *Streptomyces hygroscopicus* exhibits pelleted growth in submerged culture (O'Cleirigh *et al.*, 2005). The reason this organism has been investigated is as a result of its ability to produce the antibiotic geldanamycin (Lee *et al.*, 2006, Rascher *et al.*, 2003, DeBoer *et al.*, 1970, DeBoer and Dietz, 1976, Patel *et al.*, 2004).

2.1.2 Focus of the Work

There are a number of key objectives which are the focus of the experimental work undertaken in this document. The main aim however, is to assess the applicability of adsorbent resins in processes for the recovery of geldanamycin. Adsorbent resins are gaining considerable application in bioprocessing fields and antibiotic recovery is no different. It is believed that a potential exists for solid phase-mediated recovery of geldanamycin, and if so, it could benefit product yield and recovery efficiency.

In order to accurately assess the application of adsorbent resins it will first be necessary to develop methods for the detection and quantification of compounds, and in particular, geldanamycin in *S. hygroscopicus* fermentations. To that end, there are perhaps two major goals of the work.

The first is the development of methods which will allow the determination of compound production. *Streptomyces* can produce an array of products, and like many of them, geldanamycin is an antibiotic which possesses a bioactive effect against certain microorganisms. By developing methods which can accurately assess the presence of bioactive compounds in samples, one will be better equipped to establish product levels. It may also be advantageous to develop methods for the accurate identification and quantification of single compounds. Again, since *Streptomyces* can produce an array of products, it may be necessary to be able to determine the concentration of geldanamycin alone in order to accurately determine product levels. There are a number of methods which could be employed, however the most important factors which should be considered is that the techniques employed should be robust, high throughput and accurate.

Once accurate determination of production can be achieved assessment of the applicability of adsorbent resins can be suitably accomplished. The recovery of antibiotics traditionally takes place in a single DownStream Process, whereby, post-fermentation, the broth is recovered clarified and treated to recover product. Therefore, in the investigation of adsorbent resins it will be desirable to assess them in such an application. A preliminary investigation will be aimed at answering the question 'can adsorbent resins be used to recover geldanamycin from fermentation broth?'. If this question can be answered the applicability of the resins will have been determined. If the answer to this question is yes, then it would be beneficial to examine more ways in which these resins can be applied.

In modern bioprocessing strategies, movement is towards novel reactor design and recovery methodologies, which facilitate more that one unit operation in a single stage or configuration. Such techniques often take the form of an integration of fermentation and product recovery, and are termed *In-Situ* Product Recovery techniques. These novel approaches cut down on processing and time requirements. They constitute a means of retaining product levels and may even result in an increased level of production or product recovery. It would be desirable to examine the *In-Situ* application of these adsorbent resins and determine if hey can lead to

effective recovery of product and moreover, lead to increased product recoveries.

Section A:

Development Of Analytical Methods For The Assessment Of Geldanamycin

Chapter 3. Microtiter plate-based assay for the assessment of bioactivity

3.1 Introduction

Many biological assay techniques have been developed to assess the bioactivity of compounds. This entails determining the potency of chemical compounds against microorganisms (Sin and Wong, 2003). When applied to antibiotic fermentations, these methods use the bioactivity of the broth as an indication of antibiotic production. Traditional approaches involved the use of the disk diffusion assay technique, where the susceptibility of an organism to a sample would result in a zone of inhibition, with a magnitude related to the amount of bioactive compound present in the sample (Selvakumar *et al.*, 1999). The disk diffusion assay proves unreliable in certain applications (Swenson *et al.*, 1989) and can lead to interpretational problems, including in-growth in the zone of inhibition, whereby sparse growth of organism occurs within the zone of inhibition (Piliouras *et al.*, 2002) and subjectivity associated with visual assessment, such as interpretation of where inhibition zone boundaries are located (Deighton and Balkau, 1990). DeBoer *et al.*, (1970) took steps to standardise the assessment of the zone of inhibition and reported results in biounits, defined as the amount of antibiotic necessary to produce a 20 mm zone of inhibition under standard conditions.

Using the disk diffusion method is time consuming, material intensive and, as a result, movement has been towards more standardised and high-throughput methods of bioactivity assessment (Brown, 1988). A number of microtiter plate-based assays have been developed for screening of the antimicrobial activity of natural products (Devienne and Raddi, 2002), determination of the antimicrobial susceptibility patterns of microorganisms (Jones and Dudley, 1997), determination of microorganism adherence (Deighton and Balkau, 1990) and for the quantification of biofilm formation

inhibition (Stepanovic *et al.*, 2000). When assaying bioactivity using microtiter plate-based techniques, ambiguities may be encountered in the determination of biomass growth trends and in the calculation of the bioactive effect itself. These difficulties arise because standardisation of the response of microorganisms in these systems is difficult.

Assays for monitoring biomass growth and death using optical density (Archer *et al.*, 1996), turbidity (Nayak *et al.*, 2002) or absorbance (Lopez-Garcia *et al.*, 2003), relate bioactivity to a decrease in the measured absorbance. Such turbidity based methods often assume a linear relationship between test organism growth and absorbance. Antoce *et al.*, (1997), suggested that direct assessment of bioactivity based on turbidity can be a source of computational error and, as a result, employed calorimetric methods instead, in the determination of the inhibitory effect of C1-C4 n-alcohols on yeast growth.

A number of methods for the examination of sample bioactivity or for assessing microorganism susceptibility, report their findings as Minimum Inhibitory Concentration (MIC) (Benincasa *et al.*, 2003, Kiehn *et al.*, 1982, Stock *et al.*, 2003, Waites *et al.*, 2003). Often MIC assessments from different sources are vaguely defined and thus results may be subject to interpretational errors. For example, Lopez-Garcia *et al.*, (2003), defined MIC as the lowest compound concentration that resulted in no growth at the end of the experiment, in their examination of antifungal activity of compounds, whereas Devienne and Raddi (2002) define MIC as the concentration at which there was a sharp decline in the absorbance value, in their screening for antimicrobial activity.

This Chapter describes a method to calculate bioactivity of samples, which is designed to yield a quantitative measure of efficacy. The main aim of the work was to develop a means of examining the production of bioactive compounds in *Streptomyces hygroscopicus* fermentation broths. The development of a microtiter plate-based assay method, free from mathematical inaccuracies in relation to the calculation of biomass growth,

which also provides a strategy to successfully calculate the MIC of a bioactive compound, would be of significant benefit in this pursuit.

3.2 Materials and Methods

3.2.1 Strain and media

Streptomyces hygroscopicus var. geldanus (strain NRRL 3602 obtained from ARS Patent Culture Collection, Peoria, Illinois, USA) was used throughout this assessment. Spores were produced on Bennett's medium agar containing: technical agar No.3 (Oxoid, Basingstoke, England), 20 g/l; yeast extract (Oxoid), 1 g/l; 'Lab-lemco' beef extract (Oxoid), 1 g/l; N-Z-amine A (Sigma-Aldrich, Dublin, Ireland), 2 g/l and dextrose monohydrate (Riedel-de Haën, Seelze, Germany), 10 g/l. Spores were recovered using resuspension solution containing: yeast extract (Oxoid), 3 g/l; bacteriological peptone (Oxoid), 5 g/l and $MgSO_4 \cdot 7H_2O$, 1 g/l. The fermentation medium was Bennetts liquid medium containing: yeast extract (Oxoid), 1 g/l; 'Lab-lemco' beef extract (Oxoid), 1 g/l; N-Z-amine A (Sigma-Aldrich), 2 g/l and dextrose monohydrate (Riedel-de Haën), 10 g/l.

3.2.2 Antibiotic fermentations and organism preparation

A spore inoculum of *S. hygroscopicus* was used to inoculate fermentations and was prepared by culturing the organism on static cultures of Bennett's medium agar, in 5 L Erlenmeyer flasks, for 21 days at 28°C. The spores were recovered by washing with resuspension solution at 100 rpm for 1 hour at 4°C. Bennett's media was then inoculated at 1% using a spore suspension of approximately 10^7spores/ml and incubated at 28°C at an agitation of 150 rpm for at least seven days.

At later stages in the project some modifications were made to the standard production medium, and it was found that higher yields of geldanamycin were achieved using modified Bennett's medium (using 20 g/l to 50 g/l dextrose monohydrate instead of 10g/l). Methods such as the production of spore stock and the general *Streptomyces hygroscopicus* var. *geldanus* fermentations are generally conserved throughout this

document and the above sections should be referred to when considering fermentation conditions.

3.2.3 Microtiter assay medium requirements

YEPD media was used as nutrient source in the bioassay and contained: yeast extract (Oxoid, Basingstoke, England), 10 g/l; bacteriological peptone (Oxoid), 20 g/l; dextrose monohydrate (Riedel-de Haën, Seelze, Germany), 20 g/l. The disk diffusion assays were performed on YEPD medium agar containing: technical agar No.3 (Oxoid), 20 g/l; yeast extract (Oxoid), 10 g/l; bacteriological peptone (Oxoid), 20 g/l; dextrose monohydrate (Riedel-de Haën), 20 g/l.

3.2.4 Microtiter assay test organisms

Three test organisms were used in the assay, *Bacillus subtilis* strain 1650 (NCIMB Ltd. - National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland), *Escherichia coli* strain 9485 (NCIMB Ltd.) and bakers yeast *Saccharomyces cerevisiae* obtained in Active Dried Yeast (ADY) form (DCL Yeast Ltd, Surrey, England). The test organisms were grown in YEPD media cultures, for 24 hours, until in a log phase of growth was achieved. The cells were then harvested, resuspended in 40% (w/v) glycerol (BDH laboratory supplies, Poole, England), dispensed into 1ml aliquots, frozen and stored. A 1 mi-aliquot of test organism was thawed and added to 9 mis of sterile water for use as inoculum in the assay. After thawing, a short lag period is observed, however the impact of this is minimised by the 24 hour incubation period of the assay. These test organisms were selected in order to examine broad spectrum bioactivity, on Gram positive, Gram negative and eukaryotic microorganisms.

3.2.5 Microtiter biomass standard curve generation

To develop the standard curves of biomass concentration versus turbidity, a test organism was grown for 24 hours in YEPD media. This stock culture was then serially diluted in spent media to give a range of samples for biomass concentration and turbidity analysis.

Biomass concentrations were determined using dry-weight analysis. Clean, labelled, glass universals were dried in an oven (100°C, 24 hours). These were placed in a desiccator, weighed and retained for later use. 10 ml–aliquots of the culture samples were centrifuged at 3500 rpm for 10 minutes. The supernatants were discarded and the pellets were retained and resuspended in ethanol. This biomass slurry was transferred to the glass universals, placed in a 100°C water-bath and the ethanol evaporated. The universals were dried for 24 hours and again cooled and weighed. The dry-weight biomass concentration was determined by subtracting the weight of the glass universal from that of the glass universal plus dried biomass. The analysis was performed in duplicate.

The turbidity of the culture samples were recorded using a Tecan, Spectra Classic, A-5082 plate reader and associated data retrieval software (Tecan, Mannedorff, Switzerland). 300 µl of the test culture was added to the wells of a sterile, polystyrene 96-well microtiter plate (Sarstedt, Wexford, Ireland) and the turbidity read at 570 nm. The analysis was performed in duplicate.

Biomass concentration was plotted against turbidity to generate standard curves. This process was performed for each test organism and the results are shown in Figure 3.1. The equations of the curves of best fit (obtained from a polynomial regression fit of the data, using SigmaPlot Regression Wizard, from Systat Software UK Limited. London, UK), for each standard curve, are given in Table 3.1.

3.2.6 Microtiter bioassay

50 μl of sample was added to the wells of a microtiter plate. This was followed by the addition of 200 μl of YEPD medium and 50 μl of test organism inoculum. The contents were mixed by drawing the solution up and down in a multipipetter a number of times. The plates were aseptically read in the plate reader at 570 nm and the turbidity recorded. The plates were incubated at 30°C for 24 hours, and the turbidity read again. These turbidity values were converted to biomass concentrations and used in the calculation of bioactive effect. For assaying of fermentation samples, the control was 50 μl of Bennett's medium containing: yeast extract (Oxoid), 1 g/l; 'Lab-lemco' beef extract (Oxoid), 1 g/l; N-Z-amine a (Sigma-Aldrich, Dublin, Ireland), 2 g/l; dextrose monohydrate (Riedel-de Haën), 10 g/l.; for non-fermentation derived samples, the control was 50 μl of sterile deionised water.

3.2.7 Calculation of the bioactive effect

Having established the turbidity values prior to and immediately following incubation, the bioactive effect of the sample on test organism growth could be calculated. Turbidity values were converted to biomass concentrations using the established standard curves. Where bacterial growth was completely retarded, no increase in turbidity would result, therefore signifying no increase in biomass concentration during incubation. On this basis, an equation was developed to describe the level of growth retardation for a sample. This equation took the following form:

$$R = \frac{(C_{24} - C_0) - (T_{24} - T_0)}{(C_{24} - C_0)} X100, \qquad \text{(Eq. 3.1)}$$

where: R is the Retardation of biomass growth (%), $(C_{24} - C_0)$ is the biomass growth in the control wells (g/l) determined by subtracting initial biomass concentration in the control wells from that after incubation for 24 hours at 30°C and $(T_{24} - T_0)$ is the biomass growth in the sample wells

(g/l) determined by subtracting initial biomass concentration in the sample wells from that after incubation for 24 hours at 30°C.

3.2.8 Dose-response curve determination

Bioactivity analysis of a single sample yields a result for the retardation for the test organisms' growth at that concentration only. To establish the complete relationship between organism growth and sample bioactivity a dose-response curve should be determined. A dose response curve is graphical representation of the quantitative relationship between the amount, or dose, of an administered agent, and the biological response resultant in the organism under investigation. To obtain the data for a dose-response curve, a series of sample dilutions were assayed in accordance with the method applied for single sample analysis. Retardation of biomass growth was plotted versus the common log of bioactive compound concentration to give the dose-response curve. A regression of the concentration dependent region is incorporated for use in the determination of the MICs.

3.2.9 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

See Chapter 4 for HPLC methodologies

3.3 Results and discussion

3.3.1 Biomass concentration determinations

When absorbance based assays are used to assess biomass growth, results are often presented as absorbance fluctuations (Turcotte *et al.*, 2004, Das *et al.*, 1998, Pitts *et al.*, 2003). If using similar methods to establish the bioactive effect of a sample on test organisms, it is important to note that accurate prediction of the bioactive effect will only be accomplished if biomass growth trends share a linear relationship with absorbance / turbidity fluctuations, or if efforts are made to linearise the relationship, or work within a linear region of response. Figure 3.1 clearly demonstrates the non-linearity of absorbance with respect to biomass concentration. Welkos *et al.*, (2004) also noted discrepancies in their results when using absorbance as an analytical means, and as a result, altered their analysis processes to use a fluorescence based approach in their examination of *B. anthracis* germination.

In order to accurately determine the bioactive effect of a sample, from turbidity data, it was necessary to develop a method which would allow the conversion of turbidity values from microtiter plate wells to biomass concentration. This was achieved using biomass conversion standard curves established for all test organisms and shown in Figure 3.1. The equations of the line of best fit for each standard curve (Table 3.1) allow the calculation of biomass concentration directly from recorded turbidity. Using these standard curves removes the limitations associated with the previous turbidity based methods.

The assay is limited to the model organisms *B. subtilis*, *E. coli* and *S. cerevisiae*, since standard curves have been generated for these organisms only. Extending the array of test organisms for which bioactivity can be assessed simply involves the construction of biomass standard curves for all new test organisms and using this to determine the bioactive effect.

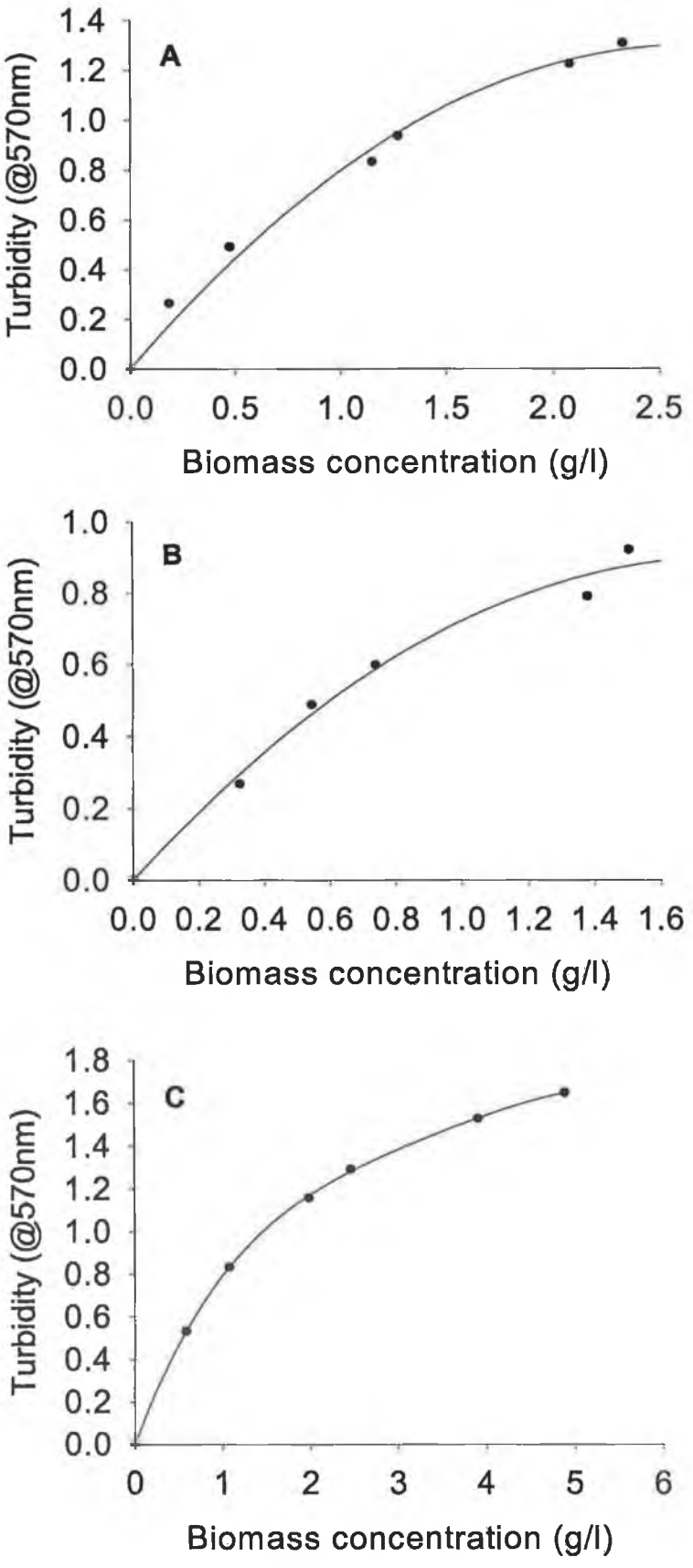


Figure 3.1: Microtiter standard curve for the estimation of (**A**) *B.* subtilis, (**B**) *E. coli* and (**C**) *S. cerevisiae* biomass concentrations from turbidity

Table 3.1: Equations for the determination of biomass concentration from turbidity for three test organisms, where X is the biomass concentration (g/l) and OD is the turbidity at 570 nm

Test Organism	Conversion Equation	Valid Biomass Concentration Range
B. subtilis	$X = 0.9949(OD)^2 + 0.4841(OD)$	0 - 2.4 (g/l)
E. coli	$X = 0.8768(OD)^2 + 0.8714(OD)$	0 - 1.5 (g/l)
S. cerevisiae	$X = 0.9261(OD)^4 - 1.6011(OD)^3 + 1.5843(OD)^2 + 0.5645(OD)$	0 - 5.0 (g/l)

3.3.2 Dose-response curve generation and MIC calculation

The effective concentration range of a bioactive compound on an organism can be represented by a dose-response curve. In this method, a log-linear plot of bioactive compound concentration versus retardation of biomass growth, returns a sigmoidal dose-response curve, comprised of a concentration range of no response, a concentration dependent region and a region of saturated response. It is from the concentration dependent region that MIC values and effective concentration ranges can be determined. Dose-response curves have been explained previously by Sarangapani *et al.*, (2002), and are comparable to the exposure time-response curves determined by Welkos *et al.*, (2004) in their examination of inhibition of *B. anthracis* germination and the drug potency curves derived by Antoce *et al.*, (1997), for the determination of MIC from specific growth activity and specific growth retardation.

As indicated previously, there is significant ambiguity in relation to the definition of Minimum Inhibitory Concentration. The phrase "Minimum Inhibitory Concentration" for a bioactive compound can be argued to indicate the minimum compound concentration at which there is any inhibition of growth or the minimum compound concentration at which there is total inhibition of growth. For reasons of clarity it is proposed that

the following terminology be applied in relation to MICs. Three distinct evaluations of MIC are necessary to avoid ambiguity. MIC_0, (highest bioactive compound concentration which results in no retardation of biomass growth), MIC_{50} (the actual bioactive compound concentration which results in 50% retardation of biomass growth) and MIC_{100} (the lowest bioactive compound concentration which results in 100% retardation of biomass growth). Such values afford an understanding of the impact a given sample has on biomass growth.

Determination of MIC_0, MIC_{50} and MIC_{100}, results in the following benefits to bioactivity assessment. MIC_{50} is a classically measured value in the assessment of bioactivity and allows the comparison of activity of samples based on a 50% retardation of test organism growth. Evaluating MIC_0 and MIC_{100} values allows the complete characterisation of the dose-response of an organism. These MIC evaluations not only supply the compound concentrations below which no bioactivity is detectable and above which complete retardation of biomass can be achieved, but connecting these two points allows the determination of the concentration dependent range of a sample, i.e. the range over which bioactivity changes with respect to compound concentration.

A regression of the concentration dependent, region of the sigmoidal dose-response curve is used to determine the three indicated MIC values. Figure 3.2 shows the analysis of the dose-response curves of *B. subtilis*, *E. coli* and *S. cerevisiae* using a commercially available detergent, Parazone™, with active ingredient Sodium Hypochlorite. The basis of the assessment was the Sodium Hypochlorite concentration as estimated as 5% from the formulation of Parazone™. The data resultant from each analysis is summarised in Table 3.2.

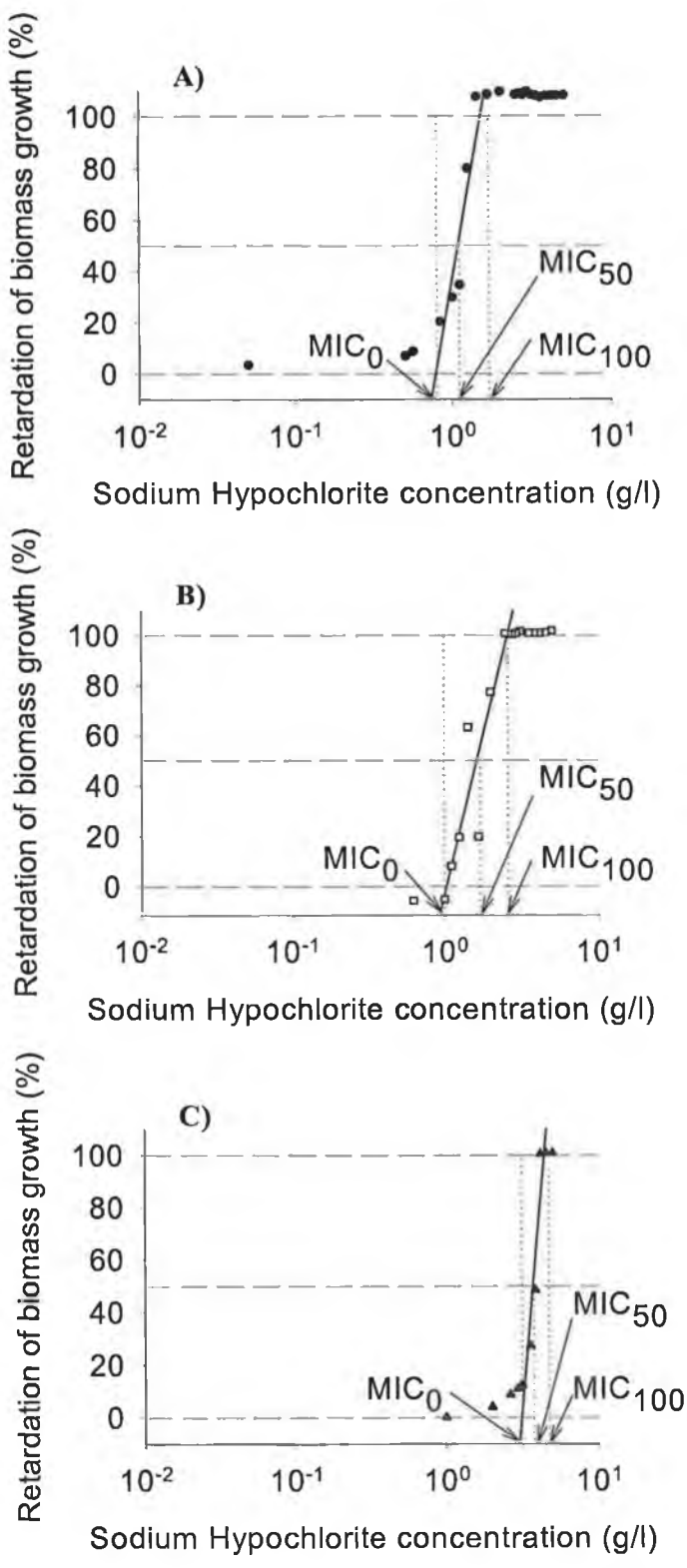


Figure 3.2: The dose-response curve of *B. subtilis* (**A**), *E.* coli (**B**) and S. *cerevisiae* (**C**) to Parazone™

MIC_0, MIC_{50} and MIC_{100} are determined from the intercept of 0, 50 and 100% retardation of biomass growth reference lines, with a regression of the concentration dependent region of the dose-response curve.

Table 3.2: Data acquired from the dose-response curves, indicating the bioactivity of sodium hypochlorite (as the active ingredient in Parazone^{TM}) against *B. subtilis*, *E. coli* and *S. cerevisiae*

Test Organism	MIC_0 (g/l)	MIC_{50} (g/l)	MIC_{100} (g/l)
B. subtilis	0.61	1.02	1.70
E. coli	1.03	1.63	2.58
S. cerevisiae	3.11	3.74	4.67

3.3.3 Method validation

Since the method was primarily developed for the examination of bioactive compound production in fermentations and the assessment of fermentation broth samples, it was decided to validate the method using a fermentation broth sample. A Day 7 sample of *S. hygroscopicus* fermentation broth was examined. The regression of the concentration dependent region of the dose-response curves for the fermentation sample is given in Figure 3.3, and the predicted MIC values summarised in Table 3.3.

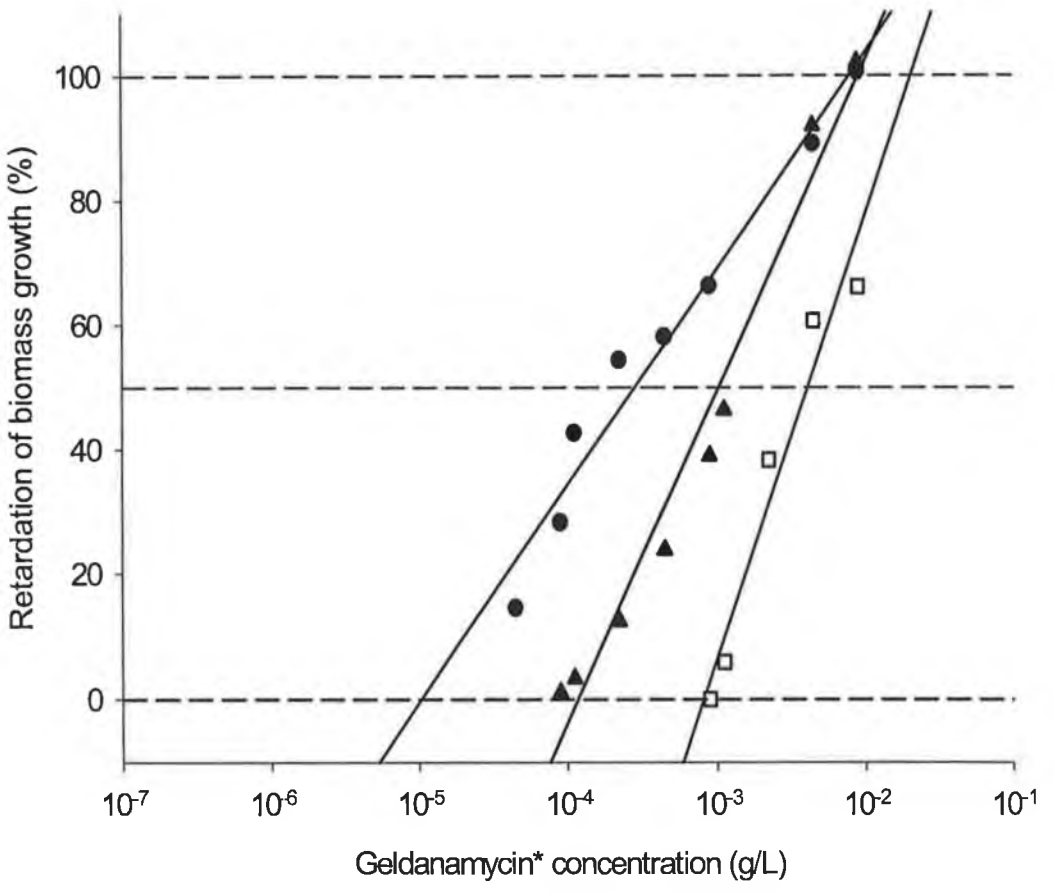


Figure 3.3: Effect of day seven Bennett's media fermentation sample on biomass growth. (●)*B. subtilis*, (□) *E. coli*, (▲) *S. cerevisiae*, (—) regression of the dose-response region for each test organism

Table 3.3: Data acquired from the dose-response curves, indicating the bioactivity of geldanamycin* against *B. subtilis*, *E. coli* and *S. cerevisiae*

Test Organism	MIC_0 (g/l)	MIC_{50} (g/l)	MIC_{100} (g/l)
B. subtilis	0.00001	0.0003	0.0080
E. coli	0.00074	0.0038	0.0200
S. cerevisiae	0.00012	0.0010	0.0092

The above analysis was performed on a fermentation broth sample thus the assessed bioactivity may incorporate the bioactive effects of geldanamycin and any other bioactive compounds present. DeBoer *et al.*, (1970) examined purified geldanamycin and determined that the MIC of

the compound, against a range of test organisms, ranged from 0.1 g/l to 0.002 g/l, with some species of *Bacillus* having MIC values in the order of 0.025 g/l. These values are higher than the MIC values obtained for the crude geldanamycin fermentation broth samples assessed in this study. This indicates that apart from geldanamycin, there may be other bioactive compounds present in the fermentation broth.

3.4 Conclusion

In any antibiotic production process it is important to be able to establish how the production is progressing and the yields of the product of interest attained. The most frequently employed technique has been the disc diffusion assay. These types of assays employ solid culture, diffusion-based methodologies, to assess the potential of a sample to limit test organisms growth. A zone of growth inhibition of a suitable test organism, proportional to the bioactive efficacy of the sample, is returned. This is essentially a qualitative assessment, allowing the determination of the presence of a bioactive effect, and as a result, the accurate quantification of this effect is difficult, and open to subjective error. As a result, movement has been towards more robust, high throughput methods for bioactivity assessment, which frequently involve the use of turbidiometric readings.

Previous methods of direct assessment of bioactivity based on turbidity had been found to be a source of computational errors, and with this in mind, it was decided to develop a method that utilised a more mathematically stringent assessment of bioactivity. The conversion of turbidity readings to biomass concentrations using biomass standard curves removed the errors associated with the non-linearity of the relationship between turbidity and test organism biomass growth, thereby allowing the more accurate assessment of bioactive ranges. The methods employed for the development of turbidity to biomass concentration standard curves had to be readily adaptable and standardised to facilitate a later increase in the number and type of microorganisms against which bioactivity could be examined.

Although numerous methods exist for the reporting of bioactivity, the calculation of Minimum Inhibitory Concentrations has been the standard for antibiotic susceptibility testing (Ceri *et al.*, 1999). Such methods provide a limited amount of information regarding the activities with respect to test compound concentrations (Lowdin *et al.*, 1998). The method described in this work allows the determination of the dose-

response curve of test organism growth with respect to bioactive compound concentration. From analysis of the dose-response curve, data can be gathered on MICs and used to compare the susceptibilities of different test organisms to the same sample. The evaluation MIC_0, MIC_{50} and MIC_{100} yield a greater understanding of dose-response behaviour for a bioactive compound. Calculation of MICs, using the intersection of fixed reference lines with the concentration dependent region, of the dose-response curve, ensure the results obtained are more mathematically stringent than previous MIC determinations, which may be arbitrary by definition.

The developed method delivers quantitative results for the determination of the bioactive range of a sample, against a variety of test microorganisms. Such a technique is important in this body of work, since it facilitates the monitoring of production of bioactive compounds by *S. hygroscopicus* in fermentation samples, and returns clear and well defined data in relation to MICs. The method may also have application in the examination of medical antibiotic dosing. Accurate establishment of the effect of a variety of drug concentrations on microbial growth, as achieved by this method, may provide more meaningful information about optimal dosing strategies than determinations obtained with a single concentration (Lowdin *et al.*, 1998). The method is high throughput, simple and robust, applies greater mathematical rigour to the establishment of bioactive ranges and MICs, than previously employed methods and can be extended to increase the spectrum of test organism subjects.

It was assumed in the bioactivity analysis of fermentation samples; that geldanamycin was the sole contributor to the bioactive effect. However, although the fermentation process was optimised for the production of geldanamycin, it is important to note other bioactive compounds may be produced by the organism, and impact on the retardation of biomass growth. *Streptomyces* are the largest antibiotic-producing genus in the microbial world and the natural products, including antibiotics, produced by the genus include, geldanamycin, streptomycin, elaiophylin and erythromycin to name but a few (DeBoer *et al.*, 1970, Watve *et al.*, 2001,

Fazeli *et al.*, 1995). This belief was verified by the assessment of geldanamycin fermentation broth samples, which returned lower MIC results than those values cited by DeBoer *et al.*, (1970) for purified geldanamycin. As a result of this, the bioactive effect determined and quantified by the bioassay, may incorporate the effects of other bioactive compounds present in the sample. It would therefore be necessary to employ an alternate, compound specific, method in order to analyse samples for geldanamycin concentration only.

Chapter 4. Strategy development for the analysis of geldanamycin in fermentation broth samples

4.1 Introduction

In the previous Chapter, a method to assess bioactivity in fermentation samples was developed. This method comprised a high throughput bioassay which allowed the determination of the relative potential of samples to inhibit growth of target organisms. Although this method was considerably more robust and quantitative than previously employed disk diffusion assays, it was still not a specific assay for geldanamycin. The nature of the assay was to determine the minimum inhibitory concentration of samples. Since *Streptomyces* by their very nature are prevalent producers of an array of bioactive compounds (Crueger and Crueger, 1982), it was not possible to elucidate whether all of the bioactive effect noted was attributable to geldanamycin. To resolve this issue, it was decided to examine the applicability of High Performance Liquid Chromatography (HPLC) for the analysis of fermentation samples, and the determination of geldanamycin concentration.

HPLC techniques employ highly sensitive detectors in conjunction with small bore HPLC columns, with small diameter column-packing particles, and high pressures, to obtain the flowrate necessary for short analytical times (Aszalos *et al.*, 1982). HPLC has been used for both the purification and separation of a number of different antibiotics (Cantwell *et al.*, 1984, Joshi, 2002, Loadman and Calabrese, 2001) but of importance to the work carried out in this chapter, is the application of HPLC techniques for identification and quantification of the antibiotic geldanamycin. Agnew *et al.*, (2001) had previously published a method which employed HPLC to examine the levels of geldanamycin and its derivative, 17-(allylamino)-17-demethoxygeldanamycin in human plasma samples. Although the source material in their work was not fermentation broth, this method still provided

a good basis for the development of a suitable HPLC procedure to determine the geldanamycin concentration of samples from *Streptomyces* fermentation broths.

HPLC analysis methods are an expedient means of efficiently identifying and resolving single compounds in multi-component systems. Up until this point, fermentation broth samples were analysed using the bioassay detailed in Chapter 3. It was therefore envisaged, that the development of a suitable HPLC process would reduce the time constraints and analytical limitations associated with this assay. For this reason, it was decided to pursue HPLC as the primary method for sample analysis.

4.2. Material and Methods

4.2.1 *Streptomyces hygroscopicus* var. *geldanus* antibiotic fermentations

The general methodologies applied to generate antibiotic containing fermentation broth are outlined in Chapter 3.

4.2.2 High Performance Liquid Chromatography (HPLC) Method Development

Agnew *et al.*, (2001) described a HPLC method for the identification of geldanamycin in plasma samples. A Hewlett-Packard 1050 HPLC system (Wilmington, DE, USA) was employed and utilised a Kingsorb C-18 Reverse Phase HPLC column (Phenomenex, Cheshire, U.K.) with dimensions of 150 mm x 4.6 mm, and used a stationary phase pore size of 3 µm and associated Phenomenex Security Guard system as the pre-column. Geldanamycin U.V. detection was achieved at 308 nm using a HP1050 diode-array detector (Wilmington, DE, USA). The mobile phase used by Agnew *et al.*, (2001) contained 50% (v/v) acetonitrile-25 mM sodium phosphate buffer (pH 3.00), containing 10 mM triethylamine, and was delivered at a flow-rate of 1 ml/min, for a run time of 25 minutes. Although the method was applied to plasma samples, it was a good basis for the development of a HPLC procedure for the analysis of geldanamycin in fermentation broth samples.

The preparation of the mobile phase employed by Agnew *et al.*, (2001) was more difficult than was desired and contained constituents which may not have been necessary in a HPLC method for the assessment of geldanamycin in fermentation broth samples. It was desirable to employ a less complex mobile phase, which was easier to prepare, and whose application would not negatively impact on sample resolution and identification.

It was therefore decided to take measures to remove the sodium phosphate and triethylamine portions of the mobile phase and replace them with ultra-filtered H_2O. These constituents essentially serve to improve retention time and separation in mixtures containing acids and bases, and address issues of compound peak tailing. It was believed that, since the sample material for analysis of geldanamycin in fermentation broths was different to the plasma samples under analysis by Agnew *et al.*, (2001), these additives may not be as required for the analysis of fermentation broth samples.

The proposed new mobile phase comprised of 50:50 (v/v) ultra filtered H_2O : acetonitrile (Lennox Chemicals Ltd., Dublin, Ireland). In order to preserve the column longevity, a Security Guard HPLC guard cartridge system (Phenomenex, Cheshire, U.K.) was also used. The HPLC system employed for sample assessment was a Merck-Hitachi LACHROM 7000-series HPLC system, comprising of a D-7000 interface device, L-7200 auto-sampler, L -7400 U.V. detector and a L -7100 isocratic pump system (Hitachi Ltd., Tokyo, Japan). Figure 4.1 outlines the effect of mobile phase alteration on the geldanamycin chromatographic results.

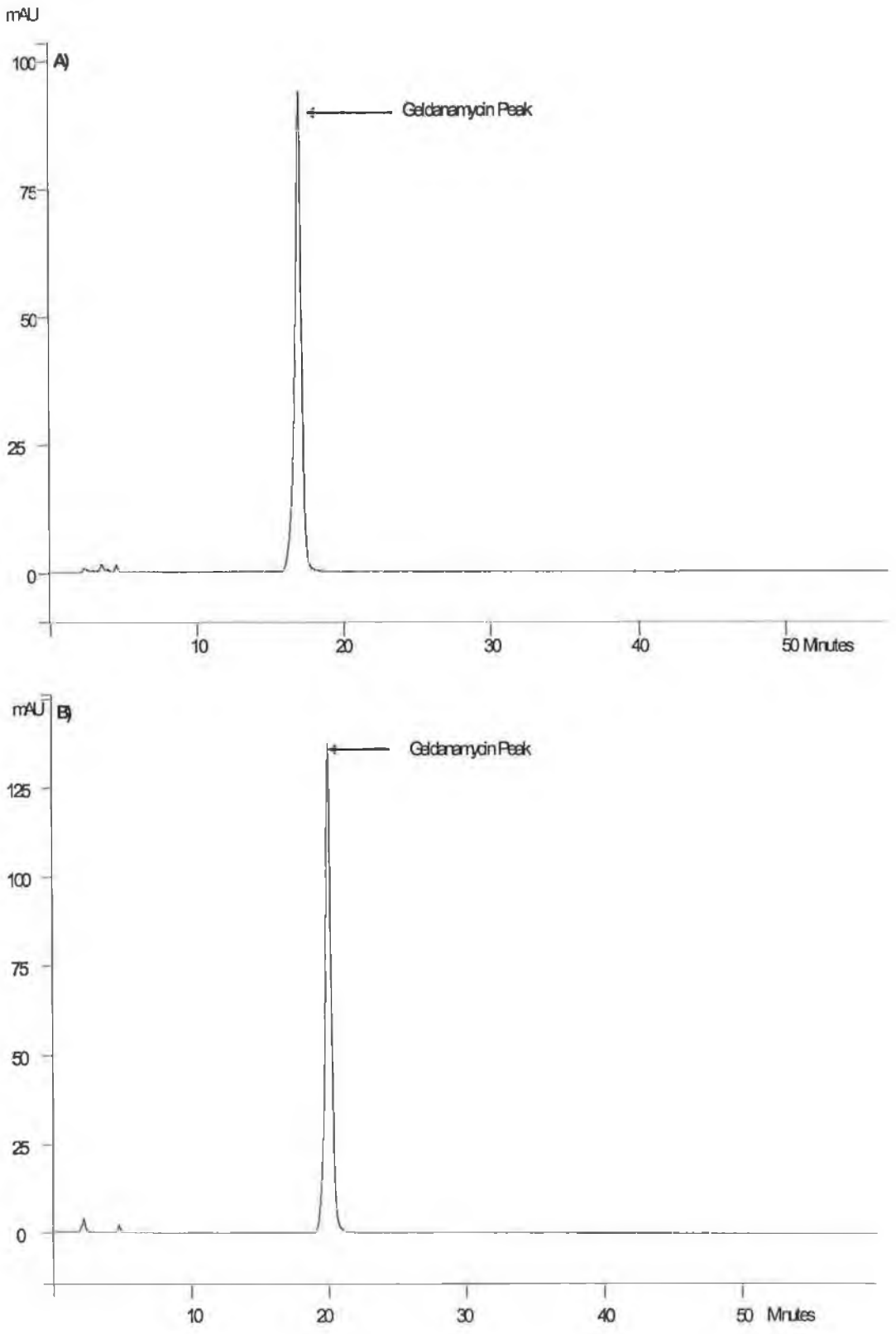


Figure 4.1: Comparison of mobile phase on analysis of a geldanamycin standard. **A)** mobile phase employed by Agnew et *al.,* (2001), **B)** substituted mobile phase of 50% H_2O : 50% acetonitrile

The main aim of altering the mobile phase was to reduce the complexity of the preparation procedure and thus limit the potential for variation between HPLC analyses.

It was therefore necessary to determine if by altering the mobile phase, compound retention or analysis time was adversely effected. Figure 4.1 shows that the geldanamycin peak could be effectively eluted, and was done so in a timely fashion using the new mobile phase. From this result, it is clear that using the new mobile phase would reduce the preparation time and cost involved in employing the HPLC process. Having established that geldanamycin could be eluted via the HPLC process it was necessary to employ this technique for the identification and quantification of geldanamycin in fermentation samples.

4.3 Results and Discussion

4.3.1 Analysis of Geldanamycin

Geldanamycin is produced as part of the metabolic processes of *Streptomyces hygroscopicus*, and is excreted into the fermentation broth. Nonetheless, it was necessary to verify its presence in the fermentation broth samples being generated. In order to achieve this, it was necessary to assess the adsorption spectrum of a fermentation broth sample.

A 3 ml sample of fermentation broth was aliquoted into a quartz cuvette (Hellma, Hellma U.K. Ltd., Essex, U.K.) and analysed using a Shimadzu U.V.-160 A, U.V. to Visible (U.V.-Vis) recording spectrophotometer (Shimadzu Europa GmbH, Duisberg, Germany) over a wavelength range of 200 to 500nm (Figure 4.2).

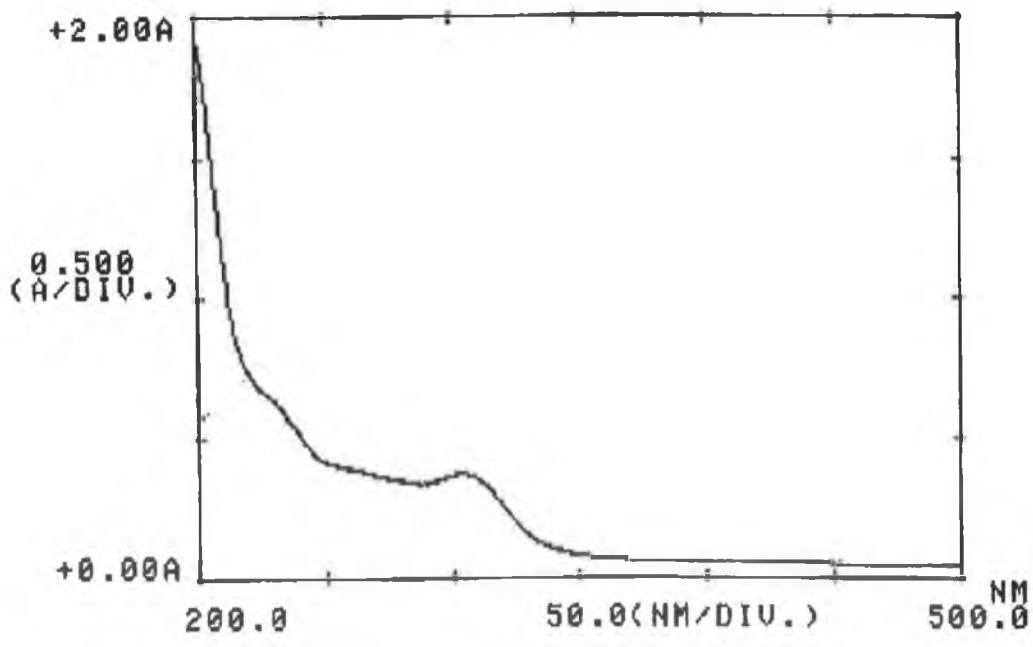


Figure 4.2: Adsorption spectrum of *S. hygroscopicus* fermentation broth

Analysing the absorption spectrum printout, an absorption maximum was noted around 300 nm. This could be related to the geldanamycin

adsorption maximum of 308 nm or 305 nm described by DeBoer *et al.*, (1970) and Alvi *et al.*, (1995), however a more definitive assessment of geldanamycin was desirable.

Since a simple HPLC method for examination of samples containing geldanamycin had been established, it was decided to employ this method for the analysis of fermentation broth samples, with the primary aim of identification and quantification of geldanamycin.

Since the sample source was fermentation broth, there would also be a number of 'contaminating' compounds inherently present in the fermentation medium. These contaminating compounds would include fermentation by-products, proteins, minerals, vitamins and carbohydrate. Throughout this Chapter, contaminating compounds will be considered as being compounds other than geldanamycin, the compound of interest. Definitive identification of geldanamycin was therefore important.

A fermentation sample was analysed via HPLC at 308 nm, and upon examination of the chromatograms, two major peaks were noted. The first was believed to be attributable to poorly retained, less hydrophobic fermentation compounds and mobile phase constituents and were considered contaminating compounds. The second was believed to be that of geldanamycin.

Since this was still not conclusive evidence of the presence of geldanamycin, the accurate determination of the presence of geldanamycin in the fermentation broth was still required. It was believed that this would be best achieved using a combination of chromatogram and adsorption spectrum comparison. HPLC employing a Photo Diode Array (PDA) detector (Varian ProStar 330, Varian Inc., California, U.S.A.) was the apparatus employed to achieve this goal.

Photo Diode Array detection is employed to analyse samples over the entire U.V.-Vis spectrum. Unlike with conventional U.V.-Vis detectors, where only one datum point can be acquired at a time, PDA detectors are

generally comprised of several hundred or thousand detectors and can therefore acquire data for many wavelengths simultaneously (Choi, 2004). This essentially acts to form a 3D-landscape of a resolved compound based on retention time and adsorption wavelength. A single peak, representing a resolved compound can be selected and its adsorption spectrum delivered at the retention time at which it was resolved. PDA detection is frequently used to monitor and identify bioproducts.

Vandana *et al.*, (1996) detailed the use of PDA detection for monitoring taxol extraction from bark and Mierzwa *et al.*, (1988) described the utilisation of PDA apparatus for detection of, and discriminating between, analogues of the antibiotic blasticidin. PDA detection can also be used to monitor reaction products. den Brok *et al.*, (2005) described the use of PDA detection for the monitoring of the formation of degradation products of the compound C1311, a lead compound in a novel group of anticancer agents. In order to accurately identify geldanamycin in fermentation broth samples three samples were analysed via HPLC with PDA detection. The samples were a geldanamycin standard (0.01 g/l), a fermentation broth sample and a fermentation broth sample spiked with geldanamycin standard.

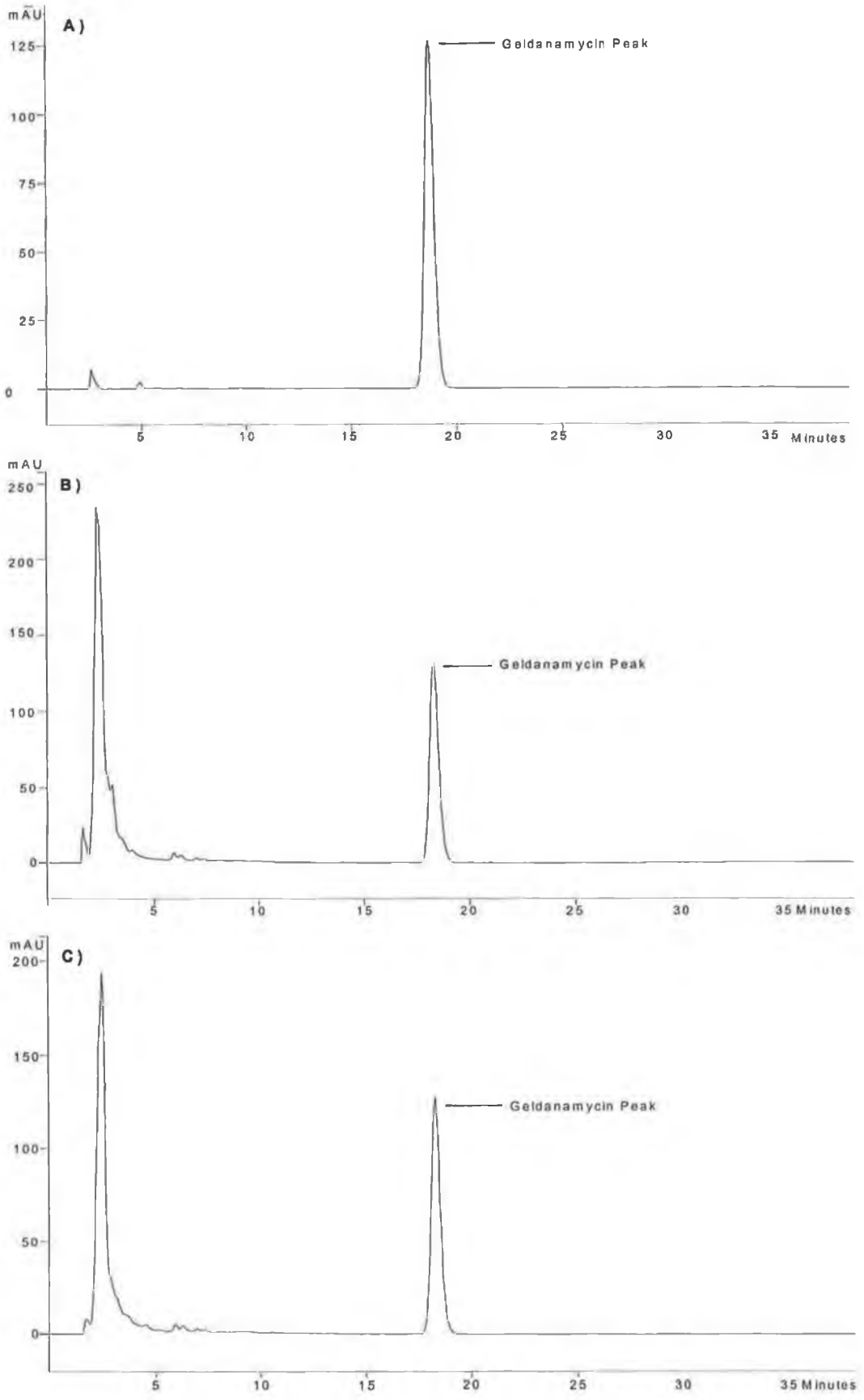


Figure 4.3: Chromatographic identification of geldanamycin. **A)** geldanamycin standard, **B)** fermentation broth sample and **C)** fermentation broth sample spiked wlth geldanamycin

Figure 4.3 shows the HPLC chromatographic results at a detection wavelength of 308 nm. It was found that the major peak present in a fermentation broth sample was consistent with that of geldanamycin through comparison with the chromatogram of geldanamycin standards. Examination of the chromatogram of the spiked fermentation broth sample showed integration of the geldanamycin standard peak and the major peak present in the fermentation broth sample. This gave further indication that the peak resolved at approximately 20 minutes was that of geldanamycin. Further proof of this was gained from comparison of the adsorption spectra of the 20 minute peak obtained using PDA detection.

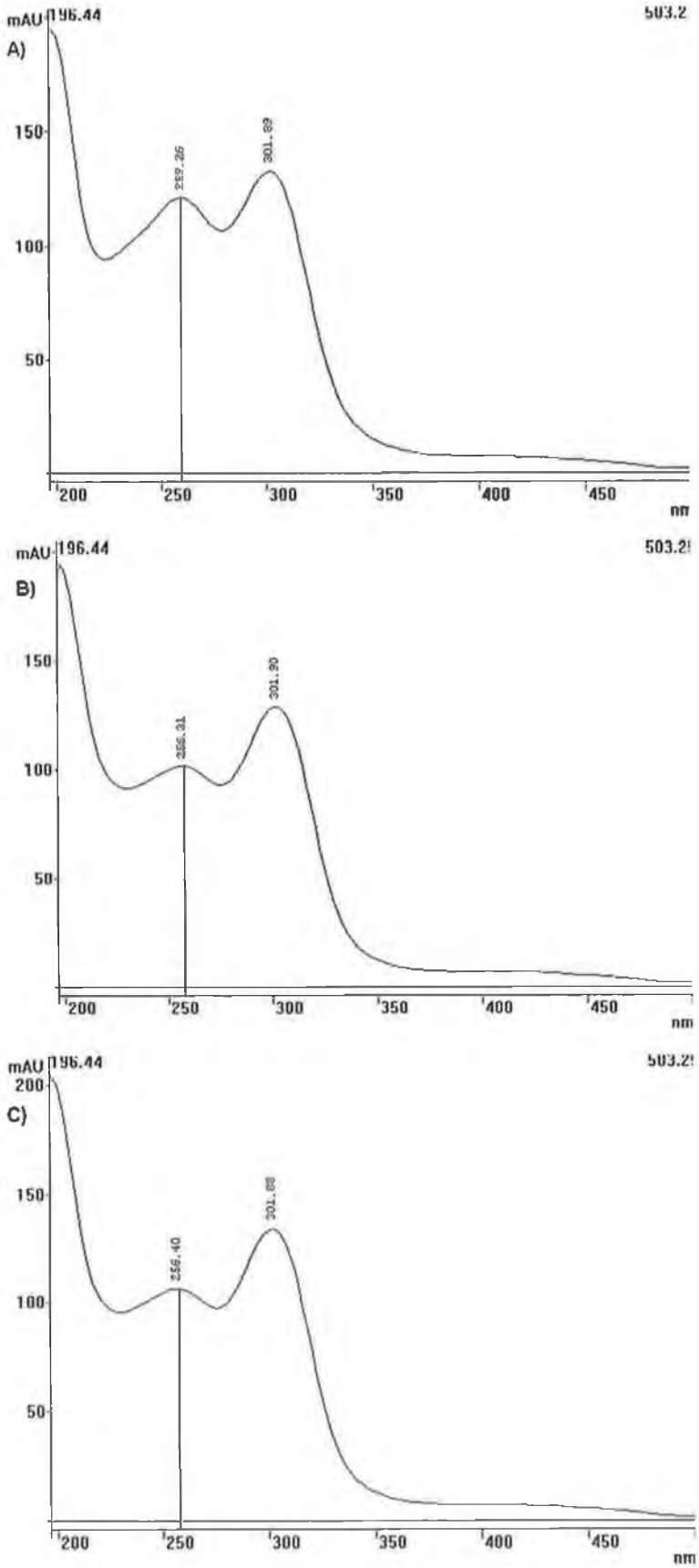


Figure 4.4: Adsorption spectrum comparison and identification of geldanamycin. **A)** geldanamycin standard, **B)** fermentation broth sample and **C)** fermentation broth sample spiked with geldanamycin

Figure 4.4 shows the comparison of the adsorption spectra of the major peak in the fermentation broth sample with that of a geldanamycin standard. A combination of the results shown in Figures 4.2 to 4.4, gives evidence that the major peak in the fermentation broth chromatogram is that of geldanamycin. Examining Figures 4.3 and 4.4 it can be seen that the HPLC peaks and adsorption spectra of broth samples and geldanamycin standard share equivalent retention times and adsorption spectra.

Trejo-Estrada *et al.*, (1998) verified the production of geldanamycin in a similar way, comparing the U.V. spectrum of a standard to that of the compound produced by their test strain of *Streptomyces*. Since geldanamycin has a broad U.V. adsorption peak from 300 to 320 nm, and in literature, geldanamycin has been assessed at a number of wavelengths in this range (Alvi *et al.*, 1995, DeBoer *et al.*, 1970, Rascher *et al.*, 2003, Trejo-Estrada *et al.*, 1998, Agnew *et al.*, 2001).

4.3.2 Analysis of Geldanamycin in fermentation broth

Geldanamycin is excreted by *Streptomyces hygroscopicus*, during submerged culture, into the fermentation broth. The antibiotic can therefore be in the presence of a variety of fermentation compounds, from unfermented carbohydrates and precursors to proteins. Analysis of geldanamycin in fermentation samples was initially performed via HPLC analysis using filtered fermentation broth. Analysing samples in this manner was however, found to be problematic. After a number of samples had been analysed, the system would show signs of column fouling, including reduced analytical performance, increased pressure and reduced column life-span. In order to circumvent these problems, it would be necessary to gain a better understanding of how these problems occurred. It would also be necessary to determine if alterations to the HPLC method could address these issues and ultimately facilitate the development of a successful analytical process for fermentation broth samples.

In a technical report released by Mac-Mod Analytical Incorporated (2006), entitled 'protecting reversed phase HPLC columns', column fouling was highlighted as a major problem in HPLC analytical processes. They stated that most damage to reverse phase columns is caused by either particulate material plugging the inlet of the column or non-eluted compounds 'fouling' the column. They suggested that column fouling leads to an increase in process costs, associated with failure of the column and its subsequent replacement, and an increase in process time, due to the downtime caused by an increased need for column regeneration and equilibration. They also suggested that fouling may be attributed to inadequate elution of compounds from the column.

The compounds or materials responsible for column fouling are usually derived from the sample and eventually build up on the column to the point where it adversely affects chromatographic performance. There are a number of compounds and materials which can lead to column fouling, and can be comprised of particulate matter (Lindemann *et al.*, 2000) to

proteins (Waterborg, 2000, Hagestam and Pinkerton, 1985). The fermentation broth in which geldanamycin is produced can be complex, containing carbohydrates, minerals, proteinaceous material and other compounds, and it should therefore come as no surprise that fouling could occur. It was found that the major impact of fouling on the HPLC analysis of geldanamycin from fermentations broth samples mirrored those highlighted in the Mac-Mod technical report. They included prevention of sample analysis due to pressure constraints (the column optimal working pressures were below 3500 psi and the pump maximum discharge pressure was just over 5000 psi), column down time and the increased requirement for column replacement.

HPLC is based on similar principles to those of gas chromatography (GC) with the major difference being the use of liquids in place of the gas phase. Since liquids are more viscous and exhibit lower diffusion rates than gases, the separation process must be conducted at higher pressures. These higher pressures also facilitate fast sample analysis times. The pressures encountered in the system can be affected by two main sources, the mobile phase flowrate and column fouling. If one increases the flowrate to reduce sample analysis time, the pressure in the system will also increase. This is due to the fact that a greater pressure is required to move the liquid through the narrow column, and small packing material pore size, at the more rapid rate. If there is a build up of material in the column as a result of fouling, this will act as a barrier to liquid flow, and therefore increase the pressure in the system.

Increased pressures are problematic because many columns and HPLC systems do not have satisfactory life-times above 5000 psi (Hancock and Sparrow, 1984), or the complete HPLC system, including tubing and fittings, may have pressure limits below this. The technical data of the column used for geldanamycin assessment stated that, in order to prolong column life, pressure should be maintained below 3500 psi, which dictated that the typical flowrate employed was 0.5 ml/min.

After relatively few broth samples were analysed a considerable increase in the pressure in the system would be noted, which indicated it was necessary to clean the column. If cleaning did not take place the pressure would continue to increase until column or system thresholds would be reached, or poor resolution of samples would result.

The cleaning of columns was carried out to facilitate the removal of strongly retained compounds, and thus reverse column fouling and reduce system pressure. The standard cleaning procedure was rinsing the columns with 10 column volumes of the following solutions in sequence: 95% H_2O : 5% acetonitrile, 100% tetrahydrafuran, 95% acetonitrile : 5% H_2O, and then the mobile phase (which was 50% H_2O : 50% acetonitrile). If, after cleaning the column, the pressure was still high upon equilibration of the column, the cleaning was repeated. This time, however, it would be carried out with the column in the reverse orientation, and having been heated to 50°C. The heating served to aid solublisation of compounds and washing in reverse helped to flush the compounds back out of the column inlet rather than encouraging their progression further down the column.

If the cleaning of columns was insufficient to reverse the column fouling, as indicated by an insufficient reduction in system pressure, the column could no longer be employed in the analysis process and had to be replaced with a new column. This occurred on a frequent basis, and columns were being irreparably fouled at an excessive rate. It was therefore decided that a pre-treatment step was required to limit fouling and the associated damage to the columns.

4.3.2.1 Butanol extraction to aid analysis of geldanamycin in fermentation broth samples

It was decided to examine the application of solvent extraction of geldanamycin from the fermentation broth samples as a means to reduce the potential of column fouling, and to therefore increase the speed of the overall analytical process. Solvent extraction is a method frequently

employed to recover selected compounds from a stream containing many compounds. It was decided that extraction of geldanamycin prior to analysis would serve to reduce the amount of contaminating material which could pass onto the column and therefore limit subsequent fouling of the column. The extraction of geldanamycin from fermentation broth was achieved via liquid-liquid extraction of the samples using butanol as the extracting solvent, since it had been identified, in literature, as a suitable solvent (DeBoer *et al.*, 1970).

The liquid-liquid extraction process involved contacting fermentation broth with butanol in a 1 : 1 ratio in a universal. This was then agitated by hand, and then sonicated, in a sonicating water bath, for 10 minutes. The sample was then centrifuged, at 3500 rpm for 10 minutes to promote partitioning of the two phases. The butanol was recovered and ready for analysis.

Although extracting the samples with butanol reduced the amount of contaminating compounds in samples to be analysed (a reduction of the order of 20% depending on the samples being analysed) whilst maintaining an extraction efficiency of geldanamycin of the order of 95%, there were some major difficulties encountered with using this butanol extraction technique. Butanol was found to be incompatible with the HPLC analysis protocol. Butanol samples returned very poor analytical resolution and frequently resulted in fronting of samples, whereby the front part of a resolved peak (before the apex) tapers in advance of the remainder of the peak.

When butanol alone was analysed via HPLC, it would traverse the column expediently and be eluted early. However analysis of a butanol extracted sample resulted in a phenomenon of 'poorly resolved peaks' being noted. Although the majority of geldanamycin in the sample was eluted at the appropriate retention time, it was believed that some geldanamycin was 'retained' with the butanol and, was continuously transferred to the mobile phase, with elution occurring sooner than the majority of the geldanamycin. The resulting chromatograms showed these poorly resolved peaks, eluted prior to the primary geldanamycin peak. Figure 4.5

highlights the negative impacts of the presence of butanol on HPLC analysis of fermentation broth samples.

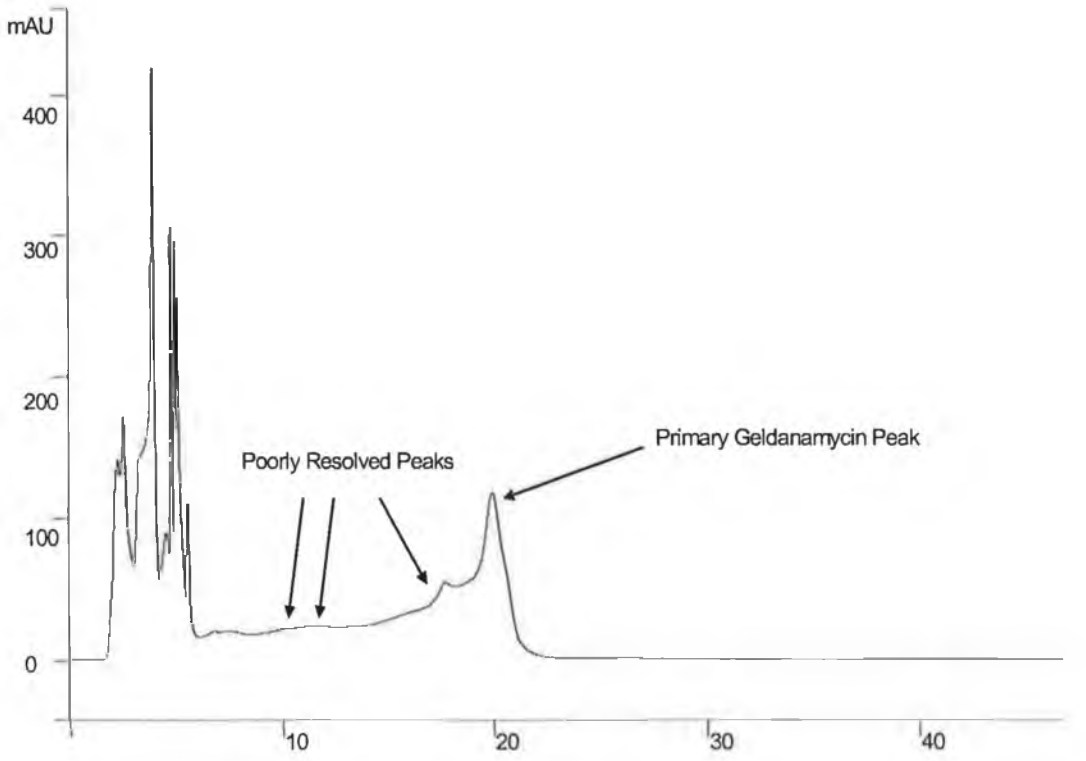


Figure 4.5: Effect of butanol on sample analysis and resolution

As a result of these problems it became clear that analysis of butanol extracted samples was not viable. In order to address these issues, the butanol would have to be removed from the samples and replaced with a suitable solvent, which was inert with relation to the HPLC system. The most suitable means of removing the butanol was deemed to be through evaporation techniques.

The first method of butanol removal attempted was flash evaporation, which involved rapid removal of solvent through the use of high temperatures. Butanol evaporates at a temperature of approximately 117°C. Since geldanamycin is a thermolabile compound, elevating a sample to this temperature would be detrimental. However, it was theorised that if the butanol could be evaporated quickly then the time the sample would spend at elevated temperatures would be minimised.

The flash evaporation process involved the following. 1ml of a butanol extracted fermentation broth sample (i.e. the butanol phase) was aliquoted in 20 ml glass test tubes. The test tubes were then lowered into a silicone oil bath, heated to 170°C. After approximately 10 to 20 seconds the butanol would evaporate. In initial applications of this technique, a rapid recondensing of the butanol was encountered. This was overcome by using smaller glass test tubes (8 ml), which could also be immersed deeper into the bath. This alteration also reduced the evaporation time.

Although the technique resulted in the evaporation of the butanol, it was not a successful method, since the heat required for butanol evaporation resulted in geldanamycin degradation. Also, the evaporation was frequently violent, resulting in eruption of sample from the evaporation vessel (see Figure 4.8 for comparison of the effect of butanol removal methods on geldanamycin signal).

The poor performance of the flash evaporation method was attributed to the high temperatures required to evaporate butanol. It was therefore decided that removal of butanol should take place at lower temperatures. This was achieved using a rotary vacuum evaporation method.

The rotary evaporation method involved placing a butanol extracted sample into a round bottom flask and coupling it to the evaporation and condensing apparatus (the unit and apparatus are displayed in Figure 4.6). The flask was then lowered into a water bath maintained at 40°C, and rotated. At this point a vacuum pump connected to the unit, capable of exerting a vacuum of - 0.98 bar, was switched on and the unit slowly sealed to create a vacuum. The sealing processes took on average 5 minutes, this slow sealing process was required to prevent suction of the sample out of the flask. The evaporation of the butanol took approximately 10 minutes, with the evaporated butanol vapour being recovered using a condenser, with circulation of a polyethylene glycol-water mixture, maintained at approximately - 4°C, using a Frigomix S-1, recirculating, cooling, waterbath (B. Braun, Melsungen, Germany). An in-line cold trap,

immersed in liquid nitrogen, prevented any butanol vapour which may have escaped from the condensing apparatus, from entering the pump.

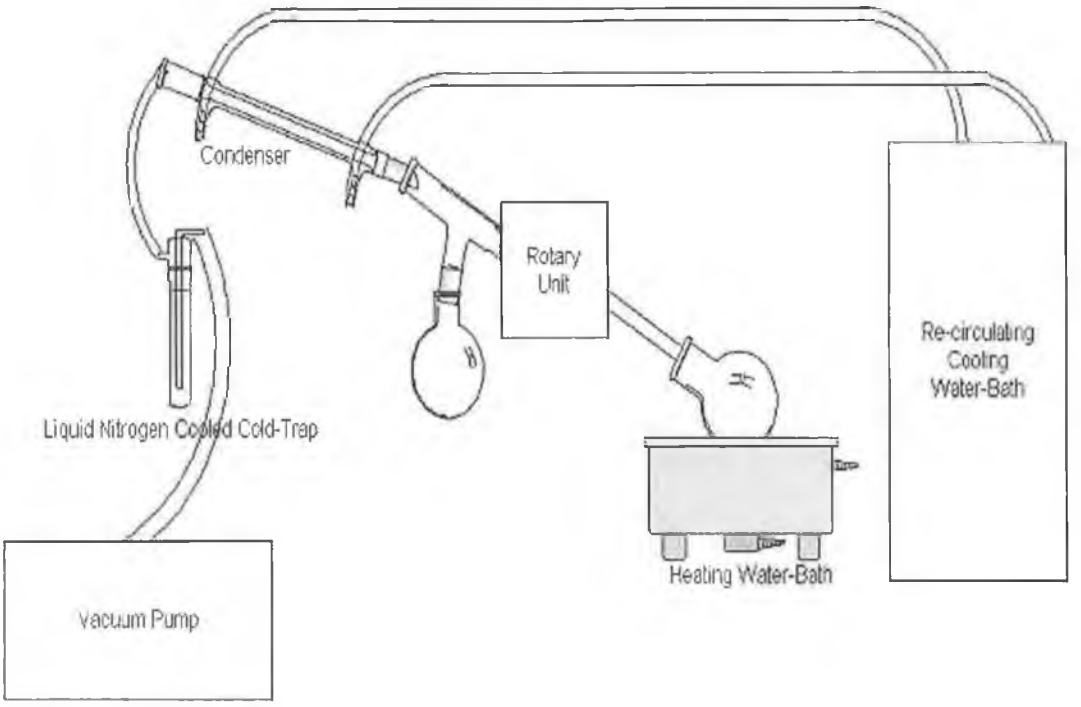


Figure 4.6: Schematic of the rotary evaporation unit employed for the removal of butanol from samples

The rotary evaporation process was successful at removing the butanol without signal loss, however, the main problem encountered with this method was sample throughput. Using the rotary evaporation method for large sample numbers was both time and labour intensive and required a large working volume. It was decided to examine other methods for butanol removal.

The final method for butanol removal examined was low pressure evaporation. This method was analogous to rotary vacuum evaporation, but involved the construction of a stainless steel evaporation chamber, and a schematic of the apparatus is provided in Figure 4.7.

The low pressure evaporation method involved evaporation of 1 ml samples of butanol extracted samples, however, it was possible to treat a greater number of samples in a single batch. The samples were added to

8 ml glass test tubes. The test tube openings were covered with tin foil and a small perforation placed in each one to allow solvent vapour removal. The test tubes were then placed into a test tube rack (with space for 72 samples) and lowered into the evaporating vessel. The vessel comprised of a stainless steel, fully sealable unit, with an internal volume of approximately 0.1 m^3, and which was evacuated using a vacuum pump. A layer of silicone oil (approximately 1 inch deep) covered the bottom of the chamber, and aided heat transfer. The chamber was heated to 40°C, via an integrated heating mantle, whilst the vacuum was applied. Once at 40°C, the heating was ceased and the evaporation continued for a further thirty minutes, after which the samples were removed, completely dry.

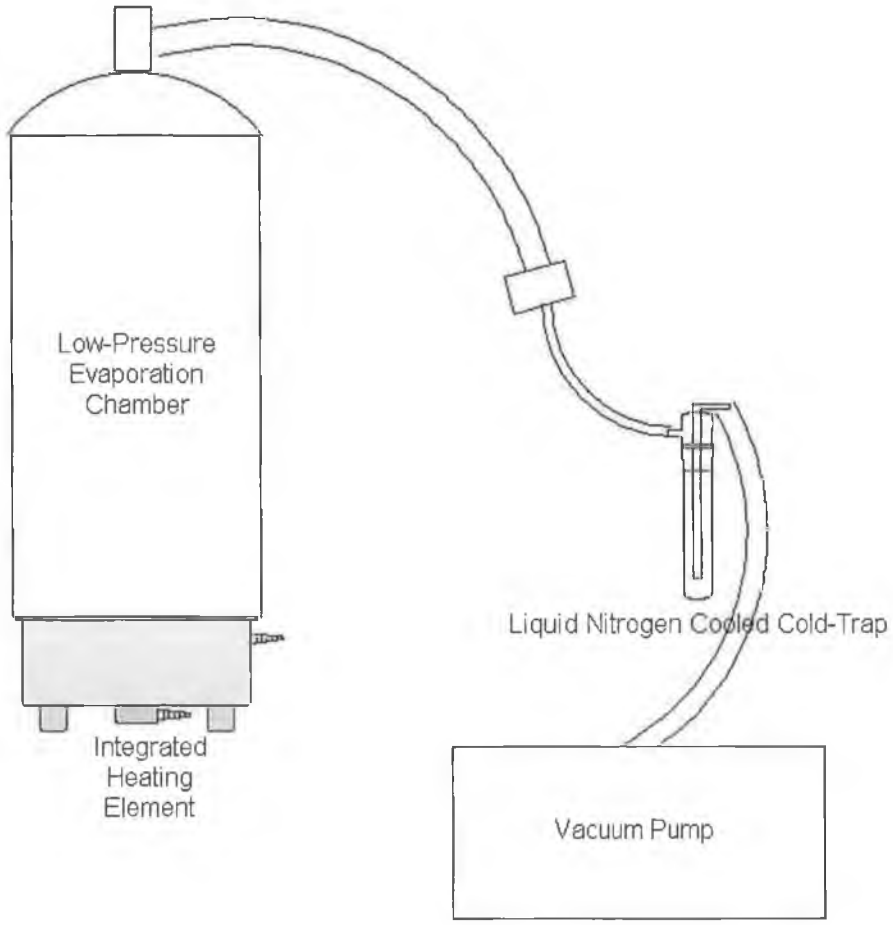


Figure 4.7: Schematic of the low pressure evaporation unit employed for the removal of butanol from samples

This method proved to be the best of the alternate methods examined. The temperatures and process times involved were not excessive and the

method allowed the evaporation of a large number of samples at once. Figure 4.8 compares geldanamycin signals resultant from treatment using each method.

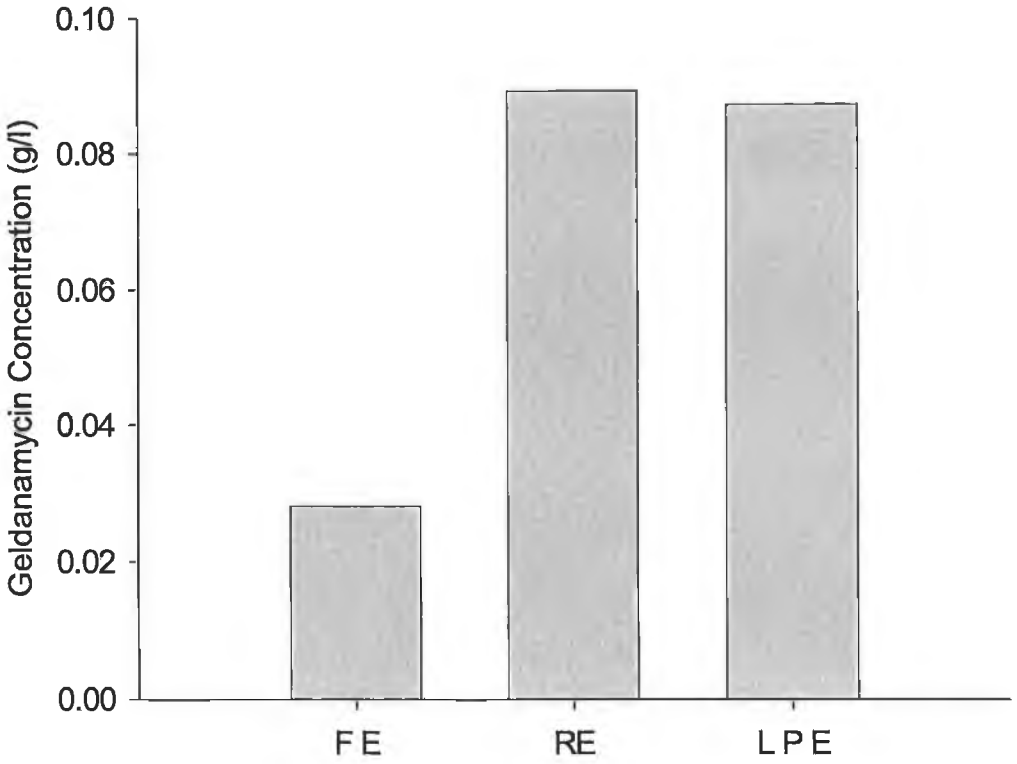


Figure 4.8: Effect of Butanol removal method on geldanamycin signal. FE) Flash Evaporation, RE) Rotary Evaporation, LPE) Low Pressure Evaporation

From these results it was now possible to carry out a butanol-mediated liquid-liquid extraction of the fermentation broth samples. Doing so would reduce the level of contaminating compounds present in the samples to be analysed, and would therefore reduce the potential for fouling of the columns, and would in turn, reduce column downtime and damage. The extracting solvent could then be removed and substituted with a solvent which was more suitable to the HPLC analytical method. The solvent employed was a 50% : 50% (v/v) mixture of H_2O : acetonitrile. This was also the HPLC mobile phase, and therefore its application would be inert in the analysis process.

4.3.2.2 Optimisation of analysis of geldanamycin from fermentation broth samples

The HPLC method was developed to be the primary means of assessing the presence and concentration of geldanamycin in fermentation samples. It was therefore apparent that the HPLC analysis of samples was the most likely 'bottleneck' of the project work and in order to address this, high throughput analysis of samples would be required. Although low pressure evaporation could successfully treat a large number of samples in a single run, each sample had to undergo solvent extraction, solvent removal and resuspension prior to analysis. This represented a drain on time and resources, and although the method addressed the issues of column fouling, it did not increase sample throughput.

Column fouling and low sample throughput can be considered as interlinked problems. Fouling results from interactions between the compounds in samples with the column stationary phase. Low sample throughput is essentially dictated by the analysis time required for a sample, which in turn is associated with the flowrate of the mobile phase. The maximum flowrate which can be employed is dictated by the pore size of the stationary phase, which, if small, may have a greater potential to clog and become fouled. As a result of this interrelationship, it was decided to examine modifying the HPLC apparatus as a means of tackling both the column fouling and sample throughput issues.

The packing phase pore size of the HPLC column employed for sample assessment was 3 µm. The technical specifications of the column and pressures resultant, meant that the maximum flowrate utilisable was 0.5 ml/min. Using this method the runtime of each sample was 60 minutes, with the geldanamycin peak identified at approximately 20 minutes. In HPLC, short analytical times and high speeds are achieved by high solvent / mobile phase flow velocities, and because of the fast flowrate a high pressure exists at the column inlet (Aszalos *et al.*, 1982). Essentially, in HPLC analyses, the pore size of the stationary material plays an inherent role in the pressure limitations and therefore the flowrate

achieved. The HPLC column employed could also be supplied by Phenomenex in a 5 µm pore size. Using the 5 µm pore size column reduced the pressure in the system, and allowed the HPLC process to be run at a flowrate of 1ml/min. This facilitated a reduction in the runtime, allowing samples to be run in 20 minutes, with the geldanamycin peak being eluted at approximately 10 minutes (Figure 4.9).

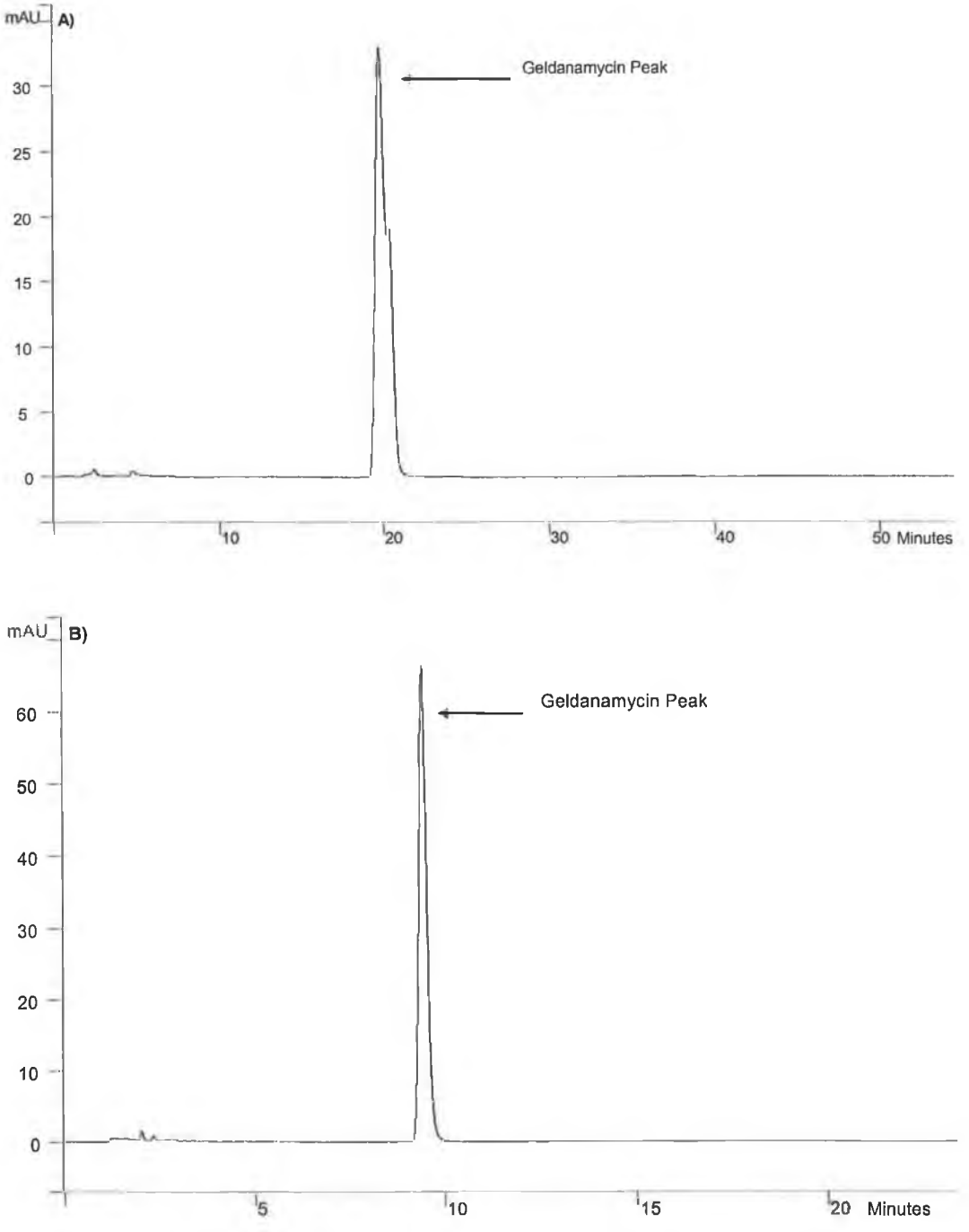


Figure 4.9: The effect of column alteration on analytical success. **A)** 3 µm pore size column, **B)** 5 µm pore size column

A further benefit of employing the column with a larger stationary phase pore size was that there was an inherent reduction in the risk of clogging and therefore fouling of the column. If the pore size is larger, the compounds being applied to the column will not be associated or retarded to the same degree as with a smaller pore size stationary phase. This

reduces the residence time of compounds and reduces liquid flow pressure and fouling events.

Since HPLC analysis of samples was one of the most central aspects of any particular experiment carried out, it was decided to put in place further measures to optimise the process. Using the security guard cartridge unit supplied by Phenomenex, it was possible to stack guard columns. It was decided to use two guard columns to reduce the risk of contaminating compounds passing through from the sample to the analytical column. When the first guard column becomes saturated with fouling material it can be changed, and one can be sure that the second guard column has acted a 'safety net', limiting access of any fouling compounds which may have traversed the first guard column, from gaining access onto the analytical column.

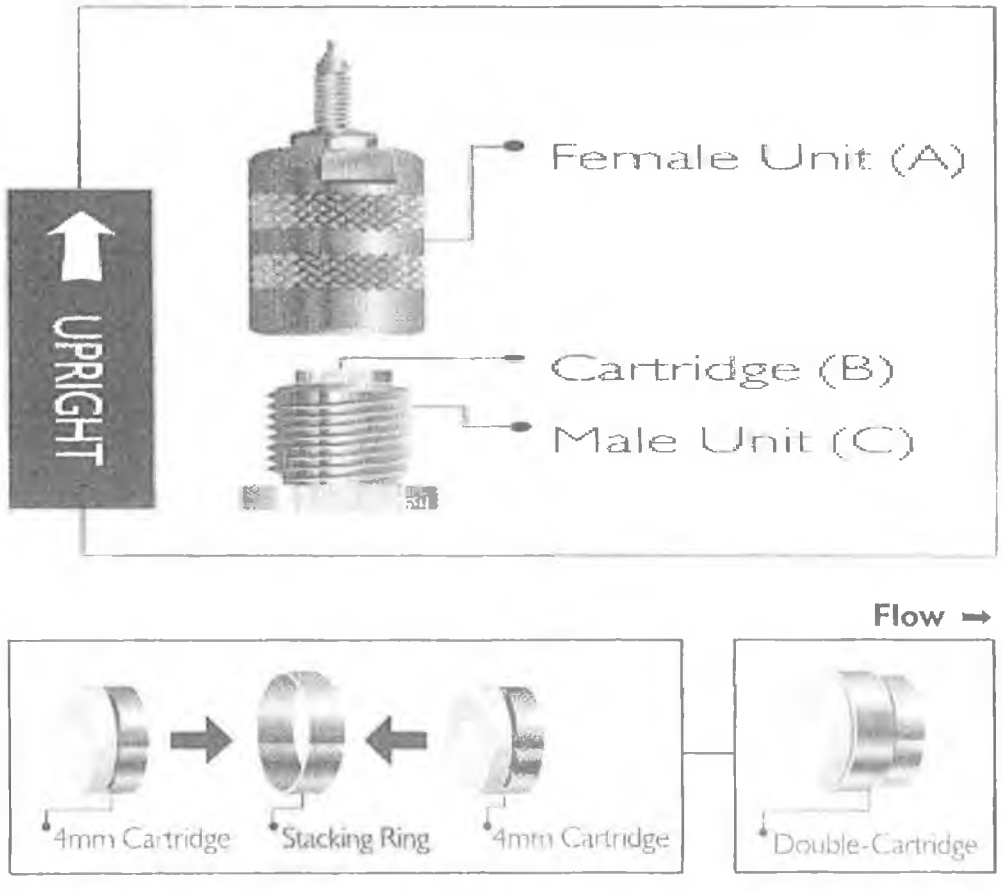


Figure 4.10: Schematic of the Phenomenex Security Guard guard column system, and diagrammatical representation of guard column stacking

4.3.2.3 The effect of fermentation broth dilution on analysis

Since employing a HPLC column with a larger stationary phase pore size in conjunction with stacking of guard columns reduced column fouling and downtime, it was believed that it may again be possible to attempt to reduce sample preparation requirements and revert back to analysis of broth samples which have not been extracted. In order to limit the potential of column fouling it was decided to dilute the fermentation broth samples prior to analysis. By diluting the fermentation broth, the amount of contaminating materials, or compounds capable of causing fouling, could be reduced. Dilution of samples would also provide a means of reducing the number of process steps required for analysis of each sample, therefore reducing the total processing times associated with sample analysis.

In order to determine if dilution was a viable means of assessing geldanamycin concentration in fermentation broth samples, it was decided to examine what effect dilution of fermentation broth in H_2O had on the chromatographic results returned. Fermentation broth was diluted in H_2O, to give a range of samples and the linearity of dilution examined. This would indicate the robustness of the dilution process, and help determine its suitability for application in the analysis of future samples.

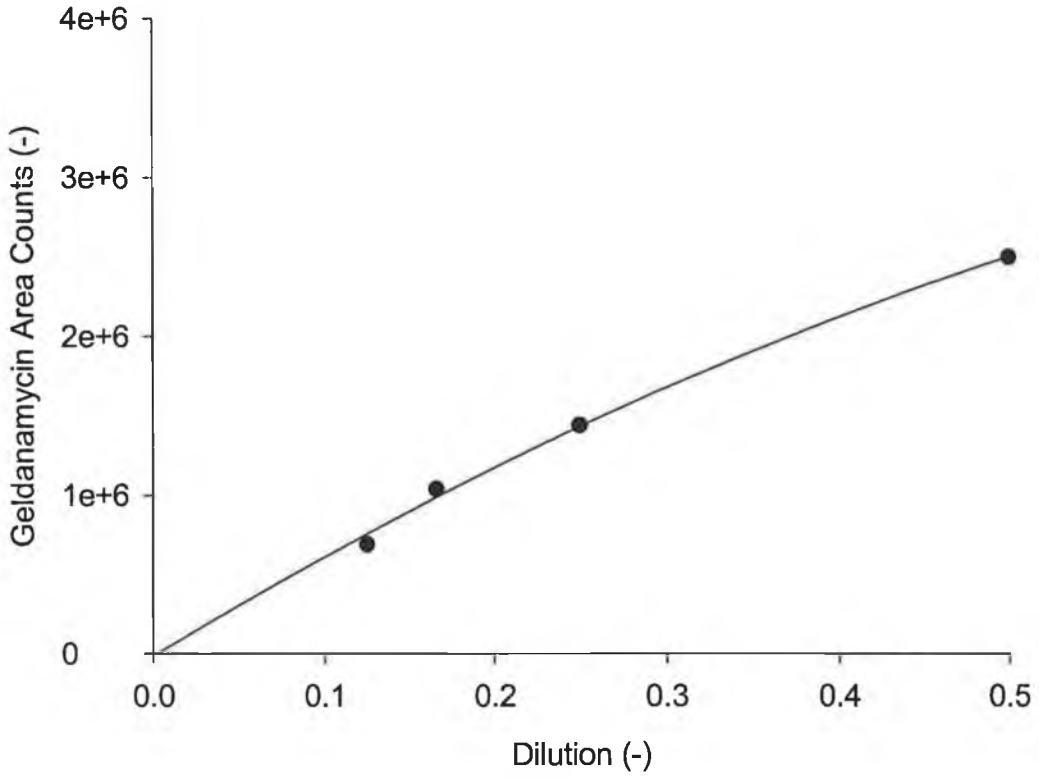


Figure 4.11: The effect of serial dilution of fermentation broth in H_2O on geldanamycin signal

From Figure 4.11 it can be seen that dilution of fermentation broth in H_2O returns a non-linear geldanamycin signal with respect to concentration. This result suggested that the soluble saturation levels of geldanamycin in H_2O were low, and that in neat broth, the geldanamycin signal will be underestimated. This theory is corroborated by the fact that no technical data could be found for the solubility of geldanamycin in H_2O. Instead, solvents such as DMSO (dimethylsulphoxide) are more commonly employed. Also, InvivoGen (San Diego, C.A. USA) noted issues with aqueous solubility, identifying a derivative of geldanamycin, 17-DMAG (17-(Dimethylaminoethylamino)–17–demethoxygeldanamycin), as the first water soluble derivative. Further corroboration of the limited solubility of geldanamycin is gained from the work of Barril *et al.*, (2005) and Hwang *et al.*, (2006). Both groups detail how one of its analogues, 17-AAG exhibits poor solubility.

In order to address this issue, it was decided to attempt to increase the solubility of geldanamycin in the aqueous phase by addition of a polar solvent. Kandori *et al.*, (2002) found that addition of a polar solvent such as acetonitrile could bring about conformational changes in the target molecule which could lead to an increase in its adsorption, in their studies of adsorption behaviour of bovine serum albumin onto synthetic adsorbents. Grezegorczyk and Carta, (1996) also suggested that the solubility of compounds in aqueous solutions could be altered by the addition of solvents.

It was believed that by addition of acetonitrile to the sample, the issue of reduced solubility could be addressed, and may lead to an increase in the geldanamycin signal. In order to achieve this, samples were serially diluted in 50% H_2O : 50% acetonitrile (v/v) and 100% acetonitrile. Acetonitrile was chosen as the diluent since it was the solvent component of the mobile phase and was miscible with the fermentation broth. The results were compared to those derived from dilution of samples in 100% H_2O.

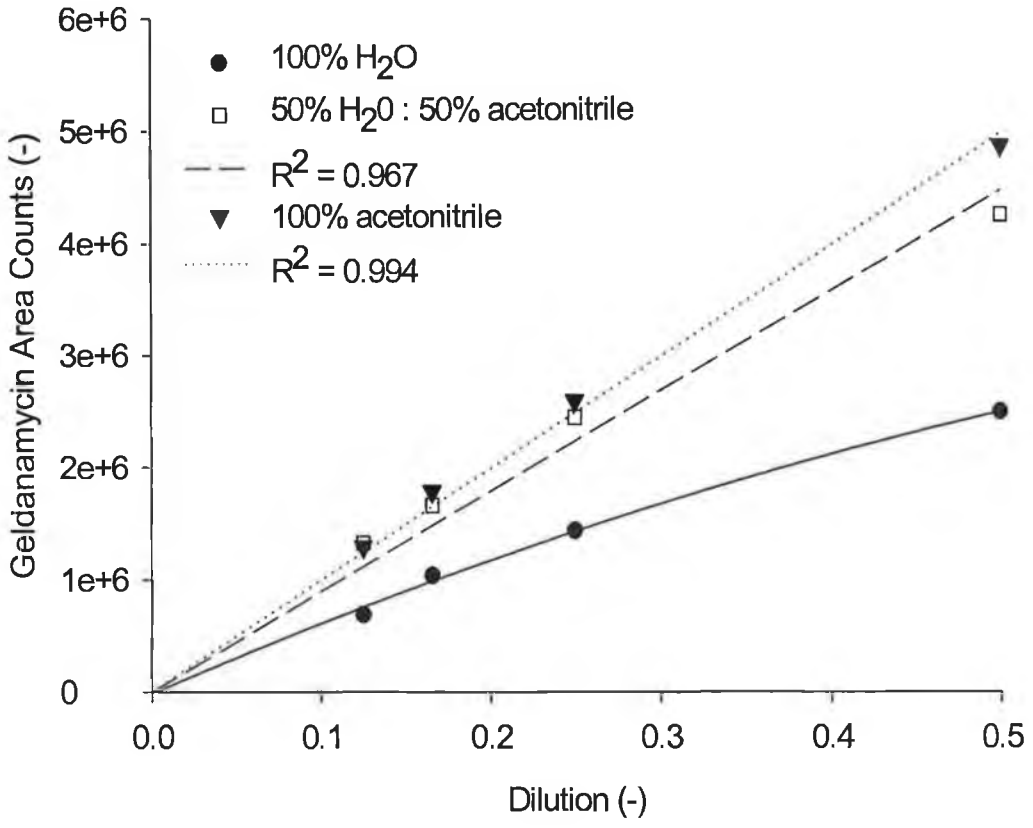


Figure 4.12: Effect of diluent on geldanamycin signal

From Figure 4.12 it was found that addition of acetonitrile to fermentation broth samples increases the linearity of the dilution curve. It is also evident that addition of acetonitrile increases the magnitude of the geldanamycin signal and thus calculation of the concentration of geldanamycin in undiluted fermentation broth samples.

Examining the samples diluted in acetonitrile, it can be seen that when a sample, diluted 1 in 2, and a sample, diluted 1 in 8, are back calculated to give a result for an undiluted sample the results would be comparable. This indicates that the benefits of acetonitrile addition, to the determination of geldanamycin in fermentation broth samples, can be gained even at low levels of acetonitrile addition. This trend is confirmed by the fact that even dilution in 50% H_2O: 50% acetonitrile shows a linear dilution pattern and high geldanamycin signal.

In the analysis of fermentation broth samples, the geldanamycin signal results were occasionally lower than would have been expected, or fluctuated between analyses. In order to attribute low signal results and poor reproducibility to poor solubility of geldanamycin in aqueous phases, it was decided to compare treatments of 'stabilised' versus 'unstabilised' samples. The general analysis of fermentation samples involved filtering of the sample prior to HPLC analysis in order to limit fouling. It was theorised that this could be a source of error. If the geldanamycin in broth samples was of low solubility, then potential existed for some of the poorly solublised geldanamycin to be to be retained by the filter. If this occurred it may be possible, by extension, that 'removal' of geldanamycin could also occur during sample preparation or analysis.

To establish if this was possible, a comparison of treatments was set up. The first sample was filtered (0.22 µm pore size nylon filters, PALL Scientific) and then diluted in acetonitrile prior to HPLC analysis. The filter paper was then washed in one sample volume of acetonitrile and the filtrate examined. The second sample was diluted in acetonitrile prior to filtration and analysed. Again the filter paper used was washed in one sample volume of acetonitrile and the filtrate analysed. Performing this analysis would determine if the geldanamycin was poorly soluble, if there was potential for it to be 'lost' during HPLC sample filtration and if acetonitrile could stabilise the geldanamycin in the sample so as to limit these negative effects on sample analysis and analytical reproducibility. Table 4.1 summaries the results.

Table 4.1: Comparison of the effect of dilution of acetonitrile pre and post filtration on the stability of geldanamycin signal

Sample	Filter then Dilute (Geldanamycin Area Counts)	% of Total Signal	Dilute then Filter (Geldanamycin Area Counts)	% of Total Signal
Broth	4694130	53.73	10993645	94.35
Filter Wash	4100790	46.27	658325	5.65
Total Signal	**8794920**	**100**	**11651970**	**100**

From Table 4.1 it can be seen that the difference in the total geldanamycin signals returned from the two treatments indicates that even though the filter was washed, residual geldanamycin may be retained on the filter. The quantities retained on the filter used to filter the pre-diluted samples are low, thus any remaining geldanamycin would be even less. The results indicate that addition of acetonitrile increases the solubility of geldanamycin in aqueous broth samples. Those samples which are filtered prior to acetonitrile addition have considerable quantities of geldanamycin retained on the filter. This again indicates that poorly solublised geldanamycin may be removed through filtration and that addition of acetonitrile increases the level of geldanamycin solublisation in the fermentation broth samples and therefore benefits accurate sample analysis.

The work performed in this section involved determining if it was possible to reduce the potential of column fouling, increase the ease of sample treatment, and facilitate a high sample throughput, via dilution of fermentation broth samples. The results of this assessment indicate that it is possible to dilute fermentation broth samples for these purposes, however, it was noted that that the diluent employed could influence the results returned. During the assessment an issue regarding the solubility of geldanamycin in fermentation broth samples was encountered. It was believed that due to the potentially limited solubility of geldanamycin in aqueous systems, analysis of broth samples directly would require treatment, in the form of suitable dilution, in order to return accurate and reproducible results. It was therefore decided to examine the effect of different diluents on the chromatographic assessment of geldanamycin in fermentation broth samples.

It was decided to carry out assessments aimed at gaining a greater understanding of the implications of dilution of fermentation broth samples. By carrying out such an assessment, it was believed that the dilution process could be standardised, and could therefore be used for the analysis of all subsequent fermentation broth samples.

Firstly, the impact of the diluent on the reproducibility of analysis of a sample was assessed. Obtaining reproducible results confers a degree of consistency and accuracy to the assessment of geldanamycin in fermentation broth samples. The assessment involved dilution of fermentation broth samples in 100% H_2O, 50% H_2O : 50% acetonitrile and 100% acetonitrile. These diluents were used because they were compatible with the mobile phase and had been involved in the previous assessment of the impact of fermentation broth dilution on analysis.

Each set of sample dilutions were run in triplicate, denoted Run 1, Run 2 and Run 3 over a period of 45 hours. The samples were analysed in the order; 100% H_2O diluted samples, 50% H_2O : 50% acetonitrile diluted samples and 100% acetonitrile diluted samples. In order to determine the fluctuations in repeated analysis over time, the average geldanamycin area counts for each sample were calculated and the deviation from that for each sample determined. This process serves the combined purpose of assessing the reproducibility of analysis, in conjunction with determining the stability of a sample over the analysis period with respect to the diluent used.

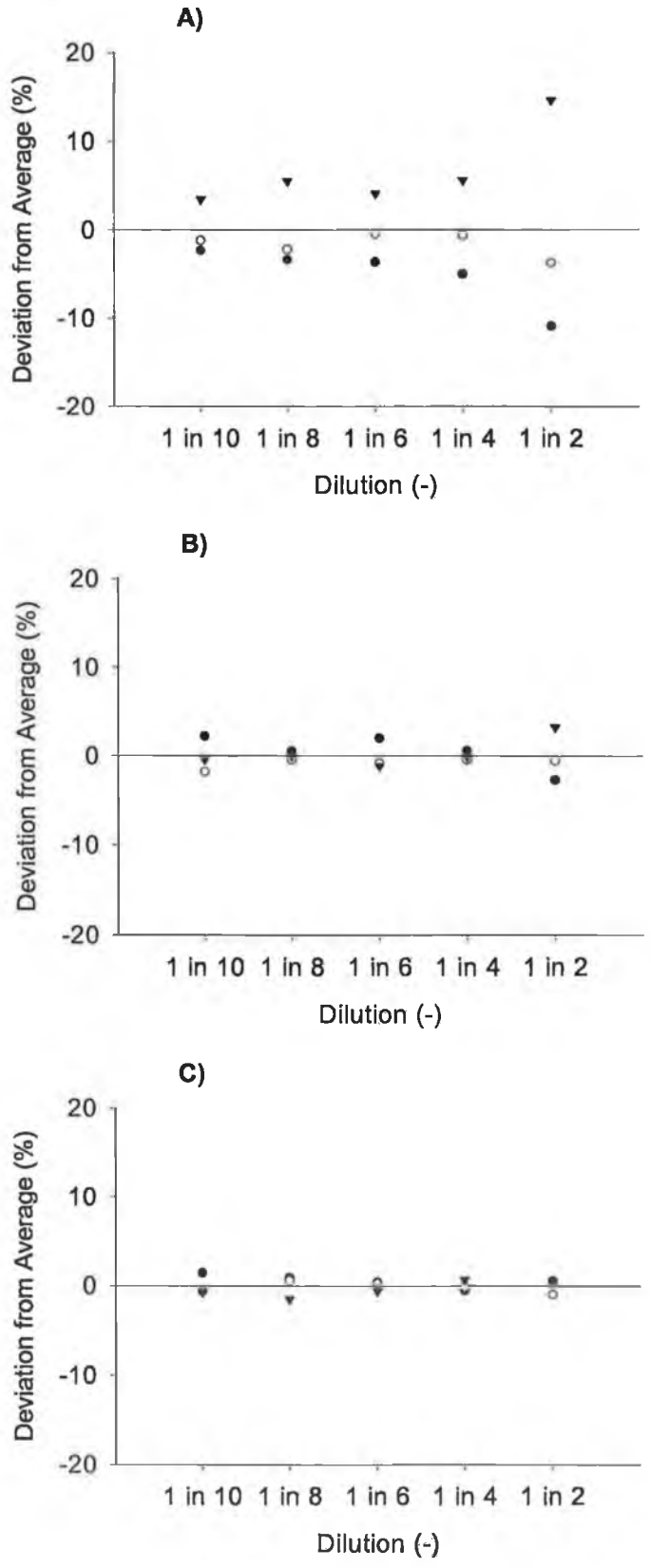


Figure 4.13: Effect of diluent, dilution and sample incubation time on reproducibility. **A)** 100% H_2O **B)** 50% H_2O : 50% Acetonitrile, **C)** 100% Acetonitrile. ● = Run 1 (1^{st} injection of samples), ○ = Run 2 (2^{nd} injection of samples) and ▼ = Run 3 (3^{rd} injection of samples)

From Figure 4.13 it can be seen that the diluent used to dilute fermentation broth can impact on the reproducibility of sample analysis. It is seen that those samples diluted in 100% acetonitrile return the best signal stability over the 45 hour analysis period. Dilution in 100% H_2O returned considerable deviation in signal from the average signal result for the three sample injections.

The second assessment, of the impact of the diluent on analysis of geldanamycin in fermentation broth, was focused on determining how the diluent impacted on geldanamycin calculations. It was desirable for dilution of samples to be used to prepare all subsequent fermentation broth samples for HPLC analysis. From the initial assessment of dilution, it was noted that addition of solvent increased solubility of geldanamycin in samples and therefore returned higher results. It was therefore decided to examine this further and ultimately, establish which diluent should be used in future sample analysis protocols.

The assessment involved diluting samples in 100% H_2O, 50% H_2O : 50% acetonitrile and 100% acetonitrile. These samples were analysed using HPLC and the geldanamycin signal determined. These results were then multiplied by the dilution factor, to return a result for geldanamycin concentration in an undiluted sample. The average of these results, for each diluent used, was calculated and the deviation of samples from the average was determined. This would give an indication of the effect of diluent on calculation of geldanamycin signal as well as providing an understanding of the reproducibility returned from dilution in each diluent.

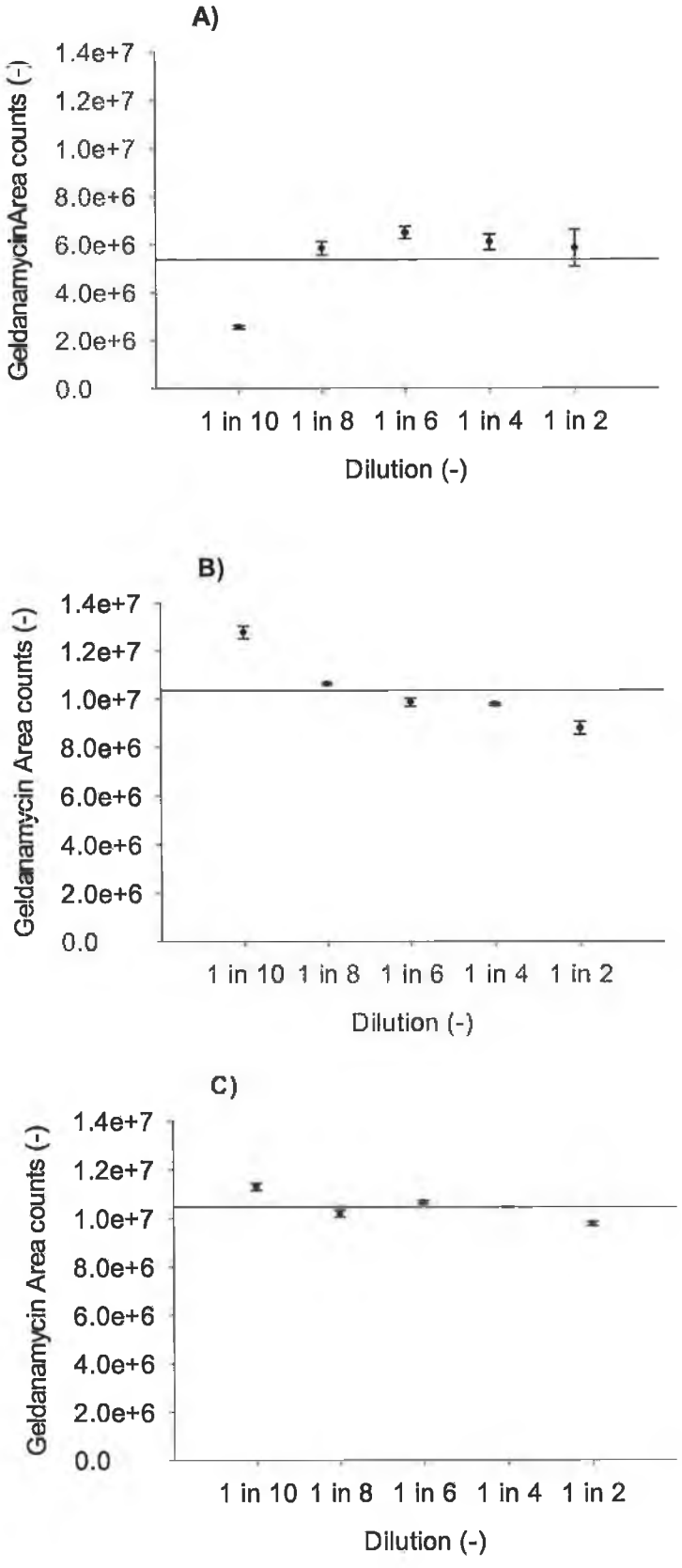


Figure 4.14: Dilution linearity check and the effect of diluent utilised. **A)** 100% H_2O **B)** 50% H_2O :50% Acetonitrile, **C)** 100% Acetonitrile, with error bars indicating the deviation from the average geldanamycin area count for each dilution

It can be seen from Figure 4.14 that 100% acetonitrile and 50% H_2O :50% acetonitrile return similar evaluations for geldanamycin in the fermentation broth. There is significant loss however in those samples diluted in 100% H_2O. When 100% acetonitrile and 50% H_2O : 50% acetonitrile are used as diluents the deviation form the average is minimised compared to that returned when 100% H_2O is used as a diluent

Combining the results of the assessment of the effect of diluent on analysis of geldanamycin in fermentation broth, it is clear to see that dilution in 100% acetonitrile is the best means of diluting fermentation broth samples prior to analysis using HPLC. This diluent continually returns the highest signals for geldanamycin, and also the best analytical reproducibility and consistency. It was therefore decided that subsequent analysis of fermentation broth samples, for the determination of geldanamycin, would all be conducted through dilution of the samples in 100% acetonitrile.

4.3.3 Stability of geldanamycin during analysis

As outlined a number of times in this Chapter, the primary concerns, relating to development of a successful analytical process, were efficient and expedient analysis of fermentation broth samples and minimisation of analytical column damage. This chapter has described the processes undertaken to achieve these goals, and a point has been reached, whereby a suitable column and operating conditions, in conjunction with a suitable sample preparation method, have been identified, and can be successfully employed for the analysis of geldanamycin in fermentation broth.

There is however, another consideration remaining. The analysis of large sample numbers was now possible, however, it was important to establish if the time between sample preparation and sample analysis could impact on the results returned. It was frequently the case that analysis of a series

of samples would require preparation of all samples and their analysis, via HPLC, in a single batch. This could involve an analysis period of 20 hours, during which time, the samples would be maintained at room temperature. It was therefore decided to examine the stability of geldanamycin samples over time, and determine if the idle time between when a sample is prepared for analysis, and when it is actually analysed, could impact on the results returned.

The assessment involved periodic injection of a geldanamycin standard over a 20 hour period and again after 72 hours. The sample was maintained in the HPLC autosampling unit at a temperature of approximately 22°C (room temperature). The effect of incubation time on signal was determined and is displayed in Figure 4.15.

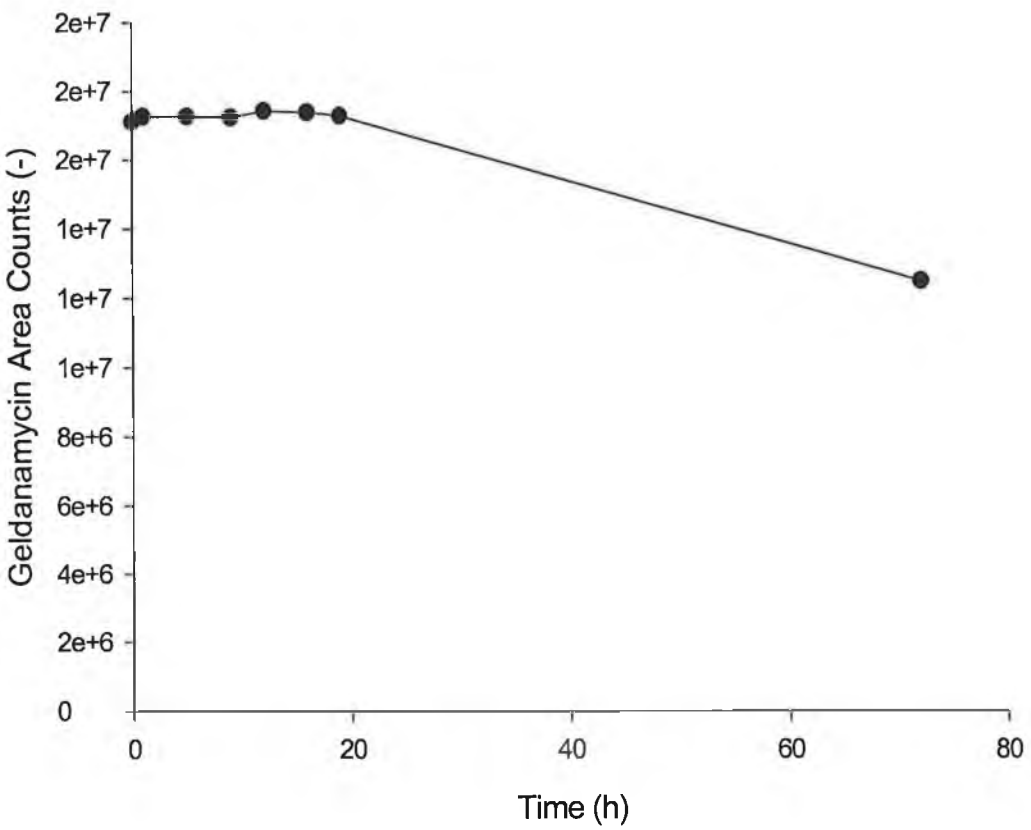


Figure 4.15: Effect of 22°C incubation on geldanamycin signal

From Figure 4.15 it is clear to see that there should be no detrimental effect to signal and sample analysis up to 20 hours after preparation for analysis. This is an important result since it indicates that analysis of samples, as soon as they are prepared, is not required. Samples injected after a 20 hour period do however, risk loss of geldanamycin signal. This was evident when a sample, injected 72 hours after preparation, returned a 30% loss in signal.

4.4. Conclusion

The main aim of the work carried out in this chapter was the development of a suitable HPLC method to accurately assess geldanamycin in *Streptomyces hygroscopicus* fermentation broth samples. It was desirable to have a method which increased upon the qualitative, and broadly quantitative, attributes of the bioassay detailed in Chapter 3, and which could deal with an increased analysis sample load.

The basis for the HPLC method was derived from Agnew *et al.*, (2001). Initially, filtered fermentation broth samples were injected without further pre-treatment, onto the HPLC column. This approach to geldanamycin analysis was found to be problematic. The two main problems encountered were that the presence of a variety of fermentation compounds in the broth samples resulted in fouling of the column. This occurred due to strong association of compounds with the stationary phase, and resulted in an increased need for column cleaning and ultimately, caused irreparable damage to the column. It was also noted that samples did not always return consistent results.

It was decided to address these issues by employing solvent extraction. Solvent extraction could be used to recover the antibiotic into the solvent phase and therefore reduce the contaminant loading of the samples and by extension, the potential for column fouling. The solvent employed was butanol. Although it was found that butanol could be used to efficiently extract geldanamycin, the analysis of butanol extracted sample was inaccurate, and poor sample resolution was frequently encountered. As a result, efforts had to be made to develop a method for its removal, and subsequent resuspension of the extract, in a more suitable solvent.

A number of methods were employed to remove the butanol. These included rotary vacuum evaporation, flash evaporation and low pressure evaporation. Of the methods examined, low pressure evaporation was found to be favourable, since it could not only be used to remove the butanol with minimal loss of product, but could also be used to treat a

large number of samples in a single run. The samples would then be resuspended, prior to HPLC analysis, in 50% H_2O : 50% acetonitrile, which was a suitable resuspension liquid since this was also the mobile phase applied in the HPLC process.

Although the above method could be successfully employed to reduce the potential for column fouling, the time taken to prepare samples was still a limitation with regard to high throughput of samples. In HPLC processes, analysis can be influenced by a number of factors. For example, sample throughput is influenced by sample runtime, which is dependent on the mobile phase flowrate, which is a function of the pressure in the system, which is dictated by the pore size of the HPLC stationary phase. As a result of these somewhat complex interactions, it was believed that increasing sample throughput may be achieved through increasing the pore size of the stationary phase of the HPLC column.

Fortunately, the column employed could also be supplied with a stationary phase pore size of 5 µm. The column was commissioned and it was found that there were no negative effects to sample analysis as a result of its application. Application of this column reduced the pressure in the system, and allowed a flowrate of 1 ml/min to be achieved. This reduced the runtime by more than half, and thus increased the sample throughput. An added advantage of using the new column was that, having a stationary phase with larger pore size, meant that material was less likely to clog the pores and therefore foul the column.

Since the new column would be more robust to column fouling, it was decided to re-examine, the analysis of broth samples without solvent extraction. As highlighted previously, the solvent extraction process was successful, but it still leads to sample preparation times in excess of 40 minutes. Reverting back to analysing broth samples, with minimal pre-treatment, would significantly reduce this time expenditure. Nonetheless, some pre-treatment was required. It was decided to dilute the samples prior to analysis, this step requires minimum of time input, but serves to reduce the risks of column fouling. The dilution of the samples was

examined and it was found that dilution in water was not a successful means of attaining reproducible results.

Samples diluted in water did not return a linear dilution relationship, and further investigation indicated that geldanamycin had a limited solubility in aqueous systems. In order to accurately examine geldanamycin in fermentation broth samples, it was necessary to add solvent to the sample. An investigation of possible diluents took place, and it was found that diluting in acetonitrile helped to increase the detectable geldanamycin in fermentation broth samples and facilitated a more robust analysis. It was decided, from the results of these assessments, that all subsequent analysis of geldanamycin broth samples should be diluted prior to analysis in acetonitrile, to prevent poorly soluble geldanamycin from being omitted for the analysis and to facilitate a more accurate assessment.

The final examination of the HPLC analysis of geldanamycin took place with respect to the stability of the compound over an analytical period. It was commonplace, due to the generation of large numbers of samples, that a batch of samples for HPLC analysis would be required to run overnight, thus the samples would experience an 'idle time' between preparation and analysis of in excess of 12 hours. Examining the effect of room temperature storage (22^{o}C) of geldanamycin, it was found that there was no significant loss in geldanamycin signal over an analysis period of 20 hours. It was also found, that after this time, loss of signal may begin.

Section B:

The Application Of Adsorbent Resins For The Recovery Of Geldanamycin From Fermentation Broths

Chapter 5. Applicability and characterisation of adsorbent resins for geldanamycin recovery from fermentation broth

5.1 Introduction

The antibiotic geldanamycin is produced by *Streptomyces hygroscopicus var. geldanus* in submerged culture, and is excreted into the culture fluid. The recovery of geldanamycin therefore involves its isolation from a number of other compounds, including proteins and sugars, which are also present in the fermentation broth. In the previous Chapters, methods were developed to allow the determination of the presence of geldanamycin in fermentation broth and its quantification. As a result of developing these methods, it was possible to pursue the primary goals of the project. In this Chapter, the applicability of adsorbent resins, for use in a Solid Phase Extraction (SPE) method for removal of geldanamycin from fermentation broth, is addressed.

Solvents have traditionally been used to obtain primary separations in processes aimed at the recovery of antibiotics. Previously, butanol and chloroform have been shown to be suitable solvents for the recovery of geldanamycin from fermentation broths (DeBoer *et al.*, 1970). There are however, a number of concerns when using solvents for antibiotic recoveries. Solvent-based liquid-liquid extractions can result in the generation of solvent waste and increase the expense of sample recovery and analysis. In solvent extractions, product-containing solvent may be partially miscible with the aqueous phase (Ghosh *et al.*, 1997) forming an emulsion, which may result in the loss of product and difficulty in accurately determining product concentrations in samples.

Arias *et al.*, (1979) found solvent and resin-based extractions were both applicable when examining the extraction of antibiotics produced by

Myxcoccus coralloides. As a result, it was decided to examine the application of adsorbent resins for the recovery of geldanamycin from fermentation broths. Pyrzynska and Wierzbicki, (2005) believed that use of sorption procedures for pre-concentration or separation could be considered superior to liquid-liquid extraction due to their simplicity and ability to obtain high enrichment factors.

Adsorption chromatography is often used for the isolation and purification of fermentation products, and the correctly selected resin and optimal working conditions can lead to such excellent results that resin-based adsorption chromatography can be superior to any of the alternative methods presently available (Voser, 1982). Güzeltunç and Ülgen, (2001) found incorporating a chromatographic technique, in the form of adsorbent resins, early in the sequence of purification steps lead to higher product recovery yields. Adsorbent resin use in bioprocessing is widespread and not confined to product recovery applications. They are also used to eliminate toxic effects on growth, reduce feedback repression of production (Lee *et al.*, 2003) and ultimately extend fermentation time and increase product yield (Tolonen *et al.*, 2004).

As a result of these factors, it was deemed that an examination and characterisation of the applicability of adsorbent resins for the removal of geldanamycin from fermentation broth could provide a novel means for the antibiotics' recovery. It would also be beneficial to determine which resin, if any, warrant further examination and application in a SPE method for geldanamycin recovery.

5.2 Materials and Methods

5.2.1 *Streptomyces hygroscopicus* var. *geldanus* antibiotic fermentations

Chapter 3, section 3.2.2

5.2.2 Adsorbent Resins

Seven resins were utilised in this assessment, all chosen for their application in adsorption studies of antibiotics. Five Amberlite non-ionic polymeric adsorbent resins; XAD-4 and XAD-7 (examined by Cen and Tsao, (1993), for cyclohexamide recovery) XAD-16 (examined by Lee *et al.*, (2003), to improve teicoplanin fermentations), XAD-1600 (examined by Xie *et al.*, (2001) for the purification of cephalosporin precursors from fermentations) and XAD-1180 (examined by Ghosh *et al.*, (1997) for the extraction and purification of cephalosporin antibiotics) supplied by Rohm and Haas Company (Philadelphia, PA, USA). There were also two ion exchange resins; Sepabeads SP-850 (examined by Adachi and Isobe, (2004) for application in industrial separations) and Diaion HP-20 (examined by Okada *et al.*, (1998) for the isolation of antifungal antibiotics from culture broth) supplied by Mitsubishi (Mitsubishi Chemical Industries Ltd., Tokyo, Japan).

5.2.3 Geldanamycin analytical methods

In geldanamycin fermentations, the product may be excreted by the *Streptomyces*, into the fermentation broth, from which it must be recovered. To assess the applicability of adsorbents to recover geldanamycin solid-liquid extractions of the fermentation broth must be carried out. In order to assess the performance of the resin the geldanamycin present in two streams were routinely examined. These streams were a liquid stream, the fermentation broth, and a solid stream,

the post-adsorption resin beads. The broth levels of geldanamycin were determined via the broth dilution method outlined in Chapter 3, using acetonitrile as the diluent and analysing samples via HPLC.

The geldanamycin adsorbed by the resins was determined by washing them once with one broth volume (the volume of broth which was contacted with the resins) of distilled water to remove loosely adsorbed compounds, but not to desorb geldanamycin. This was followed by one broth volume wash with acetonitrile, which was recycled twice more to facilitate desorption of geldanamycin. This was then analysed for geldanamycin concentration via HPLC.

5.2.4 Adsorbent Resin Preparation

Prior to their application for the recovery of geldanamycin from fermentation broths, each resin underwent a pre-treatment step. The resins were washed with water to remove compounds such as sodium chloride and sodium carbonate salts, which were used to retard bacterial growth during shipping. They were then rinsed and soaked in acetonitrile (a suitable resin regenerant) until required. Prior to application with the fermentation broth, the resins where rinsed, approximately five times, in ultra pure water.

Table 5.1: Adsorbent resin properties

[1] Porosity (ml/ml) data not available, instead the data provided is for the pore volume (ml/g). [H] Predominant mode of action of resins is hydrophobic interaction, [I] Predominant mode of action of resins is ion-exchange.

Resin	Matrix	Surface Area (m^2/g)	Particle size (mm)	Porosity (ml/ml)
XAD 4[H]	Polystyrene DVB	≥725	0.49 - 0.69	≥0.5
XAD 7[H]	Acrylic Ester	≈450	0.3 - 0.85	[1]1.4
XAD 16[H]	Polystyrene DVB	≥800	0.56 - 0.71	≥0.55
XAD 1600[H]	Polystyrene DVB	≥800	0.4 ± 0.05	≥0.55
XAD 1180[H]	Polystyrene DVB	≥500	0.35 - 0.6	≥0.6
Sepabeads SP-850[I]	Polystyrene DVB	1000	> 0.25	[1]1.2
Diaion HP-20[I]	Polystyrene DVB	600	> 0.25	[1]1.3

5.3 Experimental

5.3.1 Examination of the geidanamycin adsorption capabilities of adsorbent resins

5.3.1.1 Introduction

Frequently, the first step in developing an adsorption-based purification strategy is screening of adsorbents. Grezegorczyk and Carta, (1996) examined a range of adsorbents for application in the recovery of amino acids, and suggested that unlike other adsorbents such as activated carbon, the use of polymeric adsorbents allows a greater degree of control over the adsorption process, especially with reference to control of their chemistries and pore structures. The aim of this assessment was to determine the effect of variation of resin concentration on recovery of geldanamycin, and to gain an understanding of the adsorption efflciency, based on the decrease in geidanamycin remaining in the broth. This assessment was comparable to that performed by Gökmen and Serpen (2002), who examined the adsorption of dark coloured compounds from apple juice.

5.3.1.2 Materials and Methods

A batch contacting assessment was set up for 24 hours at 4°C in 25 ml plastic universals, agitated at 150 rpm. Various concentrations of resins [0 (control), 2, 5, 10, 12.5 and 15 g/l] were added to filtered fermentation broth and the concentration of geldanamycin remaining in the broth was monitored via HPLC analysis. Figure 5.1. outlines the effect of resin concentration on recovery of geidanamycin from fermentation broth.

5.3.1.3 Results

From Figure 5.1 it can be seen that Amberlite XAD-1600, XAD-16, XAD-1180 and Diaion HP-20 all showed good absorptive capabilities for geldanamycin from fermentation broth. When present at a concentration of 10 g/l, the resins could adsorb in excess of 90% of geldanamycin present in the fermentation broth within 24 hours. Gökmen and Serpen (2002), noted that adsorption increased with increasing resin concentration and attributed this to an increase in the available adsorption sites. This is also true for the resins under examination, and indicates an equilibrium favouring adsorption onto the resins.

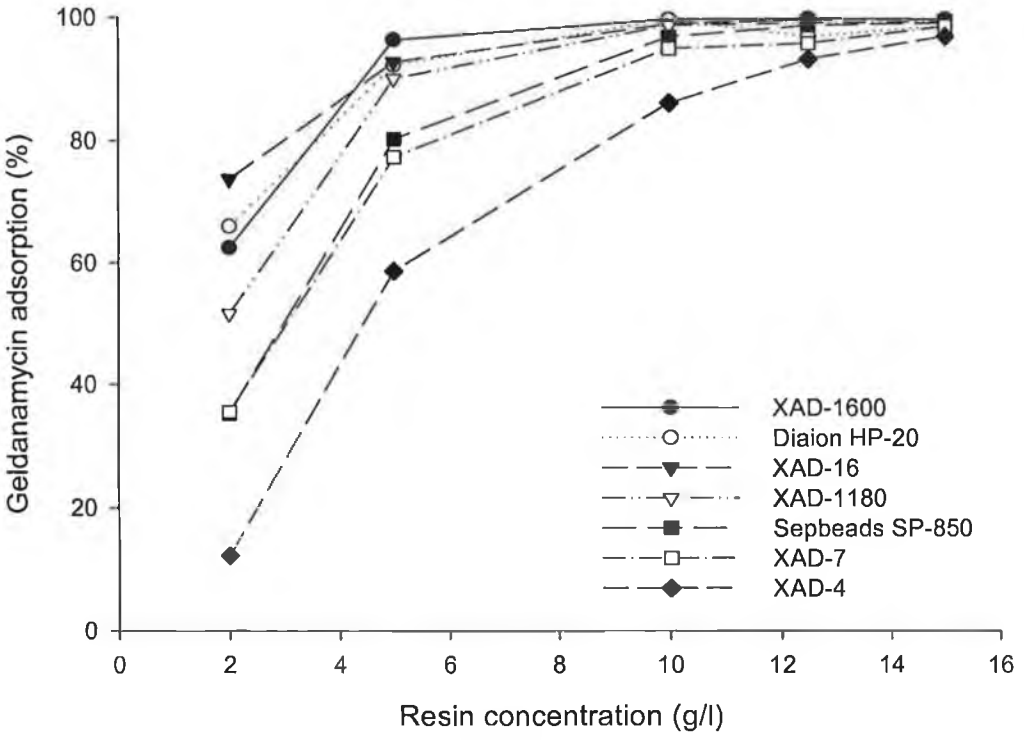


Figure 5.1: Absorbent resin performance in fermentation broth containing 47mg/l geldanamycin

Although Sepabeads SP-850, XAD-4 and XAD-7 showed an ability to adsorb geldanamycin, in this assessment, they did not display the same adsorptive performance or capabilities of the other resins under investigation. Examining seven different resins throughout the entire

assessment was deemed to be excessive. Therefore, in order to reduce the examination overheads, it was decided to remove the resins XAD-4 and XAD-7 from further examination at this point, and focus on the five remaining resins for the rest of the study. Although the performance of Sepabeads SP-850 was similar to that of XAD-7, Sepabeads SP-850 was not omitted from the study at this point because it was desirable to maintain more than one ion-exchange resin in the study.

5.3.2 Adsorption model fitting

5.3.2.1 Introduction

The use of adsorption isotherms is a favoured approach to investigate adsorption mechanisms (Ribeiro and Ribeiro, 2003). Isotherms can be used to describe how solutes interact with adsorbents and are important when examining the application of resins (Juang and Shiau, 1999). Muhammad *et al.*, (1998) assessed adsorption of heavy metals and found that adsorption isotherms could be used to describe the equilibrium relationships between adsorbent and adsorbate, and that the two main types of adsorption isotherms are the Langmuir and Freundlich isotherms. Adsorption isotherms can provide information on the adsorption capabilities, capacities and affinities of adsorbents for the compound of interest, and may be used to determine the impact of chemical, physical or environmental conditions on adsorption (Dutta *et al.*, 1999, Kyriakopoulos *et al.*, 2005, Adachi and Isobe, 2004, Jung *et al.*, 2001).

5.3.2.2 Materials and Methods

The geldanamycin adsorption isotherms for each resin were determined using similar methods as those described numerous times in literature (Veit *et al.*, 2002, Adachi and Isobe, 2004, Güzeltunç and Ülgen, 2001). Fermentation broth was serially diluted in Bennett's medium, to produce a range of samples of varying geldanamycin concentration. 5 g/l of each

resin was added to 10 mis of each dilution, in 25 ml plastic universals, and extracted. The extractions took place for 24 hours to allow equilibrium to be achieved. After contacting, the aqueous and solid phases were separated, via filtration, and recovered for geldanamycin analysis. The equilibrium concentration in the fluid (C^*_A) was determined via HPLC. The equilibrium loading concentration (C^*_{AS}) was determined by washing the resins with 10mls of acetonitrile and recycling it twice to maximise desorption. This was then analysed for geldanamycin concentration via HPLC.

The constants for each model were calculated using the experimental data. Using these constants it was then possible to generate a data set of the theoretical equilibrium concentration in the fluid, and apply this to each model. This allowed the generation of Langmuir and Freundlich isotherms which were compared with the raw data isotherm to determine which model best fit the experimental data. The models applied in this examination are based on those described in Doran, (1995).

The Langmuir isotherm model was expressed as:

$$C^*_{AS} = \frac{C_{ASm} K_A C^*_A}{1 + K_A C^*_A} \qquad \text{(Eq. 5.1)}$$

where: C^*_{AS} is the equilibrium loading concentration (g/g), C^*_A is the equilibrium concentration in the fluid (g/l), C_{ASm} is the maximum loading concentration (g/g) and K_A is an adsorption or equilibrium constant (l/g), experimentally determined and indicates the affinity of the binding sites for the compound of interest.

A plot of C^*_A versus $\frac{C^*_A}{C^*_{AS}}$, will yield a straight line with slope = $\frac{1}{C_{ASm}}$ and intercept = $\frac{1}{C_{ASm} \cdot K_A}$

The theories of the model are based on three principles:

1) The adsorbed molecules form a monolayer on the adsorbent surface
2) Each site for adsorption is equivalent in terms of adsorption energy
3) There are no interactions between adjacent adsorbed molecules

The Freundlich isotherm model was expressed as:

$$C^*_{AS} = K_F C^*_A{}^{1/n} \qquad \text{(Eq. 5.2)}$$

where: K_F is an experimentally determined equilibrium constant which increases with total adsorption capacity of the adsorbent to adsorb the compound of interest, i.e. an indicator of adsorption capacity and n is an experimentally determined, dimensionless constant which is an indication of the efficiency and energy of adsorption and which may vary along the adsorption process, i.e. an empirical constant related to the adsorption driving the value of n. If adsorption is favourable n is > 1; if adsorption is not favourable, n < 1.

The equation is linearised to give a slope of $\frac{1}{n}$ and intercept of $Log(K_F)$.

5.3.2.2 Results

Having fitted both the Langmuir and Freundlich adsorption models to the experimental data and having examined the regression co-efficients, it was found that the Langmuir model proved to be the most accurate fit (Figure 5.2 gives an example of the results for one of the resins examined, XAD-1600). It was found that all resins had a good affinity for geldanamycin with XAD-1600 displaying the largest maximum loading capacity according to the Langmuir model (Table 5.2). Ribeiro and Ribeiro, (2003), also examined the fit of the Freundlich and Langmuir models to experimental data and found that both adsorption models returned a good fit to the experimental data in the examination of XAD-7 and IRA-410 resin-mediated adsorption of erythromycin. Ramos *et al.*, (2004), detailed

phenomena in their examination of cephalosporin-C (CPC) adsorption onto Amberlite XAD-16 whereby, up to a concentration of 1 g/l CPC, the Langmuir model best fit the data, however above this concentration the Freundlich isotherm was the better fit. This indicates that the adsorption mechanisms at work are often complex and experimental examination is important when assessing the performance of a particular resin.

Table 5.2: Summary of the maximum loading concentration, C_{ASm}, using the Langmuir adsorption model for adsorption of geldanamycin by adsorbent resins

Resin	C_{ASm} (g/g)
XAD-16	0.0049
XAD-1600	0.0058
XAD-1180	0.0049
Sepabeads SP-850	0.0046
Diaion HP-20	0.0050

The maximum loading concentrations derived from fitting the Langmuir model to the experimental data are comparable to the results of the assessment of the adsorption capabilities. In that assessment it was found that a favourable equilibrium existed for adsorption of geldanamycin onto adsorbent resins from fermentation broth which contained 47 mg/l of geldanamycin.

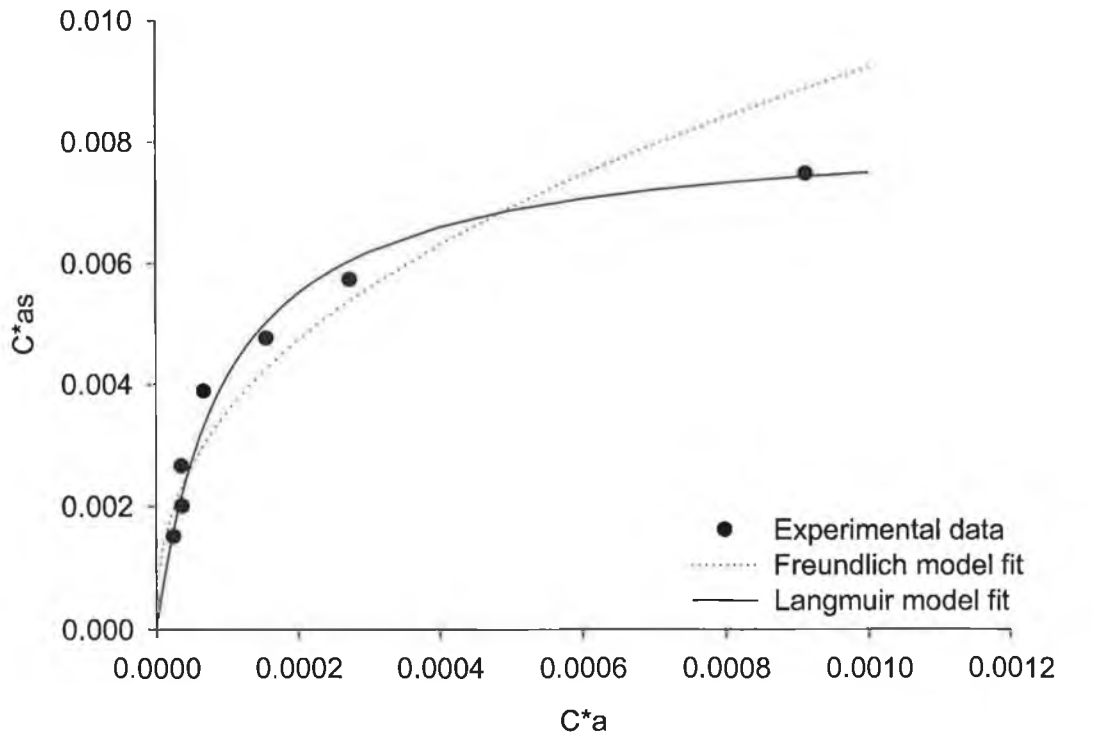


Figure 5.2: Adsorption model fitting to the experimental geldanamycin adsorption data for XAD-1600

It was also found during the assessment of the adsorption models, that dilution of the fermentation samples in Bennett's medium returned experimental results which possessed a higher regression co-efficient for the adsorption models, than if H_2O was used as the diluent. It was believed that this occurred because by diluting in Bennett's medium, a level of contaminants was maintained in the adsorption system, i.e., only the geldanamycin concentration was diluted. Dilution in H_2O however, serves to dilute all compounds present in the broth, contaminants and geldanamycin, and therefore does not maintain an environment of competition which would exist in normal fermentation broths.

5.3.3 Specificity assessment of geldanamycin adsorption by adsorbent resins

5.3.3.1 Introduction

The aim of this study was to assess the application of adsorbent resins for the recovery of geldanamycin from fermentation broth, however it was also important, to examine to what extent other compounds were adsorbed. Traditionally solvent extraction and adsorption have been of low selectivity (Payne *et al.*, 1989). As with Xie *et al.*, (2001), using crude fermentation broth posed a number of problems, primarily that there are several contaminant compounds present in the broth which can be adsorbed by the resins, as well as the compound of interest. The adsorption of contaminants may reduce the adsorptive abilities of a resin and thus limit performance in crude samples.

Also, as with most extraction systems, the ultimate goal is to increase the purity of the recovered compound of interest. The selectivity of adsorption may be limited by the non-specific nature of adsorptive interactions (Payne *et al.*, 1989), and as a result, any impurities adsorbed have the potential to be desorbed and can contaminate the product stream. Chaubal *et al.*, (1995) and Dutta and Dutta, (2006) both suggested that there is a limited knowledge of how solutes bind to sorbents, and that greater empirical knowledge is required inorder to reduce the non-specific adsorption (i.e. fouling) by components of the complex fermentation broth. It was therefore decided to assess the specificity of the resins for adsorption of geldanamycin, and determine if any resin out-performed the others.

5.3.3.2 Materials and Methods

The specificity of the resins for geldanamycin over contaminating compounds, was based on the determination of the ratio of geldanamycin adsorption to adsorption of contaminating material. The analysis utilised

HPLC to determine the ratio of compounds present in fermentation broths pre and post adsorption. Batch adsorptions were set up whereby 10 g/l of the resins were added to 100 ml of fermentation broth in 250 ml Erlenmeyer flasks and incubated at 150 rpm and 4°C. The experiment ran for 24 hours and samples were taken periodically.

The relative selectivity of the resins for geldanamycin was assessed by determining the ratio of adsorption of geldanamycin to contaminants. Contaminants were those compounds other than geldanamycin, and the adsorption was based the area counts retuned from the HPLC chromatograms. The assessment was based on the following equation:

$$R = \frac{\left(\frac{G_C - G_B}{G_C}\right)}{\left(\frac{C_C - C_B}{C_C}\right)} \qquad \text{(Eq. 5.3)}$$

Where: G_C is the amount of geldanamycin in the broth sample before adsorption, G_B the amount of geldanamycin in the broth sample after adsorption. C_C is the amount of contaminants in the broth sample before adsorption and C_B the amount of contaminants in the broth sample after adsorption. The results are displayed in Figure 5.3. When R > 1, geldanamycin is preferentially adsorbed over contaminants, when R < 1 contaminant adsorption is favoured.

5.3.3.3 Results

In the assessment of the adsorption specificity, it can be seen that selectivity increased until approximately five hours. After this time period, selectivity of adsorption was maintained at a relatively constant level.

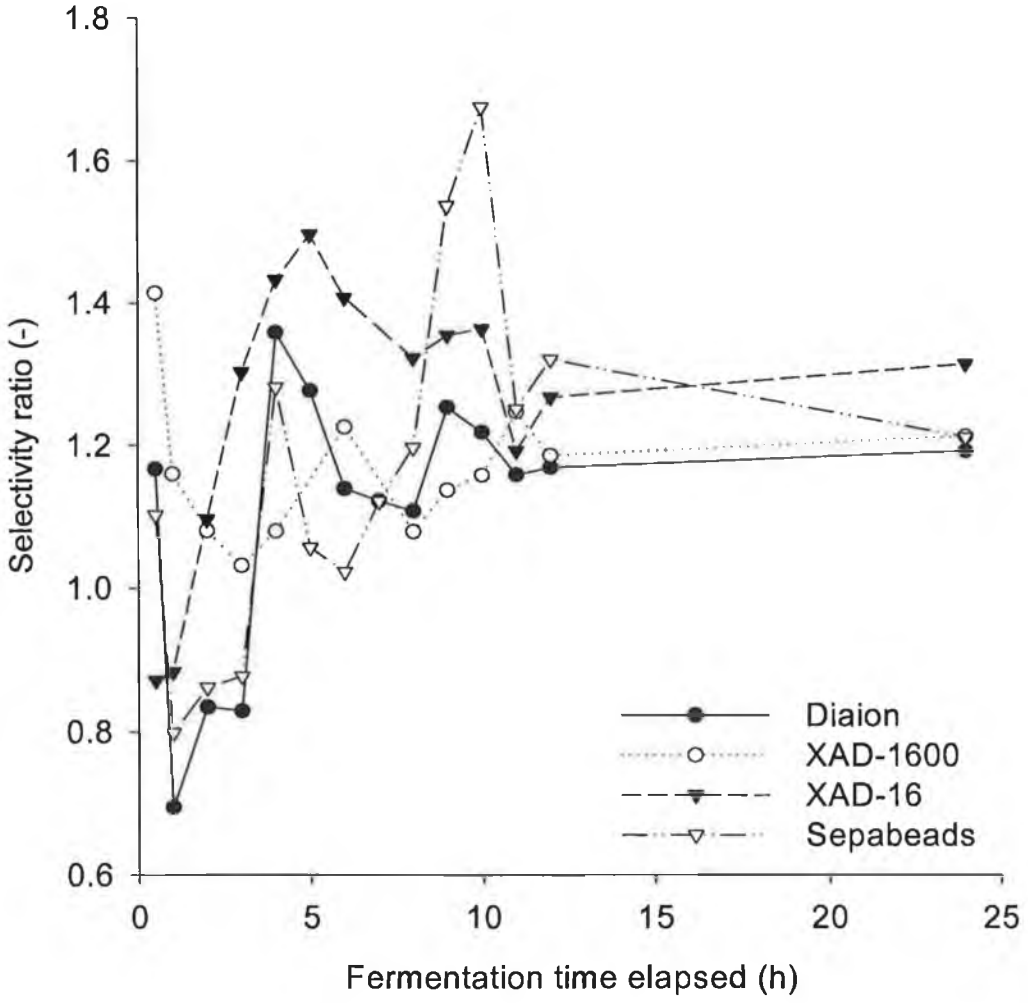


Figure 5.3: Adsorption selectivities - A ratio of adsorption of geldanamycin to contaminating compounds

All adsorbents showed preferential adsorption geldanamycin, in each case adsorbing a greater proportion of the geldanamycin present in the broth than contaminants. It is important to note that these resins had undergone no modification to increase their selectivity for geldanamycin, and such techniques may prove valuable in further recovery processes.

5.3.4 Impact of environmental conditions on adsorption

The adsorption of compounds from solutions is a complex and relatively poorly understood process. Robberson *et al.*, (2006) suggested that the adsorption of compounds could be achieved by a number of phenomena,

including electrostatic attraction between the solute and sorbent or hydrophobic repulsion of the solute from the aqueous carrier to the sorbent. These phenomena are most frequently effected by alteration in the environmental conditions of the adsorption system and among the most commonly examined environmental influencing factors are temperature and pH (Aksu, 2001, Otero *et al.*, 2004, Netpradit *et al.*, 2004, Rodda *et al.*, 1993).

Temperature, for example, is an important parameter which can influence the equilbria and kinetic rates of sorption process (ten Hulscher and Cornelissen, 1996). Srivastava *et al.*, (2006) suggested that pH can effect the adsorptive process through disassociation of functional groups on the adsorbents surface active sites, which can impact on kinetic and equilibrium characteristics. As a result, it was decided to examine how temperature and pH affect the adsorption process. It was also decided to examine the impact of modifying the pre-adsorption broth polarity, since incorporation of a polar solvent into adsorption systems had been shown to impact on such processes (Hodgkinson and Lowry, 1981, Eltekova *et al.*, 2000).

5.3.4.1 Effect of temperature on adsorption

5.3.4.1.1 Introduction

Barboza *et al.*, (2003) described, in their assessment of the recovery of clavulanic acid, that temperature sensitivities were exhibited in their processes and thus decided to assess the impact of temperature on the kinetics of adsorption. It has been reported that adsorption efficiencies and rates can be affected by altering the adsorption temperature. Gökmen and Serpen, (2002) showed that increasing the adsorption temperature resulted in increased maximum adsorption capacity of the resins. In fixed bed studies of the adsorption of phenol and salicylic acid by XAD-16 and Duolite S-861, Otero *et al.*, (2005) found that an increase in adsorption

resulted from and decrease in temperature, and Kyriakopoulos *et al.*, (2005) showed that increasing the temperature resulted in a decrease in adsorption of trifluralin and protmetryn by XAD-4 and XAD-7.

This indicates that the impact of temperature on adsorptive performance may be complex and warrant empirical assessment. As result, it was decided to examine the effect temperature had on adsorption of geldanamycin from fermentation broth, and determine if there was any benefit in performing adsorptive processes at higher temperatures.

5.3.4.1.2 Materials and Methods

The assessment included an examination of how different temperatures affected adsorption and stability of geldanamycin. Four different temperatures were assessed; 4°C, 22°C, 28°C and 37°C. A 10 ml working volume of fermentation broth in 25 ml plastic universals was used, with a resin concentration of 10 g/l and agitated at 150 rpm.

5.3.4.1.3 Results

Figure 5.4 shows the effect of temperature on adsorption by two of the resins examined over 12 hours of adsorption.

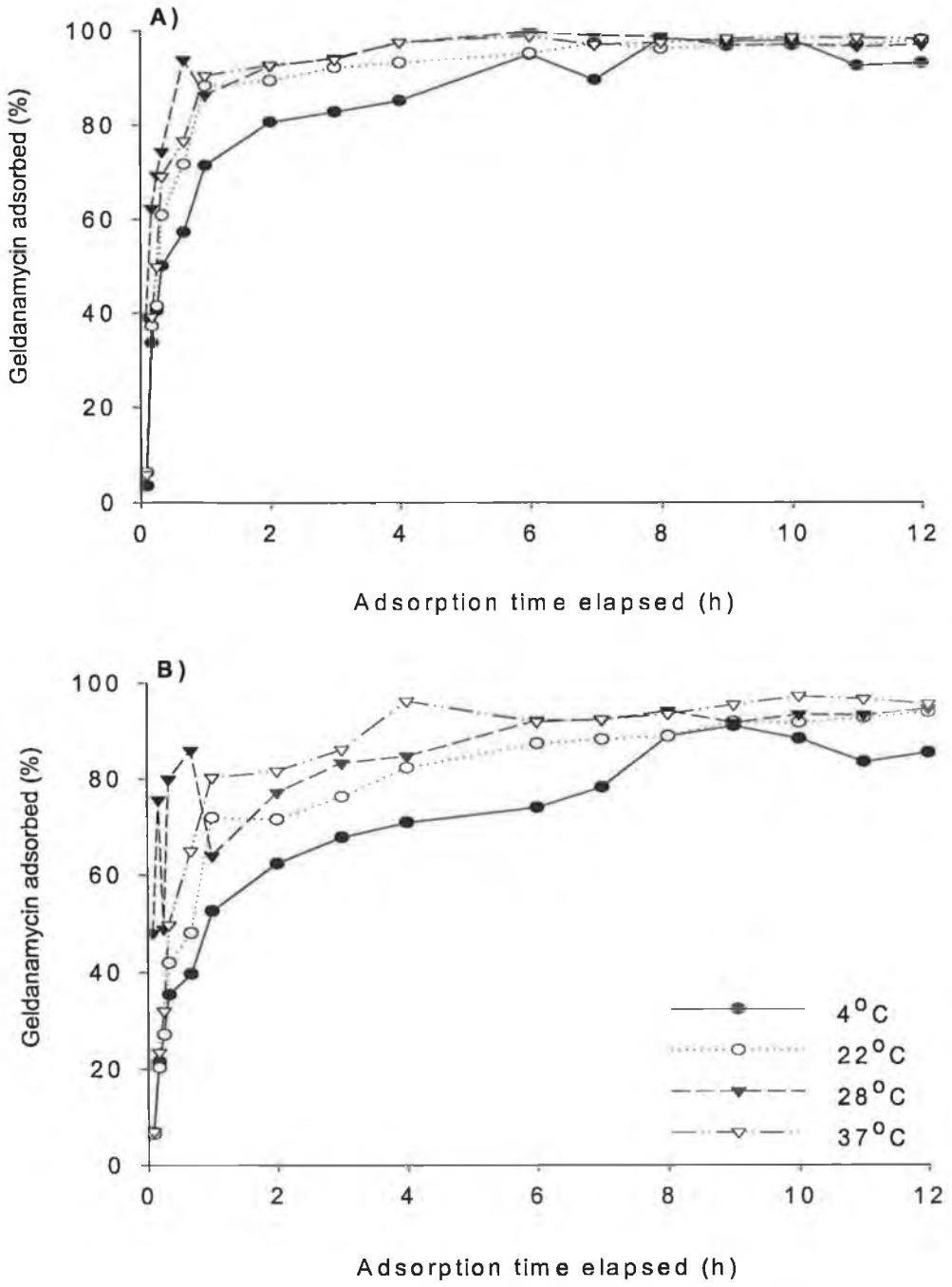


Figure 5.4: Effect of temperature on the adsorption of geldanamycin. **A)** XAD-1600 **B)** Sepabeads SP-850

Examining Figure 5.4 it was found that the rate of adsorption is most rapid over an initial one hour period, at which point there is a gradual decrease. Dutta *et al.*, (1999), observed similar phenomena in their examination of beta-lactam adsorption and suggested that the fast initial adsorption may

be correlated with adsorption in the easily accessible mesopore of the particle and the slow step to adsorption in the micropore of the microsphere typical of the resin used.

The fast initial binding and capacity displayed in Figure 5.4 concurs with the findings of the assessment of the adsorption model, whereby these resins showed a high affinity for geldanamycin adsorption according to Langmuir kinetics. In general, it was found that adsorptive performance varied over the 12 hour assessment period, however, by the completion of the assessment, adsorptive performance was found to be relatively independent of temperature. XAD-1600 and Diaion HP-20 were found to be the optimally performing resins, routinely adsorbing 95% of product, at all temperatures, and adsorbing comparable amounts of contaminants

5.3.4.2 Effect of pH alteration on adsorption

5.3.4.2.1 Introduction

In adsorption studies, the pH of the adsorption environment has been frequently found to impact on the success of the adsorption. The pH can influence the adsorption of compounds by altering charge which may impact on adsorption or even result in altering solubility (Grezegorczyk and Carta, 1996, Chaubal *et al.*, 1995). Young and Kuen-Chyr, (2000) found that removal of phenols by XAD-4 varied significantly with variation in the target solution pH. Goyne *et al.*, (2005) showed that adsorption of the antibiotic ofloxacin onto different forms of the mineral SiO_2 varied with pH, and did so most dramatically around the pK_{a2} of the antibiotic. It was therefore decided to investigate if alteration of the fermentation broth pH prior to adsorption affected the recovery of geldanamycin.

5.3.4.2.2 Materials and Methods

Assessment of the effect of pH on adsorption was carried out at 4°C and took place in 25 ml universals with a 10 ml working volume and a resin concentration of 10 g/l, agitated at 150 rpm for a period of 24 hours. The pH of the unaltered fermentation broth was 7.7 and was altered to 6.7 and 5.7 using 0.5 Molar HCL and 8.7 and 9.7 using 0.5 Molar NaOH.

5.3.4.2.3 Results

The pH of geldanamycin fermentation broth normally ranges between pH 6.5 to pH 8.0. It was noted that altering the pH to 9.7 resulted in a very significant colour change, the broth assuming a purple colouration and resulted in a degradation of approximately 40% of the geldanamycin present in control broth (pH 7.7).

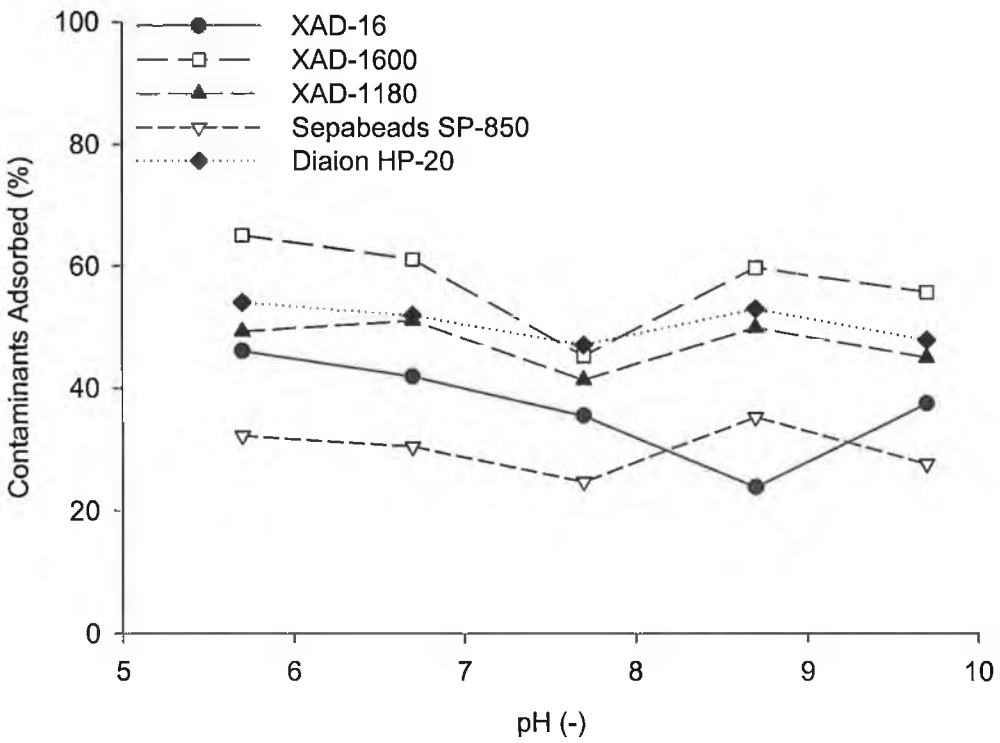


Figure 5.5: Effect of pre-adsorption pH adjustment of fermentation broth on the adsorption of contaminants over 24 hours

The assessment of resin performance was based on the percentage of geldanamycin present in pH-adjusted broth which was adsorbed. On this basis it was found that there was no significant increase in performance as a result of adjusting the pH of the broth prior to adsorption. There was some slight variation in the adsorptive performances of the resins, but in general the affect of pH alteration was minimal. Similarly, Silva *et al.*, (2004) found that monilate adsorption onto XAD-4 was independent of the solution pH over the range examined, in their remediation studies.

Examining the effect of pH alteration on the adsorption of contaminants however (Figure 5.5), showed that pH adjustment can affect the amount of contaminants adsorbed by each resin. Perhaps the most significant finding is that, except for XAD-16, at pH 7.7 (the control broth pH) adsorption of contaminants was at its minimum.

5.3.4.3 Effect of solvent addition on adsorption

5.3.4.3.1 Introduction

In Chapter 4 the importance of acetonitrile as an agent for the accurate analysis and quantification of geldanamycin in solution was highlighted. Hodgkinson and Lowry, (1981) found that incorporation of acetonitrile, which acted as a depolarising agent, aided the purification of human prolactin in their adsorption chromatography studies. Eltekova *et al.*, (2000) believed that acetonitrile molecules could effectively screen the active adsorption sites on the surface of carbon sorbents and cause a decrease in the adsorption of the solutes in their studies of adsorption of organic compounds on porous carbon sorbents.

As a result it was decided to examine the impact of modifying the adsorption environment using acetonitrile. Acetonitrile is miscible with

fermentation broth, is the preferred desorption solvent and is a constituent of the mobile phase used in the HPLC analysis of samples.

5.3.4.3.2 Materials and Methods

A working volume of 5 ml in 25 ml plastic universals and a resin concentration of 10 g/l was used. A range of samples were generated by increasing the percentage of acetonitrile added to fermentation broth from 0% to 40%. The overall adsorptive performance of the resins was assessed based on the percentage of geldanamycin adsorbed from comparable controls. The samples were incubated at 4°C for 24 hours and agitated at 150 rpm.

5.3.4.3.3 Results

From Figure 5.6 it can be seen that addition of acetonitrile at higher concentrations had a detrimental impact on the adsorption of geldanamycin. It was found that Sepabeads SP-850 showed the least loss of adsorptive performance in the presence of acetonitrile. At higher concentrations addition of acetonitrile essentially limited the uptake of geldanamycin from the liquid phase by the resins.

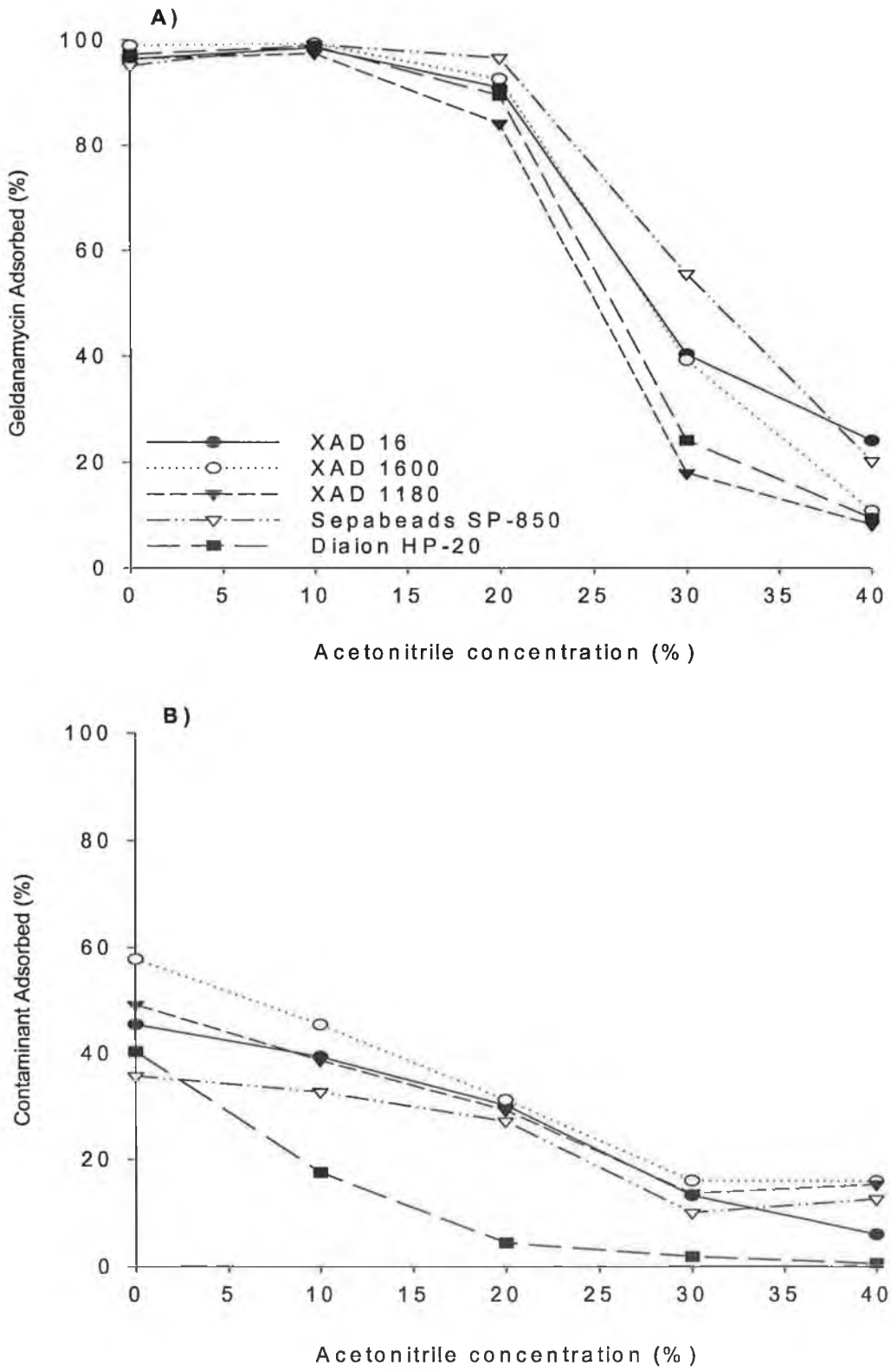


Figure 5.6: Effect of acetonitrile addition on **A)**: geldanamycin adsorption and **B):** contaminating material adsorption

Assessing the effect of acetonitrile on the adsorption of contaminants showed that, similarly to geldanamycin adsorption, increasing the concentration of acetonitrile reduced the adsorption of contaminants. The effect however was markedly more pronounced, resulting in a large drop in contaminant adsorption even at low concentrations of acetonitrile. Kandori *et al.,* (2002) suggested a reason for this. They found that incorporation of acetonitrile into an adsorption system could affect the structure of certain molecules therefore impacting on adsorption, in their studies of the adsorption of bovine serum albumin. It can thus be understood why a reduction in the adsorption of, for example, protein molecules, could be brought about via conformational alterations, as a result of acetonitrile addition.

From a combination of the results, it is apparent that by using low concentrations of acetonitrile it may be possible to optimise the adsorption process to reduce the adsorption of contaminants while maintaining a high level of geldanamycin adsorption. If one considers Diaion HP-20. It can be seen that with the addition of 20% (v/v) of acetonitrile, approximately 90% of the geldanamycin could be adsorbed. At the same time, less than 10% of contaminating material is adsorbed. This indicates great potential to preferentially adsorb the compound of interest, over contaminants, and thus facilitate the production of a purer product stream.

5.3.5 Product recovery assessment

5.3.5.1 Introduction

Up until this point, the examinations performed were aimed at determining if adsorbent resins could be used for the recovery of geldanamycin from fermentation broth. In such assessments it is important to consider not only the adsorption of the product of interest from the broth, but also its recovery from the resins into a suitable liquid phase ready for analysis and or further treatment. Therefore, in order to complete the assessment of the

applicability of adsorbent resins for the recovery of geldanamycin it was decided to examine the desorption of compounds from the resins, and determine if there was any benefit of using resins to generate a product stream of greater purity than the original fermentation broth. In this way it would be possible to determine, not only if the compound of interest could be removed from the broth by resins, but also to what extent geldanamycin and contaminant compounds were desorbed.

5.3.5.2 Materials and methods

Five resins were examined; 10 ml of fermentation broth was contacted with 10 g/l of resin for 20 hours at 4°C and agitated at 150 rpm in 25 ml plastic universals. The post-adsorption broth was separated from the resins via filtration and the two streams examined for geldanamycin concentration. The recovered resins were desorbed using three 10 ml volumes of acetonitrile, and the purity determined via HPLC analysis.

5.3.5.3 Results

It was found that 10 g/l of resin adsorbed in excess of 97% of all geldanamycin present in the broth. Desorption from the resins showed some interesting results. It was found that all resins allowed desorption of considerable quantities of the adsorbed geldanamycin. Of particular note however, was the desorption of contaminating compounds. Table 5.3 details the desorption of contaminants which were present in the control.

Table 5.3: Contaminant desorption summary. **GM**: Geldanamycin

Resin	GM Adsorbed (%)	GM Desorbed (%)	Contaminant Adsorbed (%)	Contaminant Desorbed (%)
XAD-16	98.47	72.40	97.39	27.49
XAD-1600	99.10	96.67	98.32	22.31
XAD-1180	98.69	75.97	96.36	18.24
Sepabeads SP-850	97.69	89.53	96.35	25.58
Diaion HP-20	98.03	95.61	96.83	24.63

From Table 5.3 it can be seen that less than 30% of the adsorbed contaminants were returned in the washes. Thus the recovered stream, while containing in excess of 70% of adsorbed geldanamycin, is devoid of in excess of 70% of the adsorbed contaminants. This has obvious benefits for the pursuit of a purer product stream. Again it was found that XAD-1600 and Diaion HP-20 were the best performing resins. These were among the resins which adsorbed most geldanamycin, but also allowed the most desorption of geldanamycin, whilst retaining among the most contaminating compounds.

5.4 Discussion and Conclusion

Adsorbent resins are chromatographic solids which are finding increased application for the recovery and purification of a range of bioproducts. As a result of this, it was decided to examine their application for the recovery of geldanamycin from fermentation broth. Establishing the applicability of these resins would highlight if a solid phase extraction process would be viable for the recovery of geldanamycin from fermentation broth. A series of examinations were performed, the culmination of which, indicated that adsorbent resins were capable of geldanamycin recovery and purification.

In such solid-liquid adsorption process, the separation of compounds from one phase onto another, depends somewhat on the equilibrium relationships between the two phases. Adsorption continues to take place until the distribution of the compounds remains constant. This 'transport' of compounds occurs as a result of the adsorbent having a higher affinity for the adsorbate than that of the fermentation fluid. It is therefore important to note that when assessing the performance of the resins, the results returned are indicative of how favourable is the adsorption of geldanamycin, from the broth, onto the resins. Establishing this is important for the purposes of antibiotic adsorption, since the fermentation broths generated in antibiotic fermentations often varies form batch to batch. The concentration of the antibiotic present in the broth may increase or decrease between batches and therefore impact on the amounts of antibiotic which can be adsorbed by a set concentration of resins. Examining the adsorptive performance of the resins, it was found that all resins displayed an ability to adsorb geldanamycin. It was found that 10 g/l of resins was sufficient for recovery of geldanamycin from a fermentation broth at a geldanamycin concentration of 47 mg/l, for the adsorbent resins XAD-16, XAD-1600, XAD-1180 and Diaion HP-20.

It is commonplace when assessing the applicability of adsorbents, to assess the relevance of adsorption models. An adsorption isotherm describes the relationship between the liquid phase and solid phase concentration of a solute. For the assessment of the applicability of

adsorbent resins, for the recovery of a particular antibiotic, an adsorption isotherm can be used to provide insight into adsorptive performance of the adsorbent under examination, including the affinity of the adsorbent for the antibiotic and its capacity to adsorb it. Two frequently applied adsorption models are the Langmuir and Freundlich models. It was found that both models could be applied to assess the performance of the resins, however the Langmuir model was generally the best fit. According to the Langmuir model, XAD-1600 and Diaion HP-20 possessed the greatest maximum loading capacity for geldanamycin.

The five resins examined in most detail all showed applicability for geldanamycin recovery. During the evaluation of the performance of these resins phenomena such as a fast initial adsorption followed by a slower adsorption period were observed. It is possible that such adsorption anomalies can be attributed to the physical form of the resins (pore size, volume and number) and the interactions between adsorbent and adsorbed compounds. Thus it becomes clear, that the general behaviour of the resins cannot be determined solely from their measurable physical properties (Voser, 1982) and therefore, experimental assessments are vital, in order to select the optimal resin for a particular application.

Having established that the adsorbent resins had capacity to adsorb geldanamycin from fermentation broths, it was decided to examine to what extent other compounds present in the fermentation broth were adsorbed. The success of an adsorption process can be considered to be related to the capacity and affinity of an adsorbent for the compound of interest. It was found that all resins showed some preference for the adsorption of geldanamycin over contaminants present in the fermentation broth. It was interesting to find that, in general, the selectivity of the adsorption process increased with contacting time. Reduction in the adsorption of contaminants is beneficial as it reduces the contaminants present for desorption, and may facilitate the recovery of a purer product stream.

It has been established that adsorbent resins could successfully recover geldanamycin from fermentation broth, and a number of resin had

displayed strong capacities and affinities for geldanamycin. In the assessment of adsorption or extraction processes, it is routine to examine the effect modification of the adsorption environment has on the recovery system. Two of the most commonly altered environmental parameters are temperature and pH.

Altering the temperature at which an adsorption is carried out can affect the rate of adsorption of the compound of interest. It was decided to examine the adsorption of geldanamycin at various temperatures and determine the impact on adsorption, and stability, of geldanamycin. It was found that the rate of adsorption was most rapid over an initial one hour period. At higher temperatures, adsorption of geldanamycin was more rapid and equilibrium would be established earlier. At higher temperatures, however, it was found that geldanamycin was less stable. Geldanamycin is a thermolabile compound, thus incubation of samples at increased temperatures leads to degradation. Adsorption at 22°C resulted in a rapid adsorption with minimal geldanamycin loss or degradation.

Biocompounds frequently exhibit sensitivities in environments outside their optimal pH ranges. Altering pH can therefore increase compound stability, but it has also been seen to influence adsorption of specific compounds. Assessing the impact of pH alteration, on adsorption of geldanamycin from fermentation broth, it was found that there was no significant impact on adsorption. It was noted however that at pH values of the order of pH 9.5, the stability of geldanamycin, and hence the level of recovery, would be reduced. It was also found that, for most resins, the adsorption of contaminants was lowest at pH 7.5, the pH of the unaltered fermentation broth.

In Chapter 4 it was found that addition of acetonitrile increased the liquid-phase solubility of geldanamycin in fermentation broth samples, and allowed a more accurate analysis of samples using HPLC. In literature it had also been found that incorporation of acetonitrile into the adsorption environment could impact on the adsorption of compounds, of particular note, was how inclusion of acetonitrile could interact with the adsorbent or

compounds in the fermentation system, and alter the adsorptive performance.

Examining the addition of acetonitrile into the pre-adsorption fermentation broth, it was found that, at higher concentrations, addition of acetonitrile reduced the adsorption of geldanamycin. When the effect of acetonitrile addition on adsorption of contaminants was examined, a very interesting finding was made. As the concentration of acetonitrile increased, the adsorption of contaminants decreased. This decrease occurred at a more substantial rate than the adsorption of geldanamycin. This result suggested that it would be possible to engineer an optimised adsorption system. The results indicated that at a low addition of acetonitrile, contaminant adsorption could be reduced, without a loss in adsorption of the compound of interest. This finding was of particular note for Diaion HP-20. Examining the effect of acetonitrile addition on adsorption of compounds by Diaion HP-20 it was found that as acetonitrile concentration increased, up to values of 20%, no major decline in geldanamycin adsorption, was observed, however significantly less contaminants were adsorbed. As a result of this finding, it would be desirable to examine this further, and assess acetonitrile addition, over smaller addition ranges, to determine the optimal system which would promote a more specific adsorption of geldanamycin than when no acetonitrile is added to the fermentation broth.

Since it is not only desirable to adsorb maximal amounts of geldanamycin from fermentation broth, but also, to subsequently recover the product, it was decided to assess the desorption of compounds from the resins. The assessment of the desorption of compounds showed that XAD-1600 and Diaion HP-20 allowed desorption of the greatest amount geldanamycin. It was also found that using acetonitrile to desorb compounds from the adsorbent resins resulted in desorption of between 72 and 96% of the geldanamycin and between 18 and 27% of the contaminants present in the pre-adsorption fermentation broth, for the different resins assessed. The levels of contaminant desorption are important because selectively

desorbing the compound of interest, while retaining contaminants on the resins, allows the production of a product stream of higher purity.

The benefits, with respect to product stream purity, come at the cost of resin reusability. In order to prepare the resins for reuse the strongly retained compounds must be removed using harsher methods such as acid and base washes. It would be desirable to further assess this issue of reusability. One of the benefits of using adsorbent resins over solvents in extraction processes is a reduction in costs. For an optimised process, it would be advantageous to effectively regenerate the adsorbents, and apply them in repeated extractions, without loss in adsorptive performance.

Examining the application of these adsorbent resins for the recovery and purification of geldanamycin from fermentation broth, it can be seen that there is considerable potential to optimise the process. The investigations undertaken in this Chapter were focused on a preliminary examination of resin application, however the results indicate that there are a number of facets, of the application of these resins, which could be optimised for geldanamycin recovery. Assessing the modification of adsorption in conjunction with the desorption profile of the resins, it would theoretically be possible to generate a geldanamycin product stream approximately 25-fold purer than the initial fermentation broth. This is a very significant result, however it would be necessary to verify this theory by performing sequential optimised adsorption and desorption processes, on a fermentation broth sample.

The resins employed in this study were not specifically developed for geldanamycin adsorption, nor modified to increase their selectively for geldanamycin. Modification of resins to make them more selective for a target compound is a viable means to improve the adsorption process. Pyrzynska and Wierzbicki, (2005) detailed the functionalisation of Amberlite resins with porphyrin ligands for application in vanadium species recoveries and found that a greater affinity for the loaded sorbent could be gained. Other modifications can also be made to adsorbents to confer

increased capacities or to address some other processing concerns. Jung *et al.*, (2001) examined modification of commercially available adsorbents. They modified Amberlite XAD-2 ad XAD-4 through the introduction of a porphyrin molecule and found it resulted in an increased capacity for phenol adsorption. Wang and Sobnosky, (1985) showed, that by modifying the adsorbents physically, via incorporation into a hydrogel matrix, it was possible to increase selectivity for compound of interest, to increase the stability and structure strength of the adsorbents, and facilitate application in environments of shear.

There is great potential for the use of adsorbent resins for the recovery of geldanamycin from fermentation broth. The advantages of such an extraction process are a reduction in solvent consumption, less product loss and a more controllable and standardised extraction process, and it would be desirable compare the entire pros and cons of solid-liquid mediated recovery of geldanamycin versus those of liquid-liquid mediated geldanamycin recovery. Although each resin assessed is capable of application in a solid phase extraction method for geldanamycin recovery, the two most suitable resins for further application would be XAD-1600 and Diaion HP-20. Prior to commencing this study, it was predicted that the hydrophobic interaction-based resins may perform best, since geldanamycin displays a hydrophobic nature. However, the culmination of the results of all assessments performed showed that the adsorption of compounds from their production environments is a complex procedure. Even though XAD-1600 and Diaion HP-20 exhibit two different modes of action, both have been found to be suitable adsorbent resins for further examination. This result could be rationalised based on the fact that the mode of action of adsorbents is usually complex an poorly understood, and also geldanamycin is an amphiphilic compound, thus displays a complex chemical nature.

Chapter 6. *In-Situ* Product Recovery of Geldanamycin

6.1 Introduction

Recovery of the compound of interest is a key consideration for the successful bioprocess. In bioprocessing operations, the yield of the compound of interest can be affected by exposure to detrimental conditions and processes during production, but can also be affected by the presence of the product in the production environment. The problems encountered are somewhat product specific, but commonly include end product inhibition, feedback repression and degradation. The direct or rapid removal of the product when it is formed, limits the potential for these phenomena to occur, and as a result integrated bioprocessing strategies, such as *In-Situ* Product Recovery (ISPR), have been developed to address such issues.

ISPR methodologies may be expected to improve productivity and yield via three effects: (a) minimisation of product inhibition: (b) minimisation of product loss due to cross-interaction with the producing cell (degradation, further modification) or uncontrolled loss (e.g. by evaporation); and (c) reduction of the number of subsequent downstream processing steps (Mattiasson and Holst, 1991, Freeman *et al.*, 1993). For this reason, ISPR techniques find application for the recovery of an array of products including flavour and fragrance compounds (Bluemke and Schrade, 2001) and even application in bio-catalytic reactions (Ahmed *et al.*, 2001).

If the production of the compound of interest is regulated by its own presence, increasing product yields will be problematic, since this feed-back regulation will reduce or cease metabolic processes towards production. If the organism can further metabolise the product of interest into new metabolites, its continued presence in the fermentation

environment may lead to its consumption by the producing organism. It is clear from such phenomena that the production, and therefore recovery, of the product of interest must be tightly monitored to prevent reduction in product yields. If the compound of interest is susceptible to environmental conditions such as temperature, pH or oxygen, its exposure to the production environment may result in reduced product yields. If the product is toxic to the producing organism, it may inhibit its own production. These events are commonly seen in antibiotic fermentations and the importance of application of ISPR techniques are highlighted by the belief that productivity of fermentations could be increased if product separation took place directly in the fermenter or in an external loop (van Erkel *et al.*, 2004).

It may be the case that a single methodology may not be the only method by which the compound can be recovered in such processes. The work of Choi *et al.,* (2001) focused on the integrated bioprocessing of plant cell cultures and they suggested that there are a number of ISPR techniques which can be employed for a particular application. Their findings also serve to stress that no single methodology is universally applicable.

With that in mind, it was decided to examine the ISPR of geldanamycin. Geldanamycin can be recovered from fermentation broth through solvent-mediated liquid-liquid extraction, however, in Chapter 5, the applicability of adsorbent resins for the recovery of geldanamycin form fermentation broth was assessed. It was found, in a downstream processing context, that these resins were suitable for the recovery of geldanamycin from fermentation broth, and it was therefore decided to examine their application in an ISPR context. It was desirable to determine if application of these resins in an ISPR process could facilitate direct removal of the product during fermentation, and determine if their application could increase overall yield of geldanamycin. Two resins in particular, Amberlite XAD-1600 and Diaion HP-20, were found to have significant potential for application in the recovery of geldanamycin in downstream processes and it was therefore decided that these resins would be the focus of the

assessment of the *In-Situ* Product Recovery of geldanamycin from fermentation broth.

6.2 Materials and Methods

6.2.1 *Streptomyces hygroscopicus* var. *geldanus* antibiotic fermentations

Chapter 3

6.2.2 Analysis of broth levels of Geldanamycin

Chapter 4

6.2.3 Analysis of solid phase levels of Geldanamycin

In order to determine total production one must consider the presence of two streams in the fermentation: the liquid stream, the amount of product secreted into the medium by the organism and remaining in solution/suspension, and the solid stream, the amount of product associated with cell matter, either entrapped/immobilised or retained intracellularly. When an additional solid phase is included in the fermentation system, e.g. adsorbent resins, these must be incorporated into the assessment of the solid phase-associated product levels.

Applying adsorbent resins in an ISPR process for geldanamycin recovery meant that in order to determine the amount of geldanamycin associated with the solid phase the biomass and resins were to be treated as a combined solid phase stream. Samples were centrifuged for 10 minutes at 3500 rpm. The recovered solids (biomass and resins) were then washed in one quarter fermentation volume of acetonitrile. The washing process involved resuspension of the solids with vigorous agitation followed by 10 minutes sonication to maximise mass transfer and disassociate product which is strongly associated with biomass. Lindemann *et al.*, (2000) also used sonication to increase the extraction of compounds from solids. The material was centrifuged again and the acetonitrile phase recovered. The

process was repeated once more to facilitate recovery of product and the two acetonitrile washes were pooled to form one sample for analysis via HPLC.

6.2.4 Resin preparation for ISPR applications

Addition of resins into a fermentation increases the potential for contamination of the system. When resins were added at the initiation of the fermentation, they were weighed out into the Erlenmeyer culture flask, 5 ml of sterile H_2O was added, and autoclaved (121°C for 30 minutes). The media was then added into the flasks and the fermentations inoculated, at 1%, with spore stock of approximately 10^7spores/ml. When resins were to be added during fermentation, the resins were autoclaved, in 5 ml of H_2O in glass universals, and stored until required. When it was time to incorporate them into the fermentation, the resins were added rapidly, with minimal exposure to the open environment. The resins were not autoclaved in the presence of media because it was believed this could affect the adsorption process. Marshall *et al.*, (1990) had found evidence that autoclaving resins in the presence of media components may alter the resins, reducing their ablity to bind their target molecule, rubradirin, or may promote the binding of essential metabolites required by the producer.

6.3 Experimental

6.3.1 Effect of resin addition on broth levels of geldanamycin

6.3.1.1 Introduction

In Chapter 5, it had been found that the resins were suitable for application in a DownStream Processing capacity, thus the major difference with respect to their application in an ISPR context was the adsorption would take place under fermentation conditions (agitation and temperature) and in the presence of biomass. A decrease in the broth levels of product upon the addition of resins indicates the resins are adsorbing product, and application in an ISPR capacity does not hinder their activity. It was decided to examine if this was the case with the two resins; Amberlite XAD-1600 and Diaion HP-20, which had previously been identified as warranting further examination.

6.3.1.2 Materials and methods

It would be necessary for appreciable levels of product to be present in the fermentation system in order for a noticeable effect to be elicited upon resin addition. It was decided that on Day 7, the resins would be added and the effect of resin inclusion on broth levels of geldanamycin would be assessed and compared to control levels. Two different resin concentrations, 1 g/l and 5 g/l, were employed to assess the impact of concentration. The resins were added to the fermentations and broth samples were taken daily, with the broth levels of geldanamycin being assessed by HPLC.

6.3.1.3. Results

It can be seen from both Figures 6.1 and 6.2 that the addition of adsorbent resins into the fermentation system resulted in a drop in geldanamycin concentration in the fermentation broth. This indicates that the resins were capable of adsorbing product, even in the presence of biomass and under fermentation conditions.

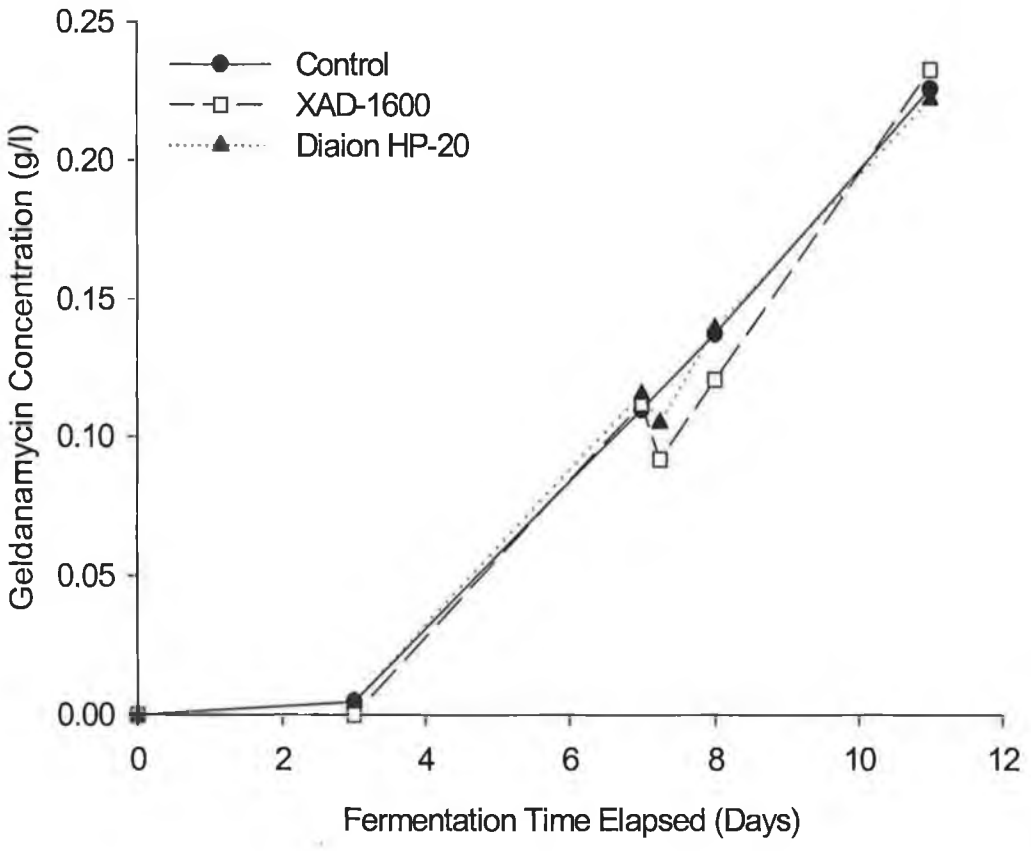


Figure 6.1: Effect of addition of 1 g/l of adsorbent resin at Day 7 of fermentation on broth levels of geldanamycin

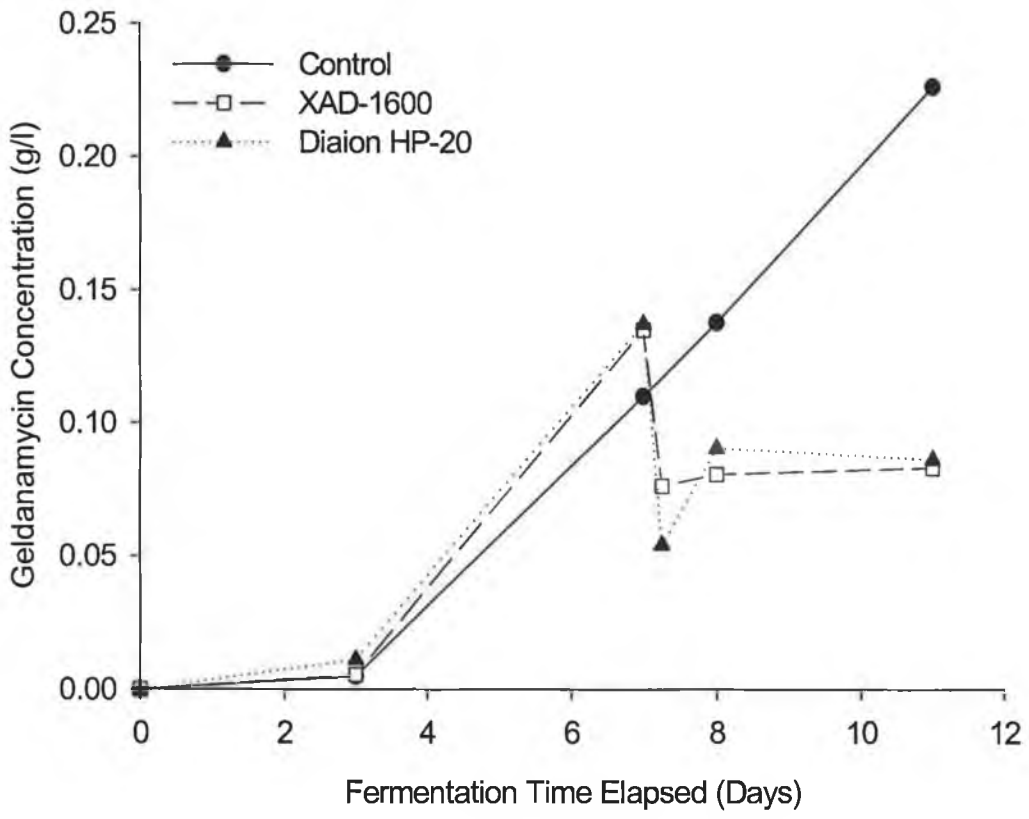


Figure 6.2: Effect of addition of 5 g/l of adsorbent resin at Day 7 of fermentation on broth levels of geldanamycin

In both Figures, after the drop in signal, resultant from the introduction of the resins, the broth levels gradually begin to increase again. This indicates that, added at low concentrations, the resins adsorb geldanamycin until saturated. Production continues, and after a period of fermentation time, achieves levels comparable to control fermentations. This suggests that there may be no detrimental effect of low level addition of resins on the metabolic and biological processes of the organism.

From Figure 6.2 however, it can be seen that although the drop is greater, since a greater concentration of resins is added, control levels of production are not re-established in the broth after the same fermentation time. This indicates that in order for broth levels of geldanamycin to be re-established in fermentations containing higher concentrations of adsorbent resins, a longer fermentation period may be required.

The above results highlight the potential application of these adsorbent resins in ISPR processes and in particular the potential for ISPR to increase geldanamycin production yields. The continual removal of product as it is formed benefits the production process with relation to limiting inhibition and degradation, and it would thus be beneficial to determine the effect of resin inclusion on total geldanamycin yields.

6.3.2 Effect of resin inclusion on total geldanamycin yields

6.3.2.1 Introduction

In production fermentations, it is common to deal with a single product stream. Prior to assessment of the applicability of adsorbent resins for the recovery of geldanamycin, only the liquid stream of the fermentation was assessed. Aiba and Okabe (1976) suggested that in actinomycete fermentations, the solid phase mycelia may contain quantities of the entrapped antibiotic. Assessing the applicability of adsorbent resins incorporates an additional stream into the process, a solid stream. These resins adsorb product from the liquid stream and it is recovered via desorption. The solid phase therefore comprises biomass for control fermentations and biomass plus resins for test fermentations.

6.3.2.2. Materials and Methods

Since it had been determined that the adsorbent resins could function in an ISPR system (i.e. adsorb product), it was decided to expand the assessment and determine if an increased amount of geldanamycin could be produced in an ISPR application of the resins. The process involved addition of resins (at 1, 5, 10 and 20 g/l) on the initiation of fermentation, and examination of geldanamycin yields on Day 7 and Day 16. Total production was assessed as a summation of the geldanamycin recovered from the fermentation broth and the solid phase of the fermentation.

6.3.2.3. Results

Examining the effect of inclusion of resins from the initiation of the fermentation, it was found that, even at as low a concentration as 1 g/l, an increase in production was achieved over those fermentations devoid of resins (Figure 6.3). It was therefore decided to examine the effect of resin addition on broth levels of production. Assessing the broth levels alone

would highlight the benefits of including a solid phase treatment with respect to the total amount of geldanamycin which could be recovered.

It can be seen from Figure 6.4 that control levels of production were achieved at lower resin concentrations. Likewise it was found that the broth levels of geldanamycin in fermentations containing higher resin concentrations were lower than control fermentations. This again demonstrates that there is residual potential for the resins to adsorb product and that there may be further benefits to product yield if the fermentation is allowed progress for longer.

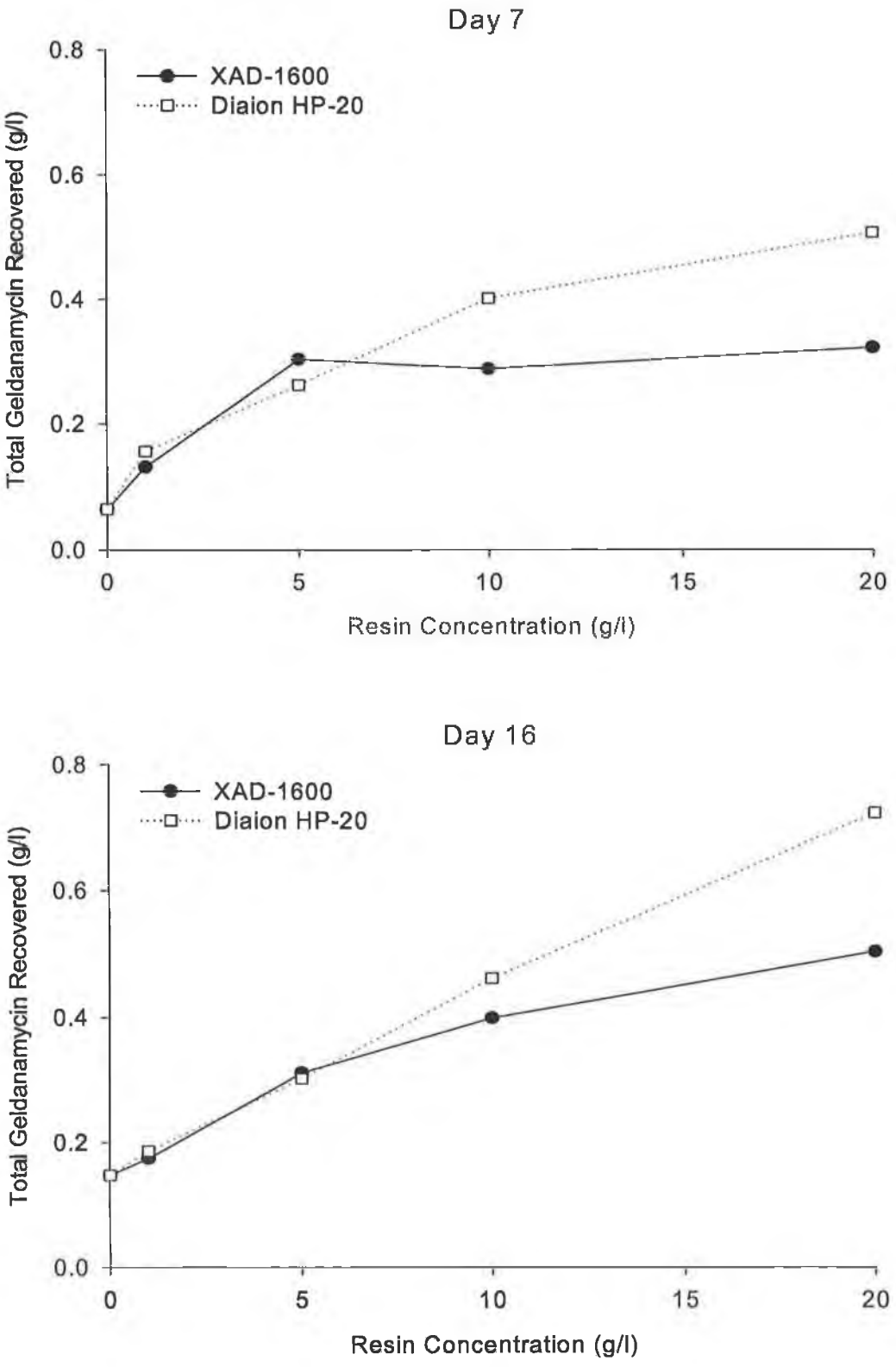


Figure 6.3: The effect of resin inclusion on total geldanamycin production

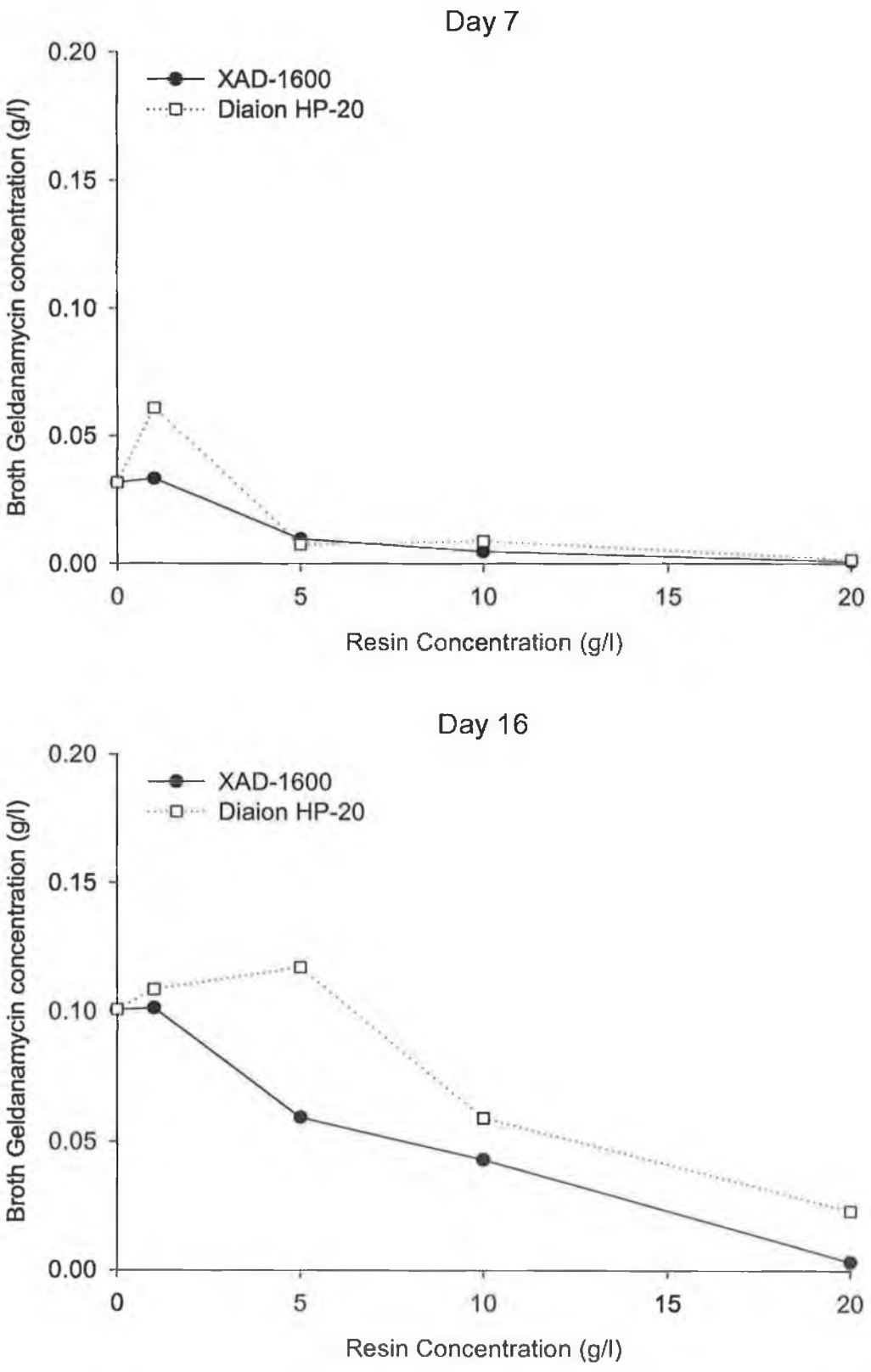


Figure 6.4: Broth levels of geldanamycin production

6.3.3 ISPR of geldanamycin for increased product yield

6.3.3.1 Introduction

The preliminary results of the effect of resin inclusion on total geldanamycin production indicated that inclusion of resins results in increased productivity over control fermentations. It was decided to perform a more in-depth examination by increasing the number of time points at which the fermentation environment was assessed. This would allow a more accurate assessment of how resins affect the fermentation and the determination of how they could be used to benefit production in an ISPR process.

6.3.3.2 Materials and Methods

It was decided to omit resin concentrations of 1 g/l and instead focus on the impact of addition of higher concentrations of resins. The results obtained to-date had indicated that there was potential for higher geldanamycin yields if higher resin concentrations were employed in the ISPR process.

Fermentations containing 5, 10 and 20 g/l of resin from initiation of fermentation were established, and were sampled at important times during fermentation. The time points selected were Day 7 (suitable product levels would be starting to accumulate), Day 11 (a mid fermentation sample) Day 16 (a suitable end of fermentation sample) and Day 21 (an extended time point to assess the implications of fermentation time on the success of ISPR and production trends with time).

6.3.3.3 Results

From Figure 6.5 it can again be seen that the inclusion of resins in fermentations has significant benefits for the production of geldanamycin.

The higher the concentration of resins incorporated, the greater the amount of geldanamycin produced, and all resin concentrations showed an increase in the production of geldanamycin over control levels. It was found that the increase in production tailed-off with respect to fermentation time and was most apparent for Diaion HP-20, which approached its maximum levels of production earlier in the fermentation process than XAD-1600.

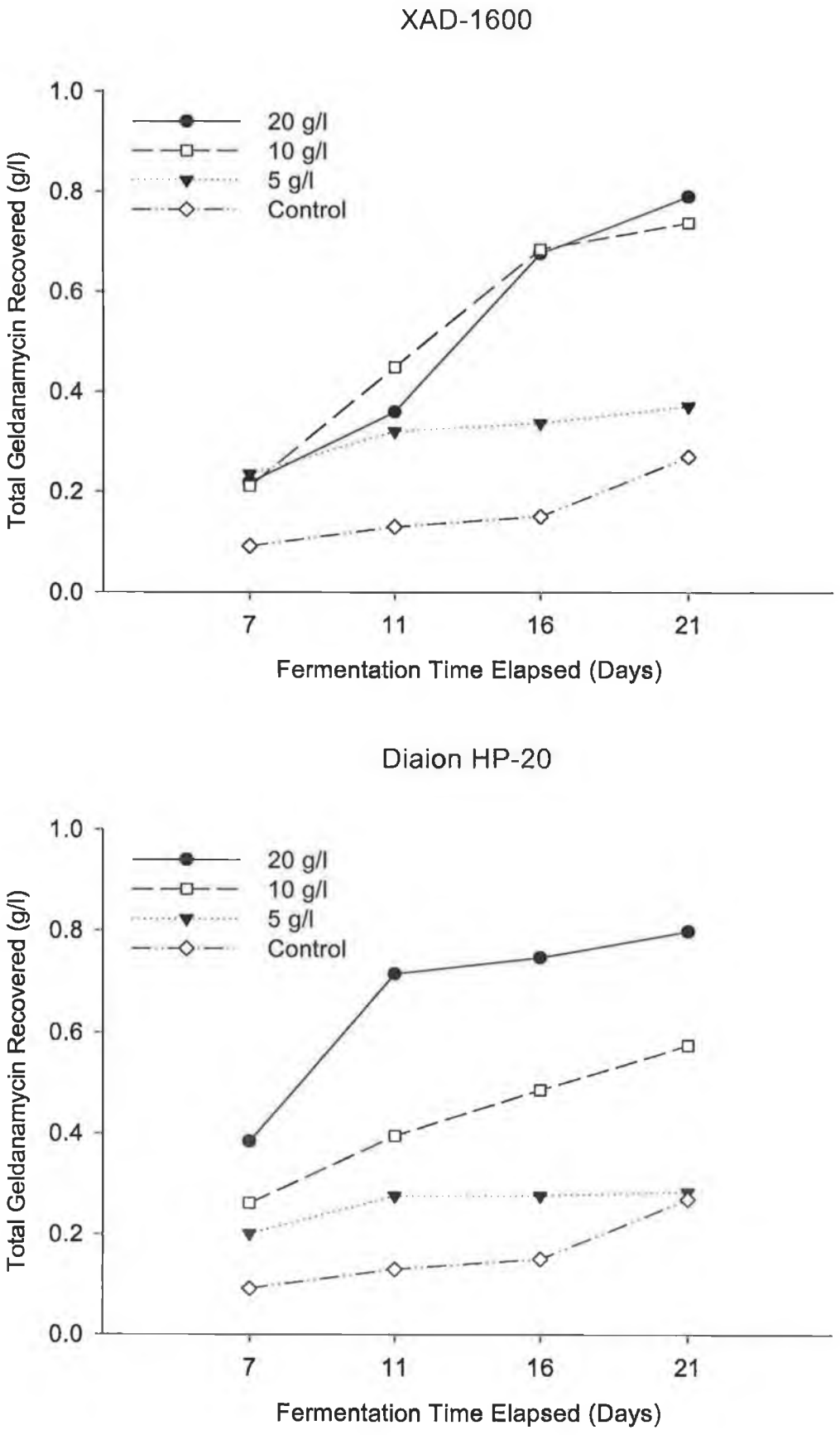


Figure 6.5: Effect of resin addition and concentration on geldanamycin recovery with time

6.3.4 Effect of resin inclusion on growth and substrate utilisation

6.3.4.1. Introduction

In the previous section the effect of addition of resins from the start of fermentation on geldanamycin production was examined and it was found that this resulted in a considerable increase in geldanamycin yield. It was decided to examine the effect of resin addition on the growth of the organism, in particular the generation of biomass. It was also decided to correlate this with the consumption of nutrients, in particular glucose, the primary carbohydrate source in the medium. Caution had to be taken during this assessment for two reasons. The resins are non-selective for the product, and use of non-specific adsorption has the draw-back of extracting other compounds, besides the desired product, from the fermentation broth (Wang and Schultz, 1981). The resins could adsorb 10 g/l of glucose from Bennett's medium, containing 50 g/l of glucose, in approximately 48 hours, under fermentation conditions of 28°C incubation, agitated at 150 rpm. This may bias the pattern of glucose 'consumption', thus one must be aware that the 'consumption' of glucose, may be partly attributable to adsorption onto the resins.

With respect to the determination of biomass growth, it was necessary to factor into account the amount of resins incorporated into a homogenous sample. Irrespective of these difficulties, it was envisaged that it would be possible to gain some understanding of the overall impact on a fermentation system resultant from the inclusion of adsorbent resins form the initiation of the fermentation. It had previously been seen that even if glucose is adsorbed by the resins, it had no major inhibitory impacts on production, thus it was important to examine just what trends were occurring in the *In-Situ* Product Recovery fermentation.

6.3.4.2. Materials and Methods

To determine the dry weight of biomass an homogenous 10ml sample of the fermentation was taken and aliquoted into a plastic centrifuge tube (Sarstedt, Wexford, Ireland) and centrifuged at 3500 rpm for 10 minutes. The pellet was resuspended in approximately 5 ml of ethanol and transferred to pre-weighed, labelled glass universals. The glass universals had been prepared cleaned, labelled and dried in a 100°C oven for 24 hours. The universals were then removed cooled, in a desiccator (to ensure they were free from additional moisture-derived weight), weighed on a three decimal place balance (Chyo Balance Corps., Japan) and retained for use. Once the resuspended pellet was recovered into the glass universals they were placed in a water bath and heated to 100°C to evaporate the ethanol. Once devoid of ethanol, the universals were returned to the 100°C oven overnight and cooled in the desiccator prior to re-weighing. The biomass concentration was determined using the following equation:

$$BiomassConcentration = (A - B)x100 \text{, where;} \qquad \text{(Eq. 6.1)}$$

A is the weight (g/l) of the dried glass universal containing sample, B is the weight (g/l) of the dried glass universal prior to sample addition and $(A - B)$ is the difference in weight of the universals after addition and drying of 10ml of sample (g). This is multiplied by 100 in order to scale-up the assessment to a g/l amount.

When this method is applied to a sample which contains resins the presence of the resins must be incorporated into the dry weight assessment otherwise a false high result would be returned. In order to factor in the presence of the resins, once a dry weight concentration (g/l) had been determined, the concentration of the resins which was added (g/l) is subtracted from this value. This gives the dry weight of biomass only (g/l).

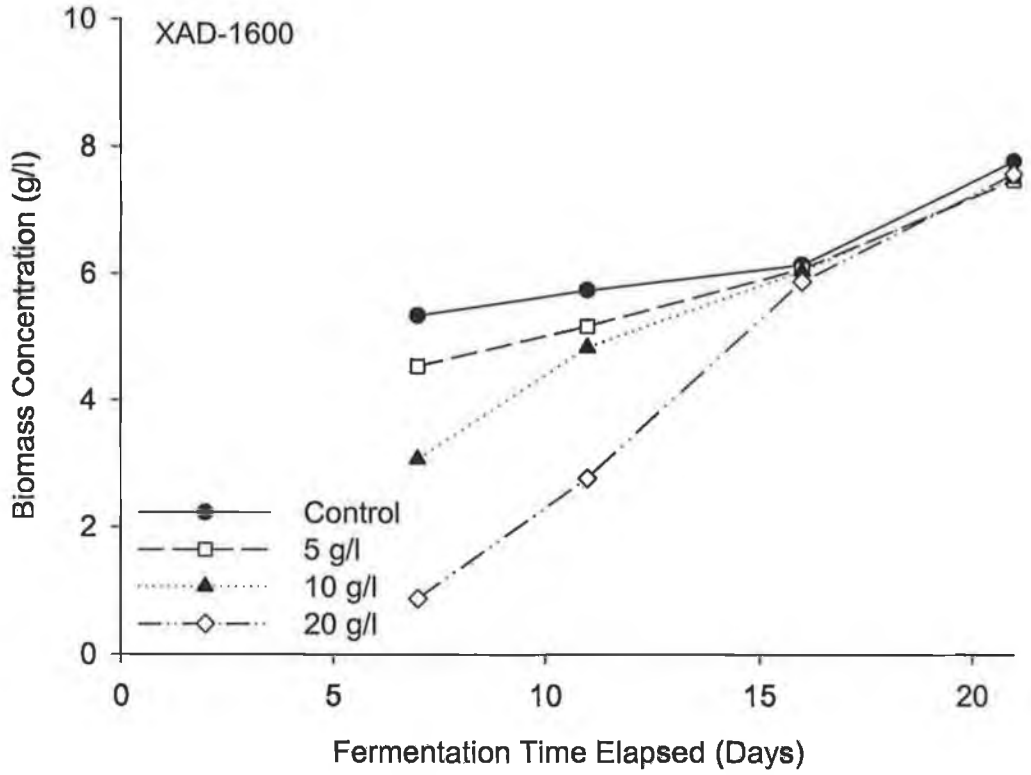
The glucose concentration in samples was determined using the GOD-PAP assay. This assay allows the quantitative determination of glucose concentration in samples via the combined action of the enzymes glucose oxidase and peroxidase. The method employed is a modification of that described in the Randox Laboratory's Ltd. assay kit (Crumlin, Co. Antrim, Northern Ireland). The method has been adapted for microtiter plate use and thus allows a higher throughput of samples in a shorter processing time. The kit comprised of a glucose standard, which is used to establish a standard curve, and reagents, which are comprised of the necessary reactants including the enzymes, in a suitable buffer. Samples of the fermentation broth were taken following centrifugation at 3500 rpm for 10 minute.

The modified assay involved addition of 20 µl of sample (diluted in distilled water, into the range of the standard curve) to a well of a 96-well plastic microtiter plate (Sarstedt, Wexford, Ireland). To this 200 µl of reagent was added. The solutions were mixed and incubated at room temperature (15 - 25°C) for 25 minutes (alternatively, the plates could be incubated at 37°C for 10 minutes to increase the speed of the analysis). The samples were then read using a Tecan, Sunrise, A-5082 plate reader and associated data retrieval software (Tecan, Mannedorff, Switzerland) at a wavelength of 492 nm. The absorbance of the glucose containing samples (standards and test samples) were measured and compared to a blank sample (distilled water). The glucose concentration was then determined using a glucose standard curve. (See Appendix for standard curves).

6.3.4.3. Results

Williams *et al.*, (1992) found that adsorbent resins, used for the recovery of the antimicrobial compound sanguinarine, could adsorb growth regulators and vitamins from the culture environment and inhibit cell growth. Examining the effect of addition of resins on *S. hygroscopicus* growth it seemed that this may also be the case, since the growth rate showed signs of reduction (Figure 6.6). With respect to final biomass yields it was found that it was possible for the fermentations containing

resins to achieve approximately the same levels of biomass generation as those without resins, however it took a longer period of fermentation time. These results indicated that when the resins were present in the fermentation environment from the initiation of the fermentation, they may have adsorbed compounds required for growth and thus slowed down the growth rate. This may be seen from the fact that the biomass concentration is low with relation to higher resin concentrations, at early stages during the fermentation.



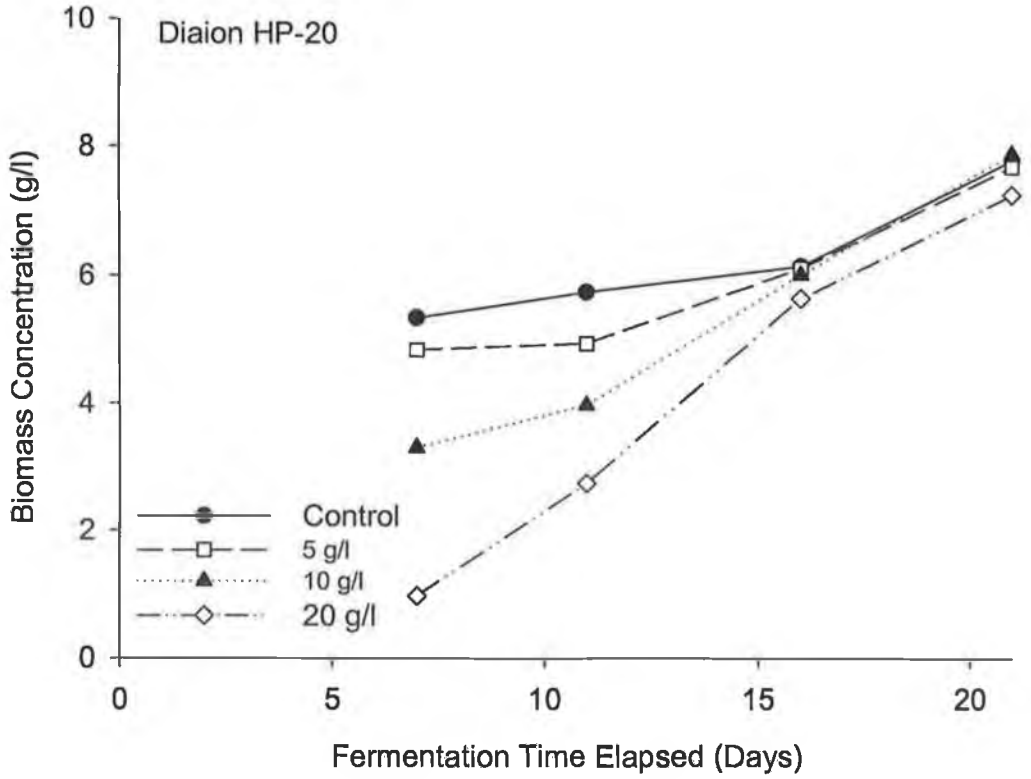
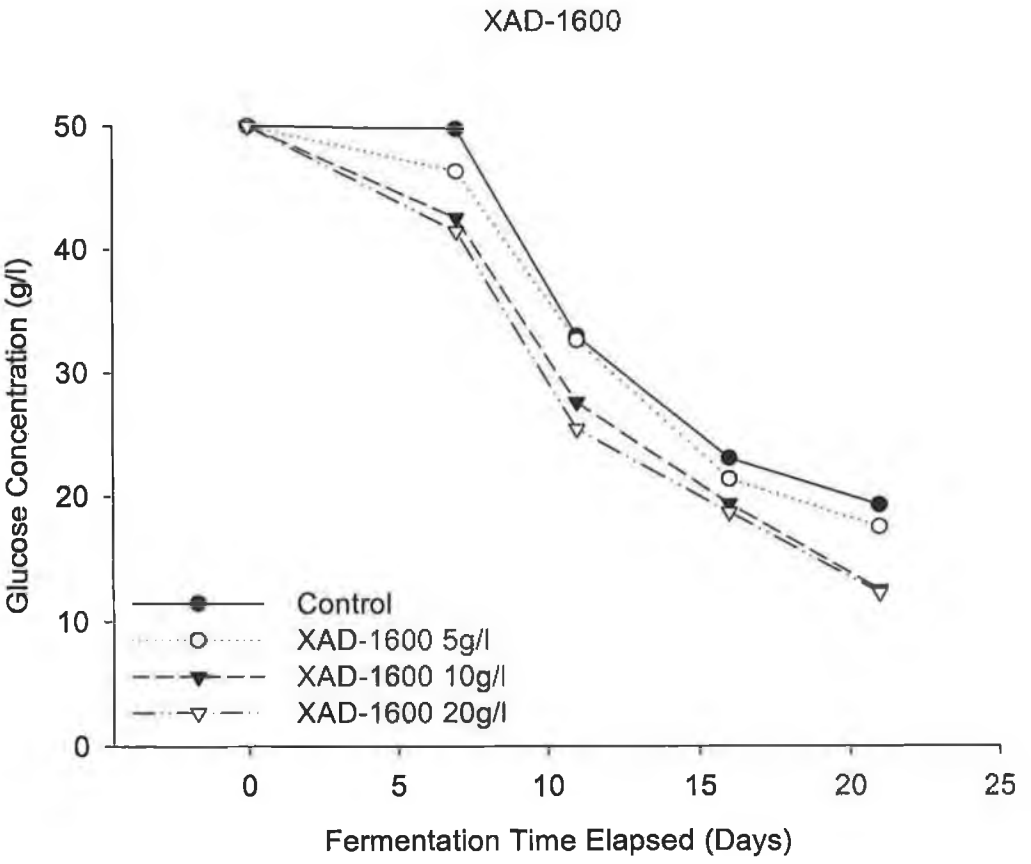


Figure 6.6: Effect of resin addition on biomass growth

Examining the effect of resin inclusion on glucose consumption, it was found that as the fermentation progressed, the concentration of glucose in the fermentation decreased. This was expected, since glucose would be consumed by the organism for growth and production, and could also be removed by the resins (Figure 6.7).



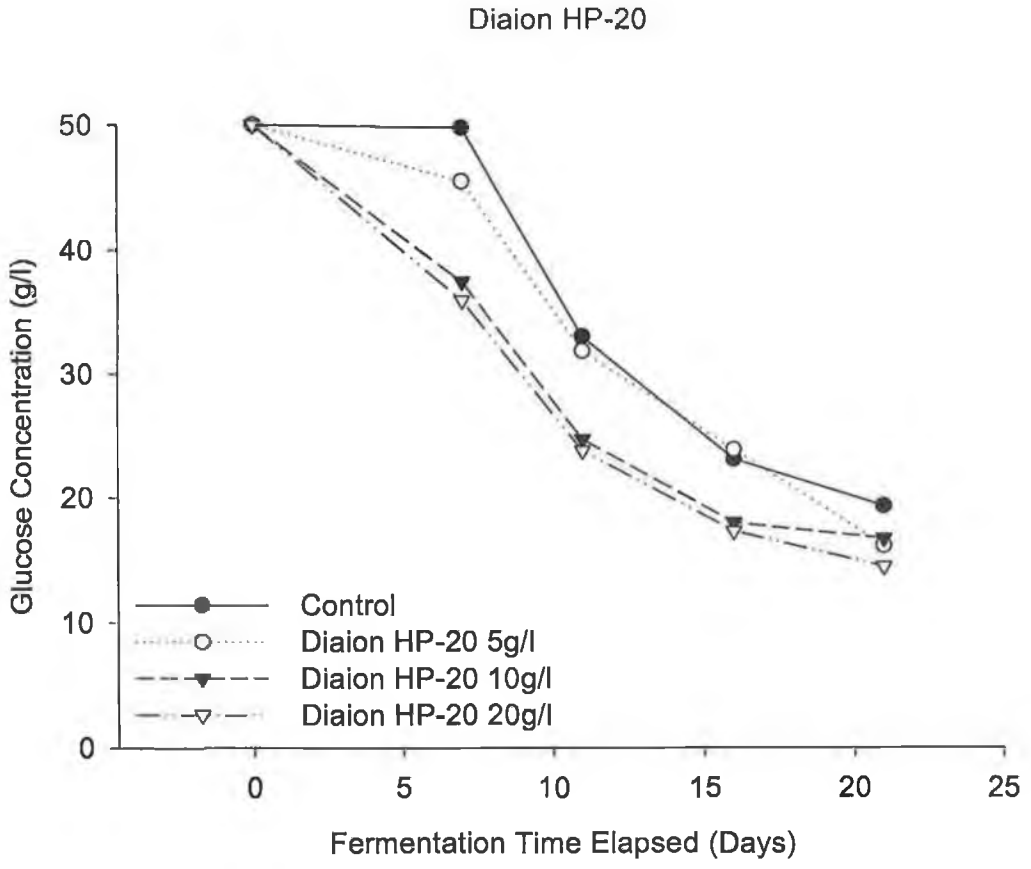


Figure 6.7: Effect of resin addition on glucose concentration

6.3.5 Effect of resin addition time on total production:

6.3.5.1 Introduction

Since it had previously been shown that higher concentrations of resins resulted in greater yields of geldanamycin, it was decided to determine the effect of resin addition time on the fermentation system. Previously addition times of Day 0 and Day 7 were examined, however it was decided to extend this assessment and examine the addition of resins at a number of important fermentation times. In this manner it would be possible to determine if addition time effected production and recovery of geldanamycin.

6.3.5.2 Materials and Methods

The experimental involved the addition of 20 g/l of resins to fermentations at the initiation of fermentation and subsequently on Day 0, 7, 11 and 16. The fermentations were allowed run until Day 21 at which point they were harvested and analysed. Resin concentrations greater than 20 g/l were not used because volumetrically, they would occupy a large amount of the fermentation environment. Increased resin concentrations may also lead to increased non-specific adsorption of nutrients, which in turn, may either deplete the environment of nutrients resulting in limitation of growth, or deplete it to levels which may favour the growth of contaminating organisms.

6.3.5.3. Results

Again it was found that the incorporation of adsorbent resins into the fermentation system increased geldanamycin yields (Figure 6.8).

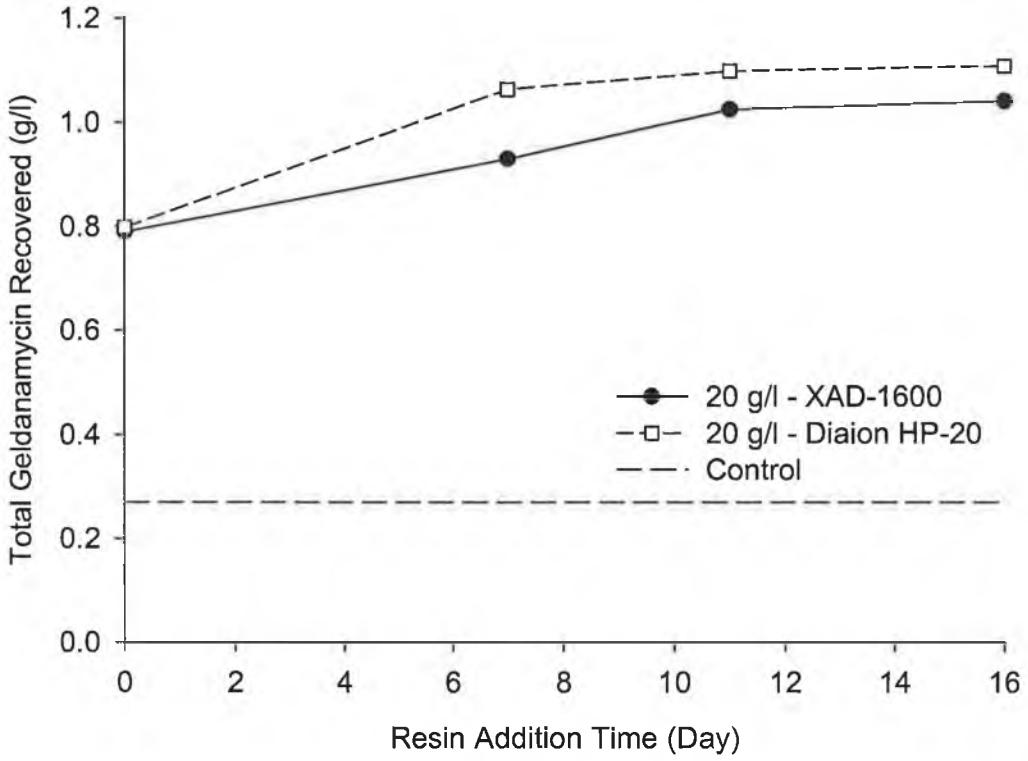


Figure 6.8: Effect of resin addition time on geldanamycin production and recovery for a Day 21 harvested fermentation. 20 g/l of each resin was employed, and ----- represents the yield of geldanamycin from resin free fermentations

It was also found that the time at which resins were added could impact on the total amount of geldanamycin recovered. Adding the resins after a period of fermentation time had elapsed aided the recovery of greater quantities of geldanamycin. The greatest benefit to production and recovery is achieved when the resins are added at approximately Day 7. Adding the resins later had minimal additional benefit to production levels over the fermentation time examined. It is also important to highlight that even if the resins are added at the initiation of fermentation, the positive effects on production levels over that obtained in control fermentations was still clear.

6.4. Discussion and Conclusion

Microbes can produce and accumulate certain substances which are toxic to the microbial cells themselves. These substances can limit and regulate their own production and negatively impact on the success of fermentations. Removal of these products from the vicinity of the producing organism as soon as they are formed would clearly be beneficial.

Having applied adsorbent resins in a DownStream Processing (DSP) application for the recovery of geldanamycin, and identifying the two optimally performing resins, it was decided that utilisation of these resins may be a viable means to achieve the goal of increased productivity. The resins could be applied in an *In-Situ* Product Recovery (ISPR) method and the effect of doing so on the levels of production and product recovery could be assessed. An initial assessment of the performance of the resins under fermentation conditions showed that their action was not hindered, and they adsorbed product, resulting in a drop in the broth levels of product.

A more in-depth assessment of the impact of resin inclusion on geldanamycin yields indicated that incorporation of the resins into the fermentation allowed the recovery of increased amounts of product over that produced in control fermentations. As the resin concentration increased so too did the amount of geldanamycin recovered. It is believed that the addition of resins may have allowed an enhanced maintenance and control of the levels of different compounds present in the fermentation system. The resins could remove geldanamycin from the production system as it was formed, preventing its further metabolism or degradation as a result of exposure to the fermentation environmental conditions.

Since the resins were not selective for geldanamycin, they may facilitate increased production by adsorption of contaminating compounds or compounds which have negative effects on production, such as precursor

or regulatory compounds. Removing compounds like these from the fermentation system would diminish their impact on production regulation. Addition of the adsorbents into the fermentation system can also result in the adsorption of nutrients (Tone et al., 1968). If adsorption and slow release of nutrient compounds occur, this too may have a beneficial impact on production. A more encompassing examination of the effect of resin inclusion demonstrated definitively that an increase in the geldanamycin was possible by addition of these resins in an ISPR mode.

It was also found that if the fermentation was allowed to progress for a number of days prior to addition of the resins, a further increase in production could be achieved. The primary reason for this was believed to be that by allowing the fermentation to progress similar to control fermentations, biomass levels build up more rapidly than they would if the resins were present from the beginning of fermentation. On addition of the resins, there is a combined benefit of the fermentation being in a more advanced state and the benefits previously attributed to addition of the resins.

Another reason for the difference in yields could be that inclusion of the resins from an early stage may result in biomass growth in the pores of the resins. This would limit the positive effects of resin inclusion through clogging of the resin pores and thus reduce the potential for geldanamycin adsorption by the resins. Adding the resins later, means that the biomass would be in a larger size and less likely to colonise the pore structures. Senthuran *et al.*, (2004) took measures to reduce the impact of microbial growth on the adsorptive performance by shielding the adsorbent in a thin layer of non-ionic polymer in their studies of integrated lactate production.

Bader, (1986) suggested that during fermentation production of antibiotics, the primary objectives during the early phase of the fermentation are to grow a large concentration of cells, develop the enzymatic pathways for antibiotic production and deplete the medium of metabolite which may be inhibitory to the production of the antibiotic. With this in mind, it is clear to see some reasoning behind the increased levels of geldanamycin

production resultant from the addition of adsorbent resins. The resins are not only capable of adsorbing and stabilising the product, thus removing it from environmental and metabolic hazards, but may also remove some product in the environment which may contribute to the inhibition of geldanamycin production.

Likewise the addition of resins at a later stage in the fermentation allows the maximum amount of biomass growth to be achieved sooner, and when the resins are added the combined effect of increased biomass concentration, removal of inhibitory compounds and recovery of product is elicited. Similarly, Marshall *et al.*, (1990) elucidated to the fact that addition of resins may benefit production in two ways, namely sequestering of product away from its sensitive producing organism and also serve to promote stabilisation of the product, and in particular, prevent its conversion to different products.

In summary, it is clear to see that the work undertaken has proven that the selected adsorbent resins display a potential for application in an ISPR methodology for the recovery of geldanamycin. The use of the resins not only facilitated recovery of the product but also resulted in an increase in the total amount of geldanamycin which could be recovered. The use of these resins in an ISPR context was a success and has considerable application possibilities for further work. There has been significant work performed in developing novel techniques using adsorbents or other methods, and also combinations of techniques, to achieve increased productivities or to address or prevent some process or metabolism-associated product loss (Zelić *et al.*, 2004, Wang, 1983, Cen and Tsao, 1993), and are of great value to the bioprocessing world.

Chapter 7. Conclusions and Recommendations

7.1 Conclusions

The aim of this project was to examine the applicability of adsorbent resins in processes for the recovery of the antibiotic geldanamycin, produced by the Actinomycete *Streptomyces hygroscopicus var. geldanus*. Adsorbent resins are gaining increased application for the recovery of antibiotics generated in a similar manner to geldanamycin and it was therefore desirable to determine if their application could lead to the successful recovery of geldanamycin. In order to achieve this however, it was necessary to have in place, methods by which geldanamycin could be assessed and monitored. It was envisaged that successful completion of these goals would complement any work performed in future assessments of geldanamycin.

Previously, work had been performed in the laboratory to establish the growth patterns of the organism, and tie this to productivity. During this period the lack of Standard Operating Procedures (SOPs), to allow the fast and accurate assessment of the progression of fermentations, and in particular the production profile of fermentations, hindered the progress of the work. Up until that point the bioactivity of samples, which served as an indication of fermentation production levels, was assessed using Disk Diffusion assays. These assays were labour intensive, consumed large quantities of materials and returned primarily qualitative results. In order to rectify this, a high throughput microtiter plated-based bioassay was developed. This assay allowed the determination of the efficacy of bioactive compounds produced in the fermentation broth, based on retardation of biomass growth. This was determined by turbidity assessment, and applying the developed technique, it was possible to correlate retardation of biomass growth with inhibition potential of fermentation samples.

The development of this assay increased the reliability and standardisation of the assessment of production over the traditional disk diffusion method, allowed higher throughput of samples and allowed a quantitative assessment to be achieved. However, it soon became clear that in order determine and asses the presence of a single compound in fermentations, namely the target antibiotic geldanamycin, it would be necessary to develop a more selective method. Since the very nature of *Streptomyces* is the production of an array of bioproducts (Glazer and Nikaido, 1995), using the developed bioassay, it could not be definitively determined that the bioactive effect assessed, was wholly attributable to geldanamycin.

In order to address this problem, an accurate, reliable, high throughput method for analysis and quantification of geldanamycin was required. High Performance Liquid Chromatography (HPLC) is frequently used for the definitive identification and quantification of compounds, and it was therefore decided to develop a suitable HPLC method for geldanamycin assessment. HPLC is often used for the quantification of bioproducts including antibiotics (Joshi, 2002, Loadman and Calabrese, 2001). However, optimisation of the process for a particular application can require considerable experimentation. The HPLC method was optimised and standardised for identification and quantification using an adaptation of the process employed by Agnew *et al.*, (2001). However difficulties arose with the preparation and analysis of samples, and considerable effort was required to optimise sample preparation and treatment.

The direct analysis of geldanamycin-containing fermentation broth samples resulted in fouling of the analytical HPLC columns, and resulted in significant costs incurred, with relation to irreparable column damage and process down-time. As a result, alterations to the analytical process had to be examined, with primary focus on reducing the level of potentially contaminating compounds, whilst maintaining the high sample throughput, inherent of HPLC processes. As with the recovery of many antibiotics, solvent-mediated liquid-liquid extraction was the first method attempted to resolve these issues. The extracting solvent, butanol, was however, incompatible with the HPLC process, and caused resolution problems.

Although the extraction process performed well, the resolution difficulties meant it was necessary to remove the butanol prior to sample analysis. A number of methods were examined and it was found that a low pressure evaporation method was best, allowing treatment of a number of samples simultaneously. This method removed the difficulties with the butanol being present in a sample, however, the time required to treat samples in such a process was excessive, a single sample taking 40 minutes to prepare.

Increasing the pore size of the stationary phase of the HPLC column was identified as the most appropriate means of resolving this issue. Increasing the pore size returns benefits on two levels, the increased pore size is less likely to become clogged and fouled and would also reduce the pressure in the system allowing faster mobile phase flowrates and therefore sample throughput. Employing a HPLC analytical column with larger pore size meant that it was possible to re-address direct injection of samples. It was decided that a minimal pre-treatment, dilution of the broth samples, would further reduce the risk of contamination, but would have minimal bearing on sample throughput.

It was during the dilution of broth samples that an interesting phenomenon was encountered. It was found that dilution of broth samples in H_2O lead to a non-linear dilution pattern. Further investigation of this indicated that geldanamycin had a limited solubility in water, and that in order for dilution to be applied for the analysis of all subsequent samples, dilution in the solvent acetonitrile was required. This proved to be a very important finding, and as a result, the analysis procedure for fermentation broth samples simply involved dilution of the broth in acetonitrile, filtration and HPLC analysis for geldanamycin concentration. This process was accurate, and facilitated the high throughput assessment of geldanamycin in fermentation broth samples.

Having developed suitable standard procedures to assess geldanamycin in *Streptomyces* fermentation samples, it was then possible to continue efforts into assessing the recovery of the product from fermentation broth

samples. Frequently in production processes, product yields can be diminished as a result of the recovery processes employed. Geldanamycin has been reported to be recovered using solvent extraction, however in pursuit of processes to aid in the recovery of geldanamycin, it was decided to examine the application of adsorbent resins. It was believed that an appropriate assessment would involve examining their application in two major bioprocessing modes, DownStream Processing (DSP) and *In-Situ* Product Recovery (ISPR).

A preliminary investigation was performed to determine the applicability of resins for the recovery of geldanamycin from fermentation broth at the end of the fermentation. Resins were added to cell-free fermentation broth and the adsorption of product assessed. A series of resins were examined, all of which had application for the recovery of antibiotics. The examination incorporated assessment of the adsorption profiles, capacities and affinities of the resins for the product. It was found that the use of resins was suitable for the recovery of geldanamycin from fermentation broth and that they displayed good affinities, loading capacities and adsorption rates, according to empirical experimentation and adsorption model fitting. The influence of temperature, pH and solvent addition was also examined, since such parameters have been found to frequently impact on the adsorption of compounds in complex systems.

Temperature is frequently seen to have an impact on the amount and rate of compound adsorption by various adsorbents (ten Hulscher and Cornelissen, 1996, Aksu, 2001, Otero *et al.*, 2004), however the effect can be somewhat specific for particular applications or compounds of interest as was seen by Gökmen and Serpen, (2002) and Otero *et al.*, (2005). In these cases, increased adsorption was brought about by opposite changes in adsorption temperature. By examining adsorption at different temperatures it was found that although all temperatures allowed the resins to recover comparable proportions of product, the rate of adsorption was generally higher at higher temperatures. Examining the impact of pre-adsorption pH adjustment, it was found that the proportion of geldanamycin adsorbed was robust to pH change, as may have been

expected, since all resins have a broad pH application range. The adsorption of contaminants however, showed the least proportion of contaminants was adsorbed at pH 7.5, a pH value typical of normal end of fermentation broth.

The discovery that acetonitrile could aid the solubility of geldanamycin in fermentation broths during sample analysis, lead to the examination of what effect inclusion of acetonitrile into the fermentation broth, had on the adsorptive performance of the resins. It was found that adsorption of geldanamycin was reduced with increasing addition of acetonitrile. The acetonitrile may have acted as a competitive force, shifting the affinity from adsorption onto the resins to remaining in the liquid phase. Of more significance was the discovery that increasing the amount of acetonitrile in the pre-adsorption broth reduced the adsorption of contaminants in a more significant manner. As a result, it would seem possible to engineer the adsorption process, based on a low level addition of acetonitrile, to favour the adsorption of geldanamycin over contaminants. This was a very interesting discovery and could have significant implications on further product purification procedures.

Finally the desorption profile of the resins post-adsorption was examined. It was found that the resins selectively desorbed geldanamycin more readily than the contaminating compounds which were more stringently retained. This would imply that a simple bind and elute process could facilitate the generation of a product stream containing large amounts of geldanamycin, and at higher purity, than that which existed in the initial fermentation broth. It would also seem likely, that purity could be further increased by combining treatments, such as modifying the adsorption environment prior to elution. The net result of the assessment of the DSP application of adsorbent resins was that it was established that resins could be applied for geldanamycin recovery, and that two resins in particular, Amberlite XAD-1600 and Diaion HP-20 performed optimally.

The final examination undertaken was an assessment of the ISPR application of the adsorbent resins XAD-1600 and Diaion HP-20. These

resins had displayed impressive activities during DSP applications and were therefore selected for further assessment on that basis. ISPR techniques have been employed for the recovery of an array of compounds and are applied for various reasons. The assessment of the ISPR application of adsorbent resins was not only focused on whether the resins could be applied in such processes and how they, or the production environment were effected, but was also concerned with determining if product yield could be increased. It was believed that ISPR application of resins may allow increased productivity through means of precursor regulation or removal of inhibitory compounds or by limiting the degradation of product during fermentation.

In order to determine if the resins were suitable for ISPR application it was necessary to validate their performance under fermentation conditions. This involved determining if they could adsorb product in the presence of biomass, at fermentation temperatures and under conditions of agitation. In order to assess this, adsorbent resins were added into the fermentations after a period of fermentation time had elapsed and the effect on broth levels of geldanamycin was assessed. It was found that the resins were capable of adsorbing geldanamycin under conditions of fermentation, including in the presence of biomass, and incubated at 28°C, and agitated at 150 rpm. This was indicated by a drop in geldanamycin concentration. Since the resins could adsorb product under fermentation conditions it, was decided to continue the assessment of their application in ISPR processes.

Adding resins at the initiation of the fermentation and assessing product yield showed an increase on control levels of production of approximately 3-fold, depending on resin type, concentration and the time of harvest. It was also noted that as the concentration of resin increased, the broth levels of geldanamycin decreased. This was indicative of the adsorption of product from the production environment by the resins. It was also found that inclusion of the resins from the initiation of fermentation resulted in adsorption of glucose and reduced the rate of biomass generation, but still

consistently returned geldanamycin yields greater than fermentations into which no resins were added.

Assessing the time at which the resins were added to the fermentation returned an important result. It was found that if the fermentation was allowed to progress for a number of days prior to resin addition, a further increase in the product yields was achieved. This was believed to be attributed, in part, to the fact that there would be an increased rate of biomass generation resultant from the resins being absent from the fermentation initially, and thus when the resins are added, there would be a combined benefit elicited.

7.2 Recommendations

This project centred on developing strategies for the successful recovery of geldanamycin from fermentation broth, using adsorbent resins, and as a result required establishing standard procedures for the treatment and analysis of fermentation samples to facilitate this. Some interesting findings were made during this time, which resulted in the development of analytical techniques for the determination of geldanamycin and novel methodologies for its recovery. Although the assessment of the application of adsorbent resins has been made, and techniques to monitor and assess geldanamycin have been developed, there are still some areas of interest which remain, and which may warrant further examination.

7.2.1 Correlation of geldanamycin production with bioactivity

Initially, production in fermentations was qualitatively assessed using the disk diffusion assay. This technique was laborious and led to the development of a microtiter plated-based assay which allowed the quantitative assessment of large numbers of samples in a relatively simple manner. Although this technique was put to limited use when it became apparent that a more accurate evaluation of the specific compounds present in fermentation samples was required, there remains some comparative work for which it could be utilised. It would be beneficial to be able to correlate geldanamycin concentration as determined by HPLC with bioactivity. In this manner the assessment of samples would be two fold, and the relationship between geldanamycin concentration and bioactive effect against test organisms, could be established.

7.2.2 Partitioning potential of geldanamycin in liquid culture

Having determined that geldanamycin has a limited solubility in aqueous solutions, and that addition of acetonitrile addresses this limitation, it would be desirable to further examine the possibility of product partitioning or

precipitation in fermentation. If this occurred in fermentation it may involve the formation of a two-phase system which in turn could have ramifications for product recovery processes. To establish if this were the case would require the production of fermentation broths of very high geldanamycin concentration and may involve treatment of the broth in a similar manner to crystallisation procedures, in an attempt to force product precipitation.

7.2.3 Column-based adsorption of geldanamycin

The assessment of the application of adsorbent resins for the recovery of geldanamycin in a DSP context was essentially a preliminary study, carried out to investigate application of the resins, and took place in batch mode. It would be desirable to explore the application of the resins in column mode, which has been cited previously in literature (Güzeltunç and Ülgen, 2001, Tolonen *et al.*, 2004, Xie *et al.*, 2001). Determination of the adsorption profiles would include the assessment and development of breakthrough curves, which in turn would indicate the saturation capacities of the resins in a similar manner to those techniques employed in the DSP application of resins. Examination of the desorption profiles, would indicate potential for further work on optimisation of product recovery, and moreover, recovery of purer product streams. Optimising a column-based adsorption system would provide information, which would be useful for the development of an external extraction loop, fitted to a fermentation vessel, and perhaps facilitate continuous removal and recovery of product.

It would also be desirable to combine some of the results discovered so far, such as the use of acetonitrile in the pre-adsorption fermentation broth, in experiments to increase selectivity of product adsorption and desorption. Again the aim of such assessments would be to produce purer product streams of high geldanamycin concentration for further study and application.

7.2.4 Optimisation of ISPR-based recovery of geldanamycin

The ISPR application of adsorbent resins has been shown to possess significant benefits for the production of increased yields of geldanamycin per-fermentation. Of particular interest remaining in this section would be the optimisation of the process parameters to increase the selectivity of the adsorption of geldanamycin whilst retaining the benefits to overall yield. It would also be interesting to assess all of the resins which were examined for DownStream Processing application, or alternatively some new resins. XAD-1600 and Diaion HP-20 were selected for examination based on their high capacities and affinities and all-round performance in DSP applications, however it may be interesting to assess the application of other resins which may be more suited to ISPR application, or more selective for geldanamycin.

Membrane fermentations and membrane recovery processes are gaining significance in bioprocessing industries, and can be used to selectively remove biochemicals directly from fermentation broths (Agrawal and Burns, 1997). A simple means by which their application in geldanamycin fermentations could be assessed would be by inclusion of adsorbent resins in dialysis tubing. In this manner, molecular weight cut-off values could be established, which would allow the transport, across the membrane, of geldanamycin and similarly sized compounds, but prevent that of larger contaminating compounds. This would limit the adsorption of contaminants and facilitate the generation of a product stream. Although this is a simple method it has the potential to be effective in delivering the desired goals, Wang, (1983) have detailed a similar idea.

There are however, a number of other means by which membranes could be utilised in such a recovery process. One could use a more specific membrane than dialysis tubing, and set more appropriate means of cut-offs than size exclusion. It is also possible to employ non-porous membranes which would allow establishment of a system analogous to membrane fermentations and facilitate direct extraction of geldanamycin into a suitable solvent, which would be retained on the other side of the

membrane without cross contamination and the associated ill-effects on biomass vitality and survival.

Another method by which the recovery of geldanamycin could be examined is through the use of encapsulation, in particular encapsulation of resins in alginate or some other polymer, or encapsulation of a suitable extracting solvent in liquid-core micro-capsules. Encapsulation of the adsorbent resins may serve to retard the diffusion of larger compounds and thus hinder their adsorption onto the resins contained within the alginate. Encapsulation of a suitable extracting solvent in a liquid-core capsule would allow the recovery of the compound of interest in a similar manner to that achieved using solvents in a liquid-liquid extraction method, but would prevent the contamination of the system resultant from direct contact with the extracting solvent.

It is also believed that modification of the adsorbents themselves may allow further development of the recovery process. The resins applied in the study are commonly available materials, and have undergone no chemical or physical modification to increase performance or selectivity. It may be possible to modify the resins through techniques such as size modification, similar to that described by Nigam and Wang, (1986) to increase mass transfer, which may in turn impact on the success of the recovery process. Alternatively, it may be possible to employ a more selective resin, conferred with specific functional groups or physical chemistries, which may increase selectivity or affinity for the product, similar to those discussed by Wang and Sobnosky, (1985) and Barboza *et al.*, (2001). It may also be possible to use a combination of techniques to elicit a more successful recovery process. Roja *et al.*, (2005) detailed the a combination of elicitation and *In-Situ* adsorption for the enhanced production and recovery of the polysaccharide arabinogalactan. The net result of these modifications would be a more focused extraction or recovery process, and perhaps lead to more adsorption of the compound of interest and less contaminants. This in turn would lead to generation of a more concentrated and purer product stream for further application or study.

7.2.5 Process economics and feasibility study

It has clearly been demonstrated from the work in this document that adsorbent resins can be used for geldanamycin recovery and have potential for further applications for the recovery, and increased yield, of geldanamycin. It would however be interesting to examine their use from an economic feasibility standpoint. It order to achieve this, a comparison of the costs associated with stream treatment, product recovery and material usage would have to be compiled, and the benefits or lack thereof, to the use of adsorbent resins assessed. Performing such an assessment would provide information of the potential for application of these resins in an industrial context, or in a scaled-up process.

7.3 Summary

The work carried out in this document details application of adsorbent resins in novel geldanamycin extraction and recovery methodologies. It also highlights factors which may influence their application and the product yields resultant. The development of analytical methodologies to facilitate, rapid and accurate quantification of geldanamycin from fermentation broths was also detailed. It is clear that considerable potential exists for the application of adsorbent resin-based processes for geldanamycin recovery and it would be of benefit to examine, some of the areas of interest, which have been identified and highlighted in this document. The potential applications of these resins are not limited to geldanamycin, and it is believed their application could be extended to other antibiotics and bioproducts.

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Appendix A – Standard Curves

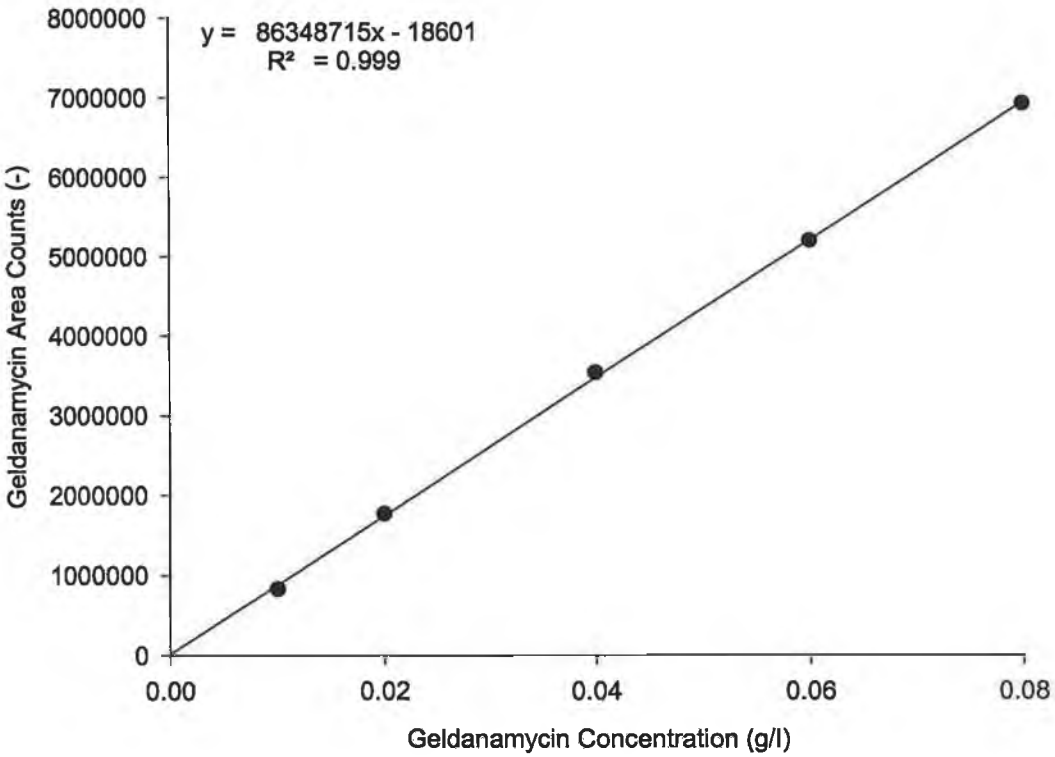

Figure A.1: Standard Curve for Geldanamycin

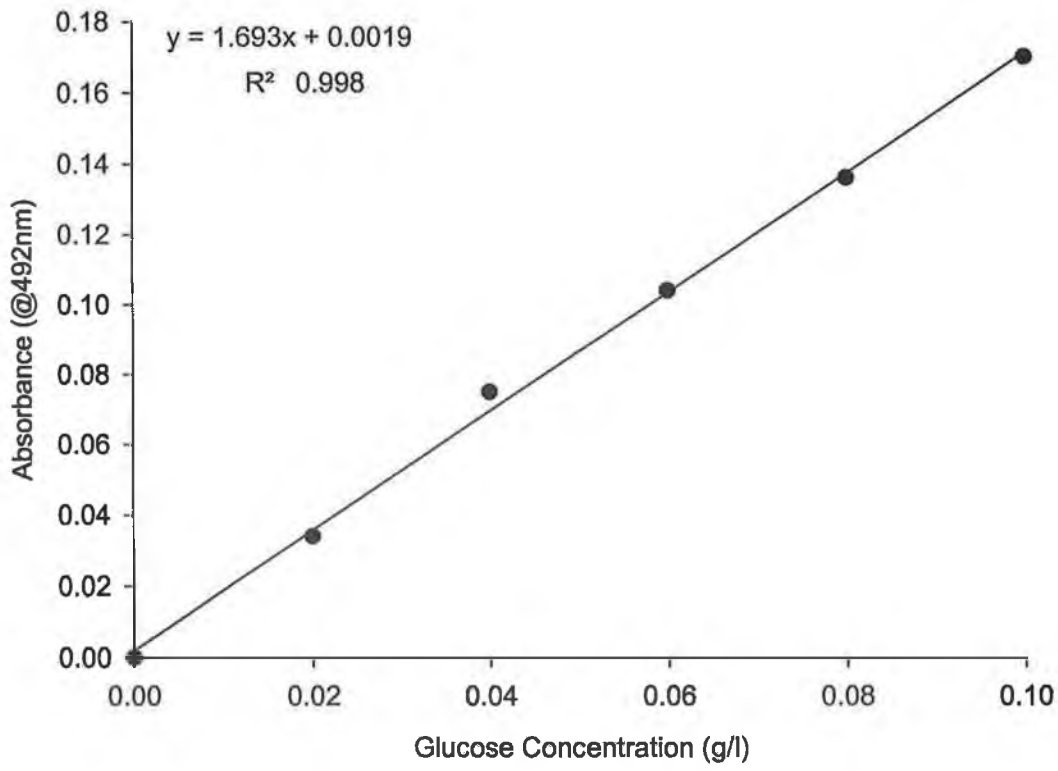

Figure A.2: Standard Curve for Glucose