Production of Pimaricin by *Streptomyces natalensis* in submerged culture.

Thesis
Presented for the Degree of
Master of Science
by Paul Mc Cabe, B.Sc. (hons.).

School of Biological Sciences,
Dublin City University.

December, 1990.

I hereby declare that the research described within this thesis is based entirely upon my own work.

Student: Paul Mc Cabe

Paul Mc Cabe.

Supervisor: _____

Dr. Gerard S. Byrne.

Internal Examiner: Thecla Ryan

Dr. Thecla Ryan.

Date: 10th December 1990

Summary

In this project, the production of the antifungal antibiotic, Pimaricin, by *Streptomyces natalensis* was investigated with a view to developing an Industrial fermentation process. A suitable medium was developed to produce dispersed growth in submerged culture, which was desirable for optimum antibiotic production. A spectrophotometric assay was successfully developed to rapidly determine the concentration of the antibiotic in culture supernates. Results from this assay were shown to be in agreement with the traditional microbiological assay. Factors affecting pimaricin production were investigated in shake flasks. Dosing the fermentation with sodium citrate was shown to have a significant effect on production. A rapid method for screening large numbers of survivors from a mutagenesis programme was developed. EMS and UV mutagenesis provided a number of isolates with increased productivity. Scale-up of the process in a 16 litre stirred fermenter proved difficult with the culture showing a strong requirement for oxygen. However, yields were equivalent to, and in some cases higher than those obtained in shake flasks. Although, improvements were made to the overall process, yields that would make the process economically viable were not obtained.

This project was funded by Biocon Biochemicals Ltd., Cork, Ireland. All of the research work was conducted at Dublin City University, formerly the National Institute for Higher Education (NIHE), Dublin.

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Gerry Byrne, for his help, guidance and constant encouragement throughout this project. I wish both he and his wife the very best in the future.

I want to acknowledge Biocon Biochemicals Ltd., for their financial and technical support during the last two years. In particular, Dr. Roland Cocker and those members of the Fermentation Development Laboratory.

I would like to thank the staff and especially the technicians of the Biology and Chemistry Departments, DCU, for all their help and advice.

Special thanks to Aidan Fitzsimons for his tireless work in preparing graphs and to Lar, the "disk doctor", for his advice on what not to do with floppy disks (they're definetly not water-proof for one thing!).

To all the postgrads. in Biology and Chemistry at DCU for making my stay there very enjoyable, especially to those Mad hikers with whom I spent many an "Arctic" expedition with. さよなら

I would also like to pay tribute to the staff of the Chemical and Life Sciences Department, Waterford RTC, for their advice and encouragement all the those many, many years ago. Long may the excellent work of the RTC's continue.

To my parents, for their moral and financial support down through the years. It is all deeply appreciated.

I must now add (one year later!!), a special thanks to Dr. Thecla Ryan, DCU, for her help in reviewing and correcting this thesis, and especially, for all those long (and we're talking real long!!) distance Faxes. I fear (keeping to true form), that I'll be cutting it fine again!
God...is it true...is it finally finished!!.

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CHAPTER 1.

INTRODUCTION

1.1. The Actinomycetes.

The actinomycetes have always been an unusual group of organisms for the bacterial taxonomists. The number of investigators studying this group of microbes has always been small and for the most part these organisms have been neglected by medical bacteriologists, physiologists and biochemists. It was only in comparatively recent times that their taxonomic position has been decided on (Breed et al., 1957). Bacteriologists considered them as bacteria and mycologists generally considered them as fungi. The diseases caused by them have been described in literature on medical mycology (e.g. Conant et al., 1954). This era is now over and the actinomycetes are generally accepted as procaryotes with filamentous growth habits. They are gram positive with DNA rich in guanine plus cytosine and they form a distinct group on the basis of nucleic acid sequencing (Nolan and Cross, 1988). They have no nuclear membrane, are sensitive to lysozyme for the most part and to the common antibacterial agents. Their cell walls also resemble those of bacteria.

Genera in actinomycetes consist of varied groups whose common feature is the formation of hyphae at some stage of development. The hyphal diameters are much smaller than those of fungi and are close to those of bacteria. The formation of hyphae is often tenuous, in some species of the genus *Mycobacterium* hyphae are never seen, whereas other species have hyphae only in young cultures. Genera of the actinomycetaceae form microcolonies which have very transitory filaments that are difficult to see. Hyphal development is more pronounced in the nocardias but they, too, fragment. The culture time at which this fragmentation occurs varies with the species and determines the types of colonies which form. Colonies in which the filaments break up early tend to be soft or mucoid whereas those whose filaments break up after

a few days have time for a branched mycelium to form and develop a harder texture. In the *Streptomycetaceae*, filament development is strong and fragmentation is rarely found. Examples are in the *Streptomyces*, *Micromonospora* and *Actinoplanes spp.*

Reproduction is usually asexual and in nonhyphal forms, asexual reproduction is by fragmentation or even by the fission of single cells. Where stable hyphae are produced, vegetative reproduction is by well-formed spores resembling fungal arthrospores, produced either free or in sporangia, as in the *Actinoplanaceae*. The free spores are in the form of sporophores and may consist of one, two or more spores in chains arising from primary hyphae. Except for the *Actinomycetaceae*, which contain aerobic or micro-aerophilic genera, the actinomycetes are generally aerobic (Gottlieb, 1973).

The genus *Streptomyces* belongs to the family *Streptomycetacea* and is an aerobic actinomycete with extensive branching substrate and aerial hyphae. Fragmentation of the substrate mycelium is rare (Gordon and Mihm, 1962) and spores are rarely produced on the substrate mycelium. The aerial mycelia usually bear long chains of spores (more than 50), but in certain species relatively short chains (5-10 spores) are the rule e.g. *Streptomyces ramulosus*. The spores are arthrospores formed by the regular septation of hyphae enclosed within a fibrous sheath (Wildermuth, 1970). In some species the sheath persists and coats each detached spore, e.g. *S. viridochromogenes* (Rancourt and Lechevalier, 1964), in others the arthrospores mature within a loose sheath and can become detached from the chain quite free of sheath material, e.g. *S. venezuelae* (Bradley and Ritz, 1968), and *S. griseus* (Vernon,1955; Williams and Sharples, 1970). This sheath is responsible for the surface appearance of streptomycete spores which may appear smooth, warty, spiny or covered with hair like protrusions when viewed under an electron microscope. As

the culture matures the spores may often appear grey in colour or remain white. The spores are heat sensitive, 70°C for 10 minutes proving lethal (Dorokhova et al., 1970). Streptomycetes are common in soil and consequently can contaminate a wide variety of plant and animal products. However, they are rarely the primary agents of spoilage and biodeterioration. There are many reports of strains isolated from diseased animals or human infections but the organisms appear to be opportunists rather than pathogens. Streptomycetes have a characteristic earthy smell which can taint drinking water and foods in cold stores. They show a pronounced antibiotic activity and are the source of several very useful antibiotics.

1.2. Antibiotics and the actinomycetes.

Actinomycetes have been described as the greatest source of antibiotics since Waksman introduced Streptomycetes into his systematic screening programme for new antibiotics in the early 1940s (Okami and Hotta, 1988). They have provided about two-thirds (more than 4000) of the naturally occurring antibiotics discovered, including many of those important in medicine, such as aminoglycosides, chloramphenicol, β-lactams, macrolides and tetracyclines. Actinomycetes produce large numbers of antibiotics with a wide variety of chemical structures (Okami and Hotta, 1988). However, not all actinomycete strains produce antibiotics and no obvious function is known for antibiotics in the life cycle of actinomycetes. Indeed, antibiotics have been regarded as typical secondary metabolites (Bu'lock, 1965). Within the *Actinomycetales*, the *Streptomyces spp.* account for approximately 93% of reported secondary metabolites (Bushell, 1982). This figure probably reflects the relative ease of isolation of the streptomycetes (Bushell, 1983), rather than the product-forming potential of members of other actinomycete genera.

Production of antibiotics by actinomycetes is characterised by strain specific production, structural diversity of the antibiotics, production at idiophase as a mixture of structurally related antibiotic metabolites, self resistance, and instability of productivity.

1.2.1. Strain specificity of antibiotic production.

It has long been known that there are actinomycete strains belonging to the same species that produce antibiotics different from one another; and also that there are strains belonging to different species that produce the same antibiotics (Lechevalier, 1975; Kurylowicz, 1976). Antibiotic production by actinomycetes, therefore is not species specific but strain specific. This means that the taxonomic characterization of actinomycete strains is not useful for the prediction of the type of antibiotics they produce. However, there should be specific genotypes conferring strain-specific antibiotic production. Such specific genotypes might correlate with specific gene clusters for biosynthetic enzymes as reported in streptomycetes that produce actinorhodin (Malpartida and Hopwood, 1986), streptomycin (Ohnuki et al., 1985) and erythromycin (Stanzak et al., 1986). If phenotypes were found and were easy to detect, they should be very useful in searching for new antibiotics.

1.2.2. Diversity of chemical structures of antibiotics.

Antibiotics of actinomycete origin show wide varieties of chemical structures encompassing aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines. These varied structures do not reflect a multiplicity of basic building blocks but rather a series of biochemical reactions such as condensation, methylation, oxidation, polymerisation and reduction. Relatively small numbers of primary metabolites serve as basic building units (Turner, 1973).

It has been shown that antibiotics are biosynthesised through pathways relating to the metabolism of sugars, shikimate, acetate/malonate, nucleosides, mevalonate and amino acids as well as through composite pathways. Antibiotics thus synthesised are usually accumulated at the idiophase (from the late log to stationary phase of growth) as a mixture of structurally related metabolites (Bu'lock, 1967). Antibiotics produced by the actinomycetes are therefore classed as secondary metabolites in contrast to primary metabolites which are produced in the tropophase or logarithimic phases of growth. Primary metabolites are those compounds which are important for growth of the organism and include enzymes and amino acids (Rose, 1979). It seems likely that structural variations of antibiotics occurs mainly in the later steps of biosynthesis. Biosynthetic enzymes involved in the later steps should vary among strains producing members of the same group of antibiotic.

1.2.3. Regulation of antibiotic biosynthesis.

Actinomycetes are usually cultivated in nutritionally rich media, and antibiotics begin to accumulate at idiophase. In addition, antibiotics are sometimes produced in nutritionally limited media or under limiting cultural conditions. This indicates that antibiotic biosynthesis is subject to various regulatory mechanisms. Carbon and nitrogen catabolites, phosphate and bioregulators may all represent major factors in these regulatory mechanisms (Demain et al., 1981).

Carbon catabolite regulation (glucose effect), which refers to the inhibition of synthesis (or activity) of catabolite enzymes by rapidly consumed carbon sources like glucose are well known in various antibiotic fermentations (Demain, 1968). Many biosynthetic enzymes (Gallo and Katz, 1972) involved in the biosynthesis of antibiotics such as actinomycin, kanamycin and puromycin, have been found to be repressed by carbon catabolites. Similarly, ammonia type nitrogen sources and inorganic

phosphate also significantly influence the production of various antibiotics. For example, a high concentration of NH_4^+ represses the catabolism of amino acids, which results in the limited supply of lower fatty acids necessary for the synthesis of the aglycones of the macrolide antibiotics (Omura et al., 1984). The use of slowly catabolised carbon (e.g. starch and soyabean oil) and nitrogen (e.g. soyabean meal) sources, NH^{++}-trapping agents (Omura et al., 1980) and phosphate trapping agents (Omura et al.. 1986) have been reported to be effective for the enhancement of various types of antibiotics.

Cations are another factor influencing antibiotic production. Metal ions, such as, Ca^{++}, Cu^{++}, Mg^{++}, Na^+ and Zn^+ have been shown to stimulate the production of aminoglycoside antibiotics (Garner et al., 1953; Demain and Inamine, 1970; Hotta and Okami, 1976).

1.2.4. Antibiotic resistance.

Antibiotic-producing actinomycetes generally possess the metabolic or structural "target" of their own antibiotics but have resistance mechanisms against them to survive in the presence of the antibiotics they produce (Demain, 1974; Vining, 1979). Consequently, resistance has been regarded as an essential factor for antibiotic production. High yielding antibiotic strains have been obtained by generating and selecting clones or mutants with higher levels of resistance compared to parental strains (Katagiri, 1954; Unowsky and Hoppe, 1978; Crameri and Davies, 1986).

Many actinomycete strains that produce antibiotics of the aminoglycoside group have been shown to have multiple antibiotic resistances (Hotta et al., 1983). Analysis of the mechanisms of the antibiotic resistance revealed that the resistance patterns of the strains tested were unique, being exclusively dependent on resistance determinants such as inactivating enzymes and ribosomal resistance. This

opened up a way of predicting the type of antibiotics that each actinomycete strain can be expected to produce. Similar antibiotic resistance patterns have been reported in macrolide antibiotic producers (Fujisawa and Weisblum, 1981). The biochemical correlation between individual resistance patterns and the types of antibiotics produced suggested a close genetic linkage between antibiotic resistance and biosynthetic genes. This has been supported by cloning experiments that revealed gene clusters comprising biosynthetic and resistance genes (Okami and Hotta, 1988). Antibiotic resistance in actinomycetes is usually stable. However, when a streptomycin producing strain of *S. griseus* was subjected to protoplast regeneration (Yamashita et al., 1985), clones with resistance to high levels of streptomycin were generated. Further study of antibiotic resistance at the genetic level may lead to the generation of highly resistant strains which may over-produce certain types of antibiotics at a commercial level.

1.2.5. Genetics of antibiotic production.

Gene manipulation and conventional genetic analysis of actinomycetes (mainly *Streptomyces*) that produce antibiotics have highlighted the possible involvement of plasmids and clusters of genes in biosynthesis of antibiotics.

Plasmid involvement was first suggested by the loss of antibiotic production after plasmid curing treatment and by genetic mapping data showing no linkage between productivity and chromosomal genotypes (Okanishi et al., 1970; Vivian, 1971; Hopwood, 1978; Okanishi and Umezawa, 1978). Detection of plasmids from various antibiotic-producing strains has strengthened the evidence for plasmid involvement in antibiotic production. However, plasmids have not been shown to be directly involved in antibiotic biosynthesis with some exceptions (Kirby

et al., 1975; Aguilar and Hopwood, 1982; Chater and Bruton, 1985). Recently, giant linear plasmids (130-590 Kb) were detected from various antibiotic-producing strains of *Streptomyces* whose antibiotic production has been suggested by genetic mapping to involve plasmids (Kinashi and Shimaji, 1987).

Development of gene technology using *Streptomyces* host-vector systems (Hopwood et al., 1985) has made it possible to clone antibiotic biosynthesis genes. It was found that antibiotic biosynthesis genes were clustered together with resistance genes in *Streptomyces* strains. These gene clusters seem likely to encompass genes directing enzymes which catalyse steps following the branching from primary metabolic pathways.

These procedures should aid in the understanding of gene organisation, structure and regulation. They may also be of use in solving specific problems in antibiotic fermentations (Baltz, 1982). However, most antibiotic biosynthetic pathways are complex processes which are poorly understood. Thus, in terms of commercial production, random chemically induced mutations continues to be the most widely applied and successful genetic procedure to improve the antibiotic productivity in many actinomycetes especially *Streptomyces* species.

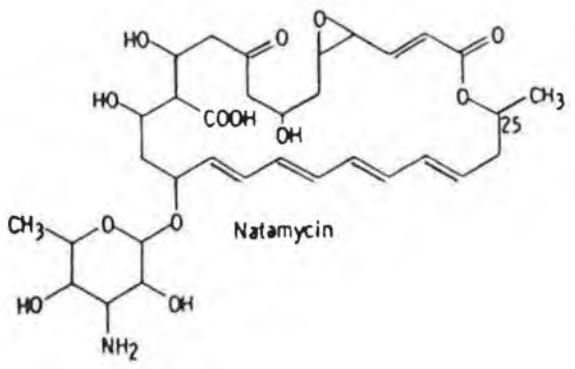
1.3. Pimaricin and the polyene macrolide antibiotics.

The polyene macrolide, Pimaricin, was first discovered in 1955 when a group of Dutch investigators isolated a new, highly active antibiotic from the culture medium of a *Streptomyces* strain (Struyk et al., 1958). The *Streptomyces* strain originated from a soil sample taken in Natal, South Africa and the strain was named *Streptomyces natalensis*. The antibiotic it produced was called "Pimaricin". However, this name was not accepted by the World Health Organisation (WHO) and had to be changed

to "Natamycin". Both names are frequently used in the literature. The antimicrobial effectiveness of this antibiotic against dermatophytes, yeast, fungi and trichomonas species was demonstrated. Further study was immediately conducted into this new antibiotic.

In 1959, American investigators isolated an antibiotic from the culture medium of a particular *Streptomyces* strain (Burns and Holtman, 1959). This organism originated from a soil sample collected in Chattanooga, Tennessee (USA). This *Streptomyces* strain was called *Streptomyces chattanoogensis* and the antibiotic it produced called tennecetin. Tennecetin exhibited antibiotic activity against a variety of fungal organisms (Welsh, 1960). However, investigations on the chemical structure of tennectin revealed the antibiotic was identical to pimaricin (Divekar et al., 1961). As a result the name tennectin disappeared from the literature. Commercially, pimaricin is now produced from either *Streptomyces natalensis* or *Streptomyces gilvosporeus*.

The polyene macrolide antibiotics form a large group of antimicrobial substances belonging to the macrolide class of antibiotics (Berdy, 1974). Their chemical structure is characterised by a 26-38-membered macrolide ring closed by a lactone bond similar to that found in nonpolyene macrolides but much larger in size. The distinctive characteristic of polyene macrolide antibiotics is a polyene chromophore of three to seven alternate double bonds that forms part of the macrolide ring. Figure 1.1. shows the structure of pimaricin, the polyene macrolide under investigation in this project. This chromophore is absent from the nonpolyene macrolides. The polyene chromophore has a strong ultraviolet and short visible radiation absorption at the 280 to 410 nm region and this facilitates characterisation of the polyene macrolide antibiotics.



Natamycin

Fig. 1.1.

All polyene macrolides show spectra of the same pattern in which the main absorption band is resolved into four tall narrow peaks. Figure 1.2. shows the spectrum for pimaricin.

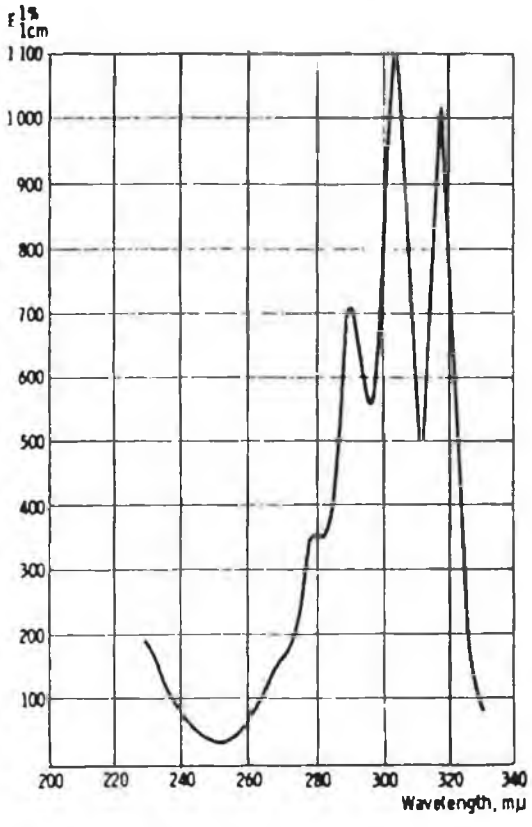


Fig.1.2.

According to the characteristic absorption peaks of the different polyenes, they may be classified as trienes, tetraenes, pentaenes, hexaenes and heptaenes (Mechlinski, 1973). Pimaricin is a tetraene. Most polyene macrolides carry an aminosugar moiety attached by a glycoside bond to the macrolide ring. Two such aminosugars moieties have been found, namely mycosamine and perosamine. With pimaricin, a

mycosamine moiety is linked to the macrolide ring which is the case for most polyene macrolides.

Biosynthesis of the polyene macrolide group has not been studied in detail (Martin, 1979). Little is known about the biosynthetic intermediates and still less about the enzymes involved and the regulatory mechanisms that control them. The complexity of enzymes and genetic controls has been discussed earlier for antibiotics produced by the actinomycetes. From the biosynthetic point of view, both polyene and nonpolyene macrolides are almost identical. Both types are formed through the so-called "Polyketide pathway" involving a head-to-tail condensation of active two- and three-carbon units to form the macrolide ring, to which the macrolide sugars are later attached. There is some evidence to suggest that all macrolides are synthesised by mechanisms similar to that in fatty acid synthesis (Omura and Takeshima, 1974). The polyene macrolide antibiotics are essentially antifungal agents in contrast to the nonpolyene macrolides which are antibacterial (Martin, 1979). It is known that the primary site of action of the polyene macrolide antibiotics are the sterols of the sensitive eucaryotic cells. The polyene-induced distortion of selective membrane permeability results in a leakage of potassium and magnesium ions and a decrease in macromolecule synthesis leading to destruction of the cells (Liras and Lampen, 1974).

In its solid state, pimaricin exhibits a crystalline structure. Temperatures up to 120°C do not impair the antibiotic's activity as long as exposure does not exceed 1 hour. This is of importance when pimaricin media are sterilised (Raab, 1972). In the dry state pimaricin is relatively stable and when stored at room temperature or at 37°C, protected from ultraviolet light, there is no loss of antimicrobial activity. Pimaricin may be inactivated under the influence of UV light with wavelengths of 300-350 nm (Posthuma, 1965a; Posthuma, 1965b). In

aqueous solutions, pimaricin reacts amphoterically. It has an isoelectric point of pH 6.5 and at pH values between 5.0 and 9.0, solutions of pimaricin are quite stable when stored in the dark. At the extreme pH ranges, pimaricin is rapidly inactivated.

Pimaricin exhibits good solubility in polar organic solvents and rather poor solubility in water. In some instances, the presence of a low quantity of water may increase its solubility (Raab, 1972). Suitable solvents for pimaricin are glacial acetic acid, methanol, butanol and propylene glycol (Clark, 1964). Methanol and butanol are used frequently when solubilising other polyene macrolide antibiotics such as candicidin (Liu, et al., 1975). Pimaricin is soluble in the higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol and various oils (Struyk and Waisvisz, 1975).

The most common method used to determine the presence of pimaricin is the microbiological assay. This is the classical method used to determine the biological activity of most antibiotics. However, as with all polyene macrolide antibiotics, pimaricin exhibits strong UV absorption and this can also be used as a basis of physical detection. Both these assay methods were investigated in detail during the course of this project. Other methods which have been used to detect pimaricin in food such as cheese, are thin-layer chromatography and liquid chromatography (Thomas, 1976).

Early studies indicated that the ability to produce polyene macrolide antibiotics is widespread among the species of streptomycetes (Ball et al., 1957). The exclusive distribution of production of polyene macrolide antibiotics among streptomycetes may be related to their postulated role as chemical components of the sheath of aerial mycelium (Cherny et al., 1972). This may have implications for recovery of polyene macrolide antibiotics from fermentation broths.

The polyene macrolides, including pimaricin, are used as antifungal agents in the treatment of external and deep seated mycoses. They have also been used in conjunction with other drugs in which they appear to facilitate a more efficient uptake of the drugs (Kobayashi et al., 1972; Medoff et al., 1972). As a therapeutic agent, polyene macrolides are somewhat toxic making it difficult to administer large doses. However, in spite of their toxicity, polyene macrolides have a good market as antifungal agents, especially for topical use (Martin, 1979). Polyene macrolide antibiotics have also found use as food preservatives in the food and dairy industries. In the dairy industry, pimaricin is applied in cheese coatings and is superior to alternative products in preventing mold formation without affecting the taste and appearance of the cheese. Because it is an antifungal agent, important ripening and flavouring bacteria are not harmed (De Ruig and Van Oostrom, 1987; De Ruig, 1987).

1.4. Growth of actinomycetes in submerged culture.

The lack of reproducibility of product formation in liquid culture is well known for the actinomycetes (Nisbet, 1982). In many cases this may be due to the diversity of hyphal morphology obtained in liquid cultures. This may range from "pellets" of mycelium up to 0.5 cm through to cultures consisting of fragments of hyphae of 2-3 mm in length. The tendency to form fragments in submerged, agitated cultures has been correlated with loss of antibiotic producing ability upon transfer from agar to liquid culture (Shomura et al., 1979). Mycelia grown in more dilute complex medium tend to be less "fragile" than those produced in medium developed for biomass production. The apparent effect of nutrient concentration on this tendency to fragment has not been fully explained.

The pelleted growth form is frequently observed in submerged liquid culture. Most pelleting strains will also produce filamentous cultures,

the morphology depending on inoculum concentration, dissolved oxygen tension, culture shear, pH and medium composition. Many studies have been carried out on fungal pellets, but very little information is available for actinomycetes (Standbury and Whitaker, 1984). The production of fungal secondary metabolites is inhibited by pellet formation (Smith and Calam, 1980), whereas pellet morphology is usually essential for tricarboxylic acid (TCA)-cycle associated organic acid secretion (Al Obaidi and Berry, 1980). It appears that similar principles apply to actinomycete cultures. In fungi and actinomycetes, pellet structure varies between members of different species, ranging from densely packed mycelium, where oxygen supply occurs by molecular diffusion to loosely structured pellets into which the penetration of currents by turbulence in the medium can increase the supply of oxygen to the interior (Pirt, 1975).

It appears that the two extremes of submerged culture morphology, pelleted and fragmented hyphae, are unsuitable for many types of product formation, particularly if antibiotic production is desired. The intermediate type of well dispersed exponentially growing mycelium is, therefore, the morphology of choice. Most streptomycetes have an oxidative metabolism, especially *Streptomyces*. Therefore the importance of aeration and agitation, in both shake flasks and stirred fermenters, cannot be understated. This is discussed in more detail later.

1.5. The fermentation process for polyene macrolide antibiotics

Development of the industrial production of polyene macrolides has been largely empirical as large-scale production was developed prior to the establishment of the biosynthetic pathways leading to the formation of antibiotics (Martin, 1979).

1.5.1. Inoculum development.

Inoculum development procedures must be standardised as these affect subsequent growth and production capabilities (Calam, 1976; Meyrath and Suchanek, 1972). As already mentioned, little information is available concerning the growth of actinomycetes in submerged culture. However, it does appear that growth morphology is similar to that of fungi and that a balance is required between pelleted and fragmented forms. In some polyene macrolide fermentations the amount of mycelia inoculated affects overall antibiotic production. In the candicidin fermentation, the larger the inoculum (washed mycelia), the higher the yield after 5 days of fermentation. A smaller inoculum could be used only if extra yeast extract was added in the production medium (Liu et al., 1975).

In the case of the pimaricin fermentation it is important to determine the optimum inoculum level and also to decide whether a mycelium or spore inoculum is more advantageous. For *Streptomyces* and other actinomycetes a spore inoculum is a convenient standardised method of inoculating culture media and spore numbers can be determined by plating out the suspension or by using counting chambers (Collins and Lyne, 1979). The inoculum itself should be designed to give optimum growth rather than antibiotic production. For this purpose the composition of the inoculum may differ from the production medium.

1.5.2. Production media.

Optimisation of medium formulation has usually been performed by changing nutritional parameters, individually or in combination.

The most frequently used carbon source is glucose. Studies have shown that glucose, or substrates which produce glucose after hydrolysis, support the highest yield of several polyenes (Brewer and Frazier, 1962; Acker and Lechevalier, 1954; Ethiraj, 1969; Abou-Zeid, 1973; Tereshin, 1976). British gum (a dextrin) was used instead of glucose in the

amphotericin B fermentation to increase the ratio of amphotericin B to amphotericin A. Starch is also a good carbon source for nystatin production (Tereshin, 1976). However, the high concentrations of glucose required for maximal polyene production in the candicidin fermentation (Liu et al., 1975), appear to inhibit antibiotic synthesis by catabolite regulation. This has been discussed as a general problem with antibiotics produced by the actinomycetes. Slow feeding of glucose, on the other hand, resulted in an increased synthesis of the two polyene antibiotics, candicidin and candihexin (Martin and Mc Daniel, 1974). Similar increases in antibiotic synthesis when catabolite repression is by-passed by glucose feeding has been described in a large number of fermentations (Demain, 1968; Martin and Mc Daniel, 1977).

Other important carbon sources are the short-chain organic acids and alcohols which are biosynthetic precursors of polyene macrolide antibiotics. Acetate, propionate, malonate, malate, lactate, succinate and citrate are unable to support antibiotic synthesis on their own (Acker and Lechevalier, 1954; Abou-Zeid, 1973). When acetate, propionate, or malonate, however, is added to a glucose basal medium, they stimulate candicidin synthesis (Martin and Mc Daniel, 1976). It is thought that glucose provides, in addition to acetate and propionate, other precursors of the aminosugar and aromatic moieties which are essential for antibiotic production.

Lower alcohols behave in a similar fashion. *n*-propanol is an affective stimulator of candicidin synthesis (Martin and Mc Daniel, 1976).

A complex nitrogen source is preferentially used in polyene macrolide production. Soya bean meal or soya peptone are probably the best nitrogen sources because of their slow hydrolysis. Other complex sources, such as corn meal, cottonseed meal, corn-steep liquor, casein and its hydrolysates, distiller's solubles and yeast extract have also

been used. Phosphate content of these media is of special relevance since phosphate is strongly inhibitory for polyene production. Also important is the content of trace metals, especially divalent cations some of which stimulate, and others which inhibit polyene production. Inorganic salts are not adequate nitrogen sources; some amino acids are good but their industrial use is uneconomical.

1.5.3. Agitation and aeration.

As already mentioned oxygen requirement for growth and antibiotic production has to be met by adequate agitation and aeration so that microbial metabolism is not limited by lack of oxygen. It is well established that fermentations of polyene macrolides are strongly aerobic processes. Studies have been carried out in shake flasks and fermenters. In flasks, the use of baffles is often required for optimal production (Mc Daniel and Bailey, 1969). For candicidin production in fermenters, a minimal dissolved oxygen level of 20% of saturation has to be maintained throughout the fermentation. Production was decreased at a dissolved oxygen tension below 20% (Ethiraj, 1969). At 40% saturation and above, the yields were constant.

Consumption of oxygen in complex media is very rapid during the growth phase, thus necessitating higher agitation rates. A mixing speed of 300 rpm. has been reported in the nystatin fermentation (Lopatnev et al., 1973). In the candicidin fermentation, 400 to 420 rpm. was considered to give optimal production (Martin and Mc Daniel, 1974). Dissolved oxygen concentrations above 50% of saturation were thus maintained (Martin and Mc Daniel, 1975).

This strong requirement for oxygen occurs only during the growth phase and the transition phase, decreasing during the antibiotic production phase or idiophase. Since auto-oxidation of most polyenes occurs easily (Martin, 1979), it seems probable that maintaining high

aeration during the production phase is unnecessary.

1.5.4. Dynamics of the fermentation.

The concept of secondary metabolism, as opposed to primary metabolism, becomes less and less adequate as progress is made in the field of regulation and the production of the so-called "secondary metabolites". Biosynthesis of these metabolites is not biochemically "peculiar" and the enzymic and regulatory mechanisms involved are similar to those of the primary metabolism of the cell. These secondary metabolites are produced by single species or strains, and have no general function in all living cells (Martin, 1979). It is therefore more proper to refer to the two phases as the growth phase and the production phase. In the production of polyene macrolides in complex medium, these two phases are clearly distinguishable (Martin and Mc Daniel, 1975).

It has been proposed that it is the specific growth rate of the culture that controls the onset of secondary metabolism (Bu'lock, 1974). The growth rate of the culture has to decrease below a certain value in order for secondary metabolism to be derepressed.

There is a correlation between the change in oxygen-uptake rate and the onset of the synthesis of polyene macrolides. This was shown for the candicidin fermentation (Martin and Mc Daniel, 1975). Cell growth increases rapidly during the growth phase and in parallel with oxygen uptake. It would appear that some essential nutrient is depleted at this time and that this is phosphate in most cases. Phosphate exhaustion occurs a few hours before the culture reaches the transition stage in the candicidin fermentation. Following phosphate exhaustion a change in the metabolism occurs. Synthesis of RNA decreases rapidly, as does the intracellular ATP pool. Both the levels of RNA synthesis and the ATP pool remain very low during the production phase. It is, therefore, speculated that either phosphate or ATP is the regulatory signal that

controls the onset of polyene macrolide synthesis. The production phase is usually accompanied by a decreased rate of metabolism and can be extended for long periods if enough glucose is supplied (Liu et al., 1975).

1.6. Regulation of biosynthesis of polyene macrolides.

Regulation of antibiotics synthesised by the actinomycetes has already been briefly discussed, however, this section describes those mechanisms which regulate the biosynthesis of the polyene macrolide group. Regulation of antibiotic biosynthesis is of great importance when considering the fermentation process of the polyene macrolide antibiotics.

In wild-type microbial strains regulatory mechanisms avoid the waste of useful metabolic intermediates. Some regulatory mechanisms have been more closely studied than others, but there is evidence which suggests that phosphate regulation, catabolite regulation and end-product regulation do play some role in the biosynthesis of polyene macrolides.

1.6.1. Phosphate.

Phosphate depresses the synthesis of antibiotics belonging to different biosynthetic groups. Biosynthesis of polyene macrolides is especially sensitive to phosphate. Phosphate concentrations of 5 mM and above strongly inhibit polyene macrolide synthesis, whereas optimal phosphate concentrations for growth are up to 300 mM (Liu et al., 1975; Martin and Mc Daniel, 1976). Industrial production of these antibiotics has to be carried out under phosphate-limiting conditions and the phosphate content of industrial media is therefore very important.

Several mechanisms have been proposed to explain the phosphate regulatory effect. These include the shifting of carbohydrate catabolic

pathways (Hostalek, 1964), inhibition and repression of biosynthetic phosphatases (Miller and Walker, 1970; Robbers et al., 1972) and limitation of the formation of inducers (Robbers et al., 1972). However, there is no clear understanding of the mechanisms involved. Phosphate in the plating media could be used to select for derepressed mutants which in turn may over-produce a particular antibiotic.

1.6.2. Catabolite Regulation.

Several researchers have observed that use of high concentrations of glucose results in a lower production of polyenes than when the same concentration of glucose is added in several fractions. Slow feeding of glucose results in an increased synthesis of the polyenes candidin and candihexin (Martin and Mc Daniel, 1974). When the glucose concentration in the fermenter was maintained at 5 g/l or 15 g/l, there was a higher production of polyenes than in the control fermentations in which glucose was added initially at 60 g/l. In slow feeding fermentations, glucose was utilised at a higher rate, but biomass accumulation was smaller suggesting that carbon was channelled into polyene formation (Martin and Mc Daniel, 1974). Periodical addition of sugars has been used in the production of the polyene macrolides amphotericin B (Brewer and Frazier, 1962). High concentrations of glucose during the growth phase probably favour a rapid glycolysis and biomass accumulation, but glucose concentrations should be low when production starts (Martin, 1979).

Thus it appears that polyene macrolide synthesis is subject to carbon catabolite regulation, this being by-passed by slow feeding of glucose. Catabolite repression can also be useful as a selection system for mutants that can produce high levels of an antibiotic even in the presence of high levels of glucose.

1.6.3. Metal ions.

It has already been mentioned that various metal ions affect the production of secondary metabolites produced by actinomycetes. Researchers have shown that some metal ions have a profound effect on the biosynthesis of candicidin (Liu et al., 1975). The maximum stimulatory effect on candicidin synthesis depended on the type of metal salts and the concentrations used. The most effective salts were ferrous sulphate, zinc sulphate and magnesium carbonate. At the metal ion concentration which gave highest candicidin yields, a significant suppression of mycelial growth of *Streptomyces griseus* was always observed. The suppression of mycelial growth was not caused by changes in other parameters, such as pH, due to the addition of the metal ion.

It was noted that, like phosphate, zinc sulphate also exerted its effect on both primary and secondary metabolic activity of the organism. The addition of zinc sulphate at the time of inoculation caused both a sharp increase in the rate of antibiotic synthesis and accelerated rate of sugar utilisation, but suppressed mycelial growth. Studies indicated that the optimum zinc ion concentration for both mycelial growth and candicidin production was around 5×10^{-6}M. Concentrations higher than this had an inhibitory effect on production.

1.6.4. Feedback Regulation.

Biosynthesis of a large number of antibiotics is sensitive to the accumulation of its own antibiotic (Jones and Westlake, 1974; Liu et al., 1977; Kominek, 1975). In the polyene macrolides, it has been reported that addition of fungicidin to a *Streptomyces noursei* culture that synthesises both cycloheximide and fungicidin inhibits production of fungicidin but stimulates cycloheximide synthesis (Spizek et al., 1965). It was proposed that fungicidin and cycloheximide are derived from a common precursor, namely malonate. Incorporation of malonate into

fungicidin is inhibited by fungicidin addition and, therefore the precursor is channelled into cycloheximide. Addition of cycloheximide, in turn, has the opposite effect. Similar results were obtained for the candihexin fermentation (Martin and Mc Daniel, unpublished results). When exogenous candicidin was added to a candihexin-producing culture of *S. viridoflavis*, the concentration of the total antibiotic in the broth was maintained, suggesting that it either causes feedback inhibition of *de novo* antibiotic synthesis or is degraded in the fermentation. Using mutation and selection it may be possible to select for mutants which have overcome feedback inhibition and continue to produce the antibiotic even in the presence of high concentrations of the antibiotic.

1.7. Strain development of antibiotic producing streptomycetes.

Induced mutagenesis remains the single most versatile and widely applicable genetic procedure for strain development to improve antibiotic yields in *Streptomyces* for several reasons. First, it is very flexible in that it can be used with any species regardless of the state of knowledge of the parameters that influence antibiotic yields. A second advantage is that methodologies for chemical mutagenesis are usually simple. Procedures can be rapidly developed which can be used for many different species. A third advantage is that it can be very efficient. With the most potent mutagenic agent, a population of cells surviving the mutagenic treatment can be obtained all of which, on average, contain one mutation affecting antibiotic yield (Baltz and Stonesifer, 1984).

The techniques associated with the isolation of improved mutants focus on the alteration of the producing organism to a culture which permits a more economic fermentation, in terms of grams of product isolated from the culture broth as well as the cost of the fermentation

(Normansell, 1984).

As described earlier, antibiotics are most frequently the end product of often very complex, multigenic biosynthetic pathways. As secondary metabolites they form a diverse group of compounds often only produced under very specific cultural conditions. Indeed, in *Streptomyces* as in other organisms, many of the largest improvements in yields are achieved by careful adjustment of fermentation conditions such as nutrient availability, temperature, pH, aeration, etc. (Kralovcova et al., 1984; Mandal et al., 1983). It is thus important to remember that improved strains may require special conditions to be able to express a beneficial mutation.

1.7.1. Genetic basis of strain improvement methods.

To generate an improved strain requires altering the informational content of the DNA in such a way as to increase the levels of enzymes involved in biosynthesis, eliminate or reduce the effects of control mechanisms, eliminate detrimental characteristics of the organism, or generate a novel and more efficient route to the desired product. All of these limitations to the rate at which an antibiotic can be produced are controlled by the genetic information of the organism, and it is here that the desired change must be induced.

It is now possible to choose from a wide variety of mutagens, ones which will give the required mutation (e.g. deletion, base change, frame-shift, duplication, clustered etc). In this project, ultra-violet light and ethyl methanesulphonate (EMS) were the two mutagens used.

UV light induces all types of base pair substitutions (Coulondre and Miller, 1977; Miller, 1983) by error-prone repair or replication of lesions which survive error-free excision. UV light is mutagenic for *Streptomyces coelicolor* (Clark and Hopwood, 1976) and *S. clavuligerus* (Saunders and Holt, 1982; Saunders et al., 1982). UV-sensitive mutants

of *S. coelicolor* have been mapped (Harold and Hopwood, 1970) and appear to be defective in pyrimidine dimer excision. It has also been demonstrated that caffeine, an inhibitor of pyrimidine dimer excision (Fong and Bockrath, 1979) has a substantial mutator effect in conjunction with UV treatment (Saunders and Holt, 1982).

Ethyl methanesulphonate is an alkylating agent which causes mutations primarily by O^6-alkylation of guanosine residues in DNA. The alkylations cause shifts in base pairing which results in GC-AT transition mutations (Drake and Baltz, 1976; Coulondre and Miller, 1977). In procaryotes, EMS generally causes such mutations by direct misincorporation of bases during DNA replication independent of error-prone repair systems (Drake and Baltz, 1976). EMS is a potent mutagen for *Streptomyces fradiae* and other *Streptomyces* species (Baltz, 1986). The development of genetic manipulation techniques for *Streptomyces* has permitted a complete reappraisal of the achievable targets in strain improvement. However, these techniques were not used in this project and are not discussed here.

1.7.2. Factors affecting screening strategy.

When devising a screening method for isolating mutants of improved productivity a number of considerations must be taken into account.

The nature and characteristics of the antibiotic itself can have a major influence on the methods used for strain improvement. In most strain improvement programmes large numbers of isolates will be screened, so rapid methods of assay, such as chemical assays, are advantageous. Direct assay of antibiotic activity using a sensitive test organism can also be adapted to very rapid screens. However, the more rapid the screen the less accurate it is. It is also important to ensure that the assay does not highlight false positives, e.g. over-production of

compounds with a similar chemistry. Direct assessment of antibiotic activity is perhaps the best way of reducing the frequency of such occurrences.

The number of genetic targets may influence the mutability of an antibiotic. The total number of genes affecting production may total in excess of 100 (at least 1% of genes in a *Streptomyces* genome). However, in any one strain, at any one time there is only a single rate-limiting step. To improve antibiotic yield this rate limitation must be overcome and this therefore, reduces the number of available targets. From this it is clear that the number of potentially beneficial mutations is small and so the frequency of their occurrence will also be small. Consequently the majority of methods for isolating improved mutants have been devised to permit screening of large numbers of isolates in as short a time as possible.

When the probability of producing an improved strain is low, as in the case of random mutation and selection, then the frequency of its occurrence in a given population is also low. Under these circumstances the screen must be designed to test large numbers of cultures in the shortest possible time. The feature common to all these screens is that accuracy and reliability will decrease as the number of strains tested increases.

1.7.3. Screening methods.

Shake flask screens have for many years been the main method employed by the fermentation industry to isolate improved mutants and is still widespread today. These techniques have been successful, but they are labour intensive and very time consuming. All shake flask screens rely on direct assessment of antibiotic yield either by biological or chemical means.

Normal usage of random screens involves the mutagenic treatment of

a population of cells (usually spores in the case of *Streptomyces*). It is important to ensure that the targets being mutagenised are uninucleate since the expression of a mutant phenotype may be masked if the culture is not homogeneous (Rowlands and Normansell, 1983). Following mutagenesis the cells are allowed to develop as individual colonies on the surface of agar and each colony is then used as a source of inoculum for a shake flask.

The use of shake flask screens as a means of assaying productivity has many advantages and disadvantages. It will permit the testing of, perhaps, several thousand cultures per week, although this may be limited by shaker space and available manpower. Liquid cultures also allow the widest variety of product assays to be used, including biological diffusion assays. Mutants isolated from this protocol which consistently over-produce the antibiotic can then be investigated at different levels of scale-up. The major drawback is the reliability with which an improved strain can be identified within a variable background. One way in which reliability can be improved is to increase the number of replicates of each culture, but of course this will reduce the total number of cultures which can be screened.

Plate-based screens allow for colonial productivity to be directly assessed. This technique allows for a dramatic increase in throughput of cultures, and although it was designed for the assessment of fungal cultures, it is also applicable to *Streptomyces* screening. Many plate-based screens exist but they all have in common the assessment of cultures growing on solid support, such as agar, and rely on diffusion of antibiotic away from the producing colony and into a population of antibiotic-sensitive organisms. The simplest plate-based assay is the "overlay technique", in which colonies of the producing culture are overlaid with agar containing the test organism. Upward diffusion of the antibiotic into the overlay results in a clear zone of inhibition. This

procedure has been used in the isolation of mutants of *Acremonium chrysogenum* (Chang and Elander, 1979) capable of over-producing cephalosporin C and for the isolation of improved mutants of *Penicillium chrysogenum* (Ball and Mc Gonagle, 1978). A variation of this technique involves the growth of the colony on an agar plug and the subsequent transfer of this plug to an agar medium containing the test organism. The antibiotic will diffuse from the plug to give a zone of inhibition. This procedure has been used to isolate improved penicillin-producing mutants of *Aspergillus nidulans* (Ditchburn et al., 1974) and for improved cephalosporin C producers (Trilli et al., 1978). It has also been used to improved streptothricin production *Streptomyces fradiae* (Prakash and Tan, 1977).

The main advantage of the plate-based screens is the large numbers of cultures that can be screened. The main limitation is that screens of this nature are generally of low resolution, and are not efficient at discerning small increments in yields. This is particularly the case when the zone of inhibition for the parental strain is large because, under the conditions provided, a further increase in productivity will only give a small increase in zone diameter. To combat this, the zone diameter can be reduced by either using a less sensitive test organism, including a product-destroying agent in the agar, or by inhibiting the growth of the producing organism. Compounds which inhibit the biosynthesis of the antibiotic e.g. phosphate can also be used.

Another limitation of plate-based screens is separating biomass (a function of colonial size and morphology) from productivity. This problem has been highlighted in a study of *Streptomyces erythreus* (Trilli et al., 1982). The specific productivity of a colony may, however, be defined in terms of "potency index" which is the diameter of the colony relative to the diameter of the zone of inhibition. This has been used

in the work described by Ball and Mc Gonagle, (1978) and Chang and Elander, (1979). The plate-based screening technique, though of limited use, has been used successfully in some *Streptomyces* (Dulaney and Dulaney, 1967; Ichikawa et al., 1971; Santos, 1974; Prakash and Tan, 1977).

Besides random screening, it is also useful to screen for "blocked mutants". Blocked mutants are those which are impaired in antibiotic production and they play an important role in strain improvement programmes. During random screening procedures mutants which are significantly depressed in antibiotic production should be isolated. The frequency of their occurrence may give an indication of the efficiency with which the mutagenic techniques being used can effect an alteration in genes involved in antibiotic biosynthesis.

Genetics and biochemical characterisation of nonproducing mutants can provide valuable information for identifying the most appropriate strain improvement techniques as well as the fermentation conditions for maximum yield. There have been several reports of such investigations in the field of antibiotic production by filamentous fungi and *Streptomyces*, (Rhodes et al., 1981).

Of far greater importance from an industrial viewpoint is that blocked mutants can have a direct influence on screening programmes when they are subjected to mutagen-induced "phenotypic reversion". The reversion or suppression of a nonproducing phenotype, usually with the use of a mutagen, can lead to the isolation of three classes of strains: those producing less than parental; those producing parental, and those producing greater than parental yields of antibiotic. The last of these is of most direct relevance to industrial strain improvement programmes. As a result, nonproducing reversions can be a source of high-yielding strains (Dulaney and Dulaney, 1967; Unowsky and Hoppe, 1978).

The use of blocked mutants enables the application of rapid

screening procedures and also this approach guarantees the isolation of mutants "hit" twice in a gene or genes involved in antibiotic biosynthesis. As described earlier, a major limitation inherent in the use of screens assessing colonial antibiotic productivity on agar is the discernibility of small increases in antibiotic yields in a high-producing background. Blocked mutants would be useful in these screens because background production is very low.

This project was concerned with the use of pimaricin as a food preservative and not as a therapeutic drug. One of the most important aspects of this project was the fermentation process and the production of the antibiotic. It was hoped to improve antibiotic production by fermentation development and also by mutation and selection to generate high-yielding mutants. The first patent on pimaricin and its commercial production is owned by the Dutch company, Gist-Brocades, N.V., who made the original discovery, and the second by a US company, American Cyanamid Corporation. Both patents comprise principally the same method of production. The patent, however, does not give precise information on the production of pimaricin (Sruyk and Waisvisz, 1975) and outside of the patent very little appears to have been published on the production of the antibiotic in submerged culture fermentation. Consequently, development of the pimaricin process in this project was modelled on improvements made to other closely related polyene macrolides. These included Candicidin (Liu et al., 1975), Candidin and Candihexin (Martin and Mc Daniel, 1974) and Amphotericin B (Cheung et al., 1975).

CHAPTER 2.

MATERIALS AND METHODS

2.1. Chemicals.

Chemicals used for antibiotic assays and other biochemical analyses were of Analar or analytical grade, unless otherwise stated. Those used for laboratory cultures were of general purpose reagent grade. Materials used for 250 ml shake flask cultures and the 10 litre fermentations were of industrial or food grade. These were obtained from Biocon Ltd., Ireland. The sources of laboratory chemicals were either B.D.H. Ltd., England, or Reidel-de-Haen AG, Germany. Routine microbiological agar and media were of Oxoid or Merck brand.

Pimaricin, under the commercial name "Delvocid" was obtained from Gist-Brocades, Delft, Holland. Delvocid contains 50% active pimaricin and 50% lactose. Methanol used for pimaricin preparation was general purpose grade obtained from B.D.H. Ltd. Water used for media preparation and for batching the 16 litre fermenter was distilled.

2.2. Bacterial culture.

2.2.1. Source of strain.

Cultures were obtained from the American Type Culture Collection, U.S.A. (ATCC) number 13326, and the Northern Research Laboratory, U.S.A. (NRRL) 2651. These were equivalent to the Central Bureau Voor Schimellcultures, Holland. (C.B.S.) 700.57. The organism was named *Streptomyces gilvosporeus* by ATCC, but as *Streptomyces natalensis* by NRRL.

2.2.2. Culture maintenance.

Stock cultures were maintained on yeast malt extract agar slopes, subcultured regularly and stored at 4°C. Cultures required 10-14 days to grow and sporulate at 26°C. White mycelia, of a white chalky texture, formed initially. In some cases this type of growth prevailed but

generally a dark grey sporulating mass appeared after 14 days incubation.

Yeast-Malt Extract Agar contained; (g/l), yeast extract, 3; malt extract, 3; mycological peptone (Oxoid), 5; glucose, 10; Oxoid agar NO.3, 20. The pH of the agar was adjusted to pH 6.5 before autoclaving. The glucose, malt and yeast extract were of food grade.
Yeast-malt extract broth was also prepared using these ingredients but without agar.

Sporulation media:
Ground Oatmeal agar (ATCC catalogue). Boil 3g of oatmeal for 1 hour in 100 mls of distilled water and filter through muslim. Use filtrate only. Add 20 g/l of agar, boil and autoclave in 20 ml glass universals.

"Sporulation" agar (g/l) (Williams et al., 1974). corn steep solids, 5; starch, 10; $(NH_4)_2SO_4$, 3; NaCl, 3; $CaCO_3$, 3; Oxoid agar No. 3, 13 (Williams et al., 1974).

2.2.3. Detection of contamination.
Bacterial contamination of stock cultures, spore suspensions or fermentation samples was tested for by plating samples onto yeast-malt extract agar and incubating overnight at 26°C.

2.2.4. Pimaricin productivity test.
Stock cultures were routinely checked for pimaricin productivity by inoculating 250 ml shake flasks containing 50 mls of standard medium (section 2.7.1.) with a standard spore inoculum (5x 10^7/ml) and incubating under standard conditions (section 2.4). Pimaricin was then assayed after 5 days growth. Under these conditions up to 400 µg/ml was obtained from stock cultures.

2.3. Spore inoculum preparation.

Streptomyces natalensis was cultured on yeast-malt extract agar slopes (section 2.2.2.) for 14 days at 26°C to give well sporulated growth. Spores and mycelia were washed from the slope with 7 mls of 0.1 M phosphate buffer, pH 7.00 and Triton X-100 (0.01% v/v). 1% (v/v) of this suspension was then used to inoculate 50 mls of medium in 250 ml shake flasks. It was later found that inoculating the flask medium directly from a slope using an inoculating loop was sufficient to give optimum growth and antibiotic production.

2.4. Shake flask cultivation.

Throughout the experimental work, shake-flask cultures were incubated at 30°C on two orbital incubators. One was an LH engineering, single-tier shaker (model MK-X), set at 250 rpm with a throw of 30mm. The second was an LH engineering, 2-tier shaker (Model MK-II/III), set at 150 rpm with a throw of 50 mm. The single-tier model had accommodation for 250 ml flasks only whereas the 2-tier model had accommodation for both 250 ml and 1 litre flasks.

2.5. Fermenter cultivation.

The fermenter used was a Microgen, New Brunswick scientific and had a 16 l gross capacity. This was run with 10 litres of media. Inoculation and sampling was carried out aseptically. The vessel was pressure-tested each time before a run commenced. This was performed by allowing a pressure (10 psi) to build up within the vessel, using air through the sparger. A pressure drop would then indicate a leak, either in the vessel or in the plumbing system. The vessel was sterilised in-place

with steam and maintained at 15 psi, and 121°C for 1 hour or longer. Components such as soya bean meal required longer sterilisation periods. The fermenter was run at 5 psi back-pressure to minimise foaming and reduce the risk of contamination. However, when addition ports were in use, during feeding experiments, a back pressure could not be used. Levels of aeration and agitation used were as indicated with individual experiments in Results section. All fermentations were run at 30°C. Foaming was controlled by the addition of KG-antifoam (Biocon, Ireland). pH was monitored continuously using an in-situ Ingold InFit 764-50 pH probe which was connected to a pH controller and chart recorder, model pH-22 (New Brunswick Scientific). Dissolved oxygen was also monitored using an in-situ galvanic probe, 900 series, which was connected to a dissolved oxygen analyzer and recorder, model DO-50 (New Brunswick Scientific).

2.6. Fermentation media.

2.6.1. Inoculum and production media (g/l).

Glucose, 30; soya peptone, 5; soya bean meal, 20; calcium carbonate, 5; approximate pH 6.80.

Materials used were of food grade and were obtained from Biocon Ltd., Ireland. This medium was used as the standard inoculum and production media for both shake-flask and 16 l fermentations.

MD01 and MD05 were used as alternative carbon sources during the development of the fermentation media (Section 3.4.2.). These are different forms of hydrolysed dextrins, derived from starch, which are graded 1 to 40 (approximately). MD01 has a dextrose equivalence (DE) of 5, whereas, MD05 has a DE of 25. MD01, therefore, is less hydrolysed than MD05 and is similar to starch. Both were supplied by Biocon Ltd.

2.6.2. Patent media (g/l).

(a) Glucose, 30; corn steep powder, 5; ammonium sulphate $(NH_4)_2\ SO_4$, 5; potassium chloride (KCl), 4; di-potassium hydrogen phosphate (K_2HPO_4), 0.2; calcium carbonate ($CaCO_3$), 8.

(b) Beet molasses, 40; lactose, 20; corn steep powder, 10; sodium sulphate (Na_2SO_4), 1; $CaCO_3$, 5.

(c) Mycological peptone (Oxoid), 5; beef extract (Gibco), 5; glucose, 10; sodium chloride (NaCl), 5.

(d) Soya bean meal, 50; soya oil, 5; corn steep powder, 1; glucose, 10; K_2HPO_4, 0.2; $(NH)_2SO_4$, 5; $CaCO_3$, 10.

(e) Soya bean meal, 50; peanut oil, 5; corn steep powder, 1; glucose, 10; K_2HPO_4, 0.2; $(NH_4)_2SO_4$, 5; $CaCO_3$, 10.

2.6.3. Source of media formulations.

Patent media were obtained from the United States Patent, No. 3,892,850 (Struyk and Waisvisz, 1975), entitled "Pimaricin and process of producing same". The inoculum and production medium was developed during the course of this project. The components of this medium were based on those used in the production of other polyene antibiotics, (Martin, J.F. and Mc Daniel, L.E. 1974).

2.6.4. Sterilisation procedures.

Media were sterilised in flasks at 121°C and 15 psi for 20 minutes. Metals which were added to the media were sterilised separately. Pimaricin was filter sterilised through 0.45 µm PTFE membrane filters, Gelman Sciences. Media in the 16 l Microgen fermenter were sterilised

by the passage of steam through hollow baffles and held at 121°C at 15 psi for 1 hour or longer if necessary.

2.7. Analytical procedures.

2.7.1. Estimation of reducing sugars.

Reducing-sugars were estimated using the DNS method (Miller, 1959). Results were expressed as reducing equivalents mg/ml, using glucose as standards.

DNS reagent (g/l).

3,5,-dinitrosalycylic acid, 10; potassium sodium tartarate, 300; sodium hydroxide, 16; were dissolved in 600 mls of water by heating without boiling. The solution was cooled and diluted to 1 litre.

Procedure.

Samples and glucose standards were diluted to 0.3-1.5 mg/ml reducing sugar equivalents. 1 ml samples were added to 1 ml water and 2 mls DNS reagent. The tubes were placed in a boiling water bath for 10 minutes and then cooled. 10 mls of water was then added to each tube, the contents mixed thoroughly and absorbance at 540 nm was measured on a Pye Unicam SP6-550 spectrophotometer. The instrument was set at zero using a reagent blank, made by adding 2 mls of water to 2 mls DNS reagent and treated in the same way as for samples. Reducing-sugars in unknown samples were determined using a standard curve of glucose 0.1-1.5 mg/l plotted against absorbance at 540 nm after DNS treatment.

2.7.2. Quantitative analysis of Pimaricin.

Two assays were used to measure the amount of pimaricin produced by the organism. The first method incorporated the classical method for assaying antibiotics, namely the microbiological assay or bioassay. The second method used was a spectrophotometric assay.

Bioassay Procedure.

This method was adapted from an bioassay already used for the detection of pimaricin, (Raab, W.P. 1972).

An overnight culture of the indicator strain (*Sacchromyces cerevisiae* ATCC 9763) was prepared. This was carried out by loop-inoculating yeast-malt extract broth (Section 2.2.2) from a prepared slope of the indicator strain and then incubating the culture at 30°C for 18-24 hours. After this incubation time 300 mls of molten yeast-malt extract agar was seeded with 0.1 ml of the indicator strain and this was then poured into a glass bottomed, autoclavable, bioassay plate (25mm x 25mm) and allowed to set. Wells were punched at regular intervals with a 7mm sterile cork borer. Particular attention was paid to pouring level agar plates using a level table and spirit level. A random pattern of numbers was used to assign samples (in triplicate) to different wells. Fermentation samples were centrifuged and the supernates diluted in methanol water (2:1). Pimaricin standards were prepared by dissolving 0.1g of Delvocid powder (Section 2.1) in 100 mls of methanol to give a stock concentration of 500 µg/ml pimaricin. Using this, a range of dilutions were prepared in methanol:water (2:1) to give between 50-400 µg/ml pimaricin standards. 150 µl of each sample and standard was placed in their appropriate wells and diffusion allowed to proceed at room temperature for 2 hours. Plates were then placed in a 30°C incubator overnight. Clear zones around the wells, where the indicator strain had been inhibited, were highlighted

by placing the plates on a light box. The diameter of each zone was measured using a vernier calipers to 0.02 mm.

Calculation:

A standard curve was plotted of log concentration versus zone diameter (mm) and unknown samples read from this.

Spectrophotometric Assay Procedure.

This method was adapted from the work carried out by other researchers on the detection of pimaricin in cheese rind (De Ruig and Van Oostrom, 1987). This assay utilizes the UV absorption "finger print" of pimaricin to measure quantities present, (see Figure 2.1.) It can detect down to 1 μg/ml of the antibiotic. Samples of culture supernates were diluted in methanol water (2:1), and the UV absorbance scanned on a Pye Unicam SP-100 UV/VIS spectrophotometer from 340 nm to 300 nm. Aqueous methanol was used to blank the instrument. The height of the characteristic pimaricin peak at 317 nm is directly proportional to the amount present between 1 and 17 μg/ml. Standard pimaricin solutions of 5 μg/ml and 15 μg/ml were run with every batch of samples. The peak heights obtained for these standards were used to calculate the concentration of pimaricin in the unknown samples.

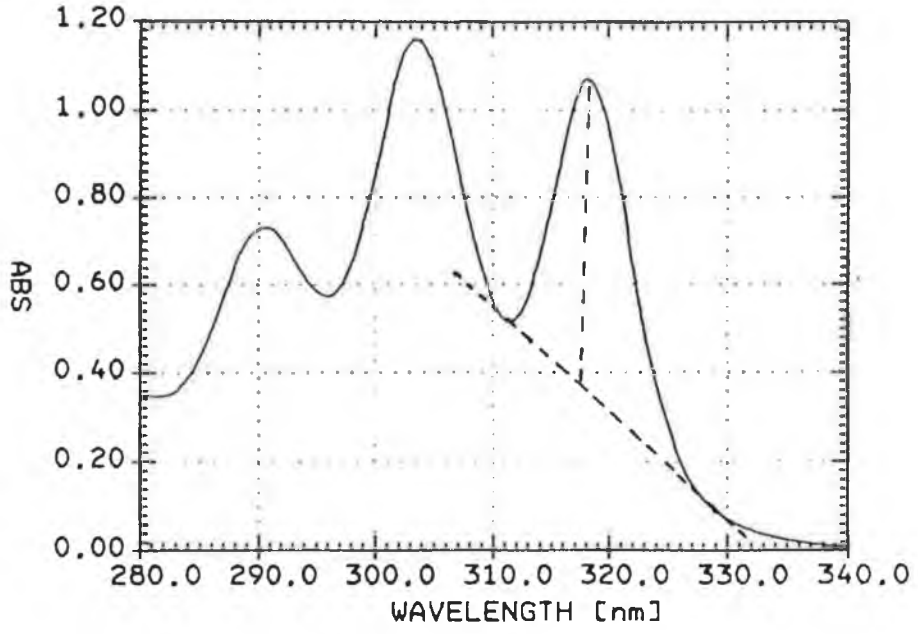


Fig. 2.1. UV absorption spectrum of Pimaricin showing measurement of peak height.

Calculation:

From the plotted chart the absorbance was examined at 317 nm (maximum point), 311 nm (minimum point) and at exactly 329 nm. A base line was drawn from the readings at 329 nm and 311 nm and the peak height at 317 nm was measured. Standards of 5 μg/ml and 15 μg/ml pimaricin were run and an average peak height for the two obtained for 1 μg/ml. This was used to calculate the concentration of pimaricin in the unknown samples as follows;

$$C_s = \frac{P_s \times C_n}{P_n} \times \text{dilution factor}$$

C_s = Concentration of pimaricin in sample (μg/ml).
P_s = Peak height of sample at 317 nm (mm).
P_n = Peak height of pimaricin standard (mm).
C_n = Concentration of pimaricin in standard (μg/ml).

2.8. Mutagenesis procedures.

2.8.1. Ultra-violet mutagenesis.

Two methods were used;

(a) Spore suspension method.

(b) Plate method.

Solutions.

A phosphate buffer (0.05 M, pH 7.00) was prepared by combining 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. Then 30.5 mls of 0.1 M Na_2HPO_4 was added to 19.5 mls of 0.1 M NaH_2PO_4, mixed well and diluted to 100 mls giving 0.05

M. The pH was checked to read pH 7.00. If required 0.01% or 0.1% of the detergent triton X-100 was added to this buffer in order to prevent clumping of spores. If, also, required 1 mg/ml of caffeine was added to this buffer (Section 3.6.1.).

Spore suspension procedure.

A slope of the culture to be mutated was resuspended in 7 mls of 0.05 M phosphate buffer containing 0.1% triton X-100. The suspension was transferred to a sterile test tube, vortexed and allowed to settle. This was carried out to separate clumps of spores from the suspension which were not desirable. Using this homogeneous spore supernate 1.0 ml was transferred to 9 mls of 0.05 M phosphate buffer containing 0.01% triton X-100. This dilution (10 mls of a 1:10) of the spore suspension was transferred to a sterile petri-dish, ready for UV treatment. The ultra-violet lamp used was a model UVG-11 minaralight lamp, short wave UV-254nm. The lamp was positioned 15 cm above the petri-dish and the suspension was exposed to UV light for a certain period (15 minutes was found to almost kill the organism completely at this height) while being stirred occasionally with a sterile glass rod. Using a automatic pipette with sterile tips, 0.1 ml aliquots were removed aseptically and added to 9.9 mls of 0.05 M phosphate buffer with 0.01% triton X-100. Serial dilutions were prepared and plated onto yeast-malt extract agar plates. These plates were incubated at 26°C to detect surviving colonies. All plates were placed in the incubator as quickly as possible to prevent photo-reactivation of the cells. When using caffeine in the plating medium, 1 mg/ml was dissolved in the agar before autoclaving.

A survival-curve for each mutated strain was prepared by plotting the number of survivors against the exposure time. The exposure time equivalent to a 90% kill was determined. Surviving colonies from this

exposure time and longer were checked for pimaricin productivity either on bioassay plates or in shake flasks. Isolates giving increased yields were subcultured onto slopes and re-checked to confirm these results.

Plate procedure.

This was carried out in a similar way to the spore suspension procedure, however the appropriate dilutions were first plated out and the plates then exposed to UV light individually. The plates were then incubated and surviving colonies checked for pimaricin productivity.

2.8.2. Ethyl-methyl-sulphonate (EMS) mutagenesis.

A slope of the culture to be mutated was resuspended as previously described with 7 mls of 0.05 M phosphate buffer and 0.1% triton X-100. Using the particulate-free spore suspension, 5 mls was added to 4.6 mls of 0.05 M phosphate buffer and 0.01% triton X-100 in a sterile reaction vessel. With the aid of an automatic pipette and extreme caution, 0.4 mls of EMS was added to the reaction vessel and this was incubated at 30°C in an oscillating water bath. At appropriate time intervals, (between 0 and 60 mins was sufficient for *Streptomyces natalensis*) 100 µl (0.1 ml) of the reaction mixture was transferred to 10 mls of 10% sodium thiosulphate in sterile glass universal bottles and left for 15 minutes. The sodium thiosulphate was used to neutralise the EMS. Serial dilutions were prepared from this in 0.05 M phosphate buffer and 0.01% triton X-100. Plates were incubated at 26°C until surviving colonies had fully grown. A survival curve was prepared and surviving colonies from a 90% or higher kill rate were checked for productivity as described previously.

A safety protocol was devised for the handling and safe disposal of EMS. All operations were carried out in a fume hood which had an

impervious surface. A covering was place over the hood surface which could soak up any spillages (Whatman Benchkote). Protective clothing, gloves and a mask were worn at all times during the operation. Sodium thiosulphate (6%) was used to soak pipette tips and it was also used to neutralise the EMS in the reaction vessel. To further minimise any risks, 2 mls of concentrated hypochlorite was added to all glass universals containing dilutions and left overnight in the fume hood. All disposable items, such as pipette tips, gloves and benchkote were placed in a biohazard bag for disposal by incineration (Leigh Environmental Co. UK). All neutralised liquid wastes were held for a long period in glass containers containing hypochlorite before being disposed of down waste disposal drains.

2.9 Routine measurements and instrumentation.

pH was measured using an Orion Ionalyser Model 501. Fermentation media and other chemicals were weighed on a Sartorious 1219 MP electronic balance (600g + 0.01g). Centifugation was carried out using a bench-top Heraeus Christ Model 6000 (maximum speed , 5000rpm or 4199g), and a Heraeus Christ model Biofuge A (maximum speed, 13000rpm or 16085 g). All aseptic procedures were conducted in a Laminar flow cabinet (Microflow pathfinder, Inter med). All microscopic examinations were carried out using a Nikon Optiphot microscope.

CHAPTER 3.

RESULTS

3.1 Growth of the Production organism on agar media.

A number of suggestions from the literature were obtained for agar media supporting good growth and sporulation of *Streptomyces* species (Williams et al., 1974; Di Marco and Pennella, 1959). These are listed below.

Plate and slope agar media (g/l):

1. Beef extract, 20; Asparagine, 0.5; Glucose, 10; K_2HPO_4, 0.5; Oxoid agar No. 3, 13.

2. Beef extract, 1.0; Yeast extract, 1.0; Glucose, 2.0; Casein hydrolysate, 2.0; Oxoid agar No. 3, 13.

3. Asparagine, 1.0; Beef extract, 2.0; Glucose, 10; K_2HPO_4 0.25; Oxoid agar No. 3, 13.

4. Corn Steep solids, 5.0; Starch, 10; $(NH_4)_2\ SO_4$, 3.0; NaCl, 3.0; $CaCO_3$, 3.0; Oxoid agar No. 3, 13.

5. Oatmeal agar (see Section 2.2.2).

6. Yeast-malt extract agar (see Section 2.2.2.).

pH of all media was adjusted to pH 6.5 with NaOH.

In this experiment media were prepared as plates and as slopes. They were then inoculated from a culture slope and incubated at 26°C under standard conditions. Growth of *Streptomyces natalensis* appeared sooner on agars Nos. 1, 2, 3 and 6 than it appeared on agars Nos. 4 and 5, eg. 2-4 days against 4-6 days, respectively. A number of different growth morphologies appeared on both plates and slopes when using these media. On plates of media Nos. 1, 2, 3 and 5, after 14 days incubation at 26°C, colonies appeared white and chalky and were embedded into the agar. Some of these colonies had a star-shape appearance and proved difficult to

break up or subculture. When growing on slopes of the same media, the organism grew in a similar fashion but covered the entire slope. On plates of media Nos. 4 and 6, after 14 days at 26°C, colonies formed a grey sporulating mat. These colonies were friable and proved easier to subculture. When growing on slopes of these media the organism covered the entire slope producing the same grey sporulating mat. This type of growth proved ideal for subculturing and for preparing spore suspension inocula for shake flasks. It was found that agar medium No. 6, yeast-malt extract agar, (Section 2.2.2) produced very dense sporulated growth and so was used routinely throughout this project when culturing *Streptomyces natalensis*.

3.2. Growth in shake flasks

There is little information available on the morphology of streptomycetes grown in submerged culture (Williams et al., 1974). It is thought that pelleting does not play as critical a role in streptomycete fermentations as, it does in fungal processes. However, researchers have demonstrated that a wide range of morphological forms are found among the streptomycetes and that these are often influenced by the concentration of spores in the inoculum, the medium composition and the shear forces operating during culture (Lawton et al., 1984). Little information appears to be currently available on the effect of morphological form on product formation by streptomycetes in submerged culture.

Initial studies on the growth and antibiotic production by *Streptomyces natalensis* were carried out using 50 ml aliquots of media in 250 ml shake flasks. The media used for these initial studies are listed in Table 3.1., and were developed using ingredients obtained from Biocon Ltd. Some of these ingredients were also used in the production of similar antifungal antibiotics (Martin, J.F. & Mc Daniel, L.E., 1974).

Table 3.1. Media used to test the growth of *Streptomyces natalensis*,

Medium	Composition (g/l)	pH_1
A	Glucose, 30; Soya peptone, 10.	5.68
B	Lactose, 30; Soya peptone, 10.	6.00
C	Sucrose, 30; Soya flour, 10.	6.25
D	Glucose, 30; Soya flour, 10.	5.82
E	Glucose, 30; Yeast extract, 10.	5.67
F	Glucose, 30; Soya bean meal, 10.	5.60
G	Glucose, 30; soya peptone, 10; $CaCO_3$, 5.	7.35

The media listed were used for both primary and secondary shake flask cultivation. A 1% inoculum, of a slope culture suspension, was used for primary flask cultures. Incubation of flasks was at 30°C, 250 rpm and growth was observed visually each day. After 3 days, a 5% vegetative inoculum from each flask was used to inoculate the secondary flasks. Incubation conditions were the same as for primary flasks. Pimaricin was assayed from samples taken after 3 days for the primary cultures and 5 days for secondary cultures using the bioassay (Section 2.7.2.). These preliminary experiments were later repeated and pimaricin was assayed using the spectrophotometric assay (Section 2.7.2.). Results from this work showed that pelleted growth was observed in all flasks except with medium F, which gave little growth and produced no pimaricin. Large pellets were associated with media A, B, C, D and E and productivity was less than 20 μg/ml. Medium G contained very tiny, dense pellets of growth and produced 350 μg/ml pimaricin. Medium G was the only medium of all 6 to contain $CaCO_3$ and it was thought that this contributed to the dispersal of the mycelia and higher antibiotic production. There was

very little difference in either growth or antibiotic production between primary and secondary flask cultures. Medium G, containing glucose, soya peptone and $CaCO_3$, was used as a starting point for subsequent studies to observe the growth and antibiotic production of *Streptomyces natalensis*.

In another experiment the ratio of these three components were varied and two other nitrogen sources were tested, yeast extract and soya bean meal. These different media combinations are listed in Table 3.2. Primary and secondary shake flasks were again used and incubation conditions were as before. The secondary flasks were inoculated with a 10% (v/v) primary vegetative inoculum after 2 days incubation. The secondary flasks were assayed for pimaricin after 3 days and the growth morphologies of each culture were recorded. The primary flasks were allowed to incubate for 1 further day before assaying and observing growth morphologies.

Table 3.2. Media used to test for pimaricin production by *Streptomyces natalensis*.

Medium	Composition (g/l)	pH_1
G (control)	Glucose, 30; Soya peptone, 10; $CaCO_3$, 5	7.45
H	Glucose, 15; Soya peptone, 10; $CaCO_3$, 5.	7.52
I	Glucose, 15; Soya peptone, 5; $CaCO_3$, 5.	7.53
J	Glucose, 30; Soya peptone, 10; $CaCO_3$, 1.	7.47
K	Glucose, 30; Soya peptone, 5; $CaCO_3$, 5; Yeast extract, 5.	7.48
L	Glucose, 30; Soya peptone, 5; $CaCO_3$, 5; Soya bean meal, 5.	7.46

Primary flask results:

Medium G, H, I, J and K gave rise to pelleted growth. The average diameter of these pellets was recorded as approximately 0.2 mm. Microscopically, these pellets appeared as clumps of densely packed mycelia with some loose hyphae at the periphery. Medium K contained particularly large pellets which may be attributed to the presence of yeast extract. Medium L contained very dispersed mycelial growth with very few pellets. Microscopically, very little clumping was observed with this medium and in general the mycelia were well separated and dispersed throughout. Pimaricin production in medium G, H, I, J and K was low with only 245 μg/ml being obtained as the highest yields. Medium L produced the highest yield of all i.e. between 300 and 350 μg/ml. The control flasks (medium G) produced less than 100 μg/ml which was lower than yields obtained for the same medium in the previous experiment. The reason for this difference is not known. The final pH readings for all primary flasks were between pH 6.98 and pH 7.43.

Secondary flask results:

The growth patterns in the secondary flasks were similar to those observed in the primary flasks. Medium G, H, I, and J produced pellets of similar sizes to those found in the primary flasks. Medium K produced larger pellets, while Medium L again gave very dispersed growth. Pimaricin production was again highest in medium L (approximately 350 μg/ml). All pimaricin assays were carried out using the bioassay (Section 2.7.2). Final pH readings for all secondary flasks were between pH 6.63 and pH 6.93. It was thought that the presence of soya bean meal in Medium L along with $CaCO_3$ may have been responsible for dispersing the growth of *Streptomyces natalensis* in submerged culture. This type of growth appears to be important for antibiotic production. Other researchers have demonstrated that glucose and soya bean meal or soya

peptone are the best carbon and nitrogen sources for the production of polyenes of the candidin-candicidin group (Ethiraj, 1969). Medium L was used as a control medium for the next stages in the study of growth and antibiotic production in submerged culture.

Studies on the performance of the organism when transferred from primary to secondary flasks were later continued using the more accurate spectrophotometric assay to analyse antibiotic yields (Section 2.7.2.). In these studies the primary flask media were prepared according to the United States Patent No. 3,892,850 (Section 2.6.2.). Other results showed that the patent media supported dense, dispersed mycelial growth, even though pimaricin production was very poor. It was hoped that some of these media would provide a well-grown vegetative inoculum for secondary flask cultivation. The secondary flask medium in this case was Medium L from the previous experiments which had already been shown to give high antibiotic yields. In this experiment the primary flasks were grown for 3 days and a 5% inoculum from each patent medium was used to inoculate the secondary flasks. These secondary flasks were then incubated for 5 days and assayed for pimaricin production. Control flasks were set up whereby both primary and secondary flasks contained Medium L (control medium).

Growth in all secondary flasks was good and consisted of dense cultures with very tiny pellets. Pimaricin yields from all secondary flasks were between 175 and 245 μg/ml. The flasks with control Medium L as the inoculum gave the highest yields (245 μg/ml). These results indicate that Medium L itself is capable of producing a good vegetative inoculum for secondary flask cultivation. With experience it was shown that the size of the inoculum should be between 5% and 10% and the inoculum incubation time between 2 and 3 days. Growth and antibiotic production appear to be more dependent on media composition than on inoculum levels or inoculum stage of growth. Further media development

studies were confined to primary flask cultures until a medium was obtained which could be scaled-up to the 10 L fermenter.

However, before proceeding with further media development work, problems were encountered with the pimaricin assaying techniques. Initial shake flask experiments relied solely on the bioassay method to determine the concentration of pimaricin produced. The bioassay, when first developed however, estimated antibiotic yields at greater than 1000 μg/ml for shake flask cultures (See Table 3.3). This was suspiciously high and because no other quantitative assay was available, at the time, it was not possible to substantiate these results. The spectrophotometric assay was, therefore, initially developed as an confirmatory assay for the bioassay. The following section deals with the development work conducted on both assays in order to obtain an overall reliable antibiotic assaying procedure.

3.3 Development and improvement of the assay techniques for pimaricin.

Two assay techniques were developed for the detection of pimaricin, both in solid agar media and in submerged culture, (Section 2.7.2.). The bioassay is the classical method for the detection of antibiotics and was used for some of the initial shake flask studies in this project. The bioassay appeared to over-estimate the level of pimaricin in the cultures. Initially the pimaricin powder, Delvocid (Section 2.1) was dissolved in aqueous methanol (1:1) with further dilutions being prepared using this solvent solution. However, it was discovered that if the standards were left at room temperature for a number of days and then assayed, using the bioassay, inhibition zones had increased in diameter by up to 5% (Note: a small increase in zone size corresponded to a significant increase in calculated potency). This seemed to indicate

that the potency of the pimaricin standards was increasing with time and that this had the effect of over-estimating the potency of the antibiotic in culture broths. It was suspected that the aqueous methanol (1:1) was insufficient to completely solubilize the pimaricin. Pimaricin exhibits good solubility in polar organic solvents but poor solubility in water (Raab, 1972). It was also found that dilutions of culture broths from shake flasks gave a disproportional decrease in zone size whereas the standard pimaricin solutions gave a linear decrease on dilution. A number of methods were tested to try and overcome this solubility problem.

Calcium chloride ($CaCl_2$), from 0.5-2.0%, was added to the aqueous methanol (1:1) when preparing dilutions. $CaCl_2$ in conjunction with aqueous methanol has been shown to increase the solubility of pimaricin from 0.15% to 1.5% (Raab, 1972). However, the use of $CaCl_2$ had no affect on zone sizes for either the standards or broth samples. It was thought that perhaps the bioassay medium itself was inhibiting the full diffusion of the antibiotic. Oxoid agar No. 1 was substituted with Oxoid agar No. 3 and all other ingredients in the bioassay medium were changed to Analar grade. However, these changes had no effect on zone sizes. Ultra-violet light at 366 nm was used to inactivate the pimaricin both in the standards and in the culture broths. Pimaricin has already been shown to be inactivated by exposure to UV (Raab, 1972). It was hoped that by inactivating the antibiotic in the culture broths, that any other antifungal chemicals present would be highlighted. These chemicals may have been contributing to the variation in zone size. However, no zones occurred after UV treatment of either standards or broths samples.

Reports in the literature outlined the use of a spectrophotometric assay to determine the concentration of pimaricin in cheese (De Ruig et al. 1987). These researchers used methanol to initially dissolve the pimaricin powder and then they prepared all subsequent standards in

methanol:water (2:1) mixture. This method of preparing standards was adopted and used in the bioassay to recheck the accuracy of previous standard curves. Results from this experiment confirmed suspicions that the aqueous methanol (1:1) was unable to fully solubilize the pimaricin. Zone sizes for the standards were now much larger than previously recorded, and could therefore be used to give an accurate measure of pimaricin concentration in culture broths.

The spectrophotometric assay was developed into a standard pimaricin assaying technique using the work of De Ruig et al., 1987. It was found that using this procedure, broths gave a proportional decrease in absorbance reading on dilution. An experiment was set up to compare results obtained from the spectrophotometric assay with those of the bioassay. The samples used were culture broth supernates which had been obtained from previous shake flask studies on media variation. They had originally been assayed using the bioassay with the standards and samples diluted in methanol:water (1:1). These were now re-assayed using both methods with methanol:water (2:1) as diluent. Methanol was used to prepare the initial Delvocid standard. The results of this experiment are shown in Table 3.3.

These results show that both the bioassay and the spectrophotometric assay agree to within 10% of each other when using the revised method for preparing standards and samples. From Table 3.3. it can be seen that the bioassay had originally been over-estimating the concentration of pimaricin in culture broths by as much as 4 times their true value. Further studies showed that using methanol/water (2:1) to prepare dilutions of sample H (Table 3.3), resulted in a proportional reduction in zone size when tested on the bioassay. It was decided to use the spectrophotometric assay on a routine basis for future assay work because of its speed and convenience. The bioassay could be used to confirm biological activity of preparations. With this reliable and

rapid method for quantifying pimaricin in submerged culture now available, it was possible to continue with further media development work.

Table 3.3. Comparison between the spectrophotometric assay and the bioassay

Sample No.	Pimaricin Concentration (μg/ml)		
	Original Bioassay (1:1)	Improved Bioassay (2:1)	Spec. assay (2:1)
A	1954.5	227.6	246.0
B	1074.1	249.5	250.0
C	1824.0	222.4	263.4
D	873.0	180.8	161.3
E	1096.5	98.7	121.0
F	1552.5	285.3	306.5
G	562.3	90.2	112.9
H	1352.2	255.3	281.6

3.4. Medium Development Studies in Shake Flasks

The control medium which gave the highest pimaricin yields (350 μg/ml) from the initial shake flask studies contained (g/l): Glucose, 30; Soya peptone, 5; $CaCO_3$, 5; and Soya bean meal, 5. The composition of this medium was then examined in more detail in order to determine the best concentration for each component. Each component of the medium was varied and, in some cases, soya bean meal was omitted to see how critical its presence was. Soya flour was also included as an additional nitrogen source to observe its effect on growth and antibiotic production. Table 3.4. lists the main variations on the control medium.

Table 3.4. Composition of the different Media to be examined:

Medium	Composition (g/l)					pH_i
	Glucose	Soya peptone	*SBM	$CaCO_3$	Soya flour	
A	30	5	5	5	-	7.13
B	30	5	10	5	-	7.05
C	30	5	5	10	-	7.25
D	30	5	5	5	1	7.12
E	30	10	5	-	-	6.84
F	30	5	5	-	-	7.49
G	15	10	5	-	-	6.89
H	15	5	5	-	-	7.41

*SBM= Soya bean meal.

pH_i = Initial pH

Flasks were inoculated and incubated under standard conditions. Samples were assayed for pimaricin after 3 days using both the bioassay and the spectrophotometric assay. These initial media experiments were conducted over a 3 day incubation period. Residual sugars were analysed using the DNS method (Section 2.7.1.) and the type of growth morphologies in each medium was observed. This experiment was repeated a second time and the results were averaged. These are listed in Table 3.5.

Table 3.5. Results of media variation on control medium

Medium	pH_i	Pimaricin Yields (µg/ml)		Residual sugars (mg/ml)
		Spec. assay	Bioassay	
A	6.50	327.6	285.9	14.20
B	6.55	263.0	285.3	11.97
C	6.63	295.3	277.0	13.90
D	6.60	321.1	304.0	13.60
E	7.12	47.1	42.4	25.95
F	7.10	168.2	166.8	27.79
G	7.10	84.1	63.3	11.63
H	7.12	168.1	160.8	8.90

Conditions: 50 mls in 250 ml flasks, 1% spore inoculum, 30°C at 250 rpm for 3 days.
pH_i = Initial pH

In general, media containing SBM as a secondary nitrogen source produced the highest yields. Varying glucose with respect to soya peptone gave poor results in the absence of SBM. These results are not included in the table because yields were particularly low in the absence of SBM. Increasing $CaCO_3$ beyond 5 g/l did not improve yields. The presence of soya flour did not have any appreciable effect on yields. It appeared that there was still about 50% residual sugar left at the end of the 3 day fermentation which indicates that maximum growth may not have been attained. Media containing SBM produced dense growth with very tiny pellets, whereas media without SBM produced much larger pellets.

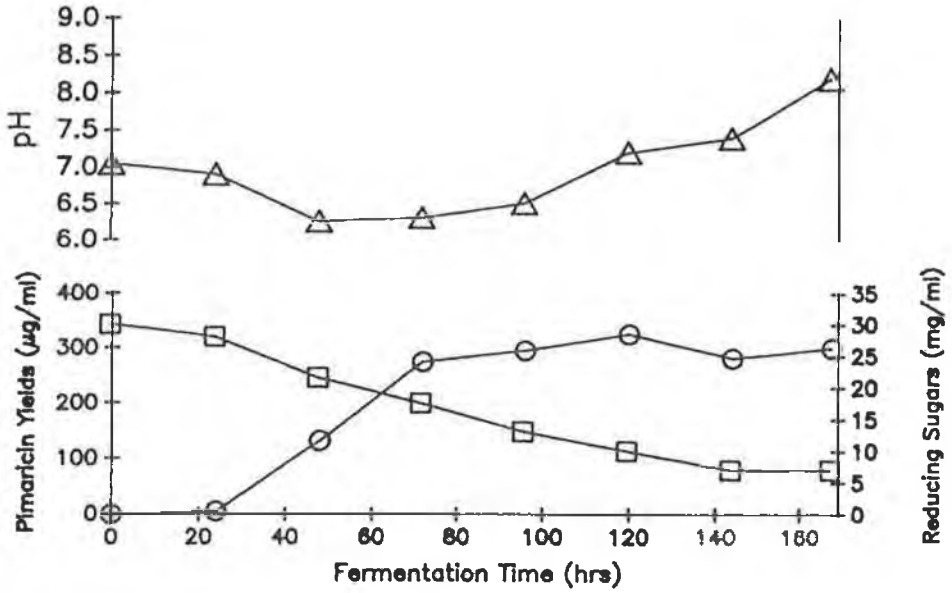
It was decided to conduct a time-course fermentation in flasks to determine at what stage antibiotic production was at its highest. This would indicate whether a 3 day incubation was sufficient.

3.4.1. Time-Course of fermentation in flasks.

Medium A (control, Section 3.4.) was selected as the best medium for this experiment. The flasks were inoculated and incubated under standard conditions. Samples of 4 mls each were aseptically removed every 24 hours and assayed for antibiotic production and residual sugars. This time-course sampling was carried out in quadruplicate and all results were averaged. These are illustrated in Figure 3.1.

Results showed that maximum pimaricin production occurred after 5 days (325 μg/ml). Beyond this time yields levelled off. Antibiotic production coincided with a decrease in residual sugars and pH, which was expected. On the basis of this it was decided to extend all shake flask incubations to 5 days. Bioassay results mirrored spectrophotometric assay results.

Fig. 3.1. Time-course of Pimaricin production in shake flasks.



○ Pimaricin Yields.

□ Reducing Sugars.

△ pH.

Conditions: 50mls in 250 ml flask, 1% inoculum, 30°C for 7 days at 250 rpm.

3.4.2. Carbon Source Studies.

In this experiment different carbon sources were used to substitute for glucose in medium A (control, Section 3.4.). These were of food grade quality and obtained from Biocon Ltd., Ireland. These included sucrose, lactose, corn starch, ground maize, MD05 and MD01. MD05 and MD01 are different grades of malto dextrins and are graded 1 to 10. 10 is equivalent to starch while 1 is equivalent to glucose. Flasks were inoculated and incubated under standard conditions (5 day fermentation). Pimaricin was assayed using both assay methods and the growth morphologies were observed for each medium. This experiment was repeated and the results averaged. These are listed in Table 3.6.

Table 3.6. Pimaricin productivity in media with different carbon sources.

Medium	Carbon Source	pH_i	pH_i	Pimaricin (µg/ml) Bioassay:	Spec. assay:	Reducing sugars (mg/ml) T_o	5 days
A	Glucose	7.02	6.90	255.0	274.0	27.3	1.2
B	Sucrose	7.36	8.74	0	0	0	2.7
C	Lactose	7.25	8.70	0	0	9.9	27.8
D	MD05	7.24	6.72	329.8	312.5	9.6	12.8
E	MD01	7.30	6.67	293.5	280.0	3.8	14.42
F	Corn starch	7.42	6.72	173.5	170.0	0	12.89
G	Ground maize	6.98	7.25	269.0	263.0	0	1.0

Conditions: 50 mls in 250 ml flasks, 1% spore inoculum, 30°C at 250 rpm for 5 days.
pH_i = Initial pH; pH_f = Final pH

Growth in media A, D, E, F, and G consisted of very dense and consisted of very tiny pellets. Media F and G were particularly viscous due to the presence of corn starch and ground maize. Media B and C supported poor growth and this was reflected in the absence of antibiotic yields

obtained for these media. Both MD01 and MD05 proved to be good carbon sources and produced the highest pimaricin yields. The control medium with glucose as the carbon source did not produce as high a yield as before. Further work was envisaged using MD05 or MD01 as carbon sources. It is possible that less available forms of carbon may be more desirable along with glucose in the medium. The glucose could favour exponential growth whereas the malto-dextrins could help prolong the stationary phase and possibly encourage greater antibiotic production.

3.4.3. Media from the U.S. Patent and its effect on pimaricin production.

A list of media was obtained from the United States Patent, No. 3,892,850 (Struyk and Waisvisz, 1975). This patent outlined the commercial production of pimaricin and listed five different media which could be used to produce the antibiotic in submerged culture. These media are listed in Section 2.6.2. Flasks were inoculated and incubated under standard conditions. Medium A (Section 3.4.) was used as the control. Pimaricin was assayed after 5 days using the spectrophotometric assay only and the growth morphologies in each medium were observed. This experiment was repeated a second time and all assay results were averaged. These results are listed in Table 3.7.

Table 3.7. Pimaricin productivity results obtained from U.S. Patent media.

Medium	pH_i	pH_f	Pimaricin (μg/ml)
control	7.16	6.60	267.5
(a)	7.13	8.10	92.0
(b)	6.56	8.10	0
(c)	5.82	8.48	98.0
(d)	7.14	7.50	233.0
(e)	7.19	7.50	221.5

Conditions: 50 mls in 250 ml flasks, 1% spore inoculum, 30°C at 250 rpm for 5 days.
Pimaricin was assayed using Spectrophotometric assay.
pH_i = initial pH, pH_f = final pH.

Visual observations of growth in the different media revealed the following: Medium (a) produced densely packed pellets; whereas Media (b), (c) and (d) produced loosely packed pellets; and Medium (e) produced large clumped growth. Similar morphologies were described previously for fungal cultures (Byrne, 1985). In general, the media described in the US patent produced poor pimaricin yields. Medium (b) contained lactose and produced no pimaricin. This was also the case when lactose was substituted for glucose in the carbon source experiment (Section 3.4.7.). The control medium still produced the highest yields and so was the medium of choice for further development studies. It was then decided to incorporate MD05 with glucose at different ratios in this medium and to observe its effect on pimaricin production. This was to follow on from the previous work conducted with the MD05/MD01 dextrins in Section 3.4.2.

3.4.4. Effect of MD05 and glucose as a combined carbon source on production.

Two sets of experiments were conducted using different ratios of MD05 to glucose in the control medium. The first experiment maintained glucose constant at 10 g/l but varied the concentration of MD05 from 5 to 30 g/l. The second experiment maintained MD05 at 5 g/l but varied the concentration of glucose from 5 to 30 g/l. Flasks were inoculated and incubated under standard conditions. Pimaricin was assayed after 5 days using the spectrophotometric assay. This experiment was repeated a second time and the results averaged. These are listed in Table 3.8.

Table 3.8. Effect of different combinations of MD05 and glucose on production.

Medium	Concentration of MD05:Glucose (g/l)	pH_i	pH_i	Pimaricin (μg/ml)
A	control	7.16	7.60	235.3
B	30:10	7.40	7.22	266.9
C	20:10	7.46	7.00	259.8
D	10:10	7.40	8.06	280.9
E	5:10	7.52	7.02	326.6
F	control	7.28	6.90	341.7
G	5:5	7.52	8.41	229.2
H	5:10	7.58	7.50	283.5
I	5:20	7.45	7.36	347.5
J	5:30	7.47	7.20	292.5

Control (g/l): glucose, 30; soya peptone; 5; S.B.M., 5; $CaCO_3$, 5.
Conditions: 50 mls in 250 ml flasks, 1% spore inoculum, 30°C at 250 rpm for 5 days.

From these results it appears that combining MD05 with glucose as a carbon source does not significantly increase pimaricin yields. However,

from the first set of results it appeared that if the glucose concentration was maintained at 10 g/l and the MD05 decreased from 30 g/l to 5 g/l, pimaricin yields increased. This trend was not substantiated in the second set of results whereby the same ratio of MD05 to glucose (5 g/l:10 g/l) gave a lower yield when compared to the control. Allowing for the inaccuracies inherent in the spectrophotometric assay it is difficult to know whether combining MD05 with glucose is consistently beneficial to improving antibiotic yields.

With the information provided by the time-course experiment and having tried unsuccessfully to increase yields using different carbon and nitrogen sources, it was decided to repeat the original studies carried out on the components of the standard control medium.

3.4.5. Further studies on the composition of the control medium.

Studies on individual components in the control medium have already been documented in Section 3.4. However this development work was conducted with only 3 day flask cultures. The time-course assay (Section 3.4.1.) indicated that maximum pimaricin production occurred after 5 days. As a result, individual components of this control medium were now looked at again in greater detail over a 5 day incubation period. In these experiments, levels of glucose, soya peptone, $CaCO_3$, and SBM were varied in relation to each other in an attempt to optimise production. Flasks were inoculated and incubated under standard conditions and pimaricin assayed using the spectrophotometric assay. The results of these studies are described in the following paragraphs.

Increasing glucose beyond 30 g/l (control level) had no significant effect on antibiotic production. Residual sugars increased as the initial glucose concentration increased, indicating that the organism was not utilising the increased level of glucose. Decreasing glucose from 30 g/l to 20 g/l caused no decrease in yields. However, below 20 g/l,

yields decreased.

Increasing soya peptone from 5 g/l (control level) to 10 g/l increased antibiotic yields by 5%. However a further increase to 20 g/l caused a decrease of 12%. There appeared to be a limit to the amount of soya peptone that could be tolerated.

Calcium carbonate ($CaCO_3$) at 5 g/l (control levels) appeared to give optimum antibiotic production. Increasing the concentration beyond this level had no effect on production, however below 5 g/l pimaricin yields dropped by 50%.

The most significant component in the control medium was found to be soya bean meal. Increasing SBM beyond 5 g/l caused a dramatic increase in pimaricin production. It was found that 50 g/l SBM gave optimum production with an increase of 175% over the control. The maximum yield obtained was 861 μg/ml compared with 312 μg/ml from the control. All components of the standard control medium were maintained at their original levels. Soya bean meal was now increased to 50 g/l for further work. Growth of *Streptomyces natalensis* in flasks with 50 g/l SBM was particularly good. There were no pellets or clumps of growth and mycelia were very dispersed. However, problems were experienced when a new batch of soya bean meal was used. Table 3.9. shows the results of flask cultures with 50 g/l of the new and old SBM in the control medium.

Table 3.9. Variation in pimaricin yields using different batches of soya bean meal.

Medium	pH_f	Pimaricin (μg/ml)
Old SBM	7.55	944.2
New SBM	8.10	236.1

Conditions: 50 mls in 250 ml flasks, 1% spore inoculum, 30°C at 250 rpm for 5 days.

Pimaricin yields had dropped to one quarter with the introduction of a different batch of SBM. Growth in the new batch was as dense and as dispersed as that found in the older batch. The reason for this variation was unknown. Further studies on the new batch of SBM revealed that 20 g/l gave optimum production with highest yields obtained of 390 µg/ml. Yeast extract and corn steep solids were also used to supplement the new batch of SBM with the hope of restoring original yields. However, both gave poor results. The composition of the new standard control medium became (g/l);

Glucose,	30;
Soya peptone,	5;
$CaCO_3$,	5;
Soya bean meal,	20;

Using this new control medium it was decided to study the effects of, inoculum level and medium volume in shake flasks, on pimaricin production.

3.4.6. Effect of Inoculum level on production.

Up to now 1% of a spore suspension from slopes had been used to inoculate shake flasks. An experiment was carried out whereby the inoculum size was varied from 0.5% to 5.0% (v/v), using the new control medium (Section 3.4.5.). The results from this study are listed in Table 3.10.

The results showed that the inoculum level had very little effect on pimaricin yields. Further experiments were conducted, whereby, flasks were inoculated directly from slopes using an inoculating loop. These studies showed that loop inoculated flasks gave the same yields as those inoculated with spore suspensions.

Table 3.10. Effect of inoculum size on pimaricin production in shake flasks.

Inoculum size % (v/v) (spore suspension)	Pimaricin (µg/ml)
0.5%	517.2
1.0% (control)	517.3
2.0%	543.1
5.0%	586.2

Conditions: 50 mls in 250 ml flasks, 30°C at 250 rpm for 5 days. Pimaricin assayed using spectrophotometric assay.

3.4.7. Effect of different volumes of media in flasks on production. In this experiment different volumes of the control medium were added to 250 ml shake flasks to see if there was any variation in pimaricin production due to different levels of aeration. Standard conditions of inoculation and incubation were used. The results of this experiment are listed in Table 3.11.

Table 3.11. Effect of different volumes of media in 250 ml shake flasks on pimaricin production.

Medium Volume (mls)	pH_i	Pimaricin (µg/ml)
25	7.30	467.9
50 (control)	7.07	434.5
100	6.55	106.5

Conditions: 1% spore inoculum, 30°C at 250 rpm for 5 days. Pimaricin was assayed using the spectrophotometric assay.

Note: Accurate, consistent pH readings of media were difficult to obtain because of the consistency of the complex media used in this project.

These results showed that 25 ml or 50 ml aliquots of media in 250 ml flasks gave optimum production. There was a sharp decrease in production when 100 ml aliquots of media were used which indicates that aeration and agitation are important factors for antibiotic production.

3.4.8. Effect of glucose dosing on production.

Slow-feeding with different levels of glucose in the broth has been shown to increase the yield of the polyene antibiotics, candidin and candihexin (Martin and Mc Daniel, 1974). It was hoped that this would also be the case for the pimaricin fermentation.

Firstly, the normal depletion of glucose was monitored in the standard control medium in flasks using the DNS method (Section 2.7.1.). This was to determine at what stage during the fermentation additional glucose could be introduced. It was found that after 72 hours residual sugars had dropped from 30 g/l to 10 g/l and levelled-off at 4 g/l for the remainder of the fermentation. It was calculated that glucose was depleted by approximately 10 g every 24 hours. The literature suggested that glucose should be maintained between 15 g/l and 5 g/l throughout the fermentation (Martin and Mc Daniel, 1974). Using this information it was decided to begin with two initial glucose concentrations in the medium, 20 g/l and 30 g/l. A stock glucose solution of 50 g/l was prepared and from this, glucose was dosed at two concentrations. One set of flasks with 20 g/l (10 mls of stock) and a second with 10 g/l glucose (5 mls of stock). The dosing scheme is summarised in Table 3.12. In order to find the dose time which would give maximum production, a range of dose times was used. Control flasks with initial glucose concentrations of 30 g/l and 20 g/l were set up to monitor normal antibiotic production. All flasks were inoculated and incubated under standard conditions. Pimaricin was assayed after 72, 96, 120 and 144 hours using the spectrophotometric assay.

Table 3.12. Glucose dosing scheme

Flask No.	Initial glucose (g/l)	Glucose dosing concentration (g/l)	Dose Times (hours)
A	30(control)	-	-
B	30	10	96, 120.
C	30	20	72,96, 120.
D	20(control)	-	-
E	20	10	72, 96, 120.
F	20	20	48, 72, 96, 120.

Results from these studies showed that dosing the fermentation with glucose at different times did not increase antibiotic production to any large extent. The dosed flasks which gave the highest yields were Flasks C and D. Pimaricin yields rose from 300 to 350 μg/ml, for C and from 375 to 425 μg/ml for D. Flask A showed a decrease in production when glucose was added and Flask B showed no change with pimaricin yields maintained at 380 μg/ml. The control flasks gave the highest yields of all with antibiotic production rising to 500 μg/ml after 96 hours. This level was maintained until the end of fermentation (144 hours).

3.4.9. Effect of ammonium chloride supplementation on production.

Researchers have shown that ammonium chloride (NH_4Cl) stimulates antibiotic production by *Streptomyces anandii var taifiensis* when used as a source of inorganic nitrogen (Kamel and Al-Zahrani, 1986). It was decided to use NH_4Cl to supplement the standard control medium or replace the soya bean meal and observe the effect on antibiotic production. The concentration range of nitrogen used by these researchers was between 40 mg/l and 360 mg/l. A stock solution of NH_4Cl was prepared to a final concentration of 360 mg of nitrogen/l which was equivalent to 1384.6 mg/l

NH_4Cl. Using this stock solution a range of concentrations of NH_4Cl was prepared in 50 mls of production medium in 250 ml flasks. A second set of flasks was prepared whereby NH_4Cl was used to replace the soya bean meal as a source of inorganic nitrogen. Control flasks were prepared in which no supplementations were made. All flasks were inoculated and incubated under standard conditions and assayed for pimaricin using the spectrophotometric assay. Results from these studies showed that supplementing the standard control medium with NH_4Cl did not significantly increase antibiotic yields. The highest yield obtained was 528 μg/ml with 200 mg/l nitrogen compared to 445 μg/ml from the control. They also showed that NH_4Cl in place of soya bean meal produced less than 80 μg/ml pimaricin.

3.4.10. Effect of Sodium acetate, sodium citrate and sodium malonate supplementation on production.

Supplementation with either of these three salts was shown to increase the production of the polyene antibiotic candicidin (Martin and Mc Daniel, 1976). These researchers suggested using between 0.1 and 20 g/l of each to supplement the production medium. Stock solutions of each were prepared at 200 g/l and dispensed in 50 mls of control medium to give the required concentrations. Each supplementation study was carried out individually. Flasks containing sodium citrate were adjusted to pH 6.5 before autoclaving. All flasks were inoculated and incubated under standard conditions. Pimaricin was assayed using the spectrophotometric assay.

Results from this study showed that supplementation of the control medium with sodium citrate, malonate and acetate did not increase pimaricin yields. Further experiments were planned whereby the control medium was dosed with sodium citrate and sodium acetate in a similar way to the glucose dosing experiments.

3.4.11. Effect of dosing with sodium citrate and sodium acetate on production.

In this experiment the initial glucose concentration used was 20 g/l and not 30 g/l as was normal for the control medium. This was to ensure that the glucose would be used up quickly allowing for earlier utilisation of the acetate and citrate. Both salts were dosed after 72 hours fermentation to give either 4 g/l or 2 g/l in each flask. Controls were set up whereby no additions were made and antibiotic production was monitored throughout using the spectrophotometric assay. These results are presented in Figure 3.2.

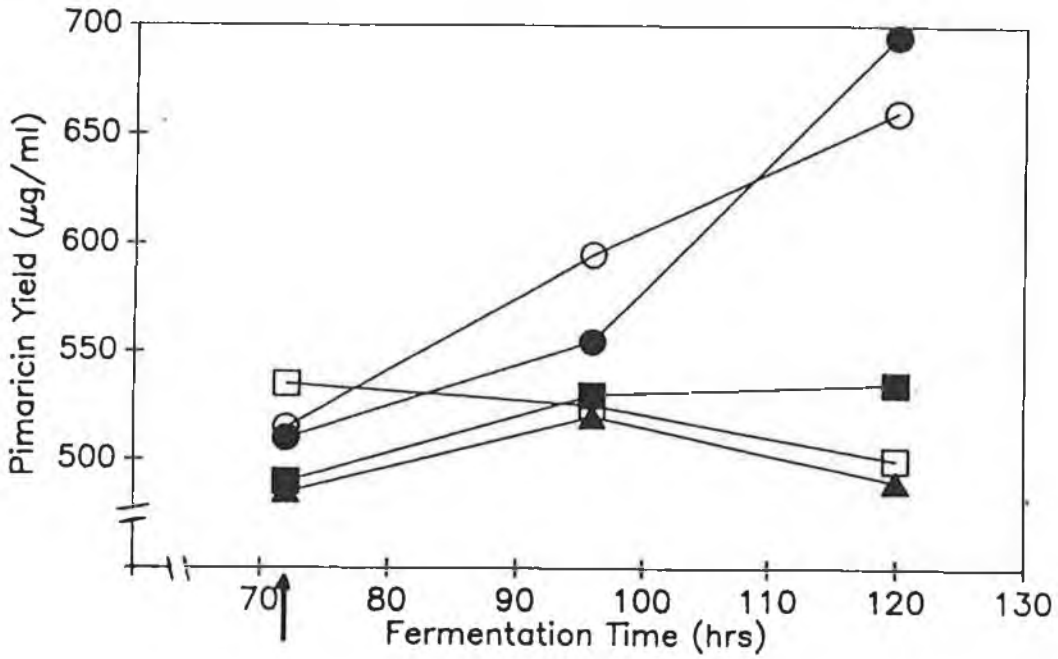
These illustrate how dosing with sodium citrate caused a dramatic increase in pimaricin production (30% increase over control). Dosing with sodium acetate on the other hand had no effect. It was decided to continue these studies by dosing with sodium citrate under varying initial glucose concentrations.

3.4.12. Effect of dosing with sodium citrate with varying initial glucose concentrations.

The concentrations of initial glucose used for this experiment were 10 g/l, 15 g/l and 20 g/l. Sodium citrate at a concentration of 2.0 g/l was dosed after 72 hours to one set of flasks and after 72 hours plus 96 hours to a second set. Control flasks were set up with initial glucose concentrations of 20 g/l and 30 g/l. All flasks were incubated under standard conditions and pimaricin assayed using the spectrophotometric assay. The results from this study are illustrated in Figures 3.3. and 3.4.

Dosing with sodium citrate clearly increased pimaricin production which confirmed the findings of the previous experiment. The highest yield obtained was 700 μg/ml when the control medium with 20 g/l glucose was dosed with 2 mg/ml sodium citrate after 72 hours and 96 hours. It now appeared that dosing with sodium citrate was definitely beneficial to the pimaricin fermentation and could be scaled-up in the 10 l fermenter.

Fig. 3.2. Effect of dosing with different concentrations of sodium acetate and sodium citrate after 72hrs.



■ 2 mg/ml sodium acetate.

□ 4 mg/ml sodium acetate.

● 2 mg/ml sodium citrate.

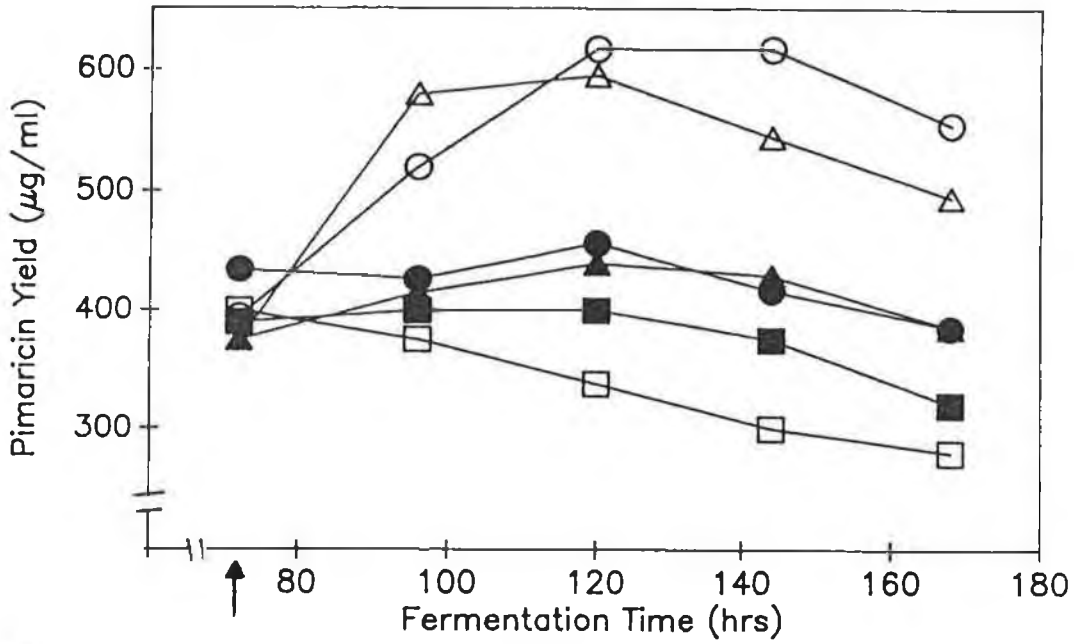
○ 4 mg/ml sodium citrate.

↑ Dose point.

▲ control

Conditions: 50 mls in 250 ml flasks, 1% inoculum, 30^{0}C for 5 days at 250 rpm.

Fig. 3.3. Effect of dosing with sodium citrate (2 mg/ml) after 72 hrs under different concentrations of glucose.



■ Control (10 g/l glucose).

□ Dosed (10 g/l glucose).

● Control (15 g/l glucose).

○ Dosed (15 g/l glucose).

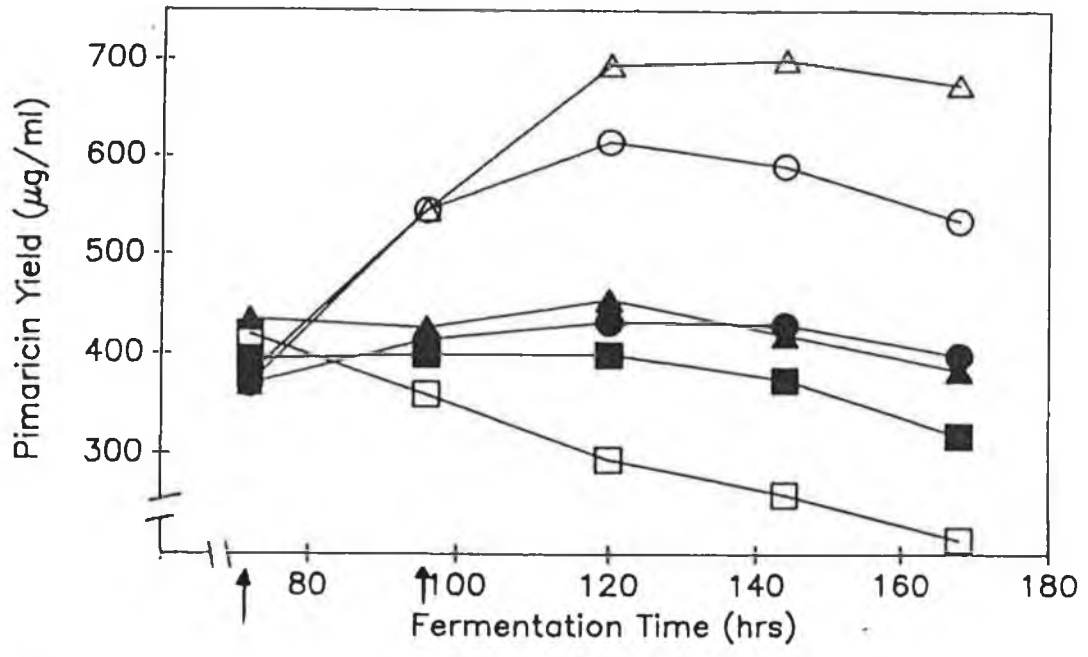
▲ Control (20 g/l glucose).

△ Dosed (20 g/l glucose).

↑ Dose point.

Conditions: 50 mls in 250 ml flasks, 1% inoculum, 30 ^{0}C for 5 days at 250 rpm.

Fig. 3.4. Effect of dosing with sodium citrate (2 mg/ml) after 72 and 96 hrs under different concentrations of glucose.



■ Control (10 g/l glucose).

□ Dosed (10 g/l glucose).

● Control (15 g/l glucose).

○ Dosed (15 g/l glucose)

▲ Control (20 g/l glucose).

△ Dosed (20 g/l glucose).

Conditions: 50 mls 250 ml flasks, 1% inoculum, 30^{0}C, for 5 days at 250 rpm.

3.4.13. Effect of dosing with zinc and ferrous sulphate on production.

Dosing with zinc and ferrous sulphate was found to stimulate the biosynthesis of the polyene antibiotic, candicidin (Chao-min Liu *et al*., 1975). These researchers found that the optimum concentration of zinc and ferrous sulphate which stimulated production was 0.5 mM. In this experiment 0.5 mM of both these salts were added separately to the control medium at T_o and after 48 hours. Control flasks were set up whereby no additions were made. All flasks were inoculated and incubated under standard conditions. Pimaricin was assayed using the spectrophotometric assay.

The results from this study showed that additions of ferrous and zinc sulphate to the standard control medium did not increase antibiotic production. Addition of zinc sulphate at T_o actually inhibited production by 50%. The control flasks with no additions produced the highest yields.

Other additions to the control medium included potassium citrate, Tween 80 and soya oil. However, in all cases, these did not increase antibiotic yields.

3.5 Strain Selection Methodologies.

Increases in pimaricin yields obtained due to media development had now been thoroughly investigated. Dosing with sodium citrate proved to be the most successful, increasing yields by 33% above the control. Target yields which were required to make the process commercially viable were between 5 and 10 g/l (5000-10000 μg/ml). In order to attain this level of production an improved strain had to be obtained. A number of methods were developed in order to select for an over-producing natural variant from the wild-type strain.

3.5.1. Resistance to pimaricin.

This is one of the classical methods for improving antibiotic yields (Calam, 1976), as poorer producing organisms are often sensitive to their own antibiotic. It was important to know the relative resistance of *Streptomyces natalensis* to its own antibiotic. The minimum inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that will inhibit the growth of a specific organism and was used here to determine how resistant our production organism was to pimaricin.

A stock pimaricin solution of 5000 μg/ml was prepared in methanol. This was used to prepare a range of standards (250 μg/ml-1000 μg/ml) in 20 ml aliquots of yeast-malt extract agar (Section 2.2.2.). The plates were poured and, when set, a suitably diluted spore suspension of *Streptomyces natalensis* was spread-plated onto each. Methanol agar plate controls were prepared to observe its effect on the growth of the organism. Positive controls were also set up whereby no pimaricin or methanol were added to the plates. All plates were incubated at 26°C for 10 days and a colony count carried out. The presence or absence of growth was then observed.

Results from this experiment indicated that the organism was sensitive to between 250 μg/ml and 500 μg/ml of pimaricin. However, the methanol had an inhibitory effect on growth and this could have distorted the results. Contamination was also observed on the plates and so it was decided to filter-sterilise the pimaricin for future MIC work. Stock pimaricin solutions of 5000 μg/ml and 20,000 μg/ml were prepared in methanol + 2% $CaCl_2$. These were sterilised through a 0.45 μm PTFE filter (Section 2.6.4.) and their concentrations checked using the spectrophotometric assay. This was to ensure that all of the pimaricin had solubilised and that filtration had not diminished potency. The results of this study showed that pimaricin concentration was not diminished by the filtration process. The 5000 μg/ml pimaricin solution

gave a concentration reading of 5089.3 μg/ml before filtration and 5357.1 μg/ml after filtration. The 20,000 μg/ml solution gave a concentration reading of 19963.2 μg/ml before filtration and 20692.6 μg/ml after filtration.

Therefore, it was possible to filter-sterilise solutions of pimaricin without losing antibiotic concentration and secondly, the antibiotic was soluble in methanol + 2% $CaCl_2$ to at least 20,000 μg/ml or 2%. This allowed for the introduction of pimaricin into agar plates using smaller sub-inhibitory volumes of methanol. Using this 20,000 μg/ml stock solution of pimaricin, a second MIC experiment was set up. The range of pimaricin concentrations used was again between 250 and 500 μg/ml. Methanol controls were again set up to assess for solvent toxicity. Results from these experiments showed that volumes of methanol from 1.4 mls to 2.0 mls in the agar medium were inhibitory to *Streptomyces natalensis*. Therefore, in order to add 1000 μg/ml or more pimaricin to YME plates, the volumes of methanol required could not exceed 1.4 mls. As a result the stock pimaricin standard was increased to 40,000 μg/ml and filter sterilised through a 0.45 μm PTFE filter. The actual concentration of pimaricin in this solution was confirmed using the spectrophotometric assay. It was decided that, rather than carry out another MIC test, 2000 μg/ml of pimaricin would be introduced into the YME plates and strains inherently resistant to this level would be screened for. Only 1.0 ml of a stock pimaricin solution (40,000 μg/ml) in 20 mls of molten agar was required to provide a final concentration of 2000 μg/ml in the plates. This volume of methanol was below the inhibitory level and did not affect the growth of the organism.

3.5.2. Effect of time on the potency of pimaricin in agar plates. Before proceeding with this selection method it was necessary to establish whether pimaricin would be uniformly distributed throughout the

agar plates and if the antibiotic would lose its potency after several days incubation at 26°C. This was to ensure that surviving colonies on these antibiotic plates were in fact resistant to 2000 µg/ml of pimaricin. An experiment was set up whereby different concentrations of pimaricin (500 µg/ml - 2000 µg/ml) were introduced into YME agar plates, five plates of each concentration. One plate from each concentration had five agar plugs removed, aseptically, and transferred to a bioassay plate. The bioassay plate was incubated under standard conditions (Section 2.7.2.) and zone sizes measured. All remaining plates were placed in a 26°C incubator and plugs were removed every 24 hours, for a total of 8 days, from each concentration to check that potency had not diminished. The results showed that pimaricin was very stable over long periods of time when introduced into agar plates, with only slight deterioration after 8 days. Results from this experiment also showed that the antibiotic was evenly dispersed throughout the entire plate. With this information YME plates with 2000 µg/ml of pimaricin were prepared and a diluted spore suspension of *Streptomyces natalensis* was plated out onto these. Control plates were prepared which contained no antibiotic. All plates were incubated at 26°C for 8-10 days. Approximately 70% of the culture did not survive, but those that did were subcultured onto YME slopes. These resistant isolates were then checked for productivity in shake flasks. In all, 50 resistant isolates were screened, most of which produced similar yields to the wildtype. However, one isolate (designated NIHE 29) produced 472.2 µg/ml compared to 361.4 µg/ml for the wild-type (a 23% increase). These resistant strains grew slowly on the antibiotic media (7 days compared to 5 days normally) but, when subcultured several times onto YME plates, productivity was still higher than the wildtype. A number of isolates, resistant to 2000 µg/ml, were found to produce no pimaricin either on bioassay plates or in shake flasks. Taking these findings together, it

appeared that there was little correlation between pimaricin resistance and production potential. It was found that none of the resistant isolates were capable of producing more than 472 µg/ml of pimaricin.

3.5.3. Agar plug/colonies onto bioassay plates.

This methodology was developed from the pimaricin bioassay to enable rapid visual appraisal of multiple isolates on plates, to be used prior to subculturing and productivity testing. Potency indices were calculated for the standard control strain (i.e. diameter of zone relative to the colony diameter). The non-producing strains mentioned above gave a zero potency index when colonies were placed onto a bioassay plate. It was hoped to easily distinguish high from low producers from amongst mutants or natural variants using this method. This selection basis was extensively studied using two experiments, involving glucose and phosphate repression of pimaricin production. These are common selection procedures used in antibiotic research (Martin and Mc Daniel, 1974, Calam, 1976).

3.5.3.1. Catabolite repression.

Addition of higher glucose levels, from 10 to 100 g/l were used to test colonies for their ability to produce pimaricin under conditions of catabolite repression, using both the agar plug method and by culturing the strains in shake flasks. In the agar plug method *Streptomyces natalensis* was cultivated for 8 days on YME agar plates which contained different concentrations of glucose. Isolated colonies were then transferred from each plate to the bioassay plate in the form of agar plugs. Potency indices (diameter of zone / diameter of colony) were then measured for each colony to observe if production had been inhibited. In the shake flask method the same strain was cultivated under standard conditions in the control medium containing different concentrations of

glucose and assayed for pimaricin as normal. It was hoped that different levels of production in shake flasks would also correlate with production on agar plates and that this would be reflected in different zone sizes for colonies growing on different concentrations of glucose. The results of this experiment are listed in Table 3.13.

Table 3.13. Comparison between antibiotic production in submerged culture and on agar medium in the presence of different concentrations of glucose.

Glucose Concentration (g/l)	SOLID MEDIA Potency Index	LIQUID MEDIA Pimaricin Yields in flasks (µg/ml)
10[1)]	3.55	161.6
20	3.38	382.7
30[2)]	3.47	348.6
50	3.39	272.1
80	3.43	191.4

1) Normal concentration of glucose used in standard plating medium.
2) Normal concentration of glucose used in control liquid medium.

These results showed that zone sizes were not affected when colonies were grown on agar media with different concentrations of glucose. However, when the strain was grown in submerged culture, glucose levels above 30 and below 20 g/l caused a decrease in pimaricin production.

3.5.3.2. Phosphate repression.

This work was carried out in a similar way to the catabolite repression studies. Additions of phosphate in the form of KH_2PO_4 (5-20 g/l) were used, both in the flask medium and in the agar plate medium. It was found that even the addition of 5 g/l KH_2PO_4 caused antibiotic yields to

drop by 60% in shake flasks. However, this decrease was not observed on the bioassay plate where zone sizes remained the same even in the presence of high levels of phosphate.

It now appeared that the agar plug method, for the rapid detection of improved strains, was of very limited use. The bioassay was not accurate to enough detect small increases in antibiotic production and did not correlate with results obtained in shake flasks. The plate method would also be of limited use when screening large numbers of survivors from a mutagenesis programme. The only accurate method was to transfer individual isolates onto slopes, cultivate these in shake flasks and then assay for pimaricin in the normal way.

3.5.3.3. Random selection.

In the absence of a selection system which could readily highlight improved variants, in the case of natural variants resistant to high concentrations of pimaricin, or mutants, it was decided to select and test isolates randomly. Mutants or variants could be chosen randomly from plates, subcultured onto slopes and then tested for productivity in shake flasks. This method is one used by many researchers (O.L. Davies, 1964) and is dependent on the availability of sufficient orbital shaker space for shake flask fermentations. Approximately 50 isolates were checked for productivity in this manner. Many of the isolates were selected from plates which had been put through a UV mutagenesis programme. These isolates were randomly chosen from different UV exposure times (Section 2.8.1.), other isolates tested were natural variants resistant to high levels of pimaricin (2000 μg/ml) and some were normal isolates under no selection pressures. Many of these isolates were cultivated both in the standard productivity medium and in medium with glucose increased to 50 g/l to detect glucose de-repressed mutants. However, no over-producers were detected with the exception of one strain

mentioned previously, designated NIHE 29 (Section 3.5.1.) which produced 23% more than the wildtype.

3.5.4. Improvement in screening methods.

It was found that the use of 250 ml shake flasks was limiting progress when screening large numbers of survivors because of the shortage of orbital shaker space. This was particularly the case when screening survivors from a UV and EMS mutagenesis programme. A new method to miniaturise this screening process was devised using 20 ml glass universal bottles. The bottles were filled with 5 mls of the standard control medium with cotton wool bungs in place of screw caps. Using cocktail sticks, colonies were aseptically inoculated, in duplicate, into the universals and incubated in the normal way, (250 rpm, 30°C for 5 days). Using this method, it was found that yields were slightly lower than those obtained in flasks (e.g. 500 μg/ml for flasks and 416 μg/ml for universals). However, results were consistent and high yielding strains could be identified. This method also allowed for the screening of larger numbers of plate isolates without the need to cultivate slopes. Any high-yielding isolates were then subcultured onto YME slopes and checked for productivity in shake flasks to confirm these increases.

3.6. Mutagenesis:

The mutagenic agents used in this project were ultra-violet (UV) light and Ethyl-methyl-sulphonate (EMS). The procedures for both of these are described in Section 2.8. The following sections describe the yield results obtained using both methods. In the case of UV mutagenesis the spore suspension method was favoured over the plate method.

3.6.1. UV mutagenesis.

Initial UV work involved screening 30 survivors using the shake flask method. All isolates tested gave similar yields to the wildtype strain but none proved better than the natural variant, NIHE 29 (Section 3.5.1.).

UV mutagenesis with caffeine in the plating medium. It has been shown that caffeine inhibited single stranded DNA repair in wildtype *E. coli* after irradiation with UV (Fong and Bockrath, 1979). Caffeine at 1 mg/ml was shown to be the most suitable concentration to add to both the diluent and plating medium. Concentrations above this inhibited the growth of *Streptomyces natalensis*. Using this procedure UV mutagenesis was carried out on NIHE 29. A total of 100 survivors were screened using the universal bottle method (Section 3.5.2.). The highest yielding mutant obtained was designated UV/CAFF. 172 and produced 483.2 µg/ml of pimaricin compared with 417.9 µg/ml for the wildtype (a 14% increase).

3.6.2. EMS mutagenesis.

EMS mutagenesis on NIHE 29.

Using the universal bottle procedure for screening, a total of 100 survivors were screened. The highest producing isolates were designated:

EMS 49 (496.1 µg/ml) and

EMS 53 (498.5 µg/ml) respectively.

Their productivity compares with 445.3 µg/ml for the control (NIHE 29). This represented a 11% increase in production.

EMS mutagenesis on a non-producing natural variant and screening for revertants:

A number of natural variants were isolated which produced no pimaricin

either in flasks or on the bioassay plate. It was hoped that some of these non-producers could be reverted using EMS mutagenesis and that some of these revertants would produce higher than normal levels of pimaricin. In all, 100 survivors were screened in universal bottles, however no improved mutants were isolated.

A second round of EMS mutagenesis was carried out on one of the EMS mutants (EMS 53). In all, 100 survivors were screened. However, no further improved isolates were obtained.

3.7. Production of pimaricin in 10 l stirred fermenters.

In general it was difficult to grow *Streptomyces natalensis* and produce optimum yields of pimaricin in the 16 l (10 l capacity) microgen fermenter. Initially, antibiotic production was low (between 200 and 250 µg/ml) compared to productivity in the shake flask controls (300-350 µg/ml). The medium used was the standard control medium that had been optimised in shake flasks (Section 3.4.5.). A 5%, 72 hours inoculum was prepared using the same medium to inoculate the vessel. Aeration was set to 1 liter of air per litre of medium per minute (10 l/min) and the agitation was set at 300rpm. Temperature of growth for both the inoculum flasks and fermenter was maintained at 30°C throughout the fermentation. Culture purity in the fermenter was monitored regularly by plating out samples onto YME agar plates and then incubating these at 30°C for 24-48 hrs. The inoculum was also checked in a similar manner. 10 mls of sterile KG antifoam (Section 2.5.) was added after inoculation and again after 24 hrs to control foaming. Because of the high concentration of soya bean meal (20 g/l) in the medium, samples had to be centrifuged in order to collect a supernate for assay purposes.

Bacterial contamination proved to be a persistent problem

throughout the microgen fermentations and normally appeared early on (between 0 and 24 hrs). The contaminant appeared to be a homogeneous culture, very likely a *Bacillus* species, when viewed under a microscope. It was thought that insufficient sterilisation of the medium was contributing to this problem. Because of the high concentration of SBM, it was decided to pre-sterilise this separately in water for 30 mins. It was possible that due to the physical nature of the SBM, heat stable *Bacillus* spores were not being destroyed. The remaining ingredients were then batched and the medium further sterilised for 60 mins. This treatment greatly reduced the incidence of contamination. However, in some cases bacterial contamination was still observed later in the fermentation (after 72 hrs.). Initial measurements with the dissolved oxygen probe (Section 2.5.) revealed that the culture was oxygen limited. Dissolved oxygen dropped from 100% saturated to between 0 and 5% after 24 hrs and remained at this level throughout the fermentation. As a result the agitation rate was adjusted. It was found that 500 rpm produced maximum pimaricin yields of 500 μg/ml after 72 hrs. Dissolved oxygen now dropped from 100% saturated to approximately 20% after 24 hrs and remained at this level for the duration of the fermentation. The shake flask controls gave yields of between 400 and 450 μg/ml. The strain used for these fermentations was an EMS mutant, EMS 53 which had already been shown to give yields of almost 500 μg/ml in shake flasks (Section 3.6.2.). Figure 3.5. illustrates the time-course for this fermentation. It was not practical to measure biomass, by using either wet or dry weights, because of the nature of the medium. The level of aeration was adjusted, but 10 l/min still gave optimum results. The graph also shows the pH and reducing sugar profile throughout the 120 hr fermentation. The pH profile was similar to that found in shake flask fermentations. pH dropped slightly during exponential growth (tropophase) and rose during the production phase (idiophase). Reducing

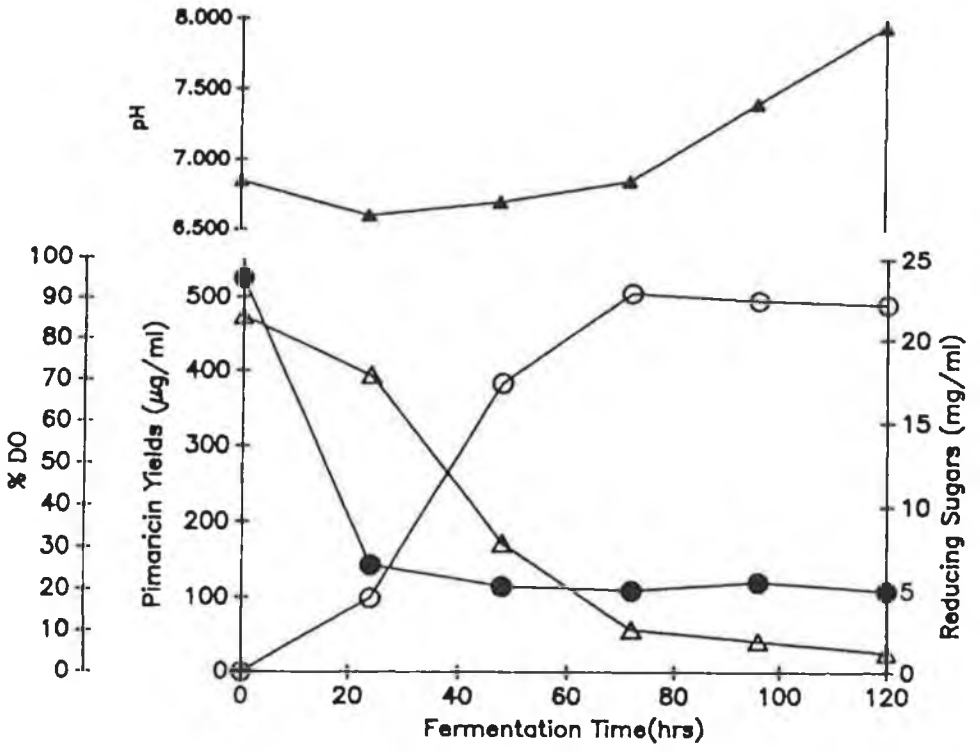
sugars dropped, during the exponential phase as glucose was utilised for growth and levelled off during the production phase. From this graph it would appear that between 48 hours and 72 hours would be the most appropriate time in the fermentation to start feeding experiments.

Experiments were also carried out whereby the inoculum level and the inoculum incubation time were varied in order to see the effect on production. It was found that 72 hours provided the best inoculum with dense, well dispersed culture morphology. Inoculum levels below 5% resulted in optimum yields being produced after 72 hrs and in some cases yields were below 500 µg/ml. Generally an inoculum level of between 5 and 10% gave the best results.

With the agitation increased to 500 rpm, extensive foaming occurred throughout the fermentation period. An antifoam probe was fitted to the vessel which was connected to a peristaltic pump and reservoir of sterile KG antifoam. This allowed automatic addition of antifoam at the precise time of foaming. However, in order to allow for addition of antifoam, a back pressure could not be placed on the vessel which increased the likelihood of outside contamination.

Glucose feeding was not introduced into the microgen fermentations because of the poor results obtained when using shake flask cultures (Section 3.4.8.). However, sodium citrate feeding was attempted. A number of runs were carried out using first, a sodium citrate dose of 2 g/l after 72 hours, followed by a continuous feed of the same salt which was delivered at approximately 0.1 g/hour. These feed quantities were chosen to mimick the conditions which had given the yield increases in shake flasks (Section 3.4.11.). However, pimaricin production was not increased beyond the yields obtained in the control flasks. A 2 mg/ml sodium citrate feed was also started after 24 hours in case that 72 hours was too late, however, no yield increases were observed.

Fig. 3.5. Time-course of Pimaricin production in the 16 l microgen fermenter.



○ Pimaricin yields.

● % Dissolved Oxygen (DO).

△ Reducing Sugars (mg/ml).

▲ pH.

Conditions: 5% Inoculum, 5 day fermentation at 30^{0}C, 500 rpm.

Studies on pH control and its effect on production were also carried out in the microgen fermenter. When the pH dropped during the exponential growth phase, the pH controller was used to prevent the pH rising again by adding in HCl. However, these experiments did not improve yields. Studies were also conducted on microgen fermentation samples to see if some pimaricin was still attached to the mycelia. It has been postulated that polyene antibiotics may play a role as chemical components of the sheath of aerial mycelium in streptomycetes (Cherny et al., 1972). Fermentation samples were filtered through Whatman No. 1 filters under vacuum and the cake washed several times with distilled water. A sample of the cake was removed, weighed, and methanol:water (2:1) was used to extract any remaining pimaricin. Results of this work were inconclusive. In some samples, small amounts of pimaricin were detected in the cake (10-100 μg/ml), in others, no traces of the antibiotic were detected. It was also found that as the fermentation time proceeded, less and less pimaricin was detected in the cake samples. It is possible that the antibiotic is shed completely from the mycelia as the culture ages.

CHAPTER 4.

DISCUSSION

In general it was found that slopes which produced a grey sporulating mat were most suitable when preparing spore suspensions to inoculate shake flasks. Both *S. natalensis* and *S. gilvosporeus* also produced a white sporulating mat. However, this type of growth did not perform well when used as an inoculum for flasks (Section 3.1.). These different patterns of growth have also been found for other *Streptomyces* species (Dorokhova et al., 1970). It was also found that yeast and malt extract were important ingredients of agar slope and plating medium if dense sporulated growth was required.

The growth morphology in submerged culture was important in relation to pimaricin production. Large pellets of growth produced low levels of the antibiotic, whereas dispersed mycelia (with some small pellets) gave optimum production in flasks (Section 3.2.). It was found that the addition of $CaCO_3$ contributed to the dispersal of the mycelia. There is little information available regarding actinomycete pellets (Stanbury and Whitaker, 1984). However, it does appear that supply of oxygen to the interior of the pellet is critical (Pirt, 1975) and it is likely that this would be restricted in the case of large pellets. Because *Streptomyces* have an oxidative metabolism, oxygen supply for growth is critical. This is particularly important when scaling up the process in stirred fermenters.

Investigations into methods to detect pimaricin in culture broths led to the development of a rapid spectrophotometric assay (Section 3.3.). Initially pimaricin was assayed using a microbiological assay, the classical method for detecting antibiotics. Because of the antibiotic's insolubility in water, the microbiological assay over-estimated the concentration of pimaricin present in samples. Poor solubility in water is a feature of the polyene macrolide group as a whole (Raab, 1972). Methanol was the solvent of choice when preparing

pimaricin standards and samples. Other solvents, which were also effective in solubilising the antibiotic, such as glacial acetic acid and glycerol were not used because they were impractical or unsafe to use routinely. By using methanol the standard pimaricin powder "Delvocid" could be completely solubilised and a methanol:water mixture (2:1) was found to be sufficient to prepare dilutions of both the standard and culture supernates. By overcoming the solubility problem, both assay techniques were found to be in agreement with each other. This allowed pimaricin to be detected quantitatively by chemical characterisation and also by it's biological activity. This rapid spectrophotometric assay was particularly useful for its ability to detect small increases in production.

The main media components which were found to be essential for both the optimum growth of *S. natalensis* and pimaricin production were: glucose, soya peptone, soya bean meal and $CaCO_3$ (Section 3.2.). Glucose proved to be the best carbon source, as reported for the production of other polyene macrolides (Brewer and Frazier, 1962; Acker and Lechevalier, 1954; Ethiraj, 1969; Abou-Zeid, 1973; Tereshin, 1976). Sucrose and lactose produced poor growth and no pimaricin when used in place of glucose in the standard medium. This would seem to indicate that the organism lacks the enzymatic ability to utilise sugars other than glucose. Corn starch and ground maize were utilised as carbon sources, however, they produced very viscous media which were difficult to process (Section 3.4.2.). MD05 and MD01 dextrins also proved to be good carbon sources and produced equivalent levels of pimaricin to glucose. MD05 was then combined with glucose in different ratios to observe the effect on production. It was thought that glucose would provide the initial energy for growth (tropophase) and once depleted, the MD05 would be more slowly utilised as a secondary carbon source, thus prolonging secondary metabolism (idiophase) and antibiotic production.

However, these studies did not show any increases in yield (Section 3.4.4.) and in all cases the control medium with no MD05 produced as much if not more pimaricin. It is possible that production of the antibiotic is under catabolite repression.

Soya peptone and soya bean meal were found to be good organic sources of nitrogen for the pimaricin fermentation as is the case with the production of most polyene macrolides (Section 3.4.5.). Soya peptone at 5 g/l was found to give optimal production, however, concentrations of 20 g/l or more caused a significant decrease in yields. It was found that increasing the soya bean meal content beyond the control level (20g/l) caused a considerable increase in antibiotic production. With a maximum soya bean meal concentration of 50 g/l, production was increased from 312 mg/ml to 816 mg/ml. It was also noticed that growth in this medium was very dense and well dispersed. However, when a new batch of soya bean meal was introduced, yields dropped again to only 216 mg/ml. The reason for this variation between batches is unknown. However, batch to batch variation in soya bean meal has been known to occur during the production of the polyene macrolides candidin and candihexin (Martin and Mc Daniel, 1974). Other complex nitrogen sources such as yeast extract and corn steep solids did not improve production either in combination with soya bean meal or on their own. Inorganic nitrogen sources such as ammonium sulphate, also, did not prove successful as a replacement for the complex nitrogen sources.

The patent describing the production of pimaricin (Struyk and Waisvisz, 1975, Section 3.4.3.) listed a series of ingredients which were designed to give optimal antibiotic production. These included the use of beet molasses, mycological peptone, beef extract, peanut oil and soya oil, dipotassium hydrogen phosphate and sodium sulphate. However, the standard control medium already in use, gave higher production. Calcium carbonate was an essential component of the production medium.

Increasing the $CaCO_3$ content beyond the standard concentration (5 g/l) had no effect on production, however, below this level or if omitted altogether, yields decreased considerably. As already mentioned, $CaCO_3$ may have an important role in dispersing the mycelia and also as a pH buffer in preventing significant fluctuations in pH.

Glucose dosing of the standard medium did not prove successful (Section 3.4.8.). Slow-feeding with different levels of glucose has been shown to increase the yield of the polyene antibiotics, candihexin and candidin (Martin and Mc Daniel, 1974). Time course studies for pimaricin production showed that most of the glucose was utilised after 72 hrs (Section 3.4.1.). This indicated the start of secondary metabolism. It was hoped that by dosing with different concentrations of glucose at different times would result in a prolonged stationary phase and extend the production of secondary metabolites. It was also thought that small doses of glucose would overcome catabolite repression. However, production was not increased above the controls. There is no clear explanation for this.

Dosing the standard medium with sodium citrate increased production from 300 mg/ml to 700 mg/ml (Section 3.4.10.). Supplementation with sodium salts such as acetate, citrate and malonate has been shown to increase the production of the polyene antibiotic candicidin (Martin and Mc Daniel, 1976). Citrate has been shown to increase the synthesis of the polyenes by providing important precursors and therefore, may have some regulatory role to play in overall biosynthesis (Volpe and Vagelos, 1973). Initially, supplementing the pimaricin medium with these salts had no effect on production. However, dosing the fermentation after 72 hrs and 96 hrs with 2 mg/ml of sodium citrate gave the increase shown. This was repeated several times and each time production was increased. From these studies, it was found that while the concentration of sodium citrate dosage was not critical, the stage of growth at which doses were

added was important.

Trace metals have been implicated as inducers or activators of secondary metabolite synthetases. Metal ions such as ferrous and zinc sulphate have a significant effect on the biosynthesis of candicidin (Liu et al., 1975). However, dosing the pimaricin production medium with these metals had no effect on production (Section 3.4.13.).

In the candicidin fermentation it was found that a larger inoculum resulted in an increase in yields (Liu et al., 1975). However, inoculum size was not found to be critical for the pimaricin fermentation (Section 3.4.6.). Inoculum levels of between 0.5% and 5.0% for flask cultures did not affect yields. In fact it was possible to inoculate flasks directly from slopes using a loop.

Resistance of the producing organism to high levels of its own antibiotic is a common selection system used to isolate possible high-yielding strains (Calam, 1976). It was hoped that a definite MIC (Minimum Inhibitory Concentration) value could be established for *Streptomyces natalensis* (Section 3.5.1.). This could then be used as a positive selection system for isolates (either induced mutants or natural variants) which could survive on plates with high concentrations of pimaricin. These resistant isolates may then produce higher yields of pimaricin in submerged culture fermentations. Initial difficulties were experienced when attempting to introduce standard pimaricin solutions into YME agar plates. Volumes of methanol above 1.4 mls were inhibitory to *S. natalensis* and this had the effect of under-estimating the MIC for the organism. This difficulty was overcome by preparing a more concentrated stock solution of the antibiotic (40,000 mg/ml) which allowed for the introduction of smaller non-inhibitory volumes of methanol into the plates. It had been suggested in the literature that pimaricin was only soluble to a maximum of 1% (v/v) in methanol (Raab, 1972). However, the spectrophotometric assay confirmed that the

concentration of the stock solution was 40,000 mg/ml (4% v/v). It was also shown that standard pimaricin (Delvocid), when introduced into agar plates, retained potency for up to 8 days (Section 3.5.2.). This stability, over time had also been found for pimaricin in culture supernates that had been stored at 4°C. However, isolates that had been shown to be resistant to 2000 mg/ml (2 g/l) on plates did not produce pimaricin even above 1g/l. One naturally occurring resistant isolate was shown to produce 23% more than the wildtype. It is possible that catabolite or phosphate repression is limiting production of the antibiotic beyond a certain level.

It was hoped that the bioassay plate technique would allow for rapid visual appraisal of isolates which showed increased productivity. Using this method, agar plugs of individual mutagenesied colonies were transfered onto bioassay plates and their potency indices measured. However, it has been found that antibiotic yield increases brought about by mutation are usually small (5-10%) and this procedure was not sensitive enough to detect this (Calam, 1970).

For this reason, phosphate and glucose (catabolite) repression were used in order to reduce the wildtype zone size on the bioassay plates, thus highlighting mutants which could overcome repression and over-produce pimaricin. It was found that increasing glucose beyond 30 g/l in shake flask medium repressed antibiotic production significantly, but did not reduce zone sizes when colonies, that had been cultivated on plates with higher glucose, were transfered to bioassay plates (Section 3.5.3.1.). Thus, there was no correlation between the effect of different glucose concentrations on pimaricin production when using both shake flasks and colonies transferred to bioassay plates. This was also found to be the case for phosphate repression (Section 3.5.3.2.). It is not known why the bioassay did not show up the repression effect. It is possible that diffusion of the antibiotic throughout the agar from a

colony is governed by other factors, such as the composition of the agar medium itself.

As a result, the bioassay plate technique was not used as a preliminary screen to detect high-yielding mutants. Isolates, now had to be randomly selected and tested for productivity in shake flasks (Section 3.5.3.3.). This method was more labour intensive and required much shaker space in order to screen enough isolates. However, for accuracy isolates had to be cultured in duplicate and for our purposes only one shaker cabinet was available with 35 flask holders. This problem was alleviated with the development of the 20 ml universal bottle method to culture isolates directly without having to first prepare slopes (Section 3.5.3.4.). This proved an ideal rapid pre-screening method and allowed a greater number of isolates to be cultured. In total, approximately 400 isolates, chosen from both UV and EMS mutagenesis were screened. In general, large shake flasks (500 ml) are more desirable for screening purposes than small flasks or test tubes which tend to give less reliable results even though more can be handled (Calam, 1970). However, results showed (Section 3.5.3.4.) that even though yields were lower in the universal bottles, they were consistent and high-yielding isolates could be identified. Ideally, approximately 1000 survivors should have been screened for each mutagenic treatment used in order to have a reasonable chance of detecting significant increases (Calam, 1970). In this project, time did not allow this. A more rapid or possibly an automatic screening system would be required in order to achieve this goal.

Both UV and EMS provided improved mutants (Section 3.6.). The percentage increase in yields (14% and 11%) was about average for small antibiotic increases when using mutation and selection (Calam, 1970). Caffeine in the plating media appeared to increase the frequency of mutations leading to higher antibiotic production. It was hoped that a

second round of EMS treatment on a previously mutated strain (EMS 49) would produce a further improved mutant. However, no improvements were made. The non-producing strain also could not be reverted. It is possible that much larger numbers would need to be screened in order to detect such improved mutants.

N-methyl-N-nitro-N'-nitrosoguanidine (NTG) is one of the most potent chemical mutagens yet discovered (Godfrey, O.W. 1974). This mutagen may have been more successful in producing mutants with improved pimaricin production. However, NTG is particularly dangerous and requires stringent safety precautions. As a result, it could not be used with the facilities which were available to us.

The pimaricin fermentation was difficult to scale-up in the 16 l microgen vessel. The culture showed a high requirement for dissolved oxygen and initial fermentation runs produced low yields because of oxygen limitations. This was demonstrated using the dissolved oxygen probe in the microgen (Section 3.7) and also using shake flask cultures (Section 3.4.7). In shake flask, volumes of media above 50 mls in 250 ml flasks showed a sharp decrease in productivity whereas 25 and 15 ml aliquots gave optimal yields. A high agitation rate (500 rpm) gave optimal yields in the microgen (500 mg/ml). However, high aeration resulted in extensive foaming which had to be controlled by automatic addition of antifoam. The medium had a high protein content (soya peptone and soya bean meal) and this may have contributed to foaming.

Periodic contamination by bacteria was also observed during the fermentations. When automatic addition of antifoam was linked to the vessel, a back-pressure could not be applied to the vessel. This would have increased the risk of contamination, especially in the latter stages of the fermentation. In general, there was no difficulty in transferring the culture from the inoculum flask stage to the microgen fermenter and the culture remained dispersed. It was also possible to inoculate the

inoculum flasks (200 mls in 1 l flasks) directly from slopes with an inoculating loop without any loss in productivity.

Feeding with sodium citrate did not prove successful when scaled-up to the microgen. Shake flask studies (Section 3.4.12) with sodium citrate dosing had been shown repeatedly to increase pimaricin production. It is not known why this increase was not repeated in the microgen fermentations.

The aim of this project was to obtain a final pimaricin yield of between 5 and 10 g/l in submerged culture fermentation. This level of production was required in order to make the process commercially viable. It was hoped to scale-up the process to production level in large scale fermenters in Biocon Ltd. The downstream processing and recovery of the antibiotic was not studied in this project. However, these aspects would also need to be thoroughly investigated in order for the process to be brought on-line commercially. Production was increased by over 50% (300 to 700 mg/ml) during the course of this project, the majority of this increase was due to sodium citrate dosing. Further studies are required in order to determine why citrate feeding in the microgen did not improve yields. Only one 16 l fermenter was available during the project and this limited the number of runs that could be carried out during the scale-up process. Mutation and selection provided a number of mutants with increased productivity. However, limited time and manpower did not allow a larger number of mutants to be screened, which could have produced even higher yielding isolates.

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