FUNCTION OF A CLONED POLYPHENOLASE IN ORGANIC SYNTHESIS

THESIS

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by

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DECLARATION

In accordance with the conditions for the award of the degree of Master of Science, I declare that the work presented in this thesis is my own original research. Any assistance with experimental procedure or technique has been declared elsewhere when necessary. Niether the whole nor any part of this thesis has been, is being or will be submitted for a higher degree from any other university.

Michael J. Naidoo

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DEDICATION

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ABSTRACT

The enzyme polyphenolase, which catalyses the oxidation of phenols to catechols and subsequently dehydrogenates these to o-quinones, is widely distributed in nature. The multicopy plasmid vector pIJ702 contains a *mel* gene from *Streptomyces antibioticus*, that codes for the production of a polyphenol oxidase. The plasmid was isolated from *Streptomyces lividans* 66 pIJ702 and subjected to a variety of mutagenic treatments in order to establish a structure-function relationship for the polyphenolase enzymes. An attempt was made to engineer the polyphenolase enzyme by localized random mutagenesis *in vitro* of the *mel* gene on pIJ702, in order to alter properties like productivity, activity and substrate specificity. It was hoped to alter the amino acid sequence of the active site of the enzyme in order to facilitate catalysis in an organic environment. The plasmid was subsequently transformed into a plasmid-free *Streptomyces* strain, and enzyme production was carried out in batch culture systems, in order to determine the effect of the height treatment, and to isolate and propagate functional polyphenolase mutants for organic synthesis.

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CHAPTER 1 GENERAL INTRODUCTION

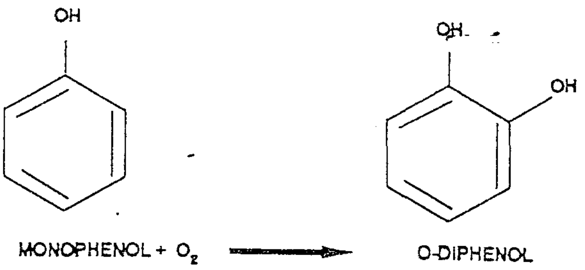
1.1 The polyphenolase enzyme

The enzyme polyphenolase, also known as polyphenol oxidase, DOPA oxidase, catechol oxidase and tyrosinase (EC. 1.14.18.1) is a copper containing monooxygenase, that catalyses the oxidation of phenols with oxygen to catechols and subsequent dehydrogenation to o-quinones (Kazandjian and Klibanov, 1985; Dietler and Kerch, 1979). The o-quinones are unstable in water and are rapidly polymerized to polyaromatic pigments, the melanins. During the oxidation of catechol to quinone, the enzyme is inactivated (Dietler and Kerch, 1979). The melanin biosynthetic pathway is a two-step enzymatic process followed by a series of chemical steps. Both enzymatic steps are catalysed by polyphenolase which has two catalytic properties: **(Figure 1)**

a) cresolase or monophenolase - hydroxylation of L-tyrosine to L-DOPA
b) catecholase or diphenolase - oxidation of L-DOPA to quinone
(Cabanes *et al.*, 1987).

Polyphenolases are metalloenzymes, containing the metal copper in the active site (Dietler and Kerch, 1979). Polyphenols are widely distributed in nature and can be obtained from microbial, plant and animal sources (Kupper *et al.*, 1989). The enzyme has a protective role in plants and animals and may have some metabolic role in bacterial systems. Polyphenolase has also been implicated with the browning reaction in plants and fruit (Wissemann and Lee, 1980). The browning reaction can be prevented by agents that inactivate the enzyme, e.g. heat.

1. Cresolase reaction.



2. Catecholase reaction.

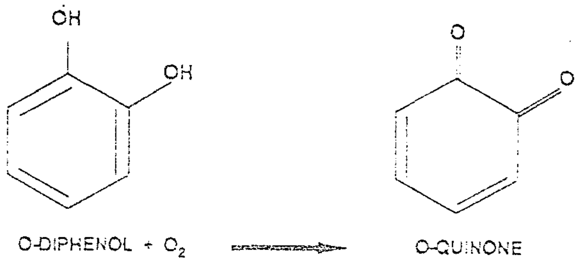


FIGURE 1

The catalytic properties of the polyphenolase enzyme (Cabanes *et al.*, 1987)

1.2 *Streptomyces* tyrosinase

The ability to synthesise melanin or melanin-like pigments is fairly common to *Streptomyces*, unicellular bacteria and fungi (Katz *et al.*, 1983). Tyrosinases from *Neurospora crassa*, *Streptomyces glaucescens*, *Mus musculus* and *Homo sapiens* have all been examined in terms of their primary structures, and are found to contain two highly conserved regions, presumed to be involved in the binding of active site copper ions (Kupper *et al.*, 1989).

Several *Streptomyces spp.* have been isolated that contain polyphenol oxidase enzymes.In *Streptomyces glaucescens*, two identical forms of the enzyme can be produced - intracellular and extracellular, with a molecular weight of 29 000 and a copper content of 0.21%. The rate of secretion of the enzyme was found to be dependant on mycelial mass or surface area, and enzyme secretion was independent of induction. Enzyme production was induced by the addition of certain amino acids, eg. methionine (Crameri *et al.*, 1982). The occurrence of identical amino acid sequences in both intracellular and extracellular tyrosinase suggested that no amino-terminal sequence was necessary for transmembrane transfer of the tyrosinase.

The tyrosinase gene has been cloned into a *Streptomyces* plasmid, so that it could be expressed extrachromosomally in *Streptomyces*. A *Bcl*I-generated fragment of DNA coding for the enzyme tyrosinase was cloned from *Streptomyces antibioticus* into SLP 1.2-based plasmid vectors (pIJ37 and pIJ41) to generate the hybrid plasmids pIJ700 and pIJ701, using *Streptomyces lividans* 66 as host. The 1.55 kb fragment was subcloned into the multicopy plasmid pIJ350 to generate four new plasmids (pIJ702 - pIJ705). pIJ350 carries a thiostrepton resistance gene and two non-essential *Bcl*I sites. All of these constructed plasmids conferred melanin production on the host strain. One of these plasmids, pIJ702, was used as a basis for the mutagenesis work in this study. pIJ702 is a useful cloning vector, containing unique sites for the restriction enzymes *Bgl*II, *Sph*I, *Sst*I, and *Cla*I. DNA insertion at any of the first three sites abolishes the *mel* phenotype, and *Cla*I insertion destroys thiostrepton resistance (Katz *et al.*, 1983) **[Figure 2]**.

The *mel* gene sequence of pIJ702 has been determined and the amino acid sequence deduced. Both intracellular and extracellular forms of tyrosinase from *Streptomyces antibioticus* transformed with pIJ702 were found to be identical and correspond to the amino acid sequence derived from the structural gene. An additional open reading frame (ORF 438) upstream of the *mel* gene was also identified that was implicated in coding for a protein that might have a signal sequence role (Bernan *et al.*, 1985). The production of tyrosinase by *Streptomyces lividans* 66 (pIJ702) was used as a model system for recombinant protein production by *Streptomyces* (Gardner and Cadman, 1990). To this end, attempts were made to optimise growth conditions and enzyme extraction processes.

Commercial interest in *Streptomyces* tyrosinase centres around its ability to catalyze reactions with phenolic compounds. Thus tyrosinase could be used for the removal of phenols from waste waters, and for the catalysis of reactions involving phenolic compounds to form novel products.

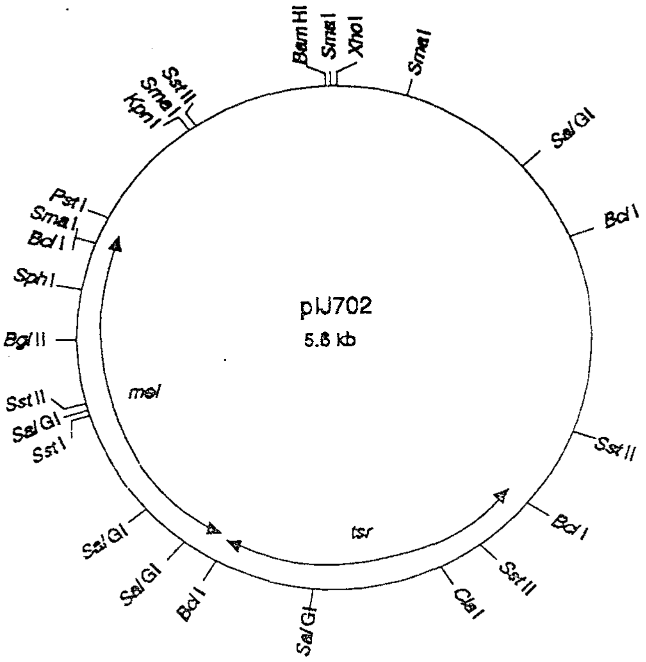


FIGURE 2 RESTRICTION MAP OF pIJ702 (Katz *et al.*, 1983)

The *mel* gene is cloned between two BclI sites. Later experiments with PCR mutagenesis used the unique sites SstI and ClaI.

1.3 Mutagenesis

In an attempt to obtain unique catalytic properties, the enzyme was subjected to a series of mutagenic treatments, firstly to alter the actual catalytic properties of the enzyme's active site, and secondly, to attempt to increase productivity of the enzyme by effecting upstream promoter mutations. Part of the strategy was to alter the amino acid structure of the protein, such that its properties in organic synthesis would change. Alteration of the sequence of amino acids would affect the hydrophobic nature of the protein. It was hoped to alter the structure of the enzyme so that it would retain functionality in an organic environment.

The use of mutagenesis to alter and to study protein structure and function is well documented (Davis *et al.*, 1986). Previously, either actual chemical modifications of the amino acid side chains that form the chemical structure of proteins, or X-ray diffraction of protein crystals, could be used to study protein structure-function relationships. The inherent limitation in these techniques is that large amounts of very pure sample are necessary for accurate analysis to be carried out. However, the application of any of several mutagenic strategies, eg. deletion analysis, insertional inactivation, site-directed amino acid alteration and localised random mutagenesis have expanded the range of techniques available to probe protein structure-function relationships (Sambrook *et al.*, 1989). The development of computer-generated alogarithms to ensemble mutagenesis (Arkin and Youvan, 1992) has further expanded this type of research.

The mutagenesis strategy adopted in this study was localised random mutagenesis. While oligonucleotide-directed mutagenesis is often preferable to random mutagenesis to ascertain the effects of changing specific amino acids on enzymatic activity, it is still useful to generate large collections of random amino acid substitutions in specific regions of a protein (Myers *et al.*, 1985).

Any mutagenic effect that leads to increased production of the tyrosinase enzyme will be of obvious importance in waste-water biotechnology, ie. the treatment of industrial and other effluent, to reduce the level of phenolic contaminants.

1.4 Biotechnological Applications

Most phenols and aromatic amines are toxic and some are even carcinogenic. Therefore, dephenolization processes involving industrial and other effluent waters are becoming commercially more significant (Alberti and Klibanov, 1981). Current methods for the removal of phenols and aromatic amines from waste-waters include microbial degradation, adsorption onto activated carbon, chemical oxidation, organic solvent extraction, membrane filtration processes and irradiation (Alberti and Klibanov, 1981 , Yun *et al.*, 1992 , Leukes, pers. comm., 1993). The physical and chemical treatments outlined are expensive and alternative biotreatment processes are the obvious solution. These include treatment of phenolic effluent with soluble and immobilised phenol oxidases. Immobilised white rot fungi has been shown to remove colour and degrade toxic compounds in phenol contaminated waters (Royer *et al.*, 1985). Treatment with Horse Radish Peroxidase (HRP) facilitated the removal of aromatic amines and phenols from industrial effluent (Klibanov and Morris, 1981). The use of tyrosinase was proposed as an alternative to HRP as it uses molecular oxygen as a cheaper alternative to hydrogen peroxide, as an oxidant (Atlow *et al.*, 1984). An ideal,strategy for treatment of phenolic waste-water would appear to be the immobilisation of microbial cells or purified enzyme onto a solid support (Livingstone and Chase, 1989 , Sun *et al.*, 1992 , Wada *et al.*, 1993). The main advantage to be realised from this approach is that the enzyme is not inactivated by irreversible substrate binding and can be used repeatedly.

1.5 Objectives of this study

An ideal set of objectives would include the production by mutagenesis of polyphenolases that have altered substrate properties and the application of promoter mutagenesis to effect altered enzyme production.

Such an enzyme could then be used against a variety of substrates to determine novel catalytic properties. Uses in waste-water treatment would include soluble and immobilised enzyme strategies against a range of phenolic contaminants. As a further end, the melanins produced by such catalysis could further be included in pharmaceutical preparations as a protective agent, and in paints and dyes as a colouring agent.

This study sought to establish introductory techniques to facilitate the attainment of the above objectives. To this end, plasmid DNA was routinely isolated, purified, subjected to mutagenesis, and then transformed into *Streptomyces* cultures. Enzyme assays were then carried out to determine the efficacy and the extent of the mutagenesis.

CHAPTER 2 ISOLATION AND CULTURE OF BACTERIAL SPECIES

2.1 INTRODUCTION

The *Streptomyces* are Gram-positive, soil microorganisms that differentiate into mycelia and spores (Schrempf *et al.*, 1988). They produce a broad range of different metabolites and bioactive compounds such as extracellular enzymes, enzyme inhibitors, pigments, cytostatics, and antibiotics (Strohl, 1992).

Streptomyces undergo a complex life cycle. The initial growth phase occurs as substrate mycelium. After growth of the colony on the solid surface has nearly ceased, which may be triggered off by nutrient limitation, the colonies develop aerial mycelia, utilizing nutrients primarily provided from the hydrolysis of the substrate. The final stage of development is the conversion of the aerial mycelia to spores, which can be dispersed to begin the life cycle again (Strohl, 1992).

Often, the secretion of extracellular enzymes and metabolites occurs after primary growth has ceased. Thus a vast array of regulatory mechanisms is required, to coordinate the various metabolic processes (Bradley, 1981). The potential for overproduction of antibiotics and novel products via genetic manipulation, and the use of *Streptomyces lividans* as a host for the secretion of recombinant proteins into culture broth, has increased interest in *Streptomyces* genetic regulation.

The *Streptomyces* strains used in this project were *Streptomyces lividans* 66 (pIJ702), *Streptomyces lividans* Tk24, *Streptomyces antibioticus* and *Streptomyces glaucescens.*

Since the main intention of this project was bioproduction of an industrially important enzyme, the culture of the *Streptomyces* was critical to realise efficient bioproduction if the enzyme.

Culture conditions were varied in terms of physical factors like aeration and temperature as well as in chemical factors like pH and medium constituents. This was done to note the effects of these variations on the production of biomass in batch broth cultures, as well as to investigate any relationship between the various factors and the production of the polyphenolase enzyme.

It must be noted that the cells in culture are in an environment that is constantly changing with respect to the presence or absence of nutrients, oxygen, secondary metabolites and toxins. Therefore, it is necessary to establish clear growth curves and to sample at various points along this curve in order to establish an all important relationship between the age of the culture and products of the enzyme of interest. For commercial reasons, it would be important to time sampling along the growth curve at the point when both biomass and enzyme production are optimal (Bourne, 1986).

In order to compare various batch cultures in broth in terms of their biomass production, it had to be ensured that each had been started with an identical number of spores in the first place. To do this, spores were harvested, pooled and stored in glycerol. This ensured that a set of inocula came from a single spore suspension.

In addition, it was found to be detrimental to our studies if the cells formed clumps due to masses of mycelia forming mycelial ball in liquid culture with shaking this. This would cause the cells at the centre to "starve" relative to the peripheral cells. This could be overcome by growing all liquid cultures with reciprocal shaking. Other ways of avoiding this problem would be the addition of sterile glass beads to the medium (Goetsch, pers. comm. 1992) or to alter the sucrose concentration of the medium (Kirby, pers. comm. 1992)

2.2. MATERIALS AND METHODS

2.2.1 Materials

All the strains, media and buffers used are listed in Appendix [A] strains used are also listed Appendix [A]. Antibiotic stock solutions were sterilised by filtration and then added to the correct final concentrates to autoclaved media. Unless otherwise stated, all media and buffers used were prepared in Milli Q water and sterilised by autoclaving at 121°C for 15 minutes.

2.2.2. Methods

Streptomyces cultures were grown at 30°C for varying periods of time, depending on the process for which the cell culture was required. Preparation of cell suspensions for the generation of protoplasts required shorter culture times. Generally, culture times varied between 4 days and 14 days.

2.2.2.1 Cultivation of *Streptomyces* on plates and slants

Streptomyces were cultured on M3 and MMT plates at 30 C for between 4 and 14 days. Cultures were stored for periods up to three months on M3 slants at which were cultured for at 30°C for 10 days and then stored at 4°C. Cultures were started using liquid inoculum suspensions and loop transfers from existing cultures, that were then spread across the surface of the agar.

2.2.2.2 Cultivation of *Streptomyces* in broth

Streptomyces were cultivated in 100 - 250 ml of MMT and M3 medium in 500 ml Erlenmeyer flasks. 20 Glass beads (0,5 cm diameter) were added after autoclaving to the media, prior to inoculation. Glass beads were sterilised separately in McCartney bottles and then added aseptically. The purpose of the glass beads was to prevent the formation of large mycelial pellets. Flasks were incubated at 30°C, with shaking at 100 - 150 rpm, for 4 - 14 days depending on the density of the cell culture required.

2.2.2.3 Preparation of *Streptomyces* spores

Spores were prepared from both slants and plates, 5 ml of sterile water was added to the cultures and the surface of the culture was then scraped with increasingly vigorous motions in order to resuspend the spores in the liquid. The mixtures were then agitated vigorously, and then filtered through a cotton-wool plug into a sterile centrifuge tube. This was done to remove mycelia. The suspension was then pelleted by centrifugation for 3- 5 minutes. on a benchtop centrifuge (Heraeus sepatech, Labofuge Ae) at 3000 rpm at room temperature. The supernatant was discarded quickly and the pellet was resuspended in the remaining droplet of water by agitation. Sterile glycerol was added to a final concentration of 20% in distilled water and the resultant spore suspension was conveniently stored at - 20°C in Eppendorf tubes.

2.2.2.4 Storage of *Streptomyces* cultures

Streptomyces cultures were stored for short periods (up to 6 months) as spores on slants. Long term storage was achieved by storage in glycerol solutions. Spores were harvested from freshly cultured slants or plates as detailed in 2.2.2.3. These were then resuspended in a sterile 20% glycerol solution in microfuge tubes and these were stored conveniently at - 20°C for long periods.

2.2.5 Culture of *Streptomyces* for DNA production , protoplast production, and enzyme assays.

Streptomyces were cultured on M3, pH 7.6 at 30°C for 5 - 7 days. An alternative medium was YEME, pH 7.6. DNA was isolated from this medium after approximately 60 hours.

Protoplasts were prepared from *Streptomyces lividans* TK21 grown in YEME pH 7.6 at 30°C for 36 - 40 hours.

Enzyme assays were carried out on all *Streptomyces* grown in MMT pH 7.6, at 30°C for 7 days. Thiostrepton was included in all of the above media at a final concentration of 5 μg/ml, when *Streptomyces lividans* pIJ702 (plasmid strain) was cultured, in order to maintain selection pressure for the plasmid .

2.2.2.6 Construction of growth curves

Growth curves were constructed of the *Streptomyces lividans* (pIJ702) culture used, as this was the culture used mainly for enzyme assays. Growth curves were constructed by measuring the change in A_{660nm} and A_{450nm} of inoculated media over a period of several days in order to establish the onset of stationery phase. Growth curves were constructed in two ways. The first method was to inoculate MMT pH 7.6 with a starter spore suspension. 2 ml aliquots of the suspension were then withdrawn at various intervals and the A_{660nm} and the A_{660nm} and A_{450nm} were recorded on a Shimadzu UV160A UV-visible recording spectrometer. An alternative method was to start cultures at staggered periods of 1 day apart, in order to obtain cultures of varying ages at the end of predetermined period, usually 10 days. At the end of this time, A 660nm and A 450nm were then measured in order to draw growth curves.

2.3 RESULTS AND DISCUSSION

Streptomyces lividans cultures grown on plates and slants usually reached confluence in 4 - 7 days, if the culture temperature was kept at a constant 30°C.

Streptomyces lividans cultures grown as mycelia in M3 and MMT broth pH 7.6 at 30°C were usually ready for harvesting after 5 - 7 days, when the culture suspension was visibly turbid. The production of pigment was usually taken as a reliable indication that the culture was ready for harvesting for enzyme assays and DNA isolation. Cultures in broth used to produce protoplasts for transformation were usually grown for 36 - 40 hours only as this was found to be optimal for spore production. It was found that for both DNA isolation and spore production, YEME medium was better than M3 as the higher sucrose concentrates assisted with pelleting and with the formation of protoplasts (Kirby, pers. comm.,1992).

In order to maintain conditions of high aerobicity, which is ideal for the culture of *Streptomyces* mycelia, the cultures were grown with constant shaking at 100 - 150 rpm on a rotary shaker. Glass beads were included in the culture media to ensure that the mycelia did not clump together and form balls, but rather grew in a dispersed fashion. Growth as mycelial balls was regarded as unfavourable, because the colonies at the centre of the ball would be subject to different physiological conditions, eg. nutrient limitations compared to the balls on the outside (Hopwood, *et al.*, 1985). Usually a suspension of inoculum in liquid was used to propagate cultures on the surface of agar, in order to ensure uniform growth.

Spore suspensions in 20% glycerol suspensions in distilled water were conveniently stored at -20°C in microfuge tubes and were found to maintain viability for periods of several years. The spores were found to be osmotolerant and could be diluted in distilled water. Spores could be spread directly onto the surface of agar plates using a sterile glass spreader or could be inoculated directly into liquid media.

The growth curves obtained for *Streptomyces lividans* indicated a long period of exponential growth, followed by a stationery phase which during secondary metabolism and enzyme production started. The visually definable production of melanin pigments co-incided with the onset of the stationery phase.

The onset of the stationery phase usually occurred after 5 days. (**Fig. 2.1.** displays a growth curve obtained by culturing *Streptomyces lividans* (pIJ702) in MMT pH 7.6 at 30° C with 100 - 150 rpm shaking, for a period of 10 days. Absorbance values were measured at 660nm, 475nm, and 450 nm. Absorbance readings at 450 nm wavelength were done primarily to follow the onset of melanin pigment production and A_{660nm} and A_{450nm} readings were done to determine the actual cell density.

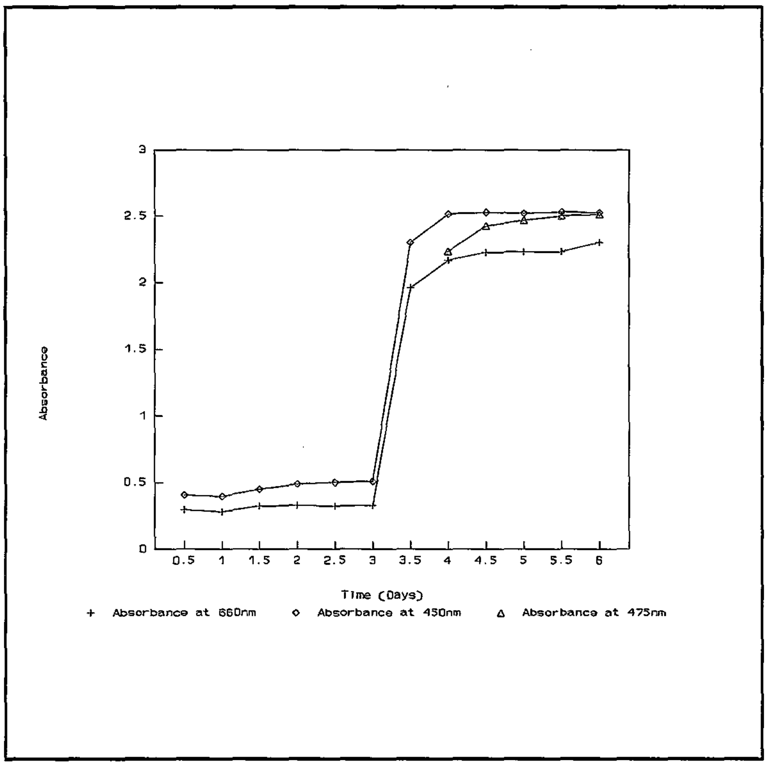


FIGURE 2.1 GRAPH OF ABSORBANCES AT VARIOUS WAVELENGTHS TO DETERMINE GROWTH KINETICS

2.4. CONCLUSIONS

It was found that the *Streptomyces* were relatively slow-growing organisms to culture (compared to *E. coli*) and usually required defined substrate media for studies on enzyme production. However, the extended stationery phase and production of secondary metabolites during this phase made the organism a convenient model system for protein engineering studies.

CHAPTER 3 DNA MANIPULATION

3.1 INTRODUCTION

3.1.1 Development

Recently, there has been a significant increase in interest in the genetics, molecular biology and physiology of the Actinomycetes, notably the *Streptomyces*. This increase in interest has stemmed from two main stimuli, viz. the development of ever-more efficient systems and strategies for the cloning of genetic elements in *Streptomyces* and the increasing importance of the commercial niche that these micro-organisms occupy. In addition to the existing possibilities for *in-vivo* genetic analysis via plasmid-mediated conjugation, and protoplast fusion, *in-vitro* techniques have allowed a more ordered genetic analysis of the production of commercially important secondary metabolites, eg. antibiotics, like the penicillins, and a wide variety of enzymes (Hopwood, *et al.*, 1895).

Plasmids are double stranded circular DNA molecules that replicate independently of the host cell's chromosome. They constitute a major portion of the genetic information in bacteria and confer many significant phenotypic properties, eg. antibiotic resistance, production of metabolites, production of pathogenic factors, conjugal properties, replication maintenance, and other not so well characterised properties, eg. production of melanin in *Streptomyces* (Stanisich, 1984).

3.1.2 Transformation

A number of examples of plasmid DNA have been found in *Streptomyces*. Some of these plasmids have been correlated with specific plasmid functions (Kirby, *et al.*, 1981). The discovery of plasmid species in *Streptomyces* and the ability to introduce plasmid DNA into into intact bacterial protoplasts has increased the range of actual mechanisms available for genetic manipulation of these micro-organisms.

Bacterial transformation is divided into three general stages: binding of the DNA to the outside of the cell, transport of the bacterial DNA across the cell envelope, and the stable establishment of the transforming DNA as a replicon within the host cell (Saunders, *et al.*, 1984).

Mandel and Higa (1970) found that high concentrations of $CaCl_2$ permitted the transformation of intact *E. coli* by phage lambda DNA. Subsequently, $CaCl_2$ treatment has been used to facilitate transfection of *E. coli* by other phages (Cosloy and Oishi, 1973). Cohen, S.N. *et al.* (1972) used $CaCl_2$ treatment to prepare *E. coli* for the uptake of plasmid DNA.

Liposomes are also used to transform bacterial cells with plasmid DNA. Liposome vesicles are formed by the dispersal of phospholipids in an aqueous solution and these vesicles encapsulate discrete volumes of aqueous fluid inside a bilayered lipid membrane. The addition of DNA solutions to a film of dried phospholipid results in the formation of liposome-encapsulated DNA which can then be fused with protoplasts by treatment with polyethylene glycol (PEG) (Makins and Holt, 1981). Both plasmid and phage DNA can be enclosed in liposomes and used to transfect *Streptomyces* protoplasts in the presence of 50% PEG. While this system is useful in the protection of transformant DNA against external DNAses secreted by *Streptomyces*, the overall efficiency of transformation was found to be low (Rodicio and Chater, 1982). *Streptomyces* protoplasts can also be induced by PEG to take up chromosomal DNA. The efficiency of transformation by linear form chromosomal DNA is lower than that for plasmid DNA (Isogai, *et al.*, 1981). Suarez and Chater (1980) describe an efficient method for the transfection of *Streptomyces* protoplasts by the DNA of temperate phages. Transfection was found to be dependant on the concentration of divalent cations in the DNA solution.

Bibb, *et al.* (1978) described a method for the uptake of plasmid DNA into bacterial protoplasts, using *Streptomyces coelicolor* and *Streptomyces parvulus*. Protoplasts are prepared by lysozyme degradation of the Gram positive cell wall in an osmotically buffered medium. Protoplasts are then exposed to DNA molecules in the presence of PEG and after uptake, spread onto suitable regeneration media. Regenerating protoplasts are screened for transformation by the formation of "pocks" by plasmid-bearing regenerants in a plasmid-free background (Bibb, *et al.*, 1977), or by screening for drug resistance markers by adding an overlay of soft agar containing a suitable antibiotic after time has been allowed for phenotypic expression (Chater, *et al.*, 1982). Bibb, *et al.* (1980) developed a system of selectable plasmid vectors suitable for the cloning of DNA fragments in *Streptomyces*. The plasmid vectors carry a methylenomycin A antibiotic resistance marker.

3.1.3 Strategies used in this study

The *Streptomyces* transformation protocols used in this study are based on the work of Bibb, *et al.* (1978), as modified by Chater, *et al.* (1982) and the host strain was *Streptomyces lividans* TK 21.

In order to adequately study the production of tyrosinase by the plasmid-bearing *Streptomyces lividans* 66 pIJ 702, it was necessary to obtain yields of plasmid DNA in the region of 100 to 200 ng/ml. Methods were optimised for the routine isolation of plasmid and total DNA from *Streptomyces*, that resulted in reasonably pure DNA. The use of organic chemicals was minimised in order to avoid deleterious effects on subsequent experiments, eg., amplification by polymerase chain reaction (PCR), or chemical mutagenesis (Fensburg, *et al.*, 1988).

All the DNA isolation protocols involved the following basic steps:

1. Centrifugation of liquid-grown *Streptomyces* mycelia to obtain a pellet.
2. Lysis of the cell walls using chemical or biological means.
3. Separation of membrane and protein constituents from the DNA.
4. Separation of plasmid DNA from chromosomal DNA.

All plasmid purification protocols are based on the relatively small size of the plasmid DNA as compared to chromosomal DNA and on the differential mobility of plasmid through agarose gels, caesium chloride gradients, and chromatographic columns (Maniatis, *et al.*, 1982).

3.2 MATERIALS AND EQUIPMENT

All media used were prepared as outlined in Appendix A. Buffers and solutions used specifically for DNA isolations were prepared as outlined in Appendix B.

Ultracentrifugation was carried out on a Beckman Optima L7 Preparative Centrifuge. Rotors used with this centrifuge were the Beckman fixed angle Type 65 and Type 35 and the Beckman vertical Titanium VTi 65.

Refrigerated centrifugation of samples larger than 2 ml were carried out on a Beckman J2-21 refrigerated centrifuge and in an Eppendorf microfuge inside a 4°C cold-room when samples smaller than 1.5 ml were used. Rotors used with the Beckman J2-21 centrifuge were the Beckman JA-14 and JA-20 fixed angle rotors.

All ultraviolet spectroscopy readings were taken on a Shimadzu UV 160 A UV-Visible recording spectrometer. 1 ml capacity Hellma quartz cuvettes were used to measure optical density (OD) in the ultraviolet (UV) range of 220 to 350 nm wavelength.

Agarose gel electrophoresis was carried out using a Hoefer Scientific Instruments MINNIE submarine agarose gel unit, model HE 33, connected to a Hoefer PS 500X 500V DC power supply pack.

Polyacrylamide gel electrophoresis was carried out on a Hoefer Tall Mighty Small vertical slab unit, model SE 280, connected to the same power pack as for agarose gel electrophoresis.

3.3 METHODS

3.3.1.1 ISOLATION OF PLASMID DNA FROM *Streptomyces lividans* 66 pIJ 702 BY METHOD I

This was an adaptation of the method of Kirby, *et al.* (1982). Colonies were first grown for 4 days on MMT plates containing 5 μg/ml thiostrepton, at 30°C. A thick inoculate was transferred to 200 ml M3 medium pH 7.6 in a 500 ml conical flask, which was then incubated for 5 days at 150 rpm shaking on an orbital shaker.

Cells were harvested by centrifugation at 15 000 g at 4°C for 15 minutes. The cell pellet was resuspended in 5 ml 0.05M Tris-HCl, sucrose 25%, pH 7.6. Cell lysis was achieved by the addition of 25 mg/ml lysozyme solution in 0.25M Tris-HCl pH 8.0. Incubation was carried out at 37°C for at least 2 hours and at 10 minute intervals, the mycelial suspension was vigorously passaged five times through a narrow bore pipette to break up the mycelial aggregates. The protoplast suspension was placed on ice for 5 minutes after which 2.5 ml 0.2M EDTA was added to arrest the action of the lysozyme. After a further 5 minutes, 5 ml 5M NaCl was added and the lysate was left on ice for a further 2 hours. The lysate was then spun at 30 000 g at 4°C for 15 minutes and the resultant pellet was suspended in 6 ml of TE buffer. 1 g/ml CsCl was added to the lysate and 0.2 ml of a 10 mg/ml stock solution of ethidium bromide was added. The density of the solution was adjusted to 1.554 to 1.574 g/ml and the solution was ultracentrifuged at 340 000 g for 4 hours at 20°C in a VTi 65 rotor, using Beckman 6 ml Quickseal tubes. Plasmid and chromosomal DNA bands were visualised under UV light of wavelength 320 nm and the plasmid band was removed using the reverse side of a Pasteur pipette. Ethidium bromide was removed by repeated extraction with isoamylalcohol according to the method of Maniatis, T. *et al.* (1982). CsCl was removed by diluting the DNA-CsCl solution in water, and then precipitating the DNA out of solution using 2 volumes of 95% ethanol. The DNA pellet thus obtained was resuspended in 0.5 ml TE and retained for subsequent experimentation. The concentration of the DNA solution was determined from the A_{260} and the sample purity was determined by determining the UV 260:280 ratios. Samples were subjected to agarose gel electrophoresis in 0.8% agarose gels in TBE in order to visually confirm the presence of plasmid DNA.

3.3.1.2 PLASMID DNA ISOLATION BY METHOD II

This method was adapted from the method of Bibb, *et al.* (1977). *Streptomyces lividans* 66 pIJ702 was cultured in 200 ml YEME medium containing 5 μg/ml thiostrepton under standard conditions for 48 to 60 hours. Mycelia was harvested by centrifugation at 16 000 g for 30 minutes at 4°C in a JA-14 rotor. The pellet was resuspended in 20 ml TE buffer, pH 8.0, containing 25% sucrose. 4 ml of 0.25M EDTA was added, followed by 5 ml of a 50 mg/ml solution of lysozyme in 0.01M Tris-HCl, 0.25M EDTA, pH 8.0. The suspension was incubated at 30°C for 30 minutes until the beginning of lysis was detected by increased viscosity. Ice-cold 34% sucrose in 20 ml TE, pH 8.0 was then added to the sample which was then mixed gently and then transferred to an ice bath. 12 ml ice-cold 0.25M EDTA, pH8.0 and 0.5 ml cold 0.01M Tris-HCl, pH 8.0 (0.5 ml) was then added. The sample was then divided into 10 ml aliquots in precooled centrifuge tubes, and to each tube, 1.4 ml of

10% (w/v) SDS, prewarmed to 50°C was added gently and mixed by inversion. 7.2 ml of ice-cold 5M NaCl was added to each tube. The sample was left on ice for 2 hours and the precipitated protein-SDS and chromosomal DNA complexes were removed by centrifugation at 29 000 g for 30 minutes at 40°C. Two volumes of cold 95% ethanol was added to the supernatant and following incubation at room temperature for 10 minutes, the precipitated nucleic acid was pelleted by centrifugation at 29 000 g as above. Pellets were dried under vacuum, washed in 70% ethanol and then pooled in 2 ml TE and purified by CsCl-ethidium bromide ultracentrifugation. 1g CsCl was added for every 1 ml of the DNA solution, to which was also added ethidium bromide to a final concentration of 250 μg/ml. The density of the solution was then adjusted to 1.554 to 1.574 g/ml and the sample was then sealed into a Beckman Quickseal 6 ml tube and spun at 340 000 g for 12 hours at 20°C. Plasmid and chromosomal DNA were visualized under UV light of 320 nm wavelength and the lower plasmid band was collected in a minimal volume. Ethidium bromide was removed as outlined by Maniatis, *et al.* (1982). The DNA was pelleted from the CsCl solution by the addition of water and 1 volume of isopropanol followed by centrifugation at 12 000 g for 15 minutes at 40°C in a microfuge. The pellet was washed twice with 70% ethanol and resuspended in 0.5 to 1.0 ml TE. Plasmid concentration was determined spectrophotometrically at A_{260} and plasmid purity was determined from UV 260:280 ratios.

3.3.1.3 PLASMID DNA ISOLATION BY METHOD III

Plasmid DNA was isolated using an anion exchange minicolumn supplied with the Qiagen Plasmid Isolation Kit (Catalogue No. 12162) as outlined in The Qiagenologist (1990). Certain steps were modified to improve the efficacy of the techniques with *Streptomyces* cultures.

Streptomyces lividans 66 pIJ702 was cultured in 200 ml M3, pH 7.6, in 500 ml conical flasks for 60 hours at 30°C with 150 rpm shaking. Cells were harvested by centrifugation at 25 000 g for 45 minutes at 40°C. The bacterial pellet thus obtained was resuspended completely in 4 ml Qiagen buffer P1. To this suspension was added 4.0 ml of Qiagen buffer P2 and incubation was carried out for 20 minutes at room temperature. The suspension was mixed gently by inversion to prevent foaming. 4.0 ml of Qiagen P3 was then added and the suspension was then mixed gently. Centrifugation was then carried out at 30 000 g for 30 minutes at 40°C. After centrifugation, the supernatant was collected gently to avoid mixing the supernatant with the potassium dodecyl sulphate layer. At this stage a second centrifugation was carried out at 20 000 g for 15 minutes at 40°C to ensure that the supernatant was completely free of suspended or particulate material which could reduce the efficiency of the subsequent column procedures. A Qiagen Tip 100 column was equilibrated with 3 ml QBT buffer which was allowed to empty from the column by gravity flow. The supernatant was then applied to the Qiagen column and allowed to enter the resin by gravity flow. The column was then washed with 10 ml Qiagen QC. DNA was eluted with 5 ml Qiagen buffer QF. The plasmid DNA was precipitated at 4°C in 2 volumes of 95% ethanol. The DNA pellet was washed in 70% ethanol and then dried under vacuum for 10 minutes, after which, it was redissolved in 1 ml TE, pH 8.0.

3.3.2 ISOLATION OF TOTAL DNA

3.3.2.1 METHOD 1

This method was based on the method of Hintermann, *et al.* (1981) and on modifications by

Krallis, (pers. comm., 1993). *Streptomyces lividans* 66 pIJ 702 and possible mutants were cultured in 50 ml M3 broth containing 5 μg/ml thiostrepton, at 30°C for 4 days with 150 rpm shaking on an orbital shaker. 1.5 ml of the culture was withdrawn and spun down at 12 000 g for 10 minutes at 40°C in a microfuge. The pellet thus obtained was washed twice in TE and then resuspended in 500 μl TE. To this was added lysozyme to a final concentration of 2 mg/ml and the mixture was incubated at 37°C for 3 hours. 30 μl of 10% SDS was then added and the protein-SDS complexes were then extracted with phenol:chloroform:isoamylalcohol 25:24:1. 2 volumes of 95% ethanol was then added to the aqueous phase to precipitate the DNA. The DNA was then washed in 70% ethanol and then vacuum dried. The DNA pellet was then resuspended in a suitable volume of TE, pH8.0.

3.3.2.2 METHOD II

An alternative method was that of (Grimberg, *et al.*, 1989) as modified by Bezuidenhout, (pers. comm., 1993). 1.5 ml of *Streptomyces* cultured as above was withdrawn and spun down at 12 000 g in a microfuge. The cells were washed twice in TE and then resuspended in 500 μl TE. To this was added lysozyme to a final concentration of 10 mg/ml and the mixture was incubated at 37°C for 3 hours with occasional shaking. After 3 hours, the suspension was pelleted by centrifugation at 12 000 g for 15 minutes at 40°C and the pellet was washed twice in pellet lysis buffer. After washing, the pellet was resuspended in 1 ml of pellet lysis buffer (Appendix B) and proteinase K was added to a final concentration of 1 mg/ml. This suspension was incubated at 56°C for 1 hour to ensure uniform membrane lysis. At the end of this time, the supernatant fluid was used without any further treatment to generate fingerprints.

3.3 PREPARATION OF PROTOPLASTS AND TRANSFORMATION

3.3.3.1 PREPARATION OF *Streptomyces lividans* TK 21 PROTOPLASTS

Streptomyces for the preparation of protoplasts was cultured as outlined in **2.2.2.5.** Protoplasts were prepared by the method of Hopwood, *et al.* (1985). Water was added to the mycelial mass which was then centrifuged at 15 000 g for 30 minutes at 40°C. The pellet thus obtained was washed in 10.3% sucrose and pelleted again. This pellet was then resuspended in 5 ml of Solution P (Appendix B) containing 3mg/ml lysozyme. The mixture was incubated at 30°C for 1 hour and the solution was passaged through a pipette every 15 minutes in order to break up the mycelial balls. A further 5 ml of Solution P was added and the solution was then applied to a 20 ml syringe containing a 2 cm cotton wool plug. Gravity filtration was carried through the cotton wool plug in order to remove any remaining mycelia. The sample was then centrifuged at 4000 rpm for 5 minutes on a Heraeus Sepatech Labofuge model AE benchtop centrifuge in order to gently pellet the protoplasts. The supernatant fluid was poured off carefully and the protoplasts were resuspended in the drop of fluid that remained. 5 ml of Solution P was added and the protoplast suspension was then divided into 5 ml aliquots and stored at -70°C. Protoplast regeneration frequencies were tested by aliquoting a suitable quantity of protoplasts onto R2YE medium and counting the number of regenerants after 4 days at 30°C.

3.3.3.2 TRANSFORMATION OF *Streptomyces lividans* TK 21 WITH THE PLASMID pIJ702 AND MUTANTS DERIVED FROM pIJ702

Protoplasts were retrieved from storage and warmed to room temperature. The microfuge was shaken to dispense clumps and 50 μl of the protoplast suspension was removed. Up to 1 μg DNA in 5 μl TE was added to the protoplast suspension, on the surface of a sterile Petri dish. 200 μl of T buffer (Appendix B) was added to this mixture, and the suspension was carefully mixed a pipette. The DNA-protoplast-buffer mixture was then carefully spread over the surface of 2 dried R2YE plates and left at room temperature to dry. Incubation was carried out for 12 to 16 hours at 30°C until growth was just visible. The R2YE plates were then overlaid with soft nutrient agar (sna) containing 500 μg/ml thiostrepton. Incubation at 30°C was continued until discrete colonies appeared on the surface of the agar (Bibb, *et al.*, 1978, Chater, *et al.*, 1982, Thompson, *et al.*, 1982).

3.3.4 AGAROSE GEL ELECTROPHORESIS

Agarose gels were prepared as outlined in Appendix B. For plasmid DNA, 0.8% agarose gels were prepared, while 2% agarose gels were prepared for the examination of PCR products. All agarose gels were run at 10 V/cm for 1 to 2 hours at room temperature in 1 X TBE. Agarose gels were stained by the inclusion of ethidium bromide to the gel mixture to a final concentration of 0.5 μg/ml (Maniatis, *et al.*,1982). Gels were visualised by UV transillumination using UV light at 300 nm wavelength on a UVP Incorporation Transilluminator, and photographed using a UVP Gel Documentation System (model GDS 2000).

3.3.5 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Polyacrylamide gels and buffers were prepared as outlined in Appendix B. The gel apparatus used was the "sturdier" vertical slab gel unit by Hoefer Scientific Instruments, model SE 400. Gels were run at 150 V for 6 to 7 hours at 25°C until the dye front had reached the end of the gel, using a Hoefer PS 500 X DC 500 V power pack. Gels were visualised by silver staining and photographed using a UVP Gel Documentation System (model GDS 2000).

Silver staining is a chemical reaction (Bassam, *et al.*, 1991) in which the silver ions are reduced by formaldehyde to metallic silver, under alkaline conditions. The gels were soaked in 10% ethanol, 0.1% glacial acetic acid (v/v in H_2O), for 3 minutes followed by a 10 minute incubation in 0.1% silver nitrate solution (w/v in H_2O), after the gels were rinsed twice with H_2O. The silver stain was then developed by incubating the gels in 1.5% NaOH (w/v), 0.01% sodium borohydrate (w/v), and 0.15% formaldehyde (v/v) for 20 minutes. Development was stopped by rinsing the gel in distilled water and analysis was carried by photographing the gels and comparing the photographs using appropriate software.

3.3.6 ULTRAVIOLET (UV) SPECTROSCOPY OF DNA SAMPLES

All DNA isolates were scanned in the UV wavelength range of 320 nm to 260 nm to obtain a representation of the DNA samples obtained. UV 260 nm readings were used to establish the concentration of the DNA and UV 260/280 nm ratios were used to assess the purity of the DNA solution (Muller, 1993).

3.4 RESULTS AND DISCUSSION

3.4.1 ISOLATION OF PLASMID DNA FROM *Streptomyces*

Plasmid DNA was isolated by the methods of Kirby, *et al.* (1982), Bibb, *et al.* (1977), and by the method outlined in ''The Qiagenologist '', 2nd Ed. (1990), supplied with the Qiagen Plasmid Isolation Kit. All three methods were successful in yielding supercoiled plasmid DNA. Figure **3.1** depicts an agarose gel showing supercoiled plasmid yielded by the respective methods. It is evident from Figure 3.1, that method III, ie. the isolation of plasmid DNA using the Qiagen anion exchange minicolumn was the most suitable method as the yield and purity of the DNA was high. Ion-exchange is a widely used chromatographic technique for nucleic acid purification, but the results have been less than optimal for the absolute

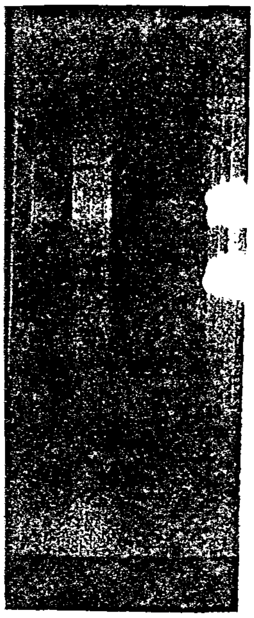


FIGURE 3.1

1% Agarose gel stained with 0.5 μg/ml ethidium bromide. Electrophoresis was carried out for 2 hours at 10 V/cm in 1 x TBE.

Lane 1 - Plasmid DNA isolated by method I
Lane 2 - Plasmid DNA isolated by method II
Lane 3 - Plasmid DNA isolated by method III

separation of DNA and RNA. Qiagen anion-exchange minicolumns are created in a unique process in which macroporous silicagel with a particle size of approximately 100 μm is covalently coated with a hydrophillic substance that prevents nonspecific binding and results in high density anion-exchange groups that allow a broad separation range of up to 1.4M salt, the elution point for double-stranded DNA (The Qiagenologist, 2nd Ed, 1990). Table **3.1** presents the $A_{260/280}$ ratios and the concentration of the DNA was calculated from the A_{260} readings.

TABLE 3.1 A COMPARISON OF THE UV SPECTRA OF THE PLASMID DNA OBTAINED BY METHODS I, II AND III

METHOD	A_{260}	A_{280}	$A_{260/280}$
I	0.300	0.138	2.17
II	0.250	0.150	1.66
III	0.438	0.238	1.84

Given that A_{260} of 1 = 40 μg supercoiled double-stranded DNA, the following plasmid DNA concentrations were obtained :

Method I A_{260} = 0.300 = 0.120 μg/ul plasmid DNA

Method II A_{260} = 0.250 = 0.100 μg/ul plasmid DNA

Method III A_{260} = 0.438 = 0.175 μg/ul plasmid DNA

The $A_{260/280}$ ratios for methods I, II, and III were respectively 2.17, 1.66, and 1.84. An OD ratio of 1.8 represents pure double-stranded DNA. Higher values represent RNA contamination and lower values indicate protein contamination (Muller, 1993). For our purposes, it was necessary to obtain DNA that was reasonably clean (OD ratios of 1.7 to

1.9), as the plasmid was to be subsequently subjected to a series of mutagenic experiments and then transformed into a new host in order to assess the effects of the mutagenic treatment. Thus all subsequent plasmid isolations were carried out as outlined in 3.3.1.3, ie. Method III. While both Methods I and II showed that there was a relationship between the efficacy of the lysozyme treatment and the presence of high concentrations of sucrose and glycine in the growth media (Schrempf, *et al.*, 1975), the Qiagen method appeared to be independant of such factors and yielded consistently good yields of plasmid DNA.

3.4.2 ISOLATION OF TOTAL DNA

Both the protocols outlined in 3.3.2.1 and 3.3.2.2 yielded sufficient DNA for further analysis and manipulation. A comparison of the concentrations of the DNA from the two methods showed that the protocol outlined in 3.3.2.2 yielded more DNA. 3.3.2.1 yielded DNA that had a final concentration of 0.04 μg/ul and 3.3.2.2 yielded DNA that had a final concentration of 0.1 μg/ml. The protocol of 3.3.2.2 was chosen in subsequent isolations because it was found to be less time consuming and it made use of no dangerous organic chemicals.

3.4.3 PROTOPLAST REGENERATION AND TRANSFORMATION OF Streptomyces *lividans* TK 21

Protoplasts prepared as outlined in 3.3.3.1 had a concentration of 1 X 10^{10} protoplasts/ml suspension. 50 μl of this suspension was used per transformation and this contained approximately 5 X 10^{8} protoplasts. Protoplasts regenerated on R2YE plates yielded a viable count of 1 X 10^8 cfu/ml or 5 X 10^6 protoplasts per 50 μl aliquot. These values are similar to experimental results obtained by Isogai, *et al.* (1981) in which they obtained about 2 X 10_8 cfu/ml from 2 day-old *Streptomyces* cultures. Regeneration frequencies observed in these experiments were approximately 1%. Several factors affect the preparation and regeneration of protoplasts. These include the age of the *Streptomyces* culture, the concentration of glycine in the medium, the dryness of the R2YE plates and the density of the protoplast suspension (Thompson, *et al.*, 1982, Okanishi, *et al.*, 1974). Provided aliquots of protoplasts were frozen gently and then stored at -70°C, there was no significant decrease in the number or percentage of regenerants on R2YE plates. Based on experimental evidence presented by Baltz and Matsushima (1981) and on the experimental data obtained in this study, protoplasts were optimally regenerated on R2YE plates that had lost about 20% weight by dehydration. The optimal temperature for regeneration was 30°C. Protoplasts were ideally prepared from mycelia grown to about late exponential phase. There was a significant decrease in protoplast regeneration frequency when mycelia in the stationery phase of growth were harvested for protoplast production.

Surface dehydration of R2YE medium is believed to facilitate protoplast regeneration by rapidly dehydrating soft agar overlays, which then minimises protoplast lysis, and provides an environment suitable for rapid cell wall regeneration. Dehydration might also function to retard diffusion of auto-inhibitory substances from developing cells, and this facilitates more efficient regeneration, particularly if these diffuse substances are autolytic proteins (Baltz and Matsushima, 1981).

For transformation, 50 ul of protoplasts, ie. 5 X 10^5 cfu/ml, was made up to 250 μl in T buffer to which 1 μg of DNA in TE was added. Then 50 μl of this suspension, containing approximately 1 X 10^6 cfu/ml was then spread onto the surface of a dehydrated R2YE plate, which was then incubated for 16 hours at 30°C or until the beginning of growth above the surface could just be discerned. A soft agar overlay was added after this time and incubation continued. 1 X 10^5 to 1 X 10^6 transformants per plate were routinely achieved, which corresponded to a transformation efficiency of 1 X 10^5 to 1 X 10^6 transformants per microgram supercoiled pIJ702 in *Streptomyces lividans* TK 21.

The factors that affect the efficiency of transformation are similar to those that affect the percentage of protoplasts regenerated, since the number of protoplasts capable of regeneration, will directly affect the number of transformants obtained.

Bibb, *et al.* (1978), Thompson, *et al.* (1982), and Isogai, T. *et al.* (1981) have conducted experiments to determine the various parameters that affect transformation efficiency in *Streptomyces*. Bibb, *et al.* (1978), showed that a definite relationship exists between polyethylene glycol (PEG) concentration and the transformation of *Streptomyces coelicolor* A3(2) by $SCP2_*$ DNA. Using PEG 1000, it was found that the optimal concentration of PEG was between 20% and 25% and all transformation experiments used 25% PEG 1000. The actual mechanism by which PEG induces transformation is unclear. It is possible that the PEG interacts with the cell membrane to make it more permeable to DNA, or that high concentrations of PEG in solution cause nucleic acid molecules to undergo a conformational change that makes them more compact, facilitating penetration into protoplasts.

Transformation efficiency may also vary with the DNA concentration (Isogai, *et al.*, 1981), but for this study an amount of 1 μg of plasmid DNA was used per transformation.

From the experimental data gleaned from this study, it is shown that plasmid DNA was introduced at high frequency into *Streptomyces*. The parameters that affected transformation, ie. growth phase of the mycelium, PEG concentration, sucrose concentration, moisture content of the medium, and protoplast density were all adjusted to allow optimally efficient transformations. This facilitated later experiments in which the isolated DNA was subjected to mutagenic treatment and then transformed into a plasmid-free *Streptomyces* strains in order to assess the efficacy of the mutagenesis treatment.

CHAPTER 4 POLYMERASE CHAIN REACTION (PCR)

4.1 INTRODUCTION

4.1.1 Development of PCR

The capacity to amplify specific fragments of DNA, made possible by the polymerase chain reaction (PCR), represents an event that has impacted deeply on the approach to both fundamental and applied biological problems (Erlich, 1989). The polymerase chain reaction invented by Kary Mullis (Mullis *et al.*, 1986) was originally applied to the amplification of the human b-globin gene and to the prenatal diagnosis of sickle-cell anaemia (Saiki *et al.*, 1985).

The polymerase chain reaction is an *in vitro* method used to amplify a segment of DNA that lies between two regions of known sequence. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies double at every cycle. This results in exponential yields of target DNA, ie. twenty cycles of PCR yields 2^{20} copies of target sequence (Erlich, 1989).

Originally, the Klenow fragment of DNA polymerase I was used to extend the DNA target sequence (Saiki *et al.*, 1985 , Mullis *et al.*, 1986, Mullis and Faloona, 1987). Since the

DNA polymerase I enzyme was inactivated at the high temperatures, required to denature the DNA template, fresh enzyme had to be added before a new round of synthesis was initiated. Yields were poor with target DNA sequences larger than 200 bp, and heterogeneously sized particles often resulted (Scharf *et al.*, 1986). Another problem encountered was that some of the products were "shuffled" and consisted of mosaics of different alleles of the target sequences, and the Klenow polymerase reaction reached an activity plateau after approximately 20 cycles (Saiki *et al.*, 1988).

These problems were solved with the introduction of a thermostable DNA polymerase (Saiki *et al.*, 1988) purified from the thermophilic bacterium *Thermus aquaticus* (Chien *et al.*, 1976). The introduction of *Taq* polymerase facilitated more efficient PCR conditions that were conducive to automation since the enzyme did not have to be replaced at every round, as the *Taq* enzyme could survive extended incubation at 95°C. Also, since annealing and extension of oligonucleotides can be carried out at elevated temperatures, mispriming is reduced, specificity is greater and the yield of the target sequence is greater. Although extremely efficient, the amplification of a target sequence by *Taq* becomes self-limiting after 25-30 cycles, and can only be increased by special methods, eg. re-amplification of an amplified target sequence, that has been diluted 1000 fold for use as template can further increase the total yield of a desired target DNA sequence (Saiki *et al.*, 1988).

4.1.2 Applications of PCR

Although the PCR is considered to be primarily a method for producing copies of a specific sequence, it has found extensive applications in other uses. Clinical uses include the diagnosis of genetic disorders (Wong *et al.*, 1987, Engelke *et al.*, 1988) and the detection in clinical samples of the presence of the nucleic acid sequences of pathogenic organisms eg. viruses like HIV in human blood samples (Kwok *et al.*, 1987, Ou *et al.*, 1988). Forensic uses include obtaining DNA-typing information from individual hair samples or from single sperm cells or diploid cells (Higuchi *et al.*, 1988, Li *et al.*, 1988). In addition, the PCR has been used in a number of molecular applications. These include the generation of sequence specific probes, the generation of cDNA libraries from small amounts of mRNA, the generation of large amounts of DNA for sequencing (Stoflet *et al.*, 1988, Innis *et al.*, 1988), the generation and analysis of mutations, and chromosome crawling (Sambrook *et al.*, 1989). Reysenbach, *et al.* (1992) used the PCR in molecular phylogenetic studies to clone and compare the 16S RNA genes of natural microbial communities. PCR has been used by Love *et al.* (1990) to analyse microsatellites in the mouse genome for size variation. Williams *et al.* 1990) describe amplification of genomic DNA using single primers of arbitrary nucleotide composition, using PCR. Chalmers and co-workers (1992) used RAPD markers for the detection of variation between and within populations of multipurpose leguminous trees of Central America and Mexico. Their results showed that variability was partitioned between and within population components, and they identified specific genetic markers. Kaemmer *et al.* (1992) also used arbitrarily primed polymerase chain reaction (AP-PCR) to determine genetic polymorphisms in its representative species and cultures of the genes *Musa* (banana). Fingerprints obtained were analyzed by computer to obtain dendograms that suggested that AP-PCR dendograms could be used to estimate intraspecific relatedness with cultivated banana species. AP-PCR and RAPDS are essentially the same application of the PCR in fingerprinting studies.

Another use of the PCR is to alter a template sequence, in order to introduce random mutations along the length of the template DNA. This mismatch-directed PCR was used to create randomly mutated sets of a specific fragment of the *mel* gene, and is discussed in more detail in the chapter on mutagenesis. The application of the PCR to an increasing range of molecular applications has a virtually unlimited future and more novel applications of the PCR, particularly in research, are being discovered (Haff, L. 1993).

4.1.3 PCR strategies for the experimental work in this study

This study focuses on the use of PCR to generate specific segments/fragments of a portion of the *mel* gene, present in the plasmid pIJ702 using primers specifically designed for this purpose.

Attempts were also made to generate sets of DNA fingerprints using a set of random primers, in order to establish relationships between mutant and wild-type *Streptomyces*.

4.1.4 Components of the PCR

Oligonucleotide primers should be at least 10 nucleotides long, and are usually between 10 and 30 nucleotides long. Usually oligonucleotides are used at a concentration of 50-100 pmol. Higher concentrations can cause ectopic priming of non-desirable sequences and limiting concentrations cause extremely inefficient PCR with low yields (Sambrook *et al.*, 1989, Erlich *et al.*, 1989).

The standard PCR buffer contains 50 mM KCl, 10mM Tris. Cl (pH 8.3 at room temperature) and 1.5 M Mg Cl_2. The pH at 72°C drops by 1 unit to approximately 7.2. The concentration of divalent cations is critical and any changes in the cation used or the concentration thereof will have a profound effect on the fidelity of the amplification. Magnesium ions are superior to manganese, and calcium ions are ineffective (Chien *et al.*, 1976).

The enzyme *Taq* DNA polymerase obtained from different suppliers has different activities. The enzyme is usually used at concentrations of 2 units per 50 μl reaction and this concentration was found to be optimal (Sambrook *et al.*, 1989, Bezuidenhout, 1993 pers. comm.). BSA may be included in PCR reactions to stabilise the enzyme.

Deoxynucleotide triphosphates (dNTPs) are normally used at a concentration of 100-400 μMol for each dNTP and pH is usually maintained at 7 (Sambrook *et al.*, 1989).

The template DNA is usually added as closed circular DNA or linear single stranded DNA or double-stranded DNA at a concentration of 10-100 ng per reaction. Control reactions are necessary to optimise the concentration of template required. Paraffin is usually added to overlay the aqueous components of the PCR in order to limit the loss of volume by evaporation during thermal cycling (Erlich, 1989).

PCR is carried out by the incubation of samples containing all of the above components, at three temperatures corresponding to the three steps in a cycle of amplification, ie., denaturation, annealing, and extension (Mullis, 1990). Cycling is usually achieved automatically using a thermal cycler. A typical reaction allows denaturing of template DNA by heating to 94°C, followed by annealing of primers to their complementary sequences, at 30-60°C, followed by *Taq* polymerase extension of the annealed primers at 72°C. The times allowed for denaturation, annealing and extension, as well as the time taken to change from one temperature to another (ramp temperature), and the actual number of repetitive cycles conducted is known as the profile. The profile varies with the type of PCR being conducted eg. specific PCR or RAPD fingerprinting and a number of trial and error experiments are necessary to establish a profile that is optimal for a particular application (Mullis and Faloona, 1987, Innes, 1990, Bezuidenhout, 1993 pers. comm.).

4.2 MATERIALS AND EQUIPMENT

The polymerase chain reaction was carried out in a Hybaid Omnigene Thermal Cycler (model HBTR3CM). *Taq* DNA polymerase was purchased from Biolabs and was supplied in storage Buffer. This buffer contained 50 mM Tris-HCl, pH 8.0 at 25°C, 100 mM NaCl, 0,1 mM EDTA, 5 mM DTT (dithiothreiotol), 50% glycerol and 1% Triton - X100.

Taq DNA polymerase was used with 10X *Taq* DNA polymerase reaction buffer supplied by Promega. This buffer contained 500 mM KCl, 100 mM Tris-HCl, pH 9.0 at 25°C and 1% Triton X-100. A tube of 25 mM MgCl2 was also supplied with the reaction buffer.

All other buffers and solutions were made up in sterile Milli-Q water. Liquid paraffin was sterilised by autoclaving in 1 ml aliquots and each aliquot was discarded after being opened. Template DNA for specific amplification was pIJ702 obtained as outlined in 3.3.1.3 and template DNA for *Streptomyces* total DNA fingerprinting was obtained as outlined in 3.3.2.2. The concentrations of both templates were varied as outlined in 4.3.3 and 4.3.5.PCR reactions were conducted in 0,5 ml sterile Locktight Eppendorf microfuge tubes.

4.3 METHODS

4.3.1 Design of primers for the specific amplification of the *mel* gene from *S. antibioticus*.

Two primers designated Tyrosinase Foward (Tyr-F) and Tyrosinase Reverse (Tyr-R) were designed. Tyr-F is an oligonucleotide primer of 21-mer and Tyr-R is an oligonucleotide primer of 20-mer. The Tyr-F primer was designed to flank the region downstream of the ORF438 region of the *mel* gene of pIJ 702 and was a 21-mer + strand sequence designed to initiate - strand synthesis. The sequence of primer Tyr-F is:

5^1 GTC GAC GAG C | | TC CAG GGC 3^1

*Sst*I

and was designed to include an *Sst*I site downstream from ORF 438 of the *mel* gene. Complete sequence of *mel* and *tsn* genes of pIJ702 are given in Appendix C. The Tyr-R primer was designed as a 20-mer-strand sequence, designed to initiate + strand synthesis, in the *thiostrepton* resistance gene of pIJ702. The sequence of primer Tyr-R is:

5^1 GT GAC AT | | C GAT GAT GCC GTC 3^1

*Cla*I

The primers flank a 1.3 kb region that includes the *mel* and *tsn* genes of pIJ702. Both primers were synthesized by the University of Cape Town by automated phosphoramidite synthesis.

Both primers were stored at -20°C and dilutions of each were stored separately, also at -20°C for regular use in the PCR. Primer Tyr-F and TYR-F were diluted to yield about 50 pmol of primer/μl.

4.3.2.1 Polymerase chain reaction to produce specific DNA fragments

A PCR reaction, to amplify a specific portion of the plasmid pIJ702, was set up, based on Saiki, R. (1989) and Mullis and Faloona (1987) with modifications proposed by Krallis, N. (1992, pers. comm) and Bezuidenhout, C.C. (1993, pers. comm.).

Table 4.1 PCR reaction indicating order of addition of reagents

REAGENT ADDED	**VOLUME (μL)**
10 X PCR Buffer	5
Milli-Q Water	37.6
Taq (5u/μl)	0.4
dNTP's (10 mM mixture of all 4)	1
Primer Tyr-F (50 pmol/μl)	1
Primer Tyr-R (50 pmol/μl)	1
$MgCl_2$ (25mM)	2
Template DNA (17-175 ng/μl pIJ702)	1
Sterile liquid paraffin	50

4.3.2.2 Optimization of PCR for amplification of a specific DNA fragment

All PCRs to amplify the *mel* region of pIJ702 had the following general profile:

Step 1 : An initial denaturation at 94°C for 300 s followed by cycling for 30-40 cycles. The cycles consisted of:

Step 2 : Denaturation at 94°C for 30s

Step 3 : Annealing at 35°C to 55°C for 30s to 60 s.

Step 4 : Extension at 72°C for 60-120 s

(Innes *et al.*, 1990).

Step 5 : This was followed by a final extension of 72°C for 240 s, and then the samples were stored at 4°C until analysis (Saiki, 1989).

The template DNA concentration was varied between 17 and 170 ng per reaction.

A number of factors affect the specificity of the amplification reaction. These include parameters like annealing temperature, template DNA concentration, and primer concentration. A series of "trial and error" experiments were conducted, in order to obtain a permutation of parameters that was considered to be optimal for the generation of multiple copies of the fragment of DNA of interest. Once obtained, such profiles were used in all subsequent PCRs with the same objective.

4.3.3.1 Polymerase chain reaction to produce fingerprints of *Streptomyces* DNA

This experiment was based on research conducted by Caetano-Annoles *et al.* (1991), Chalmers *et al.* (1992), Kaemmer *et al* (1992) and on experimental recommendations by Bezuidenhout, C.C., pers. comm. (1993).

PCR reactions for fingerprinting analysis were set up as follows:

Table 4.2 Addition of Reagents for Arbitrarily Primed PCR (AP-PCR)

REAGENT	VOLUME (μl)
10 x PCR Buffer	5
BSA	1
dNTP's (10 mM of each)	2
Sterile water	31.6
Taq (5u/μl)	0.4
Primer 8	2
$MgCl_2$	2
Acetamide	5
Template DNA (Total DNA)	1
Sterile Liquid Paraffin	50

4.3.3.2 Optimization of PCR for RAPD's

All RAPD's had the following general profile:

Step 1 : An initial denaturation at 94°C, followed by cycling for 30-40 cycles.

The cycles consisted:

Step 2 : Denaturation at 94°C for 30s

Step 3 : Annealing at 35°C to 50°C for 30s to 60 s.

Step 4 : Extension at 72°C for 60-240 s (Innes *et al*, 1990).

Step 5 : A final extension at 72°C for 240 s. Samples were then cooled to 4°C and stored until analysis (Saiki, 1989).

Template DNA, ie. *Streptomyces* total DNA was obtained at a concentration of 0,050 $\mu g/\mu l$ and a series of dilutions of crude template DNA was then made up in the range 10^{0}, 10^{-1}, 10^{-2}, in order to optimize the template DNA concentration.

Based on the results obtained, an optimum profile of the parameters for fingerprinting *Streptomyces* DNA was obtained. Once obtained, such profiles were used in all subsequent PCR's with the same objective.

4.3.3.3 Analysis of PCR results

PCR for the amplification of specific bands was tested by subjecting the PCR products on agarose gel electrophoresis on 2% agarose gels, as outlined in 3.3.4. Photographs of positive results were also recorded as outlined in 3.3.4. RAPD's were tested by subjecting the PCR products to polyacrylamide gel electrophoresis (PAGE) as outlined in 3.3.5 (Laemmli 1970). Results were photographed and analyzed as outlined in 3.3.5.

4.4 RESULTS AND DISCUSSION

4.4.1 Primer design

Both primers Tyr-F and Tyr-R showed positive results with PCR to amplify the region of interest on the *mel* gene of pIJ702. The primers were used optimally at 1/16 and 1/12 dilutions, respectively, to yield a primer concentration of 50 pmol/μl. Since the primers to a large extent determine the success or failure of amplification, design of primers was an important criterion (Kureisha and Bryan, 1992). The design of the primer reflected the following general rules:

a) primer GC content was similar to that of the fragment being amplified. For primer Tyr-F, the % GC = 76%, and for primer Tyr-R, the % GC = 56%.

b) The primers were checked against each other to ensure that they were not complementary, to ensure that primer dimer formation was reduced. The formation of primer-dimers by primers that are reasonably complementary, reduces the annealing of primer to target sequence and thus causes reduced amplification (Erlich, 1989).

In the event of primer-dimer formation, successful PCR can still be accomplished by boiling each primer prior to use for 10 minutes, and adding *Taq* DNA polymerase only after the initial denaturing step (Kureisha and Bryan, 1992).

4.4.2 Specific fragment amplification by PCR

Fig 4.1 depicts the results of an initial attempt to amplify the *mel* gene of pIJ702 downstream of ORF 438. The conditions for this PCR were as follows:

Step 1 Initial denaturation at 94°C for 5 mins
Step 2 Denaturation at 94°C for 30 seconds
Step 3 Annealing at 45°C for 30 seconds
Step 4 Extension at 72°C for 150 seconds
Step 5 Final extension at 72°C for 5 mins

Steps 2 to 4 were repeated to result in a total of 40 cycles of PCR.

From Figure 4.1, Lane 1 represents pBR 322 digested for 1 hr with *Hinf*I, at 37°O, for use as marker. the 1,5 k and 0,5 kb bands indicated, both flank the target sequence of 1 kb. Lanes 2 and 3 represent PCR product resulting from the addition of 100 pmol of primer. Lanes 4 and 5 represent PCR reactions resulting from 10 pmol of primer. No amplification was apparent. Lanes 6 and 7 depict PCR reactions using 50 pmol of primer, and 175 ng of template DNA.

It is evident from Fig 4.1 that 175 ng (Lanes 6 and 7) of template DNA

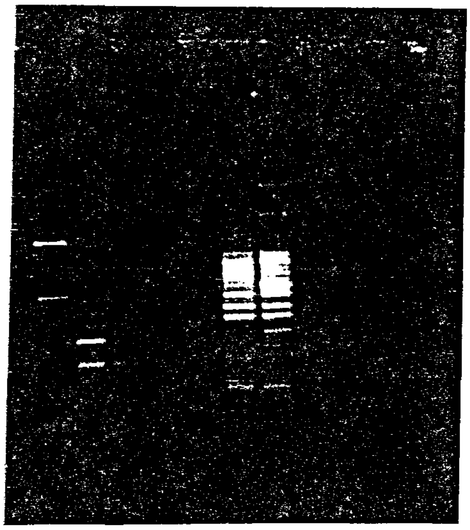


FIGURE 4.1

PCR amplification of the *mel* gene of pIJ702 using primers TYR-F and TYR-R. Electrophoresis was conducted at 1o V/cm in 1 X TBE.

Lane 1 : pBR322 DNA digested with *Hinf*I for use as marker

Lanes 2 and 3 : PCR products

Lanes 4 and 5 : No result

Lane 6 and 7 : PCR products

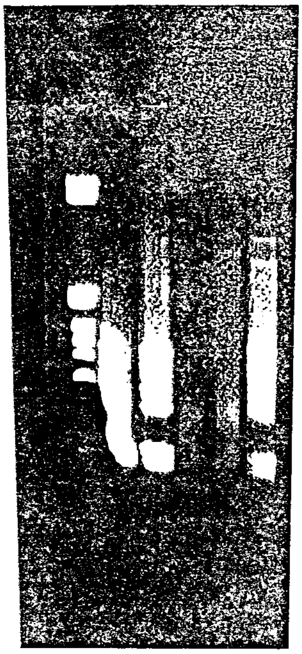


FIGURE 4.2

PCR amplification products on an agarose gel after electrophoresis at 10 V/cm in 1 X TBE. Products were the result of elevated annealing temperatures.

Lane 1 : Marker DNA as in previous gel

Lanes 2 and 3 : PCR products

Lane 4 : No amplification

Lanes 5 and 6 : PCR products

produced more of the target sequence. However, while the target sequence was amplified, other sequences that might have homology with the target sequences were also amplified, resulting in a "fingerprint" of other sequences along the plasmid pIJ702. It was therefore decided to carry out amplification of the same sequence, using 175 ng of template DNA, at a higher temperature, to ensure that unwanted sequences would not be amplified.

Figure 4.2 represents a repeat of the PCR reaction at a higher annealing temperature to eliminate non-specific amplification. The PCR reaction profile was as follows:

Step 1 Initial denaturation at 94°C for 300 seconds
Step 2 Denaturation at 94°C for 30 seconds
Step 3 Annealing at 50°C for 60 seconds
Step 4 Extensions at 72°C for 120 seconds
Step 5 Final Extension at 72°C for 300 seconds

Steps 2 to 4 were repeated to result in a total of 38 cycles of PCR.

From Figure 4.2, Lane 1 indicates marker DNA as in the previous gel. Lanes 2 and 3 represent the PCR amplification products resulting from 100 ng of template DNA. Lane 4 represents the products of a PCR reaction using 50 ng of template DNA. Lane 5 represents the PCR product from a reaction using 75 ng of template DNA. Lane 6 represents the PCR products resulting from 175 ng of template DNA. While it is evident from Fig 4.2 that the amplification of a specific band was more successful, ie. there were fewer bands present than in Fig 4.1, it is still clear that the amplification of the specific band was not totally successful.

To this end, further optimization was carried out. Elevated annealing temperatures were used in a subsequent PCR in order to narrow the range of amplification. The PCR profile for the reaction was as follows:

Step 1 Initial denaturation at 94°C for 300 seconds
Step 2 Denaturation at 94°C for 30 seconds
Step 3 Annealing at 52°C for 30 seconds
Step 4 Extension at 72°C for 90 seconds
Step 5 Final extension at 72° for 300 seconds

Steps 2 to 4 were repeated for 35 PCR cycles.

Fig. 4.3 clearly depicts the effect of the elevated annealing temperature. The amplification of unwanted sequences was greatly reduced and the target sequence was amplified preferentially, ie. it was the major product of the PCR. PCR conditions were as outlined in Table 4.1. Lanes 1-3 represent PCR product resulting from the addition of 50 pmol primer and 175 mg template DNA. The reduced number of cycles might also have had a role in improving fidelity.

A subsequent PCR reaction was carried out to demonstrate the replicability of the technique. **Fig. 4.4** depicts that under identical conditions, the primer will preferentially amplify a particular portion of the genome. Thus, the conditions outlined as for the products obtained in Fig 4.3 were deemed to be ideal conditions and were used optimally for subsequent amplification of a specific band.

The stringency of the PCR can be manipulated by changing the annealing temperature, ie. an increased annealing temperature will allow for more stringent selection of target sequences, by the specific primer. Shortening of the annealing and extension times will minimise the opportunities for mispriming and will decrease the incidence of extension of unwanted or artifactual sequences. Reduced primer and enzyme concentrations will also limit the amount of mispriming. Any change in the MgCl2 concentrations (or even replacing it with another salt) will have a distinct effect on the fidelity of the PCR (Erlich, 1989).

Manipulation of the various components of PCR can be time consuming due to the complex nature of the interactions among them. A number of permutations are possible eg. primer concentration versus template DNA concentration, annealing time versus annealing temperature, or extension time, etc. This study sought to optimise a PCR reaction to achieve a desired outcome, ie. amplification of a specific target sequence, with the minimum necessary adjustment of parameters (Sharma *et al.*, 1992).

4.4.3 RAPD Amplification of *Streptomyces* DNA

The results of an attempt to produce RAPD fingerprints of *Streptomyces* DNA is depicted in **Fig. 4.5**. RAPD fingerprints were produced in order to establish relatedness among the mutants produced. It is clear from the figure that while amplification did occur, the results of such amplification are not conclusive to any degree. This could be due to the genomic DNA isolation procedure being inefficient for *Streptomyces* cultures. Lanes 1, 2, and 3 represent different mutant organisms.

Fig 4.3 **PCR amplification of the *mel* gene of pIJ 702 using primers Tyr-F and Tyr-R. Electrophoresis was conducted at 100V in 1 x TBE.**

Lanes 1-3	PCR product
4	*Hinf*I digested pBR322 marker

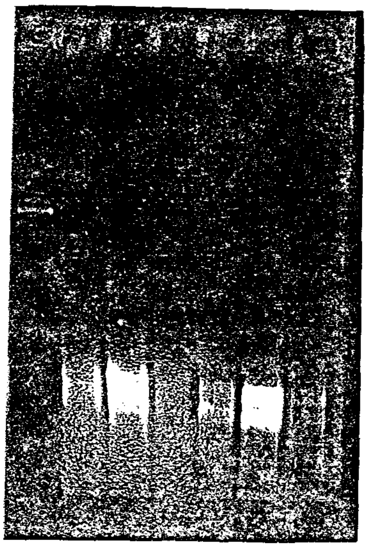


FIGURE 4.4 Repeat of the previous PCR reaction to show that the amplified band can be obtained replicably

Lanes 1 - Marker DNA

2-4 - PCR product from previous reaction

5-7 - PCR products from repeat of previous rxn

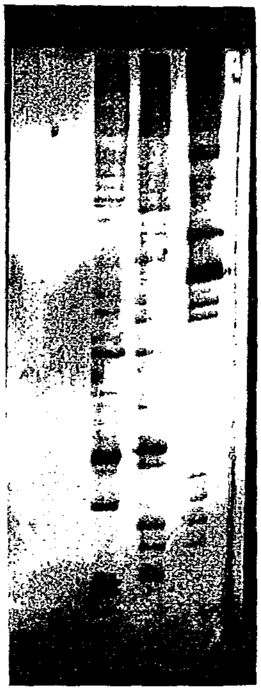


FIGURE 4.5 Polyacrylamide gel electrophoresis of *Streptomyces DNA* RAPD PCR fingerprints

Lanes 1, 2, and 3 represent different *Streptomyces mutants*

CHAPTER 5 MUTAGENESIS

5.1 INTRODUCTION

Mutations can be used to probe the relationship between structure and function of proteins (Suzutani *et al.*, 1992). They are also used to define the roles of particular protein and protein assemblies. The main reason for isolating and characterising a mutation is to assess the consequences of such mutation, i.e. to assess the phenotype. An ideal mutation experiment compares two organisms, one being mutated at a particular site, and the other lacking the mutation. Any difference between the two can thus be attributed to the mutation. Thus, the properties of normal function (or wild-type phenotype) can be determined by changing or eliminating a single gene or genetic element - (Botstein and Shortle, 1985).

Three broadly detailed categories of mutagenesis are available to the experimenter. They are *in vivo* and, *in vitro* and transposon mutagenesis. *In vivo* mutagenesis makes use of an organism that is living, usually a cell culture or bacterial culture. The mutagenesis treatment is carried out on the organism, which is then recultured under appropriate conditions to assess the effect of the mutagenesis on the phenotype of the organism.

Earliest geneticists had natural variations and spontaneous mutations at their disposal for study. The discovery that organisms exposed to x-rays, chemical mutagens and ultraviolet (UV) light yield higher frequencies of mutant progeny, led to a revolution in experimental genetics - (Botstein, *et al.*, 1985, Nicholson *et al.*, 1991, Veaute and Fuchs, 1993). Control over the process of mutagenesis allowed a systematic study of biological phenomena.

Geneticists could collect large numbers of different mutations displaying particular phenotypes and then classify these on the basis of complementation behaviour, map position, and other criteria (Botstein and Shortle, 1985).

However, *in vivo* mutagenesis has the following limitations :

There are limits on the range of phenomena which can be studied genetically since a specific phenotype is required to identify rare mutations in a mixture of wild-type and irrelevant mutation background. It is difficult to score a unique phenotype if the mutagenic treatment causes several mutations in a single gene. The simple assays designed to screen mutants are often very labour intensive. High frequencies of mutation are often associated with high killing levels of mutagens and thus greatly reduced amounts of the desired organism displaying the requisite phenotype, are often the result of an extensive *in vivo* mutagenesis treatment (Sarkar and Chakrabarty, 1979, Botstein and Shortle, 1985). The development of the concept of ''localised mutagenesis'' by Hong and Ames (1971) led to some improvement in the *in vivo* mutagenesis strategy. Specialised transducing phages carrying only a small fragment of the bacterial chromosome were mutagenised, thus limiting the effect of mutagenesis to only a few genes, while preserving the genetic integrity of the host chromosome (Davis *et al.*, 1980). Despite the advantages of this localised mutagenesis strategy, a major limitation is that many genes of interest on *E. coli* cannot be readily isolated on specialised transducing phage.

Mutagenesis as a genetic manipulation and analysis strategy, underwent further development with the realisation that insertion mutations could be induced is virtually any gene of interest by the appropriate manipulation of naturally occurring transposable elements (Scandalio and

Wright, 1991). Most spontaneous mutations in yeast and *Drosophila* are caused by the insertion of transposable elements (Finnegan and Fawcett, 1986, Boeko, 1989).

The advantages of transposon mutagenesis were first exploited in bacterial systems. Transposon mutagenesis results in a unique, single physical alteration in a gene that has been mutated. Mutagenesis can be carried out so that the number of transposons per genome is limited, usually to one transposon per genome. In addition, the insertion event can be selected by means of drug resistance or phage immunity carried by the transposon (Jorgensen *et al.*, 1979, Botstein and Shortle, 1985, Wu *et al.*, 1992). This facilitates rapid screening of a large number of potential mutants, since secondary mutation levels are low and most mutations lead to total inactivation of the gene. Transposon mutagenesis can be applied to very small genomes e.g. plasmid, and is an alternative to deletion mutagenesis in defining the extent of a gene of interest after it has been cloned - (Heffron *et al.*, 1978, Guo, *et al.*, 1983, Botstein and Shortle, 1985, King, Goodbourne, 1992).

The ability to remove an intact genome from a cell and to subject it to a variety of manipulative techniques in a test tube has opened the way for an entirely different environment for mutagenesis, i.e. *in vitro* mutagenesis.

In vitro mutagensis can be divided into three categories. They are :

(i) methods that alter the structure of DNA;
(ii) random mutagenesis;
(iii) oligonucleotide-directed mutagenesis.

The isolation of a gene from its normal cellular context, usually requires, as a prerequisite to cloning the same gene into a cloning vector, some form of structural alteration, in order to remove extragenic flanking sequences. This is usually achieved by successive deletion mutagenesis, which is then used to define the ends of the gene (King and Goodbourne, 1992). Exonuclease enzymes are commonly used to obtain specific sets of deletions (Guo and Wu, 1983). Occasionally, a specific end containing a linker or adapter sequence might be necessary in order to clone the mutagenized fragment back into a suitable vector (Luckow, 1987). These oligonucleotide insertions, called linker mutations, are especially useful in the modification of circular DNA molecules (Botstein and Shortle, 1985).

DNA can also be subjected to random *in vitro* mutagenesis using several well-characterised chemical mutagens independently or in concert to produce a range of desired effects. Commonly used chemical mutagens include 1-methyl-3-nitro-1-nitrosoguanidine (NTG) (Sarkar and Chakrabarty, 1979), ethyl methanesulfonate (EMS), (Farrance and Ivarie, 1985), hydroxylamine (HX), (Pagan and Senior, 1990), nitrous acid (NA), (Johnston, 1975), (Myers, *et al.*, 1985) and sodium bisulphite (Pine and Huang, 1987). The simplest method of random mutagenesis is to react DNA with a chemical mutagen such as nitrous acid or hydroxylamine and to clone the population of mutagenized fragments into a recombinant plasmid that carries the remainder of the wild-type genome. Recombinant plasmids carrying mutations that generate a novel phenotype can be recognised by appropriate tests. Less easily assayable phenotypes can be by DNA sequencing of random clones (Sambrook *et al.*, 1989).

A third strategy of *in vitro* mutagenesis is oligonucleotide-directed mutagenesis. This strategy is used to add, delete or substitute nucleotides in a segment of DNA of known sequence. While other methods yield mixed populations of DNA variants, oligonucleotide mutagenesis can be used to generate specific mutations, designed by the experimenter - (Mandecki, 1986), Sambrook *et al.*, 1989). The actual steps in oligonucleotide-directed mutagenesis requires that mutagenic oligonucleotide sequences be designed and synthesized, that these oligonucleotide be hybridised to target DNA and that the mutant oligonucleotides be extended by a DNA polymerase (Deng and Nickoloff, 1992). The actual complexity of the process can be increased by exploiting the error-proness of certain polymerase e.g. DNA polymerase from RNA tumour viruses (Zakour and Loeb, 1982), that lack proof-reading functions. An alternative strategy would be to manipulate the pool of deoxynucleoside triphosphates (dNTP's) so that an excess of one or an absence of one nucleoside triphosphate will profoundly affect the fidelity of gap repair or primer-directed synthesis to yield mutant DNA - (Shortle *et al.*, 1982, Lehtovaara, *et al.*, 1988, Kunkel *et al.*, 1981).

An obvious extension of the concept of oligonucleotide-directed mutagenesis, is the use of the polymerase chain reaction to generate mutant DNA *in vitro*. The PCR can be used in both random and site-directed mutagenesis experiments. *Taq* DNA polymerase lacks a 3′ → 5′ exonucleolytic editing activity and is therefore error-prone, with a measured error rate of 10^{-5} to 10^{-4} error per nucleotide synthesized (Zhou *et al.*, 1991). This error rate can be further increased by substituting $MgCl_2$ with $MnCl_2$ in the PCR buffer. The fidelity of DNA synthesis by *Taq* DNA polymerase is reduced in the presence of Mn^{2+}, giving rise to a higher error rate and increasing the overall mutation frequency. This effect is increased further still, by manipulating, in addition, the dGTP/dATP ratio to 5:1 - (Leung *et al.*, 1989). A further development on this theme allows the adjustment of the mutation frequency to the size of the target DNA, and offers an opportunity to influence the type of mutation.

This technique is based on manipulation of the dNTP pool, to result in a particular dNTP being in limiting amounts, in each of four separate PCR's. If dITP is included in the reaction, it will be misincorporated preferentially by *Taq* DNA polymerase when the limiting dNTP is incorporated. Thus, in a subsequent cycle, any of the other three dNTP's could possibly be incorporated as a complement to dITP - (Spee *et al.*, 1993).

The PCR can also be used to generate site-specific mutants. The most powerful technique, described by Landt *et al.* (1990), involves the use of a mutagenic primer to introduce a mutation into a specific site of a target strand of DNA. Improvements to this method have been proposed by Kuipers *et al.*, (1991), Mikaelian and Sergeant (1992), and Sharrocks and Shaw (1992), in which lower dNTP concentrations reduce the incidence of unwanted additional mutations.

An important goal in any mutagenesis experiment is to detect the mutations and to assess the effect of such a mutation on the phenotype of the organism. This can be done in several ways. Mutations can be detected by DNA sequencing, but this technique is laborious and time consuming. Denaturing gradient gel electrophoresis (DGGE) involves the generation of heteroduplexes between wild-type and potential mutant DNA. When such heteroduplexes are electrophoresed through a gel with an increasing denaturant gradient, abberant migration results from the mismatches at the site of the mutation. Simpler screening techniques involve the selection of possible mutants by chemical methods e.g. antibiotic selection or pigment production, followed by more complex screening of suspected mutants (Cotton and Malcolm, 1991).

5.2 MATERIALS AND EQUIPMENT

All buffers and salts used were prepared as outlined in Appendix B. The chemical mutagens ethane sulphonate methyl ester (EMS) was purchased from Sigma and stored at room temperature in the dark. Hydroxylamine was purchased from Sigma and was stored at room temperature. Sodium bisulphite was purchased from Merck and stored in crystalline form at room temperature. Great care was exercised in the handling and dispensing of all chemical mutagens, and most manipulations were carried out in a fume-hood, using hand and face protectors to minimise contact with the mutagens and to contain any aerosols generated. All *in vitro* mutagenesis experiments were performed in 1.5 ml Locktite Eppendorf microfuge tubes, which were subsequently disposed of by incineration. Test media and antibiotics for screening of potential mutants, were prepared as outlined in Appendix A.

5.3 METHODS

5.3.1 Hydroxylamine Mutagenesis

A strategy for *in vitro* hydroxylamine (NH_2OH) mutagenesis was designed around the protocol of Davis *et al.* (1980), incorporating also the research conducted by Ishii and Kondo (1975), Busby and Dreyfus, (1983) and Pagan and Senior (1990).

1 M NH_2OH pH 6.0 was made up by adding 0.56 ml 4 M NaOH to 0.35 g NH_2OH, and making up to 5 ml volume in distilled water. Hydroxylamine was added in varying amounts to yield a final concentration in the range 0.25 M, 0.5 M, and 0.75 M in a microfuge tube

containing 2 μg of DNA in 50 μl TE, pH 7.6 (Appendix B) and distilled water was added to give a final volume of 200 μl. The resultant hydroxylamine-DNA - solutions were incubated individually at temperature ranging from 25°C to 55°C in 5°C increments. A sample was also incubated at 37°C (Kirby, pers. comm. 1991). The times of incubation were varied from 15 minutes to 60 minutes in the range 15 minutes, 30 minutes, 45 minutes and 60 minutes, for each individual sample. At the end of the respective incubation time, for each of the 96 reactions carried out, the potentially mutated DNA in each sample was thus precipitated by the addition of 2 volumes of ice-cold 95% ethanol, and the resulting suspension was centrifuged at 12 000 x g at 4°C for 15 minutes to pellet the DNA. The resultant pellet was rinsed once in 70% ethanol and then resuspended in 10 ml TE. Up to 1 μg DNA in 5 ml TE was then transformed into 50 ml *Streptomyces lividans* TK_{21} protoplasts and these were spread onto regenerating media and incubated at 30°C.

Control experiments were also performed in which 50 μl of *Streptomyces lividans* TK_{21} protoplasts were transformed with 1μg of DNA that had been exposed to the same temperature as mutagenised DNA, except that no mutagen was added. Each mutagenic treatment was performed in triplicate to generate as much data as possible about the ideal condition for mutagenesis in terms of mutagen concentration, incubation time and temperature.

5.3.2 EMS Mutagenesis

EMS mutagenesis was based on the method of Stonesifer and Baltz (1985), and on research by Kunz *et al.* (1992), Pastink *et al.* (1991) and Lee *et al.* (1992).

EMS was added to 2 μg of DNA in TE pH 7.6 to final concentrations of 0.5%, 1%, 2% and 3% in a final volume of 100 μl. These EMS-DNA solutions were then incubated at a range of temperatures between 25 and 55°C, and a sample was also incubated at 37°C. Incubations were performed for 15-60 minutes after which the mutagenic reactions were terminated by the addition of 10 volumes of 0.16 M sodium thiosulphate, into each of the mutagenic reactions. The DNA was then precipitated by the addition of 2 volumes of 95% ethanol at room temperature, and pelleted by centrifugation at 12 000 x g at 4°C for 15 minutes. The pellet was resuspended in 10 μl TE pH 8.0, and 1 μg of plasmid DNA in 5 μl TE pH 8.0 was then transformed into 50 μl *Streptomyces lividans* TK_{21} protoplasts. These were then spread on R_2YE regenerating media to facilitate selection of appropriate mutants at 30°C. Control experiments were also performed at each temperature, in which DNA that had not been exposed to mutagenesis was also transformed into lividans TK_{21} protoplasts.

Each mutagenesis was performed in triplicate in order to optimize the mutagenic treatment in terms of mutagen concentration - and incubation temperature.

5.3.3 Hydroxylamine - EMS Mutagenesis

Based on the determination of optimal mutagenic treatment from 5.2.1 and 5.2.2, i.e. hydroxylamine mutagenesis and EMS mutagenesis, a third strategy was to combine both mutagens at their optimal concentrations in a single mutagenic treatment, to assess the effect of both mutagens in concert. Hydroxylamine and EMS were added to 2 μg of DNA in TE pH 7.6, in a total volume of 100 μl, so that the hydroxylamine concentration was 0.25 M and the EMS concentration was 1%. Table 5.1 outlines the order of addition of the reagents for the mutagenesis.

TABLE 5.1 : Addition of reagents for Hydroxylamine - EMS Mutagenesis

Reagent	Volume
DNA 0.1 $\mu g/\mu l$	20 (μl)
EMS	1 (μl)
Hydroxylamine	25 (μl)
Distilled Water	54 (μl)

Mutagenesis was performed using this hydroxylamine - EMS cocktail, by incubation at 50°C for 45 minutes. At the end of this time, the EMS mutagenesis was suspended by diluting the suspension into 10 volumes of 0.16 M sodium thiosulphate. The DNA was precipitated by the addition of 2 volumes of cold 95% ethanol and pelleted by centrifugation at 12 000 x g at 4°C for 15 minutes. The pellet was washed in 70% ethanol, and then resuspended in 10 μl TE pH 8.0. 1 μg of mutant DNA in 5 μl TE was then transformed into *Streptomyces lividans* TK_{21} - protoplasts. Regeneration of protoplasts was achieved on R_2YE plates at 30°C, and screening was performed to select potential mutants.

5.3.5 Primary Screening of Mutants

A simple primary screening method was carried out to select mutant phenotype transformants for further screening. After 16 hours at 30°C on R_2YE media, *Streptomyces lividans* TK_{21} protoplasts were overlaid with soft nutrient agar (Appendix A) containing 500 $\mu g/\mu l$ thiostrepton and a further incubation was carried out at 30°C for 2-4 days.

Any protoplasts that survived the soft nutrient agar-antibiotic overlay, were then replicate plated onto M_3 and MMT media (Appendix A), containing 0.01% tyrosine and further incubation was carried out at 30°C for 2-4 days, until the *Streptomyces* began producing visible pigment. Any transformants with phenotypic variation were taken to be potential mutants and were then subjected to enzyme assays in order to determine the actual efficacy of the mutagenic treatment (Hopwood *et al.*, 1985).

5.3.4 PCR Mutagenesis

The PCR was used to generate mutant copies of the *mel* gene of pIJ702 using specific primers designed in 4.2.1. A standard PCR reaction was carried out as outlined in 4.2.2, with modifications suggested by Leung *et al.*, (1989) in order to increase the error frequency of *Taq* DNA polymerase. $MnCl_2$ was added to a final concentration of 0.5 mM to replace $MgCl_2$. Prior to the mutagenesis, the plasmid pIJ702 was linearized with the restriction enzyme *Cla*I and then subjected to 35 cycles of PCR using primers Tyr-F and Tyr-R (4.2.1).

The mutagenised fragment which was designed to contain *Sst*I and *Cla*I terminii, was then ligated with wild-type pIJ702 plasmid that had been digested with the restriction enzymes *Sst*I and *Cla*I. Restriction digests and ligation reactions were carried out as per Sambrook *et al.*, (1989) - and the restriction enzymes and T_4 DNA ligase was used as per manufacturers instructions (Boehringer Mannheim) in the specific buffers supplied. The mutagenised fragment was then transformed into *Streptomyces lividans* TK_{21} protoplasts in a standard transformation reaction as per Chapter 3. Protoplasts were regenerated on R_2YE plates at 30°C prior to mutagenic screening. The strategy followed in this mutagenic procedure is outlined in Fig. 5.1.

5.4 RESULTS AND DISCUSSION

Several hundreds of apparent mutants were obtained from the various mutagenic strategies. Since the primary screening system outlined in 5.3.5 identified only phenotypically apparent potential mutants, no definite conclusions could be made about the frequency or efficacy of mutagenesis. However, the observed mutagenesis frequencies were used as a basis for optimizing the mutagenic treatments, in the absence of any other. Observed frequencies ranged between 0.1% and 1% of the transformation frequency as determined by control transformation experiments.

This raises two important considerations concerning the effectiveness of this screening procedure. Firstly, a large number of functional enzyme mutants might have been inactivated by mutations in the thiostrepton resistance gene and these mutants would not have survived the stringent antiobiotic selection overlay. Secondly, a high background might have resulted from plasmid DNA that had not actually been affected by mutagenesis, but was successfully transformed into the plasmid-free strain. These cells containing wild-type plasmid would survive the antibiotic selection procedure of primary screening and would be counted as "apparent" mutants.

A way to overcome this would be to actually screen every single transformant, in order to determine the effect of the mutagenic treatment. This would consist of isolating each transformant independantly and then subjecting each one to batch culture in order to conduct enzyme assays. In order to place the magnitude of the task in context, - it should be considered that each mutagenic treatment would then comprise the following steps :

(i) isolation of plasmid DNA
(ii) mutagenesis of plasmid DNA
(iii) transformation of plasmid DNA
(iv) culture of all potential mutants separately
(v) enzyme assay of each separate mutant

It is obvious that such a strategy, while more accurate, would be a labour-intensive, time consuming task of Herculean proportions. For the purposes of this study, therefore, the simple initial screening technique, followed by enzyme assay of only survivors of the first screening, was considered to be adequate.

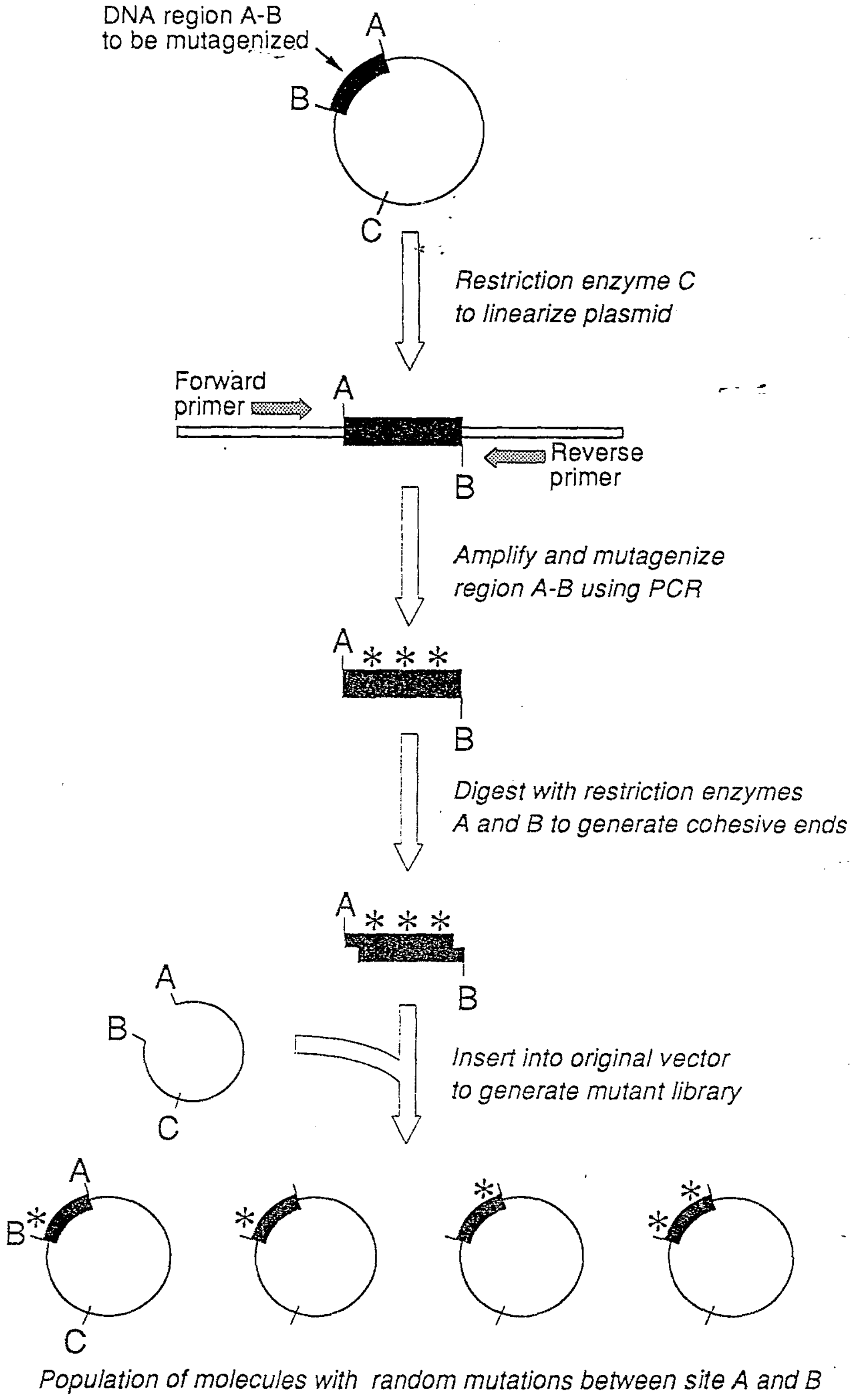


FIGURE 5.1

A general scheme of the method used to mutagenize a defined region of DNA contained in a plasmid using PCR (Leung *et al.*, 1989)

5.4.1 Optimal Hydroxylamine Mutagenesis

Based on the apparent mutation frequencies, 1% survivors were obtained using hydroxylamine under the following conditions. Hydroxylamine was added to 2 μg DNA in TE pH 7.6 in a total volume of 100 μl, to a final concentration of 0.25 M. The mutagenic reaction was carried out by incubation at 50°C for 60 minutes. Under these optimal conditions, hydroxylamine mutants designated HX-M_n, where n was an arbitrary number assigned to each possible mutant, were generated routinely in successive rounds of mutagenesis. **Plate 5.2** displays Hydroxylamine mutants on M_3 plates, showing production of pigment.

5.4.2 Optimal EMS Mutagenesis

A maximum of 0.5% survivors were obtained under the following optimal mutagenesis condition. EMS was added to 2 μg DNA in TE pH 7.6 in a total volume of 100 μl, to a final concentration of 1%. The mutagenic reaction was carried out at 50°C for 30 minutes. Under these conditions, EMS mutants designated EMS-M_n, where n was an arbitrary number assigned to each possible mutant, were generated routinely in successive rounds of mutagenesis. Plate 5.2 displays EMS mutants on M_3 plates, showing production of pigment.

5.4.3 Hydroxylamine-EMS Mutagenesis

Using optimal conditions for both mutagens as defined in 5.4.1 and 5.4.2, mutation survival frequencies of at least 1% were obtained. Plate 5.2 displays Hydroxylamine-EMS mutants on M_3 plates, showing production of pigment.

Although DNA sequencing is necessary to determine the actual effect of the EMS-Hydroxylamine cocktail on the plasmid DNA, it is known that EMS acts primarily to introduce ethyl groups at guanine N-7 (Farrance and Ivarie, 1985). The majority of loose pair changes would therefore be GC---AT transitions (Pastink *et al.*, 1991). Hydroxylamine produces G to A transitions exclusively and is useful for localized mutagenesis where heavy mutagenesis is required (Davis *et al.*, 1980).

5.4.4 PCR Mutagenesis

While this strategy yielded large numbers of potential mutants that passed the initial screening test (> 2%), subsequent replicate plating displayed no unusual phenotypes, and enzyme assays revealed that the transformants were producing wild-type enzyme. It is possible that the ligation reactions were not entirely successful in joining mutant fragments to digested plasmid DNA. Thus wild-type plasmid may have recircularized and was then transformed into *Streptomyces lividans* TK_{21} protoplasts. Further manipulation of Mn^{2+} concentrations and dNTP concentration might also have improved the mutagenic "yield".

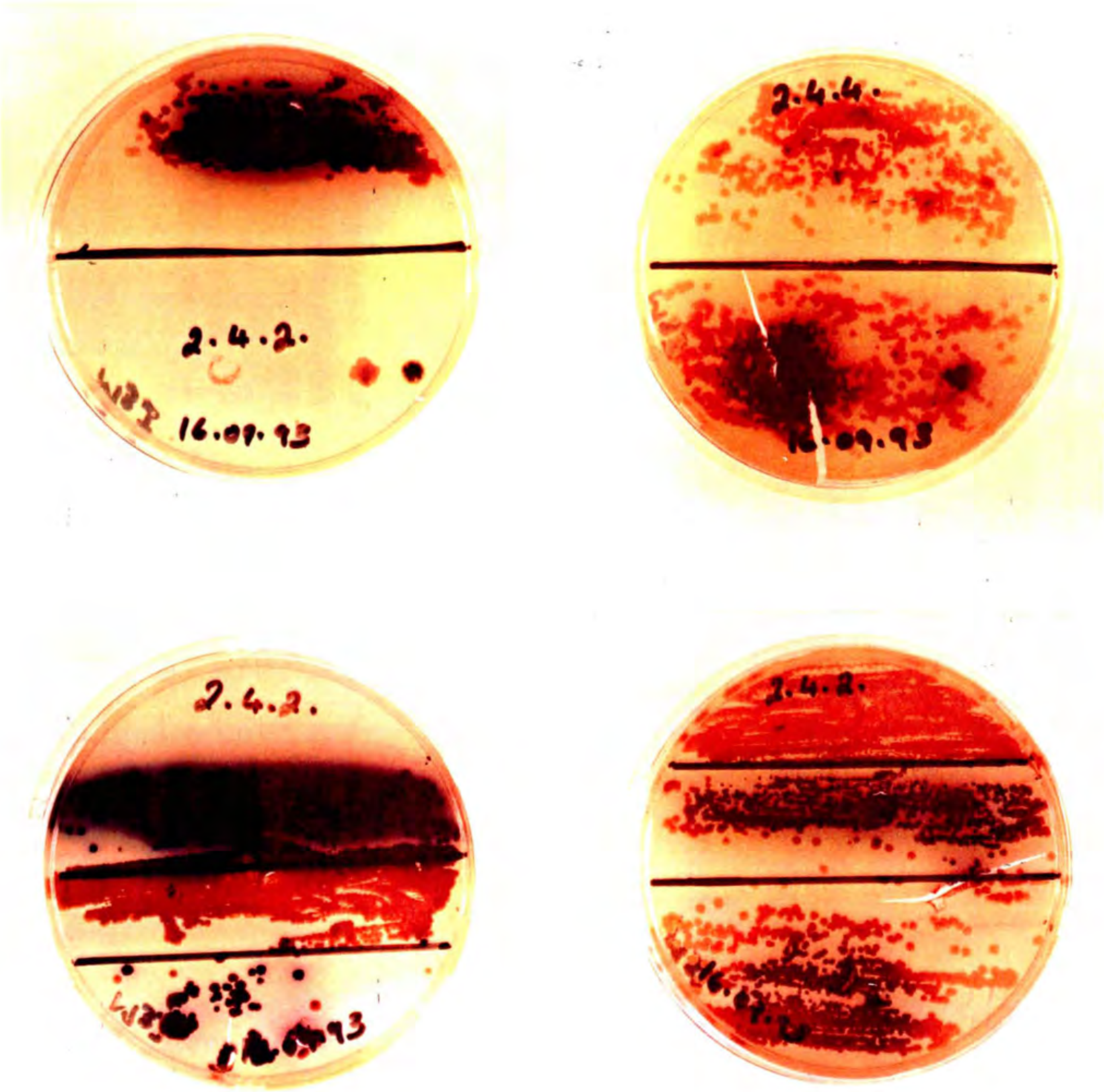


PLATE 5.1 HYDROXYLAMINE AND EMS MUTANTS ON AGAR PLATES

Top Left - Hydroxylamine mutants on M3 plates
Top right - EMS mutants on M3 plates

Bottom - Hydroxylamine-EMS mutants on M3 plates

CHAPTER 6 ENZYME STUDIES

6.1 INTRODUCTION

The polyphenolase enzyme is a copper-containing mono-oxygenase that catalyses the o-hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones (Dietler and Lerch, 1979). The reaction is a two step process, with the hydroxylation of phenols with O_2 to catechols, and subsequent dehydrogenation to o-quinones (Kazandjian and Klibonov, 1985). The enzyme is widely distributed in nature and is responsible for the formation of melanin pigments (Huber **et al.**, 1985).

The polyphenolase enzyme is present in many species of *Streptomyces* (Hintermann *et al.*, 1985). The nucleotide sequence of the polyphenolase gene from *Streptomyces antibioticus* has been cloned and expressed in *Streptomyces lividans,* using the plasmid-vector pIJ702. This plasmid has been isolated, subjected to mutugenesis and then re-transformed into a plasmid-free *Streptomyces* strains. The enzyme assays conducted in this series of experiments were an attempt to screen the possible mutant plasmids for their enzyme production properties.

Enzyme activity determinations were carried out and from these, Michaelis-Menten kinetic characteristics of both mutant and wild-type enzyme were determined. These determinations formed the basis of a screening system to detect mutant enzyme (Ingraham, 1957, Burton *et al.*, 1993). The activity of the enzymes were determined against a range of phenolic organic substrates (Davis and Burns, 1990, Mason, 1947). A lag phase was observed when assaying the enzyme against phenolic substrates, but this was not the case in reactions with catechols (Garcia *et al.*, 1987). It was also found that the enzyme was inactivated by enzyme-substrate binding and that once bound, this inactivation was irreversible (Asimov and Dawson, 1950). The actual mechanism and reaction kinetics are complex due to the dual catalytic properties of the polyphenolase enzyme, i.e. cresolase and catecholase. Melanin biosythesis pathways used in this study are based on research by Mason (1947), Cushing (1948), Cabanes *et al.* (1987) and Garcia-Carmona *et al.* (1987).

Prior to enzyme activity determinations being made, actual enzyme production had to be optimized. Enzyme production was carried out optimally according to recommendations made by Dr P.A. Goetsch (pers. comm. 1992). Subsequently, methods for the extraction of enzyme were also optimized. The actual enzyme assays presented in this study represent the results of series (at least 3 sets) of observations made under essentially similar conditions.

6.2 MATERIALS AND EQUIPMENT

Streptomyces strains were cultured for enzyme production as outlined in Chapter 2. *Streptomyces* strains used were *Streptomyces lividans* (pIJ702) and *Streptomyces lividans* TK21.

All media and buffers were made up as outlined in Appendices A and B.

Dihydroxyphenylalanine (L-DOPA) was purchased from Sigma (catalogue number D9628). Phenol was purchased from Holpro (catalogue number 76692M). p-Cresol was purchased from BDH Chemicals (catalogue number 27821). L-tyrosine was purchased from Merck (catalogue number 144361).

Enzyme extractions were achieved using a Yeda Press or by ultrasonication. All enzyme activity determinations were carried out by recording absorbance changes on a Shimadzu UV160A UV-visible recording spectrophotometer.

6.3 METHODS

All enzyme assays were carried out in triplicate and results expressed represent the mean value of such determination. Controls were also conducted, in which either enzyme or substrate were excluded from the assay mixture, and both types of controls were included in each set of determinations.

The enzyme assays were carried out on a range of substrates. The enzyme used was either crude enzyme extract from *Streptomyces lividans* (pIJ702) or one of several possibly mutant enzymes from *Streptomyces lividans* TK21.

6.3.1 Production of polyphenolase by *Streptomyces lividans* pIJ702 at different pH values

Streptomyces were cultured as outlined in Chapter 2, in MMT medium for enzyme production. The pH of the media was varied from pH 7.0 to pH 8.0 in 2 unit increments in order to establish an optimum pH for enzyme production. Repeated experiments were carried out in order to establish an optimal pH for enzyme production.

6.3.2 Extraction of Enzyme

Cells for enzyme production were cultured as outlined in Chapter 2. Usually, an enzyme assay required *Streptomyces* in 200 ml of MMT media (Appendix A), cultured in 500 ml conical flasks, with at least 150 rpm shaking to ensure aeration. Cells were harvested by centrifugation at 20 000 g for 30 mins at 4°C. The cell pellet was resuspended in 0,1 M sodium phosphate buffer pH 7.2 (Appendix B). Usually a volume of 5-10 ml was used to resuspend the pellet completely (Bernan *et al.*, 1985).

6.3.2.1 High Pressure disruption of cells

The resuspended pellet was then subjected to high pressure disruption using a Yeda Press and compressed nitrogen applied to the sample in the Yeda-cell. At least five passages at 2000 Psi pressure, were required to disrupt the cells and release the enzyme. The entire process was carried out with the Yeda-Press submerged in ice to maintain enzyme stability. The disrupted suspension was then centrifuged at 30 000 g, to pellet the membrane debris. Enzyme assays were conducted on the supernatant (Barner, 1991, Goetsch P.A. 1991, pers. comm.).

6.3.2.2 Ultrasonication

An alternative strategy was to disrupt the cells by ultrasonication (Crameri *et al.*, 1990). The cell pellet from 6.3.1 was resuspended in 0,1 M sodium-phosphate buffer, pH 7.2. A volume of 5-10 ml buffer was used, depending on the size of the cell pellet. The suspension was then subjected to repeated ultrasonication. Ultrasonication times were 30s bursts at 150W, with 30s changeover times for cooling. A minimal treatment was used to disrupt cells. The disruption process was followed microscopically. Cell samples were kept on ice during and in-between ultrasonication.

6.3.3 Enzyme Assays

6.3.3.1 The activity of polyphenolase on L-DOPA

Both wild-type and mutant enzymes were reacted against L-DOPA substrate. A standard DOPA reaction used 10 mM DOPA in 0,1 M sodium-phosphate buffer pH 6.0. 100 μl enzyme extract was added to 10 mM L-DOPA in 3 ml of 0,1 M sodium-phosphate buffer, pH 6.0. The absorbance (A) 475 nm was monitored over 5 mins, and the change in absorbance per minute was recorded. Using an extinction co-efficient of 3600 $M^{-1}.cm^{-1}$, the enzyme activity was calculated and expressed as units per ml sample. 1 unit of activity is defined as the amount of enzyme catalysing the formation of 1 micromol of dopachrome per minute under assay conditions (Gardner and Cadman, 1989, Katz *et al.*, 1983, Mason *et al.*, 1947).

6.3.3.2 The activity of polyphenolase on L-tyrosine

Both wild-type and mutant enzymes were reacted against L-tyrosine as substrate. Enzyme activity was determined from a change in optical density (A280nm) in a reaction mixture containing 0,5 mM tyrosine. 100 μl of crude enzyme extract was added to 0,5 mM tyrosine in 3 ml 0,1 M sodium phosphate buffer, pH 6.0, and the change in optical density (A280nm) was measured over 3 minutes. Change in A280nm per minute was calculated. One unit of tyrosine causes an increase in absorbance at 280 nm of 0,001 per minute at pH 6.5, at 25°C in a 3 ml reaction mixture containing L-tyrosine. Based on this, the enzyme activity was calculated and expressed as activity units per ml extract (Atlow *et al.*, 1984, Waite and Tanzer, 1981, Cabanes *et al.*, 1987 and Wada *et al.*, 1993).

6.3.3.8 The activity of polyphenolase on p-cresol

Wild-type and mutant enzymes were tested against p-cresol substrate, based on a method proposed by Kazandjian and Klibanov (1985). 100 μl of crude enzyme extract was added to 10 mM p-cresol in 0,1 M sodium phosphate buffer and the absorbance at 395 nm (Abs 395 nm) was measured for 3 minutes. Activity was calculated as for 6.2.3.2 (Cushing 1948, Waits and Tanzer 1981, Garcia-Carmona *et al.*, 1987).

6.3.3.4 The activity of polyphenolase on phenol

This assay method based on Atlow *et al.*, (1984) and Burton *et al.*, (1993) used 10 mM phenol as substrate, in a 3 ml 0,1 M sodium phosphate buffer, pH 7.2. Change in absorbance at 475 nm was measured for 3 minutes, after the addition of 100μl of crude enzyme extract, and this was used to calculate enzyme activities of both wild-type and mutant enzymes as in 6.3.3.2.

6.4 RESULTS AND DISCUSSION

6.4.1 Enzyme Extraction

The amount of enzyme obtained and the activities of the crude enzyme extract was similar for both techniques of enzyme extraction, ie. high pressure extraction using the Yeda-Press and cell disruption using the ultrasonicator. However, the method of ultrasonication was preferred, as it was less time-consuming. Optimally, ultrasonication was performed on cell pellet that had been resuspended in 10 ml 0,1 M sodium-phosphate buffer, pH 7.2. Optimal ultrasonication times were 30 s bursts over 5 minutes, with 30 s cooling intervals in ice.

6.4.2 pH optimization

Repeated culture of *Streptomyces lividans* pIJ702 at pH values ranging from pH 7.0 to pH 8.0 revealed that enzyme and biomass production was optimal at and above pH 7.6. In all subsequent experiments, the enzyme production studies were carried out at pH 8.0, using 200 ml of MMT in 500 ml culture vessels.

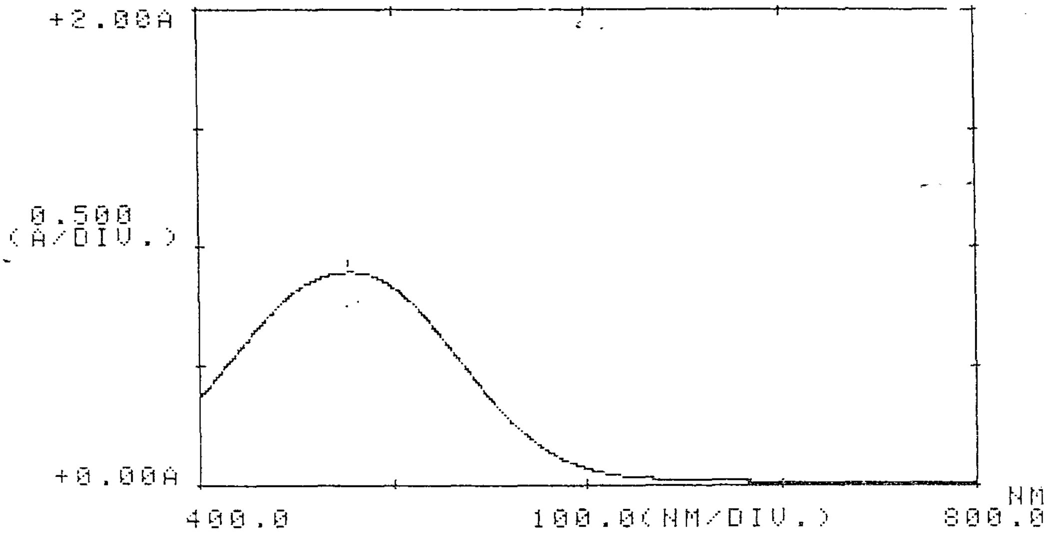
6.4.3 Results of enzyme assays

Fig 6.1A depicts the absorbance spectrum from 800 nm to 400 nm of the products formed by the reaction of wild-type enzyme and L-DOPA. The peak absorbance was measured at 475 nm wavelength. **Fig 6.1B** depicts the absorbance spectrum from 800 nm to 400 nm of the products formed by the reaction of mutant 2421A enzyme and L-DOPA. The peak absorbance was measured at 530 nm wavelength.

Fig 6.2A depicts the absorbance spectrum from 800 nm to 400 nm of the products formed by the reaction of mutant 2421B enzyme and L-DOPA. The peak absorbance was measured at 530 nm.

Fig 6.2B depicts the absorbance spectrum from 800 nm to 400 nm of the products formed by the reaction of mutant 2427 enzyme and L-DOPA. The peak absorbance was measured at 350 nm.

Both mutant and wild-type enzymes were extracted as outlined in 6.4.1. **Plate 6.1** depicts a typical batch culture experiment, including wild-type, mutant and control organisms. From plate 6.1, it can clearly be seen that the actual pigment product produced was different, which was used as the basis of the second screening process. Subsequent to this, actual enzyme assays were conducted on potential mutants to determine the efficiency of the mutagenesis programme. Attempts to culture *Streptomyces* in Mcartney bottles were abandoned because of low enzyme yields.



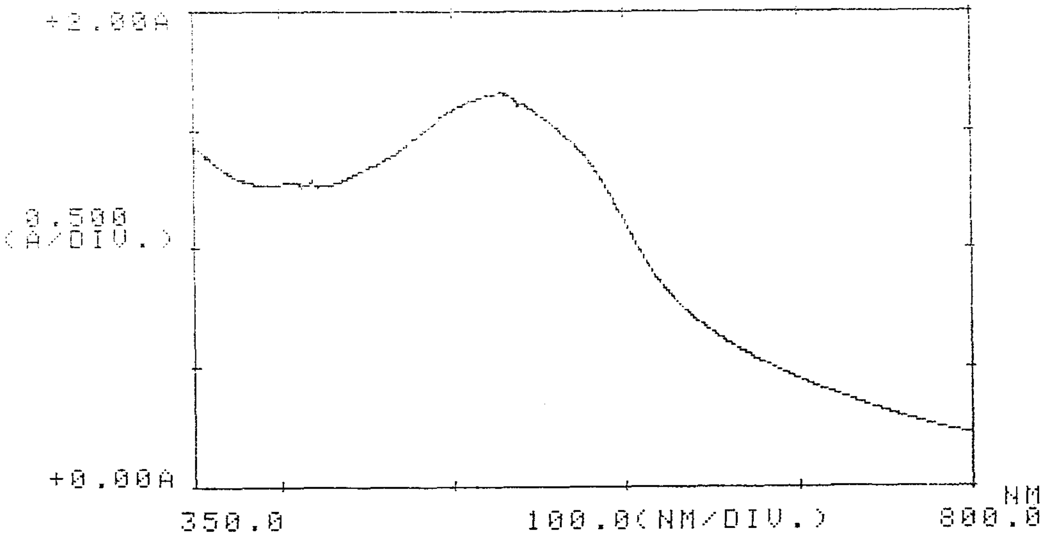
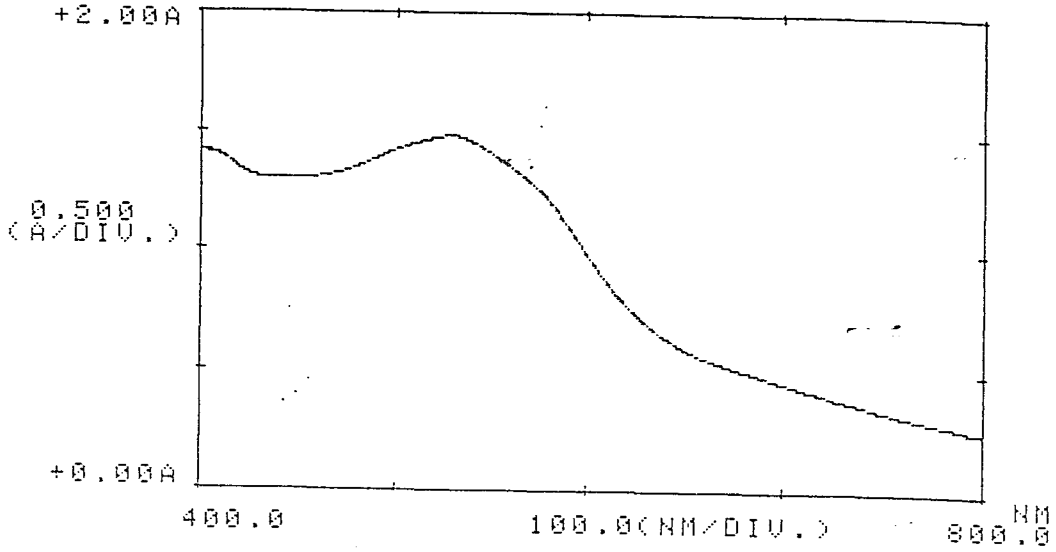


FIGURE 6.1 ABSORBANCE SPECTRA OF THE PRODUCTS OF A POLYPHENOLASE - L-DOPA REACTION

Top - Spectrum of products produced by the action of wild-type polyphenolase on 10 mM L-DOPA. The spectrum was read from 800nm to 400nm wavelength.

Bottom - Spectrum of products produced by the action of mutant 2421A polyphenolase on 10 mM L-DOPA. The spectrum was recorded between 800nm and 350nm wavelength.



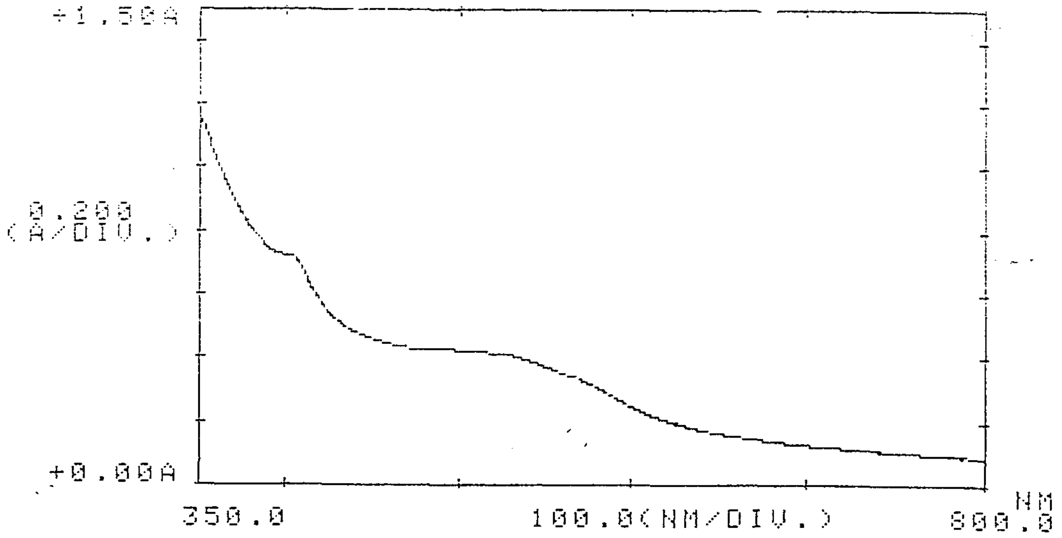


FIGURE 6.2 ABSORBANCE SPECTRA OF THE PRODUCTS OF A POLYPHENOLASE - L-DOPA REACTION

Top - Reaction of mutant 2421B polyphenolase with 10 mM L-DOPA. The spectrum was recorded from 800nm to 400nm wavelength.

Bottom - Reaction of mutant 2427 polyphenolase with 10 mM L-DOPA. The spectrum was recorded from 800nm to 400nm wavelength.

PLATE 6.1 CULTURE OF STREPTOMYCES FOR ENZYME PRODUCTION

Top - Culture of *Streptomyces* in Mcartney bottles

Bottom - Culture of *Streptomyces* in 500 ml conical flasks showing mutant. control and wild-type cultures (left to right).

Fig 6.3 is the graph of a typical "dopachrome assay" used to determine the activity of the crude enzyme extract (Burton *et al.*, 1993, Gardner and Cadman, 1990). After repeated assay, the activity of the wild-type polyphenolase enzyme was found to be 30 units/ml/minute. 1 unit of activity is taken to be the amount of enzyme that is required to catalyse the formation of 1 μmol of dopachrome per minute at 25°C (Gardner and Cadman,1990).

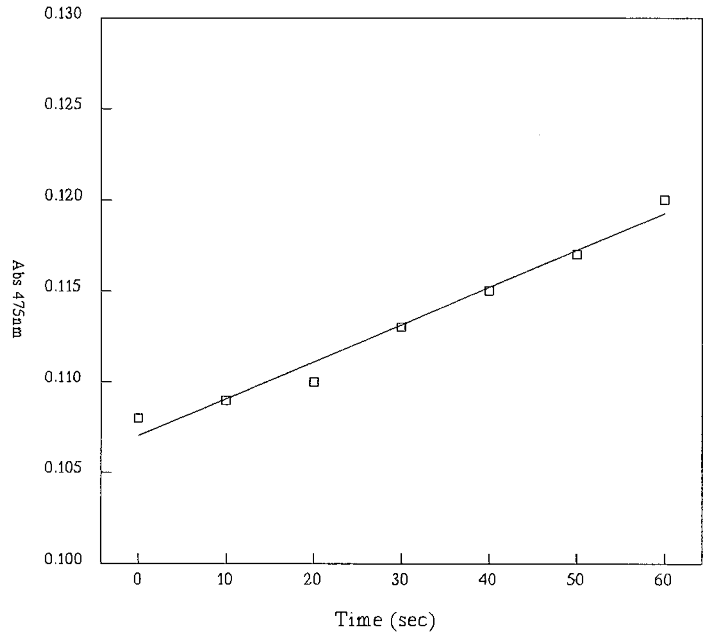


FIGURE 6.3 GRAPH OF ABSORBANCE VERSUS TIME FOR L-DOPA

Fig 6.4 A and **B** depicts Michaelis-Menten kinetic curves for the reaction of wild-type polyphenolase on L-DOPA. **Fig 6.4-A** is a plot of V against L-DOPA concentration, ie. [s]. **Fig 6.4-B** is a Hanes plot of [S]/V against [S] for L-DOPA. Hanes plots were used instead of Lineweaver-Burke plots, in order to avoid placing too much emphasis on measurements taken at low concentrations, which are not very accurate (Burton, 1993. pers. comm.).

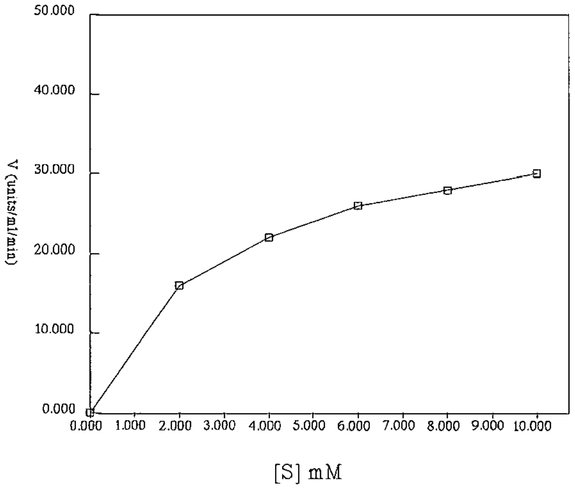


FIGURE 6.4 A V VERSUS [S] FOR L-DOPA

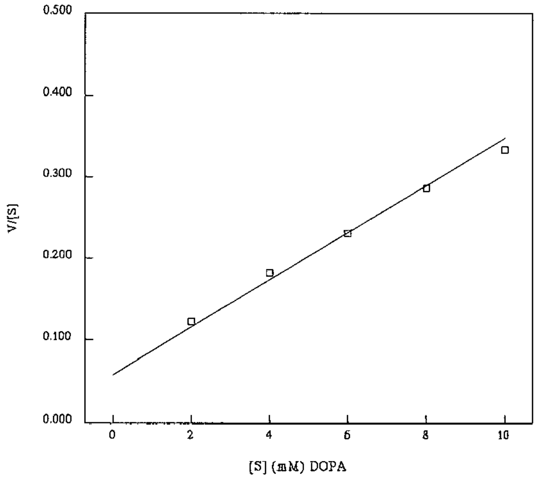


FIGURE 6.4 B GRAPH OF [S]/V VERSUS V FOR L-DOPA

Michaelis-Menten kinetic theory is based on the general reaction mechanism :

$$E + S \quad ES \quad E + P$$

The equation corresponding to this reaction is :

$$V = Vs/(K_m + S)$$

where V = initial reaction rate

S = initial substrate concentration

V = maximum velocity

K_m = Michaelis constant

Both V and K_m are peculiar to a particular system, and may be used to characterise the catalytic activity of an enzyme (Burton, 1993 pers. comm.).

Fig. 6.5 depicts Michaelis Menten and Hanes plots of the activity of wild type polyphenolase on L-tyrosine substrate. **Fig. 6.6** depicts the same plots for the activity of wild type polyphenolase on phenol, and **Fig. 6.7.** depicts kinetic plots for the activity of wild type polyphenolase on p-cresol substrate. Activity determinations for the activity of wild type enzyme on each of the four substrates tested are presented in **Table 6.1**

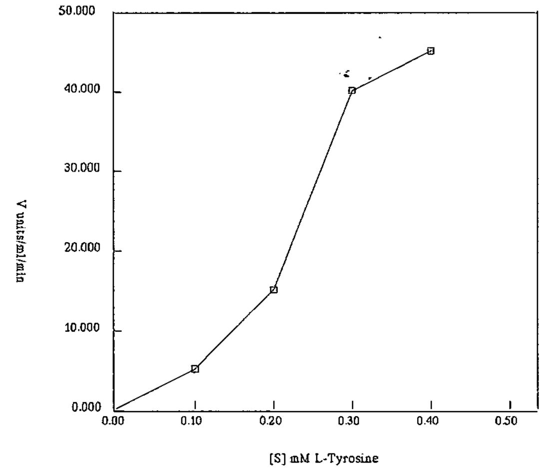


FIGURE 6.5 A V VERSUS FOR L-TYROSINE

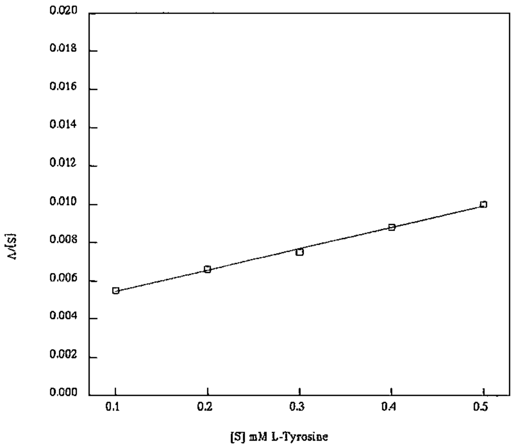


FIGURE 6.5 B [S]/V VERSUS [S] FOR L-TYROSINE

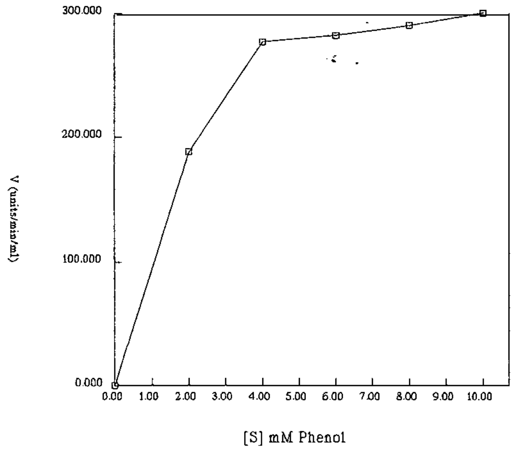


FIGURE 6.6 A V VERSUS [S] FOR PHENOL

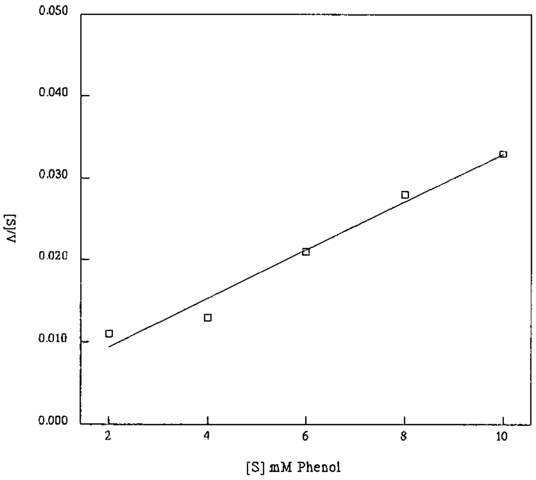


FIGURE 6.6 B [S]/V VERSUS V FOR PHENOL

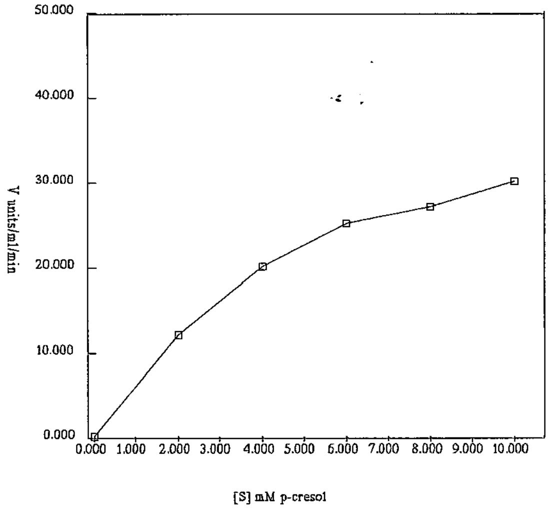


FIGURE 6.7 A V VERSUS [S] FOR p-CRESOL

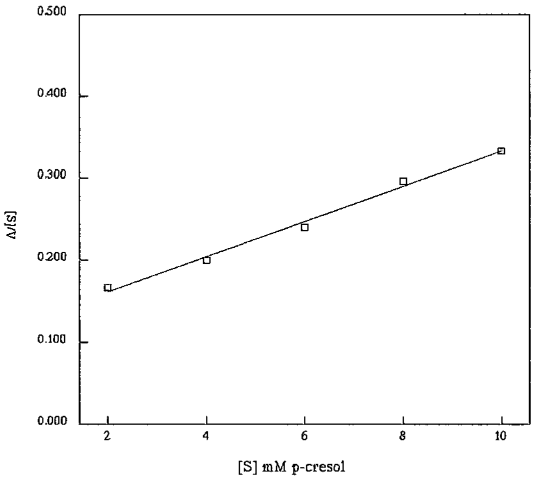


FIGURE 6.7 B [S]/V VERSUS V FOR p-CRESOL

Table 6.1 **Activity (V) of crude extract wild-type polyphenolase enzyme on different substrates**

SUBSTRATE	ACTIVITY (units/min/ml)
10 mM DOPA	50
0.5 mM L-tyrosine	50
10 mM phenol	300
10 mM p-cresol	30

Mutants 2421A, 2421B and 2427 were all obtained using the Hydroxylamine-EMS mutagenesis procedure. The mutants numbered 2421 A, 2421 B and 2427 displayed no detectable activity against L-DOPA, p-cresol or phenol substrates. This represents a significant difference from the wild-type which displays a detectable activity against the same substrates. However all of them displayed an activity against L-tyrosine, that appeared to be different from wild-type enzyme (Ingraham, 1957). In effect, this would imply that the mutagenesis strategy was efficacious, ie. mutant enzymes were produced that had different substrate properties from the wild type.

Figures 6.8, 6.9 and 6.10 - display Michaelis Menten and Hanes plots for the activity of mutants 2427, 2421 B and 2421 A respectively, on L-tyrosine substrate. Activity determinations were carried out and are presented in Table 6.2.

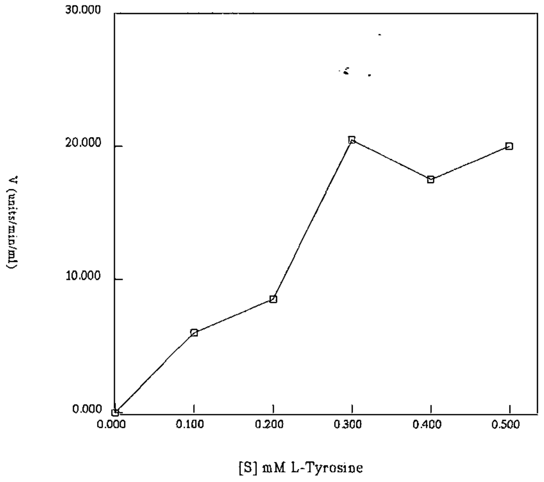


FIGURE 6.8(A) V VERSUS [S] FOR m2427 ON L-TYROSINE

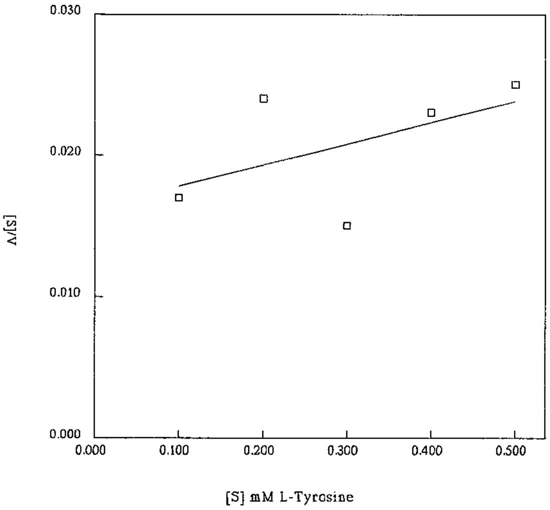


FIGURE 6.8(B) [S]/V VERSUS V FOR m2427 ON L-TYROSINE

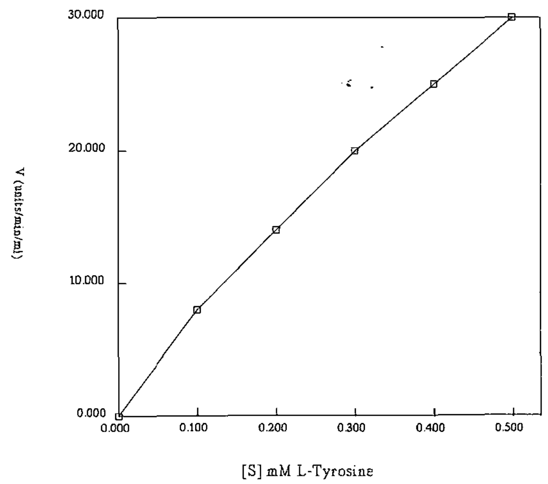


FIGURE 6.9(A) V VERSUS [S] FOR 2421B ON TYROSINE

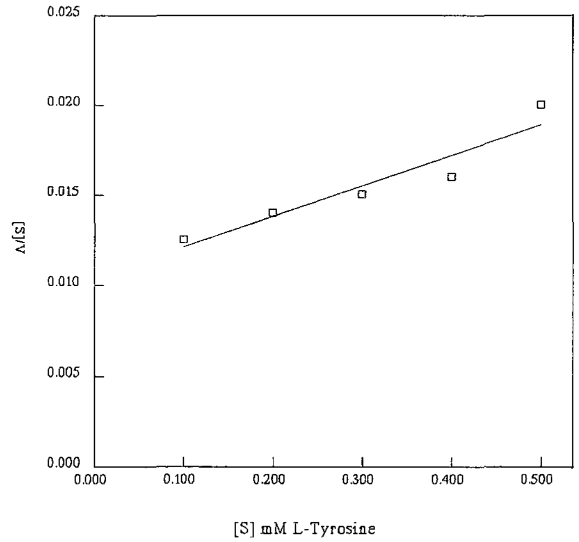


FIGURE 6.9 (B) [S]/V VERSUS [S] FOR M2421B ON L-TYROSINE

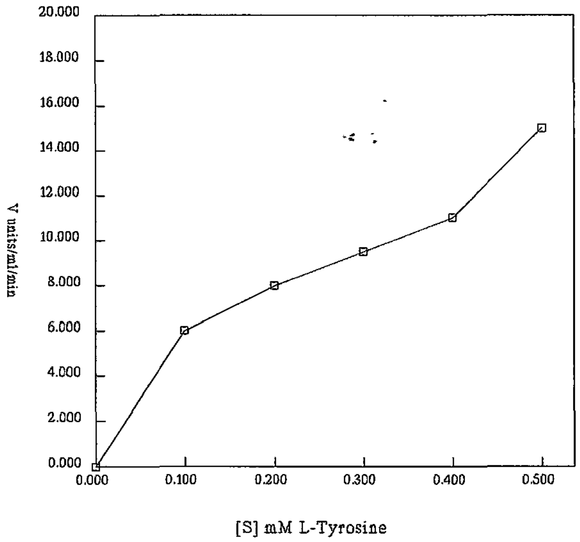


FIGURE 6.10 (A) V VERSUS [S] FOR m2421A ON L-TYROSINE

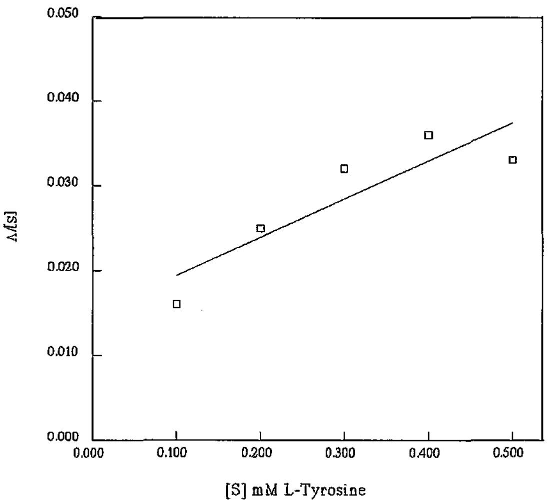


FIGURE 6.10 (B) [S]/V VERSUS [S] FOR m2421A ON L-TYROSINE

Table 6.2 Activity of crude extract mutant polyphenolase enzyme on 0.5 mM L - tyrosine

MUTANT	ACTIVITY (units/ml/min)
2427	20
2421 B	30
2421 A	20

Table 6.3 presents a comparison between the activity (V) of wild-type and mutant enzymes against L-tyrosine substrate and the apparent K_m/V values, determined from the Hanes plots.

Table 6.3 Comparison of wild-type and mutant enzymes by kinetic parameters

ENZYME SOURCE	ACTIVITY	K_m/V
mutant 2427	20	0.0163
mutant 2421 B	30	0.0104
mutant 2421 A	20	0.0149
wild-type	50	0.0043

From Table 6.3, it can be seen that K_m/V values are different for mutant and wild-type enzymes, for L-tyrosine substrate. This would indicate that the mutant enzymes isolated have different substrate affinities for L-tyrosine as compared to each other and to wild-type enzyme.

This could indicate that the active site amino acids of the mutant enzymes have been altered such that the substrate affinity and the substrate specificity of the enzymes have been altered. To determine the exact extent of such alteration, the primary structure of the mutant enzymes would have to be determined by sequencing and tertiary structure models can be deduced from such sequences using appropriate computer software.

CHAPTER 7 CONCLUDING REMARKS

From the results presented in Chapter 6, it is clear that some mutants were isolated that display distinctly different substrate affinities to the wild-type enzyme.

While it might be argued that oligonucleotide directed mutagenesis might have provided a more efficacious mutagenesis system, it must be remembered that this is an introductory study, intended to establish guidelines and parameters about the *in-vitro* mutagenesis of pIJ702.

Extension of this work might include oligonucleotide directed site-specific mutagenesis to alter specific portions of the *mel* gene and then to determine the effect of such alterations, by gradient gel electrophoresis and sequencing of mutant clones. Such mutants can then be subjected to large scale fermentation in order to produce appreciable quantities of the polyphenolase enzyme.

The development of *E. coli* - *Streptomyces* vectors, for the expression of *Streptomyces* genes in *E. coli* (della-Cioppa *et al.*, 1990), greatly extends the range of a mutagenesis research project in *Streptomyces* since it reduces the time taken to screen potentially mutant clones (overnight versus four to seven days).

Such a development can only have a beneficial impact on a project of this scale.

The optimization of a PCR-mutagenesis procedure also presents an attractive and safe alternative to chemical mutagenesis to achieve saturation mutagenesis. Such work will produce numerous clones that can be separated by gradient gel electrophoresis prior to sequencing.

Understanding the actual relations between amino acid sequence and protein structure and function is a daunting prospect because of the complexity of protein structures. Given the twenty commonly occurring amino acids, there are 20^{100} possible sequence permutations for a 100-residue protein (Degrado, *et al.*, 1989). However the increase in knowledge of the application of mutagenic strategies to investigate molecular phenomena, has added greatly to our armamentarium, in grasping such solutions.

APPENDIX A

Media, antibiotics and strains

NB. DW = Distilled Water

Malt 3 medium (M3)

Difco malt extract 24 g

Oxoid yeast extract 5 g

Tyrosine 0.05%

Adjust pH to 7-8 with 1N NaOH

For plates, add 15 g oxoid No 1 agar, before autoclaving.

Minimal Medium (MM) (Hopwood, (1967)

Agar 10 g (can be left out for broth)

L-asparagine 0.5 g

$K_2HPO_4.7\ H_2O$ 0.2 g

$FeSO_4.7H2O$ 0.01 g

DW 1 l

pH adjusted with 1N NaOH

Glucose (added as a 50% solution after autoclaving) 10 g

MMT (for tyrosinase expression and melanin production)

Supplement 1 l MM with the following sterile solutions:

Difco casamino acids (30%) 20 ml

Tiger Milk 7.5 ml

L-tyrosine (0.75%) 50 ml

1% CuCl 10 ml

NB: each solution was autoclaved separately before addition to MM.

Tiger Milk

L-arginine 1000 mg

L-cystine 750 mg

L-histidine 750 mg

DL-homoserine 750 mg

L-Leucine 750 mg

L-phenylalanine 750 mg

L-proline 750 mg

adenine 150 mg

uracil 150 mg

nicotinamide 10 mg

DW to 100 ml

Yeast Extract Malt Extract Medium (YEME)

Difco yeast extract 3 g

Difco Bacto peptone 5 g

oxoid malt extract 3 g

glucose 10 g

sucrose 340 g

DW to 1 l, autoclave, then add:

2.5 M $MgCl_2.6H_2O$ 2 ml

For preparing protoplasts, add also:

20% Glycine 25 ml

Soft Nutrient agar (SNA)

Difco nutrient broth powder 8 g

Agar 3 g

DW 1 l

Add thiostrepton to a final conch of 500 $mg.ml^{-1}$ after autoclaving.

R2YE medium (Okanashi, Hopwood and Wright, 1978).

Sucrose 103 g

K2S04 0.25 g

$MyCl_2.5H_2O$ 10.12 g

Glucose 10 g

Casaminoacids 0.1 g

Oxoid Bacto Agar No. I 22 g

DW to 800 ml

After autoclaving, while the agar is still liquid, add the following sterile solutions (each autoclaved separately):

KH_2PO_4 (0.5%) 10 ml

$CaCl_2.2H_2O$ (3.68%) 80 ml

L-proline (20%) 15 ml

TES buffer (5.73%, pH 7.2) 100 ml

Trace element solution 2 ml

IM NaOH 5 ml

Yeast extract (10%) 50 ml

NB Only Oxoid Bacto Agar No. 1 should be used for maximum efficiency. Pour and store under UV at room temperature for 2 weeks to dry until plate has a fine wrinkled appearance, or for 2-3 days at 37°C.

Luria Broth (LB)

Tryptone 10 g

Yeast extract 5 g

NaCl 10 g

DW 1 l

LB agar (LBA)

Add 1.5% agar to LB

Antibiotics

Thiostrepton (Sigma Chemical Co.)

Make up as a 50 mg.ml^{-1} stock solution in DMSO and add to media after autoclaving at a final concentration of 5 mg.ml^{-1}.

Ampicillin (Boehringer Mannheim)

Make up as a 25 mg.ml^{-1} stock solution in DMSO and add to media after autoclaving at a final concentration of 25 mg.ml^{-1}.

Trace Element solution

$ZnCl_2$ 40 mg

$FeCl_3.6H_2O$ 200 mg

$CaCl_2.2H_2O$ 10 mg

$MuCl_2.4H_2O$ 10 mg

$Na_2B_4O_7.10HO$ 10 mg

$(NH_4)_6 Mo_7O_{24}.4H_2O$ 10 mg

DW to 1 l

NB All media and constituents were sterilised by autoclaving at 121°C for 15 mins. Antibiotic solutions were sterilised by passage through a disposable 0.22 μM micro filter unit.

Bacterial strains

Streptomyces lividans 66 Tk_{24}

Streptomyces lividans 66 Tk_{24} (pIJ702)

Streptomyces antibioticus

Streptomyces glaucescens

Strains were cultivated as outlined in Chapter 2. Growth characteristics and specific requirements are also discussed in Chapter 2.

APPENDIX B

Buffers and Solutions

Unless specifically stated otherwise, all buffers and solutions were made up in Milli Q water, which was subsequently sterilised by autoclaving at 121°C for 15 mins. pH adjustments were achieved by using IN NaOH and IN HCl of the best possible quality.

Sodium Phosphate buffer 0.1M

		$NaH_2PO_4.H_2O$	Na_2HPO_4 (g.$l^{-1)}$
pH	7.2	4.44	10.20
	6	13.92	1.75

Dihydroxyphenylalanine (L-DOPA)($CaH_{11}NO_4$ (Sigma Chemical Co)

Made up as a 10 mM solution in 0,1 M sodium-phosphate buffer, pH 6.0

Lysozyme solution (Boehringer Mannheim)

Usually prepared as a 25 mg.ml^{-1} solution in 0.1 M Tris-HCl, pH 8.0.

Tris-EDTA buffer 1 (TE1), pH 8.0 or pH 7.6

A standard buffer, used for most DNA manipulations

Comprises Tris 10 mM

EDTA 1 mM

pH adjusted to 8.0 or to 7.6 by the addition of 1N HCl

Qiagen Buffer P1 (stored at 4°C)

50 mM Tris-NCl, 10 mM EDTA, pH 8.0

Add 100 mg.ml^{-1} RNAse A (supplied with buffer)

RNAse activity (ex beef pancreas) at 100 mg.ml^{-1} = 7 U.ml^{-1}

Qiagen buffer P2 (stored at RT)

200 nm NaoH, 1% SDS

Qiagen buffer P3 (stored at RT)

2.55 M Potassium acetate pH 4,8

Qiagen buffer QBT (stored at RT)

750 mM Nacl, 50 mM MOPS, 15% ethanol, 0,15% triton X100, pH 7.0

(MOPS = 3-n-morpholinopropanesulfonic acid, Sigma Chemicals Company)

Qiagen buffer QC (stored at RT)

1.0 M Nacl, 50 mM MOPS, 15% ethanol, pH 7.0

Qiagen buffer QF (stored at RT)

1.2 M Nacl, 50 mM MOPS, 15% ethanol, pH 8.0

Hydroxylamine solution

1M NH_2OH, pH 6,0

Add 0.56 ml 4 M NaOH to 0.35 g NH_2OH, then add DW to 5 ml

TBE buffer (RT)

Tris 89 mM or 10.8 $g.l^{-1}$

Boric acid 89 mM or 55 $g.l^{-1}$

EDTA 2 mM 0.931 $g.l^{-1}$

DNA sample loading buffer (stored at 4°C) (for agarose gel electrophoresis)

Bromophenol blue 0.25% (w/v)

Glycerol 30% in distilled water

Xylene cyanol 0.25%

Ethidium Bromide (10 mg/ml) (stored at 4°C)

usually included at a final concentration of 0.5 $mg.ml^{-1}$ in the agrose gel solution.

Polyacrylamide gel electrophoresis (PAGE) buffers and solutions (Laemmli, 1970)

Gel loading buffer (stored at 4°C)

Bromophenol blue 0.25% (w/v)

Xylene cyanol FF 0.25% (w/v)

Glycerol 50% in distilled water

Acrylamide stock solution (30%)

Acrylamide 29.2% - 29.2 g

Bis-acrylamide 0.8% - 0.8 g

Distilled water to 100 ml

Resolution gel buffer (stored at 4°C)

Tris 1.5 M or 18.17% w/v (36.3 g/200 ml)

SDS 0.4% w/v pH 8.8

Reservoir Buffer (10X)

Tris base (0.067 m) or 4.07% (w/v)	0.25 M 3 g/l
Glycine (0.238 M) or 17.87% (w/v)	0.192 M 14.43 g/l
SDS 1% (w/v)	0.1% 1 g/l
	10 ml of 10% SDS/l

SDS sample buffer

Tris base (pH 6.8) 0.125 M

SDS 4% (w/v)

glycerol 20% (w/v)

2-mercaptoethanol 10% (v/v)

Stacking gel buffer (stored at 4°C)

Tris base (0.5 M) or 6.06% (w/v)

SDS 0.4% (w/v)

pH adjusted to 6.8

Destaining solution for protein

10% glacial acetic acid 100 ml

DW 400 ml

50% methanol 500 ml

Makes 1 l and acts as a fixing agent for proteins

Coomassie Blue Stock (Staining Solution)

1% Coomassie Blue R-250 2g/200ml

Stir and then filter to remove solids

Coomassic Blue staining solution

Coomassie blue stock solution 62.5 ml

Methanol 250 ml

Glacial acetic acid 50 ml

DW 137.5 ml

Polyacrylamide gels were prepared as follows:

Solution	**Resolving gel (10%)**	**Stacking gel (5%)**
Acrylamide solution (30%)	12 ml	2 ml
Resolving gel buffer	8.2 ml	-
Stacking gel buffer	-	3 ml
DW	15.622 ml	6.23 ml
Ammonium persulfate (10%)	160 μl	64 μl
TEMED	18 μl	13 μl
Total:	36 ml	12 ml

Protoplast (P) buffer (Hopwood *et al.*, 1985)

Sucrose 103 g

K_2SO_4 0.25 g

$MgCl_2.6H_2O$ 2.02 g

Trace element solution 2 ml

Water 800 ml

Autoclave in 80 ml aliquots, then add to each aliquot:

K_2PO_4 (0.5%) 1 ml

$CaCl_2.2H_2O$ (3.68%) 10 ml

TES buffer (5.73%, pH 7.2) 10 ml

Tris-maleic acid buffer

1 M tris base

adjust to pH 8.0 by adding maleic acid

T buffer (transformation) (Hopwood *et al.*, 1985)

10.3% sucrose 25 ml

Trace element solution 75 ml

2.5% K_2SO_4 1 ml

DW 75 ml

Autoclave as part A

then to 9.3 ml of part A, add the following sterile solution:

5 M $CaCl_2$ 0.2 ml

Tris-maleic acid buffer 0.5 ml

Prior to use in transformation experiments, add 3 parts by volume of part A to 1 part by mass of polyethyleno glycol (PEG) 1000.

Pellet lysis solution

10 mM Tris-HCl (pH 8.0)

10 mM NaCl

10 mM EDTA

APPENDIX C

TERMINOLOGY

The references to the melanin gene in this text are references to a chromosonally derived gene that has been cloned into the plasmid vector pIJ702.

Optical density readings included absorbance ant transmission readings and were done at several wavelengths in order to obviate the mycelial effect.

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