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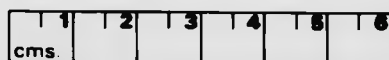
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THE PRODUCTION OF CATECHOLS
IN GLUCOSE FED-BATCH CULTURE USING
WHOLE CELLS OF PSEUDOMONAS PUTIDA.

by

Gary Kevin Robinson

This thesis is presented for the degree
of Doctor of Philosophy
Department of Biological Sciences
University of Warwick

November, 1988

To my parents, family and Helen,
without whom I couldn't have started,
let alone finished this thesis.

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I am indebted to Helen for moral support, advice and sympathy, especially in the preparation of this thesis.

I acknowledge financial support from the Science and Engineering Research Council and Shell Research Ltd.

DECLARATION.

The work in this thesis was the result of original research conducted by myself under the supervision of Professor Howard Dalton and Dr. P.J. Geary. All sources of information have been specifically acknowledged by means of reference.

None of the work presented in this thesis has been used in any previous application for a degree.

Gary Kevin Robinson.

SUMMARY.

The production of catechols, especially substituted catechols, has attracted industrial interest for the synthesis of high value-added compounds. Chemosynthetic routes are often complex and alternative methods using whole-cell biocatalysis are being investigated.

The aim of this project was to investigate the production of catechol and 3-methyl catechol using benzene or toluene respectively, as the substrates. Different mutants were used for the bioconversion of toluene and benzene to their respective catechols. Both mutants were derived from the same Pseudomonas putida strain, namely P. putida 2313 which already lacked the extradiol cleavage enzyme, catechol 2,3-oxygenase and was able to accumulate 3-methyl catechol when fed with toluene in the presence of glucose. However P. putida 2313 still possessed catechol 1,2-oxygenase allowing the organism to grow on benzene. After mutagenesis P. putida 6(12), an organism lacking the intradiol cleavage enzyme, catechol 1,2-oxygenase, was selected for further study.

P. putida strains 2313 and 6(12) were used as the biocatalyst in glucose-limited fed-batch cultures to achieve overproduction of either 3-methyl catechol or catechol. Under the conditions used, 11.5 mM (1.27 g/l) 3-methyl catechol and 27.5 mM (3 g/l) catechol were produced. Subsequently, two different product removal systems were employed in the 3-methyl catechol biotransformation and one of these, using an activated charcoal recycle column, resulted in the product yield being doubled.

In conclusion, it was shown that both catechol and 3-methyl catechol can be produced using a whole-cell biotransformation. 3-Methyl catechol was shown to be more toxic than catechol and the primary, though probably not exclusive, site of toxicity was the initial aromatic dioxygenase. Although overproduction of catechols was shown it would appear that the commercial production of these intermediates using a wholly biocatalytic route is limited by their inherent instability and resulting toxicity.

ABBREVIATIONS.

h	Hour
s	Second
min	Minute
rpm	Revolutions per minute
Pa	Pascal
Km	Michaelis constant
w/v	Concentration, weight by volume
v/v	Concentration, volume by volume
PAH	Polycyclic aromatic hydrocarbons
kd	Kilodaltons
NCIB	National Collection of Industrial Bacteria
ATCC	American Type Culture Collection
kb	Kilobase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
C12O	Catechol 1,2-oxygenase
C23O	Catechol 2,3-oxygenase
FAD	Flavin adenine dinucleotide
BCG	Benzene <u>cis</u> -glycol (<u>cis</u> -1,2-dihydroxycyclohexa-3,5-diene)
TCG	Toluene <u>cis</u> -glycol (<u>cis</u> -1,2-dihydroxy-3-methylcyclohexa-3,5-diene)
BTG	Benzene <u>trans</u> -glycol (<u>trans</u> -1,2-dihydroxycyclohexa-3,5-diene)
2,4D	2,4 Dichlorophenoxyacetic acid.
PMS	Phenazine methosulphate

NTG	N-Methyl-N'-nitro-N-nitrosoguanidine
LB	Luria broth
LA	Luria agar
MS	Mineral salts medium
mA	Milliamps.
NBT	Nitroblue tetrazolium
GC	Gas chromatography
HPLC	High pressure liquid chromatography
GC/MS	Gas chromatography/Mass spectrometry
dO ₂	Dissolved oxygen tension
OD	Optical density

CHAPTER 1. INTRODUCTION.

1.1 BIOTRANSFORMATIONS.

The term biotransformation may be considered as the selective, enzymatic modification of defined pure compounds into defined pure products using whole or part of a defined enzyme pathway. The desired product may be either a pure compound, a mixture of defined compounds, a cellular fraction or the biomass itself. Each type of product may be synthesised via transformation, degradation or de novo formation.

Throughout the twentieth century biocatalysts have been used for the production of various desirable compounds such as organic acids, solvents, amino acids and antibiotics. The large-scale production of solvents and organic acids using micro-organisms has been exploited at various times. Perhaps the most illustrative example of a microbial process which has, at times, been operated commercially is the acetone:butanol fermentation. Prior to World War I, acetone was imported from Germany. At the onset of hostilities, high quality acetone was required for the production of explosive cordite and as a propellant for artillery shells. Coincidentally, Chaim Weizmann had developed a microbial process for the production of acetone:butanol using Clostridium acetobutylicum as the biocatalyst and starch as the substrate. As a consequence of the demand created by the war, Weizmann developed the production of acetone:butanol into a large volume industrial process. Immediately after the war, butanol displaced acetone as the desirable product. Butanol was used in the production of urea /

formaldehyde resins, plasticizers and brake fluids. Additionally, the large amounts of gas (H_2 and CO_2) generated in the process and the residual solids (rich in vitamin B_2) increased the commercial viability of the process. Following World War II, the petrochemical industry developed rapidly and it was soon able to produce many organic acids and solvents considerably more cheaply than by microbial routes. Although processes such as the acetone:butanol fermentation have recently been re - evaluated (Gibbs, 1983) it would seem that when competing with petroleum - derived sources of the same products, such fermentations can only be economically viable if they consume biological wastes and agricultural surpluses. Thus their future is as likely to be determined by governmental policies, as by any progress made in increasing the efficiency of substrate conversion or product recovery. Despite the majority of solvents and organic acids being derived from petrochemicals, certain organic acids are produced using micro-organisms. Citric acid, an essential ingredient in foods is predominantly produced from molasses by Aspergillus niger. The world market for citric acid is 175,000 tons/year, for sales of \$259 million (Eveleigh, 1981)

Prior discussion has focussed upon the possibility of producing low cost, high volume feedstock chemicals which are conventionally derived from petrochemicals. The reverse situation, where the majority of current production is microbially derived, specialised, high cost intermediate/low volume chemicals is also true. Amino acids and antibiotics are inherently difficult to produce chemically. Antibiotics, although amenable to chemical modification, are very complex

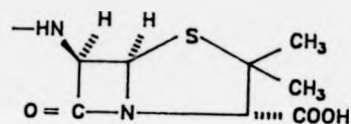
organic structures which are synthesised readily by a diversity of micro-organisms such as Penicillium sp., Cephalosporium sp. and Streptomyces sp. The broad specificity of the antibiotic synthesising systems enables the production of structurally related antibiotics rather than single entities. Consequently, depending on the media composition mixtures of antibiotics may be formed and this is outlined in Figure 1.1.

Although much simpler chemical structures, amino acids pose radically different problems. Two enantiomers (D and L forms) of each amino acid may exist, only one of which (the L form) is biologically active. If a chemosynthetic route is used for amino acid production, a racemic mixture of both D and L forms is produced, thus reducing the effective yield and necessitating a purification step. Although methionine is made synthetically, lysine (40,000 t) and glutamate (300,000 t), are made by fermentation (figs. from Eveleigh, 1981). The use of a microbial route obviates the need to separate the two enantiomers because only one form, the biologically active L form, is produced. As a consequence, since the 1950's bacteria such as Corynebacterium sp., Brevibacterium sp. and Bacillus sp. have been used to meet the world demand for amino acids.

Chemosynthesis has always been pre-eminent when considering methods to produce desired organic compounds. Synthetic organic chemistry has developed rapidly since the advent of the petrochemical industry and is usually the method of choice, whether using synthetic intermediates of natural or chemical origin. More recently, advances in applied microbiology and especially enzymology have enabled flexible synthetic routes to be evolved, using the disciplines of applied microbiology and/or chemistry.

Figure 1-1. Natural and biosynthetic penicillins
produced by *Penicillium* and *Cephalosporium* species.

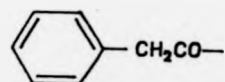
6-Aminopenicillanic acid



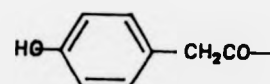
Penicillin

Side chain

G



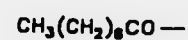
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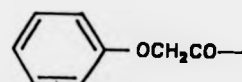
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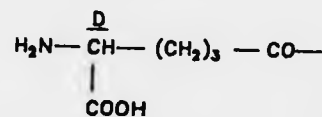
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Biotransformations may be carried out with either pure cultures of micro-organisms, plant cell cultures or with purified enzymes. The features of enzymes which make them attractive for the synthesis of organic compounds are as follows:-

(i) Reaction specificity - the reaction catalysed by an enzyme is usually highly specific and consists of a single reaction type. This eliminates side reactions if only one enzyme and substrate is employed in the biotransformation.

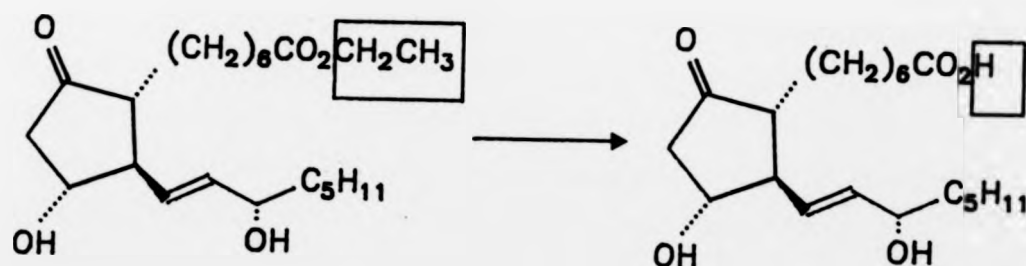
(ii) Regiospecificity - the substrate molecule is usually attacked at the same position irrespective of the presence of equivalent functional groups.

(iii) Stereospecificity - the asymmetric environment of an enzymes active site enables it to distinguish between enantiomers of a racemic mixture. Consequently, only one enantiomer is preferentially attacked.

(iv) Mild reaction conditions - unlike chemical reactions, which may need heat to accelerate a reaction, biological catalysts are very efficient at binding substrates under mild conditions (temperature below 40°C, pH around neutrality, atmospheric pressure). Such conditions facilitate the synthesis of very labile compounds without undesired decomposition or isomerization. An example of the latter is the de - esterification of prostaglandin - E_1 ethyl ester by yeast at pH 7.0 to give the natural product with minimal dehydration in the β -hydroxyketone unit (Figure 1.2).

The four major properties outlined above enable biocatalysts to be utilised for organic synthesis which are difficult using

Figure 1.2. Yeast catalysed de-esterification of
Prostaglandin-E₁-ethyl ester.



C·J· Sih et al., 1975.

existing chemosynthetic routes. This is best illustrated using examples which are of commercial importance:-

(i) Functionalization of a certain non - activated carbon.

Using micro-organisms, certain functional groups can be introduced at non - activated positions in a molecule, a feat difficult to accomplish using chemical reagents. The cornerstone for today's interest in biotransformations concerned the functionalization of steroids. The anti-inflammatory activity of corticosteroids depends on an oxygen function at position 11 of the steroid nucleus. Using chemical reagents, cortisone acetate was prepared at very low yields from deoxycholic acid by a synthesis requiring 31 steps. Nine steps were necessary to convert the 12 - hydroxy group to the 11 - keto group (Sarret, 1946). This synthesis was greatly simplified by using direct microbial 11 - hydroxylation of progesterone into 11 - alpha - hydroxyprogesterone (Figure 1.3). This was first accomplished by Peterson and Murray (1952) using Rhizopus sp. As a result of much development work the 11 alpha - hydroxylation of progesterone by strains of Rhizopus nigricans remains the most successful microbial hydroxylation in commercial operation, with yields of over 90% of 11 alpha - progesterone indicated (Nominé, 1980). Subsequently, it has become evident that, providing the micro-organism is chosen correctly, any carbon atom in the steroid skeleton can be accessed (Smith, 1984).

(ii) Selective conversion of a functional group among several groups of similar reactivity.

The regioselectivity imparted by using a biocatalyst

enables groups of similar reactivities, within the same molecule, to be differentiated. An example of this is provided by the second step of commercially synthesised ascorbic acid (Figure 1.4). Although six hydroxyl groups are present, early work using Acetobacter suboxydans showed it possessed a highly selective sorbitol dehydrogenase able to exclusively catalyse the oxidation of position 2 of D-sorbitol to yield L-sorbose (Reichstein and Grüssner, 1934).

(iii) Resolution of racemates.

The use of a biocatalyst provides the possibility to modify only one enantiomer of a racemic mixture. An early example of this was shown by Warburg (1906) who prepared L-leucine in about 83% yield from D,L-leucine propylester by a biotransformation using a pancreatic extract. Since Warburg's discovery, the use of hydrolytic enzymes to resolve racemic mixtures of D,L-amino acid derivatives has progressed significantly. The production of L-lysine by Toray Industries (1970) used an L-specific alpha-amino-ε-caprolactamase in its chemoenzymatic synthesis. The three step process, outlined in Figure 1.5, uses cyclohexane as the starting substrate. D,L-alpha-amino-ε-caprolactam, obtained from cyclohexene, is then acted upon by cells of Candida humicola containing L-specific alpha-amino-ε-caprolactamase to produce L-lysine at 98-100% optical purity. The D-alpha-amino-ε-caprolactamase is re-racemized by Alcaligenes faecalis cells containing an alpha-amino-ε-caprolactam racemase.

(iv) Introduction of a chiral centre.

Figure 1-3. The direct 11-hydroxylation of progesterone catalyzed by *Rhizopus* sp.

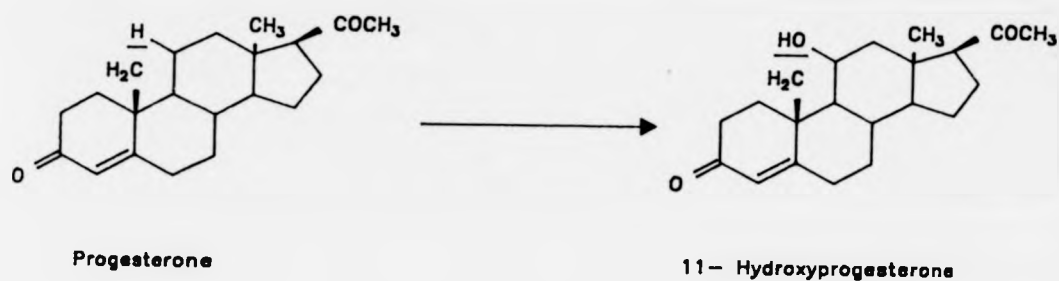


Figure 1-4. Microbial dehydrogenation of D-sorbitol to L-ascorbic during L-ascorbic acid production.

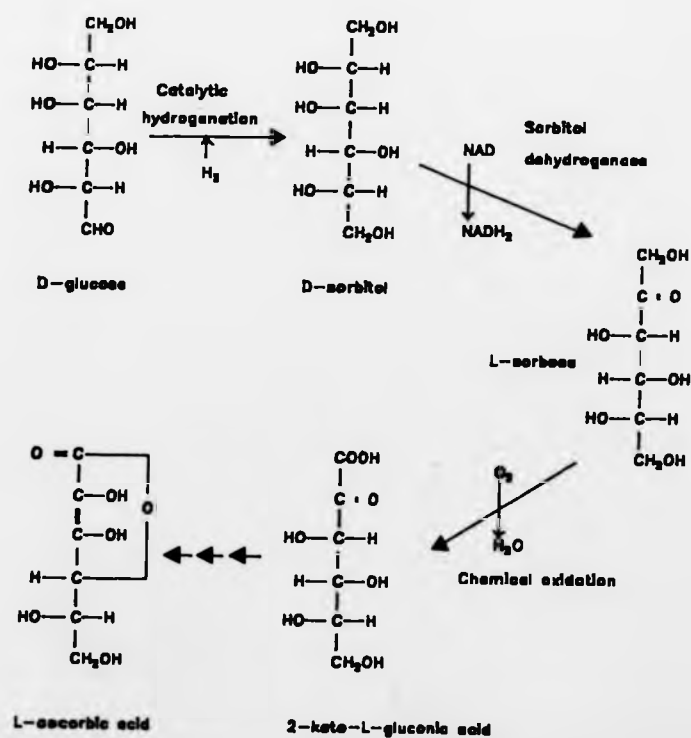
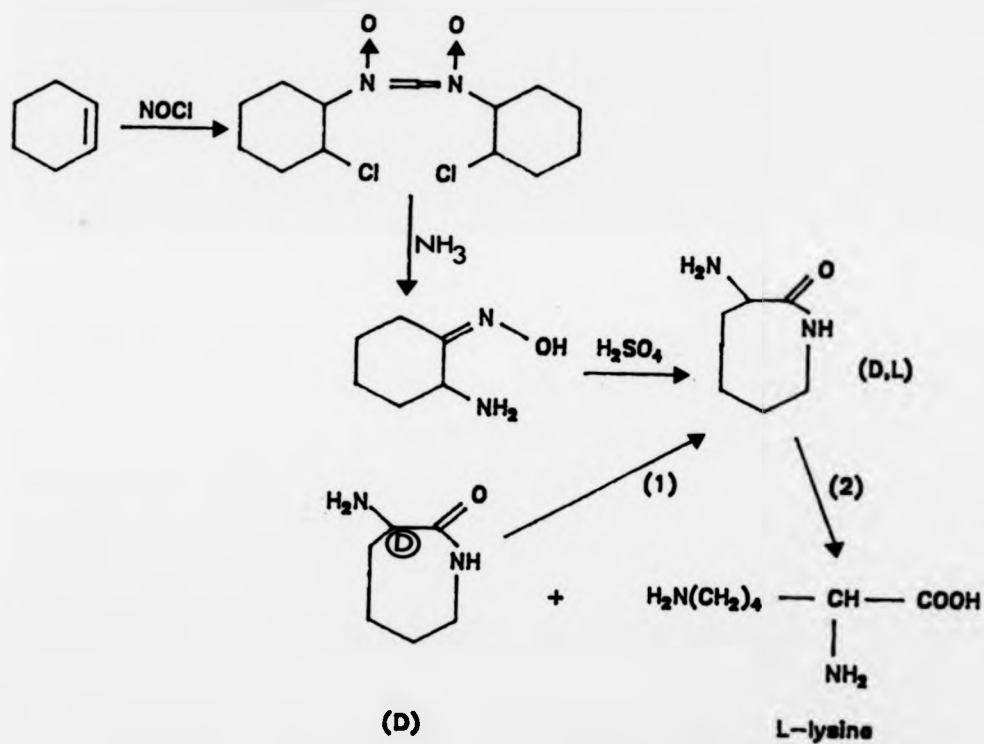


Figure 1-5. Chemoenzymatic synthesis of L-lysine

(Toray process)



(1) Amino lactam racemase.

(2) L-Amino-lactam-hydrolase.

Apart from using enzymes to modify only one enantiomer of a racemic mixture, enzymes may also be used to produce chiral products from prochiral substrates. Examples of the latter include the asymmetric addition of NH_3 or H_2O to fumaric acid to yield L-aspartic acid and L-malic acid respectively (Figure 1.6). Both of these reactions can be performed on a very large scale to give in excess of 40 tons/month of the desired product.

The advantages and benefits of using a biocatalyst for the production of low and high volume chemicals are evident from the few examples cited above. Having decided to use a biocatalytic route the chemist / microbiologist is faced with the choice of employing micro-organisms or isolated enzymes. The commercial availability and cost of the required enzyme compared with the availability of a suitable micro-organism will often be the deciding factor. The primary advantages and disadvantages of using whole - cell systems as opposed to enzymes are outlined in Table 1.1

1.2 CATECHOLS - PROPERTIES, USES AND METHODS OF MANUFACTURE.

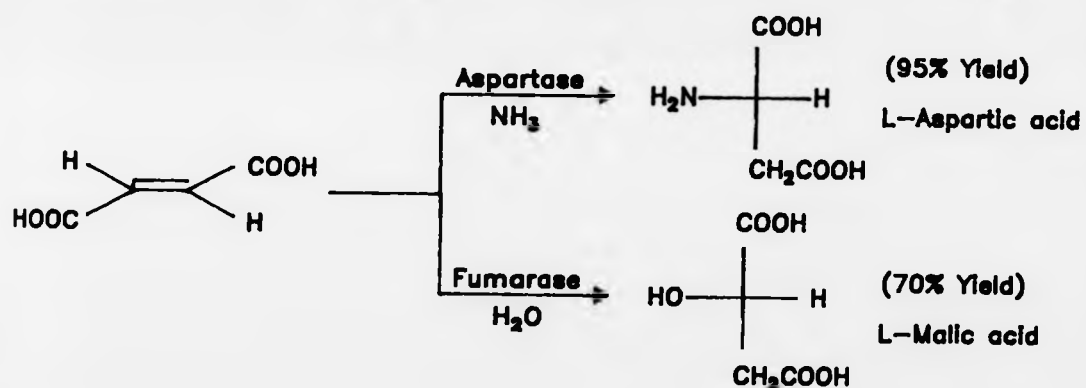
The present study aimed to produce catechols from benzene and toluene using a biotransformation route. Catechols are known to be toxic compounds which participate in a wide variety of chemical reactions. This section considers the properties of catechols which may be of relevance in the present study and the synthetic routes used in their large scale production.

Table 1.1 - Some advantages and disadvantages of using whole-cell systems as opposed to enzymes.

BIOTRANSFORMATION SYSTEM	ADVANTAGES	DISADVANTAGES
<u>Whole cell</u>	<p>Inexpensive</p> <p>Cofactor regeneration <u>in situ</u></p>	<p>Large amounts of glassware and/or fermentor.</p> <p>Possible requirement for aseptic conditions</p> <p>Side reactions may interfere or dominate product and/or substrate and/or cosolvent may disrupt (membrane bound) enzymes.</p>
<u>Enzyme</u>	<p>Simple apparatus</p> <p>Asepsis not required</p> <p>Specific for reaction</p> <p>Cosolvents better tolerated.</p>	<p>Expensive</p> <p>Addition of enzyme cofactors required or Enzyme cofactor must be recycled</p>

Adapted from Butt and Roberts (1987).

Figure 1-6. Asymmetric addition reaction to fumaric acid to yield either L-aspartic acid (Chibata et al., 1976) or L-malic acid (Yamamoto et al., 1976).



1.2.1 General Properties.

Catechol (Figure 1.7) is a crystalline compound with a phenolic odour and sweet and bitter taste. It was first obtained in 1839 by dry distillation of catechin, found in the aqueous extract of catechu (extract prepared from the heartwood of Acacia catechu). Catechol forms monoclinic crystals which are colourless and pure samples can be prepared by sublimation.

1.2.2 Chemical Properties.

Dihydroxybenzenes undergo all the typical reactions of phenols, some of which are summarised in Figure 1.8. A feature of all dihydroxybenzenes is that they are all weak dibasic acids having two dissociation constants (catechol - dissociation constants at 30°C - $K_1 = 7.5 \times 10^{-10}$, $K_2 = 8.37 \times 10^{-13}$). Catechol is able to complex most metallic salts and can be more easily oxidised than phenol. The first step in the oxidation of catechol is the formation of the semiquinone radical (Figure 1.9A). In strongly alkaline solution, the resonance-stabilised symmetrical anion (Figure 1.9B), is formed, whereas in strongly acidic media, the diprotonated radical (Figure 1.9C) is produced. Further oxidation leads immediately to the o-benzoquinone (Figure 1.9D).

Catechols are easily oxidised to o-benzoquinone using ferricyanide or Fremys salt (potassium nitrosodisulphonate $[(KSO_3)_2NO]$). An important reaction of catechol in the present study is the ease with which it auto-oxidises, exemplified by the formation of the oxygen-carbon coupled product in alkaline solution (Figure 1.10). Further oxidation and coupling can lead to polymeric material, thus

Figure 1.7 Catechol.

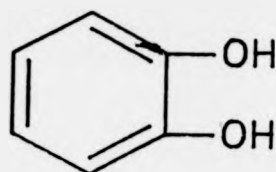
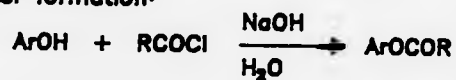
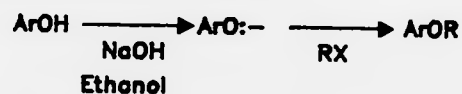


Figure 1.8 Chemical reactions of phenols.

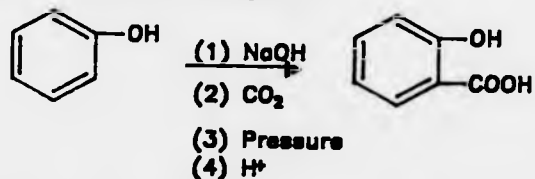
(A) Ester formation.



(B) Williamson ether synthesis.



(C) Kolbe - Schmidt carboxylation



(D) Oxidation to quinones.

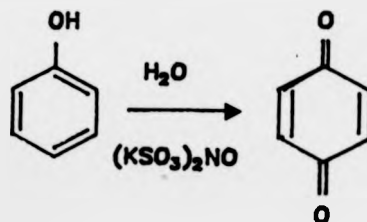


Figure 1.9. Intermediates in the oxidation of catechol.

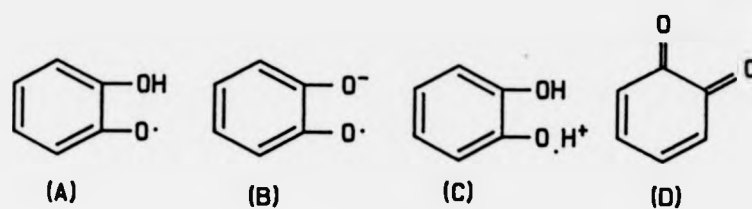
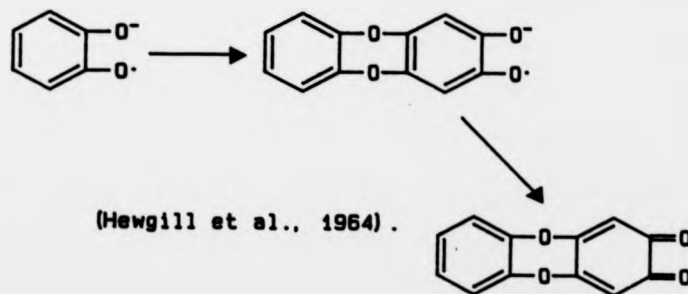


Figure 1.10. Aerial oxidation of catechol in alkaline soln.



coniferyl alcohol gives a compound resembling natural spruce lignin on oxidation either by enzymes or manganese dioxide (Freudenburg, 1962).

Dihydroxybenzenes, like phenols, possess hydroxyl groups which strongly activate the aromatic ring for electrophilic aromatic substitution. As a result, dihydroxybenzenes are highly reactive substrates for electrophilic halogenation, nitration and sulphonation as well as for coupling with diazonium salts to produce azo dyes. The electron densities at the aromatic nucleus explains the relative reactivity and regioselectivity in electrophilic substitution. With catechol, the electron densities at the aromatic nucleus directs monosubstitution reactions in the C3 and C4 positions (Figure 1.11).

1.2.3 Manufacture and Processing.

In Europe, catechol has been recovered industrially from gasworks ammoniacal liquor and, up until 1973, was manufactured from the low temperature carbonization of lignite and brown coal. This has been replaced by the distillation of coal tar and the hydroxylation of phenol using H_2O_2 . Table 1.2 shows the types of manufacturing processes and world production capacity for catechol.

Phenol can be directly hydroxylated to catechol and hydroquinone using hydrogen peroxide. The reaction occurs in the presence of catalytic amounts of strong mineral acids or of ferrous or cobaltous salts. The overall reaction is shown in Figure 1.12.

When a ferrous catalyst is used, some resorcinol is produced. The major steps of both processes are the same:-

Figure 1.11 Electron densities of catechol.

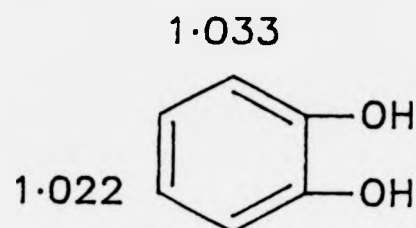


Figure 1.12. The direct hydroxylation of phenol.

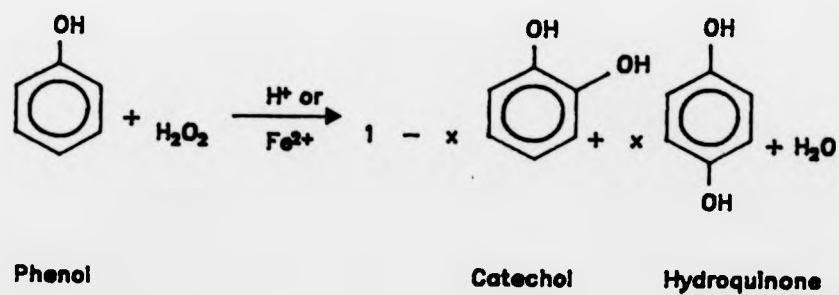


Table 1.2 - Manufacturing processes and world production capacity for catechol (Estimates for 1979).

World capacity (metric tons).	Processes	Country
> 20, 000	Phenol hydroxylation	France, Italy, Japan
	Coal tar distillation	UK, Eastern Bloc

Adapted from Varagnat (1979)

(i) Phenol is hydroxylated at 80°C, with a 70% hydrogen peroxide aqueous solution in the presence of a catalyst solution of perchloric acid or ferrous sulphate.

(ii) Phenols are extracted with a solvent after the oxidation mixture is washed with water.

(iii) Distillation is used to separate the solvent and phenol (both of which are recycled). Melted technical grade catechol is fed to a scaling machine and melted technical grade hydroquinone is solidified in a flaker.

1.2.4 Uses of Dihydroxybenzenes.

The main uses of dihydroxybenzenes (hydroquinone, resorcinol and catechol) are, in decreasing order of importance, photographic developers, tyre adhesives, rubber antioxidants, antiozonants and monomer inhibitors, wood adhesives, ultraviolet absorbers, optical brighteners and dyestuffs. Only the use of catechol will be discussed here.

The largest existing market for catechol and its derivatives is their use as rubber antioxidants and antiozonants, monomer inhibitors and, to a lesser extent, as a developer in black and white photography.

1.2.4.1 Antioxidants.

Antioxidants are substances that retard oxidation by atmospheric oxygen at moderate temperatures. Auto-oxidation can simply be described as the initiation of radical formation and their propagation (Figure 1.13).

Auto-oxidation is a free radical chain reaction and can be

inhibited at the initiation and propagation steps. Phenols, such as catechol, inhibit auto-oxidation at the propagation step. This is outlined in Figure 1.14

1.2.4.2 Antiozonants.

Many highly unsaturated elastomers are susceptible to crack development when stressed in an ozone-containing atmosphere. Such cracks can occur at low atmospheric ozone concentrations and can be a major cause of rubber failure in outdoor applications. The addition of antiozonants retard this cracking although the mechanism by which they work is unknown. Four main theories for their mechanism of action exist and these are dealt with in a review by Nicholas et al., 1978.

1.2.4.3 Photography.

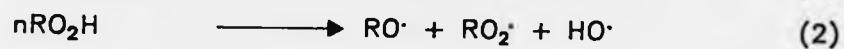
Although used less than hydroquinone, catechol is used as a developer in black and white photography. Its function is to reduce the exposed silver halide grains in a photographic emulsion at a faster rate than that of the unexposed grains, thus providing a very fine grain and high contrast image.

Apart from the large volume uses of catechols the catechol moiety is indigenous to a very large family of commercially important organic compounds, some of which are shown in Table 1.3.

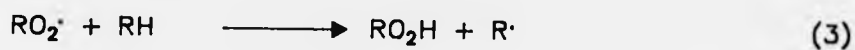
1.2.5 THE ROLE OF MICRO-ORGANISMS IN THE PRODUCTION OF CATECHOLIC COMPOUNDS.

The examples of catechol - based products outlined in this

Figure 1-13. A simplified scheme for auto-oxidation.



Propagation :



Termination:

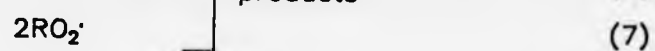
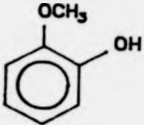
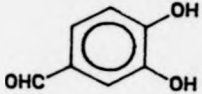
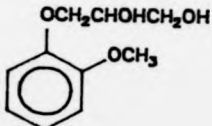
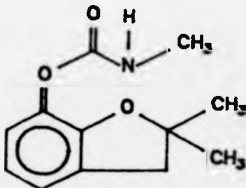
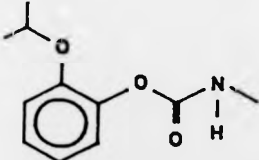


Figure 1-14. The effect of antioxidants on the propagation step.



AH = Catechol

Table 1-3- Catechol derivatives having specialist uses-

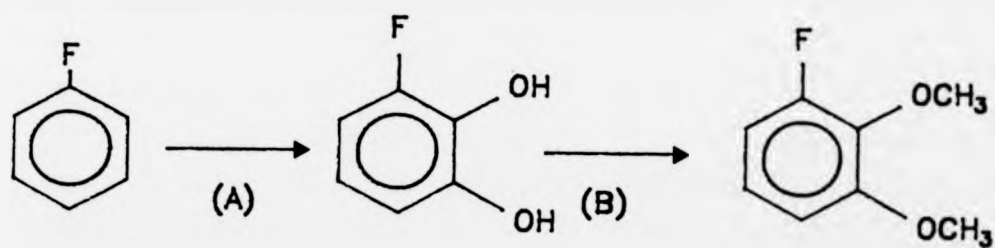
Compound	Name	Uses
	Guaiacol	Synthon for the preparation of synthetic flavourings
	Protocatechuic aldehyde	Synthon for preparing piperonal (Ingredient in cherry and vanilla flavourings).
	Guaiacol glycerol ether	Used in preparing expectorants
	Carbofuran	Insecticide
	Propoxur	Insecticide

section reflect relatively high volume chemical intermediates which may be synthesised using conventional chemical techniques. Although the catechol moiety can be produced using micro-organisms, current bio-organic processes are targetted towards high value products and reactions where non-enzymic chemistry is perceived to be weak. The production of catechols variously substituted with halogens have been shown to fulfill the afore-mentioned criteria. Whether the process is biological or chemical in nature is usually of little consequence provided that it is profitable.

The catechol moiety is indigenous to a large family of pharmaceutically active drugs, not least of which are the adrenergic catecholamines (Kirk and Creveling, 1984). 3-Fluoroveratrole, a catechol derivative, is a valuable intermediate for drug synthesis and is related to the corresponding fluorodimethoxy-phenyl ethylamines (Ladd and Weinstock, 1981; Ladd *et al.*, 1985). The purely chemical synthesis of 3 - fluoroveratrole, however, is both lengthy and involves expensive fluorinated starting materials. Gibson *et al.* (1968) showed that Pseudomonas T-12 was able, by virtue of the relaxed substrate specificity of the toluene dioxygenase, to introduce molecular oxygen into fluorobenzene. The resulting product, 3-fluorocatechol, can easily be recovered from the medium and can easily be converted to 3-fluoroveratrole by chemical methylation (Figure 1.15).

The example shown illustrates the possible advantages of using a microbial route to aid the synthesis of complex organic compounds. The present study investigates the possible problems of using a biological route and examines product yield, product

Figure 1.15. Bio-organic route to the synthesis
of 3-fluoroveratrole.



(A) *Pseudomonas* T12 + O₂

(B) (CH₃)₂SO₄

toxicity and product removal.

1.3 THE DIVERSITY AND ORIGIN OF AROMATIC HYDROCARBONS IN THE ENVIRONMENT.

The presence of aromatic compounds in the environment was first demonstrated by Kern (1947), who showed the presence of chrysene (1,2 benzphenanthrene) in soil. The subsequent development of more sophisticated analytical procedures facilitated the discovery of a plethora of organic compounds in the biosphere. The elucidation of the individual structures of all of the aromatic compounds in soil and sediment samples is almost impossible, and even in crude oil, where large amounts of material are available, only a few individual compounds have been identified (Coleman et al., 1969; Coleman et al., 1973).

The origin of aromatic hydrocarbons in the environment has been a contentious issue. Two distinct classes of compounds may be considered to be present and these are the persistent recalcitrant compounds of indirect biological origin and the rapidly turned over aromatics of direct biogenic origin. Blumer and Youngblood (1975) investigated the source of recalcitrant polycyclic aromatic hydrocarbons (PAH) and concluded that the primary sources were forest and prairie fires. These would have a wide range of combustion temperatures facilitating the production of the classes of compounds outlined in Table 1.4.

Hites et al., (1977) agreed that the polycyclic aromatic hydrocarbons in the environment arose from the deposition of combustion - generated airborne particulate matter. Unlike Blumer and Youngblood, they suggested that the origin of these

Table 1.4 - The distribution of aromatic compounds expected at various combustion temperatures.

TEMPERATURE	AROMATIC COMPOUNDS EXPECTED
2000°C	Principally unsubstituted polycyclics
400 - 800°C	Principally unsubstituted compounds but the presence of alkyl substituted molecules.
80 - 150°C	Polycyclic aromatics with 2-3 alkyl substituents as major products.

Adapted from Gibson and Subramanian (1984).

compounds was not natural sources but that they arose from anthropogenic combustion of fossil fuels. Evidence for this was presented which showed that PAH present in the sediment correlated closely with those calculated to have arisen due to anthropogenic burning of fossil fuels. If Blumer and Youngblood were correct, and forest fires were the origin of sedimentary PAH, levels of these hydrocarbons would remain constant throughout, a fact not supported by the data presented by Hites et al. (1977). Other, more recent, sources of aromatic hydrocarbons include fungicides, herbicides and general biocides used in modern agriculture. These have been chemically produced and often possess halogenated substituents, a factor which enhances their recalcitrance and allows them to persist in the environment (Steiert and Crawford, 1985). The less recalcitrant and simpler aromatic compounds which occur in the environment, such as toluene, benzene, ethylbenzene, xylenes and naphthalene probably arise due to anthropogenic synthesis. Although these compounds are not intended to enter the biosphere the high global production figures outlined in Table 1.5 illustrate that even a minimal deposition will affect the environment.

Aromatic compounds of direct biogenic origin are almost exclusively produced by plants or bacteria which suggests that the formation of aromatic rings from non-benzenoid precursors is restricted to these organisms. In agreement with this fact is that animals must depend upon their food for supplies of aromatic amino acids and a number of vitamin factors (vitamins E, K and p-aminobenzoic acid). Except for the biosynthesis of the oestrogens from non-aromatic steroids by mammals, plants and bacteria are the sole source of aromatic compounds.

Table 1.5 - Industrial production of aromatic hydrocarbons.

PRODUCTION (billions of lb.)			
HYDROCARBON	1980	1979	1978
Benzene ¹	1.56	1.67	1.49
Toluene ¹	NA	1.01	1.05
Ethylbenzene	7.61	8.45	8.39
Styrene	6.90	7.48	7.19
p - Xylene	3.83	4.65	3.52
m - Xylene	0.99	10.8	1.01

Key:- ¹ - Billions of gallons NA - Not available

Adapted from Gibson and Subramanian (1984).

There are three major pathways for the synthesis of aromatic rings. One of these sequences, commonly referred to as the shikimic acid pathway leads from carbohydrate through a series of six alicyclic stages (one of them shikimic acid) to about six or seven primary aromatic compounds (Figure 1.16). These are converted to the three aromatic amino acids (phenylalanine, tyrosine and tryptophan), the ubiquinones, several vitamins (vitamins K and p-aminobenzoic acid), to lignin and to an immense variety of so-called secondary metabolites which includes the majority of known alkaloids and natural pigments eg. morphine, colchicine and codeine (Figure 1.17). Secondary metabolites only occur in a limited number of organisms, rather than universally, and their function where known, appears not to be that of an intermediate in metabolism.

A second pathway, the polyketide pathway, uses acetate and malonate residues which are aligned in a regular head-to-tail fashion (Figure 1.18). These cyclise by two main methods, Aldol-type condensation and Claisen acylation reactions (Figure 1.19), eventually forming many of the secondary metabolites. Certain secondary metabolites are known, or at least suspected, to function as excretory or detoxification products, storage forms of valuable materials and germination inhibitors in seeds. One very evident type of function is that of many plant pigments, for which the anthocyanins and flavonoids may serve as typical examples. As an example of the latter, at least seventy different indole alkaloids are known to occur in one single higher plant, the pink periwinkle, Catharanthus roseus.

The third, and least well understood, pathway for the biosynthesis of aromatic compounds starts with mevalonic acid

Figure 1-16. The Shikimic Acid Pathway.

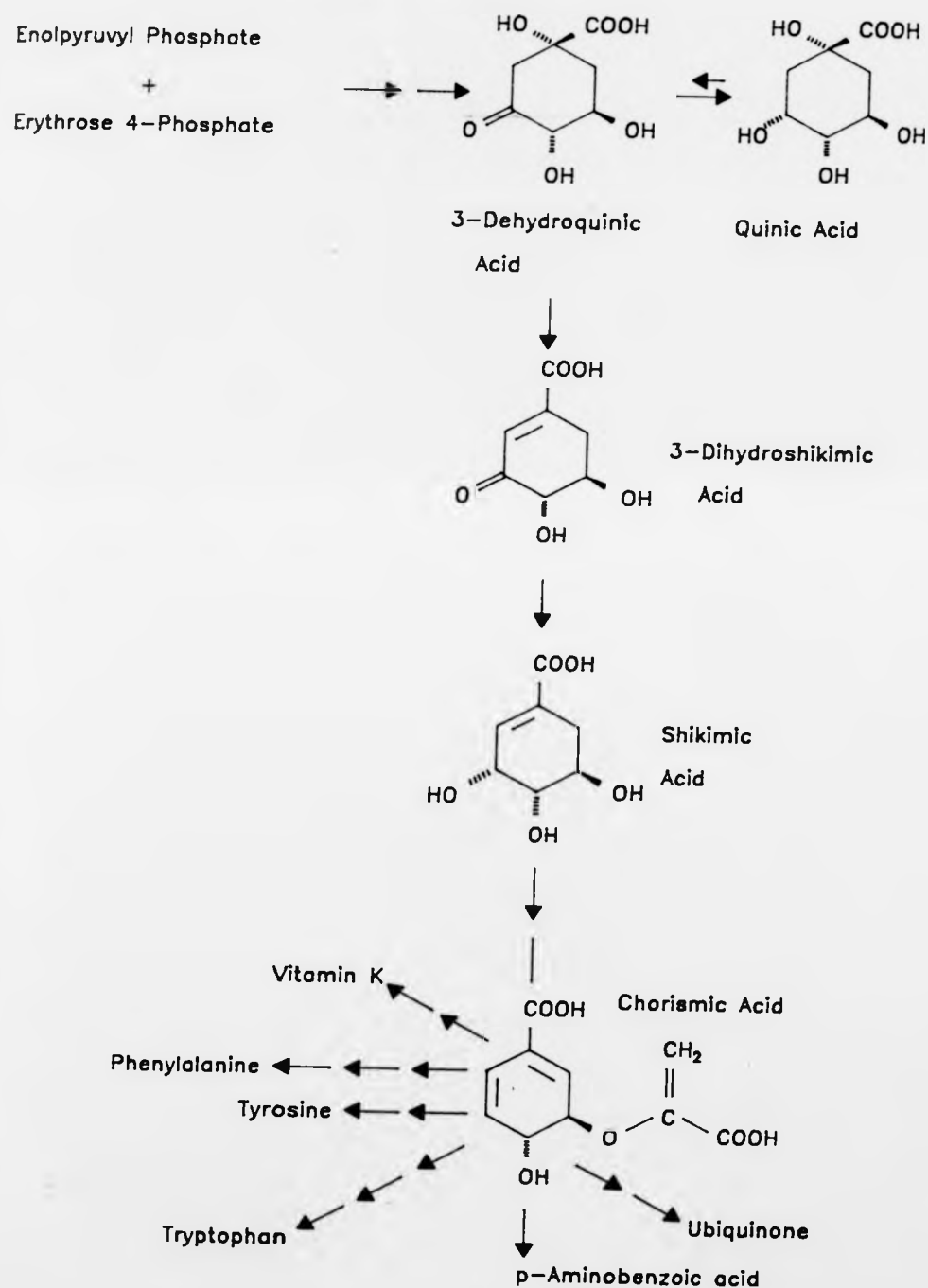
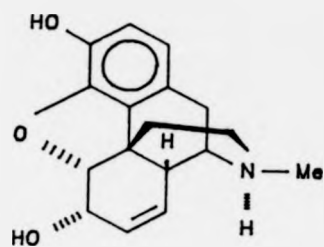
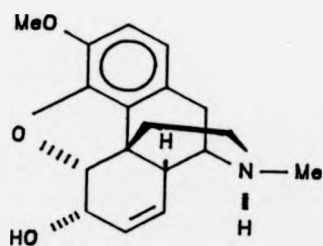


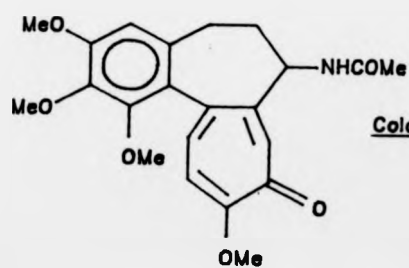
Figure 1-17. Examples of products formed from the shikimic acid pathway.



Morphine



Codeine



Colchicine

Figure 1-18. Synthesis of polyacetate for
use in the polyketide pathway.

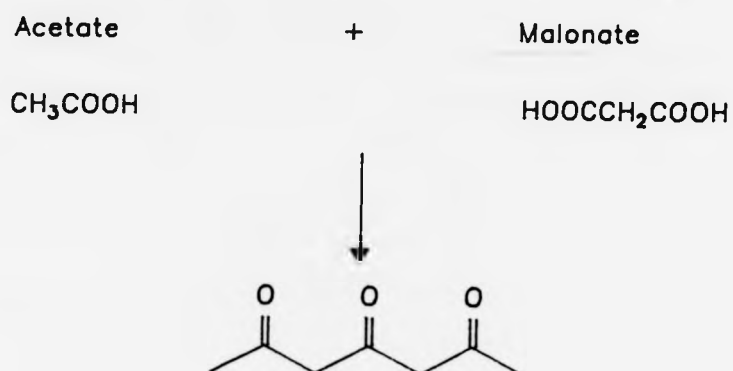
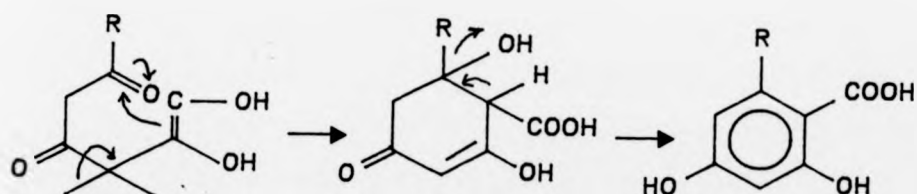
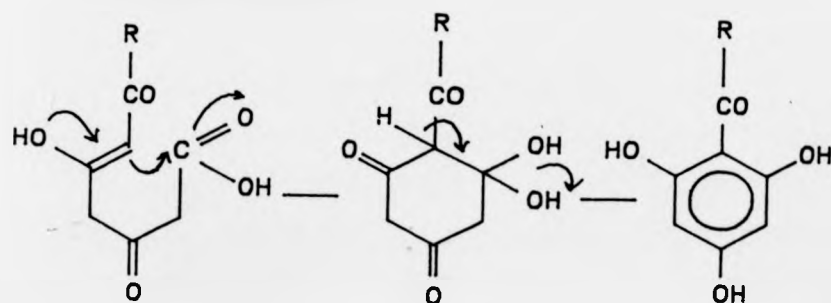


Figure 1-19. Cyclization mechanisms in the
polyketide pathway.

(1) Aldol-type condensation.



(2) Claisen acylation.



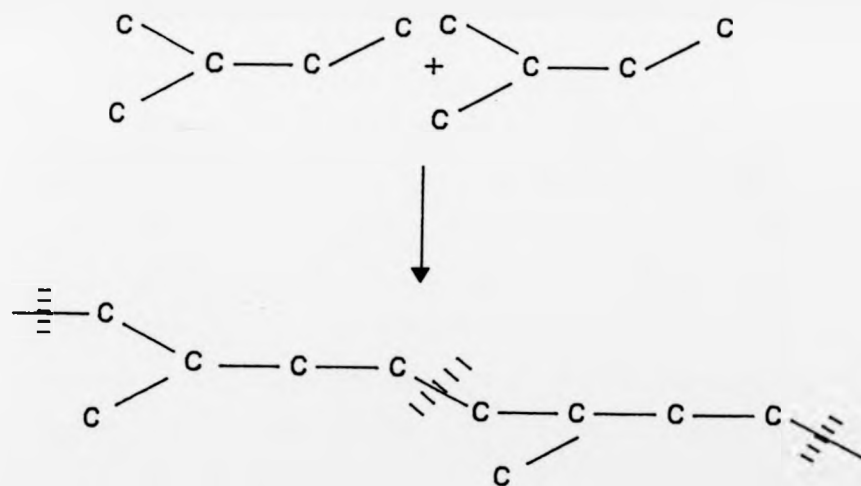
and yields a variety of mono-, sesqui-, di-, and triterpenoids, carotenoids and steroids. Typically, the majority of aromatic substances in this group have isoprenoid structures (Figure 1.20), and are used in the synthesis of hormones and carotenoids.

The three pathways mentioned demonstrate that many aromatics have a direct biogenic origin. These compounds are rapidly turned over and, as a consequence, do not form a reservoir of persistent aromatic compounds. However, the diversity of benzenoid compounds produced by plants and bacteria support the contention that micro-organisms must have evolved complex mechanisms to ensure the recycling of aromatic carbon. Such metabolic versatility has also become important for the degradation of both simple and complex benzenoid compounds which arise from anthropogenic sources.

1.4 AROMATIC UTILIZING BACTERIA.

Next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in the biosphere. Benzene and related aromatic structures possess large negative resonance energy, rendering them thermodynamically stable and chemically inert. This is due to the complete delocalization of electrons in the π -system as compared to an imaginary cyclohexatriene. Despite this, many micro-organisms are able to utilize aromatic compounds, achieving complete mineralization of the benzene nucleus. The ability of micro-organisms to utilize aromatic compounds for growth is now well established. As early as 1928 Gray and Thornton showed that bacteria were able to use

Figure 1.20. Isoprenoid structures involved in
the mevalonic acid pathway.



a diversity of organic compounds and found that, of 245 soil samples examined, approximately 60% contained bacteria that were able to degrade either naphthalene, phenol or cresol.

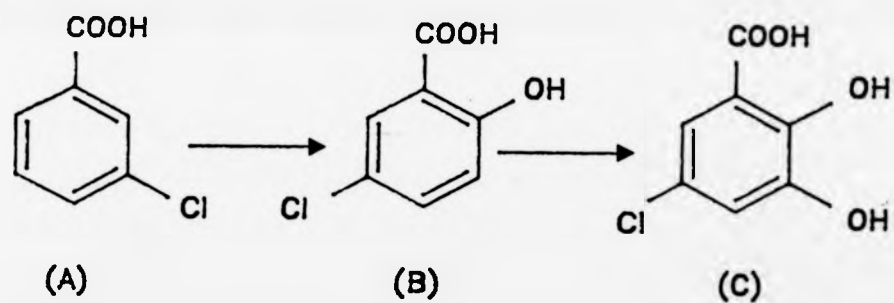
Pseudomonads are a group of soil micro-organisms which exhibit exceptional metabolic diversity, some species being able to use 60-80 different compounds as sole source of carbon and energy. For this reason, they have been much studied by microbial biochemists as biological material for the elucidation of the special metabolic pathways involved in the dissimilation of different classes of organic compounds. In particular, Pseudomonas species are able to use a range of aromatic substrates and several catabolic pathways of various degrees of complexity may occur within a single strain. This serves to verify the choice of this genus as archetypal for the study of aromatic utilization.

There is a paucity of reports on Gram-positive, aerobic bacteria which are able to degrade aromatic compounds. Despite this, studies using bacteria belonging to the genus Bacillus have shown they possess various pathways for the dissimilation of aromatics. Although similarities exist with other organisms, for example, the degradation of tryptophan by Bacillus megatorium (Bouknight and Sadoff, 1975), Pseudomonas fluorescens (Palleroni and Stanier, 1964) and Xanthomonas pruni (Brown and Wagner, 1970), certain bacilli have evolved unique degradative sequences not observed in other bacterial genera. This was exemplified by the work of Spokes and Walker (1974) who investigated chlorophenol and chlorobenzoic acid co-metabolism by a Bacillus sp. grown on benzoate. Previous work using Arthrobacter sp. (Horvath and Alexander, 1970), P. fluorescens

(Ichihara et al., 1962) and Azotobacter sp. (Walker and Harris, 1970) had shown chlorobenzoate to be metabolized via 3- or 4-chlorocatechol. Spokes and Walker (1974) showed that their Bacillus strain metabolized chlorobenzoate via 5-chlorosalicylate and 5-chloro-2,3-dihydroxybenzoate (Figure 1.21). Subsequently, Spokes and Walker showed that benzoate was metabolized via an analogous route. Although salicylic acid had previously been shown as an intermediate in the degradation of benzoate by Azotobacter vinelandii (Walker and Harris, 1970) and Pseudomonas convexa var. hippicurum (Bhat et al., 1959), the following intermediate was shown to be catechol. Therefore, evidence shown by Spokes and Walker (1974) that benzoate was metabolized via 2,3-dihydroxybenzoate demonstrated that certain bacilli possess a novel pathway for the degradation of benzoate. More recently, the ability of Bacillus strains to degrade salicylic acid via gentisate has been investigated. Crawford et al. (1979) studied the catabolism of salicylic acid by three strains of B. megaterium and eleven strains of B. brevis. Of the strains investigated, only two strains metabolized salicylic acid via the catechol branch of the β -ketoadipate pathway. The remaining strains catabolised salicylic acid via the gentisate pathway, of which three variants existed.

The pathways of aerobic metabolism of aromatic compounds are now well documented and will be discussed at length in the following sections. In contrast, the biochemical strategies involved in the anaerobic degradation of aromatics are fundamentally different and are still not completely understood. Consequently, the bacterial catabolism of aromatics in anaerobic environments will not be covered here and the reader is directed

Figure 1-21. Pathway for the degradation of chlorobenzoate catalysed by a *Bacillus* strain. (Spokes and Walker, 1976).



(A) 3-Chlorobenzoate

(B) 5-Chlorosalicylate

(C) 5-Chloro-2,3-dihydroxybenzoate

to the review by Evans (1977) for further information.

1.5 AEROBIC PATHWAYS OF AROMATIC METABOLISM.

1.5.1 Preparation for ring fission.

Aromatic compounds which contain a benzenoid nucleus are chemically inert. The benzene ring, with six π electrons in a cyclic conjugated system, is extremely stable and does not show any of the behaviour characteristic of alkenes. Micro-organisms are exceptional in their ability to invest energy in reactions that reduce the resonance energy barrier to permit fission of the aromatic ring. Subsequently, the energy invested is repaid by further cleavage reactions that yield energy, ultimately by furnishing substrates for the tricarboxylic acid cycle.

Prior to ring cleavage occurring the aromatic ring is activated by enzymatic hydroxylation, the pattern of hydroxylation being dependent upon the substrate and micro-organism under consideration. Regardless of the latter the ring fission substrate usually contains at least two hydroxyl groups in the ring structure, either ortho or para to each other (Dagley, 1971).

Consequently, diverse aromatic substrates are channelled into a few central pathways. This has several benefits, namely, a reduced genetic load, the simplification of regulatory circuits and the economisation of energy which would have to be invested if a novel, multi-step pathway were needed for complete mineralization of each aromatic substrate.

1.5.1.1 Hydroxylation by flavoprotein monooxygenases.

Flavoprotein hydroxylases/monooxygenases catalyse the introduction of a second hydroxyl group into a monohydric phenol to form the corresponding catechol. This involves a four-electron reduction of dioxygen, in which one atom of oxygen is added to the aromatic substrate and a second is reduced to water at the expense of a reduced pyridine nucleotide (Figure 1.22).

These enzymes are highly specific for their natural substrates, thus 4-hydroxybenzoate 3-hydroxylase is a different enzyme from either 3-hydroxybenzoate 4-hydroxylase or 4-phenylacetate 3-hydroxylase. Several examples of this class of hydroxylase have been purified to homogeneity and, in some cases, have been crystallised. One of the most extensively studied enzymes has been 4-hydroxybenzoate 3-monooxygenase which catalyses the reaction shown in Figure 1.23. The enzyme is a dimer with each monomer containing 394 amino acids which bind one FAD. Sites for binding NADH and 4-hydroxybenzoate (which cause a conformational change when bound) have been examined and a channel through which O_2 reaches the active site has been revealed (Wierenga *et al.*, 1982).

Other types of flavoprotein monooxygenases form different hydroxylated derivatives. 4-Hydroxyphenylacetate-1-hydroxylase catalyses a hydroxylation of the substrate and simultaneous shift of the side chain to form homogentisate, a key ring fission substrate (Figure 1.24). Salicylate hydroxylase catalyses the formation of a different ring fission substrate, catechol, from salicylate with the simultaneous loss of CO_2 (Figure 1.25).

1.5.1.2 Hydroxylation by Cytochrome P-450 dependent

Figure 1.22. Reaction catalysed by a typical flavoprotein monooxygenase.



Figure 1.23. The reaction catalysed by 4-hydroxybenzoate 3-monooxygenase.

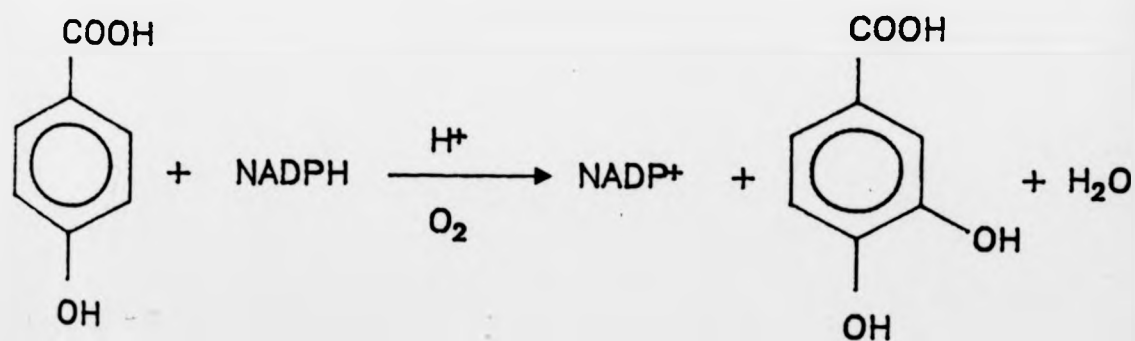


Figure 1.24. The reaction catalysed by 4-hydroxy-phenylacetate-1-hydroxylase.

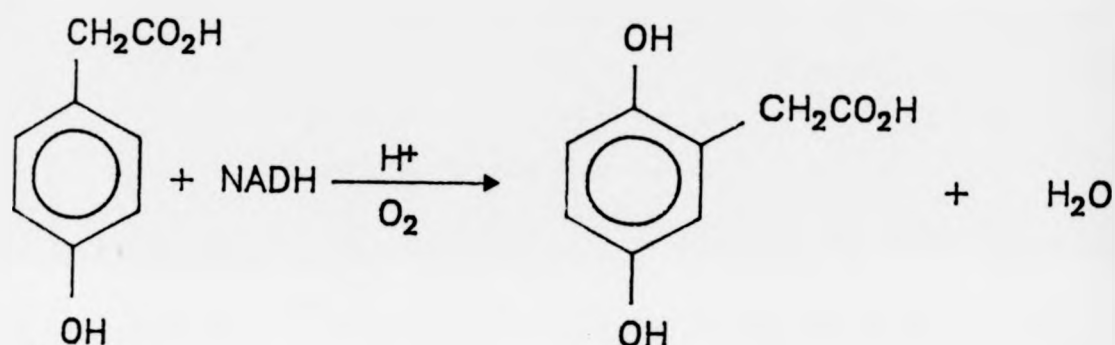
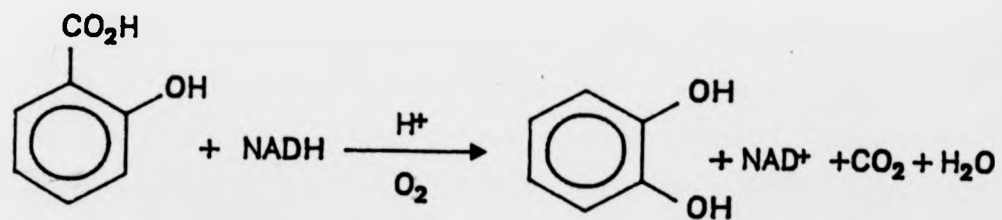


Figure 1.25. The reaction catalysed by salicylate hydroxylase.



monooxygenase.

Both mandelate 4-hydroxylase from Pseudomonas convexa and benzoate 4-hydroxylase from Aspergillus niger were reported to possess cytochrome P-450 as a prosthetic group (Dagley, 1978). Initially, this was thought to directly implicate cytochrome P-450 dependent monooxygenases in aromatic oxidations. However, it is now evident that cytochrome P-450 dependent monooxygenases are not used in degradative aromatic metabolism. Certain bacterial P-450 dependent monooxygenases have been characterised but these catalyse, among other things, the hydroxylation of steroids, saturated long - chain fatty acids, alcohols and amides and will not be discussed further.

1.5.1.3 Dihydroxylation of the benzene nucleus.

A third mechanism of hydroxylation of aromatics results in the ultimate formation of ortho - dihydric phenols, by the simultaneous incorporation of both atoms of molecular oxygen. For many years it was assumed that the formation of catechol from benzene proceeded via a mono-hydroxylated intermediate. Simultaneous adaptation studies (Wieland et al., 1958) did not support this hypothesis and an alternative mechanism was sought. Ayenger et al. (1959) showed that crude extracts of Aerobacter aerogenes contained an enzyme which catalysed the oxidation of benzene trans-glycol (BTG) to catechol (Figure 1.26). Marr and Stone (1961) suggested that this compound, BTG, was an intermediate in the dihydroxylation of the benzene nucleus. Jerina et al. (1968) later demonstrated that benzene was oxidised by liver microsomes to form an epoxide in an oxygen dependent reaction. The 1,2- epoxide was then hydrolysed to form

BTG which was subsequently dehydrogenated to yield catechol (Figure 1.27). However, Gibson et al. (1968) demonstrated that bacteria effected benzene oxidation by a different mechanism. They showed that a strain of Pseudomonas putida, isolated on ethylbenzene, would also grow with toluene and rapidly oxidised benzene and catechol. However, this strain did not oxidise BTG although it would oxidise the cis- isomer using benzene cis-glycol(BCG) dehydrogenase (Gibson et al., 1968).

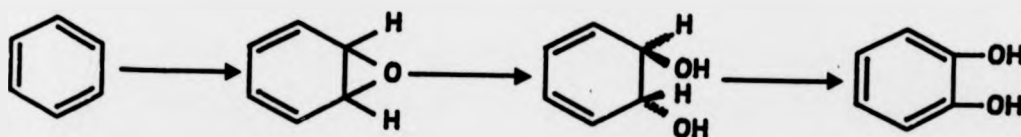
Proof that BCG was an intermediate during catechol formation from benzene was shown unequivocally by the use of P. putida 39/D a mutant lacking the glycol dehydrogenase enzyme (Gibson et al., 1970). When P. putida 39/D was grown on glucose in the presence of benzene and $^{18}\text{O}_2$, $^{18}\text{O}_2$ labelled BCG accumulated and both hydroxyl groups contained oxygen derived from molecular oxygen. Högn and Jaenicke (1972) supported this finding using a benzene-utilising strain of Moraxella B. Enzymatic oxidation of benzene in an $^{18}\text{O}_2$ atmosphere resulted in both hydroxyl groups of catechol being equally labelled, precluding an epoxide as an intermediate because hydrolytic cleavage would have led to a trans opening of the epoxide ring. If the latter had occurred it would have resulted in the formation of BTG with only one hydroxyl group containing $^{18}\text{O}_2$. Dihydroxylation of the aromatic nucleus has now been shown for benzoic acid (Yamaguchi et al., 1975), o-phthalate (Keyser and Ribbons, 1975), naphthalene (Laborde and Gibson, 1976), benzene (Axcell and Geary, 1975) and pyrazon (Sauber et al., 1977).

Following formation of the cis-glycol intermediates dehydrogenases catalyse their oxidation to catechol.

Figure 1-26 **Proposed reaction catalysed by**
***Aerobacter aerogenes*.**



Figure 1-27 **Reaction scheme proposed by Jerina et al (1968)**
for the oxidation of benzene by liver microsomes.



Dehydrogenases catalysing the oxidation of benzoate cis-glycol (Reiner, 1972), benzene cis-glycol (Axcell and Geary, 1973), toluene cis-glycol (Rogers and Gibson, 1977) and chloridazon cis-glycol (Eberspächer and Lingens, 1978) have been purified. A summary of the physico-chemical properties of the various enzymes are presented in Table 1.6. The data shows that all the enzymes are tetramers, utilize NAD^+ as their primary electron acceptor and, in the case of naphthalene cis-glycol, toluene cis-glycol and the B enzyme of chloridazon cis-glycol, oxidise a wide range of cis-glycols.

The physico-chemical and immunological properties of various glycol dehydrogenases have been compared by Patel and Gibson (1976). Naphthalene cis-glycol dehydrogenases from P. putida NP, Pseudomonas sp. (NCIB 9816) and a Nocardia species were isolated and compared. Immunodiffusion experiments did not provide evidence for any serological relationships between the enzymes from Nocardia sp. and P. putida NP. In contrast, the enzymes from Pseudomonas sp. (NCIB 9816) and P. putida NP showed similarity in their immunological properties although spurs formed in the experiments showed the enzymes from P. putida NP contains antigenic determinants not shared by the enzyme from Pseudomonas sp. (NCIB 9816). The similarities between the two strains were further supported by the apparent K_m for naphthalene cis-glycol ($2.0 \pm 0.4 \times 10^{-5}\text{M}$), the optimum pH (9.0), molecular weight (110 ± 10 kd.) and their substrate specificities for various cis-glycols. Surprisingly, the physico-chemical similarities were not supported by the heat stabilities of the enzymes. The naphthalene cis-glycol dehydrogenase prepared from P. putida NP retained complete

Table 1.5 Comparative physicochemical data for various *cis*-glycol dehydrogenases.

<i>cis</i> -glycol dehydrogenase	Organism	Mr (kdi)	K _m for <i>cis</i> -glycol	K _m for NAD ⁺	pH optimum	Reference.
Benzene	<i>Pseudomonas</i>	440	0.286 mM	43.5 μ M	7.9	Arcell & Geary (1973)
Chloridazon (A)		220	0.25 mM	ND	9.5	Eberspächer & Lingens (1978)
(B)		*	1.00 mM	*	7.0	
Benzoate	<i>Alcaligenes eutrophus</i>	94.6	0.20 mM	0.15 mM	8.0	Reiner (1972)
Naphthalene	<i>P. putida</i>	102	29 μ M	0.80 mM	9.0	Patel & Gibson (1974)
Toluene	<i>P. putida</i>	104	2 μ M	0.66 mM	9.6	Rogers & Gibson (1977)

NB (1) Chloridazon *cis*-glycol dehydrogenase was isolated as 2 isofunctional enzymes (A) and (B)..

All enzymes are NAD⁺ specific and possess 4 subunits.

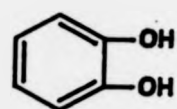
activity for >60 mins. after being heated at 50°C. Similar incubation, using the enzyme from Pseudomonas sp. (NCIB 9816) resulted in complete inactivation after approximately 5 mins.

In the same study (Patel and Gibson, 1976) the cis-glycol dehydrogenases of P. putida biotype A grown on benzene or toluene were examined. Immunodiffusion experiments indicated both enzymes were closely homologous. Additionally, the enzymes had similar pH optima (8.8 ± 0.1) and molecular weights (155 ± 5 kd.). The enzymes showed differences in their affinities for the substrates on which the organisms were grown. As in the example outlined previously, the physicochemical similarities did not extend to the thermal stability of the two enzymes. After 10 mins. incubation at 50°C, no benzene cis-glycol dehydrogenase activity could be observed. Contrary to this, complete inactivation of toluene cis-glycol dehydrogenase did not occur until after 60 mins. at 50°C. These findings indicate that the enzymes from P. putida biotype A grown on benzene and toluene may represent homologous proteins. The dissimilarities in their properties may represent minor differences in their primary structures and therefore in their protein conformations.

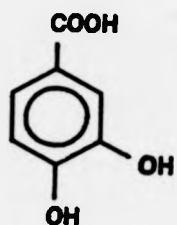
1.6 THE ROLE OF HYDROXYLATED COMPOUNDS IN AROMATIC METABOLISM.

All of the hydroxylations so far discussed illustrate a central principle in aromatic metabolism, namely, that all compounds are activated by hydroxylation to form ortho or para dihydroxybenzenes prior to ring cleavage. All catabolic pathways converge upon relatively few di- or tri-hydric phenols of which the most important are outlined in Figure 1.28. Of the ring

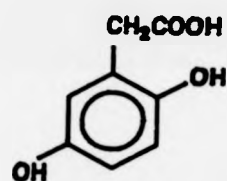
Figure 1-28 Central Intermediates of aromatic catabolism.



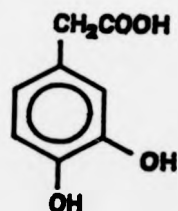
Catechol



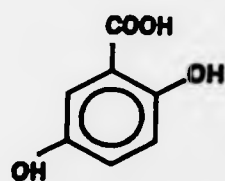
Protocatechuic acid



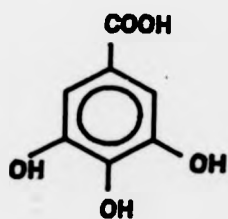
Homogentisic acid



Homoprotocatechuic acid



Gentisic acid



Gallic acid

fission intermediates shown, catechol occurs most frequently as a central metabolite in the oxidation of aromatic substrates. Some of the aromatic substrates which converge upon catechol are outlined in Figure 1.29. The use of gentisate as a ring fission intermediate is rare, only serving for the degradation of certain alkyl phenols which can be attacked by monooxygenases to give a second hydroxyl, para to the first (Hopper and Chapman, 1970). Homogentisate has been implicated as a central intermediate in the degradation of 1-phenyldodecane (Sariaslani et al., 1974). Sariaslani et al. (1974) showed that phenylacetate was formed from 1-phenyldodecane by Nocardia salmonicolor and this was subsequently oxidised to homogentisate prior to ring cleavage. Protocatechuate plays a central role in the degradation of meta and para cresol and o-phthalic acid. 3-O-Methyl gallic acid has received attention recently, due to its formation by lignin - degrading organisms, implicating it as a ring fission substrate for the degradation of lignin monomers (Donnelly and Dagley, 1981; Kersten et al., 1982)).

The majority of aromatic catabolic pathways converge upon relatively few ring fission substrates. Very few exceptions to the rule are known but Crawford et al. (1979) showed that the catabolism of 5-chlorosalicylate did not entail the formation of a di- or tri-hydric ring fission substrate. These workers showed that a Bacillus isolate from the Mississippi River (tentatively identified as a strain of B. brevis) possessed a specific 5-chlorosalicylate 1,2-dioxygenase, a novel enzyme able to cleave the aromatic ring while it was substituted with only one hydroxyl group (Figure 1.30). A second exception was shown by Kiyohara and Nagao (1977). Phenanthrene - grown Aeromonas sp.

Figure 1-29. The role of catechol as a central metabolite
in the bacterial degradation of benzenoid compounds.

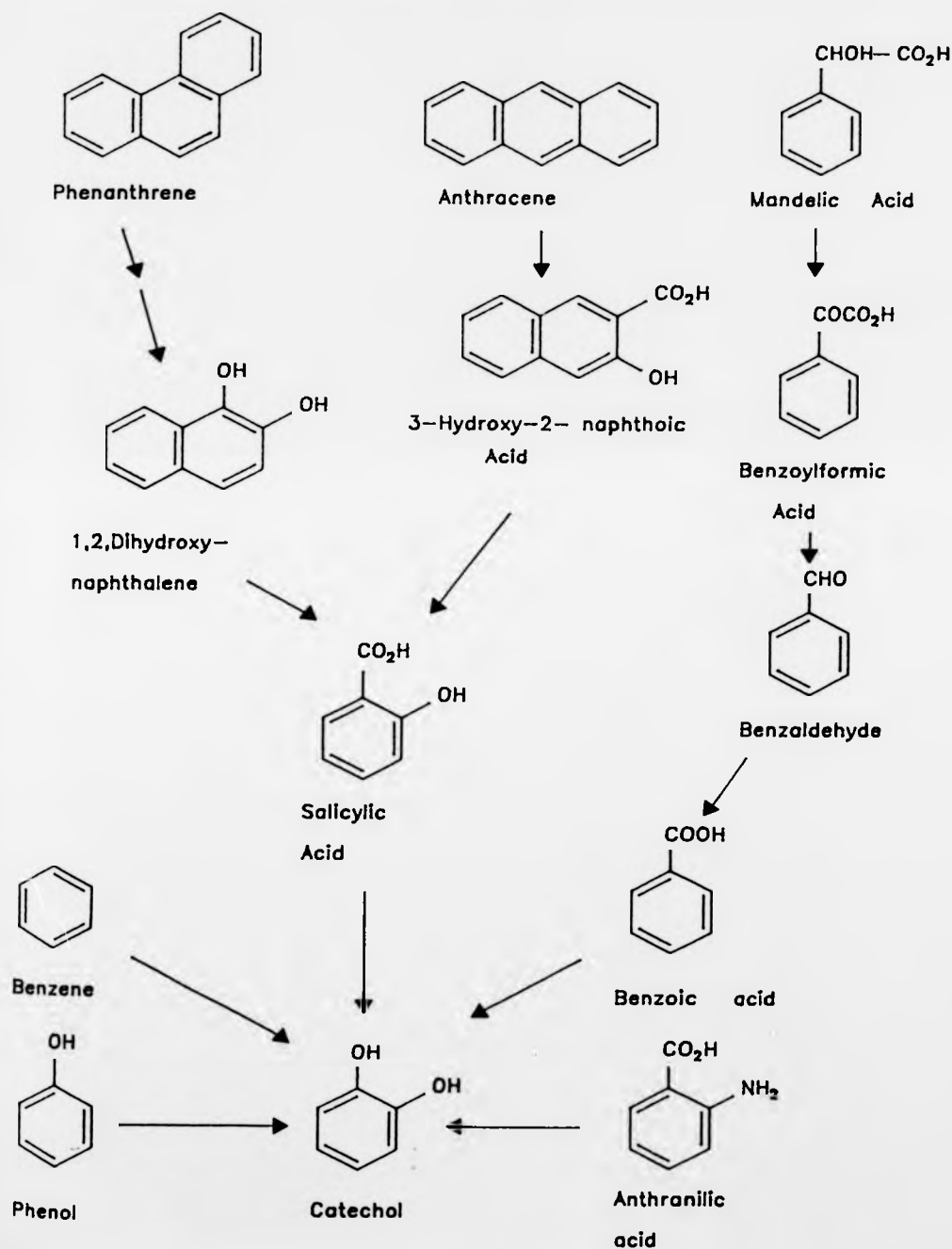
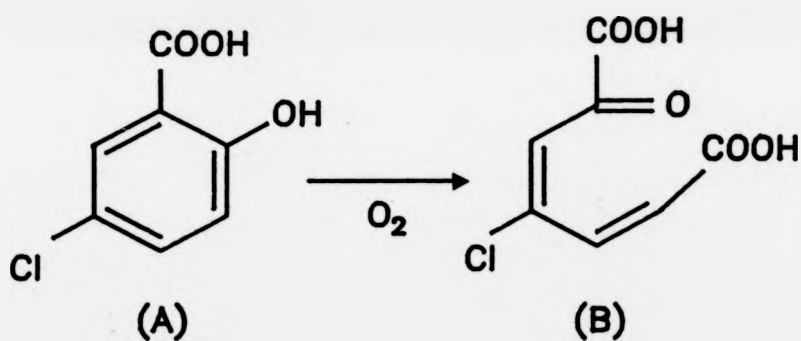


Figure 1.30 **Reaction catalysed by 5-chlorosalicylate**
1,2-dioxygenase.



(A) 5-Chlorosalicylate

(B) 7-Carboxy-4-chloro-2-ketohept-3,5-dienoic Acid

S45P1, form 1-hydroxy-2-naphthoate as an intermediate. Although the latter possessed only one hydroxyl group the enzyme, 1-hydroxy-2-naphthoate 1,2 dioxygenase, catalysed ring cleavage by using the benzene ring as a substitute for one of the usual two hydroxyls. A further example was provided by Que (1978) who showed that 2-aminophenol was slowly cleaved extradiolally by catechol 1,2-oxygenase.

1.7 PATHWAYS OF AROMATIC RING CLEAVAGE.

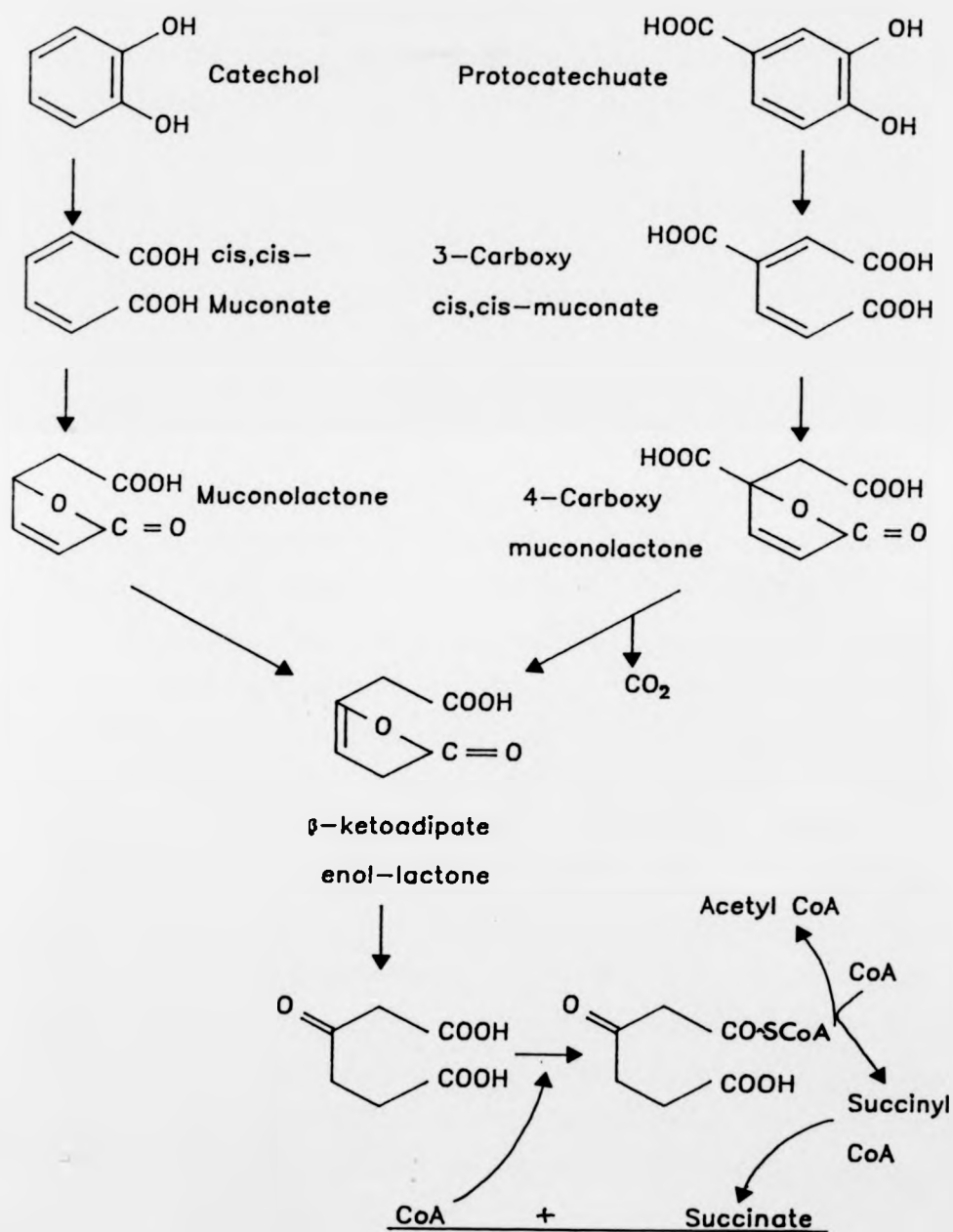
Initial attack by an oxygenase on a benzene nucleus consumes NADH that could otherwise be used to generate ATP during re-oxidation via the electron transport chain. By contrast, ring fission reactions themselves neither consume nor generate energy. The reactions which follow ring fission serve largely to break up the carbon skeleton of the aromatic nucleus, eventually yielding intermediates of the tricarboxylic acid cycle. Cleavage of the aromatic nucleus may occur either by intradiol (ortho) or extradiol (meta) fission.

1.7.1 Intradiol cleavage.

Intradiol cleavage occurs between two neighbouring hydroxylated carbon atoms to produce a dicarboxylated product. Cleavage of catechol by catechol 1,2-oxygenase (C12O) yields cis,cis-muconate as outlined in Figure 1.31. The cleavage enzyme, catechol 1,2-oxygenase (EC 1.13.11.1) was first isolated and purified from Pseudomonas fluorescens strain 23 (ATCC 11250) by Hayaishi et al., (1957). They showed that both oxygen atoms incorporated into the muconate were derived from atmospheric oxygen and the enzyme possessed no haem or flavin component. The

enzyme was found to have a narrow substrate range, failing to cleave substrates such as gentisate and homogentisate. Physical studies indicated that the cleavage enzyme possessed 2g atoms iron/mole enzyme and the molecular weight was 81 kd. Subsequently, a catechol 1,2-oxygenase was purified from P. arvilla C-1 by Nakai et al. (1979). This enzyme consisted of two non-identical subunits and contained 1g atom iron/mole enzyme based on a molecular weight of 60 kd. The iron in the enzyme was present in the ferric state and is a substrate binding site, hence it is essential for activity. Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) catalyses the conversion of protocatechuate to β -carboxymuconic acid with the incorporation of two atoms of molecular oxygen (Figure 1.32). The enzyme has been obtained in crystalline form from *p*-hydroxybenzoate-induced cells of P. aeruginosa (ATCC 23975) (Fujisawa and Hayaishi, 1978). The molecular weight is approximately 700 kd. and contains about 8g atoms iron/mole, eight substrate binding sites and appears to consist of eight subunits (Fujisawa et al., 1972).

Both catechol and protocatechuate are converted to a common intermediate, β -ketoadipate enol-lactone by a series of three chemically analogous reactions (Figure 1.33). Thus, oxygenative cleavage between the hydroxyl groups of catechol yields cis,cis-muconate and β -carboxy-cis, cis muconate from protocatechuate. Subsequently, the muconates are converted to muconolactone and gamma-carboxymuconolactone respectively. The latter is decarboxylated, forcing the migration of the double bond within the lactone to yield β -ketoadipate enol-lactone. Deprotonation of the gamma carbon of muconolactone also yields

Figure 1-33. The β -ketoadipate pathway.

the same product by a similar mechanism. Eventually, the lactone is hydrolysed enzymically to β -ketoadipate, a substrate suitable for thiolytic fission after esterification with coenzyme A.

Although both branches of the pathway consist of similar chemical reactions, the enzymes of the two pathways differ in some physical properties and are highly specific for the intermediates of the appropriate pathway (Ornston, 1966a, 1966b). Thus, protocatechuate dioxygenase from P. putida cleaves catechol at 2% of the rate at which it cleaves its natural substrate. Conversely, catechol 1,2-oxygenase will not act upon β -carboxy- cis,cis-muconate, nor will the carboxymuconate-lactonizing enzyme catalyse the lactonization of cis,cis-muconate. The following enzymes in the pathway, muconolactone isomerase and carboxymuconolactone decarboxylase are also specific for their own substrate. Furthermore, the differences between the two groups of enzymes were further investigated by Stanier et al. (1970). These workers investigated the immunological cross-reactivity of two ortho pathway enzymes, muconate-lactonizing enzyme and muconolactone isomerase from different organisms. Both antibodies were prepared using purified enzyme preparations from P. putida PRS 1. Although the experiment showed there to be a degree of antigenic relatedness between the isofunctional enzymes from different species, it was shown that no cross reaction occurred with the analogous enzymes of the protocatechuate pathway, carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase. Despite performing similar chemical reactions and sharing some physico-chemical characteristics (Maegher et al., 1972; Patel et al., 1973), the immunological data suggested

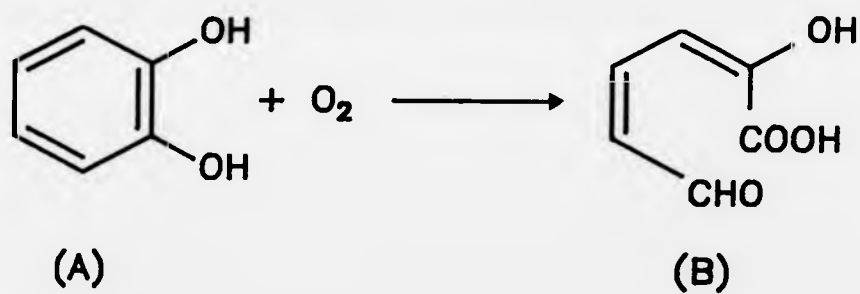
there had been extensive evolutionary divergence between the catechol and protocatechuate branches.

1.7.2 Extradiol cleavage.

The extradiol cleavage of catechols occurs between two carbon atoms, only one of which is hydroxylated. The other carbon atom is either unsubstituted or substituted with a group other than a hydroxyl group. The hydroxyl groups of ring cleavage may be either ortho or para to each other. The first evidence for the existence of a pathway other than the β -ketoadipate pathway for catechol degradation was reported by Dagley and Patel, (1957), who isolated a compound formed from the oxidative cleavage of protocatechuate by a p-cresol grown Pseudomonas species. This product was subsequently identified as 2,4-lutidinic acid, which was formed by the chemical reaction of the ring cleavage product with ammonium ions (Trippet et al., 1960). This suggested that the cleavage had occurred between C-4 and C-5 of protocatechuate to yield 2-hydroxy-4-carboxymuconic semialdehyde. The extradiol cleavage of catechol itself was shown by Dagley and Stopher (1959) using cell extracts of a cresol-grown Pseudomonas sp. They were able to identify the ring fission product as 2-hydroxymuconic semialdehyde (Figure 1.34).

Catechol 2,3-oxygenase (EC 1.13.11.2) has been obtained in crystalline form from extracts of P. putida (arvilla) mt-2 (ATCC 23973) grown with benzoate as the major carbon source. The enzyme is extremely unstable in the presence of air (Kojima et al., 1961; Taniuchi et al., 1962) and contains 1g atom of iron/mole enzyme, based on a molecular weight of 140 kd. (Nozaki et al., 1968). The iron seems to be in the ferrous form and

Figure 1.34 **Reaction catalysed by**
catechol 2,3-oxygenase.



(A) Catechol

(B) 2-Hydroxymuconic semialdehyde

oxidation by air or H_2O_2 leads to inactivation of the enzyme. The enzyme appeared to consist of 3-4 subunits (Nozaki *et al.*, 1968). Recently, Nakai *et al.*, (1983) confirmed that catechol 2,3-oxygenase from P. putida (arvilla) mt-2 (ATCC 23973) consisted of four identical subunits, with each subunit containing 1g atom of iron essential for activity. A variation in specific activity was found to correlate with the iron content; the fully active preparations appearing to contain 4g atoms of iron/ mole of enzyme.

Unlike the intradiol cleavage enzyme, catechol 2,3-oxygenase has broad substrate specificity (Nozaki *et al.*, 1970). The relative maximum rates of substrate oxidation (in parentheses) are as follows: catechol (100), 4-methylcatechol (100), 3-methylcatechol (62) and protocatechuate (0.15).

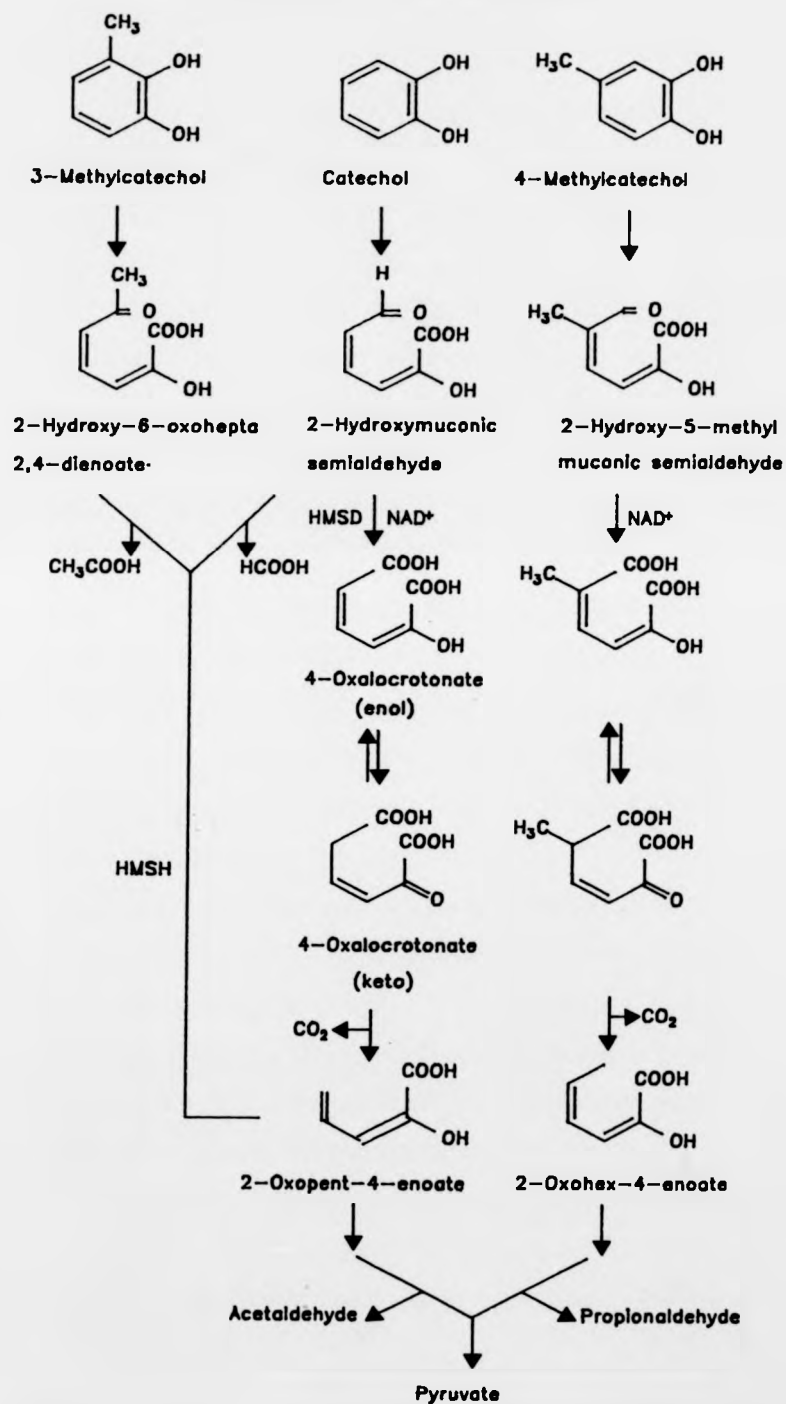
Once formed, 2-hydroxymuconic semialdehyde can be metabolized by either a hydrolytic or oxidative route. The physiologically important reaction in the degradation of 2-hydroxymuconic semialdehyde is an oxidation to 4-oxalocrotonate by an NAD^+ -dependent aldehyde dehydrogenase. This has been shown in an Azotobacter sp. (Sala-Trepat and Evans, 1971), a naphthalene-grown Pseudomonad (Catterall *et al.*, 1971) and in P. putida mt-2 (Sala-Trepatt *et al.*, 1971). However, the rate of 2-hydroxy-6-ketohepta-2,4-dienoate metabolism was NAD^+ -independent, suggesting that it was hydrolysed to 2-keto-pent-4-enoate as proposed earlier by Bayly *et al.* (1966) and Bayly and Dagley (1969).

Clear resolution of the two pathways came from further work by Bayley and Wigmore (1973) using hydrolase-defective mutants of P. putida PsU. These studies showed that the catabolic route

was governed by the growth substrate. Thus, initial hydroxylation catalysed by the non-specific phenol hydroxylase yielded a catechol, variously substituted, depending on the growth substrate. Moreover, catechol was formed from phenol, 3-methylcatechol was formed from either *o*- or *m*-cresol and 4-methylcatechol was the product when *p*-cresol was the growth substrate. Catechol can be metabolized by either the hydrolytic or the dehydrogenation route. 3-Methylcatechol can only be metabolized by the hydrolytic pathway and 4-methylcatechol can only be degraded via the dehydrogenative pathway (Figure 1.35). Harayama *et al.* (1987) demonstrated the same situation in *P. putida* mt-2 and showed that 3-methylcatechol was oxidised via the hydrolytic route because the presence of a methyl group at C-6 after cleavage precludes its oxidation by 2-hydroxymuconic semialdehyde dehydrogenase. However, catechol and 4-methylcatechol, once cleaved, can be oxidised by either the hydrolytic or dehydrogenative pathway. Murray *et al.* (1972) had shown that benzoate or *p*-toluate grown *P. putida* mt-2 had both the hydrolytic and dehydrogenase activities, but that the activity of the latter exceeded that of 2-hydroxymuconic semialdehyde hydrolase. This showed that both catechol (the product of benzoate) and 4-methylcatechol (the product of *p*-toluate) were metabolized preferentially via the oxalocrotonate route. Only when the levels of catechols are extremely high can the hydrolytic pathway become operative. This was explained by Harayama *et al.* (1987) who showed that 2-hydroxymuconic semialdehyde dehydrogenase had a much lower K_m (2 μM) compared with that of 2-hydroxymuconic semialdehyde hydrolase (>50 μM).

Figure 1-35. Meta-fission pathways for degrading catechol.

3-methylcatechol and 4-methylcatechol.



1.7.3 Regulation of the intra- and extradiol cleavage routes.

As discussed previously, catechol is a central metabolite in the degradation of many aromatic compounds. In some organisms, catechol is metabolized by the intradiol pathway, in others by the extradiol pathway, while some bacteria have the genetic capacity to degrade catechol by either pathway. The ability of an organism to use both pathways was first shown by Davies and Evans (1964) who showed that a single Pseudomonas strain possessed the ability to form the enzymes of both the ortho and meta- cleavage pathways when grown under different conditions. When grown with naphthalene or salicylate, the organism converted the growth substrate to catechol which was dissimilated via the meta pathway. Growth on benzoate led to the dissimilation via the ortho pathway, once catechol had been formed. This was later verified by Williams et al. (1975) who reported that growth of Pseudomonas P_G on naphthalene resulted in the formation of meta pathway enzymes whereas growth on benzoate, salicylate or catechol resulted in the induction of ortho pathway enzymes.

Early investigations into the chemical basis for the selective use of cleavage mechanisms (Seidman et al., 1969) suggested that aromatic growth substrates bearing electron-withdrawing groups, such as protocatechuate or cinnamate were metabolized by the intradiol cleavage route whereas aromatics substituted with an electron-donating alkyl groups led to the induction of the extradiol cleavage enzymes. The authors suggested that the intralactonic rearrangements were hindered when an electron-donating group was present. This is

because intradiol cleavage necessitates either nucleophilic attack (in lactonization) or deprotonation (in isomerization), chemical reactions not required in the extradiol pathway. The inductive mechanism used seems to select the enzymes of the metabolic pathway requiring the least activation.

The physiological basis for the choice of pathway was first elucidated by Feist and Hegeman (1969a, 1969b). Using P. putida strain U, they found that catechol could be utilised by either the intra- or extradiol pathways, the growth substrate being the factor determining which pathway is used. In P. putida U, catechol formed from phenol is metabolized by meta pathway enzymes, but if benzoate is the precursor of catechol, the intradiol cleavage pathway is used. Using phenol hydroxylase- and 2-keto- 4-hydroxy valerate aldolase-defective mutants, Feist and Hegeman concluded that separate pathways were used depending on the prevailing induction conditions. Phenol induced all enzymes of the meta pathway such that all catechol formed was immediately consumed by the high levels of catechol 2,3-oxygenase induced. However, benzoate only induced the two enzymes required for catechol formation, and low levels of constitutively-produced catechol 1,2-oxygenase yielded cis,cis muconate. The cis,cis muconate so formed then induced the synthesis of high levels of catechol 1,2-oxygenase, so that the induction of the complete pathway was essentially a two-step process. Catechol itself does not induce the meta cleavage enzymes and therefore, only the ortho pathway was induced during accumulation of this ring fission substrate.

Murray and Williams (1974) and Bayly and McKenzie (1976) clarified the findings of Feist and Hegeman (1969). Both groups

reported that catechol could induce ortho and meta pathway enzymes in P. putida U (NCIB 10015). The results of Bayly and M^CKenzie (1976) are especially relevant to the present study because they showed that mutation of either cleavage pathway leaves an alternative pathway which will function and permit growth whether the growth substrate was phenol or benzoate. In the present study, the accumulation of catechol from benzene and 3-methylcatechol from toluene, was sought. If the conclusions of Bayly and M^CKenzie were correct, accumulation of catechol from benzene may not be achieved simply by mutating one cleavage pathway. Both intra- and extradiol routes would need to be mutated to prevent catechol inducing the alternative pathway. Overproduction of 3-methylcatechol from toluene differs from catechol because the former possesses a methyl group. This usually precludes its oxidation by catechol 1,2-oxygenase because of a very low affinity for methylcatechols or the production of 'dead-end' intermediates (Dagley, 1971). Despite this, previous reports suggest that it is possible for catechol 1,2-oxygenase to catalyse the cleavage of substituted catechols (Fujiwara et al., 1975; Hou et al., 1977).

It is evident therefore, that where an organism produces an intermediate which is common to the metabolism of two growth substrates, the pathway used will be dependent primarily on the substrate used for growth. However, this can be changed either by mutation of genes encoding specific enzymes or by loss of a plasmid that encodes at least some of the enzymes of one of the pathways.

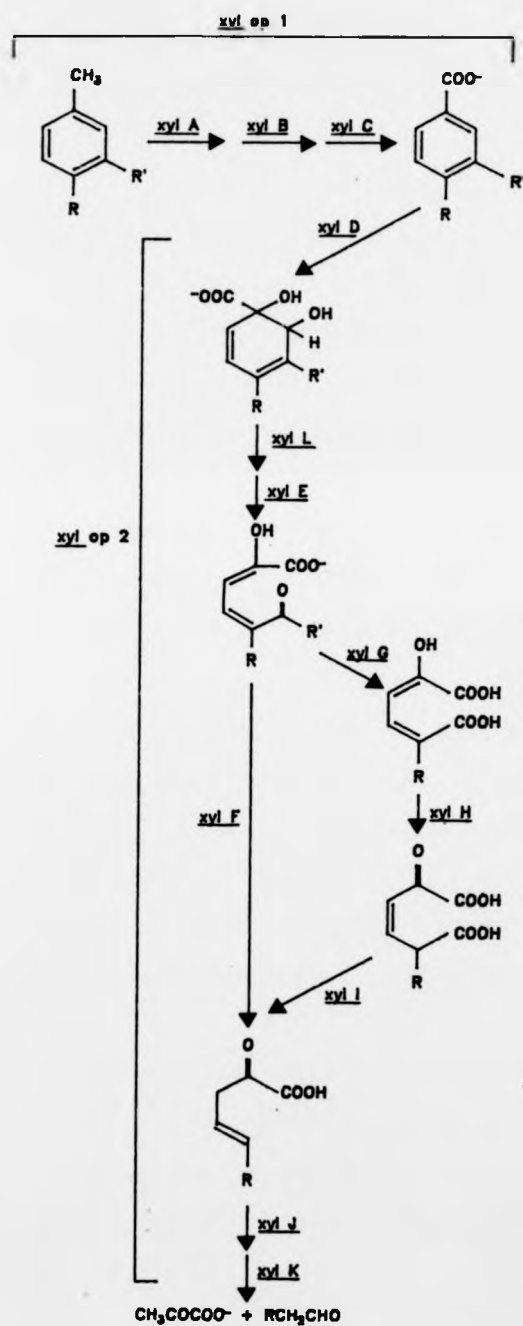
1.8 THE ROLE OF PLASMIDS IN AROMATIC BIODEGRADATION.

Many bacterial genes are not part of the main chromosome but are on separate covalently closed circular pieces of DNA called plasmids. These are not essential for normal cell function and can be stably inherited without being linked to the chromosome. Plasmids encoding the degradation of natural and synthetic organic compounds have been characterised in many bacterial genera other than pseudomonads, including Beijerinckia (Kiyohara et al., 1983), Agrobacterium (Montoya et al., 1977; Schardl and Kado, 1983), and Flavobacterium (Negoro et al., 1980).

Many types of catabolic plasmids have been shown to occur in Pseudomonas species, including those coding for the degradation of camphor (CAM), octane (OCT), salicylate (SAL), naphthalene (NAH) and xylene/toluene (TOL). Only the latter will be discussed here. The TOL plasmid represents a group of plasmids which encode enzymes involved in the degradation of hydrocarbons such as m and p-xylene, 1,2,4-trimethylbenzene, 3-ethyltoluene, toluene and their corresponding alcohol and acid derivatives.

The plasmid encoded toluene degradative pathway was first demonstrated by Nakazawa and Yakota (1973). Using P. putida mt-2, they showed loss of the meta pathway enzymes when mutant derivatives were grown on benzoate. Williams and Murray (1974) subsequently showed that the gene cluster of the meta pathway enzymes was resident on a plasmid. The TOL plasmid consists of two operons which encode the enzymes for the so-called upper and lower pathway as outlined in Figure 1.36. The first three steps (xyl op 1) effect oxidations of the methyl group to a carboxylic acid group, constituting the upper pathway. The lower pathway (encoded by xyl op 2) effects the cleavage of the aromatic

Figure 1-36- The xylene/toluene degradative pathway encoded by the TOL plasmid.



nucleus via the meta cleavage route. TOL plasmids ranging in size from about 40-300 kb have been identified from different strains of Pseudomonas (Duggleby et al., 1977; Williams and Worsey, 1976). The genetic organization of the TOL plasmid has been deciphered both by restriction endonuclease mapping and by transposon mutagenesis and by gene cloning (Downing and Broda, 1979; Franklin et al., 1981).

1.9 FUNCTION AND COMPOSITION OF OXYGENASES.

Oxidations involving direct combinations of oxygen with carbon are not commonly encountered in biological systems. It is much more usual for substrates to be oxidised by removal of hydrogen atoms which are then transferred to NAD or NADP. Oxygen exists in a triplet ground state: it has two unpaired electrons and the molecule is paramagnetic. Since organic compounds are in a singlet state, concerted reactions with oxygen are spin forbidden; and in order to react, oxygen must be activated by absorbing radiation to attain a singlet state or by combining with an enzyme. The transition elements have one or more unpaired electrons in d-orbitals, thereby permitting direct reactions with oxygen because they are not spin forbidden. Activation of the aromatic nucleus therefore involves an enzymic step with the enzyme itself having a transition element as an integral component.

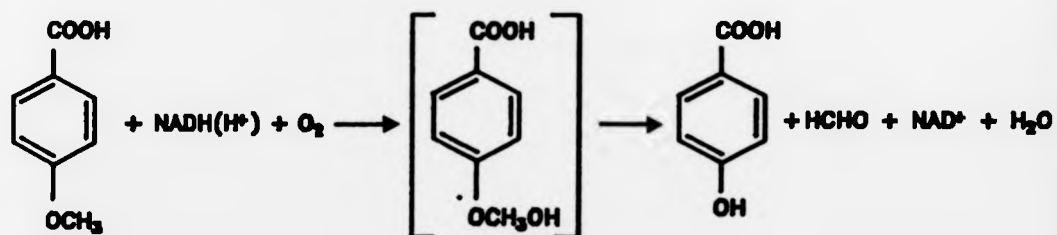
In aromatic biodegradation, attack on an aromatic nucleus may be catalysed by one of three types of enzyme, namely an oxidative demethylase, a monooxygenase or a dioxygenase. Although brief mention will be given to the first two types of

enzyme only the dioxygenases will be considered in detail.

1.9.1 Oxidative Demethylases.

Work carried out by Bernhardt et al., (1971) using a strain of P. putida grown on 4-methoxybenzoate succeeded in the isolation of an O- demethylase system. The enzyme has subsequently been characterised extensively and found to catalyse a number of different reactions. The enzyme itself consists of an NADH-dependent reductase (NADH-putidamonooxin oxidoreductase) and an iron containing acid-labile-sulphur containing monooxygenase (putidamonooxin). In the presence of the NADH-putidamonooxin oxidoreductase, NADH and O₂, putidamonooxin acts as a monooxygenase on its physiological substrate, 4-methoxybenzoate. It catalyses the O-demethylation of this substrate by oxidative attack at the methoxy group of the substrate as shown in Figure 1.37. The authors proposed that the hemiacetal product (shown in brackets) spontaneously decomposed to formaldehyde and 4-hydroxybenzoate. It has been proposed that the O-demethylase system may attack methyl groups directly attached to the aromatic nucleus. Bernhardt et al., (1971) provided supportive evidence for this when he showed that 4-methylbenzoate could be oxidised to 4-carboxybenzylalcohol. Besides the alkyl moiety of the substrate and of other benzoic acid derivatives, the aromatic ring is also hydroxylated by this fairly non-specific enzyme if 4-hydroxy or 3-hydroxybenzoate is used as a substrate analogue (Bernhardt et al., 1973). More recently, it has been shown that putidamonooxin can be directed to a dioxygenase reaction using substrate-induced modulation (Wende et al., 1982). Using 4-vinyl benzoic acid, the

Figure 1-37 Monoxygenase reaction catalyzed by
4-Methoxybenzoate O-demethylase



putidamonooxin was able to catalyse the formation of 4-glycyl benzoate, a reaction outlined in Figure 1.38 and showing the incorporation of both atoms of molecular oxygen to the side chain.

1.9.2 Flavoprotein Monooxygenases.

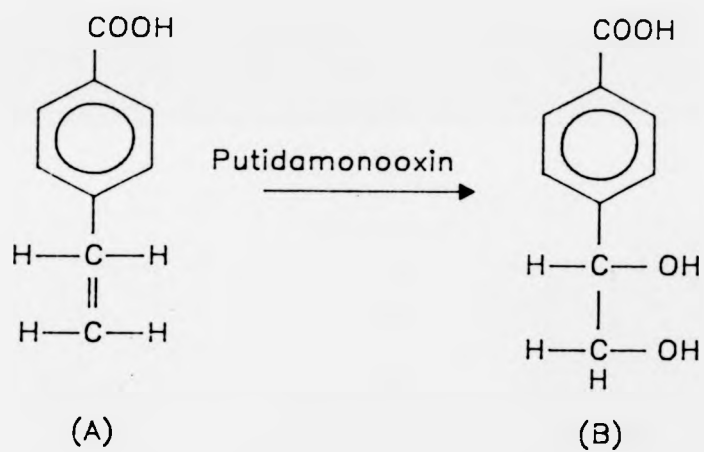
Flavoprotein monooxygenases are widely distributed in the pseudomonads, their function usually being to introduce a second hydroxyl group into a monohydroxy acid. An example of the type of reaction catalysed by this group of enzymes is shown in Figure 1.23. Enzymes such as 4-hydroxybenzoate monooxygenase have been studied in detail by Ballou (1982) who showed that the FAD is first reduced by either NADH or NADPH. Subsequently, NAD^+ leaves the active site before oxygen is bound to form a hydroperoxide with reduced FAD. Using phenol monooxygenase, Ballou proposed the mechanism outlined in Figure 1.40. When a basic amino acid, in the active site of the enzyme, removes a proton from the hydroxyl group of phenol, electrons become available at the adjacent carbon atom of the aromatic ring for attack on the distal oxygen atom of the hydroperoxide. The bond between oxygen atoms is broken, a pseudobase (HO^+FADH) is formed and water is released to give FAD.

1.9.3 Aromatic Dioxygenases.

1.9.3.1 Benzene Dioxygenase.

Gibson *et al.*, (1968) isolated a strain of *P. putida* that would grow with ethylbenzene as the sole source of carbon and energy. This organism would also grow with toluene and toluene-grown cells rapidly oxidised benzene and catechol. Cell

Figure 1.38 Reaction catalysed by putidamonooxin
after substrate-induced modulation.



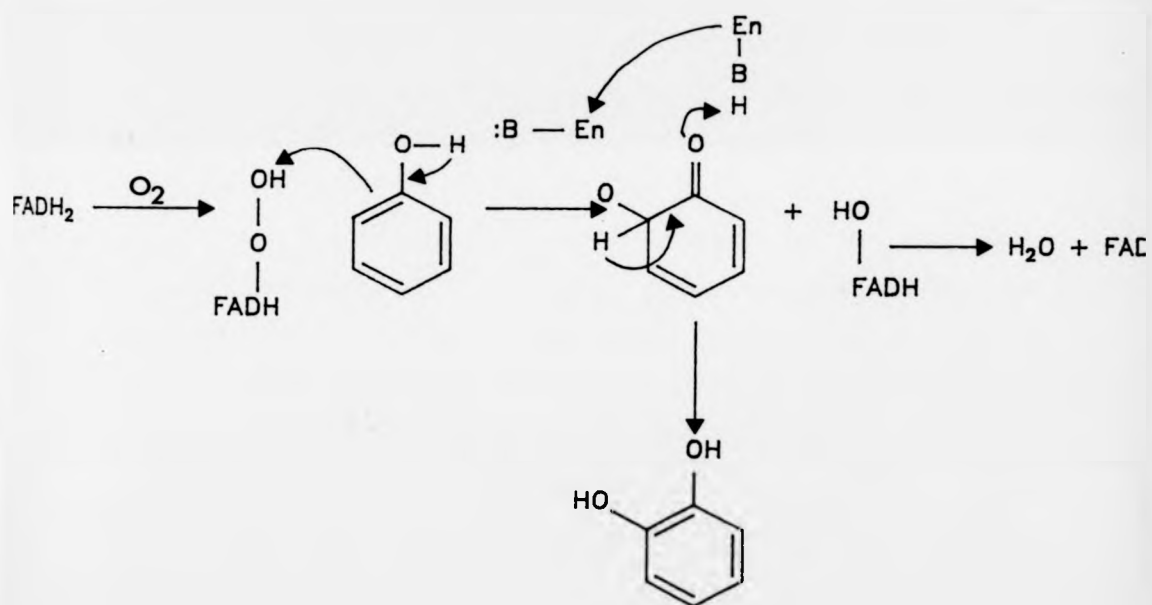
(A) 4-Vinyl benzoate

(B) 4-Glycyl benzoate

No Fig
1-39

No Fig
1-39

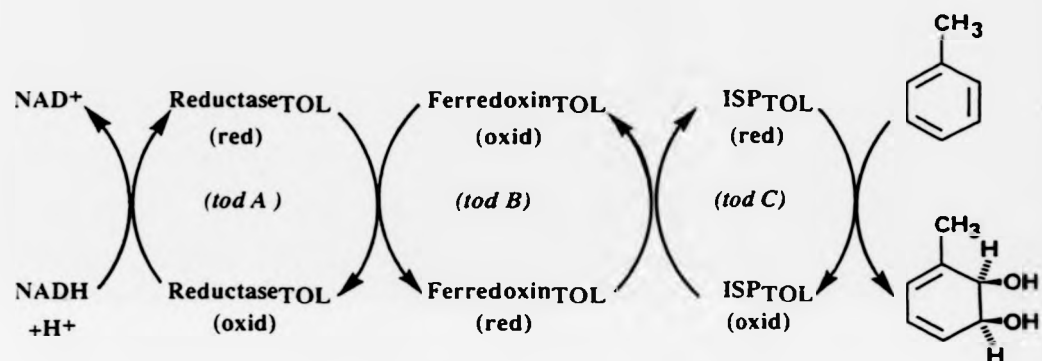
Figure 1-40. Proposed reaction mechanism for
phenol monooxygenase.



extracts of *P. putida*, after growth on toluene, catalysed the oxidation of benzene to benzene cis-glycol (BCG). This activity was dependent on molecular oxygen, reduced NAD and ferrous iron. In 1975, Axcell and Geary isolated a *P. putida* strain able to grow on benzene as sole carbon and energy source. The benzene dioxygenase system was isolated and resolved into three components, all of which were required for enzymatic activity. The components, termed A₁, A₂ and B, were further purified and analysed. A₂ was found to be unstable and was not characterised in detail. A₁ and B were iron-sulphur proteins. A₁ was identified as the terminal oxygenase component, having a molecular weight of 215.3 kd. and containing two (2Fe . 2S^{*}) clusters. This molecular weight was later challenged by Zamanian and Mason (1987) who showed that the subunit composition of A₁ was $\alpha_2 \beta_2$ with the α component having a Mr = 54,500 whilst the β component had a Mr = 23,500. The theoretical molecular weight (156 kd.) was in close agreement with that measured for native A₁, 168 kd., but differed significantly from that shown by Axcell and Geary (1975). Zamanian and Mason suggested that the difference in molecular weight was attributable to the presence of dithiothreitol added during purification, possibly reducing some thiol cross linkages causing protein unfolding.

Component B had a Mr = 12,300 and contained one (2Fe . 2S^{*}) cluster, indicating that it was a plant-type ferredoxin and served to transfer electrons from A₂ to the terminal oxygenase component A₁. By analogy with monooxygenase systems, protein A₂ is proposed to function as the NADH-protein B oxidoreductase.

Figure 1.41 Subunit composition of toluene dioxygenase.



Further work by Geary and Dickson (1981) showed that the terminal oxygenase component, when in the oxidised state, had two ferric iron sites suggesting that either the two iron-sulphur centres within each molecule are inequivalent, or the two iron atoms within each centre are inequivalent. The latter postulate was suggested to be more likely on the basis of the spectrum of the reduced protein which showed a single ferric component. Geary and Dickson also proposed a role for the exogenous iron in the benzene dioxygenase system. It was known that iron was needed for correct enzymic function and the authors proposed that the inequivalence of the two ferric iron atoms within the oxidised centre could cause distortion. Subsequently, exogenous ferrous iron atoms would be able to come sufficiently close to effect oxygenase activity by transferring electrons to bound oxygen.

1.9.3.2 Toluene Dioxygenase.

The reaction carried out by toluene dioxygenase is analogous to that shown previously for benzene. Indeed, Axcell and Geary (1975) and Zamanian and Mason (1987) reported benzene dioxygenase was able to catalyse the conversion of both toluene and benzene to their respective glycols. The composition of the protein is shown in Figure 1.41. It contains a flavoprotein and two iron-sulphur proteins. The flavoprotein - ferredoxin reductase has been purified to homogeneity (Subramanian *et al.*, 1981) and shown to be similar to those shown previously for camphor (Tsai *et al.*, 1971) and alkane mono-oxygenase systems. Its function is to transfer electrons to component 2, ferredoxin_{TOL}. The latter protein, characterised as a plant

type ferredoxin has a molecular weight of 15 kd. (Liu et al., 1978), contains one ($2\text{Fe}.2\text{S}^*$) cluster and is similar to protein B previously described for benzene dioxygenase. The function of ferredoxin_{TOL} is to transfer electrons to the terminal dioxygenase component, ISP_{TOL}. This was first purified by Subramanian et al., (1979), has a molecular weight of 151 kd. and contains dissimilar subunits with molecular weights of 52.5 kd. and 20.8 kd., giving an $\alpha_2\beta_2$ configuration. The ISP_{TOL} component contains 4g atoms iron and acid-labile-sulphur which seem to be present in two ($2\text{Fe}.2\text{S}^*$) clusters. These physical characteristics demonstrate its similarity to the A₁ component in benzene dioxygenase and immuno cross-reactivity experiments have confirmed that a high degree of homology exists (Zamanian and Mason, 1987). ISP_{TOL} is able to accept two electrons and, as with the A₁ component of benzene dioxygenase, it is catalytically inactive unless exogenous iron is supplied.

The enzyme mechanism for toluene dioxygenase (or benzene dioxygenase) has not been elucidated. Observations suggest that ferredoxin_{TOL} reductase and ferredoxin_{TOL} serve as electron transport proteins. Each iron-sulphur cluster in the ISP_{TOL} component then accepts one electron, reducing the enzyme and facilitating the binding of toluene. Once bound, electrons are then transferred to iron at the active site.

It has not been established that toluene binding is the factor which determines electron transfer from the iron-sulphur centre. Bernhardt and Meisch (1980) have suggested a possible method of oxygen activation for the benzoate-O-demethylase monooxygenase system. The terminal oxygenase, putidamonooxin, is

composed of a trimer of subunits, each containing a $(2\text{Fe}.2\text{S}^*)$ centre and requiring exogenous iron for activity. Bernhardt and Meisch (1980) suggested that dioxygen activation was achieved by the uptake of one electron from the reduced iron-sulphur centre and one electron from the exogenous iron.

1.10 INFLUENCE OF SIDE CHAINS AND MODIFICATION OF SUBSTITUENTS BEFORE RING CLEAVAGE.

The principal reactions involved in the transformation of benzene ring substituents include β -oxidation, oxidative demethylation, epoxidation of carbon-carbon double bonds and hydroxylation. These metabolic steps are representative of the few mechanisms used to deal with a diverse array of chemicals. The bacterial species involved and the size and type of substituent governs whether the side chain remains intact, or is transformed or eliminated before ring cleavage.

1.10.1 Methyl, Methoxy and Carboxy Substituents.

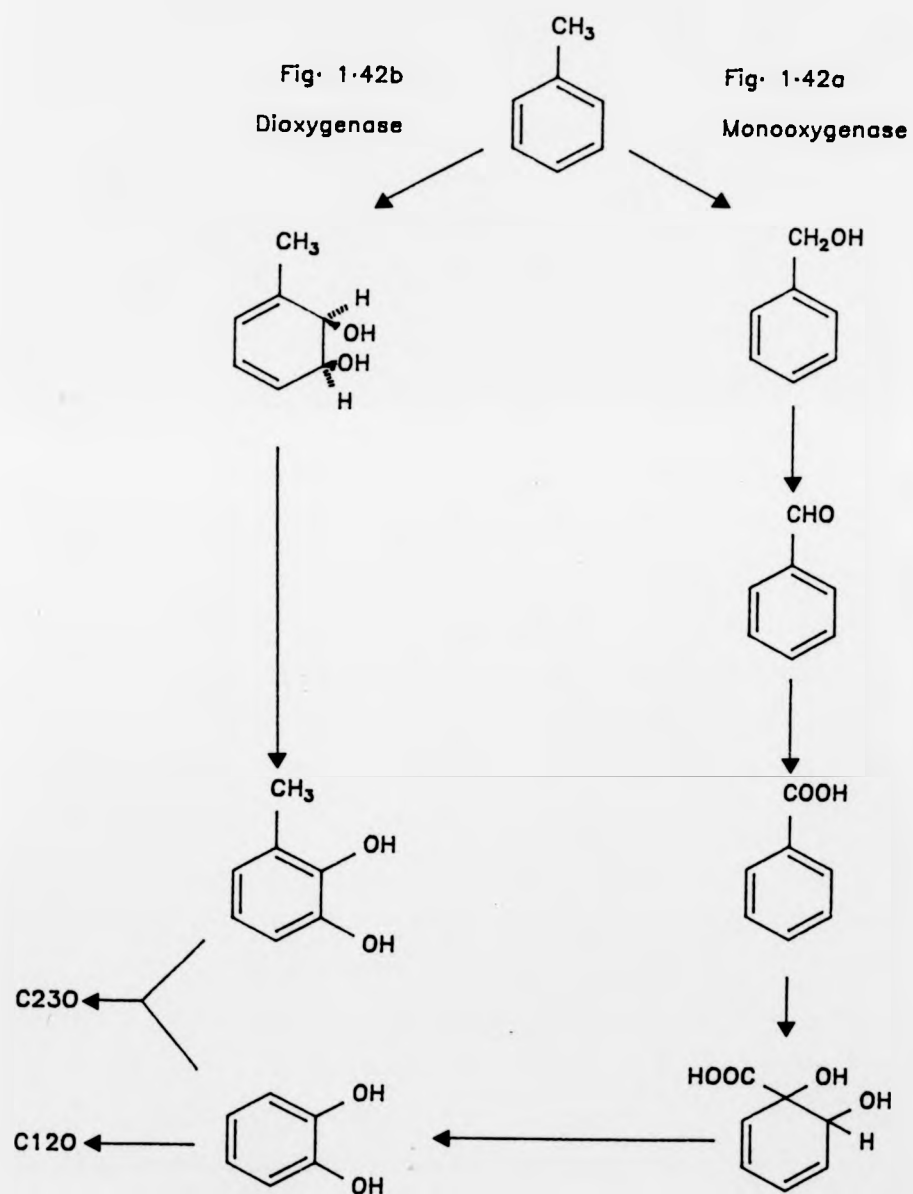
Methyl groups may either be oxidised or remain intact during hydroxylation of the aromatic nucleus. Kitagawa (1956) showed that a strain of P. aeruginosa was able to oxidise toluene, benzyl alcohol, benzaldehyde and benzoate at approximately equal rates (Figure 1.42a). More recently, Worsey and Williams (1975) showed that P. putida (arvilla) mt-2 used a similar route for the catabolism of toluene but that the monooxygenase was encoded on a TOL plasmid. Toluene may also be degraded via dihydroxylation of the aromatic nucleus, leaving the methyl moiety intact. Claus and Walker (1964) first concluded that this may occur using a Pseudomonas sp. and an Achromobacter sp. from

soil (Figure 1.42b). Subsequently, the pathway has been verified and the intermediate, toluene cis-glycol, has been identified. Similarly, larger alkyl side chains may either remain intact during the formation of the corresponding catechol or undergo oxidation prior to catechol production. In the latter case, carboxylic acids are then formed by oxidation of the terminal methyl group. Provided that extensive branching does not exist, the larger carboxyalkyl substituents can undergo β -oxidation, eventually yielding phenylacetic or benzoic acid. Direct oxidation of the aromatic nucleus of alkyl benzenes yield a substituted cis-glycol. However, it has been shown that longer chain alkyl benzenes may prevent attack on the aromatic nucleus. Isopropyl benzene and n-butyl benzene have been shown to be oxidised by toluene-grown cells but the relative rates were much slower (Gibson et al., 1968). Work using microbial cells isolated on aromatic substrates possessing longer side chains has not significantly affected the premise that longer alkyl side chains are removed prior to ring cleavage. DeFrank and Ribbons (1977) have shown that p-cymene grown P. putida strain PL form p-cumate prior to the formation of the dihydrodiol and 2,3-dihydroxy p-cumate, thus the isopropyl group remains intact during cleavage. Similarly, Catelani et al. (1977) showed that the quaternary carbon in tert-butyl benzene remained intact during ring cleavage by Achromobacter A₂.

A carboxyl substituent of an aromatic acid may remain unaltered before ring cleavage, forming protocatechuate or it may be eliminated as in the TOL plasmid-encoded degradation of xylene or toluene.

Methoxy-substituted aromatics are generally dealkylated to

Figure 1-42. Possible pathways for the degradation of toluene.



give the parent phenol, with the concomitant liberation of the alkyl moiety as an aldehyde. Thus, vanillic acid is converted to protocatechuate by the loss of its methoxy group in the form of formaldehyde. Such oxidative demethylation has previously been discussed (Section 1.9.1).

1.10.2 Halogen substituents.

The elimination of halogen substituents from aromatics is of special interest because their presence usually adds to the recalcitrance of hydrocarbons. Many of the haloaromatics in the environment arise by chemical synthesis and, as such, must be viewed as xenobiotic compounds. These xenobiotics can be metabolized fortuitously with incomplete degradation or they can be totally degraded. This may occur via a sequence of co-metabolic transformations through participation of more than one organism or by the complete catabolic machinery of a single organism and thus be utilised as sole carbon and energy source.

Many haloaromatics are degraded largely by fortuitous metabolism. The enzymes which normally serve a physiological role can often act on xenobiotic compounds provided structural analogy allows substrate binding and comparable reactivity of functional groups. It is well established that enzymes are not absolutely specific with respect to substrate binding so that substrates with xenobiotic structural elements may be bound. This facilitates the degradation of xenobiotics and allows the manufacture of high value-added halo-organic compounds using a biocatalyst. An example of the broad substrate specificity which may be manipulated in this way is provided by the methane monooxygenases of Methylococcus capsulatus. The data presented

in Table 1.7 illustrate that halogenated alkanes can be converted but the relative velocities of oxidation of substituted methane decreases sharply with the nature of the substituent.

The enrichment procedure used largely governs whether a particular isolate is able to co-metabolise a halo-aromatic compound. Thus, toluate-oxidising bacteria exhibit higher co-metabolic activities on chloro-substituted analogues than ordinary benzoate degraders. This is illustrated in Table 1.8 showing that Alcaligenes eutrophus, induced on benzoate, has a low turnover rate of substituted benzoates. Kinetic analyses have shown that reaction rates are predominantly decreased by the steric effects of the substituents (Reineke and Knackmuss, 1978). In contrast, dioxygenation of substituted benzoic acids by toluate-grown cells of P. putida mt-2 is mostly undisturbed by the steric effects of chlorine as a substituent. Elimination of the halogen substituent is essential for the complete mineralization of the substrate and can be accomplished in a number of ways. The mechanism of direct dechlorination by microbial monooxygenases is suggested to follow the NIH-shift. This has been studied with aromatic substrates labelled in specific positions with deuterium. A frequent consequence of hydroxylation is an intramolecular migration or shift of the group displaced by hydroxyl to an adjacent position on the aromatic ring (Daly et al., 1972). Initial dehalogenation of halo-aromatic compounds have rarely been found and these cases are suggested to follow the pathway outlined in Figure 1.43. This pathway has been suggested to operate in Pseudomonas sp. B13 during adaptation to 2-fluorobenzoate as sole carbon source

Table 1.7 - Relative activities of oxidation of methane and substituted methane by whole cells of *Methylococcus capsulatus*
(based on data from Stirling and Dalton, 1979, 1980)

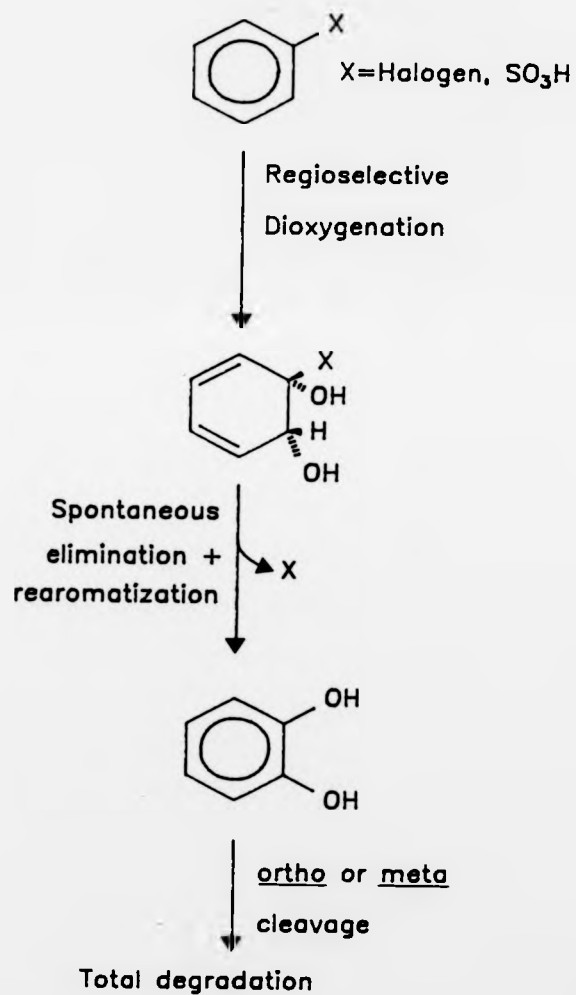
SUBSTRATE	RELATIVE ACTIVITIES
CH ₄	100
CH ₃ -Cl	49
CH ₃ -Br	25
CH ₃ -OCH ₃	2

Table 1.8 - Relative rates of dioxygenation of substituted benzoic acids by whole cells of *Alcaligenes eutrophus* B9, *Pseudomonas* sp. B13 and *Pseudomonas putida* mt-2 (data from Reineke and Knackmuss, 1978).

Substrate	Relative rates of dioxygenation (V _{max})		
	<u><i>Alcaligenes eutrophus</i> B9</u> (benzoate induced)	<u><i>Pseudomonas</i> species B13</u> (3-chlorobenzoate-grown)	<u><i>Pseudomonas putida</i> mt-2</u> (3-methylbenzoate grown)
Benzoic acid	100 (30)	100 (160)	100 (86)
3-Methyl	25	13	78
3-Chloro	15	31	48
3-Bromo	8	9	34
4-Methyl	1	1	63
4-Chloro	1	1	32
4-Bromo	Not tested	1	22
3,5-Dimethyl-	1	1	27
3,5-Dichloro-	1	1	10

NR Reaction rates are expressed as a percentage of that for benzoate taken as 100%. Absolute specific activity (μmol/min/g protein) are given in parentheses for the relative rates reported as 100%.

Figure 1.43. Initial elimination of xenobiotic structural elements through regioselective dioxygenation.



(Engesser et al., 1980). The adapted strain is able to utilize 2-fluorobenzoate by using a benzoate 1,2-dioxygenase which has been drastically changed in its regioselectivity in favour of a 1,2 attack on the aromatic ring. Thus, oxygen is introduced by double hydroxylation in such a way that 97% of the organic fluorine is labilized and spontaneously eliminated. Alternatively, the haloaromatic may be oxidised directly and degraded via the corresponding halocatechol. These chlorosubstituted catechols are critical metabolites because ordinary catechol 1,2-oxygenases are inefficient catalysts for ring cleavage, especially of 3-halocatechols. In addition, catechol 2,3-oxygenases are irreversibly inactivated by 3-halocatechols. Compared to ordinary, rather specific catechol 1,2-oxygenases, ring cleavage enzymes with broad substrate specificities are required. Correspondingly, the ring-fission enzyme from an Arthrobacter sp. that can utilize 2,4-D as sole carbon source exhibits high activities with methyl- and chloro-catechols (Tiedje et al., 1969). The same is true of the subsequent cycloisomerization of halo-muconic acids, where the 2-substituted isomers require special specificities and catalytic activities. Dehalogenation and regeneration of the diene chromophore are spontaneous secondary reactions of cycloisomerization.

1.11 OXYGENASE BASED BIOTRANSFORMATIONS.

This project aims to study the production of catechols from low cost aromatic substrates such as toluene and benzene. The pathways of aromatic degradation and their key enzymes have been

discussed previously. Consideration will be given here to the problems encountered when using oxygenase based biotransformations, more specifically, their possible use in the production of catechols.

Oxygenase based biotransformations have attracted attention because they permit the synthesis of either high value-added products, difficult to achieve using chemosynthesis, or allow the production of an entirely novel product. Ideally, a microbial based system would be operated as either a continuous whole-cell system or an enzyme based system utilising only one or two enzymes. The latter system would seem the most attractive process for the reasons outlined in section 1.1. Many oxygenases are capable, by virtue of their relaxed substrate specificity, to catalyse the fortuitous oxidation of a wide range of substrates. Methane monooxygenase from Methylococcus capsulatus (Bath) is able to catalyse the oxidation of a wide range of hydrocarbons besides its native substrate, methane (Dalton and Stirling, 1982). Unfortunately, many oxygenases are multi-component enzymes, each component of which must be purified to homogeneity and subsequently recombined to attain maximal activity. Both toluene and benzene dioxygenases have three components, namely, two oxidoreductases and a terminal hydroxylase. Additionally, the production of catechols requires a second enzyme, the cis-glycol dehydrogenase. Both BCG dehydrogenase (Axcell and Geary, 1973) and TCG dehydrogenase (Rogers and Gibson, 1977) have been purified. Therefore, to produce catechol or 3-methylcatechol from benzene or toluene using a cell-free system would require extensive purification. Added to this, the initial dioxygenase is known to be very

unstable and would require adequate cofactor regeneration.

All of the above precludes the use of an enzyme based system at the present time. Currently, the use of whole cells offers the greatest opportunity to exploit oxygenase based biotransformations. The production of BCG affords the best example of what can be achieved. Using whole cells of P. putida, which lack BCG dehydrogenase, ICI Bioproducts have developed a process for the stoichiometric conversion of benzene to benzene cis-glycol (Taylor, 1982). This process is relatively simple and involves only one enzyme, benzene dioxygenase. Catechols are formed by the action of two enzymes and may be ring fission substrates for two separate cleavage pathways, either the intra- or extradiol pathway. Thus, accumulation of catechol from benzene necessitates two mutagenic steps to inactivate both catechol 1,2- and 2,3-oxygenases.

Once a strain able to accumulate the desired product has been constructed, the product yield must be maximised in a bioreactor. Inactivation of either/both cleavage enzymes results in the cell being unable to grow on the aromatic substrate and compels a co-metabolic route to be used. Therefore, a growth substrate is fed along with the biotransformation substrate. Ideally, a continuous process for the production of catechols would be employed but this was felt to be impractical. Both toluene, benzene and the catechols derived from them are toxic. In order to overproduce catechols continuously the levels of both substrate and product would have to be monitored continuously such that they could be maintained at sub-inhibitory levels. Additionally, catechols are very susceptible to auto-oxidation and would have to be removed in

situ. Other factors which may limit the application of a continuous system are the genetic instability of the mutant and factors associated with catabolite repression. Unless a constitutive mutant is constructed, as in the case of the ICI project, the growth substrate must be fed at concentrations sufficient to overcome catabolite repression but supply enough reducing equivalents for continued product formation. Thus, any growth will be minimal and the culture will rapidly be displaced by fresh medium if run continuously.

Many of the problems mentioned are removed or controlled if the system is operated as a fed-batch fermentation. In this type of fermentation overproduction of metabolic intermediates is achieved in two stages. In the first stage, the growth phase, the carbon source is supplied in excess to achieve maximum biomass. Subsequently, the production phase is initiated whereby a growth-limiting carbon source is fed along with the co-oxidation substrate. This permits induction of the toluene/benzene degrading pathway and allows regeneration of reducing equivalents in situ. As in the continuous process the limiting factor is predominantly product toxicity.

Catechols and their oxidised products are well characterised toxic species. Perhaps the most apposite research into the toxicity of catechol is that done into their effect upon lipooxygenases. Lipooxygenases (EC 1.13.11.12) catalyse dioxygenation of polyunsaturated fatty acids possessing a cis,cis-1,4-pentadiene unit to yield cis,trans conjugated diene hydroperoxides. Catechol functionality is clearly implicated in the inhibition of lipooxygenases and much of the available information has come from work done using soybean lipooxygenase

1. The following have been suggested to account for the inhibition of this enzyme by catechols :-

(i) Inhibition via reduction of enzyme-bound radical intermediates generated from the fatty acid during turnover (Tappel et al., 1953; Papatheofanis and Lands, 1985; Takahama, 1985)

(ii) Competitive reversible inhibition (Yasumoto et al., 1970)

(iii) Irreversible inhibition resulting from co-ordination of the catechol to the ferric cofactor (Galpin et al., 1976)

(iv) Inhibition of the enzyme by reducing the catalytically active ferric enzyme to the catalytically inactive ferrous form (Kemal et al., 1987)

Additionally, Kemal et al. (1987) suggested that irreversible inactivation of the enzyme may occur due to air oxidation of the catechols generating H_2O_2 , a potent irreversible inactivator of soybean lipoxygenase 1 (Mitsuda et al., 1967).

Although toluene/benzene dioxygenase is very different from lipoxygenase the modes of action of catechol clearly have some relevance to multi-component enzymes which rely on the oxidation and reduction of iron. Moreover, the production of H_2O_2 on air oxidation will be vastly increased when seeking to overproduce catechols.

Catechols are easily oxidised to their corresponding semi-benzoquinones and benzoquinones. It is well documented that these species may have a multiplicity of effects on cellular

processes and components. For a detailed review of such effects the reader is directed to Leyden Webb (1966).

AIMS OF THE PROJECT.

This project aimed to analyse toxic product removal during microbial biotransformations. The model system chosen was the investigation of the whole-cell production of catechols using mutant strains of Pseudomonas putida. Catechols were chosen for study since they are central intermediates in aromatic metabolism, are toxic to bacterial cells at relatively low concentrations and are used in the production of high value-added compounds. Areas to be investigated in the project were as follows:-

- (i) Biochemical characterisation of the organisms used.
- (ii) Genetic manipulation of the organisms to facilitate production of catechols.
- (iii) Development of a fermentation system to optimise product formation.
- (iv) Investigation of factors which reduce the longevity of the biocatalyst.
- (v) Implementation of product removal systems to enhance product formation.

CHAPTER 2. MATERIALS & METHODS.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 Organisms.

The organisms used throughout this study were Pseudomonas putida strains 2331, 2313, 4(2), 6(12), 4(1) and R3. Strains 2313 and 2331 were a gift from Dr. S. Hagedorn, Celanese Research Co. (Summit, NJ, USA). P. putida 2331 was able to utilise toluene and benzene as sole carbon and energy source and possessed both catechol 1,2-oxygenase and catechol 2,3-oxygenase. P. putida 2313 was unable to utilise toluene as sole carbon and energy source because it lacked catechol 2,3-oxygenase. However, P. putida 2313 still possessed catechol 1,2-oxygenase enabling it to grow on benzene. P. putida 4(2) and 6(12) were putative mutants of P. putida 2313 which lacked catechol 1,2-oxygenase. P. putida 4(1) was a mutant of P. putida 2313 which lacked toluene/benzene dioxygenase. P. putida R3 was a spontaneous revertant of P. putida 2313, able to utilise toluene as sole source of carbon and energy.

2.2 Media.

A basic mineral salts medium (MS medium) was used throughout these studies for the routine growth of the organisms. Cells were grown at pH 6.8 and 30°C on a medium containing (g/l): KH_2PO_4 , 2; NH_4Cl , 3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4; trace-element solution, 2 ml/l. The trace-element solution was as described by Vishniac and Santer (1957) but containing only 0.22% (w/v)

ZnSO₄·7H₂O. Solid media was prepared by the addition of 20 g/l Difco Bacto-agar to the medium before autoclaving.

Cultures were maintained on Luria broth (LB) buffered at pH 6.8 and containing (g/l):

NaCl, 10; yeast extract, 5; Bacto-tryptone, 10; glucose, 1.

Solid medium was prepared by the addition of 20 g/l Difco Bacto-agar to the medium before autoclaving.

2.3 Measurement of cell density and growth rates.

Cell density was measured routinely as the optical density at 540 nm. The total carbon content of cell suspensions were determined using a Beckmann Model 915-B total carbon analyser (Beckman Instruments Inc., Fullerton, California, USA). Samples were washed twice in KH₂PO₄ (0.01% w/v) before resuspending in distilled water. The cell carbon content was assumed to be 47% of the dry weight (Van Dijken and Harder, 1975) and thus the dry weight of the cell suspensions could be estimated. By dilution of chemostat-grown cells, a curve of optical density at 540 nm. against dry weight was prepared for P. putida 2313. This gave an average dry weight value of an A₅₄₀ of 0.5 of 142 mg/l.

2.4 Cultivation of P. putida strains.

The organisms were maintained on Luria agar and stored at 4°C. Cultures were sub-cultured every 4 weeks. When required, cultures were plated onto MS medium containing 0.1% (w/v) glucose and incubated in the presence of an aromatic substrate

at 30°C. The MS + glucose plates were placed in an airtight "Tupperware" container which contained 1-5 ml of the appropriate liquid aromatic in a universal taped to the side.

Batch and fed-batch cultures of *P. putida* were grown on MS medium in either a L.H. 500 series II fermentor (L.H. Engineering, Stoke Poges, Bucks.) with a working volume of 0.9 l. or an M.B.R. MCS 10 Bioreactor (Sultzer UK Ltd., Farnborough, Hants.) with a working volume of 10 l. Cultures were stirred at 500 - 700 r.p.m. The temperature was maintained at 30°C \pm 0.2°C and the pH at 6.8 \pm 0.1 by the automatic addition of 1 molar KOH and HCl. Foaming was prevented by the automatic addition of 10% (w/v) polypropylene glycol. Air flow was set at 500 ml/min in the smaller bioreactor and 1000 ml/min in the larger bioreactor. Additionally, either toluene or benzene was supplied in the vapour phase with a second air stream diverted through a reservoir containing the aromatic substrate. Thus, in the case of the fed-batch studies conducted in the large bioreactor, toluene or benzene was fed at 200 ml/min. prior to, and 400 ml/min. after induction of the aromatic-utilising pathway.

In all large volume studies the fermentor was operated in a fed-batch mode. The inoculum was grown in Luria broth until it reached mid-logarithmic phase and 100 ml. of the culture was inoculated into 5 l. MS medium containing 5 g/l glucose. After overnight growth (13 hours) the dissolved O₂ (dO₂) was monitored to elucidate the time of glucose exhaustion. When the latter was observed a glucose feed was initiated (1-1.4 g/l/hr) and the aromatic feed was started.

2.5 Tests of growth specificity.

The ability of various strains and mutants to utilise a wide variety of substrates was tested by their ability to grow on the substrates in either liquid or solid MS medium. In liquid medium, cultures were inoculated into 50 ml. MS medium contained in a suba-sealed 250 ml. Erlenmeyer flask. These were reciprocally shaken at 200 r.p.m and incubated at 30°C. All aromatic substrates were added to give a final concentration of 5 mM, although this was dependent upon the solubility of the substrate. Cultures were incubated for up to a week and any that grew were viewed under a microscope to assess contamination. The ability to grow on solid medium was assessed by streaking the isolate onto MS agar plates which were subsequently placed in a "Tupperware" container into which a universal containing 1-5 ml. of aromatic substrate was placed. Non-volatile, solid carbon sources were added directly to the medium at concentrations between 0.05-0.2% (w/v). All plates were incubated at 30°C.

2.6 Preparation of cell suspensions and cell extracts.

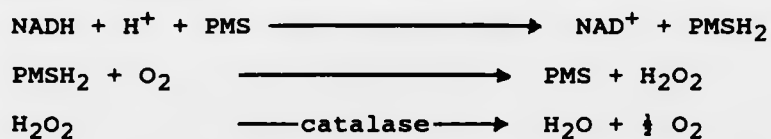
Cells grown in the fermentor were harvested at 4°C by centrifugation for 10 minutes at 20,000g. After washing the cells twice with 20 mM potassium phosphate buffer, pH 6.8, the cells were resuspended in the same buffer. When necessary, cells were drop frozen in liquid nitrogen and stored at -20°C prior to use.

Cell-free extracts were prepared using cell suspensions prepared as above, except that, for the toluene and benzene dioxygenase assay, cells were resuspended in a stabilization

buffer of 20 mM potassium phosphate (pH 7.2), containing 10% ethanol, 10% glycerol, 10 mM DTT and 5 mM NADH. The cell suspension was disrupted by two passages at 1.37×10^8 PA through a chilled French pressure cell (Aminco, Maryland, USA). Debris and unbroken cells were removed by centrifugation at 100,000g for 30 min. This gave a red/brown supernatant, which was decanted off, and a dark brown pellet, which was discarded. The soluble extract was then immediately used for assays or frozen in pellet form by drop-wise addition to liquid nitrogen. The pellets were stored at -20°C .

2.7 Respiration studies.

The ability of the organisms to oxidise various substrates was investigated by measuring the stimulation of oxygen uptake on addition of the substrate to cell suspensions. Oxygen consumption was measured using a Clarke-type polarographic oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) polarised to -0.6V . The temperature was controlled by circulating heated water through a water jacket. The dissolved oxygen concentration in air-saturated buffer was determined by the method of Robinson and Cooper (1970). The fall in oxygen is measured on addition of NADH to a reaction mixture containing phenazine methosulphate (PMS) and catalase due to the following reaction sequence:-



The measured decrease in oxygen concentration was therefore proportional to the quantity of NADH added. The electrode was calibrated to zero O_2 content by the addition of a few crystals of sodium dithionite to the reaction vessel containing 3 ml. of the appropriate buffer.

Assays were routinely carried out at $30^\circ C$ in a stirred reaction mixture containing 2.80 ml. pre-equilibrated 20 mM potassium phosphate buffer, pH 6.8, 0.1 ml. cell suspension and 0.1 ml. substrate solution. In the case of substrates readily soluble in water, 0.1 ml. of 150 mM solution was added to give a final concentration of 5 mM in the reaction mixture. Saturated solutions were used where it was impossible to obtain concentrations of 150 mM. Rates of oxygen uptake were corrected for the endogenous respiration rate.

2.8 Enzyme assays.

All enzyme assays were routinely performed at $30^\circ C$ using a Pye Unicam double beam SP8-200 spectrophotometer with integral chart recorder. Cuvettes of 1cm. light path were used throughout.

2.8.1 Toluene dioxygenase.

Two different assay systems were used in the study of this enzyme.

(a) Polarographic assay.

Activity of cell extracts was determined polarographically by measuring toluene-dependent oxygen uptake by a modification

of the procedure of Beechey and Ribbons (1972). The reaction mixture (3 ml.) contained extract; 0.3 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 μmol NADH; 100 μl toluene saturated buffer, in 50 mM potassium phosphate buffer (pH 7.2). The extract was incubated with FeSO_4 for 5 min. in the dark prior to addition to the reaction vessel. The order of addition was treated enzyme, NADH and then toluene. The rates of oxygen consumption were corrected for the endogenous rate in the absence of toluene.

(b) Spectrophotometric indole assay.

The method of Jenkins and Dalton (1985) was used. The assay is based on the ability of several bacteria, which oxidise aromatic hydrocarbons to cis-dihydrodiols, to oxidise indole to cis-indole dihydrodiol. The latter then undergoes spontaneous elimination of water to form indoxyl, a yellow dye which can be monitored spectrophotometrically at 400 nm. Reaction mixtures (3 ml.) in 25 mM potassium phosphate buffer, pH 7.2, contained 0.3 μmol $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 μmol NADH; cell extract; 17 μmol indole. Absorbance at 400 nm. was measured against a blank, placed in the spectrophotometer reference beam, containing all ingredients except indole.

2.8.2 Toluene cis-glycol (TCG) dehydrogenase.

The enzyme was assayed by a modification of Reiner (1972). Assays were routinely carried out at pH 8.0 and 30°C. Reaction mixtures in a total volume of 3 ml. 20 mM Tris-HCl buffer (pH 8.0) contained 1.5 μmol TCG; 1.5 μmol NAD^+ and cell extract. NADH formation was followed at 340 nm ($\epsilon = 6.2 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$). The blank contained all ingredients except the substrate (TCG).

2.8.3 Catechol 1,2-oxygenase (EC 1.13.11.1).

The assay for this enzyme was based on the method of Hegeman (1966). Reaction mixtures (3 ml.) contained 4 μmol EDTA, 0.3 μmol catechol and extract, in 67 mM potassium phosphate buffer, pH 7.2. The formation of cis,cis-muconate was followed at 260 nm. in quartz cuvettes ($\epsilon = 1.69 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$).

2.8.4 Catechol 2,3-oxygenase (EC 1.13.11.2).

The method of Feist and Hegeman (1969) was used. Reaction mixtures in a total volume of 3 ml. 33 mM Tris-HCl buffer (pH 7.6) contained 0.2 μmol catechol and extract. The rate of accumulation of 2-hydroxymuconic semialdehyde was followed at 375 nm ($\epsilon = 2.97 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$).

2.8.5 Protocatechuate 3,4-oxygenase (EC 1.13.11.3).

The method of Stanier and Ingraham (1954) was used. Reaction mixtures (3 ml.) in 66 mM potassium phosphate buffer (pH 7.0) contained 5 μmol protocatechuic acid and extract. The rate of accumulation of β -carboxymuconic acid was followed at 290 nm ($\epsilon = 1.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$).

2.9 Mutagenesis.

Mutagenesis of Pseudomonas putida strains 2331 and 2313 was performed using N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Both strains were grown in LB to mid-logarithmic growth phase and NTG

was added to a final concentration of 50 µg/ml. After incubation to obtain > 90% kill the cells were harvested by centrifugation, washed in MS medium and grown overnight in Luria broth. The culture was serially diluted and plated onto Luria agar. The resulting colonies were picked off and replica plated onto MS plates containing 0.2% glucose and incubated in the presence of either 0.05% (w/v) benzoate or toluene or benzene vapour. Additionally, the colonies were replica plated on MS medium without glucose in the presence of the aromatic carbon sources. Any mutants able to grow on glucose but not the aromatic substrates were subjected to further characterisation.

2.10 Screening methods used for mutant selection.

2.10.1 Catechol spray test.

The presence of the extradiol cleavage enzyme, catechol 2,3-oxygenase, was assessed by spraying colonies with a solution of 100 mM catechol. Thus, after the colonies were incubated with the aromatic carbon source, the colonies were sprayed and left for 5 mins. The presence of the enzyme was indicated by a yellowing of the colony and surrounding area, indicative of the extradiol cleavage product, 2-hydroxymuconic semialdehyde.

2.10.2 Production of indigo.

The presence of either toluene or benzene dioxygenase was assessed qualitatively by plating the colonies on MS media containing 0.2% (w/v) glucose and 1 mM indole. The plates were incubated in the presence of either toluene or benzene and the possession of a functional dioxygenase was indicated by the

fortuitous oxidation of indole leading to the formation of indigo as reported by Ensley et al. (1983).

2.10.3 Production of auto-oxidised catechol compounds.

The overproduction of catechol and 3-methyl catechol was initially determined by incubating the putative mutants on MS medium in the presence of 0.2% (w/v) glucose and toluene or benzene. Overproduction of catechol or 3-methylcatechol was indicated by allowing the products to auto-oxidise forming a dark area around the colony.

2.11 Analytical methods.

Catechol, 3-methyl catechol and the cis-glycols were determined by HPLC using a Lichrosorb RP18 column (4.5 mm x 250 mm) and a 254 nm UV - monitor (LKB 4, Bromma, Sweden). Samples were eluted isocratically using methanol/water (6:4) as the mobile phase. The flow-rate used throughout was 0.5 ml/min.

Catechol and benzene were also determined by gas chromatography using a 0.31 mm x 25 m capillary column. The column was supplied by Hewlett Packard and had a film thickness of 1 micron crosslinked with 5% phenylmethyl silicone.

Toluene was determined by gas chromatography on a 4 mm x 1.5 m column packed with 10% Dexsil 410 GC on Chromosorb W. The carrier gas was nitrogen (20 ml/min), the oven was maintained at 100°C and the flame ionisation detector was heated to 250°C.

The identity of 3-methyl catechol and other aromatic intermediates was confirmed by GC/MS using a WCOT fused silica column (0.33 mm x 24.5 m) with a film thickness of 0.12 µm. The

product was extracted into acetone, unless otherwise stated, and injected into the column. The carrier gas was helium (1ml/min), the oven was maintained at 120°C and the source was heated to 200°C. The mass spectra of the peaks were obtained using a Kratos MS25RFA mass spectrometer.

Protein was determined using Bio-Rad Protein dye reagent (Bio-Rad Labs., Watford, Herts., England) and bovine serum albumin as standard.

Glucose was determined enzymatically according to Sigma diagnostic procedure no. 510 (Sigma, Poole, Dorset, England).

Ammonia was determined according to the method of Chaney and Marbach (1962).

[NB. The aromatic products formed during the experiments often interfered with the assays for glucose and ammonia. Therefore, prior to assay, the samples were treated with Amberlite XAD-4 (1:10 w/v) to remove all traces of interfering aromatics. Amberlite XAD-4 was obtained from BDH Chemicals, Poole, Dorset, England.]

Inorganic phosphate was determined by the method of Murphy and Riley (1962).

Gluconate was determined according to the method of Boehringer Mannheim, Lewes, Sussex, England (Methods of Enzymatic Food Analysis).

The concentration of 2-ketogluconate was determined by a method based on that of Lanning and Cohen (1951). 0.5 ml of fresh o-phenylenediamine dihydrochloride was added to 1 ml of culture supernatant. This was heated in a boiling water bath for 30 mins. and the optical densities of the solution were measured at 330 nm and 360 nm. The ratio of E_{330}/E_{360} was determined

to establish it was approximately 1.51 ± 0.07 . Interfering sugars such as glucose alter the ratio to 2.5. A calibration curve for 2- ketogluconate in the range 0 - 50 μg was constructed and the E_{330} used to establish the concentration of 2-ketogluconate present in the samples.

Catechol and 3-methylcatechol were measured semi-quantitatively by the aminoantipyrine method of LaRue (1964). The test reagent contained 4- aminoantipyrine (1 mg/ml) and sodium carbonate (2% w/v) dissolved in 0.02 M sodium hydroxide. The solution was used as either a spray reagent for colonies or added to culture supernatant (1ml sample + 3 ml reagent) and measured spectrophotometrically at 515 nm. When used as a spray reagent the overproduction of catechol was indicated by the formation of a red halo around the colony.

2.12 Extraction of catechols from activated charcoal.

After completion of the recycle runs the activated charcoal was recovered from the system. The charcoal was washed with water to remove any loosely attached debris. The charcoal was placed in a continuous solvent extraction unit (EX10/95; Patterson Scientific, Harrow, Middlesex, England) fitted with a condenser and 1-L round bottomed flask. The adsorbed products were eluted with 500 ml ethyl acetate. Ethyl acetate in the flask was heated to boiling (70 - 80°C) and continuous extraction was maintained for 24 - 36 hours. Any further concentration was achieved by rotary evaporation at 35°C.

2.13 Viable Cell Counts.

Total viable counts were determined after suitable serial dilution of the sample in 5 mM MgCl_2 and plating out onto LA plates. Revertant cell counts were performed in the same way using MS plates as the plating medium followed by incubation in the presence of either toluene or benzene.

2.14 Small scale isolation of plasmid DNA.

In order to rapidly screen cells for the presence of extrachromosomal DNA, mini plasmid preparations were performed as described by Kado and Liu (1981). 3 ml of an overnight culture were pelleted by centrifugation in a microcentrifuge (MicroCentaur, MSE, Sussex, UK), and resuspended in 1 ml of E buffer [40 mM Tris-acetate and 2 mM sodium EDTA buffered to pH 7.9]. The cells were then lysed by adding 2 ml of lysing solution [3% (w/v) SDS and 50 mM Tris (pH 12.6)] which was mixed by repeated inversion. The solution was heated to 55°C for 20 min. in a water bath, and 2 volumes of phenol-chloroform solution (1:1 v/v) were added. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation. Avoiding the precipitate at the interface, the upper aqueous phase was removed and extracted with 2.5 volumes of cold ethanol (-20°C) and left at -70°C for 60 mins. The resulting DNA pellet was analysed on agarose gels.

2.15 Agarose gel electrophoresis.

'Mini-gels' were poured in mini-gel tanks (Cambridge

Biotechnology Laboratories, London, UK) and were routinely used for the rapid analysis of DNA samples. 5% (w/v) agarose was used with Tris-borate buffer, pH 8.3 containing (g/l): Tris, 10.8; boric acid, 5.5; disodium EDTA, 0.95. Before loading the gel, 0.25 volume tracking dye containing Bromophenol blue and glycerol (50% v/v) was added to each DNA sample. Electrophoresis was carried out at a constant 40 mA for about 45 min. After electrophoresis, gels were stained using ethidium bromide (about 10 µg/ml buffer, final concentration) and photographed using a UV transilluminator with a Polaroid camera.

2.16 Polyacrylamide gel-electrophoresis.

Non-denaturing gel electrophoresis was carried out on 5-15% gradient slab gels with a stacking gel concentration of 5%. The resolving gel buffer consisted of 3 M Tris/HCl pH 8.8, whilst the buffer for the stacking gel consisted of 0.5 M Tris/HCl pH 6.8. Protein samples were mixed with 0.5 ml of sample buffer before being loaded onto the gel. Sample buffer consisted of Tris/HCl pH 6.8 (0.972 mols); glycerol (0.1 mols); DTT (100 mmol) and 0.2% (w/v) bromophenol blue. Electrophoresis was carried out in Tris (1.5 M) buffer at 20 mA for 3-4 hours.

2.17 Activity stain for the detection of NAD(P)⁺ - dependent alcohol dehydrogenases.

Activity stains were carried out on non-denaturing gels. The gels were immersed in the activity stain which consisted of 100 ml Tris/HCl buffer (pH 8.0) containing PMS, 3.5 mg; Nitroblue

tetrazolium (NBT), 20 mg; and NAD^+ , 116 mg. After approximately 5 min. incubation in the dark at room temperature the gel was examined for non-specific binding of the NBT to protein bands. If no bands were visible, then the substrate (1 ml of a 10 mM or saturated solution) was added. Any active bands appeared after 5-10 mins.

2.18 Chemicals.

Most chemicals were obtained from the following manufacturers: Sigma (London) Chemical Co. Ltd., Poole, Dorset, UK; Fisons Scientific Apparatus, Loughborough, Leics, UK; Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK; BDH Chemicals Ltd., Poole, Dorset, UK.

3-Methylcatechol was obtained from Phase Separations Ltd., Queensferry, Clwyd, UK. Toluene cis-glycol and benzene cis-glycol were gifts from Dr S.C. Taylor, ICI, Billingham, UK. Granular activated charcoal (4-8 mm) was obtained from Fluka Chemicals Ltd., Glossop, Derbys, UK.

CHAPTER 3. STRAIN CHARACTERISATION
& MUTAGENESIS.

CHAPTER 3.STRAIN CHARACTERISATION & MUTAGENESIS.INTRODUCTION.

A diversity of aromatic compounds both exist and persist in the environment. Consequently, it is relatively easy to isolate micro-organisms able to catabolise simple benzenoid compounds. The catabolic pathways outlined in the introduction illustrated the idea of the catabolic funnel, whereby a myriad of both mono- and polynuclear aromatics converge upon a few central intermediates prior to ring fission. The present study aimed to overproduce catechol and 3-methyl catechol, both ring cleavage substrates, from benzene and toluene respectively. To achieve overproduction of 3-methyl catechol required a mutation in only one of the cleavage routes, namely the extradiol dioxygenase, catechol 2,3-oxygenase. Production of catechol from benzene required both the intradiol and extradiol cleavage routes to be blocked at catechol 1,2-oxygenase and catechol 2,3-oxygenase respectively.

Following mutagenesis, the putative mutants selected for process development were further investigated to ascertain their temperature and pH optima. Further analyses were also undertaken to determine the trace elements which were essential for growth on toluene and to establish whether any or all of the mutants possessed a TOL like catabolic plasmid. The latter was determined because possession of a TOL plasmid may pose additional difficulties when operating a biotransformation process. Ideally, any plasmid present at such a low copy number

would need to be stably maintained throughout the process to prevent the appearance of plasmid free cells which possess an altered phenotype detrimental to the biotransformation.

The process to be developed in the present study was viewed as a model system to investigate the problems of aromatic biotransformations and product toxicity arising thereafter. It was thought unlikely to develop a process which was cost competitive with traditional chemosynthetic routes for unsubstituted catechols. However, the relaxed substrate specificity of the initial dioxygenase may permit the fortuitous oxidation of a range of substituted aromatic compounds, yielding catechols of commercial interest whether as desired products themselves or as synthons which can easily be modified using existing chemical technology. The use of toluene and benzene in this study allowed the above rationale to be investigated because organisms able to grow on these, minimally substituted, substrates should possess the dioxygenases with the widest substrate specificity.

RESULTS AND DISCUSSION.

3.1 Growth substrates of Pseudomonas putida strains 2313 and 2331.

Of the many isolates initially screened for their ability to grow on a variety of aromatic compounds three isolates were chosen for detailed study. P. putida 2331 was able to grow well on toluene and benzene as sole carbon and energy source. P. putida 2313, although unable to grow on toluene, was still able

to grow on benzene. P. putida R3, was a spontaneous revertant of P. putida 2313 able to grow on both toluene and benzene. Other growth substrates were tested and these are shown in Table 3.1.

The results presented in Table 3.1 verified that P. putida 2313 was unable to grow on either toluene cis-glycol or toluene. These results supported the contention that P. putida 2313 lacked the extradiol cleavage enzyme catechol 2,3-oxygenase but still possessed the intradiol cleavage enzyme, catechol 1,2-oxygenase, enabling it to grow on both benzene and benzene cis-glycol. Although the ability to grow on the ring fission substrates, catechol and 3-methylcatechol, was tested in flask culture they were difficult to interpret due to the pronounced auto-oxidation giving rise to discolouration of the medium.

It is interesting to note that P. putida 2313 is able to grow on benzyl alcohol, benzaldehyde and benzoate, typical intermediates of a toluene monooxygenase pathway. However, the inability to grow on toluene itself suggests the absence of the initial toluene monooxygenase or reflects the fact that ability to grow on these intermediates is unrelated to a specific aromatic degradation pathway.

The ability of P. putida 2331 and R3 to grow on toluene, benzene and benzoate indicated the strains possess both intradiol and extradiol cleavage enzymes. Previous reports (Ribbons, 1970; Nakazawa and Yokota, 1973; Murray and Williams, 1974) had suggested that both of the ring fission enzymes may operate concurrently. If this was shown to occur in the organisms under investigation it may hinder the screening and selection of mutants able to overproduce catechols. As a result, P. putida 2331 was grown on toluene, benzene and benzoate and

Table 3.1. Aromatic growth substrates of *P. putida* 2313, R3 and 2331.

SUBSTRATE	<i>P. putida</i> R3	<i>P. putida</i> 2331	<i>P. putida</i> 2313
Toluene	+	+	-
Benzene	+	+	+
Benzoate	+	+	+
Benzyl Alcohol	+	+	+
Benzaldehyde	+	+	+
TCG	+	+	-
BCG	+	+	+

Growth was measured after 48 hours incubation in MS medium containing the substrates at a final concentration of 5 mM. All cultures were incubated at 30°C and growth was measured spectrophotometrically at 540 nm.

the activities of the ring fission enzymes measured (Table 3.2). The results indicated that both enzymes existed in the same strain but only one cleavage route was induced on any particular substrate. The results also verified that in the wild - type organism the substituted catechol, which arises from toluene, was metabolised exclusively via the extradiol route whereas unsubstituted catechol, an intermediate of benzene and benzoate metabolism, was metabolised via the intradiol cleavage route. It must be remembered that these findings only reflect the situation prevalent in the wild-type organism. The absence of either cleavage route may permit the use of the alternative route in any mutant derivatives. Although evidence exists that 3-methylcatechol can be cleaved via the intradiol route (Hou *et al.*, 1977 and Fujiwara *et al.*, 1975) it was thought unlikely to hinder mutant construction. However, catechol may be cleaved by either cleavage route and this necessitated the mutagenesis to be directed towards the extradiol cleavage route before the intradiol cleavage route.

3.2 Construction of a mutant able to overproduce catechol.

Initial attempts to obtain a mutant able to overproduce catechol were directed towards P. putida 2331, a strain known to possess both the intra- and extradiol cleavage enzymes. For overproduction of catechol to occur both fission routes needed to be inactivated as catechol can be metabolised via either pathway. It was thought it may be possible to inactivate both enzymes in one round of mutagenesis.

P. putida 2331 was mutagenized with NTG as outlined in

Table 3.2. Intradiol and extradiol activities of *P. putida* 2331 grown on different aromatic substrates.

SUBSTRATE	Catechol 1,2-oxygenase activity	Catechol 2,3-oxygenase activity
Toluene	0	210
Benzene	614	0
Benzoate	639	0

NR. All activities expressed as nmol catechol/min/mg. protein.

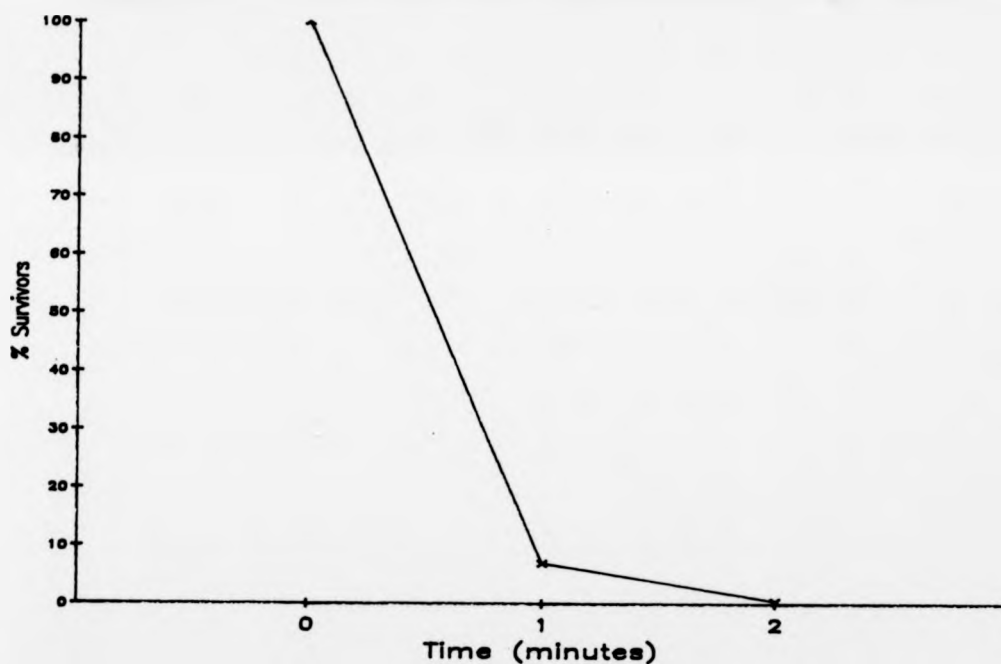
Activities were measured after growing the cells for 48 hours on MS medium containing the aromatic substrates at a final concentration of 5 mM. Cell extracts were prepared as described in Materials & Methods. Catechol 1,2- oxygenase and catechol 2,3-oxygenase were assayed using the methods outlined in Materials & Methods.

materials and methods (Chapter 2). Previously, attempts had aimed at 60% kill to minimise the possibility of lethal mutations arising in central metabolism. This approach had yielded very few mutants and, as a consequence, a kill in excess of 90% was sought. NTG was used at a final concentration of 50 $\mu\text{g/ml}$. and the killing curve for *P. putida* is shown in Figure 3.1. A kill in excess of 99% was achieved after 2 mins. and from this mutagenesis 1000 colonies were initially screened for their ability to grow on aromatic compounds of interest. The frequency of different mutants obtained is outlined in Table 3.3.

Analysis of the frequencies of different classes of mutants revealed several important facts. Firstly, the very low frequency of mutants unable to grow on benzoate probably reflects the number of degradative pathways which have benzoate as an intermediate e.g., toluene monooxygenase, mandelate and other compounds. Further analyses of the benzoate⁻ mutants revealed that all possessed a degree of leakiness enabling some growth on benzoate. Additionally, the inability to isolate a mutant unable to grow on benzoate and either toluene or benzene suggested that catechol, the presumed ring fission intermediate of benzoate, was able to be metabolised by either catechol 1,2- or catechol 2,3-oxygenase or, has as been shown previously (Bayly and McKenzie, 1976), the growth substrate may induce a separate catechol 1,2-oxygenase than that induced when growing on either toluene or benzene.

The mutants unable to grow on toluene and/or benzene were screened for the presence of catechol 2,3-oxygenase using the catechol spray test (see Materials and Methods). All mutants were grown on glucose in the presence of either toluene or

Figure 3.1 - Killing curve for *Pseudomonas putida* 2331.



Cells were grown overnight in Luria broth and 1 ml. was then sub-cultured into 50 ml. of the same medium. Optical density of the culture was followed until it reached mid-logarithmic growth phase. At this point, NTG was added to a final concentration of 50 ug/ml.

Figures were obtained after screening 1000 presumptive mutants for their ability to grow on the aromatic substrates outlined. Toluene and benzene were supplied in the vapour phase as outlined in Materials & Methods. Benzoate was present in MS agar plates at a final concentration of 5 mM.

Presumptive mutants were plated on to MS agar + 0.2 % (w/v) glucose in the presence of either toluene or benzene vapour. After 48 hours growth colonies were sprayed with a 0.1 M solution of catechol and left for 10 mins. at room temperature. Catechol 2,3-oxygenase activity was characterised by the formation of a yellow compound indicative of 2-hydroxymuconic semialdehyde.

Table 3.3. Frequency of mutants obtained after NTG mutagenesis of *P. putida* 2331.

PHENOTYPE	FREQUENCY (%)
Auxotrophic	1.6
Benzene ⁻	1.2
Benzoate ⁻	0.3
Toluene ⁻	1.0
Toluene ⁻ /Benzene ⁻	0.4
Benzene ⁻ /Benzoate ⁻	0
Toluene ⁻ /Benzoate ⁻	0

Table 3.4. Results of catechol spray test for the presence of catechol 2,3-oxygenase in presumptive mutants of *P. putida* 2331.

PHENOTYPE	NO. MUTANTS	C230 ⁺	C230 ⁻
Benzene ⁻	10	6	4
Toluene ⁻	8	2	6
Toluene ⁻ /Benzene ⁻			
Benzene ⁻	4	2	2

compounds akin to 2 - hydroxymuconic semialdehyde, the cleavage product formed by catechol 2,3-oxygenase.

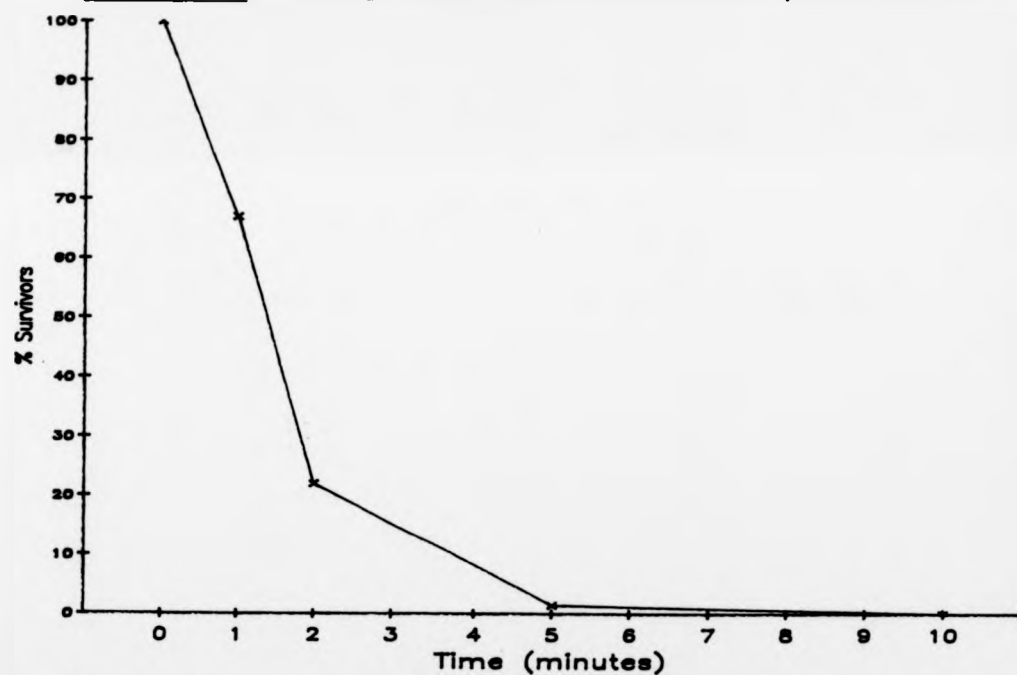
The inability to isolate a catechol producing mutant of P. putida 2331 emphasised the need to remove both catechol 1,2- and catechol 2,3-oxygenase. This would have necessitated further characterisation of suitable catechol 2,3-oxygenase mutants of P. putida 2331 before initiating another round of NTG mutagenesis. It was therefore felt more productive to use a mutant which already possessed a stable mutation in one of the catechol oxygenases.

P. putida 2313 was known to lack catechol 2,3-oxygenase and was able to overproduce 3-methylcatechol. Using this strain another killing curve was constructed and the results of this are shown in Figure 3.2. Using the data obtained, 89% kill was achieved after 4 mins. and 1000 colonies were screened for their ability to grow on benzene and benzoate. The frequencies of mutants obtained are shown in Table 3.5.

The results of the mutagenesis outlined in Table 3.5 show that the absence of one cleavage pathway, the extradiol cleavage route, significantly enhances the number of putative mutants unable to grow on a particular substrate. The mutants obtained were further characterised using the methods outlined in Materials and Methods. The results of the mutant screening are shown in Table 3.6. The aim of the screening programme was to obtain a mutant able to accumulate catechol from benzene and/or benzoate. The mutant sought should ideally fulfill the following requirements:-

- (i) The ability to produce indigo from indole when

Figure 3.2 - Killing curve for *Pseudomonas putida* 2313.



The experiment was performed as outlined previously for Figure 3.1.

Table 3.5. Frequency of mutants obtained after NTG mutagenesis of
P. putida 2313.

PHENOTYPE	FREQUENCY
	(%)
Auxotroph	3.3
Toluene ⁻	All toluene ⁻
Benzene ⁻	2.1
Benzoate ⁻	1.1

The frequency of presumptive mutants were obtained as outlined previously in Table 3.3.

Table 3.6. Frequency of *P. putida* 2313 mutants obtained after screening.

PHENOTYPE	FREQUENCY (%)
Indigo ⁺ /Catechol ⁺ /Ant. (B) ⁺ /Ant. (b) ⁺	0.2
Indigo ⁺ /Catechol ⁻ /Ant. (B) ⁺ /Ant. (b) ⁻	0.1
Indigo ⁻ /Catechol ⁺ /Ant. (B) ⁻ /Ant. (b) ⁺	0.8
Indigo ⁺ /Catechol ⁺ /Ant. (B) ⁻ /Ant. (b) ⁺	0.2
Indigo ⁺ /Catechol ⁻ /Ant. (B) ⁻ /Ant. (b) ⁺	0.1

Key:-

(1) Indigo - Ability to produce indigo from indole when incubated with benzene.

+ = Indigo

- = No indigo

* = No growth

(2) Catechol - Ability to grow on 2 mM catechol as sole carbon and energy source.

(3) Ant. - Overproduction of catechol from benzene(B) or benzoate(b) when the supernatant was assayed with amino-antipyrine reagent.

incubated in the presence of the aromatic substrate. This would indicate the mutant still possesses a functional dioxygenase.

(ii) The inability to grow on catechol indicating neither the intra- nor extradiol cleavage routes are intact.

(iii) The ability to produce catechol from either benzene or benzoate.

It must be noted that the use of the aminoantipyrine assay, whether as a spray on the colonies or as an indicator of overproduction using culture supernatants, only served as a qualitative measure of catechol production.

From the putative mutants obtained in this section several were used for further experiments. These were as follows:-

P. putida 2313 / 4(1) - a mutant lacking toluene/benzene dioxygenase.

P. putida 2313 / 4(2)

P. putida 2313 / 6(12) - presumptive mutants able to accumulate catechol from benzene (C12O⁻, C23O⁻)

Throughout the present mutagenesis protocol no mutant able to accumulate catechol from benzoate could be identified, further supporting the contention that the cleavage route for benzoate is under separate inductive control to those of either benzene and/or toluene.

3.3 Process considerations.

3.3.1 Medium Composition.

Prior to any large - scale cultivation of micro-organisms the pH, temperature and trace element requirements were investigated. Using a mineral salts medium P. putida R3, the wild - type revertant of P. putida 2313, was inoculated into a flask containing 5 mM toluene and different trace element mixtures. The results of the 48 hr. incubations are shown in Table 3.7.

The results of the growth experiments indicated that only iron was an obligate requirement for growth on toluene. This finding was further supported by the results of the control experiment which showed that the omission of iron in a glucose grown culture of P. putida R3 reduced, but did not prevent, growth occurring. The obligate requirement for iron when growing on toluene probably reflects the well documented fact that both toluene and benzene dioxygenase require exogenous iron for optimal catalytic activity (Axcell and Geary, 1975; Yeh et al., 1977).

3.3.2 Temperature optima.

The optimum growth temperature of all strains was investigated prior to their use in a bioreactor. This was investigated by measuring the specific growth rates of all mutants (P. putida 2313, R3, 4(1) and 6(12)) growing in a complex medium at temperatures between 25 and 45°C. Ideally, the growth rates on toluene should have been measured but this



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Table 3.7. The effect of different trace elements being omitted from the growth medium of *P. putida* R3 and *P. putida* 2313.

STRAIN	OMISSION	OD ₅₄₀
R3	Nothing	0.491
R3	ZnSO ₄ ·7H ₂ O	0.348
R3	CaCl ₂	0.440
R3	MnCl ₂ ·4H ₂ O	0.447
R3	FeSO ₄ ·7H ₂ O	0.034
R3	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.555
R3	CuSO ₄ ·5H ₂ O	0.461
R3	CoCl ₂ ·6H ₂ O	0.575
2313	Nothing	0.056
2313	Nothing + 0.1% (w/v) glucose	1.21

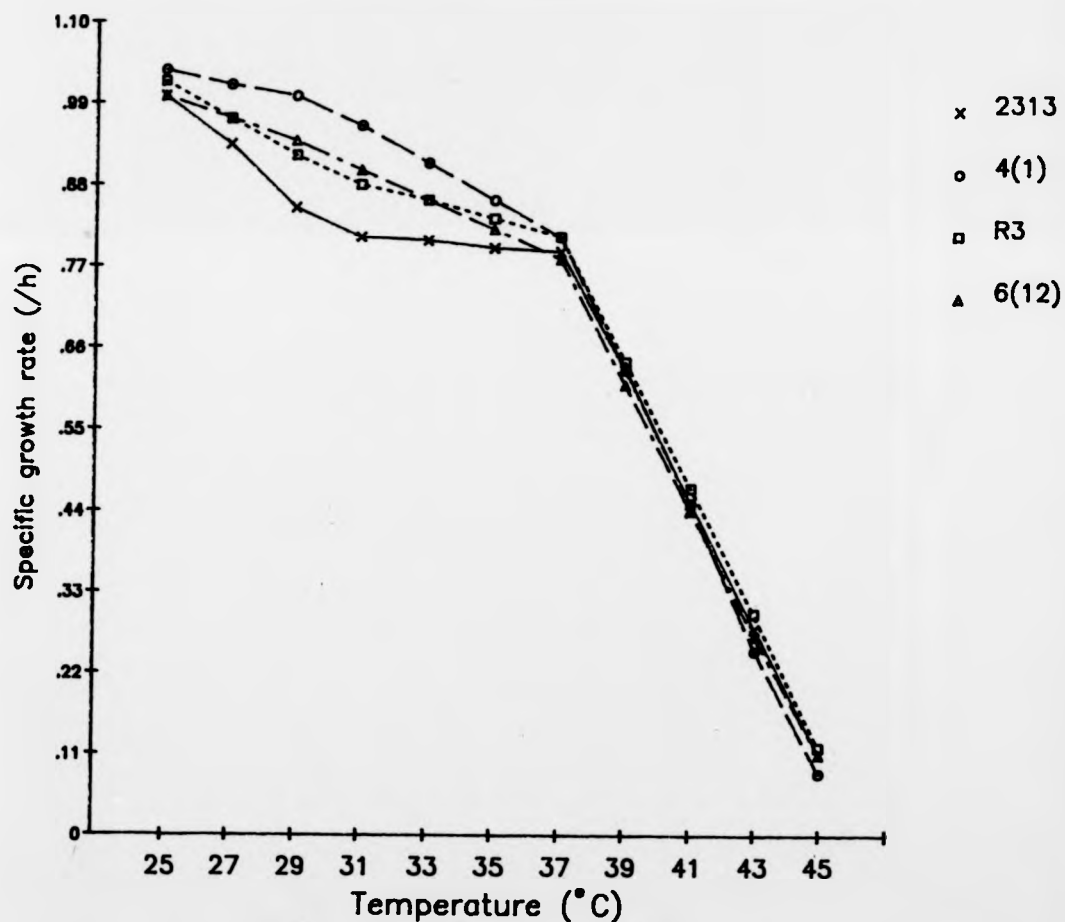
Cells were grown for 48 hours in MS medium containing toluene at a final concentration of 5 mM. Following this, 1 ml. was sub-cultured into modified MS medium lacking the trace elements outlined. After 48 hours the optical densities of the cultures were measured.

would have precluded measuring the effect of temperature on mutant derivatives. Therefore, all mutants were inoculated into Luria broth and the results are presented in Figure 3.3. The data shows the optimum growth temperature was in the range 25 - 30°C and this temperature range agrees closely with that shown in the literature (Bergeys Manual of Determinative Bacteriology). Additionally, recent work has shown that, using a resting cell system, the optimum temperature for catechol production was 30°C (Shirai,1987).

3.3.3 pH Optima.

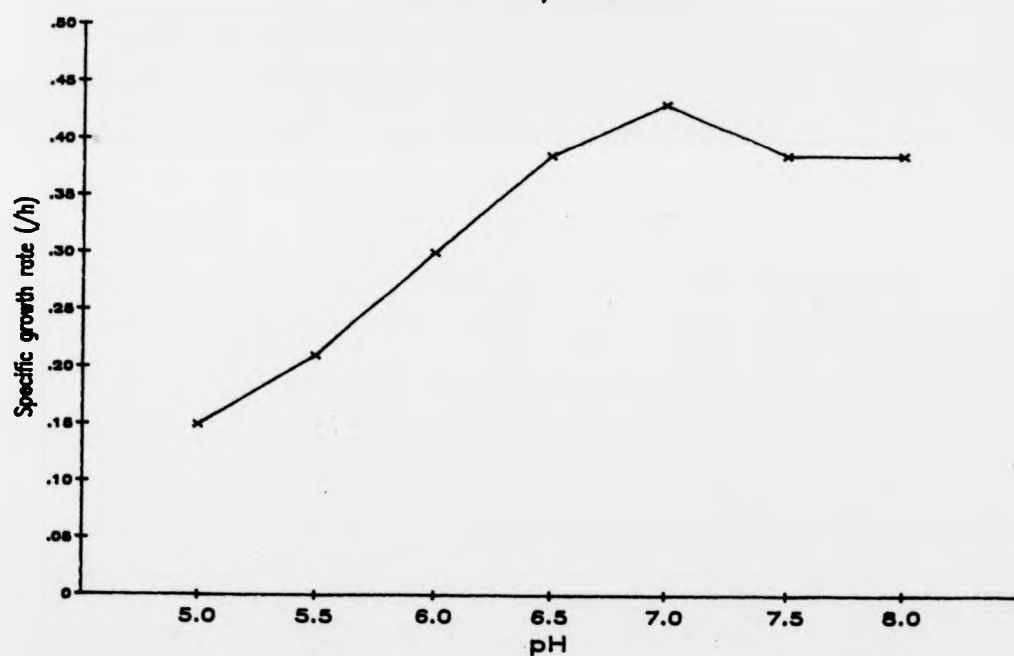
According to Bergeys Manual of Determinative Bacteriology, all species of pseudomonads can grow well at neutral or alkaline pH. This experiment aimed to show whether P. putida R3 grew well on toluene at pH values around neutrality. The experiments were done in shake flasks using 5 mM toluene as sole source of carbon and energy. The results presented in Figure 3.4 show that the optimum pH for growth on toluene was around neutrality although they were able to grow well in in the region 6.5 - 8.0. The production of catechols cannot be studied at alkaline pH values because of problems associated with auto-oxidation, resulting in the polymerisation of the product. Although the auto-oxidation cannot be eliminated at pH values around neutrality the pH chosen for all further studies was 6.8.

Figure 3.3 - The effect of temperature on the specific growth rates of *P. putida* strains 2313, 4(1), R3 and 6(12).



Cells were grown in Luria broth and the growth was monitored throughout at 540 nm. Growth curves were constructed (not shown) and specific growth rates were calculated.

Figure 3.4 - The effect of pH on the specific growth rate of *P. putida* R3.



P. putida R3 was grown in 50 ml. MS + 5 mM toluene in 250 ml. suba-sealed flasks. 1 ml. of mid-logarithmic phase culture was inoculated into the same medium which had been adjusted to the appropriate pH by the addition of 1M KOH and 1M HCl.

3.4 Elucidation of the Presence of large TOL - like Catabolic Plasmids.

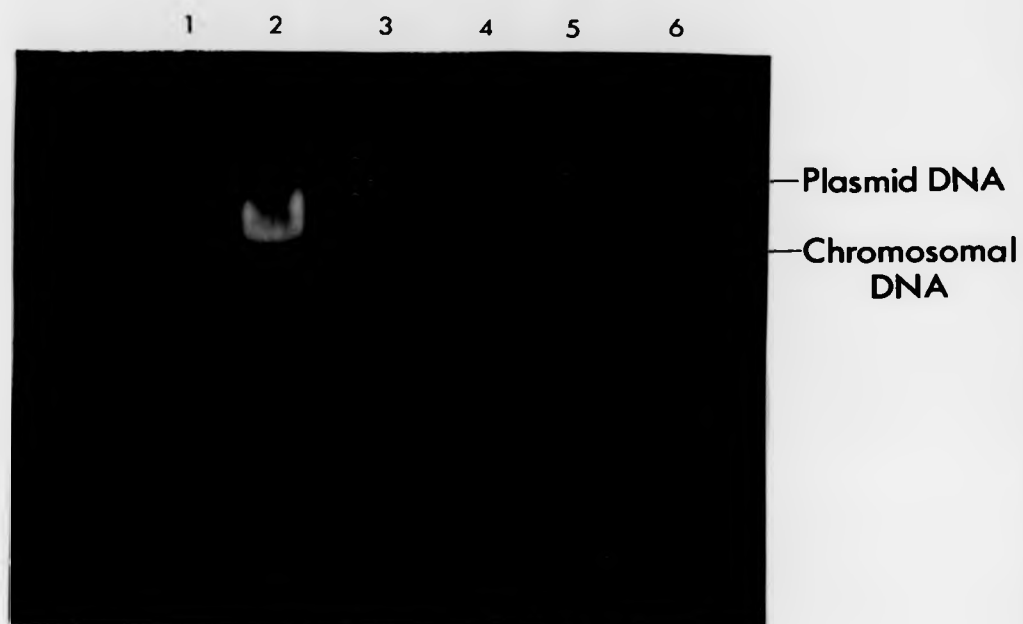
Catechols maybe formed as an intermediate in several different catabolic pathways. Some of the routes of formation, and degradation can be encoded by large catabolic plasmids such as TOL which encodes the enzymes catalysing the degradation of xylene and toluene. In the present study, overproduction of catechols was attempted by creating mutants lacking particular cleavage enzymes. Obviously, the phenotype of some mutants may change if a single copy number plasmid such as TOL was not stably maintained throughout the biotransformation. Consequently, it was necessary to establish whether any of the mutants under investigation possessed plasmids.

Small scale plasmid preparations were done using the method of Kado and Liu (1981) and the results are shown in Figure 3.5. The results showed that a large plasmid is present in all strains and the plasmid from the positive control, pWVO from P. putida mt-2, migrated the same distance as those present in all other strains. The size of plasmid pWVO is known to be 117 kb. and, although by no means definitive, the evidence shown indicated the derivatives of P. putida 2313 contained a plasmid of similar size.

3.5 Examination of the Catalytic Utility of P. putida 2313 and its derivatives.

Toluene and benzene were the substrates used in all subsequent biotransformations. The catechols derived from these

Figure 3.5. Small-scale plasmid preparation of *P. putida* 2313, R3, 6(12), 4(1) and mt-2.



Key:- Lane 1 - *P. putida* 2313.
Lane 2 - *P. putida* 4(1).
Lane 3 - *P. putida* 6(12).
Lane 4 - *P. putida* R3.
Lane 5 - *P. putida* mt-2 containing pWVO.
Lane 6 - Lambda/Hind III.

substrates, 3-methylcatechol and catechol, were the toxic model compounds to be investigated. However, the dioxygenase enzyme catalysing the incorporation of both atoms of molecular oxygen into the aromatic substrate is known to have a relaxed substrate specificity. This feature should permit substrates possessing different substitutions to be fortuitously oxidised and, in some instances, yield catechols of commercial interest. This section deals with investigations into the following:-

(i) Can aromatic compounds possessing different side chains serve as growth substrates for either P. putida R3 or P. putida 2313?

(ii) Is toluene grown P. putida R3 able to oxidise variously substituted aromatic compounds?

(iii) Can any catechols be detected using P. putida 6/12 cultures growing on glucose in the presence of substituted aromatics?

Studies into these areas would yield valuable information on both the catabolic versatility of the organisms and the rationale to be adopted for the production of substituted catechols using a biocatalytic route.

3.6 Growth studies using P. putida R3.

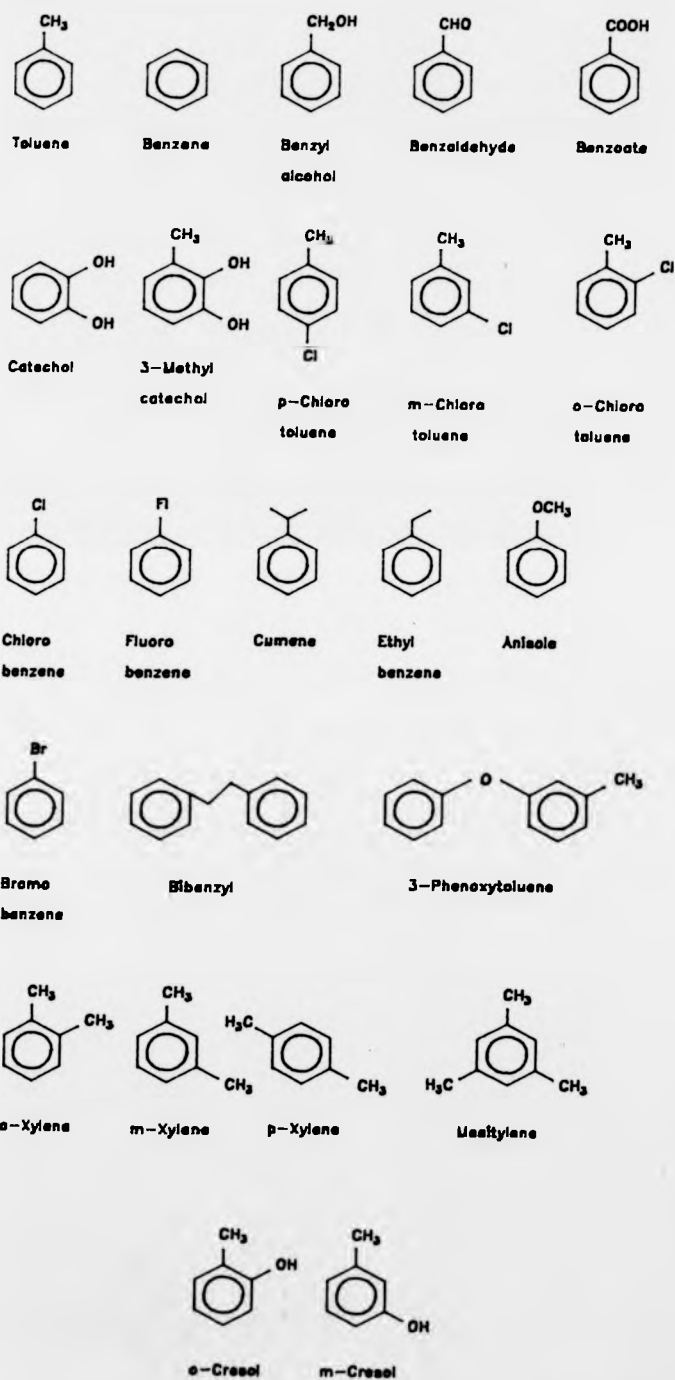
P. putida R3 was streaked onto mineral salts agar plates

containing the substrate under investigation. The substrates investigated are shown in Figure 3.6. From the qualitative data obtained it was difficult to establish any structure - activity relationships. Potentially, the pattern and nature of the substituents of all growth supporting compounds could permit both dioxygenase and monooxygenase pathways to be used. Additionally, linear alkyl side chains bigger than C-2 preclude the compound being used for growth. Further analyses of the growth of mutants on the same compounds revealed more about the possible pathways used. All of the mutants (P. putida 2313, 4(1) and 6(12)) were able to grow on bromobenzene, benzoate, benzyl alcohol, m- and p-chlorotoluene suggesting the catabolic pathway for their degradation was remote from that for the degradation of toluene and benzene. However, none of the mutants, including P. putida 4(1) which lacks toluene/benzene dioxygenase, were able to grow on cumene, ethyl benzene, toluene, mesitylene, or any of the xylenes or cresols. This suggests that the absence of a dioxygenase prevented growth on typical monooxygenase substrates leading to speculation that the dioxygenase and monooxygenase are the same enzyme system or at least under the same inductive control. Additionally, the results showed that a separate cleavage pathway was not induced when growing on a 'monooxygenase' substrate.

3.7 Investigation into the substrate specificity of toluene grown P. putida R3.

Previously, an insight into the substrate specificity of the aromatic dioxygenases was obtained by comparing the ability of a

Single colonies of P. putida R3 were streaked on to MS agar plates. When assessing growth using volatile substrates, plates were incubated in 'Tupperware' boxes containing a small universal of the substrate under investigation. Catechol, 3-methylcatechol and benzoate were incorporated into the MS medium at a final concentration of 2mM. Growth was assessed after 96 hours in relation to control plates which had been inoculated but lacked any carbon source.

Figure 3-8. Substrates which support the growth of *P. putida* R3.

revertant and mutants to grow on a variety of aromatic compounds. This section investigates the ability of toluene grown P. putida R3 to oxidise a variety of aromatic substrates.

P. putida R3 was grown on toluene in chemostat culture and washed prior to measuring the stimulated oxygen uptake in an oxygen electrode when challenged with a variety of aromatic compounds. The results shown in Table 3.8 compare the ability of P. putida R3 to oxidise aromatic compounds relative to the whole cell oxidation of toluene. The data shows that typical intermediates of the toluene monooxygenase pathway, apart from benzoate, are oxidised rapidly by toluene grown cells. The inability to oxidise benzoate was not unexpected in view of the differences in composition of toluene/benzene dioxygenase and benzoate dioxygenase. The latter is composed of only two components whereas the toluene/benzene dioxygenases have three components (Axcell and Geary, 1975; Yeh et al., 1977). The other data further support the contention that toluene dioxygenase does possess broad substrate specificity which may allow the fortuitous oxidation of variously substituted aromatic compounds. Additionally, the results may be interpreted to further support the idea that the monooxygenase and dioxygenase pathways are not discrete catabolic routes.

3.8 The production of variously substituted catechols.

P. putida 6/12 was grown on glucose in the presence of a variety of aromatic compounds to establish whether the toluene/benzene dioxygenases would fortuitously oxidise the aromatic substrate, forming a substituted catechol. The presence

Table 3.8. The results of oxygen electrode studies using toluene -
grown *P. putida* R3 to assess the catalytic utility of toluene
dioxygenase.

SUBSTRATE	STIMULATED OXYGEN UPTAKE ¹
Toluene	100
Benzyl Alcohol	55
3-Methyl catechol	51 ²
Benzaldehyde	42
Ethyl benzene	37
p-Chlorotoluene	14
Fluorobenzene	12
Anisole	12
Cumene	11
Aniline	10
p-Chlorotoluene	9
Chlorobenzene	7
Catechol	4
Benzoate	0

NR. 1 Stimulated oxygen uptake assessed relative to the oxidation
of toluene (100%).

2 Not corrected for auto-oxidation.

of a catecholic compound was assessed using the aminoantipyrine reagent. Of the compounds tested only toluene, benzene, fluorobenzene, chlorobenzene, 4-picoline, 2-picoline and pyridine showed any formation of catechol. Other compounds such as aniline, cumene, ethylbenzene and anisole showed some product formation when analysed using HPLC but these remained unidentified.

The limited results obtained show that the presence of side chains complicated product identification and permitted microbial attack by routes other than those involving initial ring dioxygenation. Interestingly, the use of picolines yielded catechol type compounds. The heterocyclic nature of these compounds would preclude their degradation by characterised intra- and extradiol cleavage pathways. However, such compounds may serve as gratuitous inducers and increase the yield of more conventional catechols.

In conclusion, the results presented in this section on catalytic utility showed some interesting findings. Firstly, the substrate specificity of the initial aromatic dioxygenase is relaxed permitting growth on, and fortuitous oxidation of, a variety of aromatic substrates. Although some production of halocatechols was demonstrated the yields were not as great as the non-halogenated catechols. Consequently, production of specific catechols may best be achieved by isolating a strain able to specifically oxidise the substrate of interest, ensuring the initial dioxygenase was optimised to deal with the chosen substrate.

CHAPTER 4. DEVELOPMENT OF A PROCESS
FOR THE PRODUCTION OF CATECHOLS.

CHAPTER 4.

DEVELOPMENT OF A PROCESS FOR THE PRODUCTION OF CATECHOLS.

INTRODUCTION.

Pseudomonas putida strain 2313 was known to produce 3-methylcatechol when growing on glucose in the presence of toluene. Other workers (Jenkins et al., 1987; Taylor, 1982; Stirling and Dalton, 1979; Maxwell, 1982; Shirai, 1986; Kuwahara and Tsuji, 1976) had shown that aromatic intermediates accumulated in the medium when the substrate was co-oxidised in the presence of a suitable carbon and energy source.

Prior to investigating the ability of P. putida to co-oxidise toluene to 3-methylcatechol, the physiological effect of toluene upon the organism was studied. Many aromatic substances are known to exercise a selective toxicity on P. putida at relatively low levels (Bringmann and Kühn, 1980). Toluene is especially toxic and is used routinely to make permeable bacterial cells. This experiment aimed to assess the toxicity of toluene both in flask culture experiments and by subjecting a growing culture to gradually increasing levels of toluene in the vapour phase. To permit induction of the suite of enzymes for the metabolism of toluene, a gradual increase in the toluene feed was necessary to minimise toxicity. Optimisation of the substrate feed must fulfill the criteria of being both sufficient to permit induction but insufficient to cause inhibition.

Once these conditions had been established it was then necessary to optimise growth conditions prior to the addition of the aromatic substrate. The conditions sought were the

overproduction of high levels of product and the maintenance of a high concentration of biomass. Operating the system continuously was not considered feasible for the reasons outlined previously (Section 1.11). Therefore, two alternative systems, batch and fed-batch, were investigated for possible future use. In a homogeneous batch culture, the culture develops in time and all the phenomena observed, including a given product, represent a function of time. Different phases occur and these reflect changes in the biomass and the culture environment. After the lag phase, growth occurs at a maximum rate and finally ceases due to nutrient starvation. The period when nutrient limitation significantly affects the growth rate is limited to a short period prior to nutrient starvation. Consequently, batch cultures are subject to large fluctuations in nutritional status, that is growth with excess substrate followed by starvation. To overcome nutrient limitation in a batch culture it was important to elucidate the duration of the batch fermentation, both for purposes of possible overproduction of catechols and to ensure a glucose feed was started at the appropriate time in fed-batch fermentation.

The overproduction of aromatic intermediates, such as toluene cis-glycol (TCG) has been achieved using a fed-batch mode of fermentation under nitrogen limitation (Jenkins et al., 1987). This approach prevents catabolite repression of the oxygenase and helps overcome the problems of reversion to the wild-type and hence loss of bioconversion ability. The separation of the growth and production phases limits the problem because the control of cell growth permits a more efficient conversion of substrate to product by reducing the

amount of energy expended for growth-related purposes. It is a type of fermentation which, during the growth phase will yield a high biomass concentration whilst permitting the control of growth via regulation of an essential nutrient. In this experiment the aim was to control growth by feeding limiting concentrations of glucose.

RESULTS AND DISCUSSION.

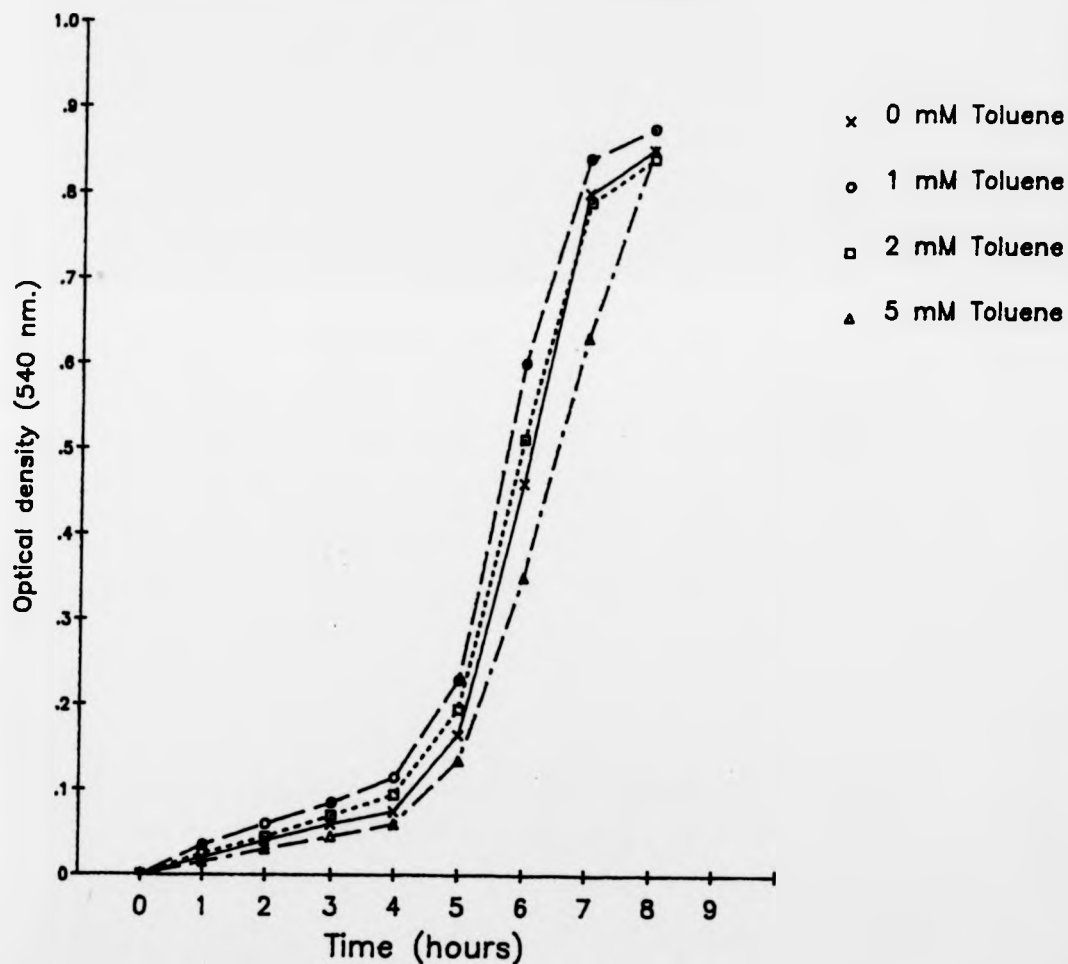
4.1 The effect of toluene on *P. putida* 2313 growing in flask culture.

Growth of *P. putida* 2313 in the presence of toluene was assessed on Luria broth with various concentrations of toluene. The results shown in Figure 4.1 show that the effect of toluene on this strain at concentrations ranging from 0-5 mM was negligible. This contradicts the findings of Bringmann and Kühn (1980) who suggested the toxicity threshold was 0.3 mM. The difference between these figures are probably due to *P. putida* 2313 being selected for growth on high levels of toluene. The only inhibitory effect observed in the present experiment was a possible lengthening of the lag phase. Apart from this all cultures had a doubling time of approximately 1 hour and attained a final cell density of 0.85 at 540 nm.

4.2 The growth of *P. putida* 2313/4(1) in fed-batch culture in the presence of increasing levels of toluene.

The toxicity of toluene in the bioreactor was investigated using *P. putida* 2313/4(1), a mutant known to lack toluene dioxygenase and obtained via NTG mutagenesis (Section 3.2). The inability to co-oxidise toluene when grown on glucose in

Figure 4.i - The effect of toluene on *P. putida* 2313 growing in flask culture.



Single colonies of *P. putida* 2313 were inoculated into 250 ml. suba-sealed flasks containing 50 ml. Luria broth and liquid toluene at the concentrations outlined.

fed-batch culture obviated any inhibition due to the accumulation of 3-methylcatechol and allowed the toluene feed to be increased till it was seen to affect growth.

The results presented in Figure 4.2 shows that growth, after overnight batch growth and measured by optical density and total cell protein, continued to rise throughout and reached maxima of 14.3 and 5.6 g/l respectively. Despite feeding toluene at 600 ml/min. for 4 hours between 21 and 25 hours, glucose continued to be limiting and ammonia continued to be depleted.

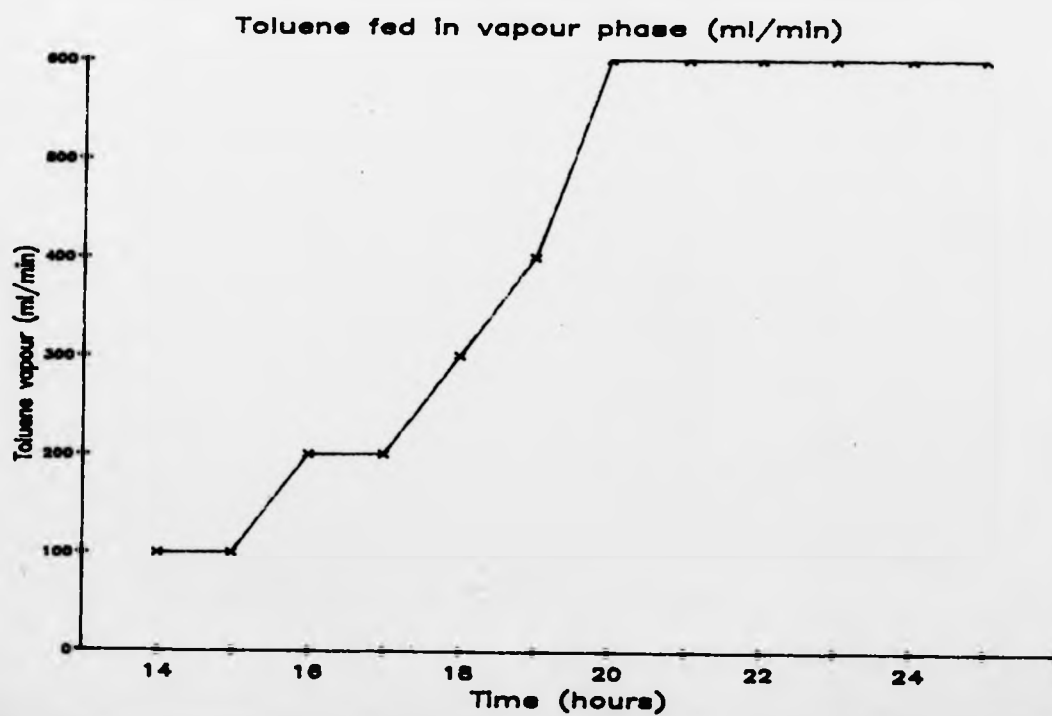
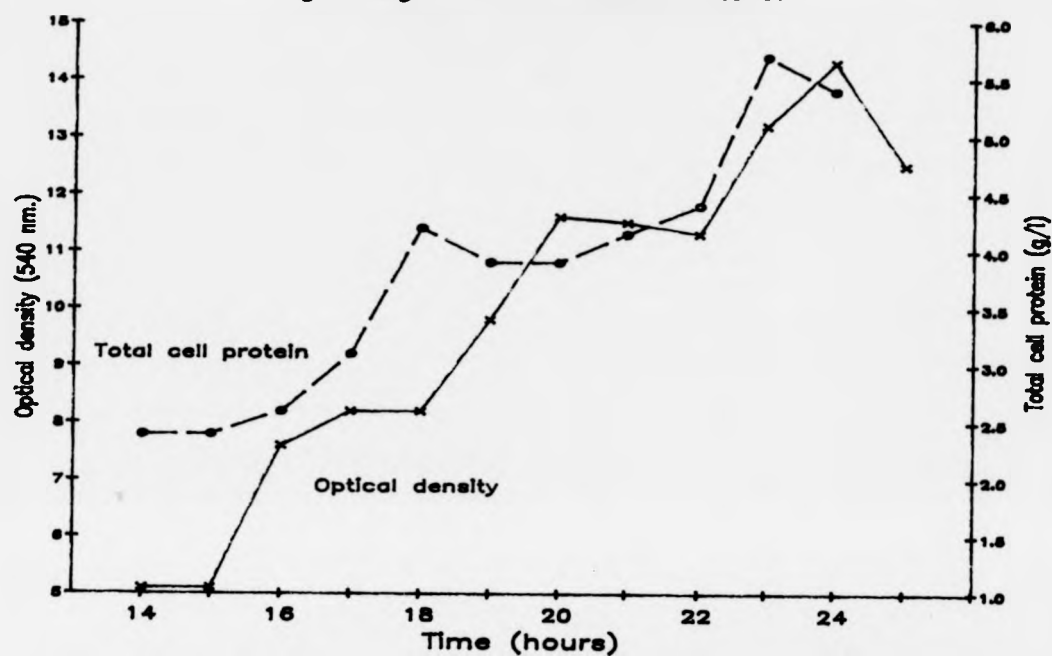
The data presented supports the conclusion that toluene would not inhibit the growth of the organism and would not contribute to any inhibitory effects when P. putida 2313, or mutants thereof, are used for the production of dihydroxylated benzene derivatives. In subsequent biotransformations the toluene or benzene vapour was fed at 200 ml/min. before induction and 400 ml/min. subsequently. Although toluene and benzene have different vapour pressures, 3.7 and 13 kPa at 25°C and have different solubilities in water, 0.052% and 0.18% at 25°C (Hoff, 1979; Purcell, 1979) it was assumed that benzene would have a similar effect to toluene within the system used. Thus, the results obtained here were implemented in both systems.

4.3 Growth of P. putida 2313 in batch culture.

The results for the growth of P. putida 2313 in batch culture are presented in Figure 4.3. After inoculation the lag period was three hours. Exponential growth phase was attained for 4 hours, verified by the rapid decrease in detectable glucose and dissolved oxygen. Although the available nitrogen

P. putida 4/1 was grown in batch culture overnight. At T=14 hrs. glucose feed was initiated at 1 g/l/h and toluene feed was initiated at a level of 100 ml/min. Over the following 10 hours the toluene feed was increased to a level of 600 ml/min. Glucose feed was maintained at 1 g/l/h throughout. Additionally, air was supplied at 1 L/min. throughout.

Figure 4.2 - The effect of toluene on *P. putida* 4/1 growing in fed - batch culture.

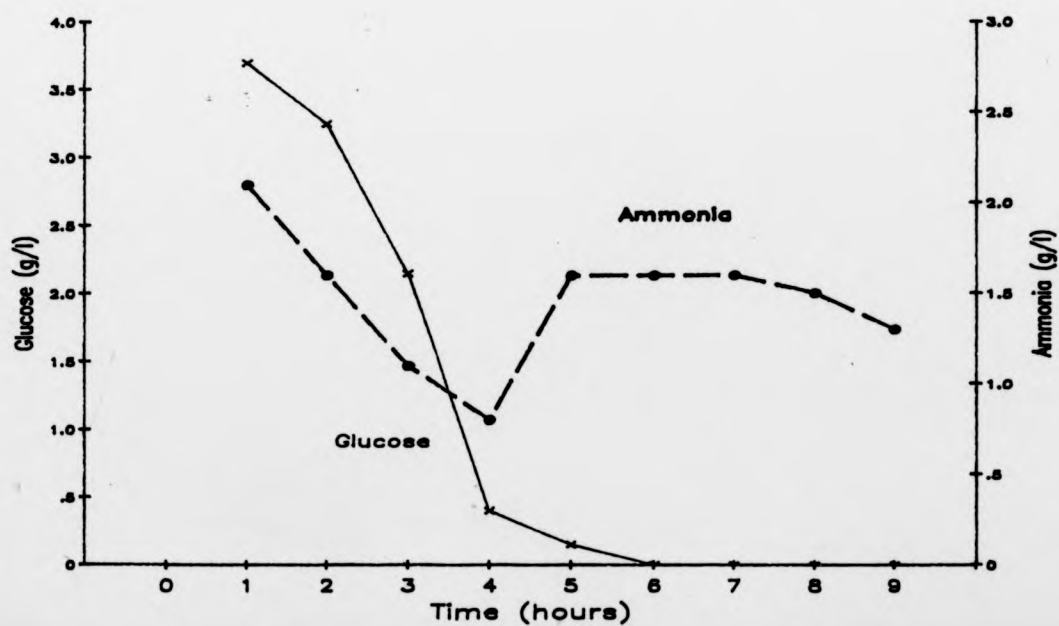
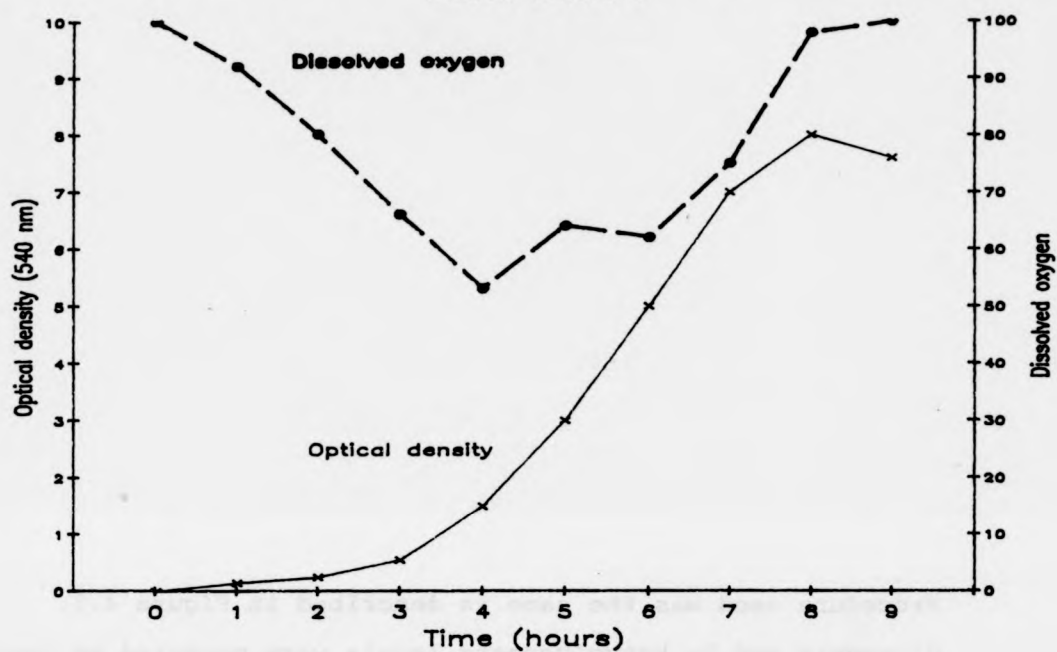


diminished it remained in excess throughout the duration of the experiment. The maximum specific growth rate was 0.69 /h and supernatant glucose became exhausted after 5.5 hours. Despite the depletion in detectable glucose growth continued for a further 2 hours to a final optical density (540 nm) of 8.2. The temporal separation in detectable glucose and the continuation of growth was further investigated. There are two routes of conversion of glucose to 6-phosphogluconate: the direct oxidative pathway, which acts on glucose extracellularly and the phosphorylative pathway, which does so intracellularly. These pathways will not be discussed in detail here and the reader is directed to Lessie and Phibbs, (1984) for further information. Vicente and Canovas (1973) suggested that the primary pathway of glucose utilization in P. putida was the direct oxidative pathway. The two successive oxidations required to convert glucose to 2-ketogluconate are carried out in the periplasm by membrane-associated, pyridine nucleotide-independent, glucose and gluconate dehydrogenases. The data presented in Figure 4.4 shows the levels of intermediates present in the supernatant. Both gluconate and 2-ketogluconate were present in the culture broth verifying that glucose was metabolised via the direct oxidative pathway. The data obtained for gluconate levels was inconclusive, probably because of interference from other substances in the assay system used. Levels of 2-ketogluconate were shown to increase rapidly to 2.5 g/l after glucose had become exhausted.

In summary, glucose was shown to be metabolised via the direct oxidative pathway. Glucose was rapidly converted to 2-ketogluconate by periplasmic enzymes. High levels of gluconate

P. putida 2313 was grown in 100 ml. Luria broth until it reached mid-logarithmic phase. This was used as the inoculum into 5L MS medium containing glucose at a final concentration of 5 g/l. Glucose and ammonia remaining in the supernatant were measured using the methods outlined in Materials and Methods.

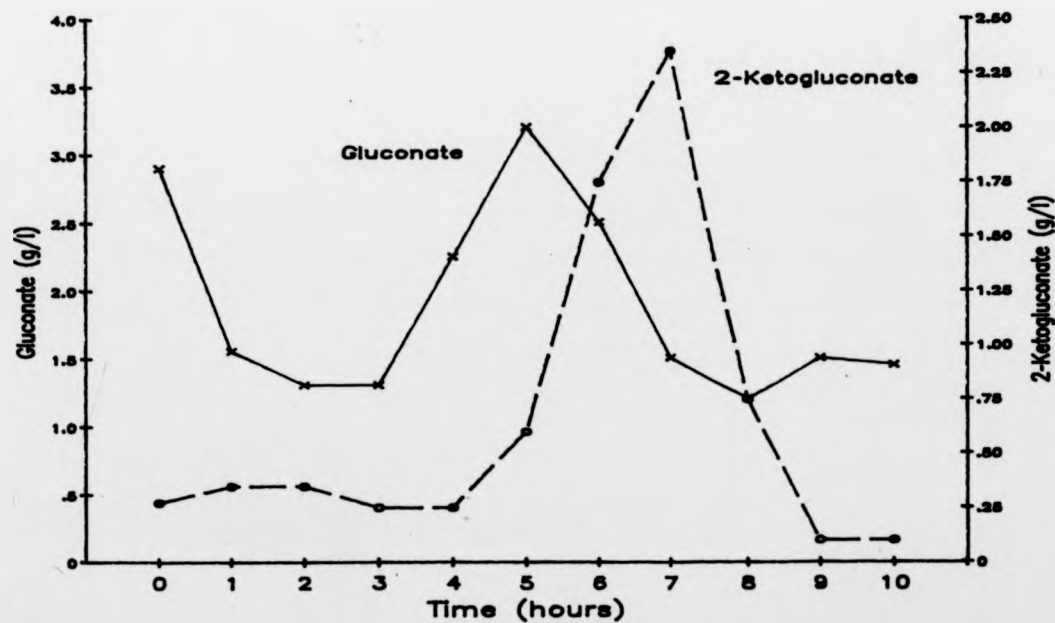
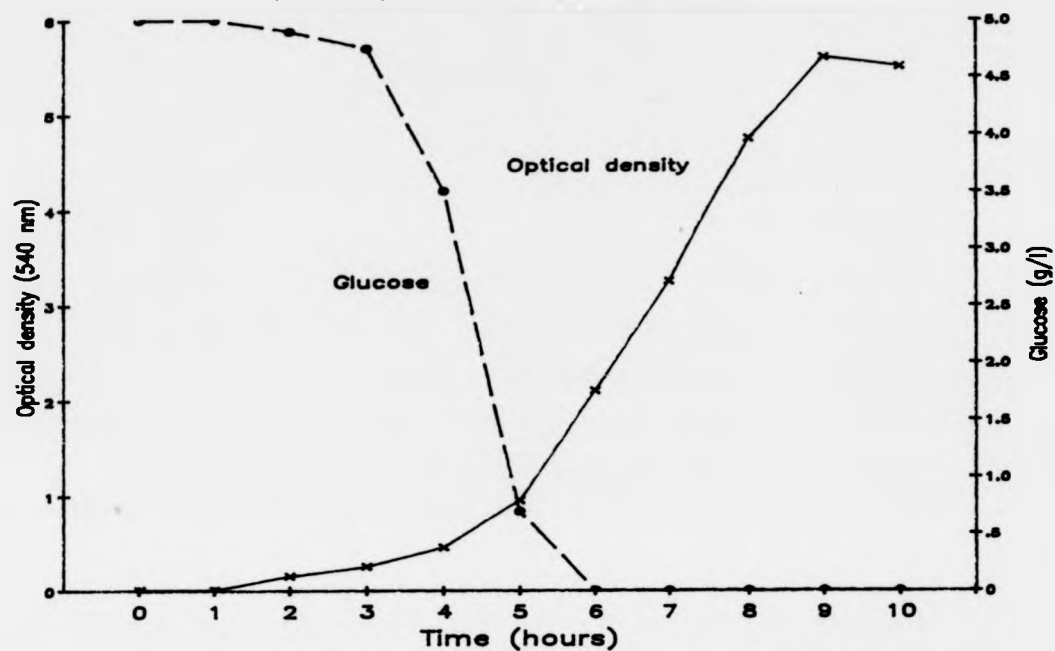
Figure 4.3. The growth of *P. putida* 2313 in batch culture.



Procedure used was the same as described in Figure 4.3.

Gluconate and 2- ketogluconate levels were measured as described in Materials and Methods.

Figure 4.4. Investigation into the glucose assimilatory pathway used by *P. putida* 2313.



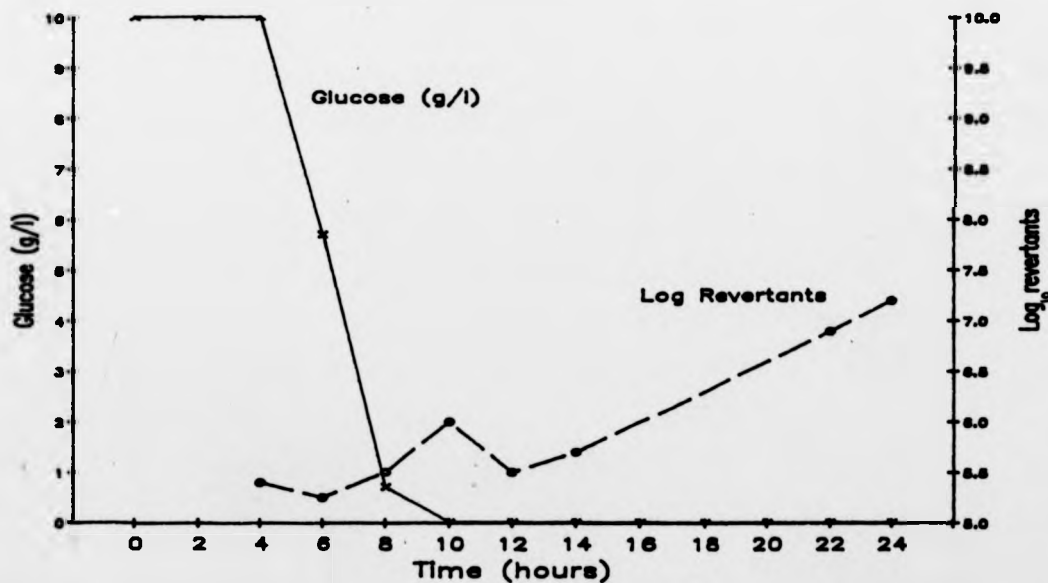
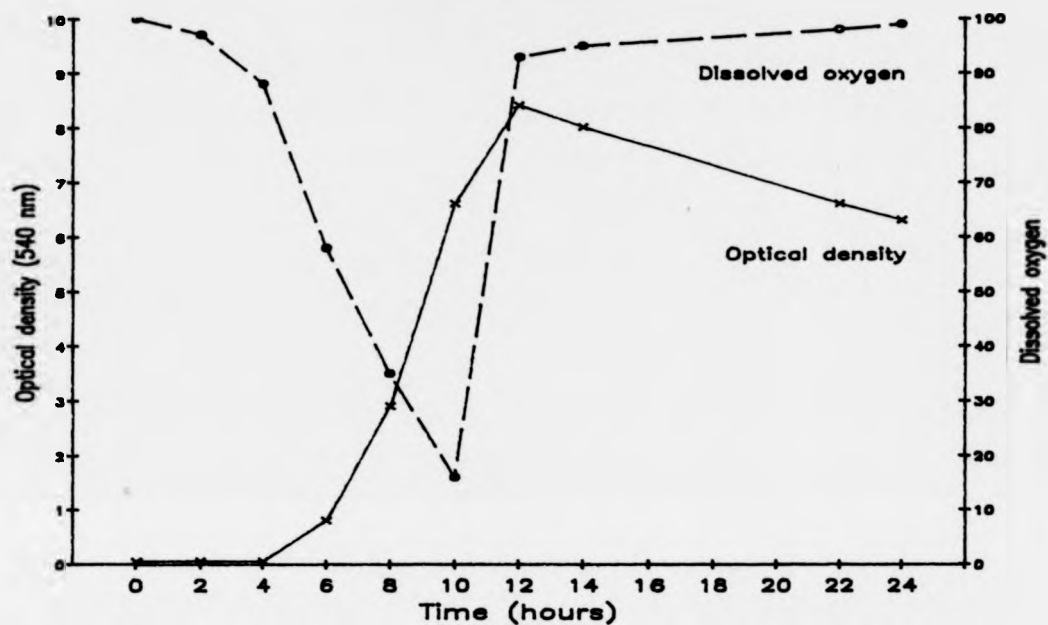
and 2-ketogluconate built up within the periplasm and diffused through the outer membrane. Subsequently, the 2-ketogluconate would be actively transported into the cell and acted upon by an ATP-dependent kinase, forming 2-keto-6-phosphogluconate which is then converted to 6-phosphogluconate.

4.4 The production of 3-methylcatechol in batch culture using *P. putida* 2313.

The fermentation described in section 4.3 was repeated to investigate whether the biotransformation could be performed in batch culture. This was done by feeding toluene in the vapour phase as outlined previously and assuming the induction period lasted no longer than 3 hours. The results of this experiment are shown in figure 4.5. The toluene feed was initiated after 4.25 hours. The specific growth rate was 0.58/h, compared with 0.69/h in the absence of toluene. Glucose became depleted in the supernatant after 9 hours although maximum biomass was not achieved until 12 hours. A rapid increase in dissolved oxygen was observed after 10 hours indicating nutrient starvation. Although toluene was fed at the rates previously described no 3-methylcatechol was detectable throughout the duration of the experiment, neither during the growth phase nor the stationary phase. The experiment was repeated but 3-methylcatechol was not produced indicating that growth of the organism and production of the catechol could not occur concomitantly. It is interesting to note that in the stationary phase the number of revertant organisms increased ten-fold due to the selective advantage of growing on toluene when the primary growth substrate was exhausted.

P. putida 2313 was grown as described in Figure 4.3. Glucose levels remaining in the supernatant were measured as described previously (Materials and Methods). Toluene vapour was fed at 200 ml/min. after 4.25 hours. After 7.25 hours this was increased to 400 ml/min. Additionally, air was supplied at 1 L/min. throughout the experiment. The levels of 3-methylcatechol were monitored throughout using HPLC as outlined in Materials and Methods.

Figure 4.5. Investigation into the production of 3-methyl catechol by *P. putida* 2313 growing in batch culture.



The inability to produce catechols in batch culture may be attributable to either non-induction of the enzymes due to catabolite repression or an inability to co-oxidise toluene in the presence of excess glucose. The latter has been shown previously by Cox et al. (1979) who demonstrated that the production of 1,2-dihydroxy-1,2-dihydronaphthalene (DHD) by P. putida 119, a mutant strain, was adversely affected by high glucose concentrations. When grown on 0.05% (w/v) glucose, in the presence of naphthalene, DHD production was ten-fold higher than when 0.5% (w/v) glucose was used.

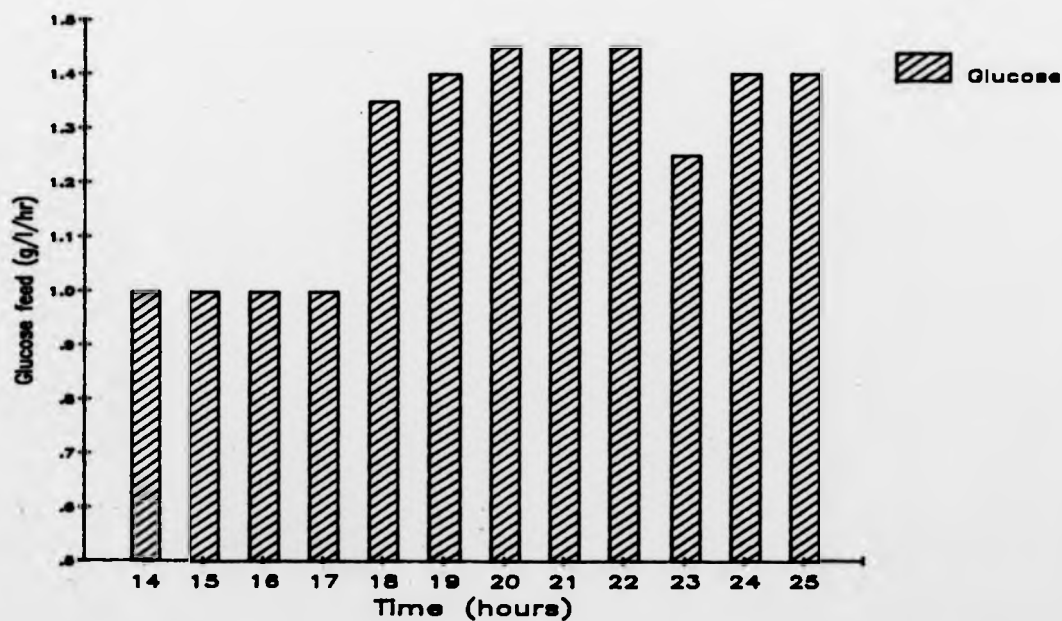
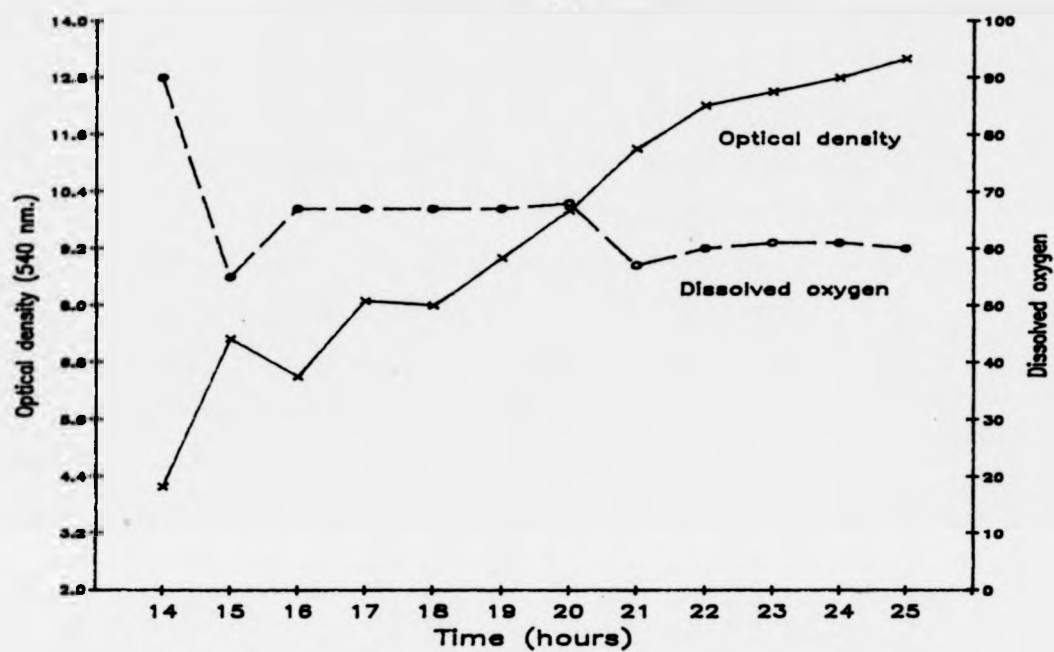
4.5 Optimisation of the growth of P. putida 2313 in fed-batch culture.

The inability to produce catechol in batch culture necessitated the development of an alternative system. Ideally, substrate-limited growth was sought to overcome any possibility of catabolite repression or inhibition of aromatic co-oxidation. Fed-batch culture allows the rate of substrate utilisation to be restricted using the substrate feed rate, thereby overcoming catabolite repression of product formation (Demain, 1972).

This experiment investigated the glucose feed required to achieve linear, glucose-limited growth. The results shown in Figure 4.6 demonstrate that when glucose was fed at 1-1.4 g/l/hr linear growth was achieved using an initial biomass concentration of 1.5-2.0 g/l. Reproducible results relied on the biomass falling within these limits to ensure that glucose would remain limiting throughout the biotransformation and growth could be controlled. The only problem foreseen was that continued growth, if maintained at the same rate, would quickly result in

P. putida 2313 was grown in batch mode as described previously (Figure 4.3). After 14 hours the glucose feed was initiated at 1 g/l/h. Over the following 10 hours the glucose feed was increased as shown in the histogram. Cell density was monitored throughout and glucose was found to be absent in the supernatant throughout the course of the experiment.

Figure 4.6. Optimisation of glucose feed to achieve linear growth of *P. putida* 2313.



depletion of the nitrogen source. This was overcome by increasing the amount of NH_4Cl initially present from 3 g/l to 4 g/l.

It is interesting to note that when the organism was grown in the batch mode, with excess glucose, its specific growth rate was 0.69/h. However, when grown with a substrate limited feed the specific growth rate was 0.13/h. These figures only serve for comparison because in true fed-batch culture the growth rate measured only reflects that obtained transiently.

It can be argued that similar growth, obtained in the presence of toluene or benzene, would prevent catabolite repression and facilitate co-oxidation of the aromatic substrate. This was investigated in the following series of experiments.

CHAPTER 5. THE PRODUCTION OF CATECHOLS
IN GLUCOSE FED-BATCH CULTURE.

CHAPTER 5

THE PRODUCTION OF CATECHOLS IN GLUCOSE FED-BATCH CULTURE.

INTRODUCTION.

The use of whole microbial cells as a catalyst for the production of aromatic intermediates has been demonstrated in many instances. Previous workers have shown that intermediates such as cis-glycols and catechols can be produced from a variety of substrates including toluene (Jenkins et al., 1987); benzene (Shirai, 1986); phenol (Nei et al., 1974); cresols (Bayly and Wigmore, 1973) and xylenes (Davey and Gibson, 1974).

The work of Jenkins et al. (1987) best illustrates the methodology employed in the present study. The authors described the production of toluene cis-glycol in glucose fed-batch culture using P. putida NG1, a mutant organism lacking the enzyme toluene cis-glycol dehydrogenase. Following exponential growth on glucose, the dioxygenase was induced by the addition of toluene and biotransformation to the glycol was carried out under glucose-limiting conditions to minimise catabolite repression of enzyme synthesis.

In the present study, the production of catechol and 3-methylcatechol was studied in glucose fed-batch culture using benzene and toluene as substrates. Up to this stage of the experimental programme studies were concerned with fed-batch fermentation systems using P. putida 2313, a mutant organism known to produce 3-methylcatechol from toluene by virtue of it lacking the extradiol cleavage enzyme, catechol 2,3-oxygenase. Growth of P. putida 2313 on benzene and benzoate (section 3.1)

showed that the organism still possessed a fully functional intradiol cleavage pathway, including catechol 1,2-oxygenase. The biotransformation carried out with toluene and P. putida 2313 aimed to show whether the intradiol cleavage enzyme, catechol 1,2-oxygenase would function in vivo to permit cleavage of catechol possessing a methyl moiety. Other workers (Hou et al., 1977; Fujiwara et al., 1975) had suggested that catechol 1,2-oxygenase would cleave 3-methylcatechol in vitro although the possible role of this in vivo was not addressed.

The production of catechol from benzene differs from the production of 3-methylcatechol from toluene in a number of respects. Firstly, P. putida 2313 is able to grow on benzene via the intradiol cleavage route. Consequently, production of catechol necessitates a further round of mutagenesis to remove catechol 1,2-oxygenase and permit overproduction of catechol. This has been shown previously by Shirai (1987) who demonstrated that a mutant of P. putida BE-81, which was obtained using mutagenesis (Shirai, 1986), could produce 3.1 mg/ml catechol when supplied with 5 mg/ml benzene.

Therefore, in this section experiments are described which outline:-

(i) The production of 3-methylcatechol from toluene using P. putida 2313 in glucose fed-batch culture.

(ii) Investigations into the overproduction of catechol from benzene using P. putida 2313.

(iii) The production of catechol from benzene using P. putida 6/12, a mutant derivative of P. putida 2313.

RESULTS AND DISCUSSION.

5.1 The production of 3-methylcatechol in glucose fed-batch culture.

The production of 3-methylcatechol from toluene using *P. putida* 2313 was established using a fed-batch culture in which the glucose feed rate was 1-1.4 g/l/hr and the toluene was fed in the vapour phase at the rates previously described (section 4.2).

The results presented in Figures 5.1.1 to 5.1.7 show the parameters measured throughout the biotransformation. Growth, monitored both by optical density and viable count (5.1.1), was shown to increase only for the initial 2-5 hours of the production phase. This suggests that although linear growth was shown previously, the concomitant feeding of toluene prevented the increase in biomass previously observed in the absence of toluene. Cell viability remained fairly constant over the first 10 hours of the production phase and only fell 2 orders of magnitude over the subsequent 12 hours. This was not attributable to toluene toxicity since it was fed at the levels used previously and shown not to cause any growth inhibition. Once the toluene feed was started growth appeared to slow down and ultimately cease. The decrease in growth during the production phase could be attributable to a higher maintenance requirement, needed to maintain concentration gradients between the cell and its exterior. Whatever the effect upon the culture, whole cell measurements of biomass and viability showed that the

overproduction of 3-methylcatechol led to the inhibition of growth.

Cessation of growth was also indicated by a rapid drop in the dissolved oxygen tension after the glucose feed had been initiated in the production phase. After a further two hours the dissolved oxygen tension rose rapidly to 80% (Figure 5.1.2), and continued to rise reaching a plateau of 90% after 8 hours of production. Coincident with all the afore - mentioned data, measurements of supernatant glucose showed that soon after the glucose feed had been started there was no detectable glucose present in the supernatant (Figure 5.1.2). Subsequently, five hours into the production phase, the level of glucose in the supernatant rose to 27 mg/l and, although it dropped, still persisted in the medium.

Measurements of the levels of supernatant toluene showed that induction of the toluene utilising pathway had occurred within 3 hours of the initiation of the toluene feed (Figure 5.1.3). The early excess of toluene in the supernatant soon dropped before increasing when the aromatic substrate feed was increased to the post-induction level of 400 ml/min. As the level of detectable toluene fell, 3-methylcatechol was produced rapidly and in the 2-6 hour period of the production phase the maximum rate of 3-methylcatechol production was 0.49g 3-methylcatechol/g cell carbon/hour. Product concentration reached a maximum of 10.5 mM (1.3 g/l) in this experiment whereas levels of 18 mM (2.2 g/l) had been obtained previously. This discrepancy in the amount of product was due to auto-oxidation, leading to the formation of an insoluble polymer

when stored, and has been mentioned previously in the introduction. The authenticity of the soluble product was shown by the use of GC/MS. The GC trace of the product, after extraction into acetone, is shown in Figure 5.1.4. The peak at 1'55" was analysed by mass spectrometry and found to match the spectrum of authentic 3-methylcatechol stored in the software library (Figure 5.1.5).

The concentration of toluene cis-glycol (TCG) present in the supernatant throughout is not shown but HPLC analysis revealed that it did not exceed 0.05 mM. The amount of toluene present in the aqueous phase reached a sub-inhibitory maximum of 0.7 mM throughout the biotransformation. Having established that the production of 3-methylcatechol was inhibitory to the culture an insight into the nature of this toxicity was sought. Cell-free extracts were prepared throughout the biotransformation and the specific activities of key enzymes were determined. The potential biotransformation capacity was assessed by measuring both the toluene dioxygenase and toluene cis-glycol dehydrogenase activities in crude cell-free extracts. Maximum TCG dehydrogenase activity was 62 nmol. NAD⁺/min/mg protein measured after only 4 hours of the production phase (Figure 5.1.6). This activity was coincident with the maximal rate of 3-methylcatechol production. After a further day the TCG dehydrogenase activity had only fallen 27% to 45.4 nmol NAD⁺/min/mg protein. High relative specific activities of the toluene dioxygenase in cell extracts (8.7×10^{-3} A₄₀₀/min/mg protein) again corresponded with the maximum rate of 3-methylcatechol production. However, unlike the TCG

dehydrogenase activity, exposure to 3-methylcatechol resulted in a 78% reduction in specific activity after only 8 hours of the biotransformation. Throughout the production phase neither catechol 1,2- nor 2,3-oxygenase were detected. This finding would suggest that, although revertant counts were not monitored, very little reversion occurred during the course of the biotransformation.

Whole-cell oxidation of both toluene and 3-methylcatechol was monitored during the production phase to follow induction and see whether any activity could be detected which may have reduced the final product yield. The pattern of toluene oxidation mirrored that shown previously using the spectrophotometric indole assay to monitor toluene dioxygenase activity (Figure 5.1.7). Maximum activity was coincident with the latter, reaching a level of 90 nmol O₂/min/mg. protein. This rapidly fell to 20 nmol O₂/min/mg. protein, verifying the 78% fall in toluene dioxygenase activity observed previously. Unlike the cell extract assay, the whole cell oxidation of toluene was completely absent after overnight exposure to the high product levels. This suggested that the primary site of catechol toxicity was the toluene dioxygenase but that other factors affecting cellular processes such as toluene uptake were adversely affected by prolonged exposure to 3-methylcatechol.

To monitor the possibility of reversion or enzyme recruitment the cells were assayed for oxygen uptake in the presence of 3-methylcatechol. This was expected to show that the cells were unable to use the product due to the absence of the extradiol cleavage enzyme, catechol 2,3-oxygenase. Contrary to

the results of cell extract studies it was shown that cells possessed the ability to oxidise 3-methylcatechol and the pattern of oxidation followed closely that demonstrated for toluene (Figure 5.1.7). Whole cell oxidation of 3-methylcatechol reached a maximum of 42 nmol O₂/min/mg. protein after only 2 hours of the production phase. Subsequently, this fell by 50% and eventually fell to zero. In the light of these results, both intra- and extradiol cleavage enzymes were again assayed but no detectable activity was found. The oxygen uptake observed may have been attributable to the oxidation of an impurity in the 3-methylcatechol solution. Analysis of the latter by HPLC and GC/MS revealed the substrate to be free of any interfering substances. If the activity was enzymic it was thought a stoichiometric relationship would exist between the amount of 3-methylcatechol used and the amount of oxygen consumed.

Further experiments were undertaken to establish whether a stoichiometric relationship existed between 3-methylcatechol and oxygen consumed. Measuring only auto-oxidation and the oxidation in the presence of viable or non-viable cells the results showed that irrespective of the presence, absence or viability of the cells the ratio of 3-methylcatechol : oxygen consumed was approximately 130:1 (Table 5.1). This was far too high to be considered as an enzymic reaction such as that catalysed by an oxygenase. The results suggested that the stimulated uptake could be due to the non-enzymic enhancement of auto-oxidation. This could occur at the cell surface or within the periplasmic space where localised abiotic factors such as pH may promote the polymerisation outlined in the introduction (Section 1.2).

P. putida 2313 was grown in batch mode overnight. Subsequently, glucose feed was initiated as previously described (Figure 4.6). Additionally, toluene was fed in the vapour phase from T=0 at 200 ml/min. At T=4 hours the level of toluene vapour fed was increased to 400 ml/min. Viability was assessed throughout on Luria agar. Glucose remaining in the supernatant is shown in Figure 5.1.2.

Figure 5.1. The production of 3-methyl catechol in glucose fed-batch culture using *P. putida* 2313.
Fig. 5.1.1.

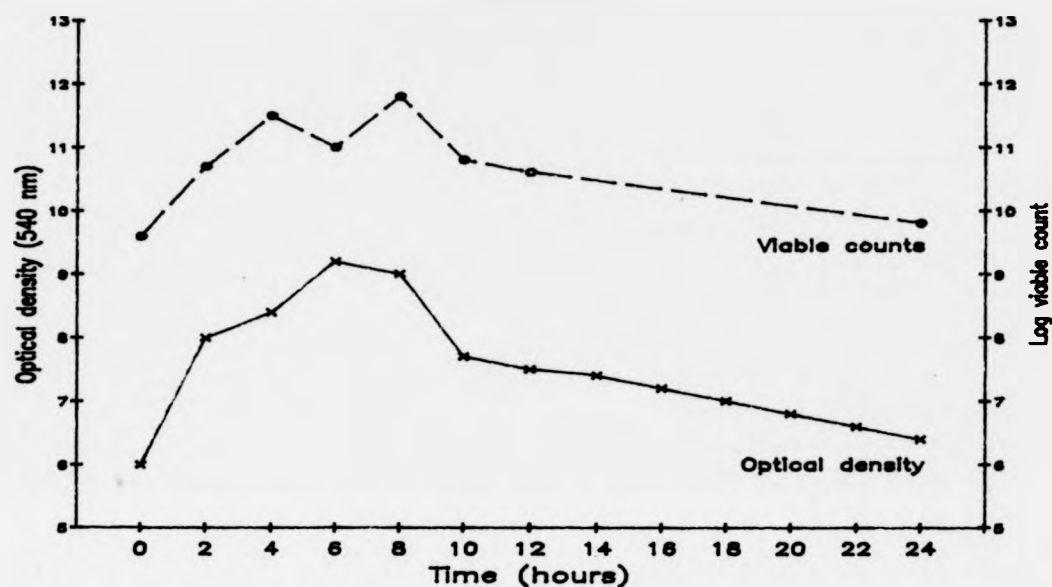


Fig. 5.1.2.

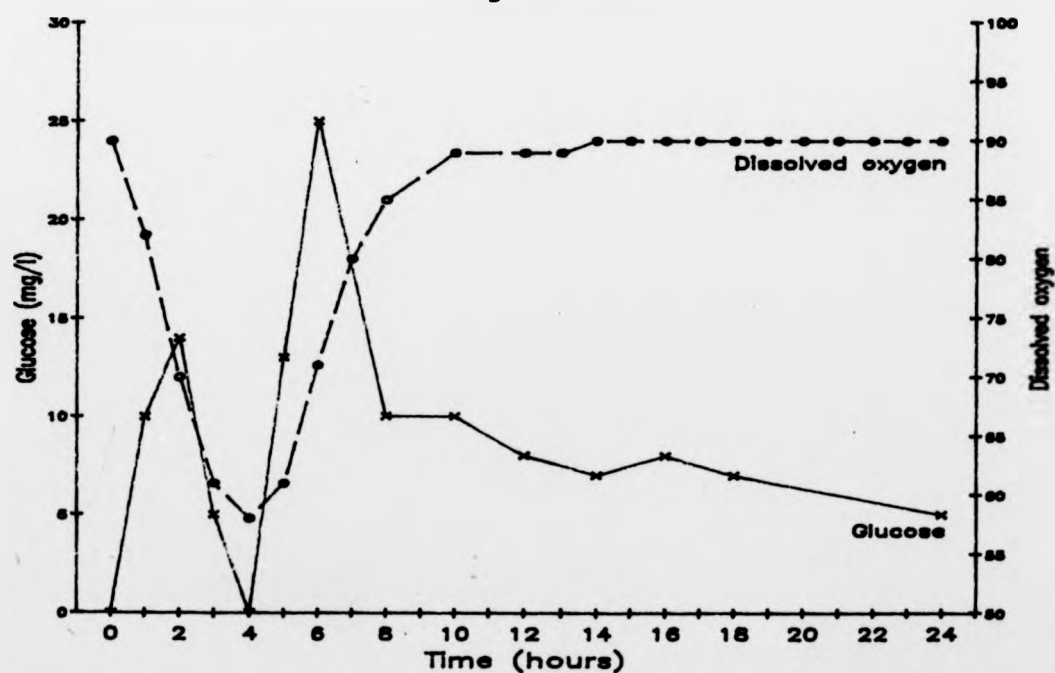


Fig 5.1.3

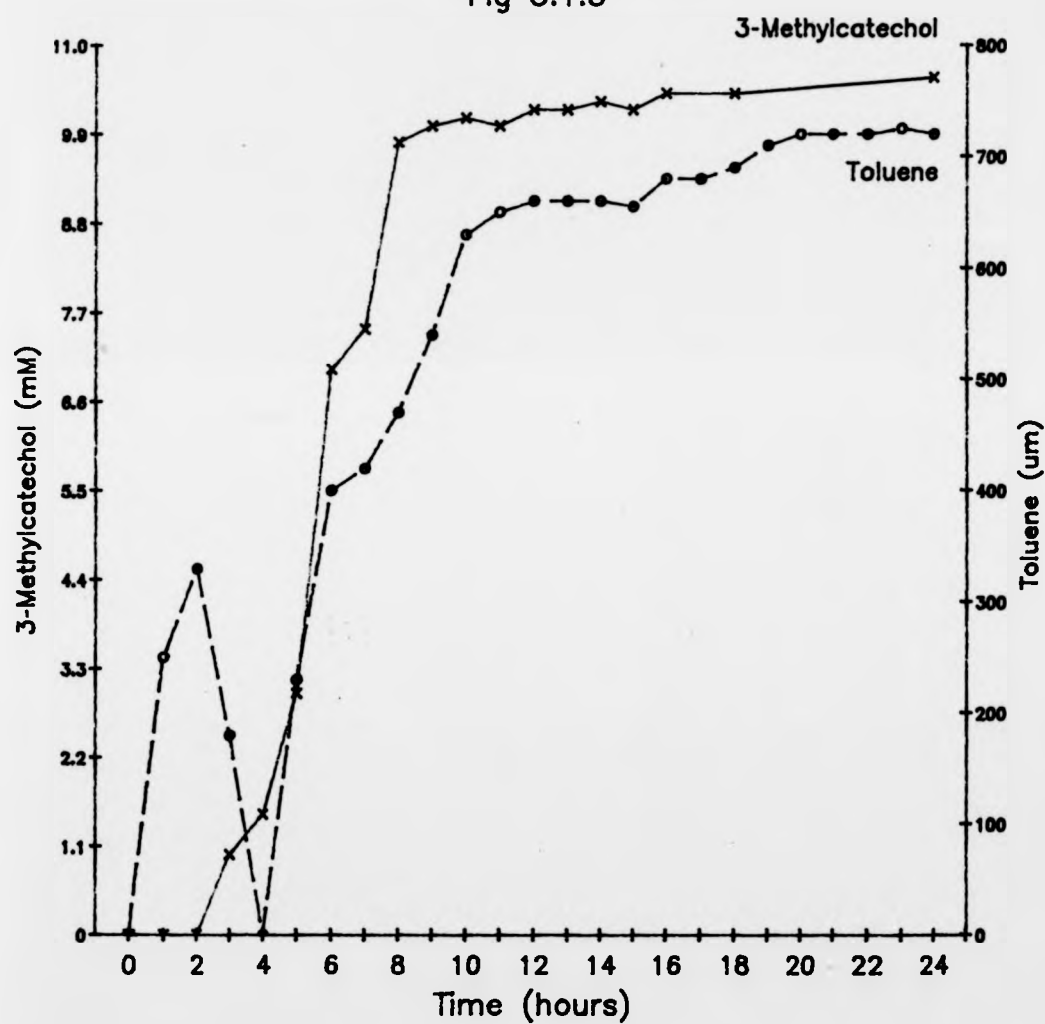
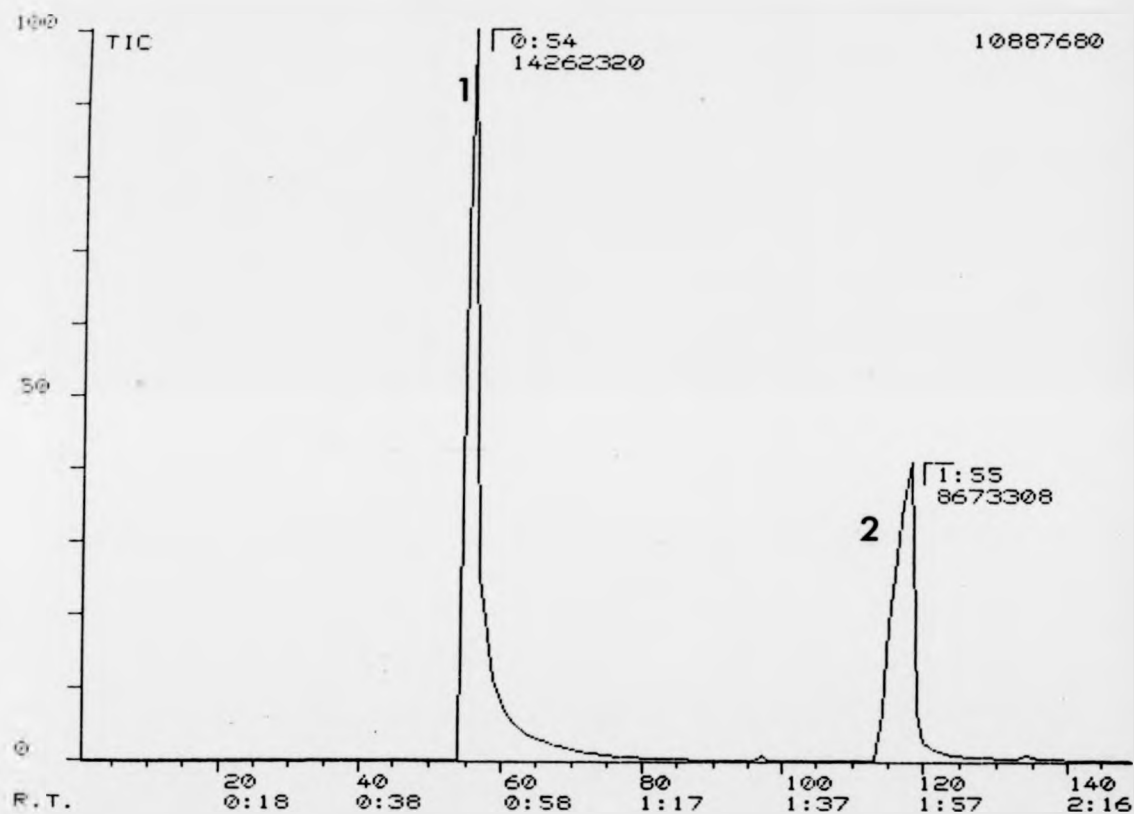


Figure 5.1.4. Gas chromatogram of 3-methyl catechol after extraction into acetone.

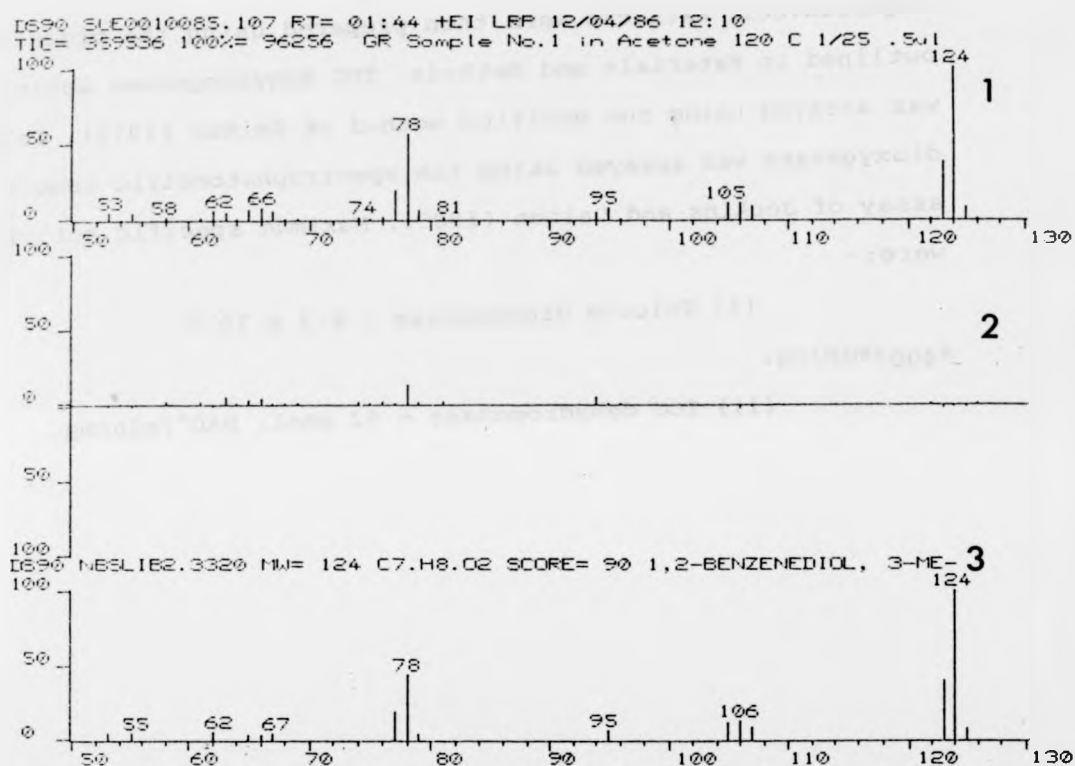
DS-90 CHROMATOGRAM REPORT RUN: SUE0010083 12/ 4/86 11:54 EI
OF Sample No.1 Std 3 - CH3 CAT 120 C 1/25 .5ul in Acetone



Key:- Peak 1 - Acetone

Peak 2 - 3-Methyl catechol

Figure 5.1.5 Mass spectrum of 3-methyl catechol extracted compared with authentic mass spectrum from data library.



Key:- Trace 1 Extracted product.
Trace 2 Difference spectrum.
Trace 3 Authentic 3-methyl catechol.

Cell samples (50 ml.) were removed from the fermentor throughout the biotransformation and were stored at 4°C until required. Cell extracts were then prepared using the procedure outlined in Materials and Methods. TCG dehydrogenase activity was assayed using the modified method of Reiner (1972). Toluene dioxygenase was assayed using the spectrophotometric indole assay of Jenkins and Dalton (1985). Maximum specific activities were:-

(i) Toluene dioxygenase - 8.7×10^{-3}

A₄₀₀/min/mg.

(ii) TCG dehydrogenase - 62 nmol. NAD⁺/min/mg.

Cell culture (5 ml.) was removed periodically during the biotransformation. After concentration by centrifugation at 20,000 g the cells were washed in 20 mM potassium phosphate buffer (pH 6.8). Cells were resuspended in the same buffer (0.5 ml.) and oxygen electrode studies were performed as outlined in Materials and Methods.

Fig. 5.1.6

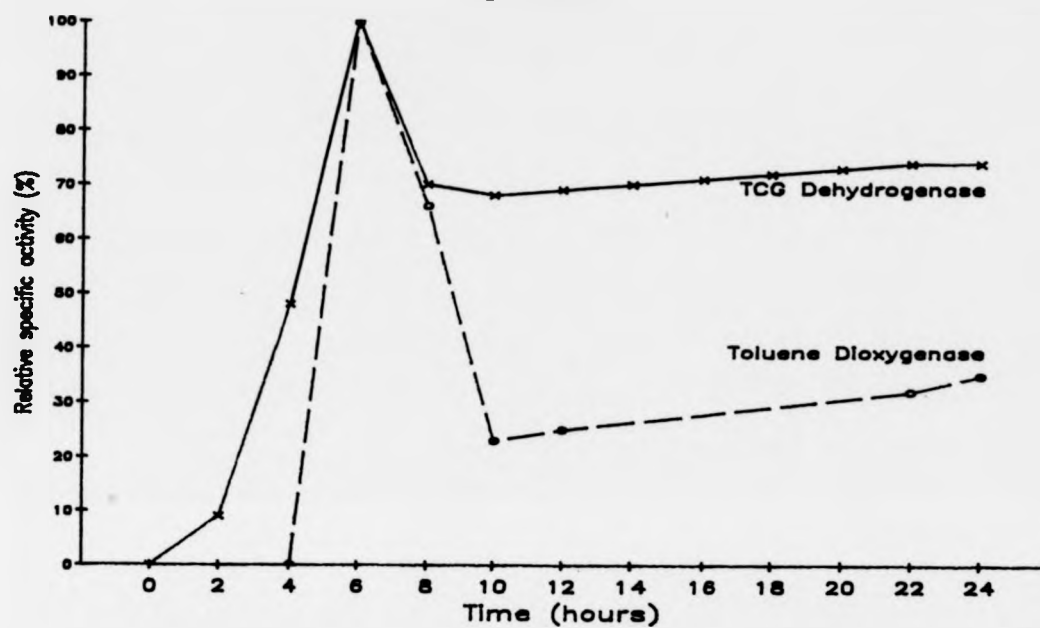


Fig. 5.1.7.

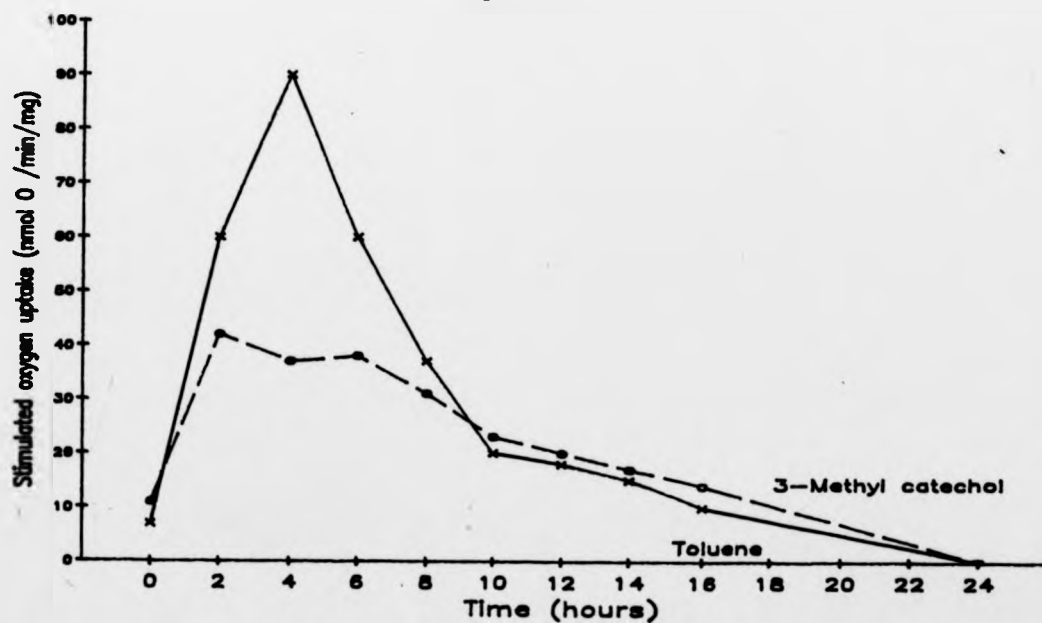


Table 5.1. Investigation into the stoichiometry of the 3-methyl catechol 'oxidising' activity.

Cells	Amt. of 3-methyl catechol used	Amt. of oxygen used	Ratio
Viable toluene oxidising	7.5×10^{-6} mol.	55.6 nmol.	135:1
Heat treated ¹	1.68×10^{-6} mol.	13.2 nmol.	127:1
No cells ²	6.81×10^{-7} mol.	5.02 nmol.	131:1

NB. 1-Cells were heat treated at 100°C for 5 min. No endogenous respiration was evident.

2-Oxygen use solely attributable to autooxidation.

Although this phenomenon was investigated further no cause could be found. Ideally, if possible, the removal of this activity would reduce any loss of 3-methylcatechol but the prevention of polymerization would seem improbable within the fermentation regime used.

In summary, the use of P. putida 2313 resulted in the production of 10.5 mM (1.3 g/l) 3-methylcatechol from toluene. The production of 3-methylcatechol ceased after 9 hours of feeding toluene and a variety of potential reasons for cessation of production were investigated. The accumulation of the product led to an inhibition of growth, viability, whole-cell oxidation of toluene and glucose uptake. Despite these effects, the prime cause of inhibition appeared to be due to an effect upon the toluene dioxygenase.

5.2 Investigation into the overproduction of catechol from benzene using P. putida 4(2) and 6(12), mutant derivatives of P. putida 2313.

In flask culture both 4(2) and 6(12) had shown enhanced levels of catechol production compared to the parent organism, P. putida 2313 (Chapter 3). Consequently, larger scale cultivation of these organisms was investigated and the results are presented here. Using the regime previously outlined for the production of 3-methylcatechol differences between the parent strain and the two mutants were evident when feeding benzene in the production phase. The results showed that induction of the benzene oxidation pathway and overproduction of catechol was

rapid using P. putida 6(12), reaching a maximum of 26 mM (2.86 g/l) after 8 hours in the production phase (Figure 5.2.1). As expected, the parent organism, P. putida 2313 showed negligible catechol production giving rise to a maximum of 0.4 mM (0.044 g/l) catechol. The most surprising result was obtained with the second presumptive mutant, P. putida 4(2), which accumulated levels of catechol intermediary to those afore mentioned. Levels of catechol for 4(2) reached a maximum of 5.6 mM (0.572 g/l) after 8 hours suggesting that inactivation of the intradiol cleavage pathway at the level of catechol 1,2-oxygenase had not been achieved. This was investigated using cell extracts prepared throughout the experiment. P. putida 4(2) had a reduced catechol 1,2-oxygenase activity which was induced later than the enzyme activity detected in the parent organism (Figure 5.2.2). The latter showed maximum activity after 2.5 hours, reaching a level of 360 nmol. catechol/min/mg. protein. P. putida 4(2) showed maximum activity after 6 hours, reaching a level of only 166 nmol. catechol/min/mg. protein. Unlike the parent organism, 4(2) was unable to grow on benzene as the sole carbon and energy source but had retained some ability to oxidise benzene, at least to the level of cis,cis-muconate. The temporal difference in induction of catechol 1,2-oxygenase may support the theory that a different intermediate of the benzene dissimilatory pathway is responsible for the induction of the intradiol cleavage pathway. Conversely, it may reflect the fact that more than one catechol 1,2- oxygenase is present, the difference being the affinity of each oxygenase for the substrate. The possibility of two separate inducers for the same catechol

1,2-oxygenases or the presence of two separately inducible enzymes has been discussed previously by Bayly and M^CKenzie (1976). The authors showed that in P. putida NCIB strain PsU-O there were two catechol 1,2-oxygenases, one induced by either catechol or cis,cis-muconate and the other induced by benzoate or some intermediate in the benzoate oxidase system. In the system under investigation it seems plausible that two analogous enzymes may exist allowing differential expression of the intradiol cleavage enzyme.

Although P. putida 4(2) shows some activity towards catechol it derives very little benefit in terms of growth in the presence of benzene. This is illustrated in Figure 5.2.3, showing that the growth curves of P. putida 6(12) and 4(2) when grown in glucose fed-batch culture in the presence of benzene were similar. Additionally, the yields were similar for both P. putida 4(2) and 6(12). The parent organism, P. putida 2313, achieved linear growth even in the presence of benzene. The growth rate, 0.295/h, compared favourably with the maximum specific growth rate shown when the organism was grown in the absence of benzene (Chapter 4).

As mentioned previously, benzene is more soluble than toluene. This is illustrated in Figure 5.2.4 which shows the benzene rapidly accumulating in the supernatant of the P. putida 4(2) culture. This reached a maximum of 4.5 mM which compared with maxima of 2.75 mM and 2.0 mM for P. putida 2313 and 6(12) respectively. These figures reflect the ability of the organisms to use the aromatic substrate. Both the parent organism and the mutant 6(12) possessed the ability to rapidly oxidise benzene.

Each culture was grown in batch mode as described previously (Figure 4.3). Glucose (1 g/l/h) and benzene (200 ml/min) feeds were started at T=0. Catechol and benzene were measured using HPLC and GC respectively as shown in Materials and Methods. Cell samples (25 ml.) were removed throughout the biotransformation, washed twice in 50 mM potassium phosphate buffer (pH 6.8) and resuspended in the same buffer (2.5 ml.). Cell suspensions were stored at 4°C until required. Cell extracts were prepared using the procedure outlined in Materials and Methods. Catechol 1,2-oxygenase was assayed using the method of Hegeman (1966).

Figure 5.2. Preliminary studies into the production of catechol from benzene in glucose fed-batch culture using *P. putida* 2313, 4(2) and 6(12).
Fig. 5.2.1.

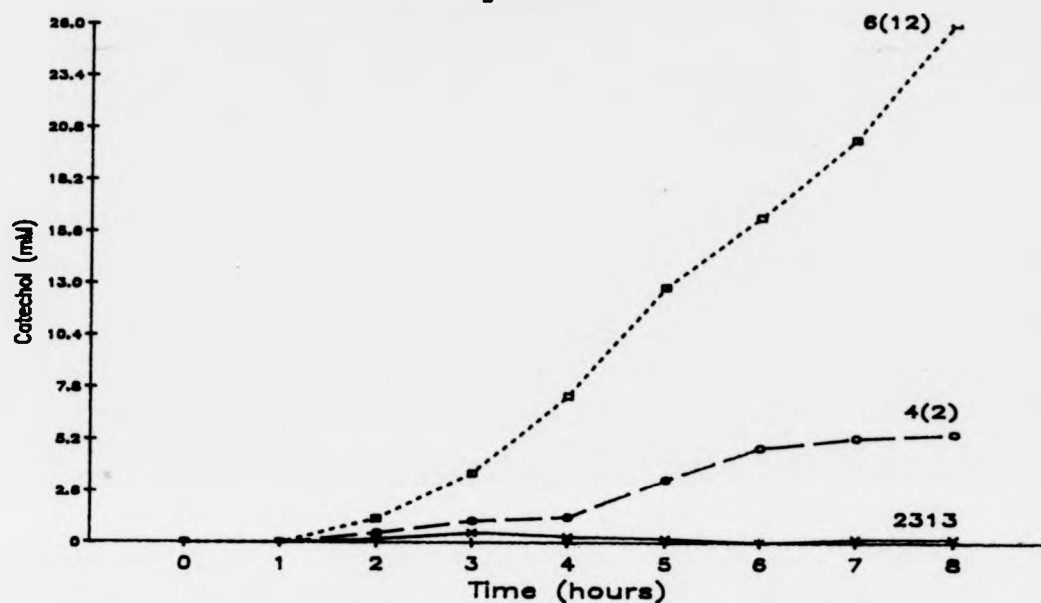


Fig. 5.2.2

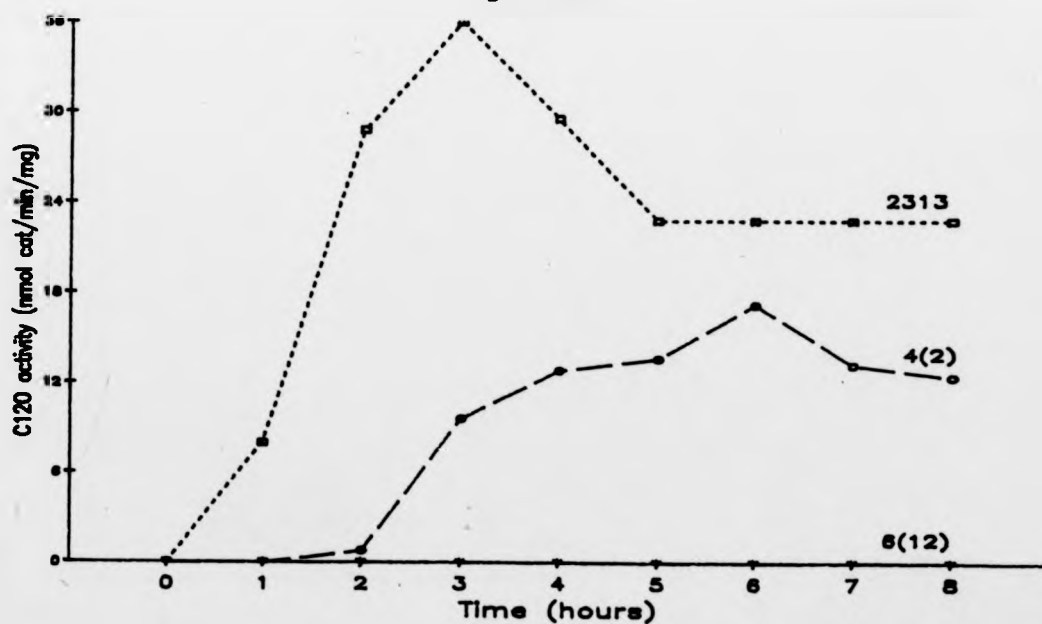


Fig. 5.2.3

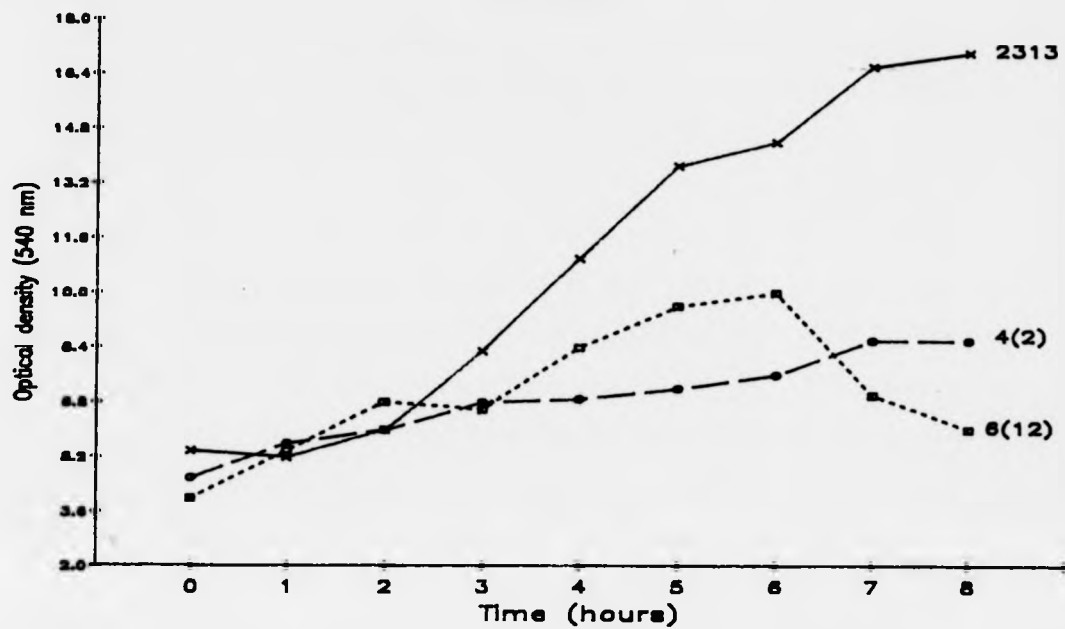
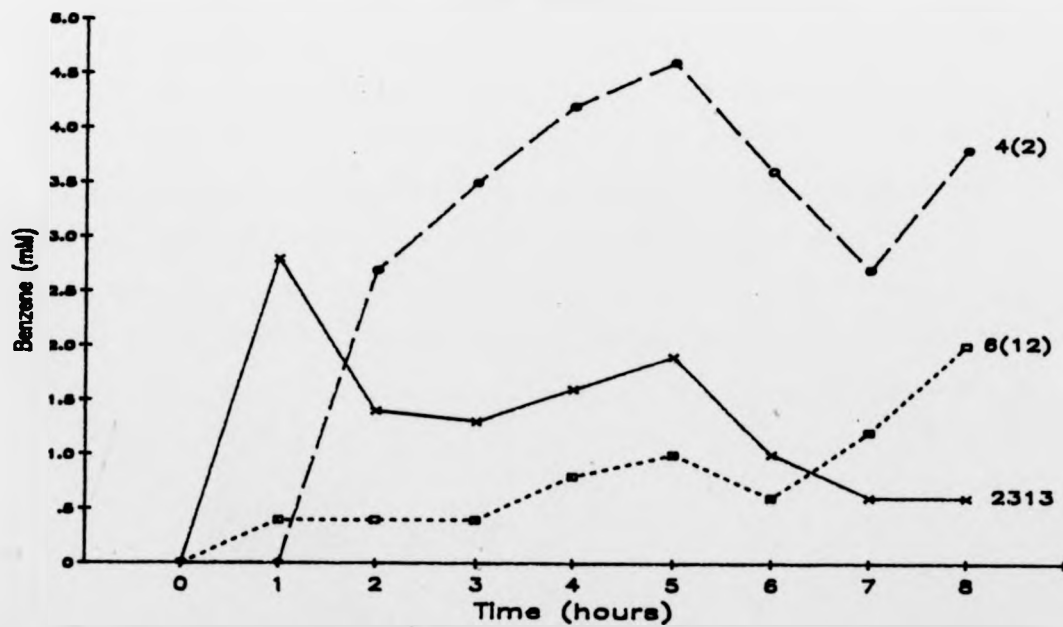


Fig. 5.2.4



However, P. putida 4(2) was unable to rapidly oxidise benzene to tricarboxylic intermediates, demonstrated by its inability to produce high levels of catechol or conversion of the co-oxidised substrate into biomass.

5.3 The production of catechol from benzene using P. putida 6(12), a mutant derivative of P. putida 2313.

The results of the previous experiment indicated that P. putida 6(12) would produce catechol from benzene. Unlike P. putida 4(2), 6(12) completely lacked catechol 1,2-oxygenase and rapidly accumulated catechol to a level of 26 mM (2.8 g/l). For these reasons P. putida 6(12) was studied in detail to assess product toxicity and compare it with the production of 3-methylcatechol by the parent organism, P. putida 2313.

The production of catechol from benzene occurred over a 10 hour period reaching a maximum of 27.5 mM (3 g/l) when the biotransformation was repeated (Figure 5.3.1). This figure corresponds very closely with that shown in the preliminary study and matches that previously reported by Shirai (1987). The level of catechol subsequently fell overnight to 18 mM (1.98 g/l). This was due to the auto-oxidation of catechol, resulting in the production of an insoluble polymer in both the aqueous phase and at the surface of the cells. The latter was characterised by the presence of a black pellet when cells from the end of the production phase were prepared by centrifugation.

The level of benzene present in the medium reached a maximum

of 3.6 mM, a level higher than the toxicity threshold suggested by Bringmann and Kühn (1980) but below the maximum solubility. Surprisingly, even after maximal production had occurred the level of benzene present in the aqueous phase did not increase, due to constant aeration and the slightly elevated temperature. During the biotransformation the maximum rate of catechol production was 0.46 g catechol/h/g total cell protein, achieved after full induction of the culture between 6 and 8 hours.

In a manner analogous to the toluene system there was little net increase in biomass after the benzene feed had been started (Figure 5.3.2). This was supported by measurements of optical density and total cell protein.

Cell viability and reversion were assessed throughout the experiment and the results are presented in Figure 5.3.3. The total number of viable cells fell by approximately 3 orders of magnitude during the biotransformation, with the largest decrease occurring after maximum product levels had been attained. The reversion frequency at the start of the production phase was 10^{-5} but this had increased to 10^{-1} at the end of the biotransformation. This suggested that the loss of bioconversion ability was ultimately due to reversion to wild-type.

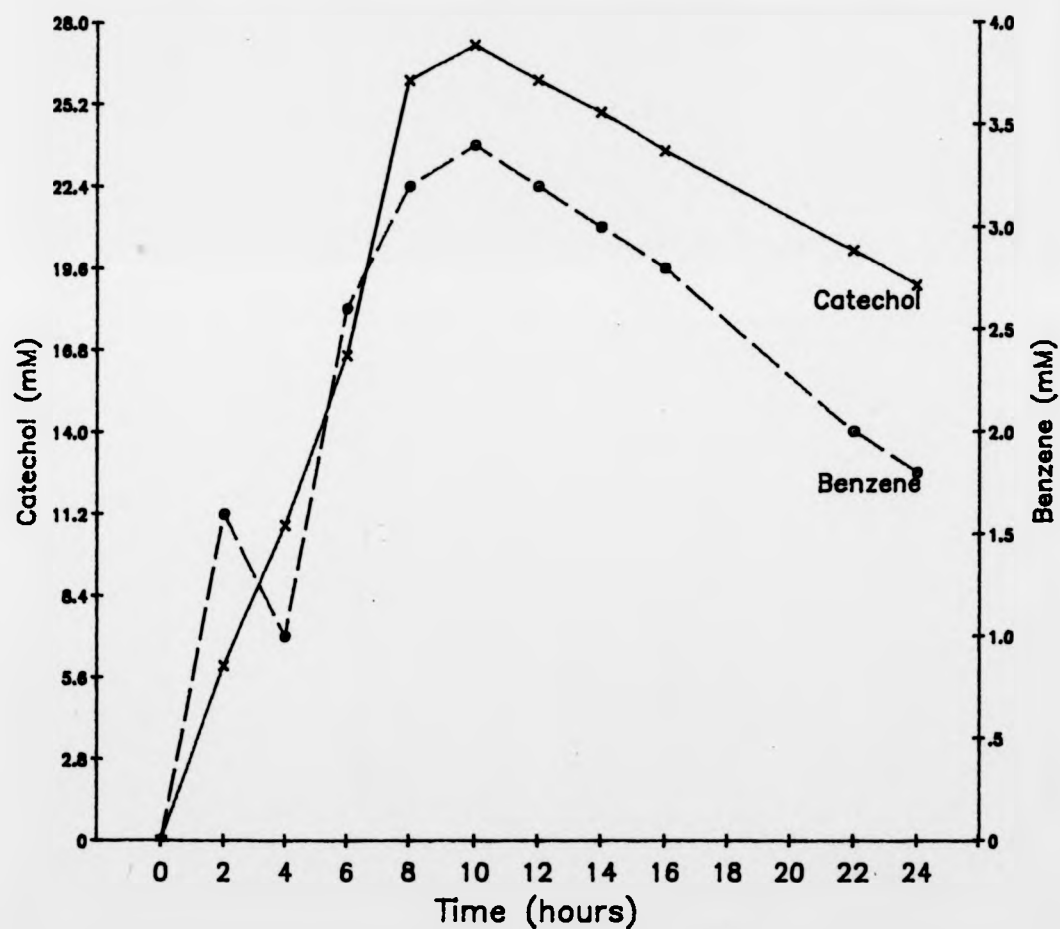
Cell extracts were prepared at intervals during the biotransformation and the specific activities of key enzymes were determined. Using both the polarographic method of Beechey and Ribbons (1972) and the spectrophotometric indole assay method of Jenkins and Dalton (1985) no benzene dioxygenase activity could be detected in any of the extracts. Maximal

benzene cis-glycol (BCG) dehydrogenase activity, using BCG as a substrate, was 54 nmol NAD⁺/min/mg protein (Figure 5.3.4) measured after 10 hours. Using toluene cis-glycol (TCG) as substrate the maximum dehydrogenase activity was 71 nmol NAD⁺/min/mg protein. Despite the fact that the organism was growing in the presence of benzene the BCG dehydrogenase showed higher activity with TCG as the substrate. This has been shown previously with naphthalene cis-glycol dehydrogenase (Patel and Gibson, 1974) and toluene cis-glycol dehydrogenase (Simpson et al., 1987). The former authors, using extracts prepared from P. putida biotype A, showed that a benzene-grown extract displayed a 1.72X higher specific activity of BCG dehydrogenase when TCG was used as the substrate compared with BCG. Simpson et al., (1987) suggested that substrates which possess a side group have a greater affinity for the enzyme by virtue of their increased hydrophobicity. In the present study, cis-glycol dehydrogenase activity with either TCG or BCG reached maxima coincident with the maximum rate of catechol production. The activity of BCG dehydrogenase fell after prolonged incubation of the cells with high levels of catechol, dropping 38% to 34 nmol NAD⁺/min/mg protein. Neither catechol 1,2-oxygenase nor catechol 2,3-oxygenase were detected throughout the production phase, despite the high reversion frequency at the end of the production phase. One possible explanation is that enzyme recruitment may have been used to cleave the available catechol, enabling it to be used by other dissimilatory routes. The most likely cleavage enzyme responsible was thought to be protocatechuate dioxygenase, an enzyme which furnishes

intermediates of the β -ketoadipate pathway. Although protocatechuate dioxygenase was assayed throughout the biotransformation no activity was found in crude cell extracts. This supports the conclusion that the reduction in catechol present in the supernatant was due to auto-oxidation and the production of an insoluble phenolic polymer.

Whole-cell oxidation of toluene, benzene and catechol was monitored throughout the production phase using the oxygen electrode. No oxidation of catechol was observed either in the presence or absence of cells, distinguishing it from the enhanced auto-oxidation shown in the toluene based biotransformation using 3-methylcatechol as substrate. This observation provides strong evidence for the view that the unsubstituted monomer is inherently more stable than the 3-methylcatechol derivative. In whole cell studies, benzene oxidation reached a maximum of 43 nmol O₂/min/mg protein after 5.5 hours but this fell sharply as catechol production reached a maximum (Figure 5.3.5). Using toluene as substrate the same pattern of whole cell oxidation was observed. Both results supported the view that the major effect of catechol was due to its inhibition of the initial oxygenative step of aromatic substrate utilisation. Unfortunately, this was not supported by in vitro assays since benzene dioxygenase could not be detected in cell extracts. Previously, when activity was observed using cells grown in the presence of toluene, the crude cell extract was prepared immediately after harvest and assayed soon after. In the present experiment using cells grown in the presence of benzene it was not possible to prepare the crude cell extract

Figure 5.3. The production of catechol from benzene by *P. putida* 6(12) in glucose fed-batch culture.
Fig. 5.3.1.



All data shown in Figures 5.3.1 - 5.3.5 was obtained using the same procedures outlined for Figure 5.1.1 - 5.1.7.

Fig. 5.3.2.

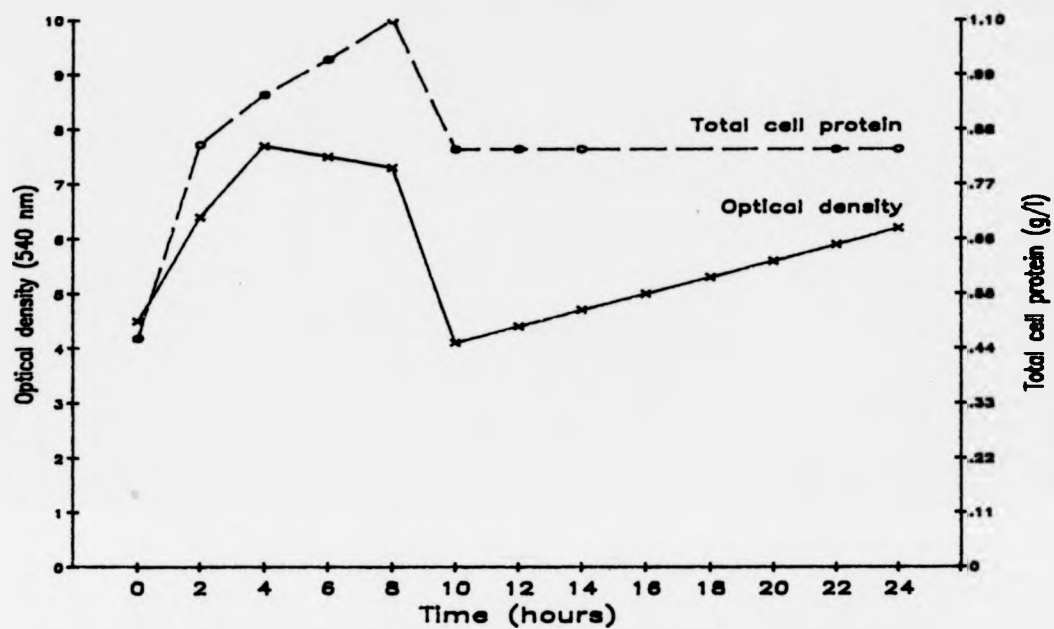


Fig. 5.3.3.

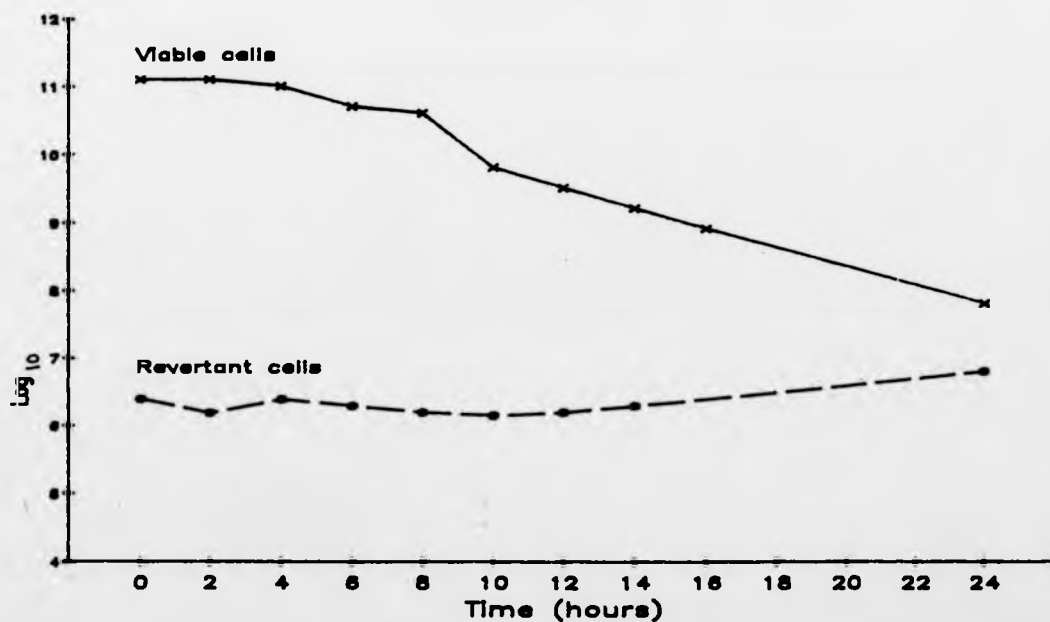


Fig. 5.3.4.

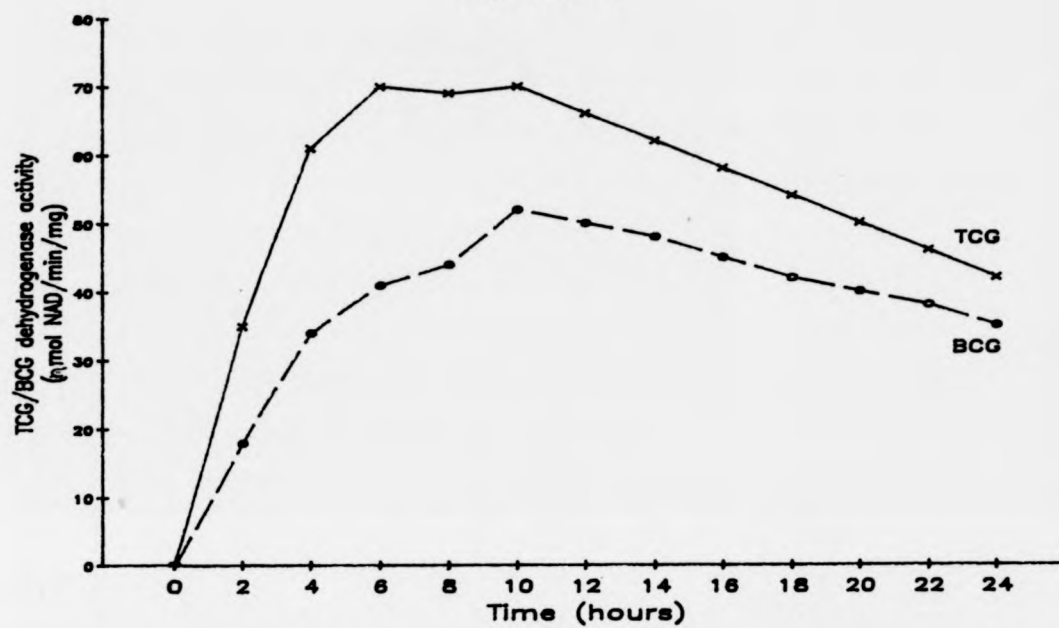
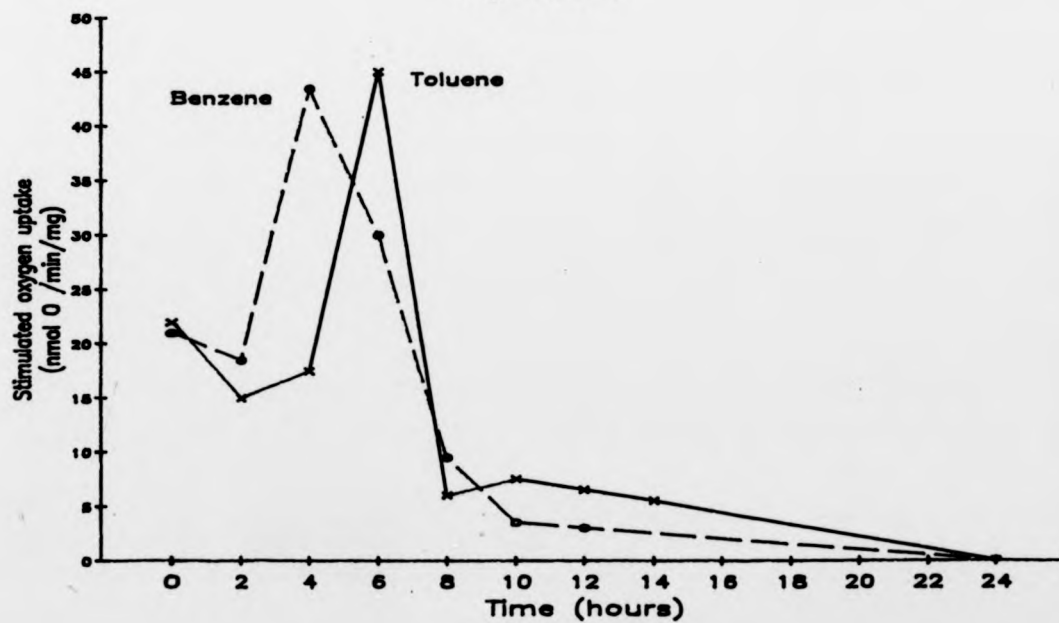


Fig. 5.3.5.



immediately. Consequently, the cells were stored at -20°C prior to use and this may have led to the lack of activity observed thereafter. Although this was the only major difference in the preparation of the extract it seems unlikely that it led to the inactivation of the enzyme in the light of the procedures adopted by other workers (Axcell and Geary, 1973; Yeh *et al.*, 1977; Zamanian and Mason, 1987). All of these workers studied either toluene or benzene dioxygenase and achieved active enzyme preparations after storing the harvested cells at -20°C prior to the preparation of the extracts.

5.4 Summary - Comparison of the production of catechol from benzene and 3-methylcatechol from toluene.

The data presented in this chapter show that similarities exist between the biotransformation for the production of 3-methylcatechol and catechol. These similarities are best outlined as follows:-

- (i) Maximum production was achieved within 10 hours of the production phase.
- (ii) Both systems achieved maximum rates of production of 0.46 ± 0.005 g product/g cell carbon/h.
- (iii) Growth ceased at the onset of the production phase.
- (iv) Whole cell oxidation of both benzene and toluene was inhibited by their respective products.

The major difference between the production of catechol and 3-methylcatechol was their final concentrations in the culture

supernatant. Catechol reached a maximum of 27.5 mM (3g/l) whereas 3-methylcatechol was only 11.5 mM (1.27 g/l). This provides strong evidence that catechol is inherently more stable than its methylated derivative. Irrespective of the final level of product attained it would seem that both products are toxic to the cell and the primary site of toxicity is the initial aromatic dioxygenase.

CHAPTER 6. THE DEVELOPMENT OF A PRODUCT REMOVAL
SYSTEM FOR THE PRODUCTION OF 3-METHYLCATECHOL.

CHAPTER 6.

THE DEVELOPMENT OF A PRODUCT REMOVAL SYSTEM FOR THE PRODUCTION OF 3-METHYLCATECHOL.

INTRODUCTION.

The results presented in Chapter 5 showed that the maximum levels of 3-methylcatechol which could be obtained by microbial fermentation were 10-18 mM (1.24-2.23 g/l). Even after prolonged exposure to maximum levels of product the viability of the cells only fell by 2 orders of magnitude. It was reasoned from this that it may be possible to remove the product in situ and maintain it at sub-inhibitory levels, promoting continued production of 3-methylcatechol and result in higher levels of product.

Many systems have previously been used to remove inhibitory products. These include vacuum fermentation (Cysewski and Wilke, 1977); flash fermentation (Ghose et al., 1984); extractive fermentation (Minier and Goma, 1982; Kuhn, 1980); dialysis fermentation (Friedman & Goden, 1970) and the use of adsorption (Wang et al., 1981) and ion-exchange resins (Tongnu and Ghose, 1981). Only the use of adsorption and ion-exchange resins were considered here because they are the easiest to employ and give an insight into whether product toxicity can be alleviated. Solid, porous adsorbents with extremely large surface areas, ranging from activated carbon to polymeric resins, have been used to remove metabolites from fermentation broth. They can be either added directly to the fermentor or be placed in a separate vessel with broth circulating through the adsorbent.

Good results have been obtained in situ for the removal of

the antibiotic cycloheximide from fermentation broth (Wang et al., 1981; Wang, 1983). The production of the antibiotic by Streptomyces griseus is feedback regulated by the product. The addition of a neutral polymeric resin to the broth reduced the amount of free cycloheximide and resulted in a 100% increase in final product yield.

Tangnu and Ghose (1981) used a Corynebacterium renale strain for the production of salicylate from naphthalene. The use of an anion-exchange resin (Amberlite IRA-400) packed in a column resulted in a doubling of salicylate produced when the broth was recirculated between the column and fermentor. Ion-exchange resins have rarely been used for the in situ recovery of ionic products because the ions that are released upon product adsorption may inhibit microbial growth reaching toxic levels in a continuous culture. Despite this, Raymond et al. (1969) showed that the presence of weakly basic polystyrene-polyamine resin (IR-45) or a macro-reticular type resin (IRA-93) in the culture could aid biotransformations. Using the afore mentioned resins the authors showed that concentrations of p-toluic acid, 2,3-dihydroxy-p-toluic acid and alpha, alpha-cis, cis dimethylmuconic acids all increased when the resin was present in co-oxidation systems using Nocardia strains able to transform p-xylene.

In the present study, the presence of catechols in the culture supernatant had interfered with glucose and ammonia assays which relied on the production of an indophenol dye. This had been overcome by the use of Amberlite XAD-4, a polymeric adsorption resin particularly effective for the removal of phenol and substituted phenols from aqueous streams. It differs

from ion-exchange resins in that it has no ionic functional groups incorporated into its resin structure, an important property when considering its possible use in situ.

The use of Amberlite XAD-4 was compared with that of granular activated charcoal. Compared to the former, the latter is inexpensive, easy to use and available in many grades and sizes. Its many uses have been well reported (Cavagnaro, 1980) but its major use has been in the water supply industry where it is successfully used for taste and odour removal. Its high effective surface area ($1050 \pm 50 \text{ m}^2/\text{g}$ in the present experiment) and relative inertness when used in biological systems has facilitated its use for haemoperfusion. Other uses have included the removal of the antibiotic patulin from cider (Sands et al., 1976) and the facilitation of enzymatic hydrolysis of cellulose (Khan et al., 1985). In the latter, the authors were able to improve the saccharification of cellulose by Trichoderma cellulases. Charcoal adsorbed both cellobiose and glucose, minimising end-product inhibition, without affecting the enzymatic hydrolysis of cellulose.

Since this process was concerned with the overproduction of an organic compound, studies were undertaken to establish whether activated charcoal would remove the product efficiently. Other factors considered were the effect activated charcoal may have upon the composition of the medium, and whether it would entrap cells and reduce the biomass present in the bioreactor.

RESULTS AND DISCUSSION.

6.1 Preliminary Studies.

Amberlite XAD-4 was selected for use because of its proven

ability to remove phenolic compounds. The first study undertaken was to establish a minimum weight:volume ratio which could be used for the removal of 3-methylcatechol. The results shown in Table 6.1 illustrate that a 1:10 (w/v) ratio was sufficient to achieve optimum removal of the product.

Having established that 3-methylcatechol could be removed from an aqueous system using the resin the efficacy of removal was assessed. Standards of both compounds (5, 10 and 20 mM) were left to adsorb overnight in the presence of Amberlite XAD-4 at a ratio of 1:10 (w/v). The concentration remaining in solution was assayed by HPLC. The resin was then placed in an equivalent volume of acetonitrile:water (7:3) and agitated for 10 minutes. The resultant solvent/product mixture was then assayed by HPLC and the results are shown in Table 6.2. A control experiment using the adsorption resin alone showed no detectable leakage of any interfering aromatic substances under the conditions described. The efficacy of removal was found to be dependent upon the concentration of product initially present. Using 20 mM 3-methylcatechol, 76% was eluted although prolonged extraction increased this recovery.

Amberlite XAD-4 is a cross-linked polystyrene type polymer which should be preconditioned to remove all traces of preservative agents and monomeric compounds. In all studies undertaken the resin was not preconditioned and the adsorption and elution conditions were not optimised. This was felt unnecessary until in situ studies had shown that Amberlite XAD-4 relieved product inhibition.

6.2 In situ studies into the use of Amberlite XAD-4 for the

Table 6.1. Effect of varying the amount of Amberlite XAD-4 on the removal of 3-methyl catechol.

Ratio (w/v)	1:1	1:2	1:3	1:4	1:5	1:10
% Remaining in solid	7.7	2.5	10.3	3.9	4.8	2.1

All samples initially contained 10 ml of 20 mM 3-methyl catechol.

Table 6.2 Preliminary study of the efficacy of catechol removal from Amberlite XAD-4 after elution with acetonitrile/water.

Compound	Concentration of standard adsorbed (mM)	% Recovery
Catechol	5	68
	10	84
	20	83
3-Methyl catechol	5	47
	10	61
	20	76

removal of 3-methylcatechol.

A comparative study of the use of Amberlite XAD-4 was undertaken using a 0.9 l fermentor operating as a chemostat. All biotransformations were done using a mineral salts medium supplemented with 4 g/l glucose. The pH and temperature were 6.8 and 30°C respectively.

The following biotransformations were set up to compare the production of 3-methylcatechol under controlled conditions:-

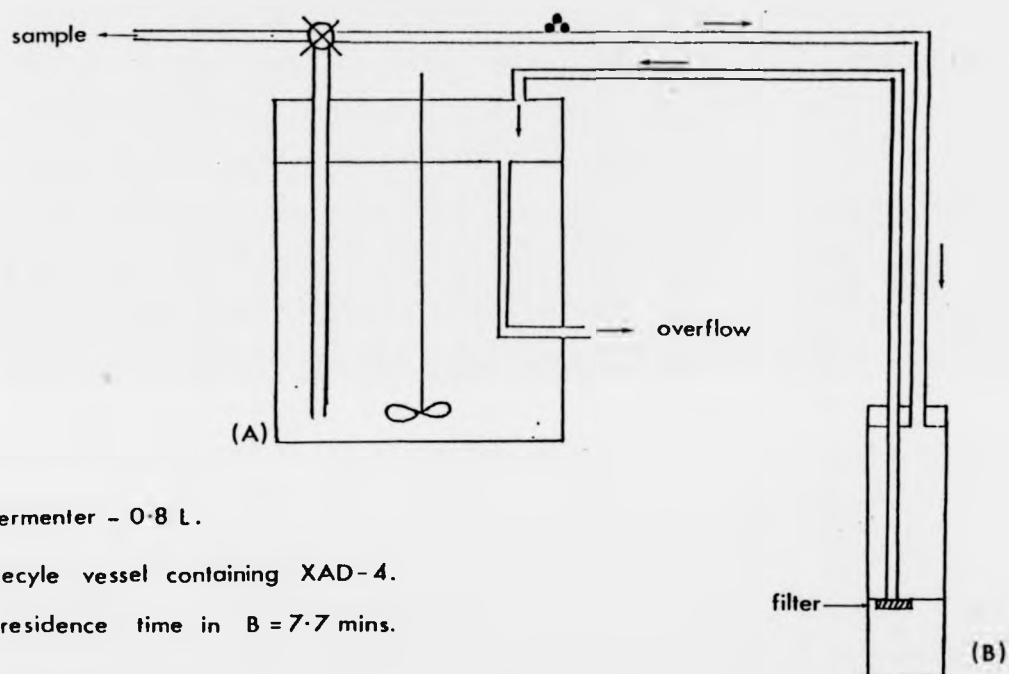
(i) P. putida 2313 grown at a dilution rate of 0.1/h in the presence of toluene.

(ii) As (i) with the inclusion of Amberlite XAD-4 (40g) in the bioreactor.

(iii) As (i) with Amberlite XAD-4 (30g) contained in a separate recycle vessel (Figure 6.1).

The results of the experiment are shown in Figures 6.2.1-6.2.3. In the absence of resin the product accumulated to a final concentration of 5.6 mM (0.7 g/l). When the resin was present within the recycle system the product reached a maximum of 3.8 mM (0.47 g/l) compared to 1.3 mM (0.16 g/l) when the resin was placed in the fermentor vessel (Fig. 6.2.1). The product concentration in the supernatant of the recycle system was higher than that expected and this was thought to be due to saturation of the adsorption resin resulting in retention of the product within the culture vessel. The data presented in Figure 6.2.2 illustrates that when the Amberlite XAD-4 was present in the recycle system the biomass present in the culture vessel decreases dramatically during the production phase. This

Figure 6.1 Schematic representation of Amberlite XAD-4 recycle system.



(A) Fermenter - 0.8 L.

(B) Recycle vessel containing XAD-4.

residence time in B = 7.7 mins.

suggested that the cells were being retained in the recycle vessel. Microscopic analysis of the resin showed that microbial cells were entrapped within the matrix of the beads. The operation of the system as a chemostat was also contributory to the dilution effect observed. The removal of cells in the recycle system was also supported by the observation that glucose levels in the supernatant were shown to increase dramatically as soon as the production phase was initiated (Fig. 6.2.3). It is interesting to compare these figures with those obtained in the system free of any adsorption resin. When the product level reached a plateau the detectable glucose increased suggesting that the viability of the cells was affected on exposure to higher levels of 3-methylcatechol.

After completion of the biotransformation the resin was recovered to assess the amount of product adsorbed. The potential of different solvent systems to remove the product were examined. The results of these extractions are shown in Table 6.3. Acetone was used to elute the product from Amberlite XAD-4 but was subsequently found to cause drifting of the retention times when assayed using HPLC. The product in the acetone fraction was therefore extracted a second time into diethyl ether and the product measured. The amount of product recovered from the recycle column (1.116 g) was greater than that obtained from the resin contained in the culture vessel (0.161 g). However, the total amount of product in the system without Amberlite XAD-4 was 0.56 g. This was greater than that recovered from the resin contained within the bioreactor and suggested that the presence of Amberlite XAD-4 was inhibitory when present in the culture vessel. It must be remembered, that

Figure 6.2. The effect of Amberlite XAD-4 upon the production of 3-methyl catechol by *P. putida* 2313.
Fig. 6.2.1

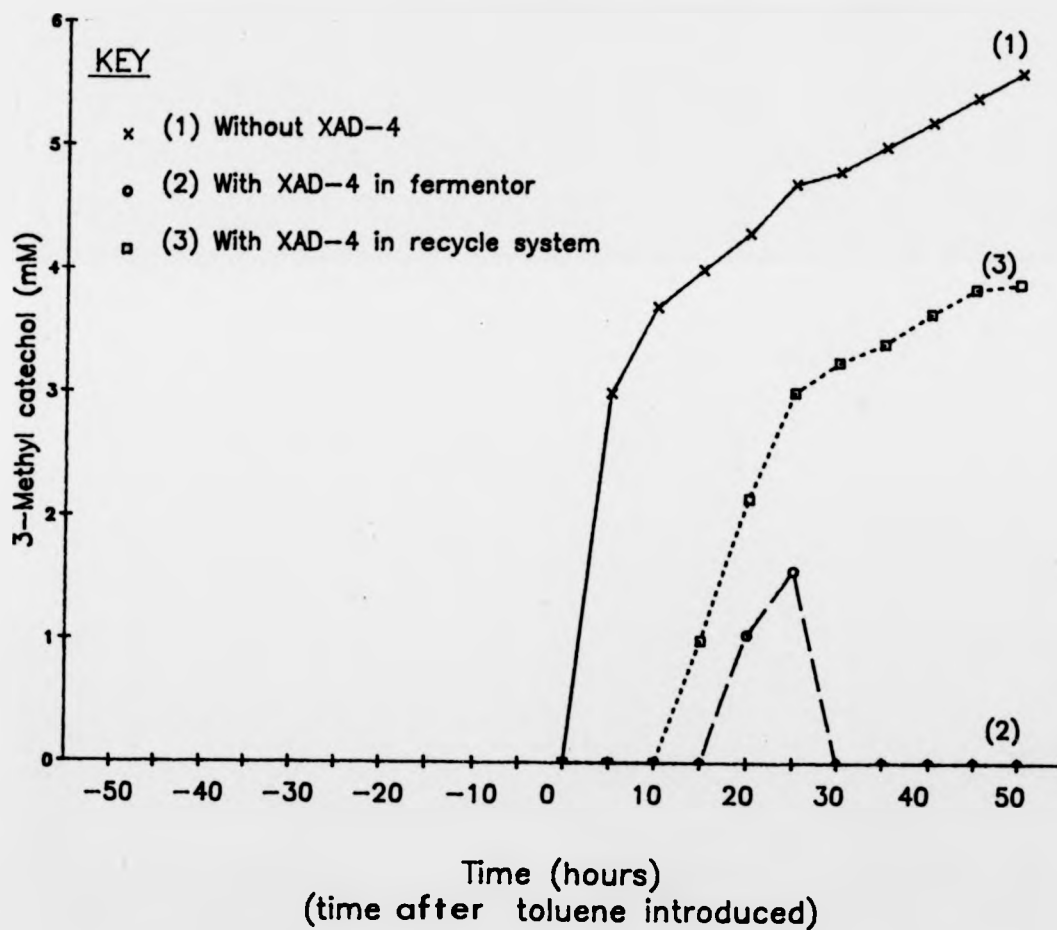


Fig. 6.2.2

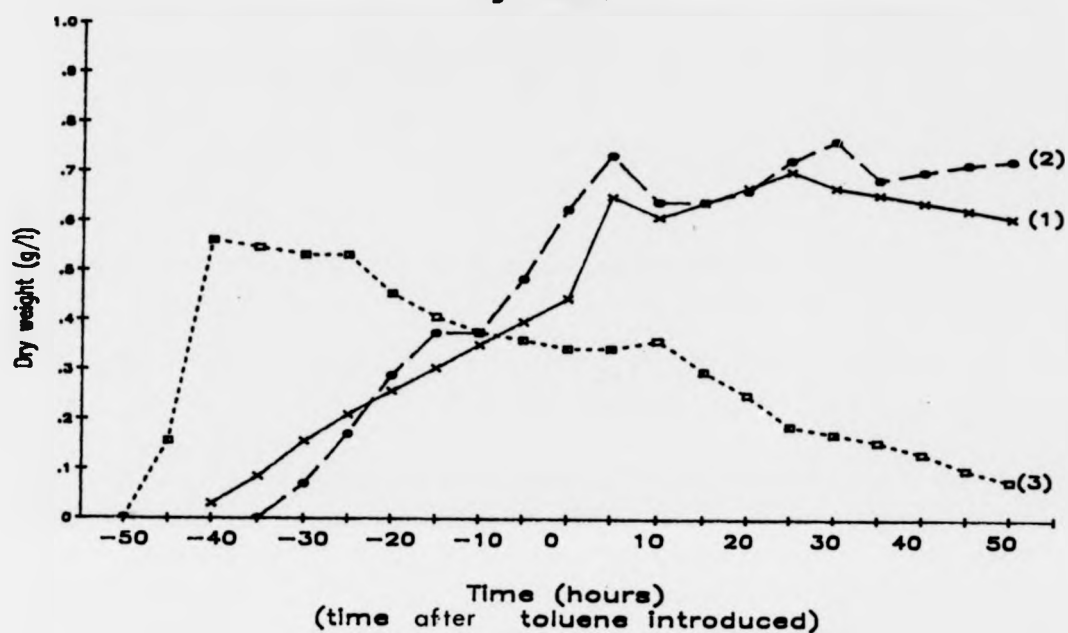


Fig. 6.2.3

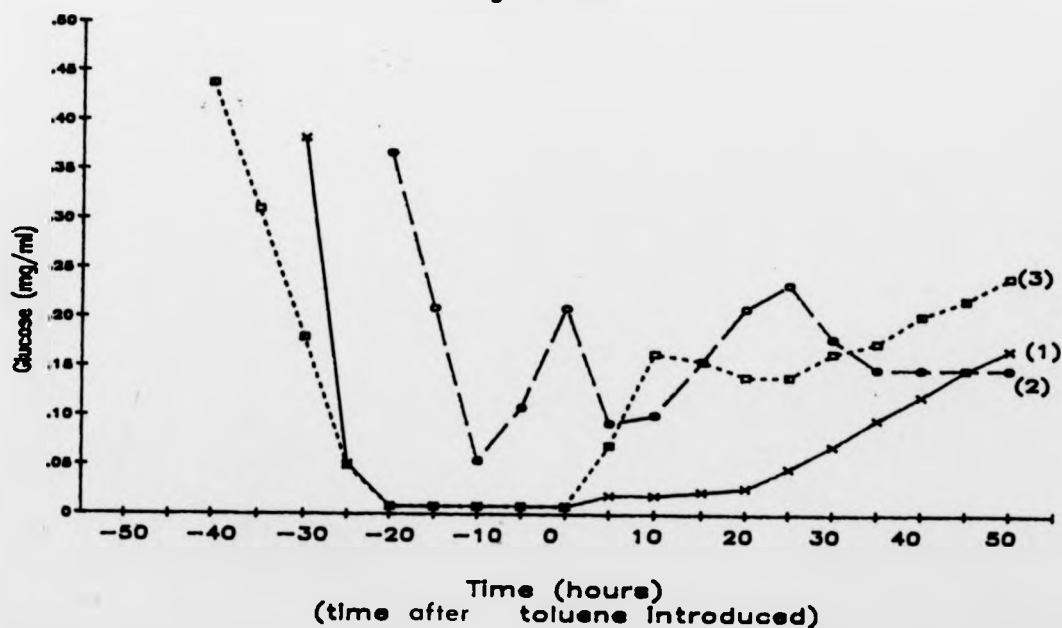


Table 6.3 Recovery of 3-methyl catechol from Amberlite XAD-4.

Solvent	Origin of XAD-4	[3-methyl catechol] (mM)	Volume (ml)	Total product (g)
Diethyl ether	(A)	26	50	0.161
Diethyl ether	(B)	180	50	1.116

Key:- (A) Chemostat containing Amberlite XAD-4 in bioreactor
without broth recycle.

(B) Chemostat containing Amberlite XAD-4 in broth recycle
vessel.

when present in the bioreactor, the Amberlite XAD-4 was present throughout the growth and production phases. This increased the amount of attrition of the resin causing possible leakage of preservative agents and monomers prior to the production phase. Although some of the preservative agents were removed in the washing steps prior to use, it was impossible to remove all traces which may leak when constantly agitated. Further evidence for the inhibition caused by Amberlite XAD-4 was shown by the concentrations of glucose in the supernatant of the three cultures. Unlike the biotransformations using the recycle system and no Amberlite XAD-4, glucose was never limiting in the culture containing the resin in the bioreactor (Figure 6.2.2). This suggested that inhibition of P. putida 2313 occurred prior to the initiation of the production phase. Separation of growth and production phases was therefore deemed necessary for optimum product recovery.

Although the system used was not optimised the data presented showed that selective removal of the product was advantageous for enhancing product yield. The total amount of 3-methylcatechol recovered from the recycle system was 1.5 g in total, a threefold increase in that obtained in its absence. Unfortunately, the prerequisites for the large-scale production of catechols using a selective removal system are a high capacity to adsorb the product and the ability to easily regenerate the adsorption resin. In the small-scale system outlined the complete elution of 3-methylcatechol could not be accomplished. Auto-oxidation of the product gave rise to a black polymer within the resin and only harsh chemical treatments such as acetonitrile:water (7:3) could remove the adhered

contaminant. The use of harsh regeneration procedures affected the composition and integrity of the resin beads and rendered them useless for further work. Considering the high cost of the resin, the small particle size and the inability to regenerate it using mild chemical conditions it was not optimised further .

6.3 The use of activated charcoal for the removal of 3-methylcatechol.

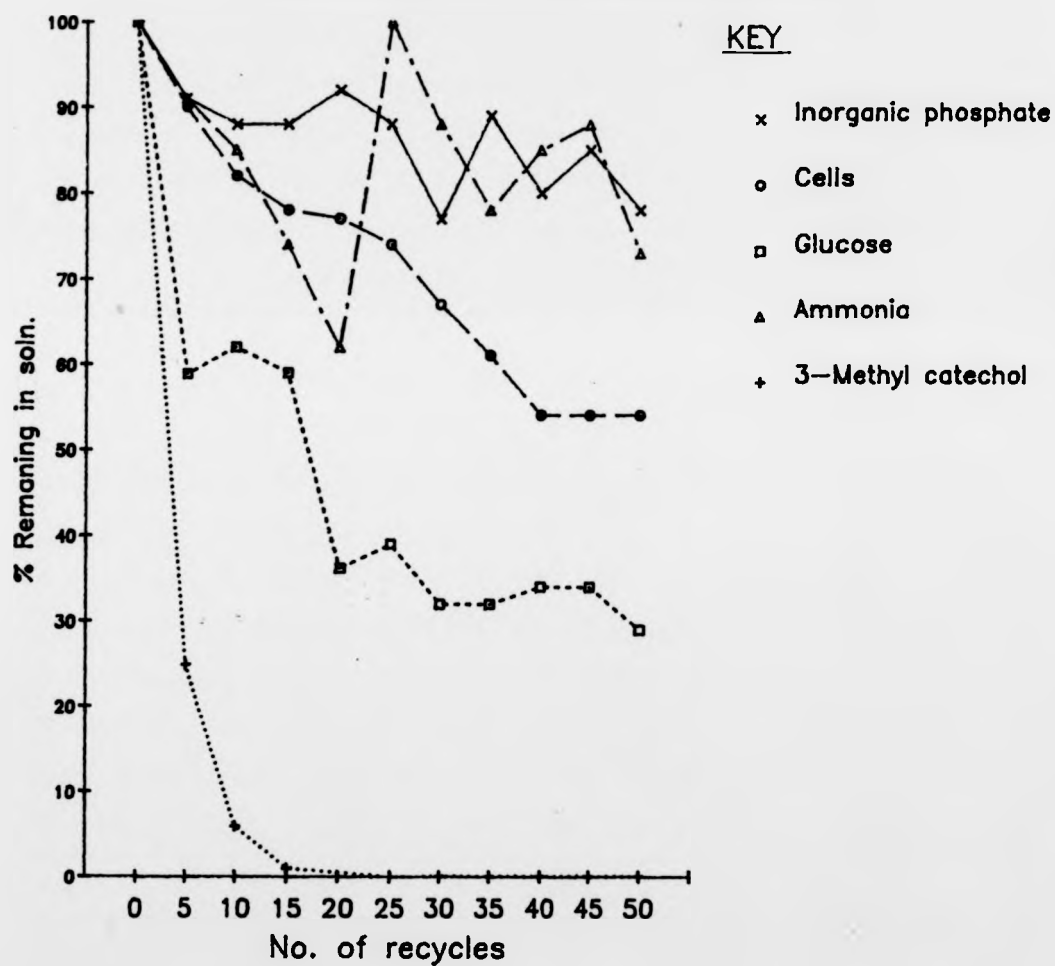
Amberlite XAD-4 was abandoned because it would be prohibitively expensive to use on a large-scale and had a very small particle size (0.35 - 0.45 mm). This caused it to act as a microbial sieve and reduced the amount of biocatalyst present in the culture vessel. A larger mesh size was unavailable and the use of activated charcoal was investigated.

Preliminary experiments were done to assess the adsorptive properties of the charcoal. The removal of 3-methylcatechol, microbial cells and several medium components was monitored and the results are presented. The data shows the rapidity with which the product, 3-methylcatechol, was removed (Figure 6.3). Glucose was rapidly adsorbed, with 70% removal occurring within 50 recycles (3.1 hours). Although this would seem to prohibit its use in a glucose fed-batch culture it must be remembered that the recycle system would only operate during the production phase and the glucose was to be fed at growth-limiting concentrations. Biomass removal posed a greater problem in that 40% removal over a 3 hour period would place serious constraints upon the use of the system should it occur in situ. It may have been possible to overcome this by reducing the bed volume of the column, thus allowing greater flow through the column and

Activated charcoal (200 g) was placed in a glass column (300 mm x 20 mm) to give a filter with a dead volume of 225 ml. Aqueous solutions (1 L) of the components under investigation were prepared as follows:- Inorganic phosphate (1 g/l), glucose (5.0 g/l), ammonia (3 g/l) and 3-methylcatechol (5 g/l).

Additionally, a 1L cell suspension of P. putida 2313 (initial $OD_{540} = 4.6$) was used in the experiment. Each solution was passed through a fresh column at 400 ml/min. and at the appropriate intervals samples were removed to assay the amount of each component remaining in solution. All analytical methods used are shown in Materials and Methods. Reduction in cell density was measured spectrophotometrically at 540 nm.

Figure 6.3. The efficacy of removal of medium components by activated charcoal.



reducing the biomass retained. Alternatively, the cells may eventually slough off when operated at different flow rates in situ.

Phosphate and ammonia concentrations were not adversely affected by repeated passage of the medium through the column. Although not measured, trace elements are known to be absorbed by activated charcoal (Heuss and Lieser, 1979). If this was shown to occur in the experiment a trace element supplement would be fed along with the glucose or the column would be pre-saturated with trace elements prior to use.

Having established that the system would effectively remove the product, the efficacy with which it could be recovered was assessed. This was done by removing the activated charcoal from this experiment and, using a continuous extraction system (see Chapter 2), extracting for 8 hours using 100% ethyl acetate. The result of this extraction was the recovery of >90% of the adsorbed product (7.5g adsorbed/7.1g recovered).

6.4 The production of 3-methylcatechol by *P. putida* 2313 using activated charcoal for product removal.

Previous attempts to achieve growth and concomitant production of 3-methylcatechol had been unsuccessful (Chapter 4). Work by Shirai (1987) had shown the production of catechol from benzene was best achieved using a resting cell culture. It was reasoned from this that the amount of glucose fed to the culture could be lowered to fulfill only the maintenance requirement. This served to allow cofactor regeneration for the biotransformation to occur and reduced the free glucose which may have been adsorbed by the recycle column.

Initially, an experiment was performed to find the minimum glucose requirement to sustain maintenance metabolism of the cells. A fed-batch culture was set up and, after overnight growth, the glucose was fed at diminishing levels until growth stopped and the stationary phase was maintained (Figure 6.4). After overnight batch growth a glucose feed of 0.5 g/l/h was initiated but this was gradually reduced to 0.16 g/l/h. This was maintained for the the final 20 hours of the experiment and the optical density and dry weights were monitored. The results showed that although the optical density dropped from 8.4 to 7.6 the cell dry weight remained constant at 2.2 g/l. In all subsequent experiments glucose was fed at 0.16 g/l/h, giving a maintenance requirement for P. putida 2313 of 0.073 g glucose/g dry wt./h.

Using the glucose feed outlined, a biotransformation system incorporating a recycle unit containing activated charcoal was set up. The recycle unit was run in parallel to the bioreactor and consisted of a glass tube (392 mm x 40 mm) containing 200g of granular activated charcoal (4-8 mm). The whole culture broth was repeatedly cycled through the column at 500 ml/min.

The level of 3-methylcatechol present in the supernatant increased rapidly when the production phase was started. When the product level exceeded 4 mM the recycling of the fermentation broth was initiated (Fig. 6.5.1). Over the following 40 hours the level of 3-methylcatechol was maintained below the inhibitory levels shown previously (Chapter 3). It had been anticipated that toluene may be stripped from the aqueous stream when the supernatant was passed over the activated charcoal. Although toluene may have been removed initially it

Figure 6.4 Investigation into the minimum glucose requirement for maintenance metabolism in *P. putida* 2313.

Fig. 6.4.1

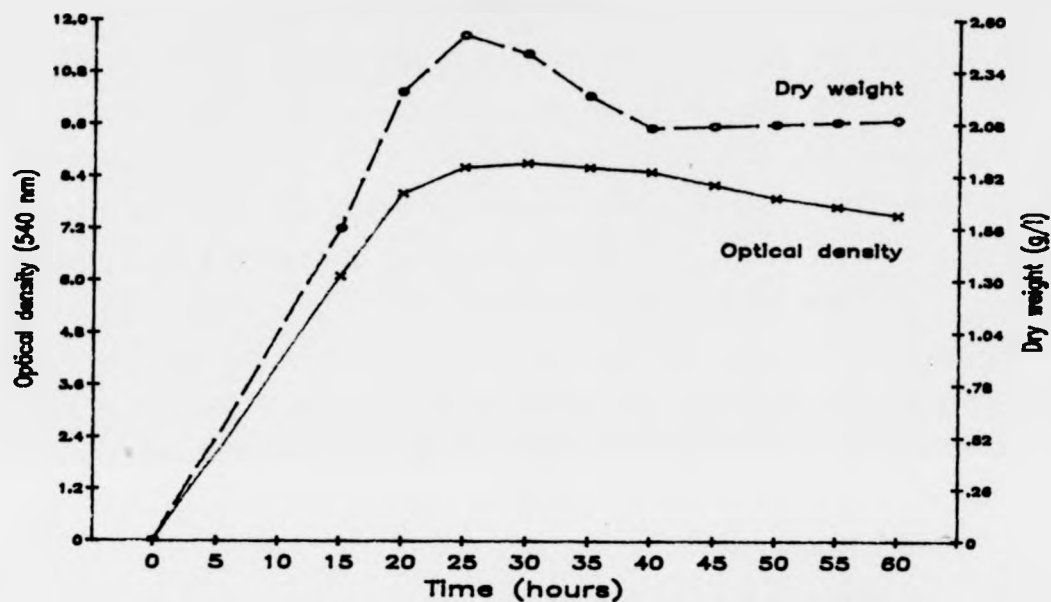
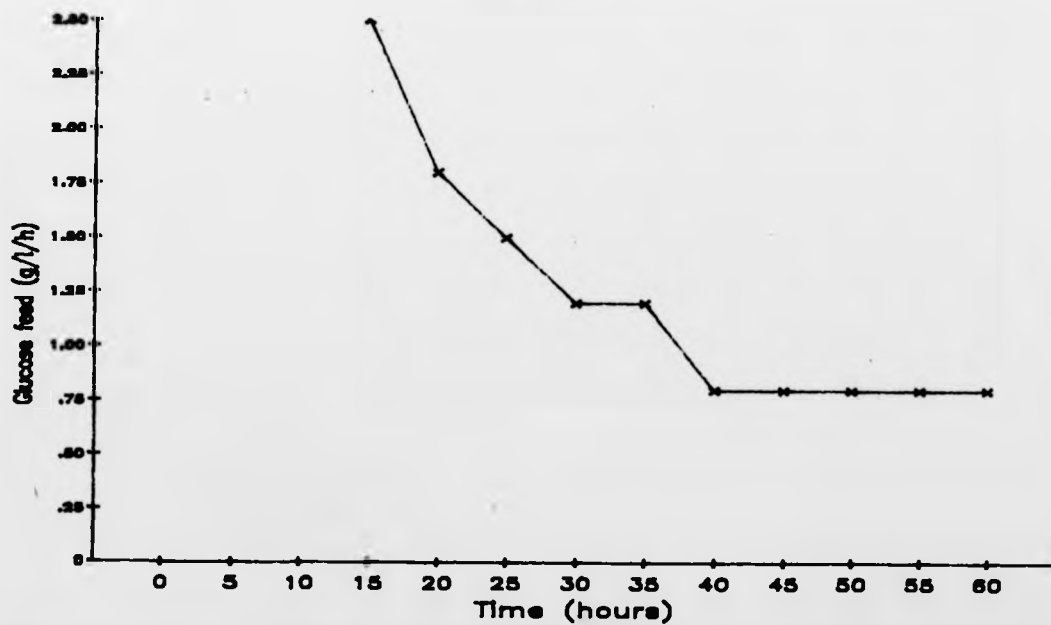


Fig. 6.4.2



remained in excess in the supernatant (Fig. 6.5.1).

Preliminary investigations had suggested that the cells were removed from the fermentation broth when repeatedly passed through the activated charcoal. Although this was supported by the measurements of optical density and dry weight at the start of the production phase the cell population was subsequently shown to increase again, probably due to cells sloughing off the column (Fig. 6.5.2). The viability of these cells may have been affected but this could not be shown in the present system because it was an axenic, closed system which prevented samples from being taken from within the matrix of the column. Throughout the production phase the dissolved oxygen tension was constant (86-90 %) reflecting the fact that the cells were in resting phase throughout the biotransformation.

The most interesting data obtained is shown in Figure 6.5.3. The figures obtained for dry weight and optical density showed that biomass fell during the first 25 hours, whereas the viability was shown to remain constant over the same time period. The number of viable cells subsequently fell by two orders of magnitude and, along with this, the level of reversion increased ten-fold such that at the end of the biotransformation process less than one in a hundred organisms were able to grow on toluene. The reduction in viability may have been due to either the retention of biomass on the activated charcoal or death and autolysis of the cells. The former was not borne out by either the dry weight measurements or optical density readings. This suggested that the biotransformation occurred primarily in the 25 hours after the recycling of culture broth had begun. Subsequently, the cells either died or reverted and

Figure 6.5. The effect of broth recycle upon the production of 3-methyl catechol by *P. putida* 2313.
Fig. 6.5.1.

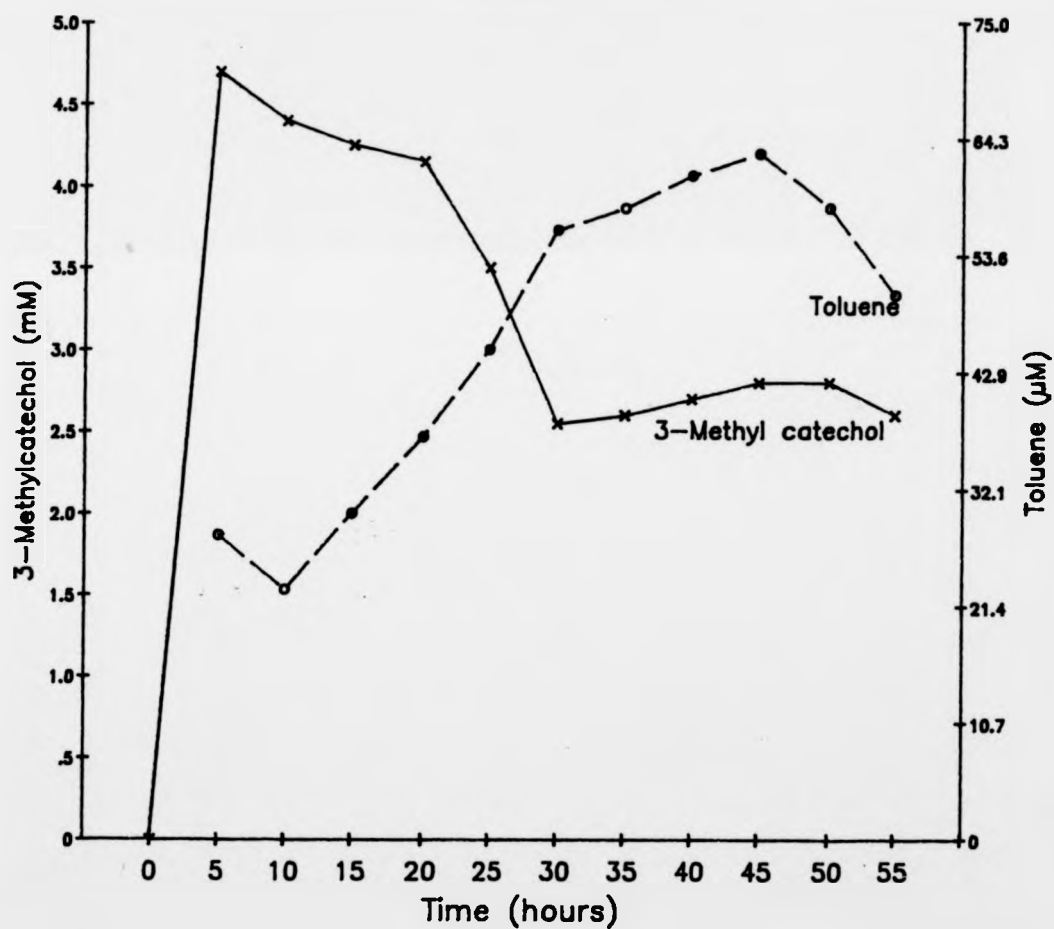


Fig. 6.5.2.

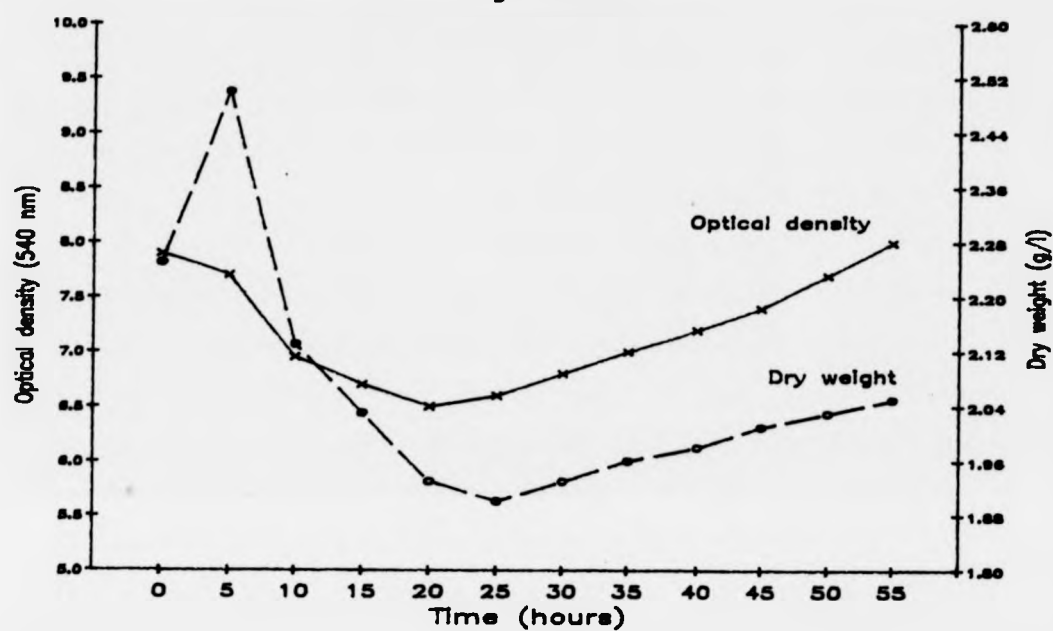
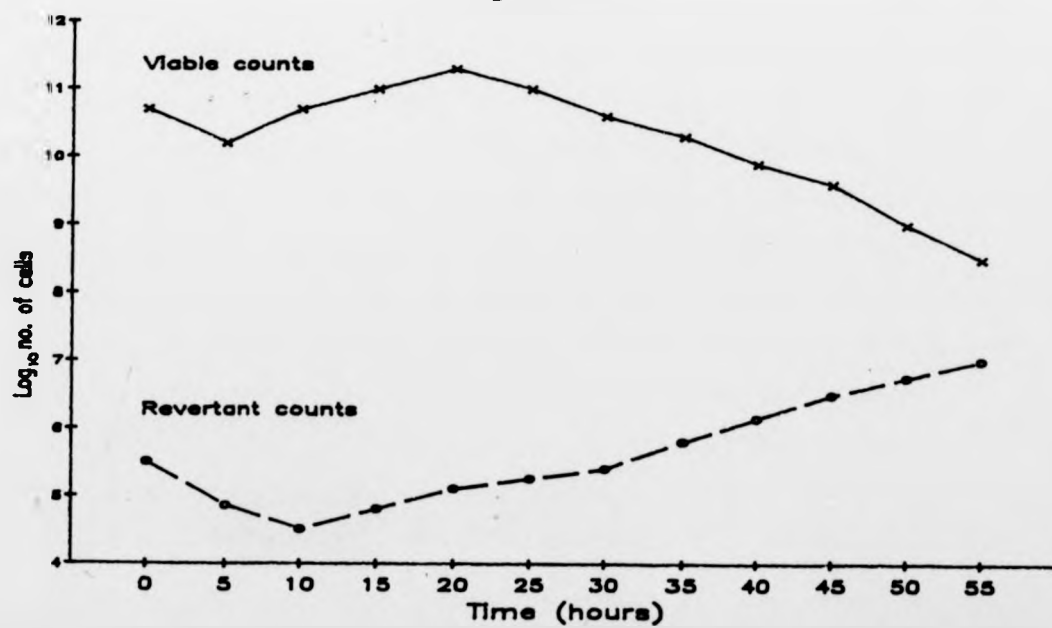


Fig. 6.5.3.



it was the revertant population which accounted for the increases observed in dry weight and optical density measurements.

After the production phase had ceased the activated charcoal was recovered and washed to remove any loosely attached debris. The charcoal was then dried and placed in 500 ml of ethyl acetate overnight. Following this, the charcoal was further desorbed continuously for a further 36 hours to remove as much product as possible. The total product recovered, as measured by HPLC, was 23.5 g; produced over 50 hours. This was produced using a 5 litre fed-batch culture and equated to 4.7 g 3-methylcatechol/litre (36.8 mM). Comparing this yield with that shown previously in the absence of any product removal system a 2-3-fold increase was demonstrated using the recycle system.

It was then considered necessary to determine whether an increase in the bed volume of activated charcoal would result in greater production of 3-methylcatechol. The biotransformation was repeated using the same conditions as above apart from a larger recycle system. The latter consisted of a larger column (800 mm x 35 mm) containing 700g of activated charcoal. Unlike the system operated previously the recycling of the culture was begun at the onset of the toluene feed to further reduce the possibility of 3-methylcatechol accumulating in the supernatant. The data obtained using a larger recycle column reiterated the findings made previously. The level of 3-methylcatechol in the supernatant was maintained below 4 mM throughout the production phase and the total cell protein remained constant at approximately 2.25 g/l (Fig. 6.6.1). However, unlike the previous biotransformation the dissolved oxygen tension was

maintained at approximately 90% until the final 7 hours of the production phase when it dropped to 50% (Fig. 6.6.2). The rapid decrease in dissolved oxygen tension is indicative of an additional requirement for oxygen. In this instance it could be due to the presence of a contaminant or, as is more likely, an increased oxygen requirement for the catabolism of toluene by a revertant population able to grow on toluene.

Work shown previously had demonstrated that the primary site of 3-methylcatechol toxicity was the initial oxidation of toluene catalysed by toluene dioxygenase (Chapter 4). In order to assess the efficacy of the recycle system used in the present experiment the whole cell oxidation of toluene and toluene cis-glycol, and the activity of TCG dehydrogenase in crude cell extracts were measured throughout the biotransformation. Unfortunately, no toluene dioxygenase activity could be detected in vitro. The data for TCG dehydrogenase activity showed that induction of the enzyme occurred within 2 hours and reached a maximum of 13 nmol NAD^+ /min/mg protein after 48 hours (Fig. 6.6.3). Surprisingly, the maximum specific activity of TCG dehydrogenase measured in this experiment was five-fold lower than that shown in the fed-batch system without the recycle system (62 nmol NAD^+ /min/mg protein). Despite this the relative specific activity of TCG dehydrogenase in the present experiment showed little inhibition throughout the biotransformation. The strongest evidence for the reduced product toxicity in the presence of activated charcoal was obtained from whole-cell studies using the oxygen electrode. In the absence of any removal system the whole cell oxidation of toluene reached a

maximum after 4 hours (90 nmol. O₂/min/mg protein) but then fell to zero in the following 20 hours. Results with the recycle system showed that the whole-cell oxidation of toluene reached a maximum after 9 hours (40 nmol O₂/min/mg protein) but the ability to oxidise toluene was retained throughout the duration of the experiment, a further 40 hours (Figure 6.6.4). Similarly, the endogenous rate of respiration followed a similar pattern throughout the biotransformation.

The activated charcoal was recovered from the recycle column and, after washing to remove loosely attached cell debris, was continuously extracted for 36 hours with ethyl acetate. The extracted material was analysed by HPLC and it was determined that 22.6g of 3-methylcatechol was recovered. This was equivalent to 4.52 g/l (36.5 mM) when the culture volume was taken into consideration. It was concluded that despite increasing the amount of activated charcoal threefold, the amount of product remained the same. Two possible explanations may account for this finding:-

(i) The use of 200g activated charcoal leads to saturation of the charcoal and the production phase needs to be longer to achieve an increase in the amount of product formed.

(ii) The production of 3-methylcatechol only occurred during part of the production phase and the maximum yield is governed by a factor aside from the extracellular 3-methylcatechol concentration.

After measuring the amount of product formed further analyses were done on the eluant. Gas chromatographic analyses

Figure 6.6. Investigation into the use of a larger activated charcoal recycle column for the production of 3-methyl catechol using *P. putida* 2313.

Fig. 6.6.1

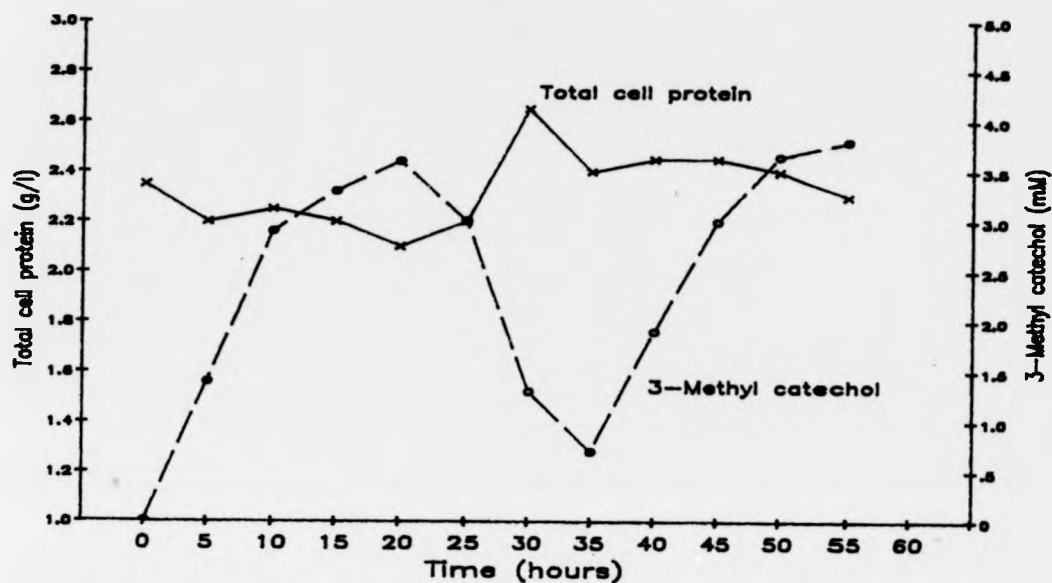


Fig. 6.6.2

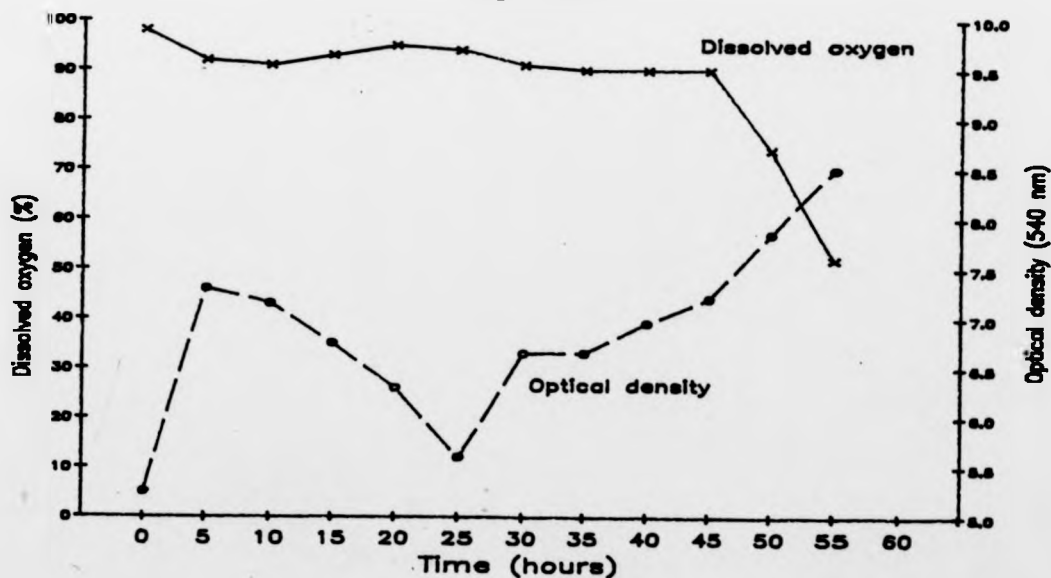


Fig. 6.6.3

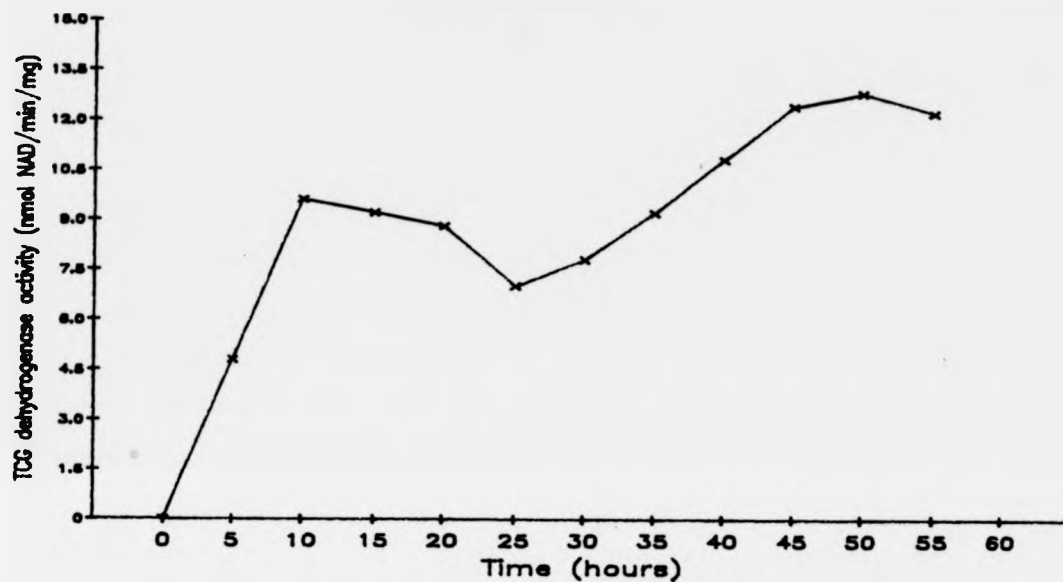
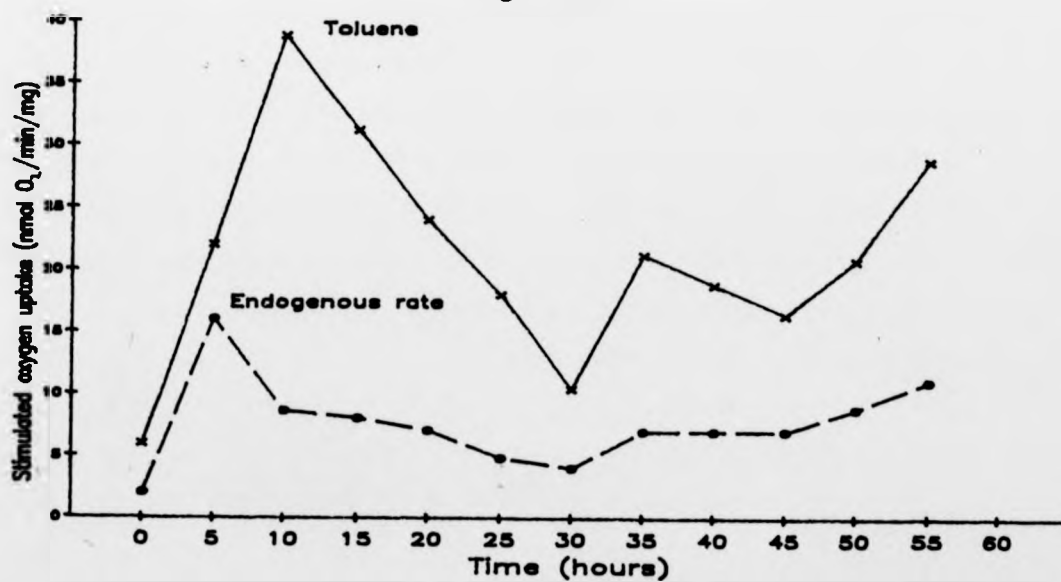


Fig. 6.6.4



using Tenax revealed that the eluant contained compounds other than those expected. The samples were further analysed using capillary GC analysis and four peaks were found (Figure 6.7). Mass spectra of the four identifiable peaks are shown in Figure 6.8. They were identified as:-

- | | |
|----------------------|---------------------|
| (i) 3-Methylcatechol | (ii) 2-Methylphenol |
| (iii) 3-Methylphenol | (iv) Benzyl Alcohol |

The presence of compounds (ii) and (iii), though unexpected, could be accounted of the chemical dehydration of toluene cis-glycol. TCG was not shown to be present in the supernatant of this fermentation but had been shown previously in the fed-batch system in the absence of a cell recycle system (Chapter 4). The presence of activated charcoal in the present experiment adsorbed the small amounts of TCG present in the supernatant.

The presence of benzyl alcohol, an intermediate of the toluene monooxygenase pathway was unexpected. A control experiment using fresh activated charcoal, under the extraction conditions employed, verified that the benzyl alcohol did not leak from the charcoal during elution. Semi-quantitative determination of the benzyl alcohol concentration showed it to be approximately 10% of the 3-methylcatechol concentration.

If it is assumed that the benzyl alcohol was formed during the biotransformation then its origin must be questioned. Although monooxidation catalysed by a dioxygenase has been noted previously (Gibson *et al.*, 1973; Ichihara *et al.*, 1962; Chapman, 1979 and Wackett *et al.*, 1988) it has never been shown to occur at the methyl substituent of toluene. Closer inspection of the

Figure 6.7 Gas chromatogram of eluant from activated charcoal recycle column.

DS90 Chromatogram report RUN: SUE0030001, 1/31/88 8:23
BF1 Gary Sample B1 70X5025 300 C 1ul 1/2 split

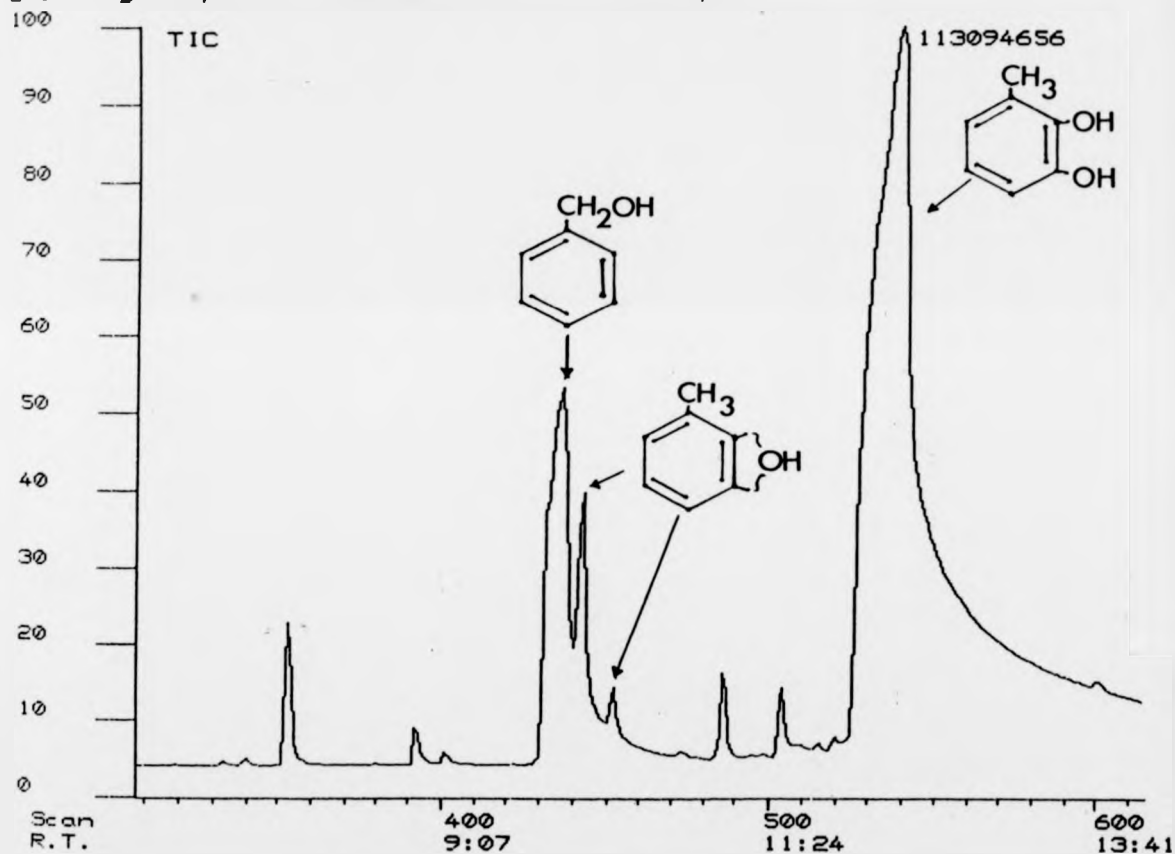
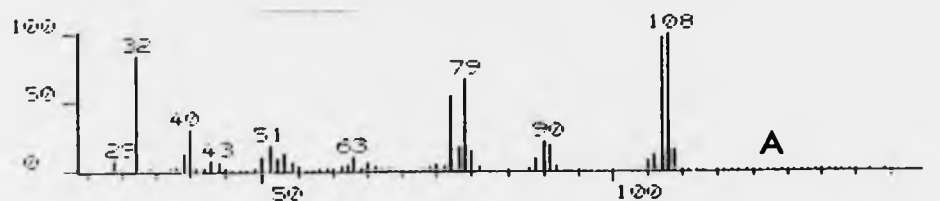
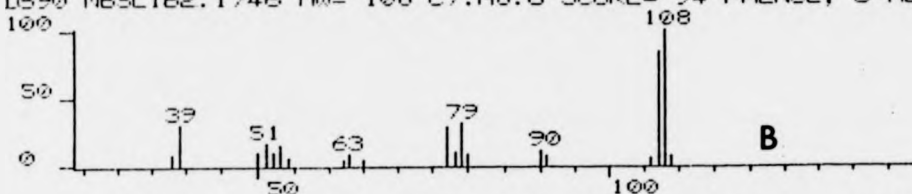


Figure 6.8 Mass spectra of compounds present in the eluant recovered from the activated charcoal recycle column.

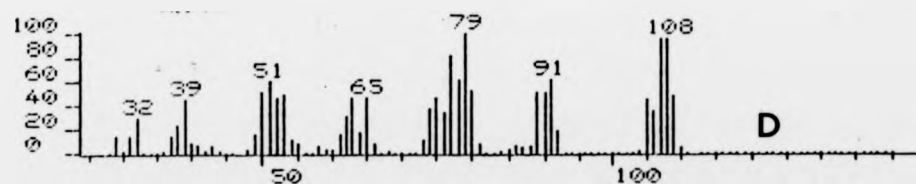
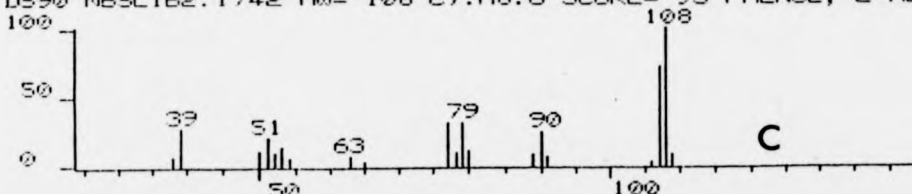
Spectra A and C are 'unidentified' compounds from the experiment. Spectra B, D and E are authentic spectra of the compounds named.



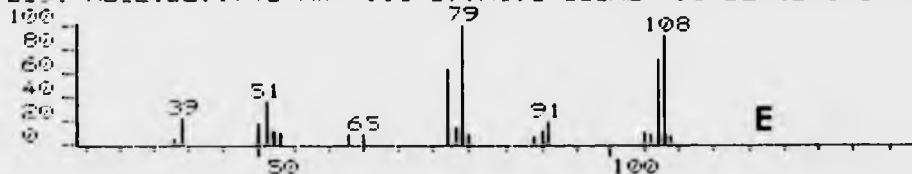
DS90 NBSLIB2.1746 MW= 108 C₇H₈O SCORE= 94 PHENOL, 3-ME-



DS90 NBSLIB2.1742 MW= 108 C₇H₈O SCORE= 93 PHENOL, 2-ME-

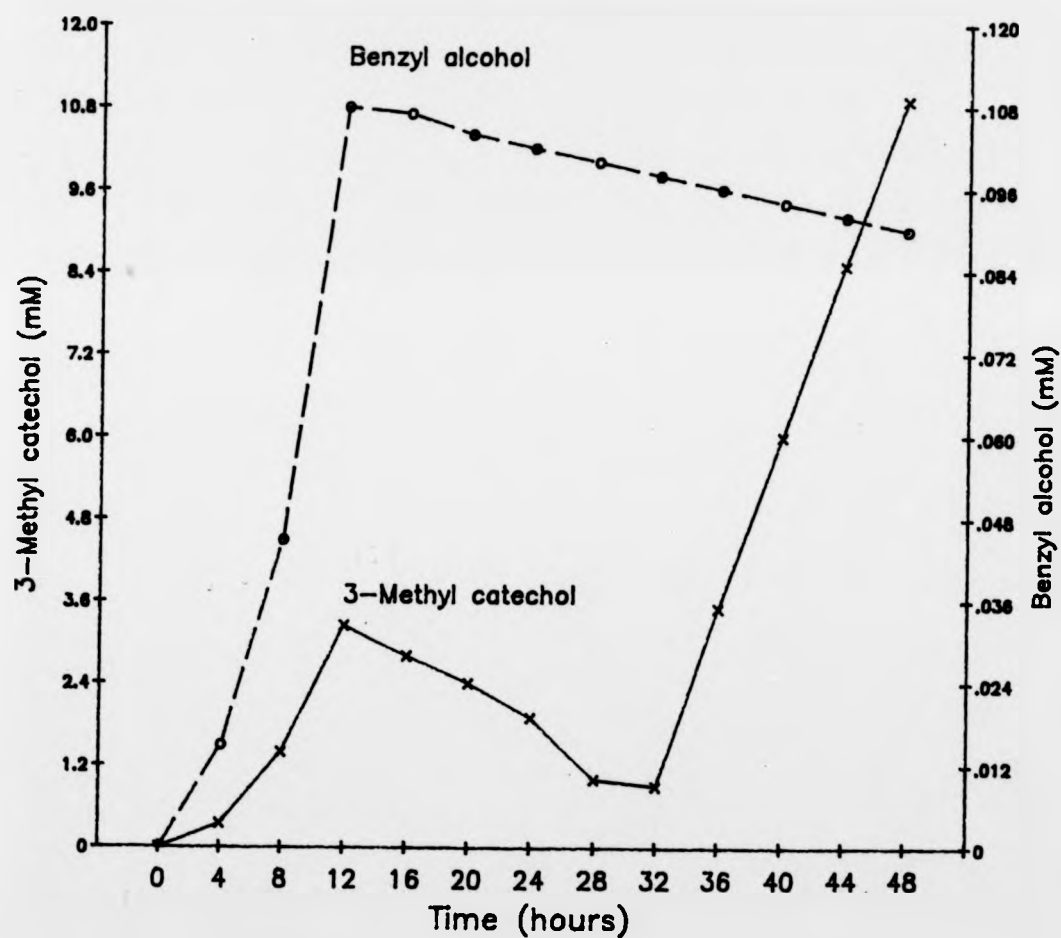


DS90 NBSLIB2.1743 MW= 108 C₇H₈O SCORE= 98 BENZENEMETHANOL



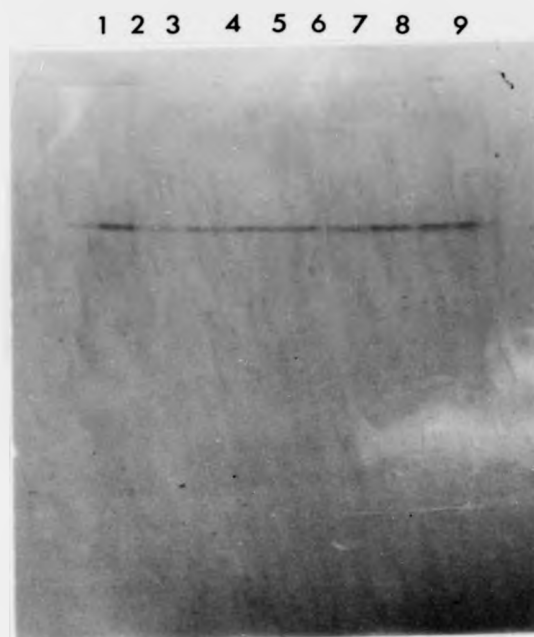
data for the present experiment (Figure 6.6.1-6.6.4) revealed an apparent biphasic biotransformation with whole cell oxidation of toluene and TCG and TCG dehydrogenase activity declining at around 25 hours but subsequently increasing again. Additionally, the dissolved oxygen tension fell at around 45 hours and the optical density increased. These findings could have been interpreted by assuming that P. putida 2313 possessed both a mono- and dioxygenase route for the utilisation of toluene. It was known that P. putida 2313 possessed a functional catechol 1,2-oxygenase when grown on benzene. Therefore, if the organism possessed the ability to utilise toluene via the mono-oxidative route it would be able to cleave the resulting benzoate via the intradiol cleavage route using catechol 1,2-oxygenase. The ability to switch between a dioxygenase to a monooxygenase catalysed reaction may have accounted for the biphasic nature of the present biotransformation. The experiment was repeated again and benzyl alcohol was monitored throughout. Contrary to expectation, the biphasic nature of the biotransformation was not shown with respect to the production of benzyl alcohol. Within 4 hours of toluene being fed the coincident production of 3-methylcatechol and benzyl alcohol was observed (Figure 6.9). Although the level of benzyl alcohol was only 0.11 mM (0.012 g/l) in the supernatant it showed that it was produced throughout the biotransformation and did not arise from an enzymatic activity induced only in the latter part of the production phase. The absence of any detectable catechol 1,2-oxygenase further supported the theory that a separate pathway was induced towards the end of the biotransformation. The presence of benzyl alcohol may reflect a non-specific oxidation of the methyl

Figure 6.9. Investigation into the production of benzyl alcohol during the production of 3-methyl catechol by *P. putida* 2313.



moiety by toluene dioxygenase. Subsequently, this may serve as a substrate for either a constitutive or inducible benzyl alcohol dehydrogenase. This was investigated using crude cell extracts which were prepared from the present study and run on a non-denaturing polyacrylamide gel. The presence of a specific benzyl alcohol dehydrogenase was assayed using the activity stain of Collins and Hegeman (1984). Although no activity was shown using benzyl alcohol as substrate a constitutive benzaldehyde dehydrogenase activity was shown (Figure 6.10). Collins and Hegeman (1984) concluded from their work on benzyl alcohol metabolism in P. putida that the benzaldehyde dehydrogenase was induced by benzyl alcohol. Additionally, the authors suggested that benzaldehyde arose due to a non-specific alcohol dehydrogenase which was able to act on benzyl alcohol.

Figure 6.10 Non-denaturing polyacrylamide gel electrophoresed for non-specific alcohol dehydrogenase using benzaldehyde as substrate.



Key:- Track 1 - 0 hours (before induction)
Track 2 - 4 hours
Track 3 - 8 hours
Track 4 - 12 hours
Track 5 - 16 hours
Track 6 - 20 hours
Track 7 - 24 hours
Track 8 - 28 hours
Track 9 - 32 hours

CHAPTER 7. PRELIMINARY INVESTIGATION INTO, AND
POSSIBLE METHODS OF OVERCOMING TOXICITY
ATTRIBUTABLE TO CATECHOLS.

CHAPTER 7.

PRELIMINARY INVESTIGATION INTO, AND POSSIBLE METHODS OF OVERCOMING TOXICITY ATTRIBUTABLE TO CATECHOLS.

INTRODUCTION.

Catechols are dihydric phenols which have been shown to be toxic to the biocatalyst used in the present study. The demonstration of product toxicity during the biotransformations studied provided the impetus for a preliminary investigation into the identification of toxic species and further confirmation of the site of action.

The first steps in the oxidation of both toluene and benzene by *P. putida* 2313 and 6(12) involves the synthesis of the appropriate cis-glycol and its subsequent dehydrogenation to form a catechol. These steps are catalysed by a dioxygenase and a dehydrogenase respectively. Toluene and benzene dioxygenase are multicomponent systems (Yeh et al., 1977; Axcell and Geary, 1975) consisting of a flavoprotein, a ferredoxin and an iron-sulphur protein. Dehydrogenases catalysing the oxidation of benzene cis-glycol (Axcell and Geary, 1973) and toluene cis-glycol (Rogers and Gibson, 1977) have been purified. The enzymes share similar properties in that they are both tetramers, utilise NAD^+ as their primary electron acceptor and oxidise a range of cis-glycols.

Catechols, in their protonated form, have been studied as effective enzyme inhibitors, most notably using soybean lipooxygenase 1 as outlined in the introduction (Section 1.11).

Although lipoxygenases differ markedly from both toluene and benzene dioxygenase, the former having a single subunit and the latter having three components, they are all non-haem iron-containing dioxygenases. Consequently, it was thought that the results obtained with lipoxygenases may offer an insight into the mechanism of inhibition in the system under investigation here.

Catechols are very unstable molecules, easily being oxidised to o-semibenzoquinone and o-benzoquinone derivatives. The formation of such compounds significantly enhances their modes of toxicity and, as a consequence, they are the most difficult inhibitors to discuss. The participation of quinones in electron transport and oxidative phosphorylation provides the opportunity for exogenous quinones to disturb these metabolic processes by substituting for, displacing or in other ways interfering with them. Additionally, quinones have the capacity to react with nucleophilic groups of amino acids to inactivate sensitive enzymes and also react with isolated amino acids, peptides and proteins (Kalyanaraman, 1987) although the physiological significance of these reactions in vivo remains unresolved.

The present work aimed to establish catechol toxicity and to investigate the conditions which enhance auto-oxidation in order to reduce or eliminate it. Previously (Chapter 4) it was shown that a cell extract prepared from toluene grown cells showed no toluene dioxygenase activity. Subsequent preparations also possessed no in vitro activity and an alternative assay system was sought. Using the oxygen electrode, it was possible to

assess catechol toxicity in relation to the whole cell oxidation of toluene and toluene cis-glycol. In order to gain an insight into the in vitro toxicity of catechols their effect upon TCG dehydrogenase was studied.

RESULTS AND DISCUSSION.

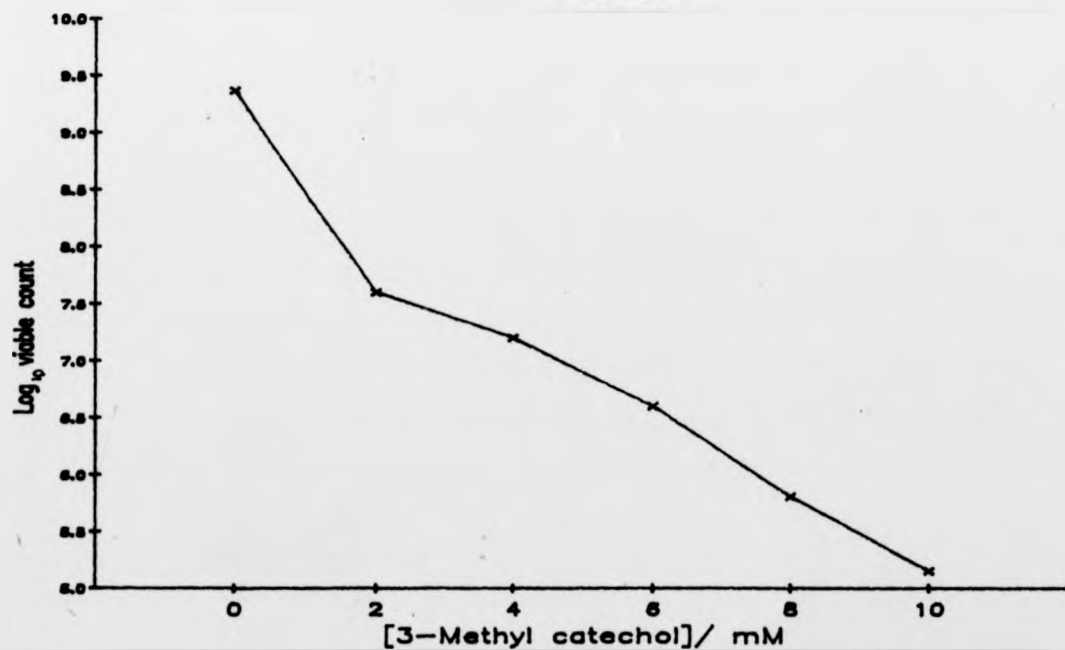
7.1 The effect of catechol and 3-methylcatechol upon the growth of *P. putida* 2313.

Before investigating any subcellular site of catechol toxicity it was important to show whether or not catechols inhibited growth in the absence of toluene or benzene. This obviated any sites of toxicity directly relevant to the biotransformation and showed that the products were inhibitory to bacterial growth. This was established by monitoring two different indicators of growth. The auto-oxidation of 3-methylcatechol gave rise to an insoluble polymer which interfered with spectrophotometric readings. As a consequence, the effect of 3-methylcatechol was assessed by measuring whole cell viability whereas the effect of catechol, which polymerised less readily, was assessed by cell turbidity. *P. putida* 2313 was inoculated into a mineral salts medium containing 0.2% (w/v) glucose and various concentrations of catechol and 3-methylcatechol in the range 0-10 mM. The results presented in Figures 7.1.1 and 7.1.2 show that both catechol and 3-methylcatechol are toxic to bacterial growth. Catechol was shown to be toxic at concentrations above 5 mM. At catechol concentrations below 4 mM it was shown that *P. putida* 2313,

Figure 7.1.1. The effect of catechol upon the growth of *P. putida* 2313.



Figure 7.1.2. The effect of 3-methyl catechol upon the growth of *P. putida* 2313.



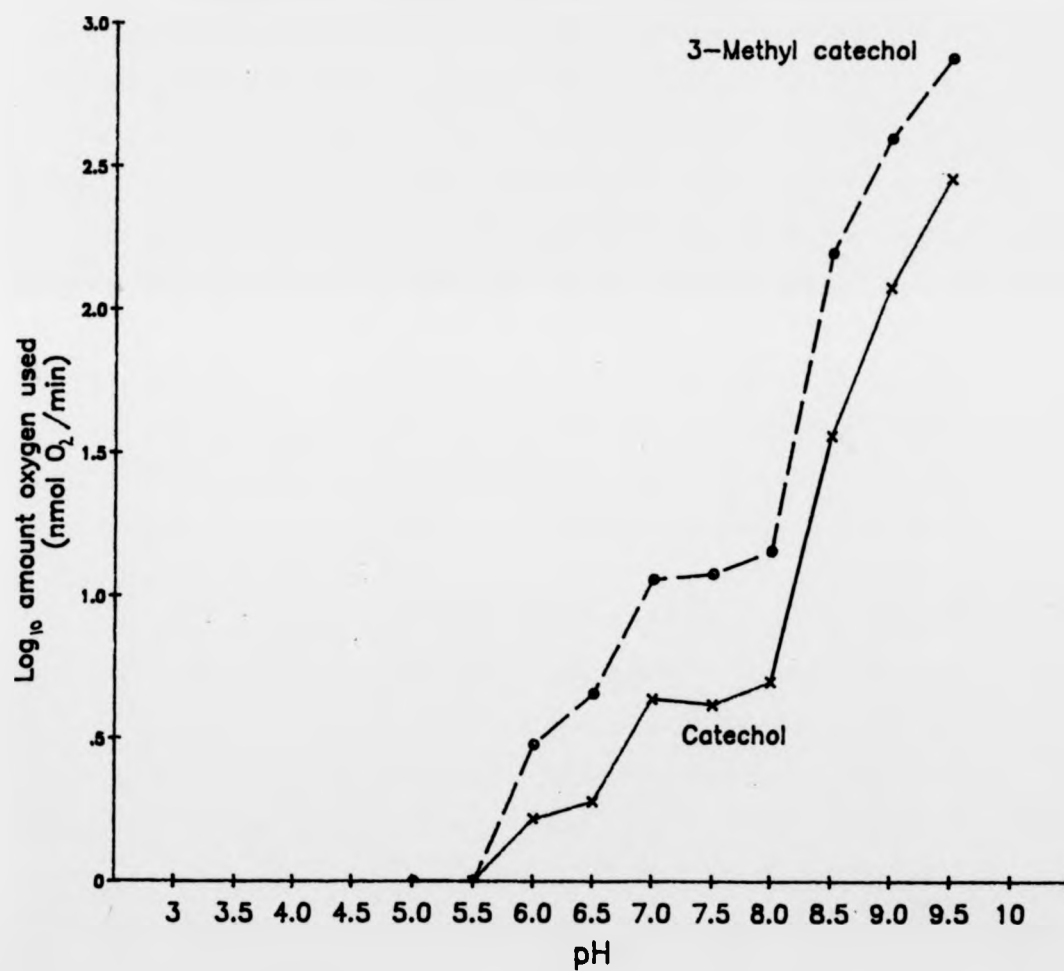
which still possesses a functional catechol 1,2-oxygenase, was able to utilise catechol as an additional carbon and energy source. 3-Methylcatechol was shown to be toxic at low concentrations, the cell viability being reduced by five orders of magnitude in the range 0-10 mM 3-methylcatechol. The increased toxicity of 3-methylcatechol probably reflects both its enhanced susceptibility to auto-oxidise and the fact that P. putida 2313 is unable to grow on 3-methylcatechol.

7.2 A preliminary study into the auto-oxidation of catechols and its possible prevention.

Previously, it was shown that catechols inhibit the growth and viability of P. putida 2313. The toxic species could be either the protonated monomer, the semibenzoquinone or benzoquinone or H_2O_2 resulting from the air oxidation and subsequent polymerisation. Air oxidation and production of H_2O_2 has been shown previously using NDGA (nordihydroguaiaretic acid), a catecholic antioxidant (Kemal et al., 1987).

The production of H_2O_2 and quinone derivatives is known to be pH dependent and an experiment was performed to measure the auto-oxidation of catechols with respect to pH. Using an oxygen electrode, the consumption of oxygen by 10 mM solutions of catechol and 3-methylcatechol were measured. The latter were buffered at various pH values in the range 3-10 and the results are shown in Figure 7.2. Both catechol and 3-methylcatechol were shown to auto-oxidise at pH values above 5.5. Additionally, it was shown that 3-methylcatechol auto-oxidises much quicker than

Figure 7.2. The effect of pH upon the auto-oxidation of catechol and 3-methyl catechol.



catechol, a fact that may explain the differences in product yield shown previously during the biotransformation.

Evidence for the autooxidative formation of H_2O_2 was obtained by an adptation of the method outlined above. Both catechol and 3-methylcatechol were buffered at pH 8.0 and left to auto-oxidise in the oxygen electrode until the level of dissolved oxygen fell below 50%. After this time a solution of either catalase or bovine serum albumen (BSA) was added to a final concentration of 130 $\mu\text{g/ml}$. The immediate generation of oxygen was then measured and found to be 141 and 29.7 nmol O_2 for 3-methylcatechol and catechol respectively using catalase. When BSA was added in the same way only 17 nmol O_2 was generated using both compounds. These results supported the contention that H_2O_2 is liberated when catechols are oxidised by air and the amount formed during auto-oxidation of 3-methylcatechol is significantly higher than that formed by catechol. Furthermore, the results suggest that the increased inhibition caused by 3-methylcatechol, both during growth and in the biotransformations, maybe attributable to toxic species during air oxidation.

It is well established that catechols are able to form many complexes with boron by virtue of the ortho hydroxyl groups (Steinberg, 1964). Although not investigated in detail here it was thought it may be possible to modify the culture medium to incorporate boron, allowing the catechols to be complexed and preventing auto-oxidation. This was preliminarily investigated by repeating the previous oxygen electrode studies over the range pH 7.5-9.0 using a boric acid/borax buffer. The latter was equilibrated at 30°C in the oxygen electrode and solutions of

catechol and 3-methylcatechol were added to a final concentration of 10 mM. No detectable oxygen consumption was measured indicating that the immediate auto-oxidation had been completely retarded, presumably by a catechol-boron complex being formed.

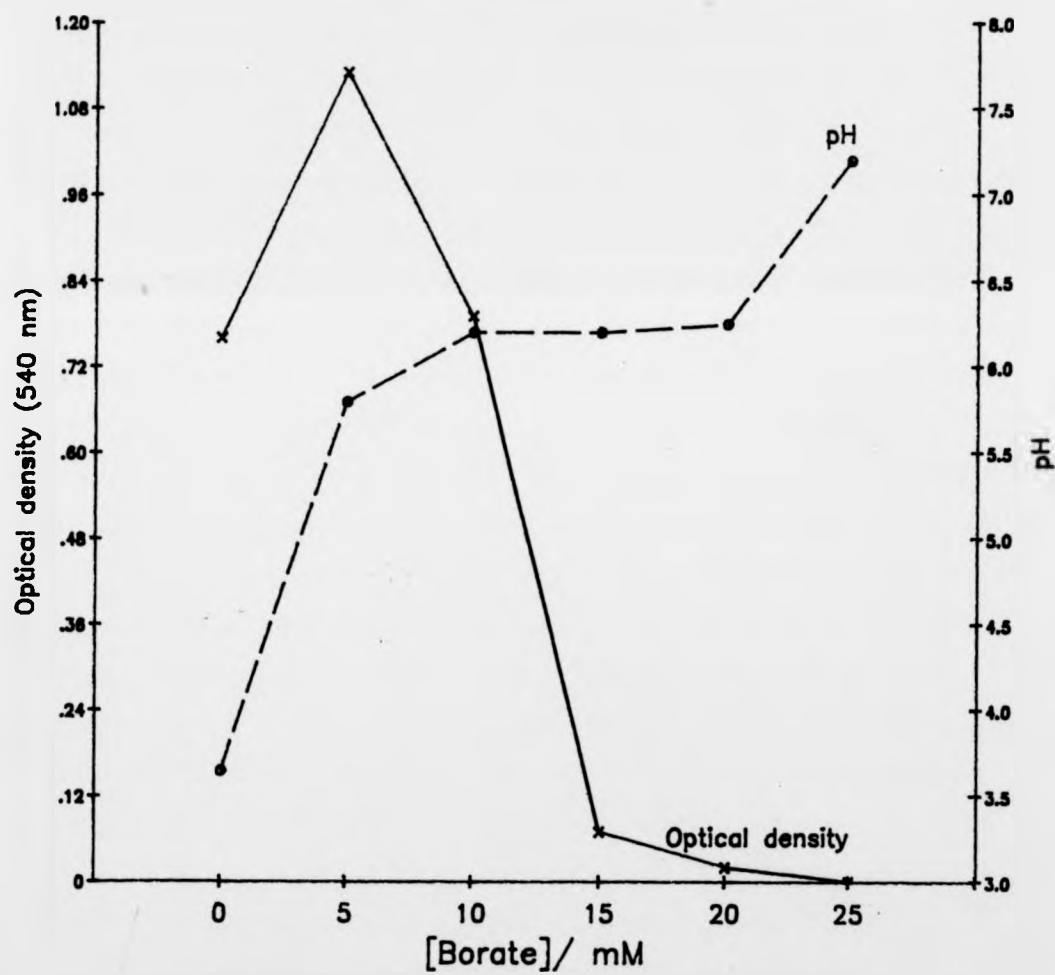
In order to assess the possibility of incorporating borate (di-sodium tetraborate) into the growth medium, *P. putida* 2313 was inoculated into flasks containing mineral salts medium supplemented with 0.2% (w/v) glucose and varying concentrations of borate in the range 0-25 mM. The results indicated that at concentrations above 10 mM, borate significantly inhibited growth (Figure 7.3). This would preclude the inclusion of borate in the growth medium but may, in a two-stage process, allow its introduction during the production phase of a resting-cell biotransformation. Assuming the stoichiometry of the catechol:borate complex is 1:1, and knowing the final product levels, such a system would require detailed investigation to prevent borate exceeding catechols as the predominant toxic species.

7.3 The effect of 3-methylcatechol on the whole-cell oxidation of toluene and toluene cis-glycol by *P. putida* R3.

P. putida R3, a spontaneous revertant of *P. putida* 2313, is able to grow on toluene and possesses a functional extradiol cleavage pathway. The inability to assay toluene dioxygenase in vitro provided the impetus to investigate the whole-cell oxidation of toluene and toluene cis-glycol. *P. putida* R3 was grown on toluene, washed and then concentrated in potassium phosphate buffer (pH 7.0). All cells used were obtained from the

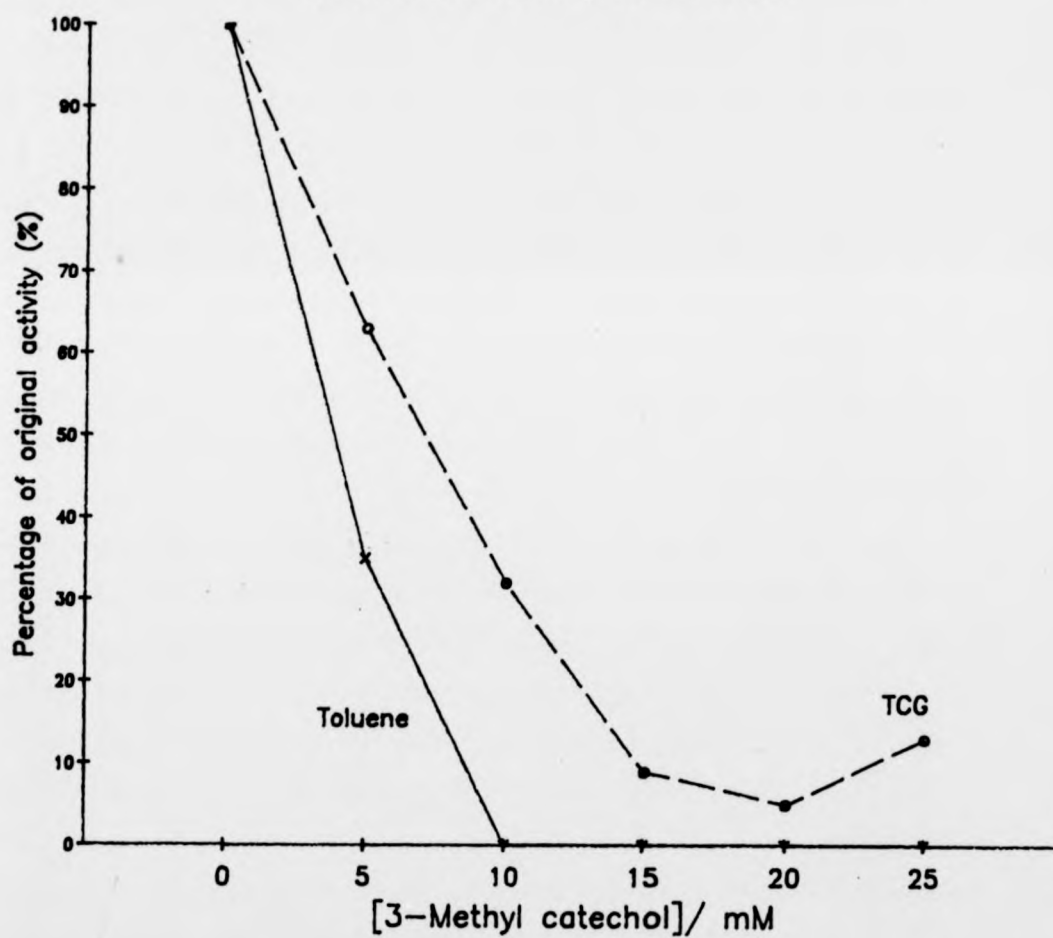
A single colony of P. putida 2313 was inoculated into 50 ml MS medium (pH 6.8) + 0.2 % (w/v) glucose and appropriate concentrations of di-sodium tetraborate. Cultures were incubated at 30°C for 48 h. and the final cell density and pH are displayed.

Figure 7.3. The effect of borate upon the growth of *P. putida* 2313.



fermentor at the same time, allowing direct comparison of the rates of stimulated oxygen uptake. The cells were introduced into the oxygen electrode and left to equilibrate at 30°C in the presence of various concentrations of 3-methylcatechol in the range 0-25 mM. Subsequently, either toluene saturated buffer or toluene cis-glycol (at a final concentration of 5mM) was added and the stimulated oxygen uptake, relative to that obtained in the absence of 3-methylcatechol, was measured. The results showed that the whole-cell oxidation of toluene was completely inhibited when pre-incubated with 10 mM 3-methylcatechol (Figure 7.4). At the same inhibitor concentration, the whole-cell oxidation of toluene cis-glycol retained 36% of its original activity. Even at 3-methylcatechol concentrations up to 25 mM the cells showed some capacity to oxidise toluene cis-glycol. The data further supported earlier findings that the primary site of 3-methylcatechol toxicity is the initial oxidation of toluene catalysed by toluene dioxygenase. This has previously been shown by Porter and Dalton (personal communication) using cell-free extracts of P. putida NCIB 11767. The authors showed that 3-methylcatechol inhibited toluene dioxygenase, assayed using the spectrophotometric indole assay of Jenkins and Dalton (1985). When pre-incubated with 3-methylcatechol, Porter and Dalton showed it non-competitively inhibited toluene dioxygenase with a K_i of 28 μ M. Additionally, these workers showed that the level of inhibition was pH-dependent with 167 μ M 3-methylcatechol causing 43, 63, 65, 82 and 91% inhibition at pH 6.4, 7.0, 7.2, 7.6 and 7.9 respectively. These findings provide further evidence that inhibition of toluene dioxygenase is due to products formed

Figure 7.4. The effect of 3-methyl catechol upon the whole-cell oxidation of toluene and TCG by toluene-grown *P. putida* 2313.



during auto-oxidation and not 3-methylcatechol per se.

7.4 The effect of catechols upon the activity of TCG dehydrogenase.

The data presented thus far has shown unequivocally that catechols are inhibitory both to growth and the biotransformations studied. The most dramatic inhibition was observed with the whole-cell oxidation of the aromatic substrates shown during the biotransformations. This was presumed to be at the site of toluene dioxygenase, a fact supported by the limited data obtained using the indole assay and the results of whole-cell oxidation studies shown in this chapter. Although the effect of catechol accumulation on the glycol dehydrogenases was not as marked as with toluene dioxygenase, the inhibition of the former was nevertheless significant. The aim of the work presented here was to establish whether the inhibitory thresholds obtained in situ bore any relation to those obtained in vivo.

Firstly, the effect of pH upon TCG dehydrogenase activity was investigated and found to be optimal at pH 8.5 (Figure 7.5). This was not used in subsequent assays because the increased alkalinity enhanced the auto-oxidation of the catechols causing interference with the assays. The reaction mixtures were routinely buffered at pH 7.5 to minimise the afore-mentioned interference, which was especially prevalent at high catechol concentrations. Furthermore, the effect of various substrate concentrations upon the glycol dehydrogenase was studied. The results shown in Figure 7.6 illustrate that TCG was inhibitory at concentrations exceeding 0.5 mM. All further studies were

Figure 7.5. The effect of pH upon the activity of TCG dehydrogenase.

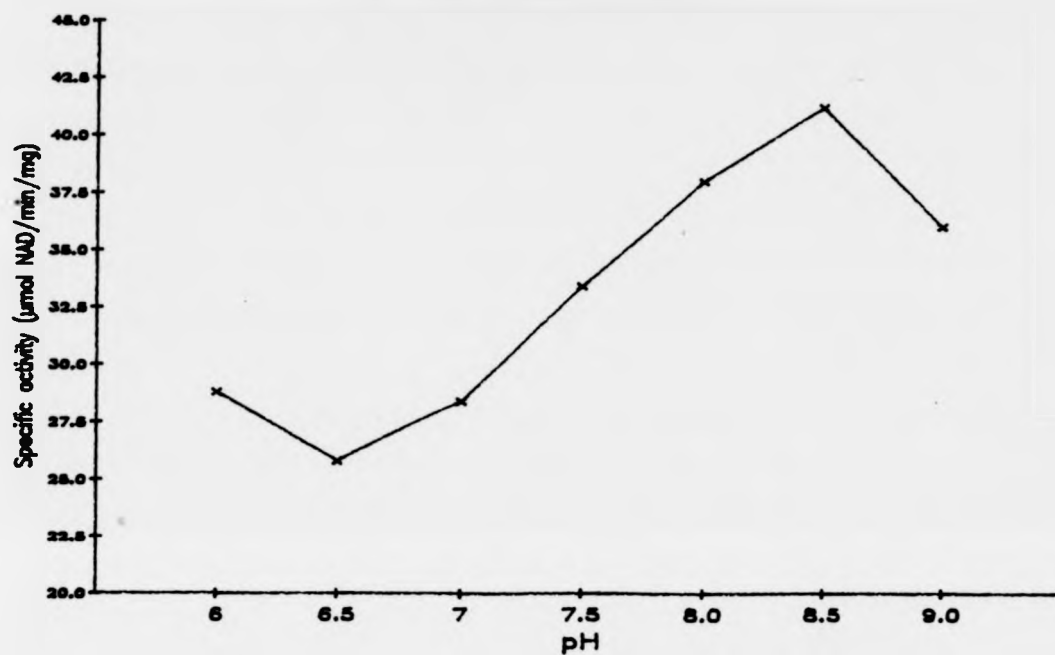
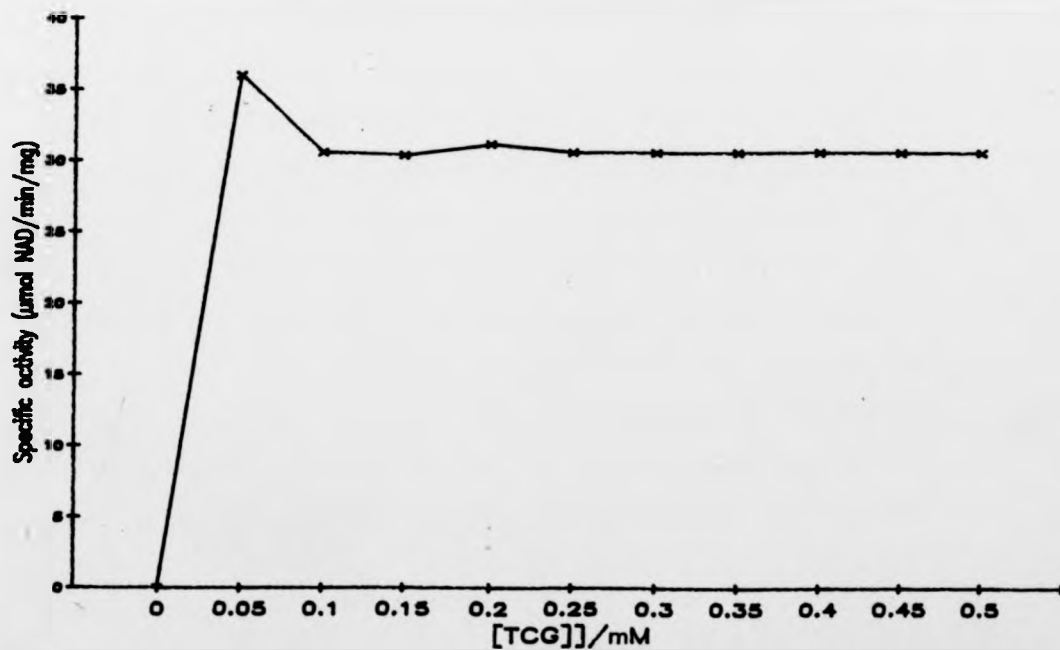


Figure 7.6. The effect of TCG concentration upon the activity of TCG dehydrogenase.



done using substrate concentrations below this level, thus minimising any inhibitory effects attributable to the substrate.

Most experiments were done using toluene cis-glycol (TCG) as the substrate but it must be borne in mind that the affinity of the dehydrogenase for benzene cis-glycol (BCG) and toluene cis-glycol (TCG) were the same. This is shown in Figure 7.7 which verified the apparent K_m for BCG and TCG was 60 μM .

The effect of catechol and 3-methylcatechol upon the glycol dehydrogenase is shown in Figure 7.8. Although the data shows deviations at higher substrate concentrations, catechol was shown to be a competitive inhibitor of TCG dehydrogenase. Unfortunately, the data obtained using 3-methylcatechol was less informative and simply showed the product was inhibitory.

The effect of 3-methylcatechol upon the activity of purified TCG dehydrogenase has previously been investigated by Simpson et al., (1987) using a heat stable dehydrogenase purified from a Bacillus. These workers showed that non-competitive or mixed inhibition was observed with 3-methylcatechol [$K_{i(E)} = 3.6$ mM; $K_{i(ES)} = 1.7$ mM] using TCG concentrations ranging from 0.05 mM - 0.5 mM. When the NAD^+ concentration was varied from 0.25 mM - 2.5 mM, mixed inhibition was observed with 3-methylcatechol and the $K_{i(E)}$ and $K_{i(ES)}$ values were 2.7 mM and 5.2 mM respectively. However, at saturating concentrations of the diol, uncompetitive inhibition was observed ($K_i = 1.2$ mM).

In the present study, inhibition data obtained using catechol showed that complete inhibition of the in vitro system would be expected at 12-13 mM catechol (data not shown). The levels of product present in the culture supernatant of the biotransformation were much higher than this and would seem to

Figure 7.7 Lineweaver-Burk plot of the oxidation of TCG and BCG by Toluene cis-glycol dehydrogenase.

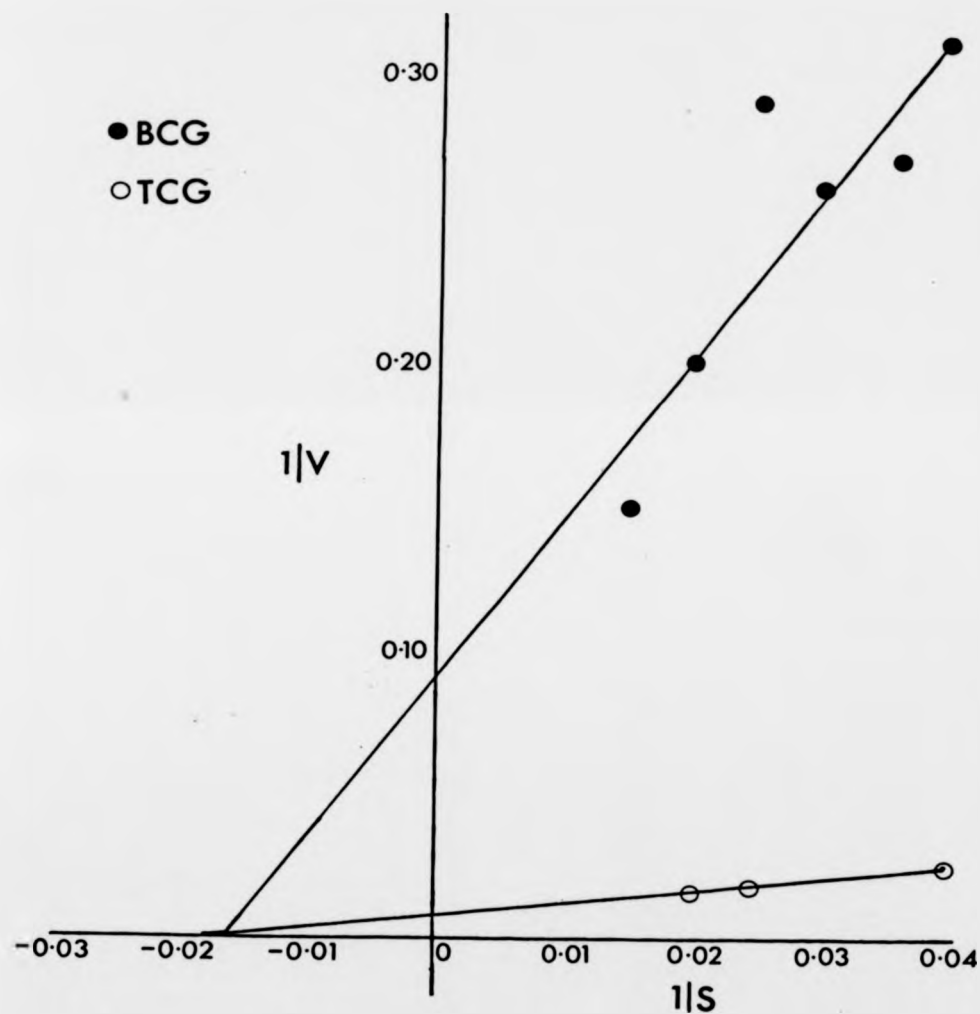
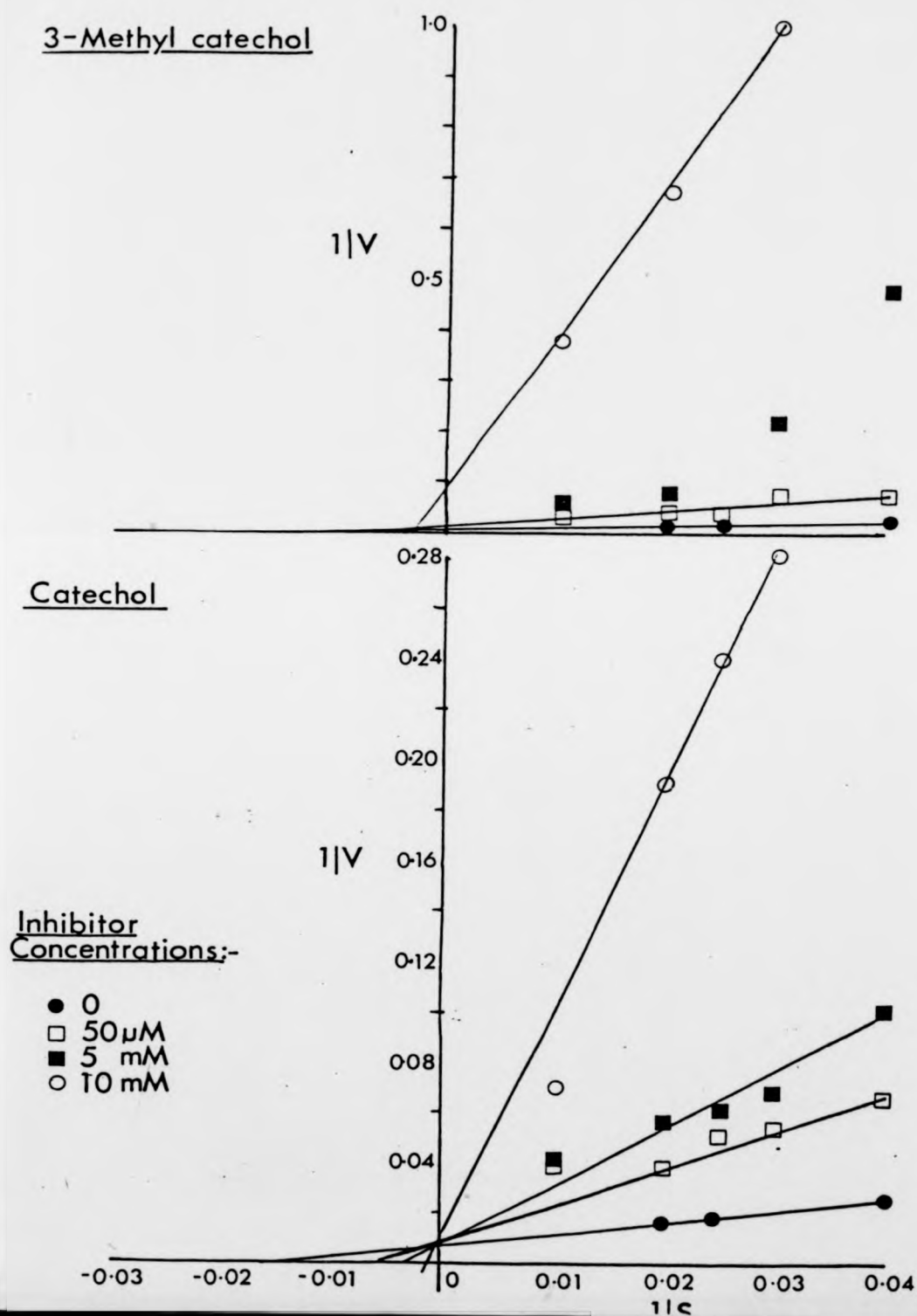


Figure 7.8 Lineweaver-Burk plot showing the inhibitory effect of catechol and 3-methyl catechol on the oxidation of TCG by toluene cis-glycol dehydrogenase.

230



indicate that the intracellular product levels were much lower than those outside the cell.

CHAPTER 8. GENERAL DISCUSSION & SUGGESTIONS
FOR FURTHER WORK.

Chapter 8.

General discussion and suggestions for further work.

The project aimed to study the bioconversion of low cost substrates to high added-value compounds. The results that emerged from this study highlighted the importance of understanding both the biocatalyst and the process prior to using biotransformations in the production of alternative, substituted catechols of commercial interest.

(i) Strain isolation and mutagenesis.

The overproduction of catechols was achieved by the use of whole-cells of P. putida which had been mutagenised using NTG. The first difficulty encountered in the present study was the selection of a strain able to accumulate high levels of catechol when fed with benzene in glucose fed-batch culture. Unlike toluene, which was shown to be metabolized exclusively via the extradiol cleavage route, benzene was able to be metabolized by the intradiol and extradiol cleavage routes. Both of these cleavage routes were shown to be present in P. putida 2313 and this necessitated a sequential mutagenesis protocol to be carried out. Although overproduction of catechol was achieved it may have been possible to increase the product yield further if a single round of NTG mutagenesis could have been used. The complex array of pathways involved in aromatic biodegradation was illustrated by the inability to isolate a strain able to overproduce catechol from benzoate. Initially, it was assumed that the absence of the intradiol cleavage enzyme, catechol 1,2-oxygenase, would result in the accumulation of catechol when fed with benzoate as opposed to benzene. However, P. putida

6(12), a mutant shown to lack catechol 1,2-oxygenase when grown in the presence of benzene was able to grow on benzoate as sole carbon and energy source. Additionally, no mutants were isolated which showed the inability to grow on benzene plus benzoate or toluene plus benzoate. These findings suggested that the pathway for the catabolism of either toluene or benzene was distinct from that for benzoate, at least at the level of the cleavage step. Whether this finding is evidence of gene duplication or simply a difference in the regulatory mechanism remains to be investigated.

(ii) Product toxicity.

Both catechol and 3-methyl catechol were shown to be overproduced from benzene and toluene respectively. Catechol reached a maximum of 27.5 mM (3 g/l) whereas 3-methyl catechol was produced at levels of 11.5 mM (1.27 g/l). The latter figure probably represents the minimum which can be expected for the reasons outlined previously (section 5.1). Despite this, it is clear that catechol is inherently less toxic to the biocatalyst than 3-methyl catechol. The reasons for the reduced toxicity of catechol are not fully understood. The evidence presented here suggests that the increased toxicity is inextricably linked to the auto-oxidation of 3-methyl catechol compared to that of catechol.

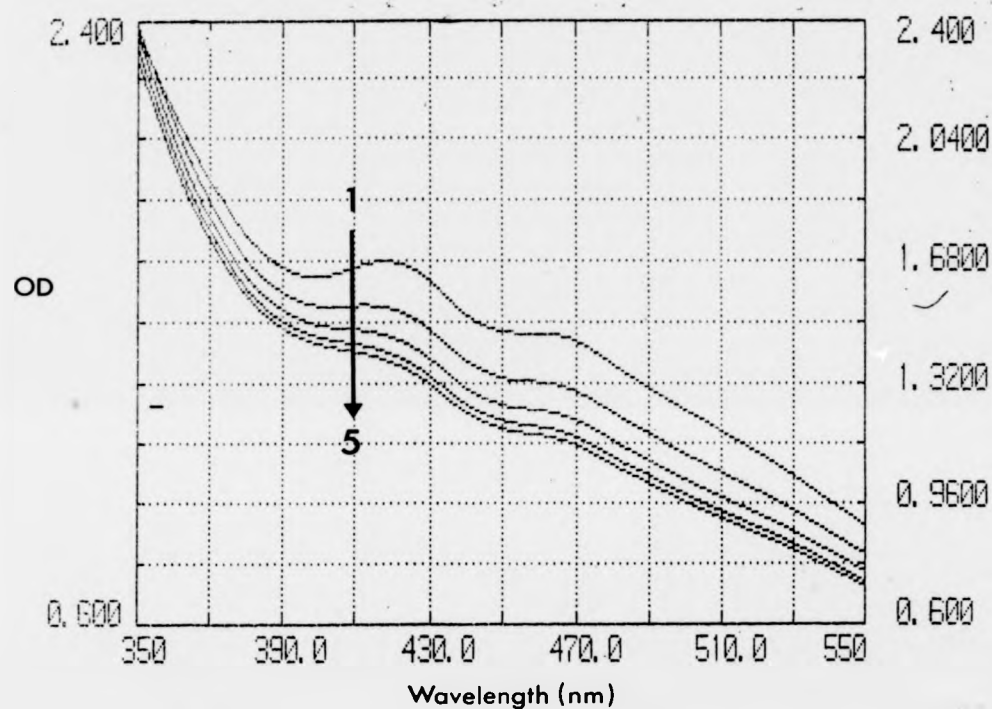
Although preliminary investigations of product toxicity are presented it was not possible to examine all possible modes of catechol toxicity. The instability of 3-methyl catechol and, to a lesser extent, catechol in aerated aqueous solutions was a recurrent problem. It seems probable that, within the fermentation regime used, both catechol and 3-methyl catechol

were oxidised to their corresponding semiquinone and quinones increasing the possible sites of toxicity. However, it seems clear that the primary site of toxicity was the initial toluene or benzene dioxygenase, as evidenced by the limited data obtained from crude cell extract assays of toluene dioxygenase and the results of investigations of the whole cell oxidation of toluene and benzene. If the data from the investigations of toxicity are assumed to be correct, the data begs the question 'what is the mode of catechol toxicity?'.

Both benzene and toluene dioxygenase are similar, multi-component enzymes having two electron transport proteins and a terminal hydroxylase. Indeed, it may be argued that both benzene dioxygenase and toluene dioxygenase are the same enzyme, a fact which could be proven by purifying the respective enzymes from the same strain after growth on benzene and toluene. Both enzymes are reported to require exogenous Fe for catalytic activity and possess components containing iron-sulphur centres. Previous reports, using lipxygenases, have suggested that the catechol moiety is able to irreversibly inactivate this enzyme by co-ordination of catechol to the ferric cofactor (Galpin et al. 1976) or by reducing the catalytically active ferric enzyme to the catalytically inactive ferrous form (Kemal et al. 1987). Lipxygenases are single subunit enzymes and cannot be said to be analogous to either toluene or benzene dioxygenase. However, it seems plausible that the catechol moiety maybe able to gain access to the holoenzyme and disrupt the redox reactions involved in electron transport from the cofactor (NADH) to the oxidation of the aromatic substrate. A possible site of action was thought to be the second component of the enzyme, component

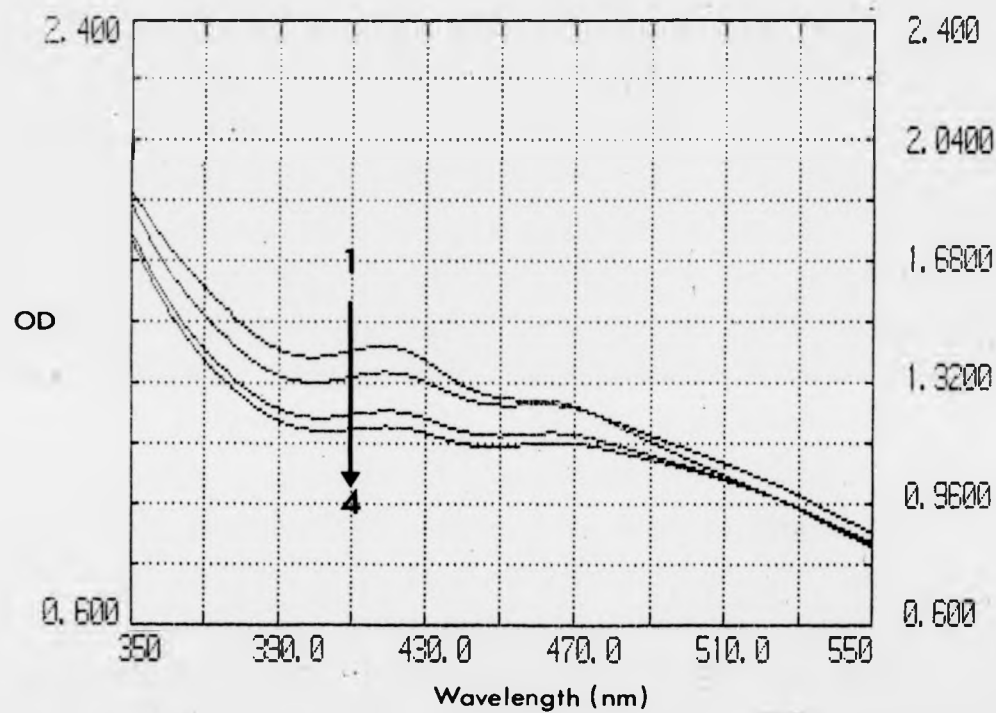
B in benzene dioxygenase and Ferredoxin_{TOL} in toluene dioxygenase. Both of these components have been characterised as plant-type ferredoxins which function to transfer electrons to the terminal hydroxylase component. All the studies presented in the present work were conducted using whole cells and no purified toluene or benzene dioxygenase was available. Consequently, it was thought that it may be possible to gain an insight into the mode of catechol toxicity by a preliminary spectroscopic investigation of the effect of catechol on an alternative plant-type ferredoxin, namely spinach ferredoxin. The spectra of these studies are shown in Figures 8.1 and 8.2. Initially, an aqueous solution of spinach ferredoxin (0.5 mg/ml) was incubated with a crystal of dithionite and the spectral changes observed (Fig. 8.1.1). The results shown indicate the ferric iron centre was reduced within 10 mins. ($OD_{419}=0.29$). Using a crystal of catechol in place of dithionite it was shown that catechol also reduced the ferric iron centre within 20 mins. ($OD_{419}=0.24$). These results showed that catechol was able to reduce the ferric iron centre of a plant-type ferredoxin and illustrates how it may effect inhibition of the initial dioxygenase. However, it must be noted that the protein sequence of spinach ferredoxins (type I and II) show no homology with that for P3, the presumed ferredoxin_{TOL} component, published recently by Irie *et al.*, (1987b). Although the iron may be present in the ferrous form in the native enzyme it must be oxidised during the catalytic incorporation of dioxygen into either toluene or benzene. Irrespective of the ability of catechol to reduce the plant-type ferredoxin component, the redox potential of catechols will probably lead

Figure 8.1 Effect of dithionite on the adsorption spectrum of spinach ferredoxin.



Key:- Spectra 1 - 5 measured every 2 minutes.

Figure 8.2 Effect of catechol on the adsorption spectrum of spinach ferredoxin.



Key:- Spectra 1-4 measured every 5 minutes.

to either the diversion or the interference of the flow of electrons from NADH to O_2 during aromatic dioxygenation.

Further in vitro studies into the effect of catechols on either the holoenzyme or components thereof would require purified toluene or benzene dioxygenase. Alternatively, it may be possible to examine the turnover of particular components of the aromatic dioxygenases using antibodies raised against the individual components. Such an approach would yield valuable information about the specific site of toxicity.

(iii) Substrate specificity.

One of the primary reasons for investigating the production of catechols was the possible application of the system for the production of commercially important substituted catechols. It was thought that the use of either benzene or toluene would select for a dioxygenase with the broadest substrate range. The preliminary investigation of the catalytic utility of the whole-cells used in this study showed that it was possible to produce catechols from a range of aromatics although none of these were definitively identified. Ideally, a biotransformation should be predictable if one is using expensive substrates to prepare specific products. To this end, it was surprising to discover that toluene did not appear to be metabolized exclusively by the dioxygenase route. Although ordinarily present in small amounts, it appeared that benzyl alcohol was produced along with 3-methyl catechol. This was initially observed in the fed-batch system which incorporated an activated charcoal column but was subsequently shown in the absence of any adsorption column. Previous workers had shown a dioxygenase functioning as a monooxygenase but had not shown it to occur at

a methyl moiety attached directly to an aromatic ring. The ability of a dioxygenase to catalyse the insertion of one atom of oxygen is not understood. Several reasons may be postulated to explain the presence of two apparent activities and these are best outlined as follows:-

(a) Both the monooxygenase and dioxygenase pathways are present concurrently. This postulate seems unlikely in view of the findings presented in section 3.6 which showed that P. putida 4(1), a mutant lacking toluene dioxygenase, was unable to grow on xylene or mesitylene, typical substrates of toluene monooxygenase.

(b) The monohydroxylated products may arise due to the activity of a specific dehydratase. This may explain the presence of the methyl phenols shown in section 6.4 but would not explain the production of benzyl alcohol.

(c) The aromatic dioxygenase is able to function as a monooxygenase. This may occur due to the proximity of the methyl group to the site of dihydroxylation thus permitting the fortuitous oxidation of the side chain. Alternatively, the dioxygenase may be directed to a monooxygenase reaction in a manner analogous to the substrate-induced modulation of putidamonooxin shown by Wende et al. (1982). This would seem unlikely in the present system which uses toluene as the sole substrate to be transformed.

(d) The aromatic dioxygenase is directed to a monooxygenase reaction by another protein. This type of modifier protein has previously been shown by Brodbeck et al. (1967) who showed that the presence of alpha-lactalbumin enabled the galactosyltransferase component of lactose synthetase to

catalyse the transfer of galactose to glucose. Although this occurs in the absence of alpha-lactalbumin it plays no physiological role due to the very high K_m for glucose when galactosyltransferase is used alone. Thus, the addition of alpha-lactalbumin increases the affinity of the transferase for glucose. More recently, another modifier protein has been characterised by Ford *et al.* (1985). These workers showed the presence of a modifier protein (M-protein) in *Pseudomonas* AM1 which altered the substrate specificity of methanol dehydrogenase allowing it to oxidise propanediol to lactaldehyde. A similar M-protein was also shown in *Methylophilus methylotrophus* (Page and Anthony, 1986). Alternatively, rather than a protein which modifies the activity of a particular enzyme towards a given substrate it may be envisaged that a protein component is able to regulate the enzyme activity in response to the conditions prevalent in the cell. Such a protein component has been characterised in the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) (Green and Dalton, 1985). These workers suggested that protein B of methane monooxygenase enabled the enzyme to function as both an oxidase in its absence and an oxygenase in the presence of protein B. Thus, in the absence of methane the soluble methane monooxygenase is uncoupled to furnish NAD^+ required for formaldehyde and formate dehydrogenases to function and prevent the accumulation of the toxic product, formaldehyde. Although the present system catalyses a very different reaction it can be speculated that the overproduction of 3-methyl catechol effects alterations in the initial dioxygenase mediated by a regulatory protein. Such a protein may promote the

synthesis of benzyl alcohol and prevent further production of 3-methyl catechol.

To gain an insight into the reactions which toluene and benzene dioxygenase will catalyse the enzyme must first be purified to see whether both the monooxygenase and dioxygenase activities are catalysed by the same protein. Additionally, the culture conditions prior to harvesting the cells could be modified to see whether both activities are only present under certain conditions. Until the reasons for the apparent duplicity in activity are resolved it would seem impossible to rationally design a biocatalytic route which leads exclusively to the synthesis of a dihydroxylated product.

(iv) Use of recombinant DNA technology.

In all studies outlined no attempt was made to use recombinant DNA techniques to achieve overproduction of catechols. This approach was initially avoided due to the technical difficulties associated with the manipulation of large catabolic plasmids, namely their large size, low copy number and lack of methodology for pseudomonad plasmid isolation. However, advances in the genetic manipulation of Pseudomonas species now offer alternative approaches to the large-scale production of aromatic intermediates and may facilitate the development of expression systems which are more resistant to catechol, allow more efficient substrate uptake and permit rapid excretion of the product. Initial attempts at genetic manipulation were concerned with the cloning and expression of plasmid borne genes such as those involved in the catabolism of naphthalene. Schell (1983) was able to clone the genes encoding the enzymes catalysing the degradation of naphthalene to 2-hydroxymuconic

semialdehyde. This fragment was introduced into pBR322 and was transformed into E. coli where the genes were expressed allowing the host strain to convert naphthalene to salicylic acid. However, this approach illustrated the problems associated with the expression of catabolic genes in E. coli. Firstly, the efficiency of expression of foreign genes in E. coli was found to be 10% of that in P. putida despite the gene dosage being ten fold higher in the E. coli strain. Additionally, the ability of the transformed E. coli to convert naphthalene to salicylate was unstable due to the deletion of internal DNA segments from the cloned fragment. Despite the problems outlined above, other workers have used similar methodologies to illustrate the value of this type of approach. Ensley et al., (1983) transformed E. coli with pE317, a recombinant plasmid constructed from pBR322 and a 10.5 kb DNA fragment from the plasmid encoding the enzymes for the catabolism of naphthalene, NAH7. E. coli strains carrying the recombinant plasmid were able to oxidise naphthalene constitutively, unlike P. putida which was inducible. Interestingly, when the E. coli was grown on a complex medium the production of indigo was observed. This was formed from a novel catabolic route using the tryptophanase of the host organism and the broad substrate specificity of the cloned naphthalene dioxygenase. Although the expression of the cloned dioxygenase was lower than that observed in P. putida, it was the first demonstration of the novel biosynthetic routes available when using recombinant DNA technology. Subsequently, Mermod et al. (1986) used a similar approach to show the synthesis of indigo from indole using a sub-cloned DNA fragment of pWWO which encoded the broad specificity enzyme xylene

oxidase. The workers constructed a recombinant plasmid consisting of pBR322 and a fragment containing xylA and the upper pathway operon promoter from pWWO.

All of the approaches outlined above used either dioxygenases or monooxygenases encoded by plasmid borne genes. In the present study, one would require a chromosomal fragment encoding both a broad specificity toluene/benzene dioxygenase and the corresponding cis-glycol dehydrogenase. The genes encoding todA, todB, and todC (assigned for reductase_{TOL}, ferredoxin_{TOL}, and ISP_{TOL}) have been identified by the isolation of the mutants lacking each component of toluene dioxygenase (Finette *et al.*, 1984). Moreover, the genes encoding the enzymes involved in the dihydroxylation of benzene to catechol have now been cloned and expressed in *E. coli* (Irie *et al.*, 1987a). Using a variety of recombinant constructs Irie *et al.* (1987a) showed that the functional region of the cloned fragment was less than 5 kb in length and the level of expression was far lower than that in the native pseudomonad with levels of catechol accumulating to only 200 mg/l. Despite the low product levels achieved, it would seem that the technology now exists to use this type of approach for the overproduction of valuable intermediates. The use of a recombinant organism offers the opportunity to couple the required broad specificity dioxygenases to strong promoters present on high copy number expression vectors. Such an approach may lead to a gene dosage effect such that the enzymes of interest form a much higher percentage of the total soluble protein and consequently increase the rate of product formation. However, although a recombinant DNA approach may yield valuable

information about the expression of pseudomonad genes in alternative hosts it seems unlikely that catechol production would be significantly enhanced. The multiplicity of presumed sites of catechol toxicity could not be overcome simply by expressing the relevant genes in an alternative host which possesses the same metabolic machinery.

(v) Use of product removal systems.

Irrespective of the approach used to achieve overproduction of catechols, the primary problem of product toxicity remains. Any oxygenase-based biotransformation obviously requires molecular oxygen and this accelerates the rate of catechol auto-oxidation. The only way of preserving the product and alleviating toxicity attributable to its instability would be to develop a system for its rapid and continuous removal. Aside from the physical adsorption method used in the present study, it may be possible to develop other product removal systems such as the use of aqueous two-phase systems. Such a system may also reduce the auto-oxidation of the product and facilitate the catechol being retained in the monomeric form. The development of alternative product removal systems pre-supposes that the intracellular catechol levels accumulate in a manner analogous to those present outside the cell. Indeed, previous measurements of the intracellular levels of both TCG and muconic acid showed them to be present at higher concentrations than those outside the cell (D. Porter and H. Dalton, personal communication). Unfortunately, no data on the intracellular catechol levels could be obtained. This leads to speculation that the internal environment of the cell may facilitate auto-oxidation of the product and result in the rapid loss of biocatalytic activity.

Consequently, the levels of extracellular product formation may not be significantly increased. Obviously, this hypothesis could be investigated if reproducible measurements of intracellular product concentrations could be made.

(vi) Final comments.

In conclusion, a wholly biocatalytic route to the formation of catechols is possible using a fed-batch mode of fermentation. The major problem associated with this route is the inherent toxicity of the products and their instability within the fermentation system used. Alternative approaches employing either recombinant DNA techniques or more efficient product removal systems may increase product yields. However, it would seem preferable to utilise the broad specificity dioxygenase for the production of the less toxic aromatic cis-glycol and then use existing chemical technology to dehydrogenate the cis-glycol to the corresponding catechol. This approach alleviates the problems associated with product toxicity and allows the catechol to be synthesised in a stringently controlled process. Additionally, the product can be recovered without recourse to expensive purification procedures.

CHAPTER 9. REFERENCES.

Chapter 9.

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