DEGRADATION OF AROMATIC COMPOUNDS AND EXOPOLYSACCHARIDE FORMATION BY *Pseudomonas mendocina* P_2d

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Certificate

This is to certify that ***Ms. Chanda Parulekar*** has worked on the thesis entitled **"Degradation of aromatic compounds and exopolysaccharide formation by *Pseudomonas mendocina* P_2d"** under my supervision and guidance.

This thesis, being submitted to the Goa University, Taleigao Plateau, Goa, for the award of the degree of Doctor of Philosophy in Microbiology, is an original record of the work carried out by the candidate herself and has not been submitted for the award of any other degree or diploma of this or any other University in India or abroad.

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Chanda

Dedicated

to my parents

CONTENTS

List of abbreviations

A	Absorbance	M	Molar
APS	Ammonium per sulphate	nm	Nanometer
BA	Benzoate agar	nM	Nanomolar
cm	Centimeter	NA	Nutrient agar
cfu	Colony forming units	*o*	Ortho
°C	Degree centigrade	O.D.	Optical density
EPS	Exopolysaccharide	*p*	Para
Fig.	Figure	PAGE	Poly acrylamide gel electrophoresis
GC	Gas chromatography	strain	*Pseudomonas mendocina*
		P_2d	strain P_2d
g	Gram (s)	%	Percentage
HPLC	High performance liquid chromatography	rpm	Revolutions per minute
h	Hour (s)	Rt	Resolution time
HCl	Hydrochloric acid	Rf	Resolution factor
HMS	2-Hydroxymuconic semialdehyde	R.T.	Room temperature
kD	Kilodaltons	SEM	Scanning electron microscopy
L	Litre	sec	Second (s)
m	Meta	SDS	Sodium dodecyl sulfate
µg	Microgram	NaOH	Sodium hydroxide
µL	Microlitre	sp.	Species
µM	Micromolar	NaCl	Sodium chloride
µ	Micron	TLC	Thin layer chromatography
mA	Milli-amperes	TEMED	Tetramethyl ethylene diamine
mg	Milligram	UV	Ultra-violet
ml	Millilitre	U	Unit
mm	Millimeter	v/v	Volume/volume
µm	Millimicron	V	Volts
mM	Millimolar	w/v	Weight/volume
MM	Mineral media	λ	Wavelength
min	Minute (s)		

List of Tables

List of Figures

INTRODUCTION

1. Incidence of aromatic compounds in environment:

Aromatic compounds are present in the environment as product of industries that are based on coal and petroleum, combustion of organic material, smoking, automobile exhaust, *etc.* and also from living systems (1,2). Plants synthesize large quantities of natural products that are biologically inert such as lignin, alkaloids, carotenoids, terpenes, *etc.* (3). Carotenoids like renieratene from sea-sponge, *Chlorobactene,* from *Chlorobium* species and terpenes such as calamenes, α-curcumenenes form the major group of compounds that contribute to aromatic hydrocarbons in nature.

Increasing use of petroleum has resulted in widespread dispersal of these compounds. The concern over the effect on environment has stimulated interest in their metabolism (1, 3, 4). Thus, pollution of the environment caused by human activities becomes an important environmental issue. Herbicides, fertilizers, fungicides and insecticides that are used to increase food production; detergents and pharmaceuticals used for health reasons; organic solvents used in industries; are synthetic xenobiotic organic chemicals that pose a

threat (5, 6, 7). The inability to degrade these compounds will lead to large quantities of carbon remaining locked up in the stable rings. Since these compounds are synthesized continuously, carbon will be out of circulation even after plant death (3). Hence, microbial degradation is very important from carbon cyclization point of view.

Aromatic compounds are divided into monoaromatic compounds, having one aromatic ring and polyaromatic compounds, having more than one ring structure. Monoaromatic compounds are further classified based on the type of substituents present in the benzene ring (Table 1.1).

Polyaromatic compounds: Naphthalene, anthracene, phenanthrene, β-naphthol, *etc.* have polyaromatic structures. These hydrocarbons are environmentally persistant and are found abundantly in aquatic and terrestrial ecosystems. These are present as natural constituents of fossil fuels and their anthropogenic pyrolysis products. The higher molecular weight polyaromatic hydrocarbons (PAH) may not pose a risk to human health, but are toxic to fish and algae (8, 9).

Table 1.1: Substituent groups on monoaromatic compounds

Substituent	Compounds	Source	Reference
Phenolic	Phenol, Catechol, Resorcinol, Cresol, Xylelol	Effluents from coal carbonization; petroleum and petrochemical industries; coking and coal gasification	10
Nitro-	3-Nitrophenol, Nitrocatechol, 4-Nitrobenzene, 2-Nitrobenzene, 2,4-Dinitrobenzene	Pesticides such as parathion; pharmaceuticals; plastic dyes; explosives; solvents; herbicides	11, 12
Amino-	Aminosalicylate, Aniline,*p*-Amino-benzoate, *o*-Aminophenol	Azo dyes and other nitro compound metabolites	13, 14
Methyl-	Toluene, Cresols, Xylelols, Alkyl benzene, Vanillic acid	Organic chemical industry solvents; fuels from petroleum	1, 15
Halogen	4-Chlorobenzoate, 2,4-Dichloro-phenoxyacetic acid, Chlorosuccinate, 3-Fluorocatechol, 3-Fluorobenzoic acid	Solvents; lubricants; insulation and hydraulic fluid; biocides; synthetic waste	6, 16
Sulfo-	Benzenesulfonate, *p*-Toluene-sulfonate	Insecticides such as Arbicarb, Parathion, Fenitrothion, Fenthion; herbicides such as Benthiocarb and fungicides such as Feaminosulf	5, 7

PAH being hydrophobic in nature, are taken up by suspended particles coated with organic matter and settle at the bottom of water. Thus, sediments of lakes, estuaries, oceans, form a major sink for PAH (17). Besides being present in petroleum, PAH are formed during incomplete combustion of almost any organic material, cigarette smoke, automobile exhaust, chimney soot, refuse burning, oil pollution and industrial processes (2).

The substitution of hydrogen of normally readily biodegradable hydrocarbon xenobiotics with structural elements such as halogens, nitro, sulfo groups leads to significantly reduced or retarded microbial breakdown. Increase in the number of aromatic rings also reduces biodegradability. As a result, these compounds tend to accumulate in the environment, for example, DDT (18). Since these compounds are used and produced on a large scale, their breakdown study is important.

Microorganisms having ability to degrade aromatic compounds are used for dissimulation of these compounds in metabolism of natural products, feed industries, water purification and sewage

treatment. Microorganisms react with xenobiotics depending on their surroundings and the structure of the compound (19). Several genera of bacteria, filamentous fungi and yeast are able to degrade aromatic compounds (20).

2. **Degradation of aromatic compounds**:

Aromatic compounds are ubiquitous growth substrates for microorganisms. Like some of the aliphatic compounds, aromatic compounds have a chemical inertness which make them 'difficult substrates'. Microorganisms utilize these compounds by dissimilating them in a step-wise manner.

(a) **Initial hydroxylation of aromatic ring structure**: Aerobic organisms introduce hydroxyl group in the aromatic ring using molecular oxygen as obligatory cosubstrate (21). The position whereby the group is incorporated decides the type of intermediate formed such as catechol, protocatechuate or gentisate. Fig.1 shows the three central intermediates formed from various aromatic compounds (22, 23, 24, 25). The hydroxyl group is introduced either in *ortho* or *para* position.

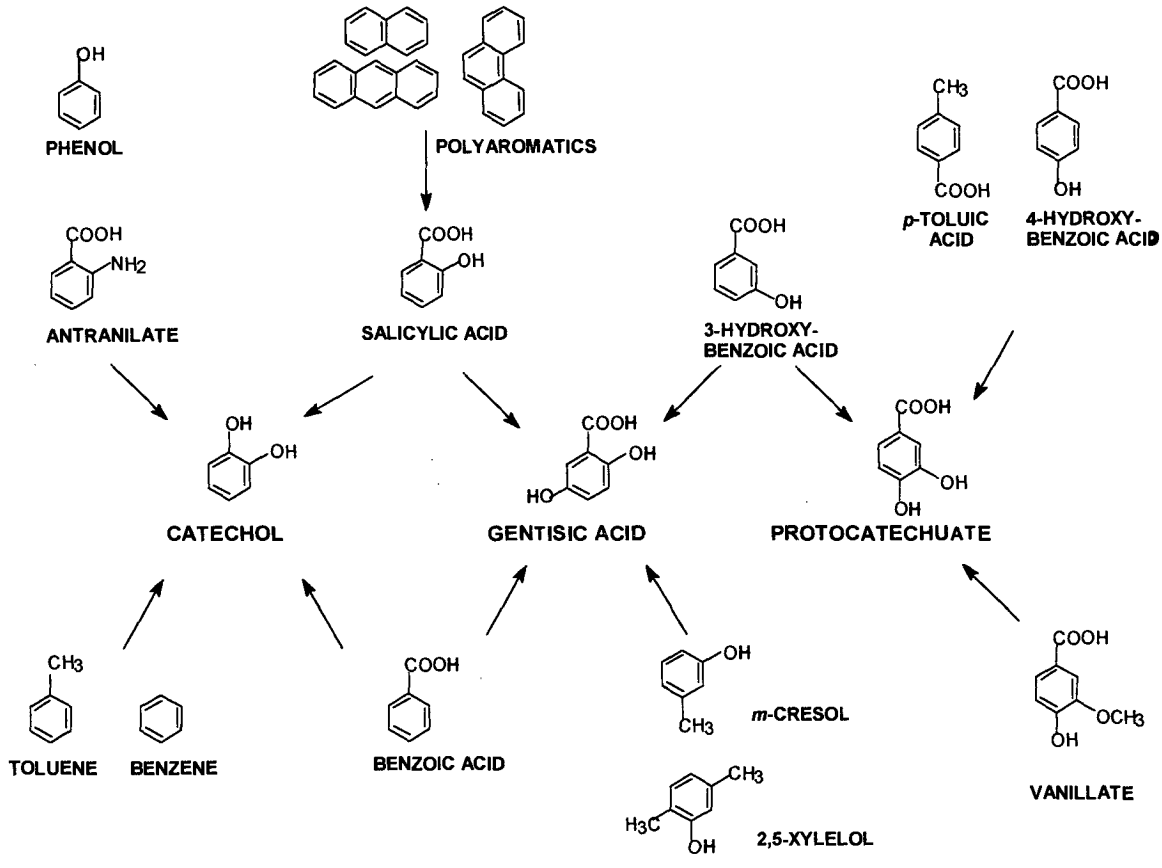


Fig.1: Formation of central aromatic intermediates from various aromatic compounds (22, 23 , 24 , 25).

In the case of compounds such as resorcinol, gallic acid, *etc.*, wherein two hydroxyl groups are already present, a third hydroxyl group must be either present or introduced into the ring before cleavage (23, 26, 27). The enzymes that bring about introduction of hydroxyl group are the hydroxylases. Hydroxylases belong to the monooxygenase group of enzymes that incorporate only one atom of oxygen per substrate molecule while the other atom is reduced to water. Coenzymes $NADH_2$ and $FADH_2$ serve as electron donors. These enzymes do not require heavy metals for activity. Oxygen is activated on reaction with reduced coenzymes and then reacts with substrate to yield monooxygenated product (28).

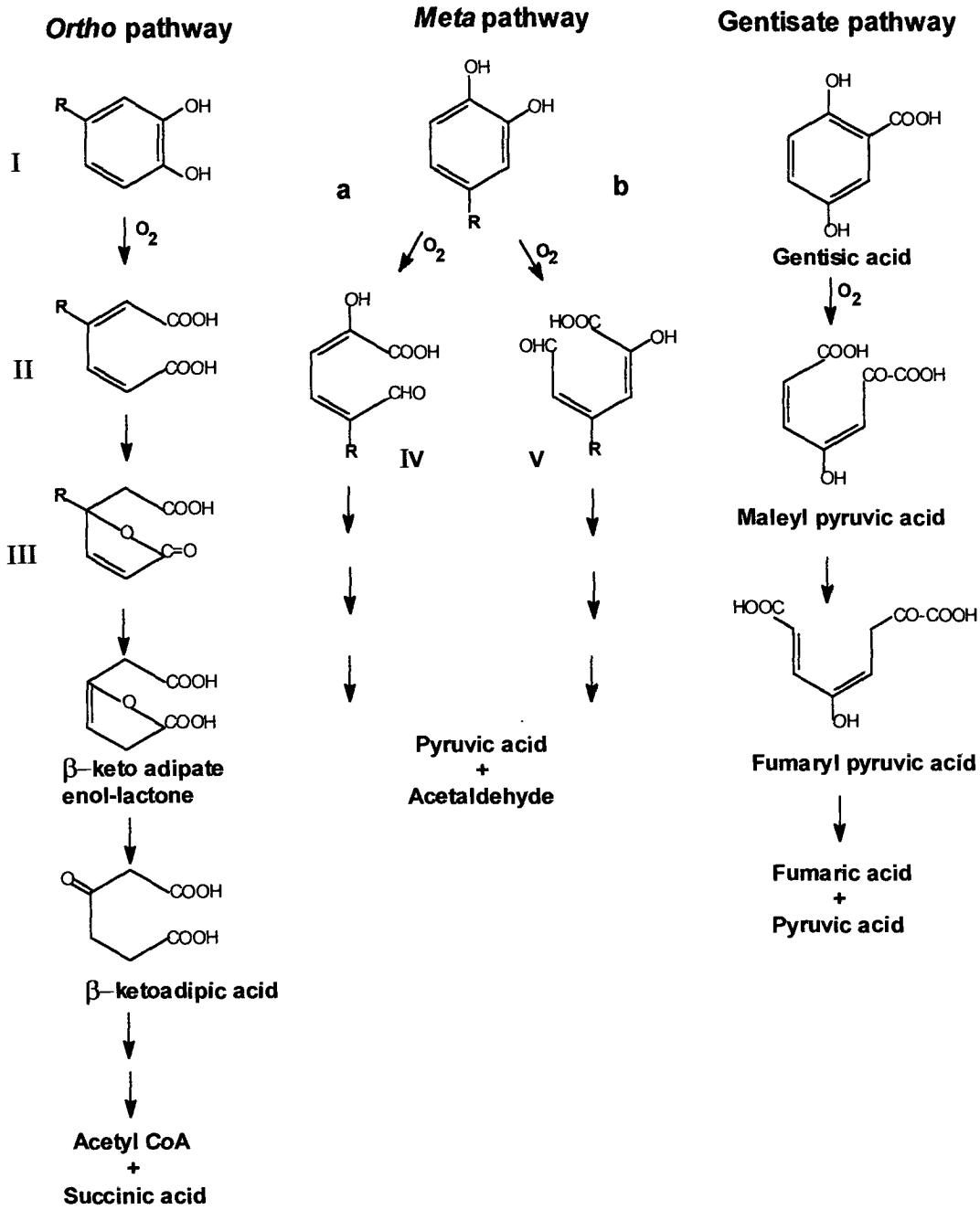
It is not clear whether a unique oxygenase system is induced for initiation and cleavage of aromatic compounds. Studies on mineralization of PAH show that more than one oxygenase system exists in individual species of bacteria (29), e.g. monophenol monooxygenase (30), *p*-hydroxybenzoate hydroxylase (31). Details of the enzyme monophenol monooxygenase are given in section 3(a).

(b) **Modes of ring cleavage**: The aromatic ring prepared for cleavage by hydroxylation is cleaved with the help of dioxygenases. Depending upon the site of cleavage, three modes are described (Fig. 2).

(i) ***Ortho ring cleavage***: Cleavage of the benzene ring is termed '*ortho*' or 'intradiol', when it takes place between adjacent carbon atoms that carry hydroxyl groups. The pathway is termed '*ortho*' or 'β-ketoadipate' pathway (32). Succinate and acetyl CoA are the end products (22, 33).

Catechol 1,2-dioxygenase brings about *ortho* ring cleavage of catechol whereas protocatechuate is cleaved by enzyme protocatechuate 3,4-dioxygenase (28, 34, 35). Cleavage between the hydroxyl groups yields cis, cis-muconate and β-carboxy-cis, cis-muconate from catechol and protocatechuate, respectively (33, 35, 36), β-keto-adipate-enol-lactone is the common intermediate. As seen in Fig. 1., catechol and protocatechuate are formed from a number of compounds that occur in the environment (37,

Fig.2: Modes of aromatic ring cleavage



R=H, I=Catechol; II=Cis,cis-muconate; III=Muconolactone;
IV= α-Hydroxymuconic semialdehyde
R=COOH, I=Protocatechuate; II=β-Carboxy-cis,cis-muconate;
III= γ-Carboxy-muconolactone; IV= γ-Carboxy-muconic semialdehyde;
V= α-Hydroxy-γ-carboxy-muconic semialdehyde

38) and also as intermediates in catabolism of more complex aromatic substrates. These intermediates get channeled *via* the *ortho* pathway. Hence, this pathway plays a major role in nutrition of metabolically versatile microorganisms.

The enzymes of this group, 1,2-dioxygenases, are referred to as red enzymes since the purified enzyme preparations are red in colour. These contain iron in trivalent state at the active centre. Due to the high spin state of this ferric ion, these enzymes show a sharp electron spin resonance (ESR) signal (28, 36, 39). Reducing agents readily inactivate the *ortho* pathway enzymes whereas oxidizing agents are inactive towards them. So also *o*-phenanthroline and 2,2'bipyridyl have no action on these enzymes. Trivalent iron chelator, Tiron, can readily inactivate the red enzymes. The trivalent iron present at the active centre of the red enzymes, bind to substrate and oxygen. Iron gets reduced transiently upon binding.

Oxygen and substrate then react to give the product and iron is released (28).

(ii) ***Meta ring cleavage***: Takes place between two carbon atoms, one of which carriers a hydroxyl group and the other carbon atom may be either substituted or unsubstituted. This kind of cleavage is also called extradiol cleavage. Catechol is oxidized by enzyme catechol 2,3-dioxygenase (23, 40, 41, 42). In the case of protocatechuate, the *meta* pathway is said to be proximal when cleavage takes place between carbon atoms of position 2 and 3 by enzyme protocatechuate 2,3-dioxygenase (43, 44, 45) and distal when the cleavage is between carbon atoms at position 4 and 5 by enzyme protocatechuate 4,5-dioxygenase (46, 47, 48, 49, 50, 51) (Table 1.2).

Iron in divalent form is present at the active centre of these enzymes, which belong to colourless group of enzymes. Fe^{+2} chelators such as *o*-phenanthroline, 2,2'bipyridyl, nitrilotriacetate, EDTA act as inhibitors.

Table 1.2 : Properties of *meta* ring cleavage enzymes

Enzyme	Molecular weight (kD)	Substrates	Reference	
Catechol 2,3-dioxygenase	140	Type I –catechol Type II-3-methylcatechol 4-methylcatechol	40, 41, 52	Fig.2a
Protocatechuate 2,3-dioxygenase	-	3 or 4 substituted catechols, 2 or 5 substituted protocatechuate, esters of protocatechuate, analogs of protocatechuate	43, 44, 45	Fig.2a
Protocatechuate 4,5-dioxygenase	140-150	Substrates with vicinal hydroxyl groups in 3 and 4 position	46, 47, 51	Fig.2b

These enzymes are resistant to reducing agents and are inactivated by oxidizing agents such as hydrogen peroxide and ferricyanide (28). Fe^{+2} ions at the active centre react with oxygen to form perferryl ion complex. Substrate molecule binds with this to form tertiary complex of iron, oxygen and catechol. Oxygen and catechol are activated and react to form catechol-peroxide. Ferrous ion is released, which then binds to oxygen molecule again. Catechol-peroxide undergoes intra molecular rearrangement to form 2-hydroxymuconic semialdehyde (HMS).

(iii) ***Gentisate pathway***: This is the third mode of aromatic ring cleavage, seen in compounds having hydroxyl groups in *para* position to one another. The pathway is also called *p*-hydroxy-phenol pathway and enzyme catalysing the reaction is gentisate 1,2-dioxygenase (23, 53). This enzyme is heat stable and inhibited by *o*-phenanthroline and 2,2'bipyridyl, like the colourless enzymes (54). Molecular weight ranges from 154 – 159 kilo-daltons (54, 55, 56).

Gentisate formed from a number of aromatic compounds (Fig. 1) is cleaved by this pathway (25, 57, 58).

(c) **Regulatory mechanism for synthesis of cleavage enzymes**: Microorganisms form cleavage enzymes depending on the growth substrate. However, more than one type of enzymes have been reported to be present during growth on a particular substrate (59, 60). Two mechanisms are put forth to explain the regulation of enzyme synthesis.

(i) ***Chemical basis***: The selection of ring cleavage mechanism was found to be dependant on the substrates and their substituents (61, 62). A study by Seidman *et al.* (62) showed that substrates with electron withdrawing groups such as protocatechuate, will elicit *ortho* cleavage enzyme synthesis while those with electron donating alkyl groups will induce the *meta* cleavage enzymes. The electron donating groups will hinder the intralactonic rearrangement in the *ortho* pathway. This is not the case in *meta* pathway where open chain hydrates are involved.

(ii) ***Physiological basis***: Feist and Hegemann (63) showed that the inducers determine the type of pathway to be employed. Phenol and its methyl derivatives induce the *meta* pathway enzyme, catechol 2,3-dioxygenase and the other enzymes for catechol. Catechol is not allowed to be accumulated by the rapid activity of the enzyme. Growth on benzoate induces the enzyme forming catechol from benzoate. Catechol is converted to cis,cis-muconate by catechol 1,2-dioxygenase which is present at low concentration. Cis,cis-muconate then induces the other *ortho* enzymes (63, 64). The *ortho* pathway is thus more specific than the *meta*.

The induction of catechol 1,2-dioxygenase synthesis is regulated by muconic acid an enzymatic cleavage product of the inducer, diphenol. Acyl substituted diphenols influences the interaction with macromolecules and thus induction of enzymes of *ortho* pathway does not take place (61, 63). A number of reports of *ortho* enzymes bringing about extradiol cleavage are cited in literature (13, 16, 65, 66).

(d) **Modifications in substituted aromatic compounds**:

Substituted aromatic compounds have to undergo side chain modification or removal of substituent group, either before or after the ring cleavage takes place. More the number of substituents, more difficult it is to breakdown the compound.

Simultaneous removal of nitrite and addition of oxygen has been reported for nitro aromatics (11, 12, 67). The product formed may be a quinone from nitrophenol or nitrocatechol (68, 69, 70) as shown in Fig. 3a or catechol from dinitro aromatics and nitrobenzene (71, 72, 73).

In *Pseudomonas pseudoalkaligenes*, the nitro group is reduced to amino group, which remains attached to the aromatic ring even after cleavage (74).

Methyl groups in the aromatic ring are modified by converting to alcohol followed by benzaldehyde, acid and finally hydroxyl group (1). Either methyl-catechol or

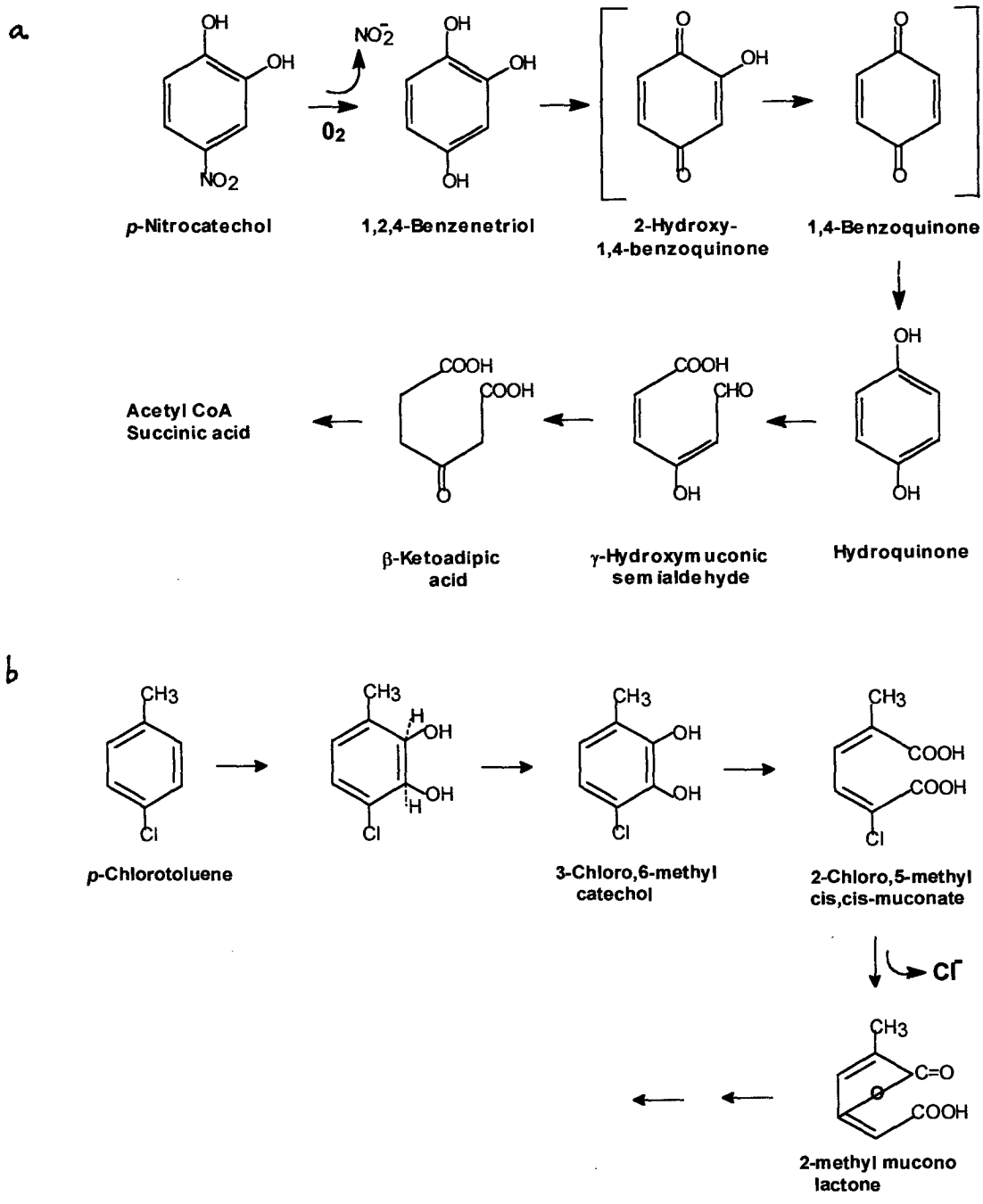


Fig.3: Degradation of (a) nitroaromatic compounds (68, 69 , 70) (b) chloro-compounds (77)

protocatechuate may be formed as intermediates which get degraded *via* the *meta* ring cleavage pathway (65, 75). *Ortho* cleavage of methyl aromatics lead to dead-end methyl lactone formation (76). *Pseudomonas* species follow modified *ortho* pathway when methyl and chloro groups are present in the same compounds e.g. *p*-chlorotoluene (77). *Pseudomonas putida* CLB 250 could grow in the presence of chloro, fluoro and bromo-benzoates (78), while *Pseudomonas cepacia, Arthrobacter globiforms, Corynbacterium sepadonium* could mineralise chlorinated benzoates (79). Chlorinated compounds result in formation of chlorocatechol that get degraded *via ortho* ring cleavage pathway (16, 40, 77, 80, 81). The chloride ion is retained during cleavage and is removed during lactonization of chloromuconate (Fig. 3b). *p*-Chlorophenol was metabolized via hydroquinone formation by *Arthrobacter ureafacians* CPR 706 (82). Chlorosubstituted vanillate is reported to form chloroprotocatechuate (83). *Meta* cleavage of chlorophenols lead to misrouting of substrate or suicide inactivation of the enzyme by highly reactive acyl chloride

(84, 85). However, some bacterial strains having resistant *meta* enzymes have been reported to utilize chlorocatechol *via meta* pathway (76, 86, 87).

Polychlorinated compounds get converted to chlorohydroxyquinol intermediates after 2 steps of hydroxylation (88). Hydroquinone formed may be either cleaved *via ortho* pathway (88, 89, 90) or *via meta* pathway (91). Chlorocatechols follow *ortho* cleavage while methyl aromatics are mineralized *via meta* cleavage.

Aromatic amino acid metabolism is a good example whereby side chains are modified to structures that can be easily cleaved. Tyrosine is metabolized *via* homogentisate, homoprotocatechuate and protocatechuate pathways (92, 93, 94) while tryptophan is converted to anthranilate and further cleaved (61, 94, 95).

Amongst the polyaromatic compounds, study of naphthalene has been valuable in understanding the

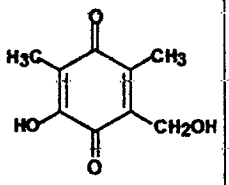
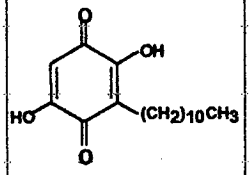
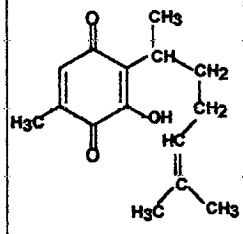
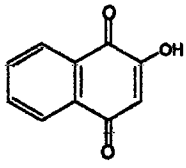
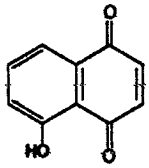
pathways of metabolism of structurally more complex polycyclic compounds. Naphthalene is either hydroxylated to salicylate and then catechol or may be converted to gentisate (96, 97, 98, 99).

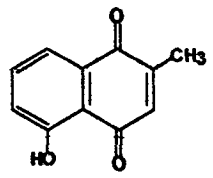
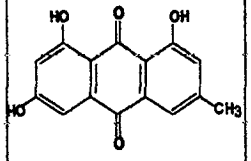
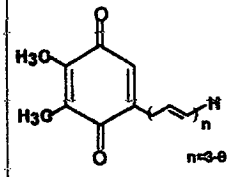
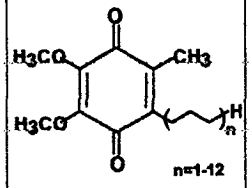
3. **Products of aromatic degradation**:

During biodegradation of aromatic compounds, number of products are formed either as byproducts or intermediates.

(a) Quinones: are non-aromatic conjugated cyclo-hexadienones. Colours of natural pigments that are quinonoid in nature, range from pale yellow to black (100, 101). Variety of quinones exist and are found in bacteria, fungi and plants. 4 groups are described for identification purposes: benzoquinones, naphthoquinones, anthraquinones and isoprenoid quinones (Table 1.3). The first three forms are hydroxylated, with phenolic properties. They are found *in vivo*, combined with sugars or in dimeric quinol formation. Acid hydrolysis is required to release them.

Table 1.3: Naturally occuring quinones and their properties (111)

Type	Example	Structure	Colour	Source	λ_{max} (nm)
Benzoquinone	Shanorellin		Orange -yellow	*Shanorella spirotricha*	272, 406
	Embelin		Orange -yellow	Berries	
	Perezone		Orange	Perezonia plant	
Naphthaquinone	Lawsone		Yellow	Henna	249, 345, 422
	Juglone		Yellow	Walnut, *Juglans regia*	

	Plumbagin		Yellow	Roots of Plumbagina -ceae plants	
Anthraquinone	Emodin		Yellow	Higher plants and fungi	223, 254, 267, 290, 440
Isoprenoid Quinones	Ubiquinone		-	Plants	270
	Plasto-Quinone		-	Plants	254, 261

Isoprenoid quinones are found in plants and are of two types, ubiquinones and plastoquinones. Reversible reduction whereby colourless quinols formed can be reoxidized by shaking in air, is a characteristic feature of quinones (100). Reduction can be carried out using alkaline sodium-dithionite sodium-borohydride, zinc and other reducing agents (100, 102, 103).

Benzoquinones and napthoquinones turn colourless or yellow-brown on reduction whereas anthraquinone with sodium-dithionite gives red colour (100). Hydroxyquinones with alkali produces deep-red to violet colour having characteristic wavelength maxima and this can be taken as an indicator for quinone (100, 103).

Presence of quinone can also be detected by reduction of a second leuco compound such as leuco-methylene blue, which turns blue on oxidation in the presence of oxygen. A number of reactions with amines giving coloured compounds are used for identification of quinones (104).

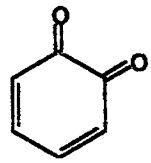
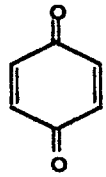
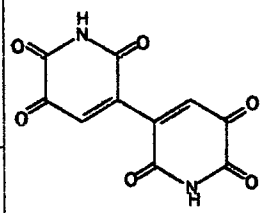
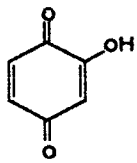
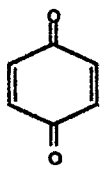
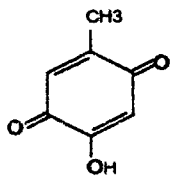
UV-Visible spectrum with the number and position of absorption bands indicate the complexity of the structure and the class of quinone.

Infra-red spectra can be used for structure determination though the Vco value alone may not be enough. *p*-Benzoquinone shows bands at 1640 and 1700 cm^{-1} while *o*-benzoquinone shows bands at 1640-1690 and 1600 cm^{-1} (100, 104).

Formation of quinones during aromatic degradation: Evans (105) first reported the oxidation of phenol giving rise to *ortho*-benzoquinone. The quinone was trapped and identified as dianilino-*o*-benzoquinone derivative. Many such reports on quinone formation are available and are tabulated in Table 1.4.

Two groups of enzymes are recognized, belonging to the monophenol monooxygenase or polyphenol oxidase group, namely catechol oxidase and laccase, that bring about oxidation of phenols, resulting in quinone formation (30, 106). These oxidative enzymes are responsible for all browning reactions

Table 1.4: Quinones formed by various microorganisms during utilization of aromatic compounds

Culture	Substrate	Quinone	Colour	Reference
Vibrio 01	Phenol	 **o-benzoquinone**	Red	105
Pseudomonas aeroginosa	Benzene		Red	112
Polysporus versicolor	Catechol		Red	113
-do-	Hydroquinone	 ***p*-benzoquinone**	Yellow	113
Arthrobacter sp.	2-Hydroxy pyridine	 **Oxidized tripyridol**	Blue	114
Bacillus sp.	Nicotinic acid			114
Burkholderia cepacia AT1100	2,4,5-Trichloro phenoxyacetate	 **2-Hydroxy-1,4-benzoquinone**	Orange	88
Bacillus sphaericus	*p*-Nitrophenol		Purple	115
Burkholderia cepacia RKJ200	*p*-Nitrocatechol		Purple	69
Pseudomonas Strain DNT	2,4-Dinitrotoluene	 **2-Hydroxy,5-methyl quinone**	-	116

throughout the phylogenetic scale. Both enzymes contain copper and described briefly below:

(i) ***Catechol oxidase***: Found in mammals, fish, insects, plants, fungi, algae, and bacteria. It acts on monophenols, diphenols and triphenols; inhibited by copper chelators such as thiourea, sodium-azide, cysteine, mercaptoethanol, *etc.* (30, 106). Substrate analogs such as benzoic acid, mimosine, *etc.* also inhibit the enzyme (107, 108).

Catechol oxidase catalyses two reactions, namely cresolase and catecholase activity. Monophenols are oxidized by cresolase to give corresponding *ortho*-quinones (30, 107, 109). These participate in the polymerisation to form a phenolic chain, as in the case of tyrosine bioconversion to melanin *via* dopaquinone, shown in Fig. 4 (110, 111).

L-Dopa, the immediate product from tyrosine oxidation plays an important role in the central nervous

system and is used in treatment of Parkinson's disease (30, 117). Tyrosinase is the most studied catechol oxidase enzyme. This enzyme, present in mammals, mushrooms, *Neurospora crassa, etc.,* has multiple forms and molecular weight ranging from 55 to 120 kD (30, 118, 119, 120, 121, 122). In another report, three allelic forms of the enzyme are described, based on the oxidation state of copper, TL, TS and Sing forms. TL has met-Cu (II) and absorbs at 700 nm. TS has oxy-Cu (II) and shows two bands at 345 and 600 nm, while the Sing form has the deoxy-Cu (I), with no absorption bands (107, 108). A tyrosinase enzyme of molecular weight 67 kD has been reported in a marine gram-negative bacterium (123).

The enzymes of this group are being applied in food processing either to prevent browning or to facilitate the reaction as in case of tea, coffee and cocoa industry. L-Dopa, melanin, food colourants, metabolites such as actinomycin chromophore are important products of this enzyme (30). Quinones find use as natural colourants such

as Monascus pigment from *Monascus ruber* (124) and in photographic development (101). Genetically engineered cultures are used for production of aromatic compounds such as quinones, catechols, indigo, *etc.* (125). The isoprenoid quinones are used as biomarkers in the study of bacterial communities (126, 127).

(ii) ***Laccases:*** Found in fungi and plants but not in prokaryotes. The enzyme has attained importance due to its involvement in demethoxylation of lignin (30). Laccases oxidise all diphenols but not monophenols. Tyrosine is not oxidized by this enzyme. The enzyme is resistant to carbon monoxide, phenyl-hydrazine, *etc.* that inhibit catechol oxidase and is more sensitive to heat. Presence of Cu^{+2}, actinomycin D and puromycin induce its synthesis (128, 129). Molecular weight of the enzyme in *Neurospora crassa* is 64.8 kD while in *Aspergillus* it is 107 – 117 kD (128, 130).

According to Nakamura (131) laccase acts on quinol to form a semiquinone. An electron transfer from substrate to copper takes place. The second step is non-enzymatic

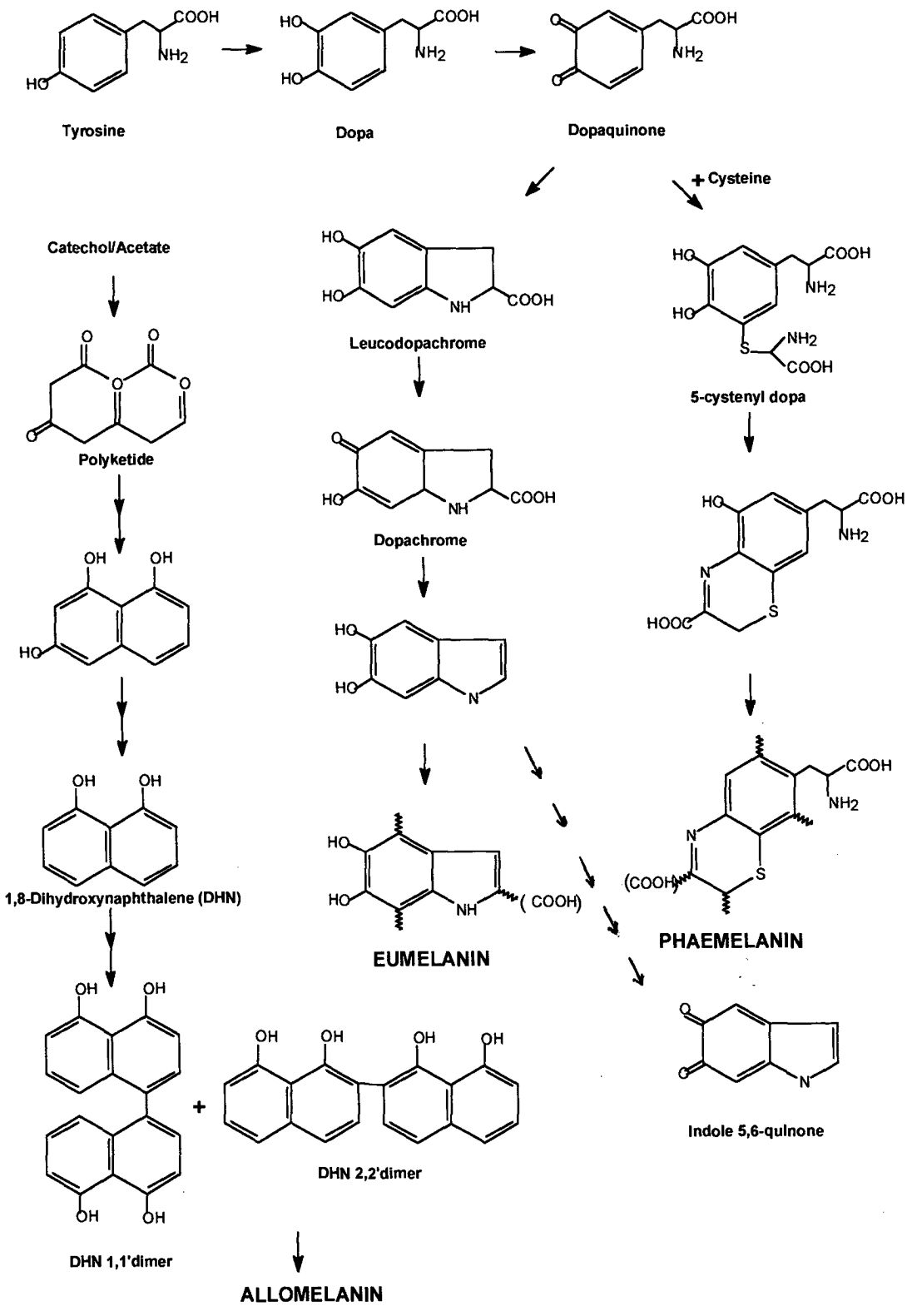


Fig.4: Pathways for melanin formation

whereby two semiquinones react to give one quinone and one quinol molecule.

(b) Melanins: This is the black pigment often encountered in microbial systems. Apart from imparting intense pigmentation, which in certain cases is of taxonomic value, melanins plays a role in increasing resistance of fungi towards lytic enzymes; detoxification of polyphenolics in root nodules; creating anaerobic condition in nitrogen-fixing cultures and protecting cells due to antioxidant, antiradical and superoxide scavanging properties (30, 110, 132, 133, 134, 135, 136).

Three types of melanins are formed:- (110, 134, 137)

(i) Eumelanin: This is a black insoluble pigment formed from tyrosine. Seen in mushrooms and potatoes. Pathway for eumelanin formation is shown in Fig.4.

(ii) Phaemelanin: This pigment is not dark brown or black but has reddish appearance. It is formed from tyrosine

and cysteine. Found in hair and feathers of fowls, encysted forms of *Azospirillum brasilense* ATCC 29145, black spots of potato, human hair, squid ink, *etc.* (133, 138, 139) (Fig. 4).

(iii) Allomelanin: This pigment is formed from nitrogen free precursors such as acetate, catechol, 1,8-dihydroxynaphthalene, *etc.* No tyrosine is involved in the synthesis of this pigment. The pathway for allomelanin formation is called the polyketide biosynthetic pathway (Fig. 4). Filamentous fungus *Alternaria alternata* forms melanin *via* this pathway (140). Allomelanin is also seen in watermelon seeds (139). Tricyclazole, Pyroquilon and Fthalide are inhibitor of this pathway. Basidiomycetes form melanin from γ- L-glutaminyl-4-hydroxybenzene (GHB) by a different route from those described above (110).

4. Genetics of aromatic degradation:

The degradation pathways for aromatic biodegradation are encoded by genes present either on chromosome or on plasmids, the

degradative plasmids. These plasmids are present not only in *Pseudomonas* but are also found in other genera such as *E.coli, Agrobacterium, Moraxella, Flavobacter, Arthrobacter, etc.* Plasmids in *Pseudomonas* species include TOL, CAM, SAL, OCT, NAH (141, 142, 143, 144). The genetic organization and regulation of genes encoding all the *meta* cleavage enzymes for catechol have been studied in *Pseudomonas picketti* PK01 (145) and *Pseudomonas putida* mt-2 (146, 147,148).

The chromosomal BenR gene is induced by benzoate and regulates its conversion to catechol (60). Benzoate also induces *meta* cleavage enzymes by binding to xylS gene on TOL plasmid. Growth on benzoate is reported to cause loss of plasmid by "benzoate curing" (148,149).

Microorganisms with multiple plasmids are constructed, which have the advantage of degrading wide range of aromatic compounds and can be used for cleaning up of oil spills (4,20,150). These organisms also serve as models for the study of metabolic pathways.

Bacterial exopolysaccharide (EPS):

The interaction of the organism with its environment influence its outer surface. The presence of toxic compounds/heavy metals, contact with surfaces, *etc.* induces the culture to alter the cell structure inorder to overcome the stress conditions (151, 152). Bacteria produce a wide variety of complex and diverse EPS, which is involved in this interaction between the organisms producing it and its environment. EPS is used to cope in various ways with the extreme environments (153, 154, 155). Bacterial EPS occurs as either capsular polysaccharide (CPS) or slime polysaccharide (SPS). CPS is intimately associated with cell surface and may be covalently bound (156). The nature of this linkage is difficult to identify. A diacyl glycerol moiety has been found in several *E.coli* strains (157), while in *Haemophilus influenzae* type b, lipids have been suggested to play a role in the linkage (158). Such linkages may be common to gram-negative bacteria (156, 157).

The two forms can be differentiated upon centrifugation whereby CPS appears in the pellet while SPS remains in the supernatant (156, 159). Either one of the two forms or both may be formed together. SPS is loosely attached to the cell surface. In strains of *Rhizobium,*

Agrobacterium and *Alcaligenes* species, more than one chemically distinct EPS are found (156).

Depending upon the chemical composition bacterial EPS may be divided as homopolysaccharide and heteropolysaccharide (160,161).

(a)Homopolysaccharides: Contain one type of monomeric units and are found in genera *Pseudomonas, Xanthomonas* and *Bacillus*. Sialic acid, levans and glucans are some of the homopolysaccharide. Glucan polymer is composed of solely glucose e.g. cellulose, made up of β-1,4-D-glucan. α-1,6-linked glucans or glucopyranosyl units branched at second, third or fourth positions belong to dextran group, which are formed from sucrose as in *Streptococcus viridans, etc.* (162). Levans are polymers of D-fructose with β-2,6 linkages.

(b) Heteropolysaccharides: These are made up of more than one monomeric unit of carbohydrate. The repeating structures are anionic in nature and made up of less than six sugars. The composition may vary depending on growth conditions (163).

Different sugars are involved in EPS formation and hence the numbers of combination are possible which contribute to the diversity in microorganisms (164). Some sugar component such as D-glucose, D-mannose, D-galactose, D-glucuronic acid occur frequently while others like L-rhamnose, L-fucose are less frequent. D-mannuronic acid and L-guluronic acid are rare (161). L-altrose, an unusual hexose was reported first in *Butrivibrio fibrisolvens* CF3 EPS (162). Alginic acid formed by *Azotobacter vinelandii* and *Pseudomonas aeroginosa* contain D-mannuronic acid and L-guluronic acid (163). *Streptococcus thermophilus* MRIC has an EPS composed of D-galactose,L-rhamnose and L-fucose (165). *Cyanospira capsulate* EPS contain glucose,mannose, fucose, arabinose and galacturonic acid in the ratio of 1:1:1:1:2 (166). Other cyanobacterial species are known to produce large amounts of heteropolysaccharides (166, 167, 168, 169, 170).

Heptoses and octonic acids mostly found in lipolysaccharide are absent from EPS (161). Other non-sugar components also contribute to structural diversity (164). Glycerol, D-glucosamine, D-galactosamine, uronic acid,*etc.* are unique to EPS. Phosphates,

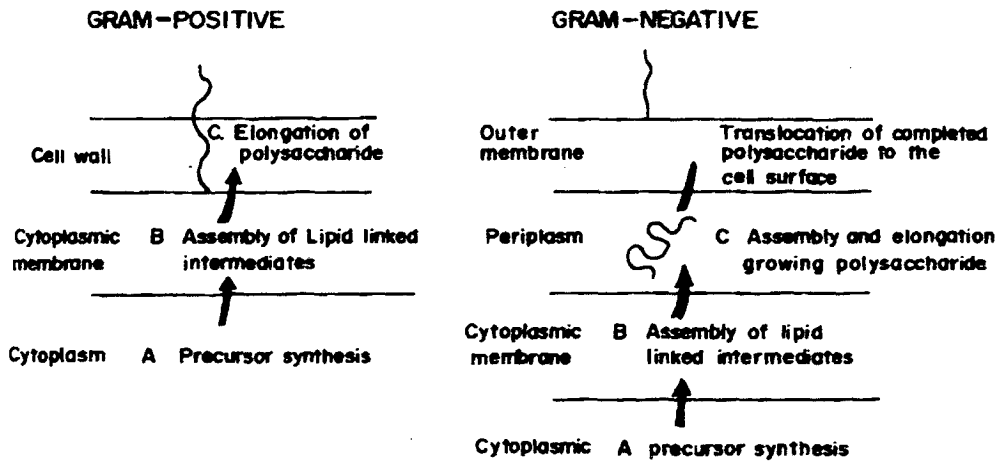


Fig.5: Exopolysaccharide biosynthesis and assembly in gram-positive and gram-negative bacteria (159)

variety of growth conditions. Two categories are described for biosynthetic process, based on the site of synthesis and the type of precursors (156,161). Cell contact with solid surface is known to stimulate EPS synthesis (151,173).

(b) **EPS synthesized outside cell**: Seen in case of gram-positive organisms. Levans and dextrans (synthesized from sucrose) are synthesized outside the cell, with the help of enzymes devansucrase and dextransucrase. Variations in the polymer are seen depending on the degree of branching and the type of linkage i.e. α 1→3 and α 1→6 (161).

(c) **EPS synthesized at the cell membrane**: This has been studied in gram-negative organisms. Nucleotide diphosphate or monophosphate sugars serve as activated glycosyl donors. The enzymes may be cytoplasmic or attached loosely to the cytoplasmic membrane. The repeating units are assembled by sequential transfer of sugars to lipid linked intermediates (174,175). The lipid involved is undecaprenol phosphate (C_{55}-P). The role of lipid is thought to be in facilitating accurate and

ordered formation of repeating unit structure, solubilization of hydrophobic oligosaccharides in hydrophobic membrane domain, transport across the membrane, *etc.* (161,175).

In case of addition of acetate and pyruvate groups, acetyl-CoA and phosphoenol pyruvate serve as precursors (161). A different system is involved in the biosynthesis of *Azotobacter vinelandii* alginate (176). Polymannuronic acid is initially synthesized and transported across the membrane. Mannuronan 5'epimerase, an extracellular enzyme, then converts some mannuronic acid to guluronic acid resulting in block structures (176).

ATP binding cassette (ABC) transporters are involved in export of EPS and forms the inner membrane polysaccharide export system. These transporters are ubiquitous and are involved in import and export system in both prokaryotes and eukaryotes (177).

The EPS of *Myxococcus xanthus* contains large amount of protein, equal to polysaccharide amount, in structures called fibrils, that make up the EPS. The EPS appears to consist of polysaccharide backbone decorated with several protein species (178). Similar structures are seen on the cellwall of another myxobacterium, *Stigmatella aurantiaca* (179).

d) **Effect of various physiological growth condition on EPS formation**: Microorganisms can convert a wide variety of substances into polysaccharides. Hydrocarbons are utilized by various polysaccharide forming organisms. There is no corelation between the composition of polymer synthesized and the growth substrate. Some strains produce more polysaccharide under nitrogen, phosphorus, sulfur or potassium limited conditions. Certain ions are known to be required for substrate uptake or as cofactors in polysaccharide synthesis (161, 180) e.g. calcium induces EPS production in growing cells. It also regulates gluconeogenic enzyme activity (152, 179).

EPS production seems to be a feature of late logarithmic or early stationary phase as seen in *Pseudomonas* species and *Aeromonas salmonicida* species (181, 182). In gram-negative bacteria, sufficient isoprenoid lipid needs to be available to allow simultaneous synthesis of peptidoglycan, lipopolysaccharide and EPS, during growth. EPS formation therefore takes place at late logarithmic phase or early stationary phase, when there is no requirement for lipopolysacharide and peptidoglycan synthesis and the precursors are thus available for EPS formation (161).

Effect of aeration on EPS production, varies from culture to culture. High aeration rates favour certain organisms while lower aeration gave higher yield in case of alginate formation by *Azotobacter vinelandii* and EPS by *Rhizobium meliloti* (183, 184).

Genetics and regulation of EPS formation:

The genes for EPS may be chromosomal or present on plasmid. In case of *E.coli* K1, the capsular EPS genes are present on chromosome at the kps locus. Twelve genes products encoded by 15kb of chromosomal

DNA are required for synthesis, polymerization, modification and translocation of CPS (185).

In *Haemophilus influenzae* type b, genes are present as tandem repeats and loss of one of the copy of the genes, results in unencapsulated bacteria (186). Whereas in *Pseudomonas aeroginosa*, the non-mucoid strains form alginate under stress conditions that amplify the EPS genes (187).

Genetic manipulation inorder to get stable production, of required type and amount of EPS synthesis, is being made use of in commercial EPS production such as Xanthan gum by *Xanthomonas campestris* (188).

Functions of bacterial EPS:

The role of the EPS depends on the natural environment of the microorganism. The ability to form EPS is related to selective pressure in environment (189). The functions of EPS are as follows:-

(a) **Protective function**: EPS surrounding the microorganisms provide protection from action of antibacterial agents, toxic metal ions, *etc.* and against desiccation and predators such as protozoa. The changes in the external osmolarity brought about by desiccation is known to triggers the capsule biosynthesis (189).

(b) **Adherence**: EPS promotes adherence of bacteria to each other and also to surfaces. They form biofilms and colonize various niches, including dental plaques. Some of the disadvantages of the biofilms are fouling of pipes in industrial processes (177), corrosion of metal surfaces such as ships (154).

Colonization and biofilms may also cause the health problems such as excess alginate formation by *Pseudomonas* species in the lungs of cystic fibrosis patients, leading to impermeability to antibiotics (190) and nosocomial infections due to colonization of catheters in hospitalized patients.

(c) **Resistance to non-specific host immunity**: CPS masks the underlying cell surface receptors thereby resisting complement-mediated killing by the host (191). The more highly charged the

CPS greater is the degree of resistance to opsono-phagocytosis (153).

Some CPS may modulate response of host to mediate an immune response by effecting release of cytokine molecules, thus disrupting coordination of hosts cell mediated immune response (192). Another possibility is formation of copious amounts of EPS leading to tolerance (153, 189, 193)

(d) **Bacteria-plant interaction**: Phytopathogenic bacteria secrete EPS that help in virulence. The CPS of *Erwinia amylovora* helps in establishing growth in plants and masks surface molecules that would elicit immune response (194). In case of symbiotic associations, CPS help to induce nodulation, invasion of nodule and development. Plant lectins recognise surface polysaccharides of *Rhizobium* species and association is formed when both are compatible (155, 189). In *Rhizobium meliloti*, the surface polymer is a succinoglycan made up of one galactose and seven glucose residues with acetyl, succinyl and pyruvyl substituents (177).

Applications of bacterial EPS:

Given in Table 1.5. Polysaccharides are important in both indus and medicine. Some polysaccharides are used for development of bacterial vaccines for prevention of infections caused by encapsulate pathogens (195). The know-how of export of polysaccharide in gra negative bacteria is used in the production of chemotherapeutic agen which are targeted to selectively disrupt capsule export and combat infections by encapsulated bacteria (177). Xanthan, produced by *Xanthomonas campestris* was the first industrial polysaccharide of microbial origin and was approved for use as food additive (196).

Apart from medical fields, EPS has wide application range in f pharmaceutical, petroleum industry, *etc.* (197, 198, 199)

Table 1.5: Applications of bacterial EPS

Organism	Polysaccharide	Use	Reference
Sclerotium glucanium	Scleroglucan	Antitumor	200
Lentinus edodus	Lentinan	-do-	200
Hansunela polymorpha	Arabitol	Sweetening agent	201
Azotobacter vinelandii	Alginate	Immobilization agent	200
Pseudomonas sp.	Gelrite	Culturing media for thermophiles	202, 203
Pseudomonas cepacia	Emulsan	Emulsifier	204
Acinetobacter calcoaceticum	-do-	-do-	205
Streptococcus equi Streptococcus zooepidemicus	Hyaluronic acid	Cosmetics, pharmaceuticals	197
Acetobacter xylinum	Cellulose	Acoustic membranes, wound dressing, heparin analogues	197
Xanthomonas campestris	Xanthan	Cosmetics, printing, pharmaceuticals, food industry, oil recovery and drilling, fire fighting fluids, pesticides	206, 197
Leuconostoc mesenteroids	Dextran	Molecular sieve, restriction endonuclease inhibitor	207, 208
Agrobacterium sp., *Rhizobium* sp.	Curdlan	Gelling agent in food industry	197
Aureobasidium pullulans	Pullulan	Packing material in food industry	197

Aim and scope

Oil spills, chemical effluents, and non-judicious use of pesticides and herbicides, cause a threat to environment. These compounds being toxic in nature are shunned by most microorganisms and very few can breakdown these esoteric compounds. This small number of microorganisms, mostly the gram-negative organisms such as *Pseudomonas* species, play a vital role in removal of compounds from environment and utilize them as carbon and energy sources (209, 96).

Acquiring biochemical properties such as enzymes and new genetic traits, enable the microorganisms to adapt to stress conditions created by presence of xenobiotics. Other mechanisms of overcoming stress are by forming a protective EPS that minimizes the toxic effects. EPS also function in adherence and protecting against desiccation (189).

It was of interest to isolate microorganisms from environments exposed to constant inflow of pollutants such as industrial effluent discharge site and areas surrounding oil wells. Microorganisms having

ability to degrade wide range of aromatic compounds would be of interest.

The study was undertaken and presented in the following chapters.

1. Screening for benzoate degraders in deep seawater, sediments and industrial effluent samples.
2. Morphological, biochemical and physiological characterization of strain P_2d.
3. Utilization of benzoate in liquid medium by strain P_2d.
4. Characterization and identification of biotransformation products of benzoate.
5. Metabolism of tyrosine by strain P_2d.
6. Characterization of EPS formed during growth of strain P_2d in benzoate medium.

MATERIALS AND METHODS

I. Screening for benzoate degrading microorganisms

The seawater and sediment samples were collected from a depth of 75-100 metres from various stations of Bombay High oil fields, during the cruise on board, Sagar Pachimi in mid November 1999 (Fig. 6). Water samples from the industrial effluent discharge sites were also collected and screened.

Water samples were plated on mineral medium (MM) (Appendix A.1) agar plates supplemented with 0.1% sodium-benzoate. For viable count on nutrient agar medium (Appendix A.2), the water samples were diluted tenfold in saline (0.85% NaCl). The soil sediments were suspended in saline at a ratio of 1:10 and incubated on Orbitek shaker for 1 h at 150 rpm. The suspension was allowed to settle and 0.1ml of clear supernatant was spread plated.

Plates were incubated at room temperature (28°C ± 2°C) for 24 to 48 h. The colonies were counted and the viable count was calculated.

II. Tests for identification of the bacterial strain P_2d

Cultural, morphological and biochemical characters: Colony characters of the culture, strain P_2d were studied by streaking the culture on nutrient agar and benzoate agar medium. Motility was studied by the hanging drop technique. Culture was stained by Gram's staining method (210) and observed under the light microscope. Standard methods were followed to study the biochemical characteristics of the culture (210).

Rothera's test: Ring cleavage of aromatic degradation was studied by modified Rothera's method (211). Strain P_2d cells grown in MM with different aromatic compounds, were centrifuged at 6000 rpm for 20 min at 4°C. Pellets were washed twice and resuspended in 0.05M phosphate buffer pH 7 to an absorbance of 4.0 at 540 nm. To 2 ml cell suspension, 0.5 ml of toluene and 3 ml of 13.3 mM catechol (final concentration = 5 mM) were added. After 3 min, the colour change was noted. Appearance of yellow colour indicates presence of *meta* ring cleavage enzymes. The mixture was further incubated on shaker

for 12 h following which, 1 g of ammonium-sulphate was added and the solution was mixed well. 5 drops of freshly prepared 1% sodium-nitroprusside solution and 0.5 ml of liquor ammonia were then added slowly along the side of the tube and the colour change was recorded. Presence of *ortho* ring cleavage enzymes results in a purple ring formation.

III. Growth of *Pseudomonas mendocina* P_2d in liquid medium

(a) Culture grown for 24 h in the presence of benzoate was used as inoculum. MM (20 ml) containing 0.3% benzoate in the culture flasks was inoculated with 5% inoculum and growth was monitored as increase in absorbance at 600 nm on Elico colorimeter (filter no. 60). To check the effect of inhibitors, 2,2'bipyridyl, at concentration of 2 mM was added to benzoate medium during growth.

Viable count of the culture was routinely observed by serial dilution in saline and plating on nutrient agar medium.

Plates were incubated at room temperature and the colonies formed were counted.

(b) Activities of enzymes involved in benzoate metabolism:

Preparation of cells: The cells, P_2d grown in benzoate medium (0.3%) for 24 h, were pelleted by centrifugation at 6000 rpm for 10 min. The pellet was suspended in phosphate buffer (0.05M, pH 7), vortexed and again centrifuged. The washed pellet was resuspended in the same buffer to an absorbance of 4 at 540 nm.

Preparation of cell free extract (cfe) (212, 63): Cells of strain P_2d were suspended in phosphate buffer containing 10% acetone to absorbance of 4.0 (42). The cells were sonicated under cold conditions at 150 mA for 5 min with pulses of 30 sec in between. The suspension was centrifuged at 16,000 rpm for 1 h at 4°C. The supernatant was used for enzyme assays.

Preparation of HMS (212, 213): Cfe of *Pseudomonas cepacia* AC1100 cells, preheated at 53°C for 10 min with 10 mM catechol in 50 mM phosphate buffer was incubated till HMS formation

stopped, as seen from absorbance at 375 nm. It was then extracted with diethyl-ether followed by acidification of aqueous layer. Again extracted with diethyl-ether and reextracted in phosphate buffer. Molarity calculated from absorbance value.

(i) Catechol 2,3-dioxygenase (40): The reaction mixture of the assay was taken in 3 ml silica cuvette and consisted of 2.7 ml of 0.05M phosphate buffer (pH 7) with 100 µl of cells or cfe and catechol (final concentration 10 mM). The increase in absorbance at 375 nm was monitored. Specific activity of the enzyme was calculated and expressed as units (U)/mg protein.

Enzyme unit ≈ increase/decrease in absorbance per min or activity per min.

(ii) The reaction mixture for catechol 1,2-dioxygenase was the same as for catechol 2,3-dioxygenase. The absorbance was monitored at 260 nm for 5 min. Cells/cfe heated at 60°C for 10 min were also used.

(iii) HMS hydrolase (63): To 2.7 ml of 0.05 M phosphate buffer (pH 7), 2.5 µM HMS and 100 µl of cells/cfe were added

and fall in the absorbance was monitored at 375 nm for 5 min.

(iv) HMS dehydrogenase (63): 2.5 μM HMS, 0.1 μM NAD and 100μls of cells/cfe were added to 2.7 ml of buffer and the absorbance checked at 375 nm.

(v) Tyrosinase assay (119): Strain P_2d grown in MM supplemented with 0.2% tyrosine for 48 h was pelleted and resuspended in 0.1 M sodium-phosphate buffer (pH 6), to get an absorbance of 4.0 at 540 nm. Substrate solution was prepared with DL-Dopa (0.8 mg/ml) in same buffer as above. Reaction mixture contained 2.9 ml of substrate solution taken in a 3 ml cuvette, incubated for 5 min at 30°C, followed by addition of 100 μl of cell suspension or cfe. The change in absorbance was read at 475 nm. The assay was carried out with culture grown in benzoate (0.3%), protocatechuate (0.1%) and glucose (0.2%) medium.

(vi) Catechol-oxidase assay (30): Strain P_2d cells were incubated at 35°C in the presence of catechol at a final concentration of 10 mM and the colour change was noted.

Inhibition of enzyme activity was studied visually. Enzyme inhibitors namely sodium-metabisulphide, sodium-bisulfide, mercaptoethanol, cysteine, thiourea, ascorbic acid, potassium cyanide (KCN), sodium-azide, at 1 mM concentration were added and the reaction mixtures were incubated at 35°C for 10 min and the colour change was noted.

Similarly the effect of these inhibitors was studied by incorporating the above inhibitors in growth medium supplemented with 0.3% benzoate and 0.2% tyrosine. Appropriate controls were kept for the experiment.

(vii) Protocatechuate 2,3-dioxygenase (43): Reaction mixture contained 2.8 ml of 0.05 M phosphate buffer (pH 7) and 50 µl of 0.1% protocatechuate solution. The reaction was started by adding 100 µl of cells/cfe and absorbance was monitored at 350 nm for 5 min. The assay was carried out with culture grown in protocatechuate (0.1%), benzoate (0.3%), glucose (0.2%), tyrosine (0.2%), 3-hydroxybenzoate (0.1%) and *p*-hydroxybenzoate (0.1%).

Benzoate cells induced in the presence of salicylate, gentisate,and protocatechuate were also assayed.

(viii) Protocatechuate 3,4-dioxygenase (35): The reaction mixture was same (vii). Activity was monitored at 290 nm.

(ix) Protocatechuate 4,5-dioxygenase (46): Reaction mixture was same as (vii) and the absorbance was read at 410 nm.

(x) Gentisate 1,2-dioxygenase (214, 53): Cells grown in the presence of various aromatic compounds were pelleted and adjusted to absorbance of 4.0.Phosphate buffer (0.05M) pH 7.4 was taken in 3 ml cuvette with 1.6 mM gentisate. Cells/cfe (100 µl) were added to the reaction mixture and activity was monitored at 330 nm. Gentisate 1,2-dioxygenase was also assayed by measuring oxygen uptake using 10 µl of 0.1 % stock substrate in 1.6 ml reaction mixture.

IV. Extraction of transformation products of benzoate metabolism

Culture supernatant (20 ml) or reaction mixtures (20 ml) containing cells in buffer incubated with benzoate or related

compounds, were extracted with 10 ml diethyl-ether. Organic layer was separated and concentrated with nitrogen gas. The aqueous phase was acidified to pH ~ 2 with 6 N HCl and centrifuged at 6000 rpm for 10 min. The supernatant was extracted with 10 ml of diethyl-ether and the ether layer was concentrated by passing nitrogen gas and analyzed by TLC.

For column chromatography, the wine-red culture supernatant from 2 L culture broth, was lyophilised to dryness using LABCONCO lyophiliser. The dried powder was then extracted with methanol. The methanol extract was concentrated and used for column chromatography.

V. Analytical techniques

a. **UV- Visible spectrophotometry:** UV-Visible scans of the culture supernatants and other samples were done using UV-1601 Shimadzu spectrophotometer.

b. **Thin layer chromatography (TLC):** The slurry, prepared by mixing 6 to 8 g of Silica Gel G (Acme's) in 15 ml of distilled water, was poured on glass plates and drawn into thin layers. The plates were air- dried and then activated at 110°C for 30

min. Samples were spotted on the activated plates using thin glass capillaries. After drying the spots, plates were developed in solvent chambers, saturated overnight. Solvent was allowed to run upto 3/4th of the plate, the solvent front was marked and coloured spots, if any, were noted. The plates were then placed in iodine chamber. Rf values were calculated using the formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Various solvent systems were developed and used for separation of transformation products:

	Solvent system	**Ratio**
I	Benzene: methanol: acetic acid	45:10:1
II	Benzene: methanol	10:45
III	Benzene: methanol: ethyl-acetate	40:60:40
IV	Benzene: acetone	90:10
V	Toluene: chloroform: acetone	40:25:35
VI	Hexane: ethyl-acetate	85:15
VII	Benzene: chloroform: xylene	40:40:20
VIII	Benzene: ethyl-acetate: acetic acid	75:24:1 24:75:1 50:50:1 40:60:1
IX	Chloroform: ether	66:33

c. Column chromatography: A 30x2 mm glass column was washed, cleaned and dried. Slurry of Sephadex LH-20 (Sigma) was prepared by mixing 8 g of the gel in 20 ml of methanol. Column was packed by adding the slurry slowly with the help of a glass rod. The packed column was equilibrated by washing with methanol, twice. Sample (0.5 ml) was loaded onto the column using a Pasteur pipette and eluted using methanol as mobile phase. Aliquots were collected as 1 ml fractions in clean test-tubes and the fractions with similar TLC patterns were pooled together.

d. High Performance Liquid Chromatography (HPLC):

Samples were analyzed on Spectra-Physics HPLC system, using a reverse phase C_{18} Nucleosil column. The system was initially cleaned using methanol before the run. The HPLC pump was programmed to give 100% of mobile phase at the rate of 1 ml per min. The UV-visible detector was switched on and allowed to stabilize. The integrator was programmed to record the data.

During standardization, the mobile phase, flow-rate, wavelength of detector and amount of sample injected were varied. Results are tabulated in Appendix B. The column was initialized with mobile phase under isocratic conditions. The samples were filtered using Millipore 0.45 μm filters and 10 μl were injected in the injection port and turned to 'inject' position while simultaneously starting the recording on the integrator. After the run time was over, the injection port was brought back to 'load' position for the next injection. Results were recorded as peaks on the chromatogram. From the data, the retention time for the peaks was obtained.

e. **Gas chromatography (GC):** Gas chromatography was carried out as per the method of Bhosle, *et. al.* (215). 5 mg of lyophilized sample was hydrolyzed with 2 ml of 2N HCl by flushing with nitrogen gas. The mixture was heated on a sand-bath for 3 h, after which the hydrolysed samples were filtered. To the supernatant, 100 μl of Inositol was added as internal standard. Sample was dried in Perfit Rotovapor bath under vacuum for 5 min. Sample was dissolved in 1 ml of distilled water and passed through Dowex resin column, then

vacuum dried for 5 min and kept in dessicator, overnight. The sample was incubated at 45°C for 30 min after adding 1 ml of 0.1 M Na_2CO_3 to it. Then, 0.5 ml of Na_2HBr_4 (20 mg/ml) was added and the sample incubated in the dark for 2-3 h. To destroy Na_2HBr_4, glacial acetic acid was added drop-wise till no more effervescence was seen. The sample was vacuum dried and 4 ml of methanol was added to the sample thrice, vacuum dried and then kept in vacuum dessicator for 3 h. 1 ml of 99% acetic-anhydride and 1 ml of 99% pyridine were added to the sample and incubated overnight at room temperature. Then the sample was vacuum dried and kept overnight in the desiccator. It was washed with 1 ml of distilled water and extracted thrice with 4 ml of 99% methylene-chloride. To the lower layer, collected in a clean test-tube, anhydrous Na_2SO_4 powder was added and then filtered through the same. Sample was collected in a flask, vacuum dried out, extracted with diethyl-ether and then flushed with nitrogen gas. Sample volume was made to 100 µl with methylene-chloride before injection. 0.4 µl of sample was injected. A capillary Gas Chromatography system of Chrompack Model CP 9002 with capillary column CP Sil 88 (25m, id =0.32mm, df=0.12) and

flame ionization detector was used at oven temperature of 70°C. The sample was analyzed for 40 min. Standard sugars and methylene chloride as blank were also run for 40 min.

f. Light microscopy of stained samples:

EPS staining (216): Smears of culture were prepared on clean slides by routine method and air-dried. The smear was flooded with saturated congo-red stain for 2 min and washed. Carbol-fuchsin was then added for 2 min.The slide was washed and air dried.

Capsule staining: was done by using Manewal's stain (Appendix C.4). A drop of congo-red was mixed with loopful of culture and a smear was made using another slide. The smear was flooded with Manewal's stain. The air-dried slides were observed under 100x objective using Leitz phase contrast microscope.

g. Scanning electron microscopy (SEM): Adopted from Specimen Preparation Methods from SEM, JEOL application note.

The culture was centrifuged at 5000 rpm for 5 min and the supernatant discarded. The pellet was dispersed in 0.05M phosphate buffer (pH 7) and smeared onto the stub. Smear on the stub was fixed in 2 ml of 2.5% glutaraldehyde fixative (pH 7.2-7.4), overnight at R.T.. The stub was placed in 0.05M phosphate buffer and then in 30% acetone. It was allowed to stand for 10 min. The dehydration procedure was repeated likewise with 50%, 70% and 90% acetone for 10 min, each and finally in 100% acetone for 30 min. The stub was then put in the critical point drying device wherein the acetone gets replaced by liquid carbon-dioxide at high pressure. This was evaporated by raising the temperature to 45°C and liquid carbon-dioxide gets converted to gaseous carbon-dioxide and escapes. The process takes 1 h. The stub was placed on the sputter coater (spi-module) specimen holder, after drying. The position of the stage is set such that the specimen is approximately 50 mm from the bottom of the sputter head. After sputtering the specimen with 10-15 nm thin film of gold, the stub was placed onto the electron microscope sample chamber and observed with JEOL- 5800 LV SEM.

h. **Infra-red spectroscopy**: Samples were mixed with KBr pellets and applied to window of FTIR 8101A Shimadzu-Fourier transform infra-red spectrophotometer.

i. **Oxygen analysis**: The rate of oxygen uptake was analyzed by Gibson Oxy 5/6 analyser. The instrument was stabilized at 8 V. Paper speed was maintained at 0.1 mm/sec. Scale was adjusted to 'zero' using zero suppression key and to 100, using the sens key. Phosphate buffer 0.05 M pH 7, was aerated for 5 min prior to use and 1.6 ml was added in the reaction chamber. Magnet speed was adjusted at 6 units. Once the baseline stabilized, 100 µl of cells, adjusted to an absorbance of 4.0 at 540 nm, were added into the reaction mixture. The endogenous oxygen uptake was recorded for 5 min, followed by addition of 10 µl of substrate into the same mixture. The uptake rate was recorded again for 5 min. The chamber was cleaned, washed twice with distilled water and 1.6 ml of buffer was added for the next reading. This procedure was repeated twice with each substrate and the mean of oxygen consumed was calculated (Appendix D).

j. Polyacrymide gel electrophoresis (217): *Sample preparation for whole cell protein profile:* Cell pellet obtained from 25 ml of culture broth was resuspended to an optical density of 4.0 at 540 nm. 100 µl of cells were treated with 50µl of sample buffer [Appendix C.1 (viii)] and 100 µl of cells were used for protein estimation by Folin-Lowry's method (218). Cells with sample buffer were boiled for 10 min in a boiling water-bath. 30 µl of loading dye solution was added to the treated sample prior to electrophoresis.

Preparation of Gels: A modified method of Laemmli (7) was followed. The glass plates after washing and drying were wiped with acetone and clamped together with spacers in place. The assembly was sealed from inside using molten 1% agar and allowed to solidify. All the solutions for separating gel (Appendix C.1) were taken in a clean beaker and mixed well. Ammonium-per-sulphate and TEMED were added, together, just before pouring the gel. The mixture was added in between the 2 plates using a 10 ml pipette, upto $3/4^{th}$ of the space. Distilled water was added above the separating gel to get a uniform

surface. Water was drained off after the gel had solidified. Similarly, stacking gel was prepared and added over the separating gel. A comb was introduced into the stacking gel and it was allowed to set. For denatured protein separations, 0.2% of sodium-dodecyl-sulphate (SDS) was added to separating and stacking gels during preparation. The lower spacer and comb were removed and the assembly was placed in electrophoretic chamber. Samples were loaded in the wells with the help of a syringe. Tank buffer [Appendix C.1 (vii)] was added to upper and lower tanks. Electrodes were connected to powerpack and the gel was run at 120 V till the tracking dye reached the bottom of gel. Plates were separated carefully after removing the spacers and the gel was put in staining solution.

Staining of gels: Protein staining was carried out using Coomassie- Brilliant Blue R-250 stain [Appendix C.2 (i)]. Gel was placed in this staining solution for 6-8 h. It was then destained in Destain solution I [Appendix C.2 (ii)] for 1 h and in solution II [Appendix C.2 (ii)] till clear bands appeared. Polysaccharide staining was done by silver staining method (92). After electrophoresis, the gel was put in fixative solution

[Appendix C.3 (i)] for 2 h with intermittent shaking. The fixative was drained out and gel was washed with deionised water for 1 h. It was soaked in dithiothreitol solution [Appendix C.3 (ii)] for 2 h and then the solution was poured out. Silver-nitrate solution [Appendix C.3 (iii)] was added for 1½ h and gel was again washed with deonised water, twice. Addition of formaldehyde solution [Appendix C.3 (iv)] resulted in appearance of dark bands within 5 min duration. Citric acid (10 ml) [Appendix C.3 (v)] was added immediately to stop the reaction and avoid the whole gel from turning dark. Gel was washed with deionised water and stored in the same.

k. **Melting point determination**: 10 mg of compound was added into a melting point capillary of 1 mm diameter. The capillary was introduced in the capillary holder of Centrofix melting point instrument and a thermometer was introduced in the thermometer holder. Temperature was allowed to rise at the rate of 3 degrees per min and the temperature where the sample melts completely was noted.

VI. Qualitative and quantitative tests for transformation products

a. **Precipitation of catechol** (219): This method was used for detection and quantitation of catechol, during growth of *Pseudomonas mendocina* P_2d in benzoate medium. Lead- acetate solution, 0.3 ml of (20% w/v) was added to 1 ml of culture supernatant. Presence of catechol gave a white precipitate, which was centrifuged at 1000 rpm for 5 min and the wet weight was noted.

b. **Colorimetric estimation of Catechol** (105): 1 ml of 10% sodium-molybdate solution was added to 1 ml of culture supernatant, followed by addition of 0.5 ml of 0.5 N HCl and 1 ml of 0.5% sodium-nitrite solution. Presence of catechol gives a yellow colour to the solution. Addition of 1 ml of 0.5 N NaOH forms cherry-red colour. Absorbance was read at 510 nm on Shimadzu UV-1601 spectrophotometer (Fig. a).

c. **Spot tests for quinones**: Red culture supernatant was subjected to the following spot tests for quinone detection.

   i) To 5 ml of supernatant, 6 N HCl was added dropwise and the colour change was observed. The solution was then

made alkaline with 10 N NaOH and again the colour change was noted.

ii) 0.1 N NaOH was added dropwise to 5ml of red supernatant and the colour change was observed.

iii) Sodium-dithionite (2%) was added to the red supernatant, mixed well and allowed to stand for 4-6 h. The colour change was noted and the solution was kept on shaker to check the effect of aeration.

iv) A pinch of sodium-borohydride was added to the red supernatant and the colour change was observed.

v) 0.5 ml of 2% potassium-iodide on acidification was added to 5 ml of supernatant and benzoate agar medium plate was flooded with the same. The observations were noted.

d. Derivatization of quinone using aniline (105):

2,5-dianilino-*o*-benzoquinone derivative: 1% aniline was added to a solution containing 25 mM catechol and culture pellet of *Pseudomonas mendocina* P_2d. The reaction mixture was incubated on the shaker at 150 rpm for 3 h. The precipitate formed at the end of the incubation period was filtered and the

residue was dissolved in diethyl-ether. Ether was allowed to evaporate and the dried sample was reextracted in petroleum ether (40°-60°C). The extract was dried to powder and weighed.

VII. Standard estimation methods

a) **Protein estimation by Folin-Lowry's method:** The protein concentration in samples was quantitated by Folin Lowry's method (218) (Fig. b).

b) **Sugar estimation by phenol sulphuric acid method:** Samples were analyzed for their sugar content by the phenol-sulphuric acid method (220). To 1 ml of aqueous sample containing polysaccharides, 1 ml of 5% aqueous phenol was added. The tubes were placed in ice and 5 ml of concentrated sulphuric acid was added quickly into the tubes. Tubes were incubated in ice for 10 min. After allowing the tubes to come to R. T., absorbance was measured at 480 nm. Standard curve was plotted using glucose (0-100 μg/ml) as standard (Fig. c).

c) **Tyrosine estimation by Arnow's method (221):** The tyrosine estimation method is based on Millon's reaction. To 1 ml solution, 1 ml mercuric-sulphate reagent [Appendix C.5 (i)] was

added. The mixture was mixed well and kept in boiling water-bath for 10 min. After cooling the mixture, 1 ml nitrite reagent [Appendix C.5 (ii)] and 1 ml D/W was added to it. The turbid solution formed was centrifuged at 6000 rpm for 5 min and the clear red solution read at 540 nm. Standard curve is shown in Fig. d.

d) **Dopa estimation by Arnow's method (221):** One ml test solution was acidified with 1 ml 0.5 N HCl and mixed well. To this, 1 ml sodium-molybdate reagent [Appendix C.5 (iii)] was added which gives yellow colour. Addition of 1 ml of 1 N NaOH gave red colour. The solution was diluted with 1 ml D/W and read at 540 nm. Standard curve is shown in Fig. e.

VIII. (a) Extraction of EPS from *Pseudomonas mendocina* P_2d cells:

EPS extraction was carried by the method described by Adhikary (170). Cells grown in benzoate medium were incubated on ice for 2 h, followed by centrifugation at 6000 rpm for 10 min. The viscous pellet was suspended in 10 ml of deionised water and kept on magnetic stirrer, overnight. Cells were separated from the solution by centrifugation at

6000 rpm for 10 min. EPS was precipitated from the supernatant by adding 3 volumes of cold ethanol and incubating at 4°C for 3-4 h. The white precipitate of the EPS was weighed.

(b) Determination of emulsifying activity of EPS:

EPS extracted from the cells incubated on ice was used to find the emulsifying activity (222). The EPS (100 µl) was dissolved in 0.5 ml deionised water by heating intermittently and the volume was then made to 2 ml. The absorbance was adjusted to 0.1 at 540 nm using Elico colorimeter (filter no.54). Hexadecane (0.5 ml) was added to the above solution and vortexed for 1 min. The 0 min absorbance (Ao) was noted and the suspension allowed to stand for 30 min (At). The absorbance was read after the incubation period and further kept for another 30 min. Percent emulsifying activity was calculated using the formula,

$$\% \text{ Emulsifying activity} = \frac{A_t}{A_0} \times 100$$

RESULTS

I. Screening for benzoate degraders in deep seawater, sediments and industrial effluent samples:

Sea water and sediment samples collected from various stations of Bombay High oil fields (Fig. 6), were analyzed for bacterial counts on nutrient agar and benzoate mineral medium (Appendix A). Viable counts of these samples, and effluent collected from a local industry are given in Table 3.1a. Colony forming units (cfu) count for seawater ranged from 10^1 to 10^4 cfu/ml and for the soil sediments, from 10^2 to 10^3 cfu/g on benzoate agar medium.

The effluent sample had a total viable count of 2.6×10^6cfu/ml on nutrient agar medium and 1.38×10^5cfu/ml on benzoate agar medium. Isolates were picked at random and further screening was done by studying the growth in the increasing concentration of sodium-benzoate upto 1% (w/v). Cultures a, b, c, d, e, f and P_2d could grow very well in the presence of 1% benzoate (Table 3.1b) and their colony characteristics on benzoate agar medium were noted (Table 3.1c).

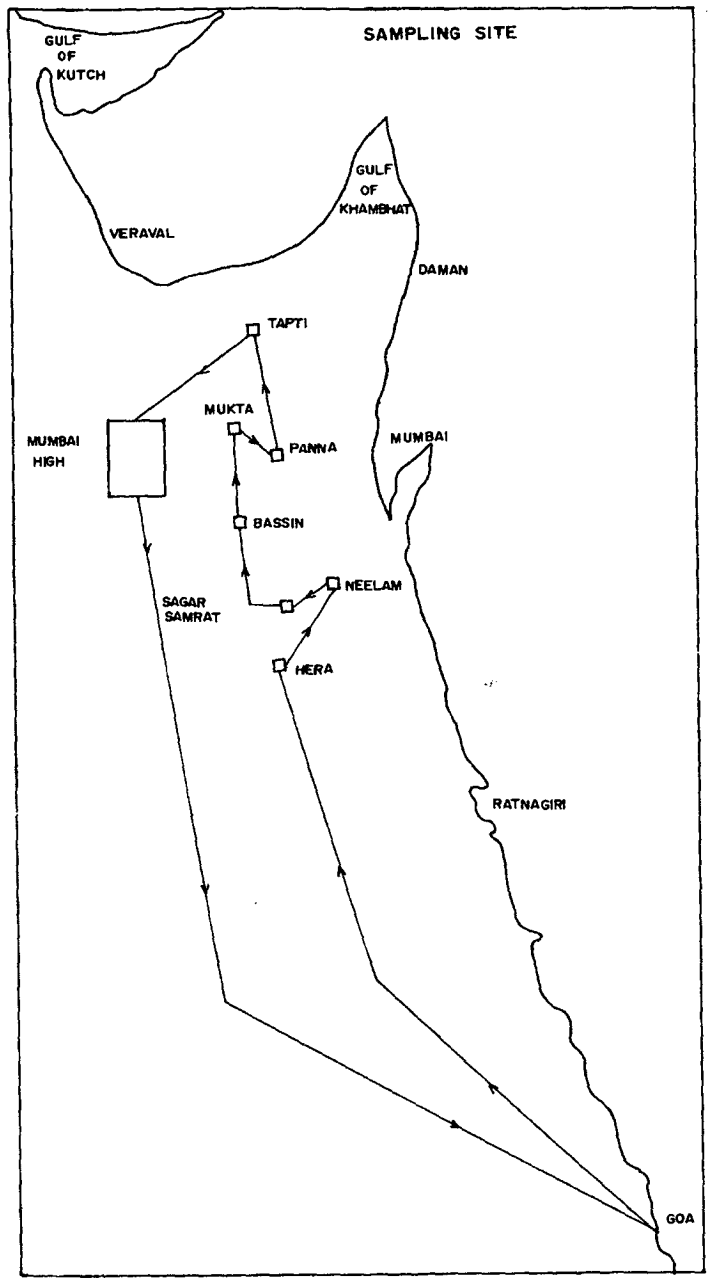


Fig.6: Sampling sites of the Bombay High oil fields

Table 3.1a: Viable counts of sea water and sediments from Bombay High and for industrial effluent

Sea-water sample cfu/ml			Sediment cfu/g		
Sampling site	**NA**	**BA**	**Sampling site**	**NA**	**BA**
BASSIN 2	9.90×10^4	-	BHN 3	2.64×10^8	4.40×10^2
BHN 2	9.06×10^4	-	BHN 8	1.54×10^8	1.10×10^2
BHN 3	3.40×10^4	1.00×10^1	BHS 3	6.70×10^7	-
BHN 8	3.45×10^4	-	BHS 10	6.38×10^8	-
BHS 3	3.20×10^4	1.00×10^1	BLQ 2	2.20×10^7	6.16×10^3
BHS 10	1.30×10^4	-	BLQ 7	9.57×10^8	2.20×10^2
HEERA 4	1.59×10^5	4.03×10^3	HRA 7	1.40×10^7	2.20×10^2
HEERA 6	2.44×10^5	1.63×10^4	HRA 8	-	3.30×10^2
HEERA 8	Matt	4.60×10^2	ICP 5	2.20×10^8	
ICP 5	1.62×10^6	1.37×10^3	ICP 7	2.37×10^{10}	-
ICP 7	7.00×10^4	-	ICP 8	7.90×10^8	-
MUKTA 8	1.16×10^5	-	MC-HRA	1.36×10^9	-
NEELAM 2	9.00×10^4	1.70×10^2	MUKTA 3	4.47×10^7	2.20×10^3
NEELAM 3	2.40×10^5	1.80×10^2	MV 8	1.05×10^{10}	1.65×10^3
NQ 10	1.60×10^4	8.00×10^1	NLM 3	2.77×10^9	5.50×10^2
NQ 3	2.50×10^3	1.00×10^1	NQ 3	8.25×10^8	-
NQ 4	5.14×10^4	-	NQ 9	8.14×10^8	-
NQ 9	8.30×10^4	3.80×10^2	NQ 10	1.10×10^9	-
PANNA 4	1.23×10^5	-	PANNA 3	7.15×10^8	1.43×10^3
SAMRAT 2	5.70×10^6	-	PANNA 4	1.65×10^7	4.40×10^2
SHP 3	1.50×10^4	-	SHP 3	1.02×10^{10}	-
SHP 7	4.10×10^4	-	SHP 7	9.68×10^8	1.10×10^3
SHP 8	4.30×10^4	-	SHP 8	6.90×10^8	-
TAPTI 3	1.93×10^5	-	SS 2	5.19×10^9	3.3×10^2
TAPTI 4	1.77×10^5	-	SS 6	8.80×10^9	-
TAPTI 9	1.55×10^5	-	TAPTI 3	7.30×10^8	-
Effluent	2.6×10^6	1.38×10^5	TAPTI 4	9.90×10^8	-
			TAPTI 9	4.36×10^8	-
			VM 2	1.85×10^7	-

Key: NA, Nutrient agar; BA, Benzoate agar; -, no growth;

Table 3.1 b: Growth of isolated cultures in the presence of varying benzoate concentrations

Source	Isolate	Growth* in MM supplemented with sodium-benzoate				Rothera's test
		0.1%	0.3%	1%	Colour of medium	
Seawater	a	+++	+++	++	White	*Ortho*
	b	+++	+++	++	White	*Ortho*
	c	+++	+++	++	White	*Ortho*
	d	+++	+++	++	White	*Ortho*
	e	+++	+++	+++	Bluish-green	*Ortho*
	f	+++	+++	+++	Light orange	*Ortho*
Sediment	g	++	+	-	White	*Ortho*
	h	++	+	-	White	*Ortho*
	i	++	+	-	Blackish-yellow	*Ortho/Meta*
	j	++	+	-	White	*Ortho*
	k	++	+	-	White	*Ortho*
	l	++	+	-	White	*Ortho*
Effluent	P_2d	++++	++++	++++	Wine-red	*Ortho/Meta*

Key: * growth was monitored visually;
-, no growth ; +, poor growth ; ++, good growth ;
+++, very good growth ; ++++, excellent growth

Table 3.1c: Colony characters of the isolates on benzoate agar

Colony character	P_2d	a	b	c	d	e
Size	0.5 mm	1 mm	0.5 mm	2 mm	1 mm	1 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Cream with orange halo	Cream	Cream	Cream	Cream	Blue
Margin	Entire	Irregular	Entire	Irregular	Entire	Entire
Opacity	Translucent	Translucent	Opaque	Translucent with opaque centre	Opaque	Transparent
Elevation	Raised	Flat	Raised	Flat	Raised	Flat
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Brittle	Mucoid
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Gram character	Gm-ve slender rods	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli

Colony character	f	g	h	i	j	k
Size	1.5 mm	Pinpoint	0.5 mm	2 mm	0.2 mm	1.5 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Cream with peach center	Cream	Cream	Cream	Cream	Cream
Margin	Irregular	Entire	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Transparent with opaque center	Transparent	Transparent with opaque border
Elevation	Flat	Raised	Raised	Flat	Flat	Raised
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Mucoid	Mucoid
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Gram character	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli	Gm-ve coccobacilli

Culture 'e' formed bluish-green colouration in the liquid medium while 'f' and P_2d formed light-orange to red colours. Each of the cultures showed the presence of *ortho* ring cleavage during growth on benzoate, while culture 'i' and P_2d showed *ortho* as well as *meta* ring cleavage enzymes to be operative. During growth on benzoate agar medium, P_2d formed yellow halo around its colonies (Fig. 7) and was selected for further studies.

II. Morphological, biochemical and physiological characterization of strain P_2d:

a. Identification of strain P_2d: Results of biochemical tests are given in Table 3.2a. Strain P_2d is a motile coccobacillus and is gram negative. The culture grows in the presence of sugars such as glucose and lactose, with acid production but no gas formation. It is oxidase and catalase positive and grows aerobically and forms acid in Hugh Liefson's medium. Culture does not grow at 4°C and at pH 3.6.

All morphological, cultural and biochemical tests of the strain P_2d were found to be similar to that described in Bergey's manual

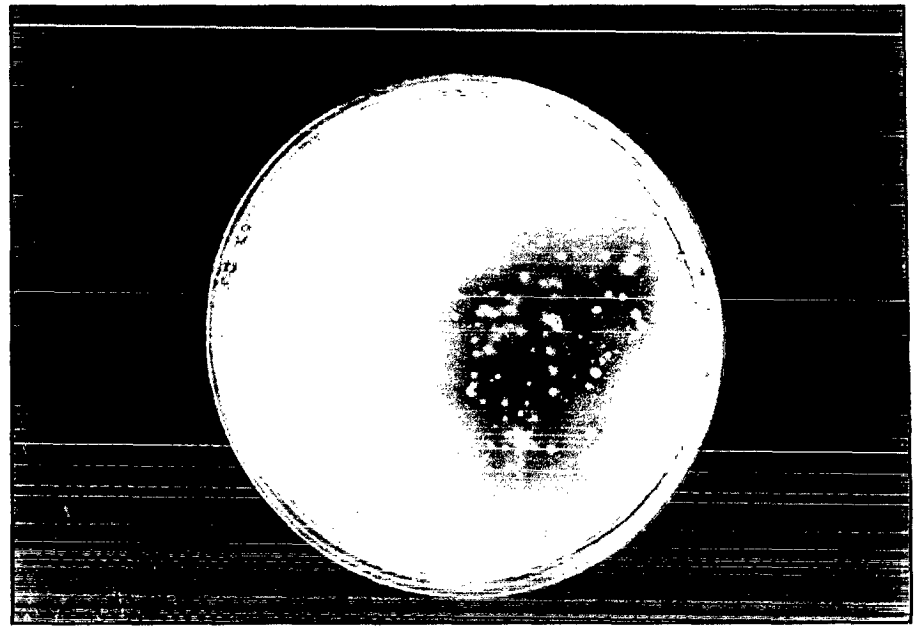


Fig.7: Growth of strain P_2d on benzoate agar medium showing the formation of yellow halo around the colonies

Table 3.2a: Biochemical characteristics of strain P_2d

Biochemical test	Observations	
Gram character and morphology	Gram-negative, short rods of 1μm length	
Motility	+	
Glucose utilization	+A	
Lactose utilization	+A	
Oxidase	+	
Catalase	+	
Methyl-red	-	
Voges Proskauer	+	
Indole	-	
Citrate utilization	+	
PHB accumulation	-	
Growth at pH 3.6	-	
Growth at 4°C	-	
	a	**b**
Denitrification after 0 h	-	+
6 h	++	-
12 h	+	-
24 h	+	-
48 h	-	-
Hugh Liefson's medium Without oil /With oil	+/-	

Key: -, negative ; +, positive ; +A, growth with acid only, no gas ; ++, strongly positive ; a, presence of NO_2 ; b, presence of NO_3

(180) for *Pseudomonas mendocina* but for the denitrification test, carried out with 24-48 h old culture, gave repeatedly negative results. Therefore, the test was carried out at intervals of 6 h, 12 h, 24 h and 48 h. Six hour old culture showed absence of nitrate as there was no effervescence with zinc dust and distinctly positive nitrate reduction test for presence of nitrites, as seen by red colour formation with sulphanilic acid and α-naphthylamine. Intensity of red colour for nitrate reduction had diminished in 12 h and 24 h old culture and no nitrate could be detected.

On the basis of the above tests, the culture was identified as *Pseudomonas mendocina* P_2d. Fatty acid profile of the culture is shown in Fig. 8. Accordingly to Microbial Identification System (MIS), the culture was identified as *Pseudomonas* confirming the identification based on biochemical tests.

b. Utilization of various aromatic compounds by strain P_2d: Growth of strain P_2d in the presence of various aromatic compounds was checked in liquid MM. The aromatic compounds were used at a concentration of 0.1% (w/v). Growth was checked visually as increase in turbidity and colour formation in the

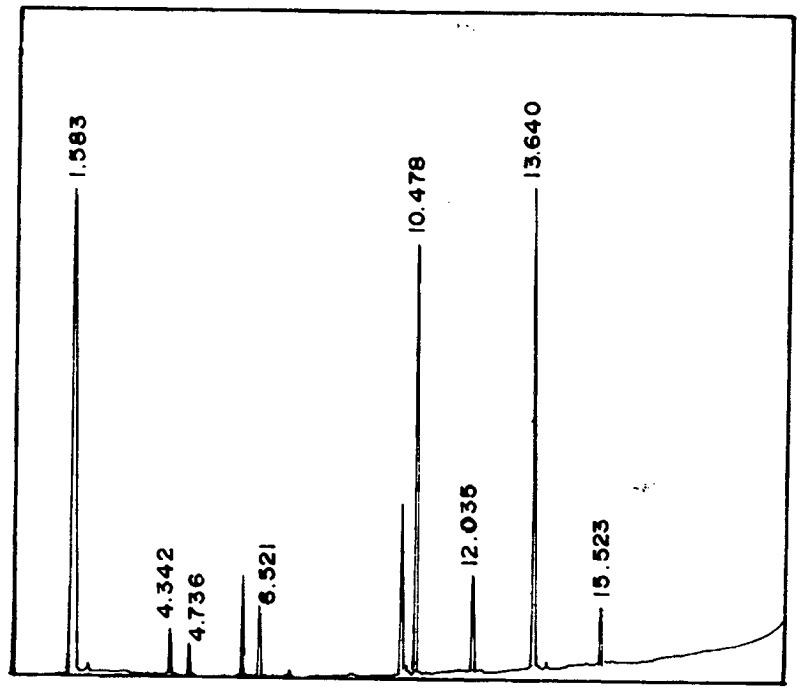


Fig.8: Gas chromatographic fatty acid profile of strain P_2d grown on NA, as obtained from Microbial Identification System

medium. The culture was sub-cultured thrice in order to confirm the growth.

Strain P_2d grew in the presence of wide range of aromatic compounds (Table 3.2b). Three types of growth patterns were observed i.e.poor growth with no colour formation in the medium, good growth with no colour formation and good growth with colour formation. The colours formed ranged from yellow, pink, red, brown, black.

Poor growth was seen in the presence of toluene, phenol, *p*-nitrophenol and *o*-chlorophenol. The medium remained colourless except for *p*-nitrophenol where the yellow colour of the substrate in the medium was retained.

Culture showed good growth in the presence of glucose, protocatechuate, xylene, salicylate, *p*-hydroxybenzoate, 3-hydroxybenzoate, nitrobenzene, *p*-chlorophenol, resorcinol, biphenyl, phthalate, anthracene, phenanthrene and β-naphthol, as sole carbon sources (Table 3.2b). No colour formation was seen in

Table 3.2b: Growth and colour formation by *Pseudomonas mendocina* P_2d in the presence of various aromatic compounds

Substrate	Growth[1]	Colour of the culture medium
Benzoate	+++	Yellow→Red
3-Hydroxybenzoate	+	White
p -Hydroxybenzoate	++	White
Protocatechuate	+++	White
Gentisate	+/-	Light brown
Salicylate	+++	White
Phenol	++	White
Catechol	++	Yellow→Red→ Black
Resorcinol	+	Brown-yellow*
o-Chlorophenol	+	White
p-Chlorophenol	+++	White
p-Nitrophenol	+	Yellow*
Nitrobenzene	++	White
Toluene	+	White
Xylene	++	White
Tyrosine	+++	Pink → Brown→Reddish-black
Tryptophan	++	White
Dopa	-	Black
Gallic acid	++	Greenish-black
Guiacol	+	Pale yellow
Pyrogallol	++	Reddish-brown
Tannic acid	+	Dark brown
Caffeic acid	+	Brown
Phthalate	++	White
β-Naphthol	++	White
Biphenyl	+++	White
Anthracene	+++	White
Phenanthrene	++	White
Naphthalene	+	White

Key: * colour of the original medium with substrate; [1] growth + to +++, increasing turbidity in minimum three successive sub-cultures; -, no growth

the medium. In the presence of 0.05% L-Dopa, the medium turned black, but no growth was obtained. Good growth as well as colouration of medium was seen in the presence of benzoate, tyrosine and catechol. Strain P_2d gave yellow, orange and red colour in the presence of benzoate. Growth in the presence of catechol formed initially yellow followed by red turning into black colouration. In the presence of tyrosine, initially the medium turned pink and then brown and finally reddish-black in colour. Gallic acid, guiacol, pyrogallol, tannic acid and caffeic acid were utilized by strain P_2d giving greenish-black, yellow, reddish-brown and brown colours.

Culture grew in gentisate at 0.01% concentration and pale brown colour of medium was retained. Higher concentration did not support the growth.

c. Oxygen uptake by strain P_2d: Results are tabulated in Table 3.2c. Strain P_2d grown in the presence of sodium-benzoate as sole source of carbon, showed a very high oxygen consumption rate of 189.4 x 10^{-6} nmoles/mg dry weight, for catechol. Surprisingly,

Table 3.2c: Oxygen uptake rates of culture grown in glucose, benzoate and tyrosine with various substrates

	Oxygen uptake ($x10^{-6}$ nmoles/min/ mg dry weight)		
Growth Substrate / **Substrate**	**Benzoate**	**Glucose**	**Tyrosine**
Glucose	29.32	21.29	ND
Benzoate	40.15	30.77	7.08
3-Hydroxybenzoate	-	-	ND
p-Hydroxybenzoate	6.6	-	ND
Salicylate	-	-	ND
Protocatechuate	5.8	-	ND
Catechol	189.4	180	ND
Resorcinol	-	-	ND
o-Chlorophenol	9.17	-	ND
p-Nitrophenol	20.11	-	ND
Nitrobenzene	-	-	ND
Toluene	1.87	-	ND
Xylene	-	-	ND
Tyrosine	-	-	24.26
β-Naphthol	2.19	-	ND
Biphenyl	-	-	ND
Phenanthrene	-	-	ND

Key: -, no uptake ; ND, not done

glucose grown cells also showed a similar oxygen uptake for catechol of 180 x 10^{-6} nmoles/mg dry weight of cells. With sodium-benzoate as substrate, an uptake rate of 40.15 x 10^{-6} and 30.77 x 10^{-6} nmoles was seen for benzoate and glucose grown cells, respectively. The uptake with glucose was low i.e. 29.32 x 10^{-6} and 21.29 x 10^{-6} with benzoate and glucose cells, respectively. A good oxygen uptake was seen for *p*-nitrophenol, while in the presence of protocatechuate, toluene, *p*-hydroxybenzoate, *o*-chlorophenol and β-naphthol, benzoate grown cells showed poor oxygen uptake. Glucose grown cells did not show uptake with these substrates.

Culture grown in benzoate and glucose showed no oxygen uptake with substrates tyrosine, biphenyl, xylene, nitrobenzene, 3-hydroxybenzoate, *p*-chlorophenol, salicylate, phthalate, anthracene, phenanthrene and resorcinol. All substrates were used at 6.25 x 10^{-4} % concentration. Substrates used at higher concentration did not yield the oxygen uptake. Tyrosine grown cells showed an uptake of 7.08 x 10^{-6} nmoles for benzoate and 24.26 x 10^{-6} nmoles for tyrosine.

Pseudomonas mendocina P_2d is a versatile culture having ability to grow in the presence of wide range of aromatic compounds. Various colourations are formed in the growth medium during utilization of some aromatic compounds such as benzoate, catechol, tyrosine, *etc.*, by the culture. Benzoate being the substrate used for isolating aromatic degraders, the physico-chemical characterization during growth in benzoate was carried out.

III. Utilization of benzoate in liquid medium by strain P_2d

a. Growth of strain P_2d at varying concentration of benzoate: Growth curves of strain P_2d at varying benzoate concentration are depicted in Fig. 9. Strain P_2d grew in the presence of 0.1% benzoate, without any significant lag phase, attaining poor yield with maximum absorbance of only 0.56 at around 12 h growth. A pale yellow colour appeared after 4 h of growth, deepened after further incubation to dark-yellow by 24 h. With higher concentration of benzoate at 0.3% cells grew well without any lag phase attaining

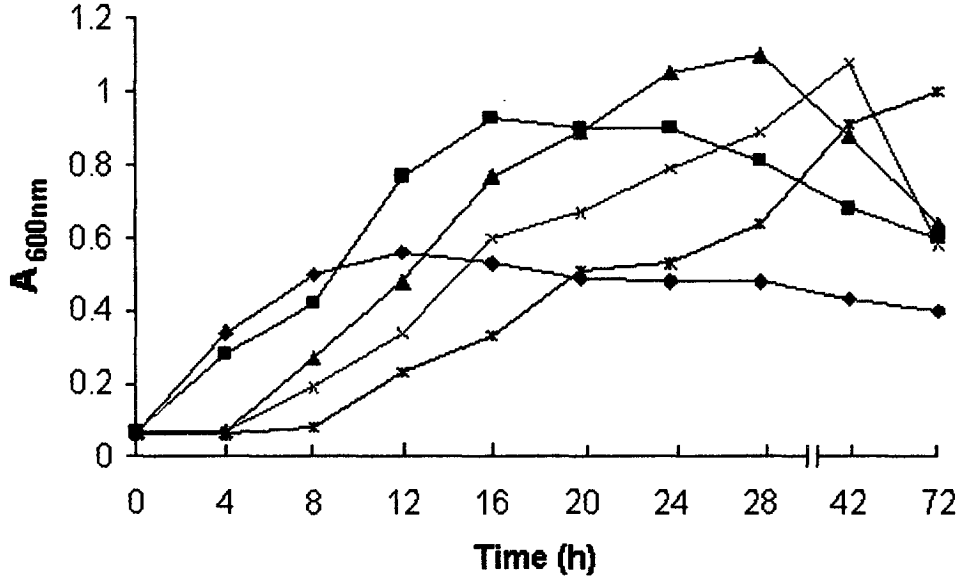


Fig.9: Growth of *Pseudomonas mendocina* P_2d at 0.1% (♦), 0.3% (■), 0.5% (▲), 0.7% (×) and 1.0% (✱) benzoate concentration

maximum good growth within 16 h. Colour of the medium changed to yellow to dark yellow within 8 h and changed further to red within 24 h (Fig.10). With increasing concentration of 0.5, 0.7 and 1% benzoate the cells showed an increasing lag of 4 to 8 h, which was followed by steady growth, maximum absorbance having reached after 28 h for 0.5%, 42 h for 0.7% and 72 h for 1% concentration of benzoate. As in 0.3% benzoate, colour change from colourless to yellow to red was observed with 0.5%, 0.7% and 1% benzoate. Higher the concentration, giving the deeper red colour, although it took longer period to appear. Growth was poor and only yellow colour was formed with benzoate above 1% concentration (Fig.11). Red colour lasted in the medium for 2-3 weeks. On the basis of above observations, concentration of 0.3% benzoate was chosen for further studies.

b. Effect of varying temperature, pH and aeration on growth and colour formation in benzoate medium:

Results of the effects of growth parameters are tabulated in Table 3.3. Growth of strain P_2d at 28°C, pH 7 and 150 rpm was as described above. Culture incubated at 37°C, showed less growth than at 28°C.

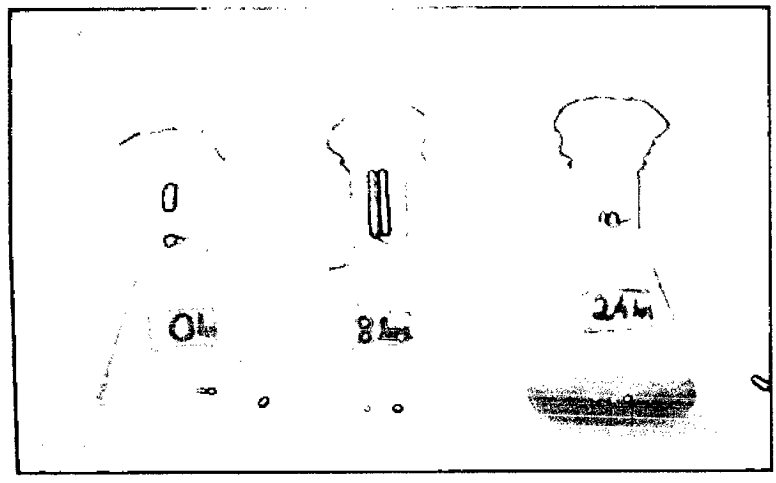


Fig.10: Variations in colour of medium at different intervals of growth of strain P_2d in 0.3% benzoate medium

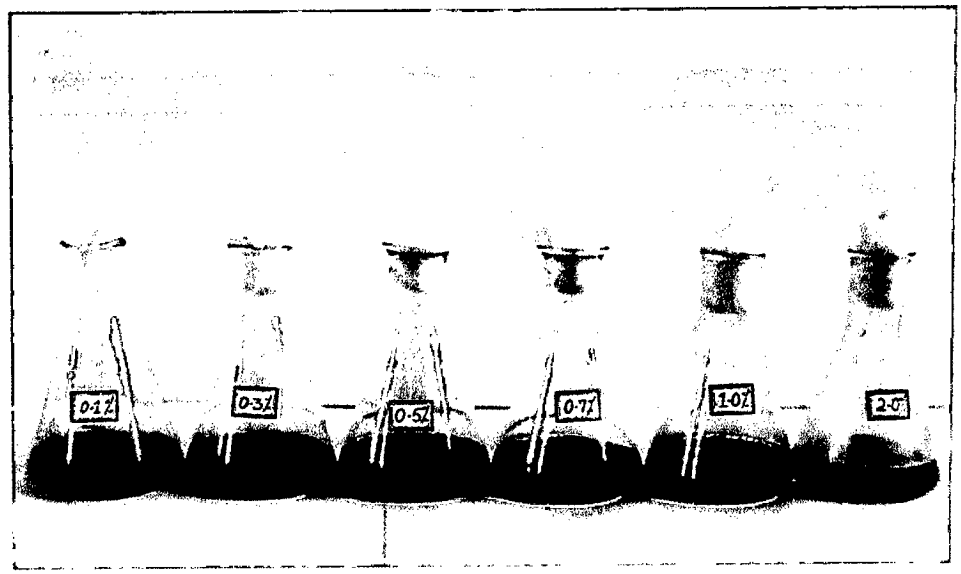


Fig.11: Strain P_2d grown at R.T. for 48 h in varying benzoate concentrations from left to right: 0.1%, 0.3%, 0.5%, 0.7%, 1.0%, 2.0% (w/v)

Table 3.3: Effect of varying temperature, pH and aeration on growth and colour formation by *Pseudomonas mendocina* P_2d incubated for 48 h in 0.3% benzoate medium

Incubation temperature	Growth*	Colour of the medium
4°C	-	Colourless
28°C	+++	Red
37°C	++	Light orange
45°C	+	Colourless
pH of medium		
3.6	-	Colourless
5.0	++	Light yellow
6.5	+++	Red
7.0	+++	Red
9.0	++++	Orange-red
10.0	++	Orange-yellow
11.0	+	Orange-yellow
rpm		
Static	+	Yellow
75	++	Light orange
150	+++	Red
200	+++	Red

Key: * growth was monitored visually; -, no growth; +, poor growth; ++, good growth; +++, very good growth; ++++, excellent growth

Growth medium was light orange in colour after 24 h of growth. At 45°C, growth was very slow and no colouration was seen in the medium even after 48 h of incubation. No growth was seen at 4°C. Growth and colour formation was optimal at 28°C.

Culture could grow at lower pH upto 5 in the acidic range and pH 11, in the alkaline range. At pH 6, the growth pattern was similar to that at neutral pH. Growth was slow at pH 5 and medium colour remained colourless. Maximum growth and turbidity was seen at pH 9 after 48 h of growth, however the medium colour changed from colourless to yellow to orange-red. At higher alkaline pH of 10 and 11, the growth was slow and medium attained faint orange-yellow colouration.

Under static incubation condition, culture showed sparce growth at the base of the flask and the colour of the medium was yellow, which turned dark yellow after 24 h but no further change was observed. At 75 rpm, growth was good and yellow colour was formed within 8 h, which later turned orange. Increasing the aeration to 150

and 200 rpm, the good growth was accompanied with formation of yellow followed by red colour (Fig.12).

The growth of strain P_2d cells in benzoate was accompanied by formation of range of colours namely yellow, orange-red, red, *etc.* that may be ascribed to the metabolic intermediates of benzoate released in the medium. It was therefore of interest to study

A) the enzymes involved in the transformation of aromatic compounds and

B) characterize and identify the transformation intermediates of benzoate.

IV. Enzymes involved in transformation of aromatic compounds

As seen by the Rothera's test, both the *meta* and *ortho* ring cleavage pathways could be detected in the culture grown in different aromatic compounds as sole carbon sources. Hence, the enzymes of both pathways in strain P_2d, grown in benzoate medium, were assayed and their specific activities determined (Table 3.4a).

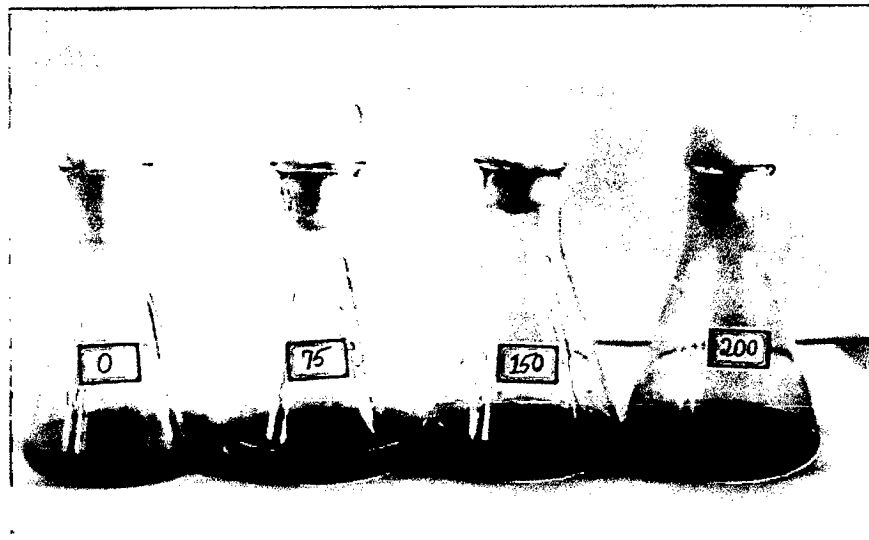


Fig.12: Colour formation by strain P_2d during growth in 0.3% benzoate medium at varying rpm, from left to right: 0, 75, 150, 200

Table 3.4a. Activities of enzymes present in *Pseudomonas mendocina* P_2d

Enzyme$^{\Delta}$	Growth Substrate				
	Benzoate	**Protocatechuate**	**Tyrosine**	**Glucose**	**4-Hydroxy benzoate**
Catechol 1,2-dioxygenase					
Untreated	(0.12)*/(0.12)	+	+	+	+
60°C	(0.018)*/(0.024)	ND	ND	ND	ND
Mercaptoethanol treated	(0.16)*/(0.12)	ND	ND	ND	ND
Catechol 2,3-dioxygenase	(0.48)*/ (1.55)	(0.018)*/(0.19)	(0.58)	(0.39)	+
Protocatechuate 3,4-dioxygenase	-	(0.148)*/(0.18)	-	-	+(0.10)
Tyrosinase	(0.09)	-	(0.18)	(0.16)	-

Key: -, negative; +, positive; Δ, Activities of protocatechuate 2,3-dioxygenase and protocatechuate 4,5-dioxygenase were not detected in any of the reaction mixtures; figures in parenthesis ( ) indicates specific activity (U/mg protein); * demonstrated with cfe; ND, not done.

Enzyme assays were carried out using whole cells as well as with cfe of cells. To study the effect of inhibitors, the cells/cfe were incubated with 25 mM mercaptoethanol for 30 min and then used for the assay. Catechol 1,2-dioxygenase activity monitored at 260 nm was seen to be same or marginally higher with mercaptoethanol-treated samples than with untreated samples. Heating at 60°C for 10 min prior to assay, decreased the catechol 1,2-dioxygenase activity to 0.024 and 0.018 U/mg for whole cells and cfe, respectively. Whole cells showed higher catechol 2,3-dioxygenase activity of 1.55 U/mg as compared to 0.48 U/mg shown by cfe. Strain P_2d cells grown in nutrient broth showed catechol 2,3-dioxygenase activity of 1.054 U/mg, while culture grown in tyrosine and glucose had activities of 0.58 and 0.39 U/mg, respectively. The protocatechuate cells showed lower activity than the above. Reaction mixture was yellow in colour at the end of the incubation period. No yellow colour was formed with mercaptoethanol and heat-treated samples, showing the inactivation of catechol 2,3-dioxygenase activity.

Cfe of benzoate grown cells prepared in buffer containing acetone, showed catechol 2,3-dioxygenase activity of 0.48 U/mg,

while cfe of protocatechuate cells showed very low activity of 0.018 U/mg. The enzymes hydroxymuconic semialdehyde (HMS) hydrolase and dehydrogenase showed a feeble activity of 0.013 U/mg for the whole cells, while cfe showed no activity for both the enzymes. No activity was observed for gentisate 1,2-dioxygenase either spectrophotometrically or by oxygen uptake analysis.

Protocatechuate 2,3-dioxygenase and protocatechuate 4,5-dioxygenase activities were absent in whole cells as well as in cfe. Cells grown in the presence of protocatechuate and *p*-hydroxybenzoate showed protocatechuate 3,4-dioxygenase specific activities of 0.18 U/mg and 0.10 U/mg respectively, whereas enzyme activity of 0.148 U/mg was obtained with cfe of protocatechuate grown cells. Culture grown in other substrates showed no protocatechuate 3,4-dioxygenase activity. Surprisingly, benzoate grown cells induced by incubation for 1 h with 0.1% salicylate showed good protocatechuate 3,4-dioxygenase activity of 0.19 U/mg while benzoate grown cells induced with protocatechuate did not show any activity.

Tyrosinase assay carried out with whole cells of culture grown in 0.2% tyrosine had a specific activity of 0.178 U/mg. Glucose grown cells showed an activity of 0.16 U/mg while benzoate cells had 0.09 U/mg specific activity. Protocatechuate grown cells showed no tyrosinase activity. Cfe of cells grown in tyrosine showed very low activity of 0.002 U/mg.

Growth and colour formation in presence of various diphenolic and triphenolic compounds such as catechol, gentisate, dopa, tyrosine, guaicol, pyrogallol, tannic acid, caffeic acid, protocatechuate indicate presence of enzyme catechol-oxidase (Table 3.2b). The enzyme assay carried out with strain P_2d cells grown in 0.3% benzoate;in the presence of catechol as substrate, gave yellow colour followed by red. Presence of copper chelators affected the pattern of colour formation (Table 3.4b)

Yellow colour was formed in the presence of all inhibitors except KCN. The presence of mercaptoethanol, cysteine, sodium-bisulfide and KCN in the medium inhibited formation of red colour from catechol as well as benzoate. Sodium-metabisulphide and

Table 3.4b: Effect of copper chelating compounds on colour formation by *Pseudomonas mendocina* P_2d cells

Compounds	Colouration	
	A	B
Thiourea	⊕	⊕
Mercaptoethanol	+	+
Cysteine	+	+
Sodium-bisulfide	+	+
Ascorbic acid	+	⊕
Sodium-metabisulfide	⊕	+
Sodium-azide	⊕	+
KCN	-	-
Control	⊕	⊕

Key: A, strain P_2d cells grown in benzoate medium and suspended in catechol (10 mM); B, Growth of strain P_2d in 0.3% benzoate medium; -, no colour; +, yellow; ⊕, yellow → red

sodium-azide inhibited red colouration in benzoate but not in catechol medium. On the other hand, ascorbic acid inhibited red colouration in catechol but not in benzoate medium. Thiourea did not affect the colouration.

Growth of strain P_2d in tyrosine gives pink→brown→reddish black colours in the medium. Incorporation of copper chelating compounds inhibited formation of these colour.

V. Characterization and identification of biotransformation products of benzoate:

UV-Visible spectrum of the culture supernatant during growth of strain P_2d in 0.3% benzoate medium is shown in Fig.13. The spectrum of uninoculated blank showed a single peak at 250 nm, which being too high is not indicated in the figure. Freshly inoculated zero h culture aliquot showed a slight absorbance at 375 nm, in addition to the peak at 250 nm. The absorbance of the peak at 375 nm increased steadily with the incubation time with a concomitant

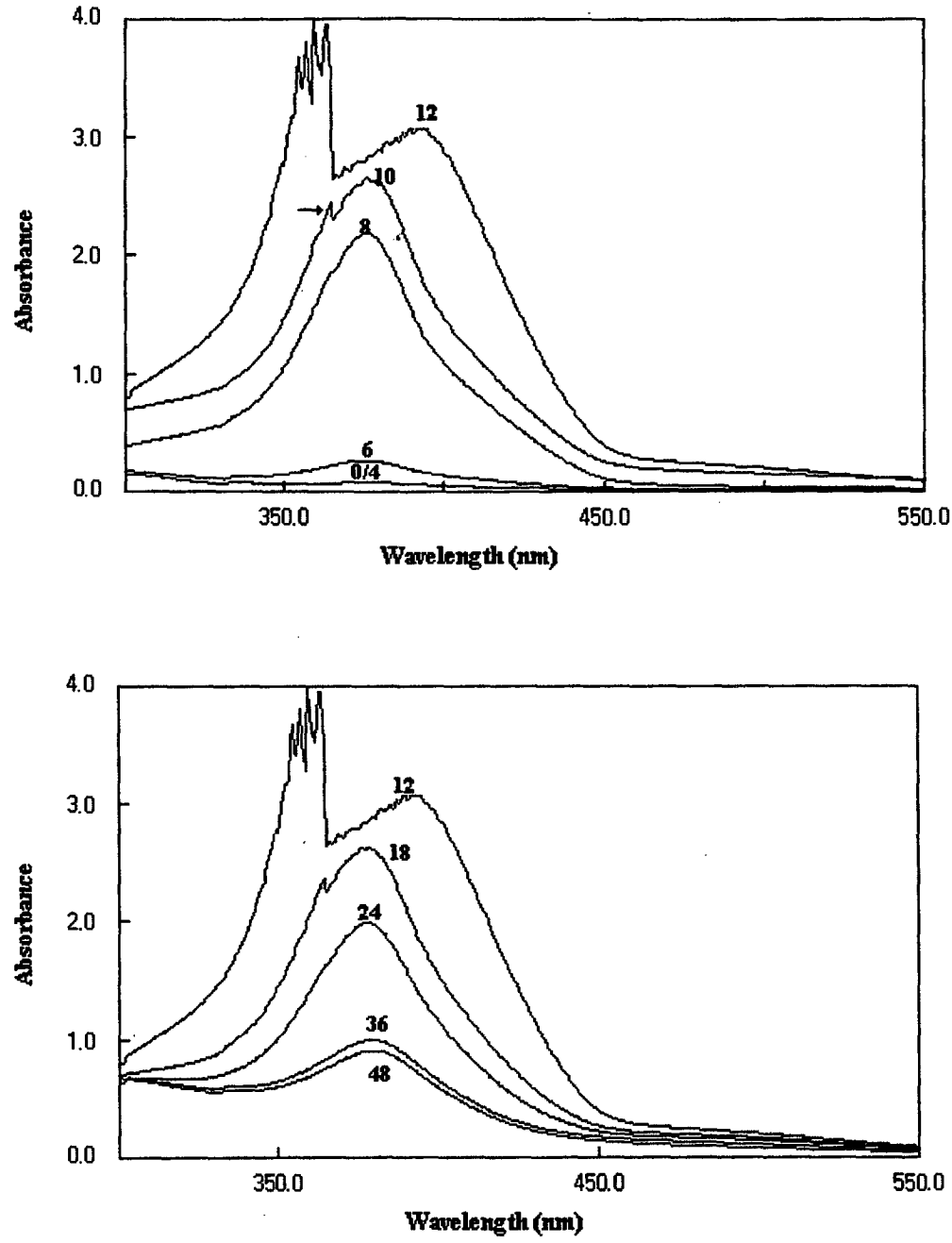


Fig.13: UV-Visible spectra of culture supernatant of *Pseudomonas mendocina* P_2d grown in benzoate medium (0.3%) for varying period (h); →, absorption peak at 360nm

increase in intensity of yellow colouration in the medium. Absorbance at this wavelength was maximum at 16 h of growth (Fig. 14) when the culture had reached the stationary phase. An addition peak was seen at 360 nm at 10 h of growth (Fig. 13). This peak increased in intensity with further incubation.

TLC pattern of ether extracts of unacidified culture supernatants is depicted in Fig. 15B1/B2. A distinct spot of Rf value 0.91 which corresponded with standard catechol, was seen in 8 h and 15 h culture supernatants and this product was termed as transformation product I (TP I). TLC of ether extracts of acidified culture supernatant at various intervals of time, developed in Benzene: Methanol: Ethyl-acetate (40: 60: 40) supported the spectral data (Fig. 15). The uninoculated medium containing benzoate, on extraction with ether gave a single spot of Rf value 0.699, corresponding to standard benzoate. Yellow coloured supernatant of 8 h old culture broth showed 2 spots of Rf values 0.69 and 0.165 on exposure to iodine vapours. The latter spot before exposure to iodine was yellow in colour and was designated as transformation product II (TPII). The 15 and 24 h old aliquots showed 3 spots of Rf values 0.69, 0.165 and

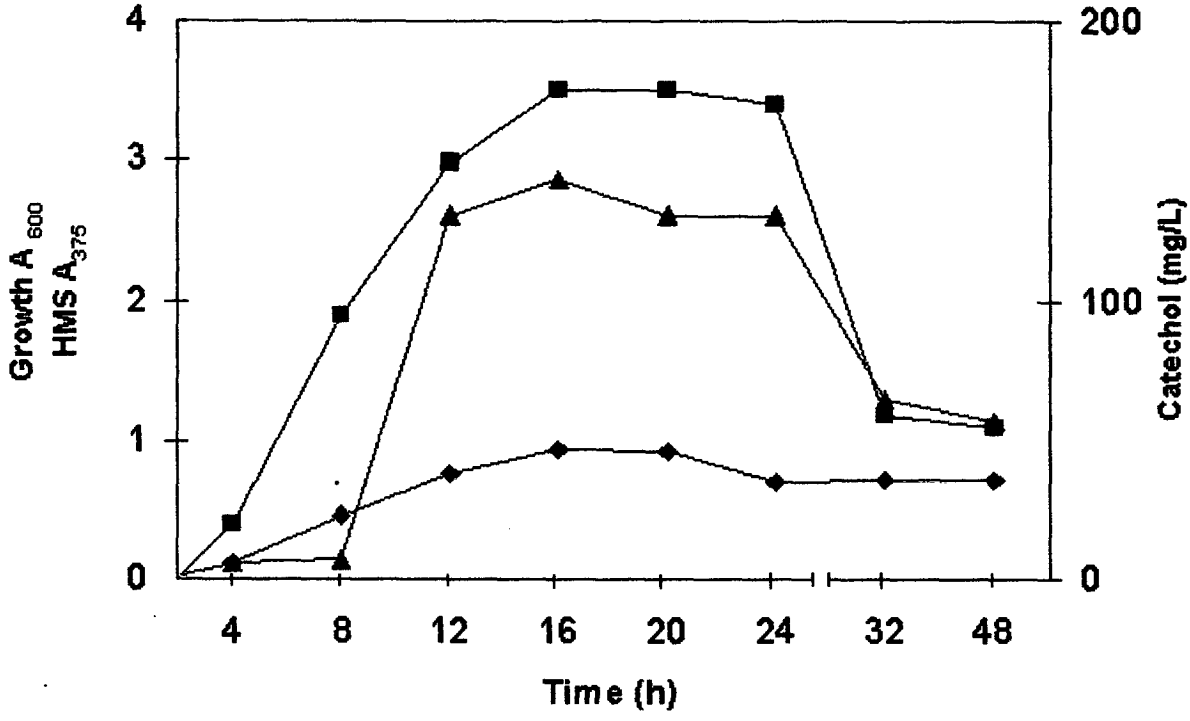
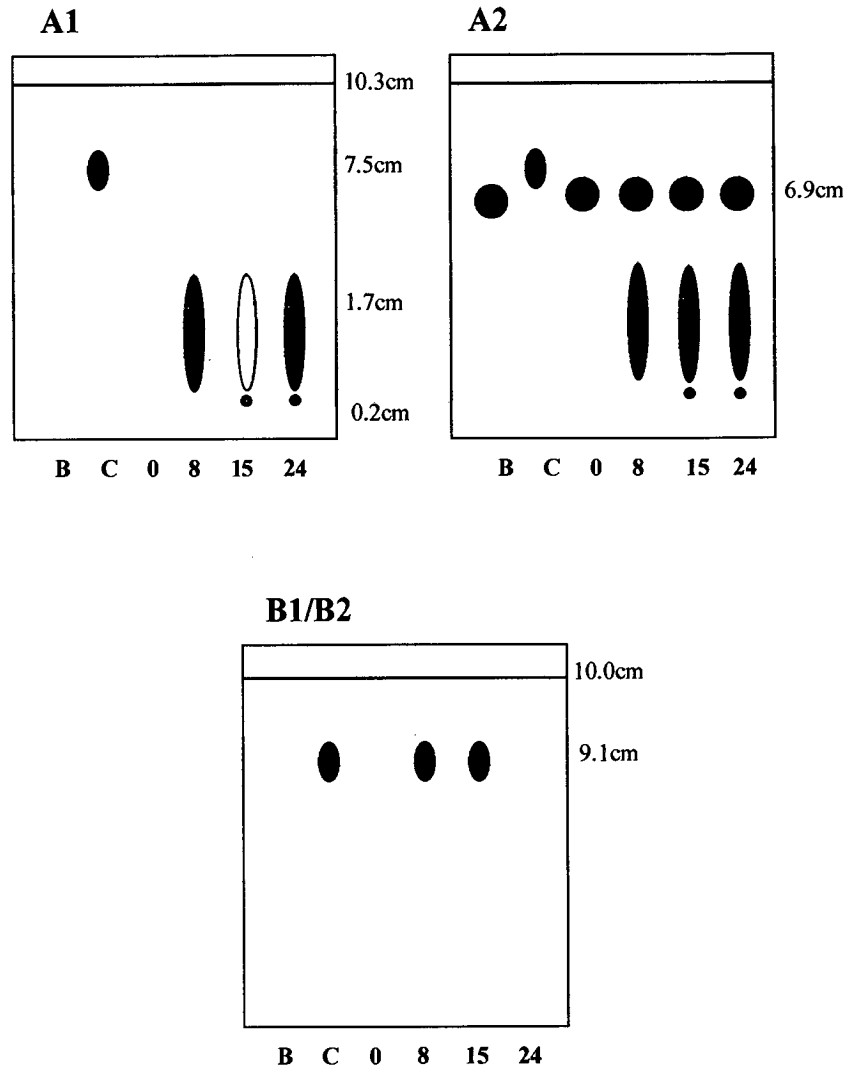


Fig. 14: Formation of catechol (▲) and HMS (■) during the growth of *Pseudomonas mendocina* P_2d in 0.3% benzoate medium (◆)



Key: B = Benzoate standard
C = Catechol standard
A1/B1 = Before iodine exposure
A2/B2 = After iodine exposure

Fig.15: TLC pattern of ether extracts of acidified (A) and unacidified (B) culture supernatant during growth in 0.3% benzoate medium at different time intervals (h)

0.02, after exposure to iodine vapours (Fig. 15A2). The spot with Rf value 0.02 was pink in colour before exposure to iodine (Fig. 15A1) and was termed as transformation product III (TPIII).

a. Transformation Product TP I:

(i) **Identification of TP I:** Culture grown in 0.3% benzoate medium for 24 h and extracted in ether showed on TLC besides the other spots, one spot of Rf 0.9, which turned black in colour (Fig.15B) and was corresponding to catechol. Although the colorimetric test did not show the presence of catechol in culture supernatant, addition of lead-acetate to the supernatant gave a white precipitate thus showing the presence of catechol in the medium. Catechol detected by this method in culture supernatant of 6 h old culture onwards was assayed gravimetrically (Fig. 14). Maximum concentration was seen at 16 h of growth, retained upto 24 h. On further incubation, a fall in the concentration was seen.

HPLC of authentic catechol gave a peak at 5.6 min (Fig.16a). The culture supernatant sample also gave a peak at

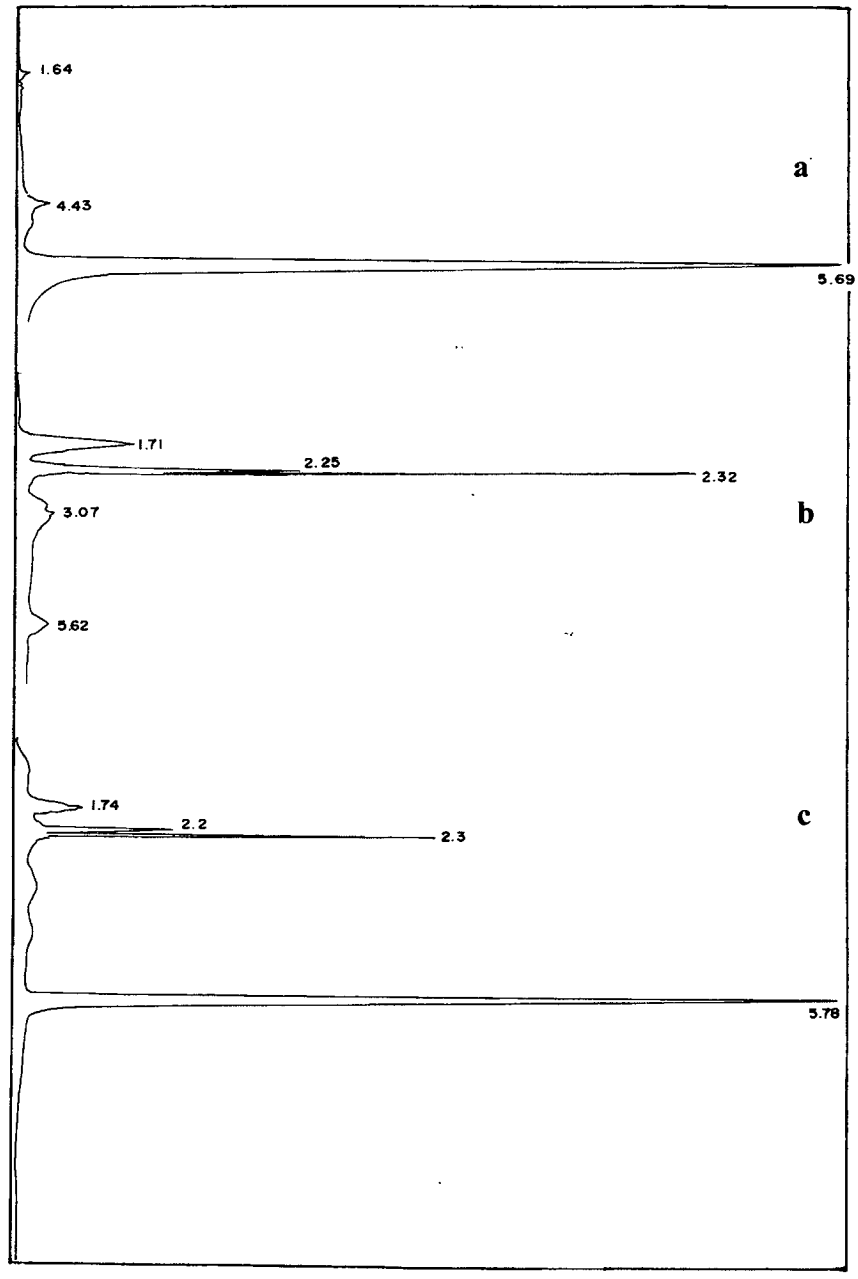


Fig. 16: HPLC chromatogram of

a. **standard catechol**
b. **red supernatant of culture grown in 0.3% benzoate medium**
c. **catechol mixed with red supernatant**

5.6 min (Fig.16b), which was accentuated on addition of catechol. (Fig. 16c).

(ii) Response of cells to TP II:

(a) Growth: Strain P_2d grew well in the presence of 0.01% catechol forming initially yellow followed by red and finally black colour in the medium.

(b) Oxygen uptake: A very good oxygen uptake was shown by cells grown in benzoate and glucose, for catechol (Table 3.2c). The reaction mixture immediately turned yellow in colour, during the analysis.

(c) Response of resting cells: Resting cells of strain P_2d with absorbance of 4.0 at 540 nm when incubated in the presence of catechol (10-25 mM) produced red to wine-red colour in 3 h with UV-Visible peaks at 230, 270, 300 375 and 402 nm (Fig. 17b), while incubation of cells with 5 mM catechol formed yellow colour with UV-Visible spectral peaks at 230 and 375 nm . The solution was white with 1 mM catechol. Above 25 mM, the

solutions turned brown to black in colour immediately (Fig.18).

Strain P_2d cells grown in 0.3% benzoate medium were incubated with catechol solutions of varying concentrations, ranging from 0-100 mM. Table 3.5a shows the amount of catechol at the end of 3 h and the colouration in the suspension. It was seen that at the end of the incubation period, no catechol was available in the reaction mixtures with 1, 5 and 10 mM concentrations whereas in 25 mM and above concentrations, residual catechol was seen. At lower concentration of catechol (5 mM), only yellow colour was formed whereas at higher concentration, yellow colouration was followed by red as seen at 10 and 25 mM concentrations.

TLC profile of red solution obtained by incubating cells with solutions of 10 to 25 mM concentration, showed three spots of Rf 0.91, 0.5 and 0.05. Spot of 0.91 Rf turned black, while spots of 0.5 and 0.05 Rf values

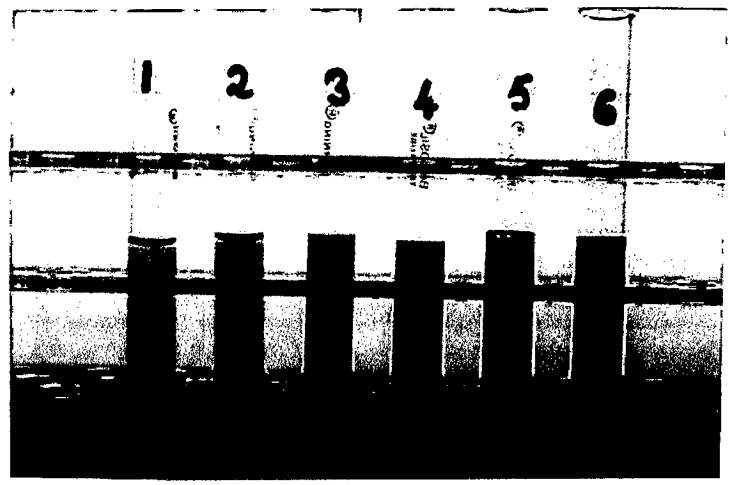


Fig.18: Range of colours obtained from catechol on treatment for 3 h of resting cells of strain P_2d with varying catechol concentration: 1, 1 mM; 2, 5 mM; 3, 10 mM; 4, 25 mM; 5, 50 mM; 6, 100 mM

Table 3.5a: Colour formation with different concentrations of catechol by resting cells of strain P_2d

Catechol (mM)		Colour obtained
0h	**3h**	**3h**
1	0	White
5	0	Yellow
10	0	Yellow→Red
25	22	Yellow→Wine-red
50	224	Brown
100	24	Black

were yellow and red, and corresponding to TP II and TP III, respectively. The solutions that turned black in colour (50 and 100 mM catechol) showed a single spot of Rf value 0.90 (Fig.19).

iii) **Detection of intermediate during conversion of catechol by cells:** Reaction mixture containing strain P_2d cells incubated with 50 mM catechol turned yellow in colour immediately. This mixture on acidification and extraction with ether, was reextracted in buffer. This solution was initially yellow in colour. After standing for 1 ½ -3 h, the solution turned dark yellow and finally orange-red in colour. Catechol was estimated at each step by colorimetric method. The conversion rate of catechol to HMS was 52.9% while that to the red compound was found to be 20.0%. Calculations are shown below:

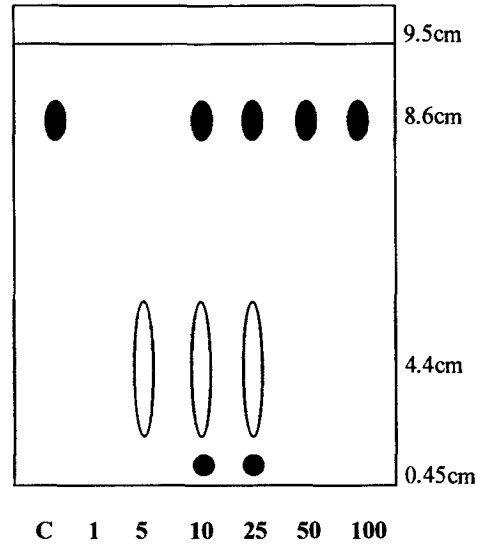


Fig.19: TLC profile of ether extracts of strain P_2d cells incubated with different concentrations of catechol (mM)

Calculations:

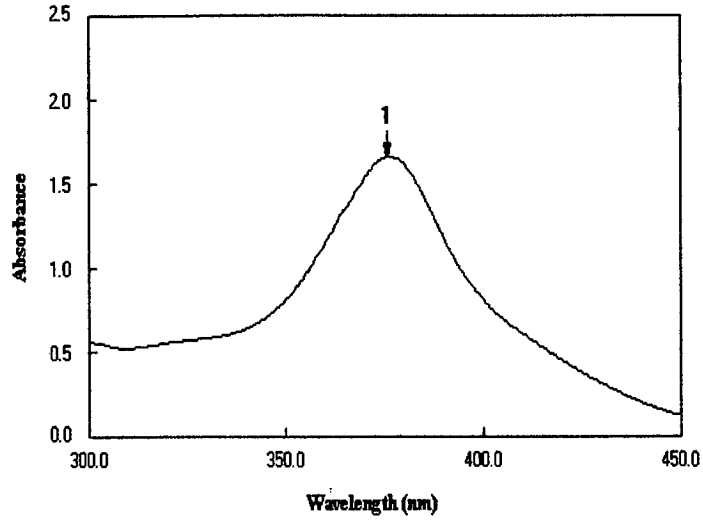
50 mM catechol + cells → 68 mM
↓
yellow colour → 32 mM ∴ amount of catechol
ether extract ← ↓ converted to HMS
before acidification yellow aqueous = 68-32= 36 mM =52.9%
↓ pH
extract with ether ← ↓
yellow solution → 10 mM
↓
orange solution → 8 mM ∴ amount of catechol
converted to quinone
= 10-8= 2 mM = 20%

b. Transformation product-TP II:

(i) **Identification of TP II**: During metabolism of benzoate by the culture, a yellow colour was formed in the medium. A distinct peak at 375 nm in the UV-Visible scan was seen with appearance of yellow colour in the medium and the absorbance was found to increase with deepening of the colour during growth (Fig.13). Formation and intensification of colour coincided with a spot on TLC, (Fig. 15) which was also yellow in colour. TLC patterns of ether extracts of culture supernatant

and reaction mixture of cells incubated with catechol, showed presence of distinct yellow spot of Rf value 0.168 in solvent system III(Fig.15). This yellow spots on elution in buffer also showed a peak at 375 nm (Fig. 20a). Culture pellet of strain P_2d suspended in 0.05M phosphate buffer containing 10 mM catechol gave yellow colour, immediately. Colour was lost on acidification and the ether extract of the acidified solution showed a peak at 305 nm in the UV-Visible scan. The ether layer on reextraction with phosphate buffer, turned yellow and gave a sharp peak at 375 nm. Standard HMS gave an identical UV-Visible spectrum with a peak at 375 nm (Fig.20b). Fig. 21a shows the HPLC profile of standard HMS and Fig. 22a shows the HPLC profiles of the red supernatant of culture grown for 24 h in benzoate medium. The chromatogram shows the two very closely situated peaks with retention time 2.25 and 2.32 min. It is noted that the latter peak of 2.32 min gets accentuated with addition of standard HMS (Fig.22b). The corresponding peak is also observed in the HPLC of yellow supernatant of culture grown in 0.1% benzoate (Fig. 21b). It is interesting to note that on decolourization of red colour with

a



b

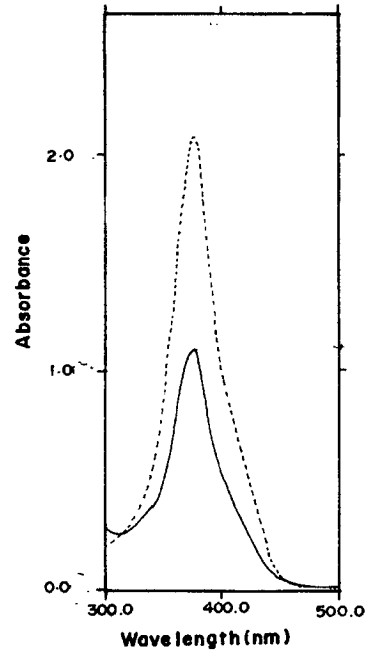


Fig. 20a: UV-Visible spectrum of TP I eluted from TLC in phosphate buffer (0.5 M, pH7), absorption peak, 1=375nm
b: UV-Visible spectrum of standard HMS from catechol using ---- *Pseudomonas mendocina* P_2d, — *Pseudomonas cepacia* AC1100

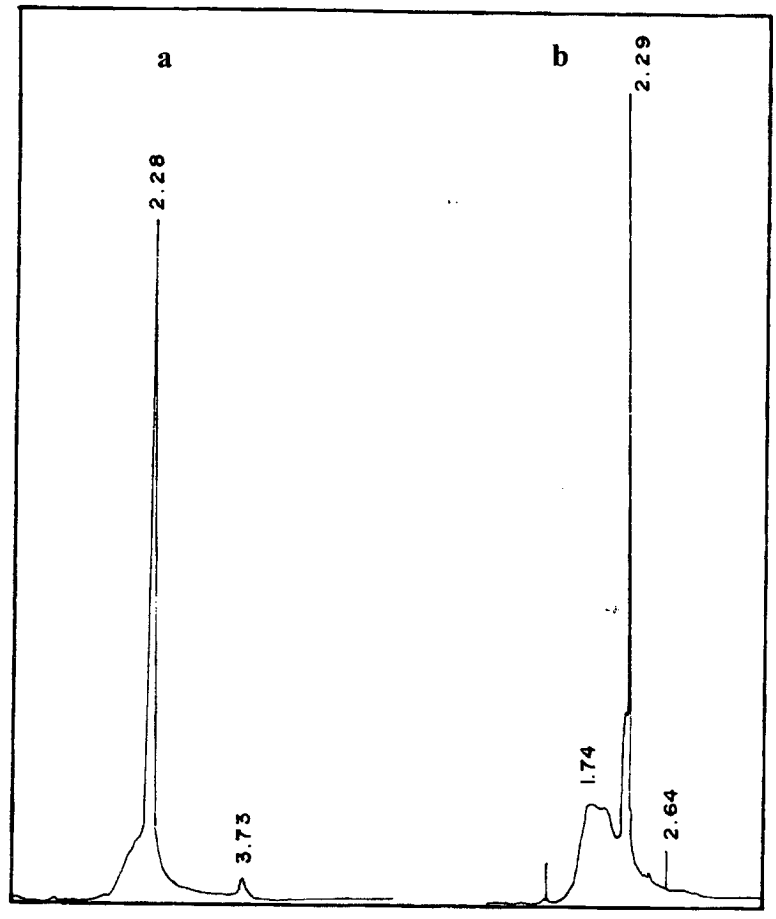


Fig. 21: HPLC chromatogram of

a. standard HMS

b. yellow culture supernatant of P_2d cells grown in 0.1% benzoate medium for 24 h

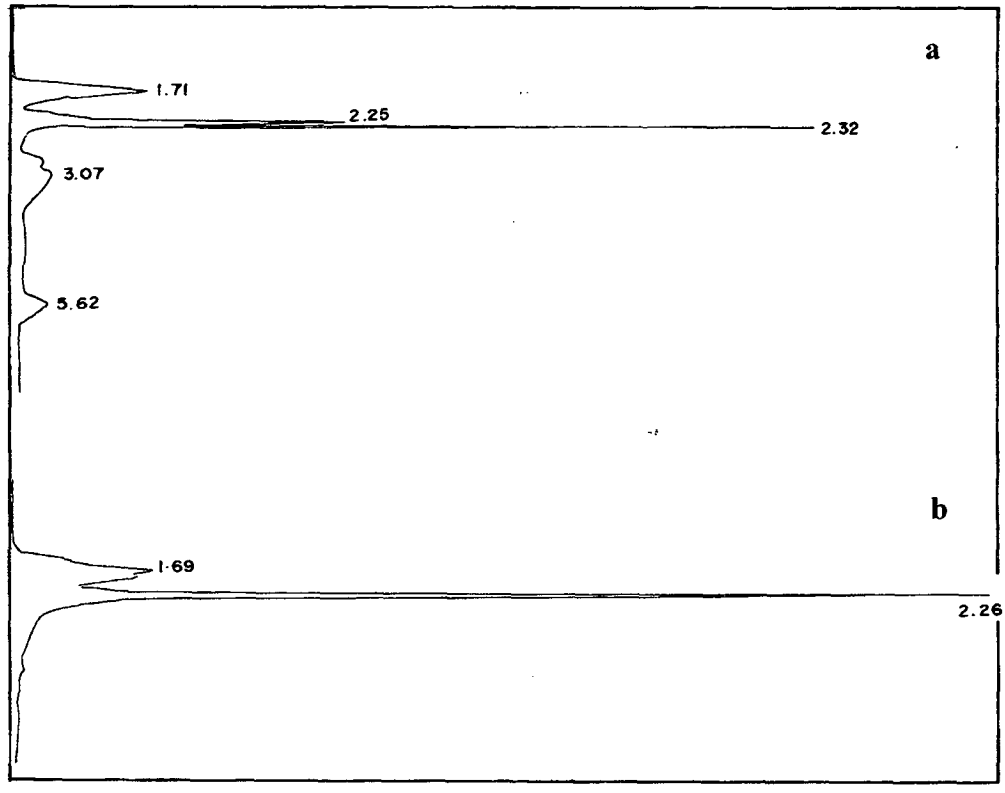


Fig. 22: HPLC chromatogram of

a. red supernatant of culture grown in 0.3% benzoate medium

b. HMS mixed with red supernatant

acid and dithionite, the peak corresponding to TP III, as given in the next section, disappears. Instead however, the peak corresponding to TP II is very prominent (Fig. 23b and c).

Thus, the TLC, UV spectral and HPLC data clearly confirm that TP II, the yellow product formed from catechol and sodium-benzoate by a strain P_2d is 2-hydroxymuconic semialdehyde (HMS), *meta* cleavage product. Presence of 2,2'bipyridyl in the growth medium containing 0.3% benzoate resulted in a long lag period of 22 h (Fig.24). No yellow colour was formed, instead the medium turned dark red after 46 h of growth. Incorporation of glucose (0.2%), a readily available carbon source, did not change the effect of 2,2'bipyridyl. UV-Visible spectrum of dark red supernatant showed peaks at 375 and 490 nm (Fig. 25). In contrast, in the control no peak was obtained at 490 nm and the absorbance at 375 nm was out of range. Hence the supernatant was diluted 10 times and the spectrum is shown in the figure.

(ii) **Utilization of TP II for growth**: Culture could utilize HMS at

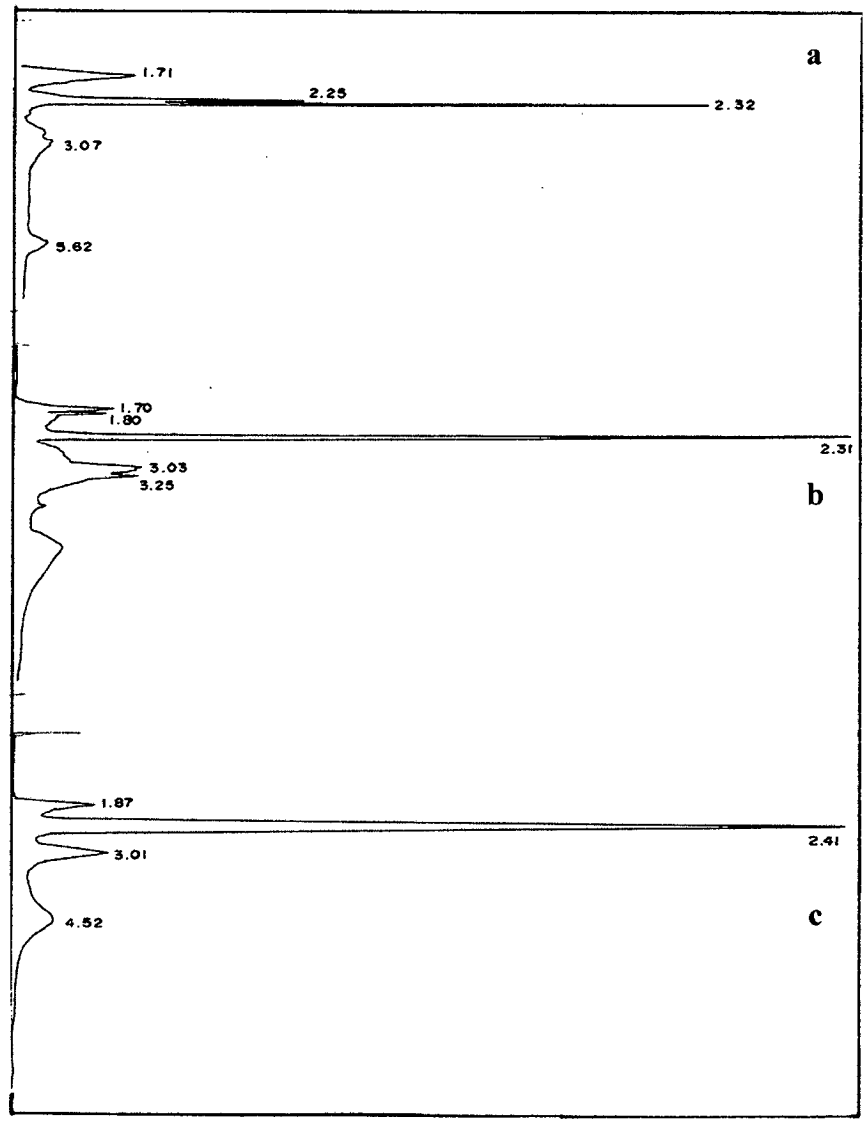


Fig.23: HPLC chromatogram of

a. **red supernatant of culture grown in 0.3% benzoate medium**
b. **red supernatant decolourized with acid**
c. **red supernatant decolourized with sodium-dithionite**

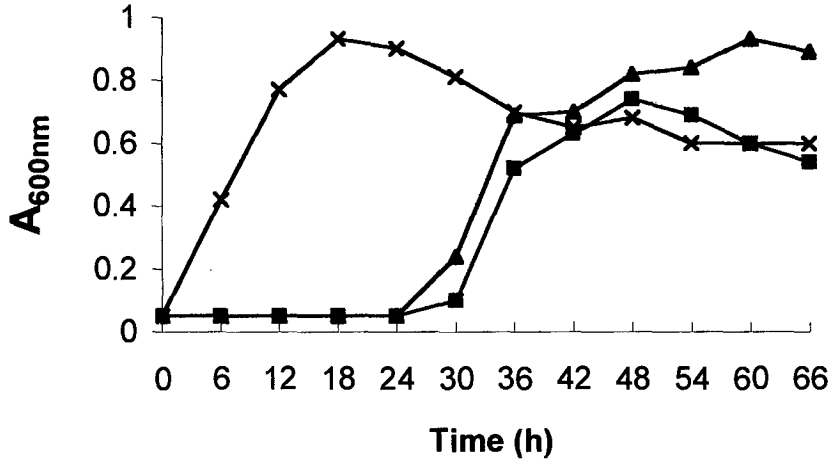


Fig.24: Growth of *Pseudomonas mendocina* P_2d in 0.3% benzoate (x), 0.3% benzoate with 2 mM 2,2'bipyridyl (■) and 0.3% benzoate with 0.2% glucose and 2 mM 2,2'bipyridyl (▲)

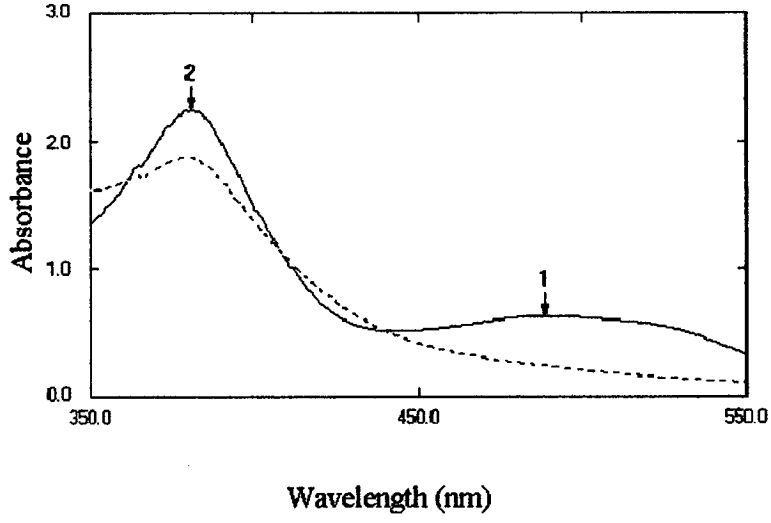


Fig. 25: UV-Visible spectrum of culture supernatant of *Pseudomonas mendocina* P_2d grown in benzoate (0.3%) —— for 48 h with 2,2′bipyridyl (2 mM) and ----- for 24 h without 2,2′bipyridyl (10^{-1}); Absorption peak, 1= 490 nm; 2 = 375 nm.

a concentration of 25 mM, as sole source of carbon with distinct increase in absorbance of 0.13 at 600 nm at 24 h of growth. Medium colour changed from yellow to white. Cells of strain P_2d grown in benzoate medium showed an oxygen uptake of 47 nmoles/min/mg dry weight with HMS (0.57 nM).

c. Transformation Product TP III:

(i) **Identification of TP III:** Red coloured supernatant formed during metabolism of benzoate was tested for the presence of quinone. The observations are tabulated in Table 3.5b. Acidification to pH 2.0 led to decolourization of the supernatant and on addition of alkali, the colour reappeared. Similarly, addition of reducing agents such as sodium-dithionite and sodium-borohydride decolourized the coloured solution and aeration caused the colour to appear. Supernatant colour was reduced on addition of hydrogen-peroxide while addition of acidified 2% potassium-iodide resulted in formation of brown colour. All the tests confirm red coloured product in supernatant to be a quinone.

Table 3.5b: Qualitative tests for quinone performed on the red supernatant obtained on growth of *Pseudomonas mendocina* P_2d in 0.3% benzoate medium

	Test	Observations
1a	Red supernatant + 6 N HCl (pH 2.0)	Red → yellow
b	(1a) above + 12 N NaOH (pH 12)	Yellow → Red
2a	Red supernatant + pinch sodium-dithionite	Red →Yellow
b	Aerate (2a) above	Yellow → Red
3a	Red supernatant + pinch sodium-borohydride	Red →Yellow
b	Aerate (3a) above	Yellow → Red
4	Red supernatant + acidified 2% KI	Red to brown
5	Red supernatant + hydrogen- peroxide	Red →yellow

Table 3.5c: 4,5-Dianilino-*o*-benzoquinone derivative obtained from catechol with and without *Pseudomonas mendocina* P_2d cells

	Dry weight of derivative mg/100ml solution	
Time of incubation	Control (without cells)	Test (with cells)
3h	4	16
24h	8	35

UV-Visible scan before and after decolourization of the supernatant showed the disappearance of peaks in the visible range (Fig. 26), thus supporting the visual observations. Separation of quinone from culture supernatant using solvent extraction was unsuccessful. Red colour remains in the medium for 2-3 weeks. The supernatants were pooled and lyophilized and the concentrated red residue obtained was loaded on Sephadex LH-20 column and eluted with methanol. The red and yellow products got eluted together and hence purified product was not obtained.

Pure *ortho*-benzoquinone derived from 50 mM catechol by aerial oxidation gave a peak of retention time 2.1/2.2 min, on HPLC (Fig. 27a). Corresponding peak was also seen in red supernatant (Fig. 27b). On addition of the pure *ortho*-benzoquinone to red supernatant, peak accentuation was seen (Fig. 27c). Decolourization of red supernatant with acid and sodium-dithionite led to the disappearance of the peak (Fig. 23b & c).

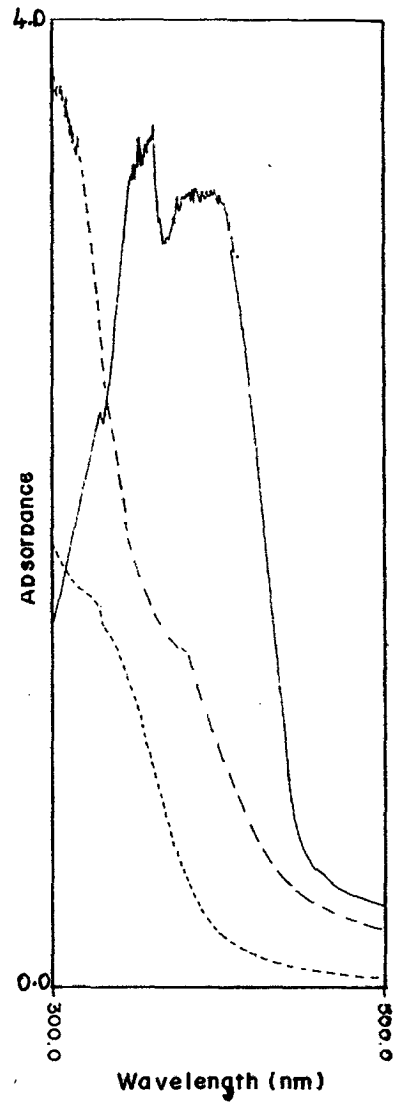


Fig. 26: UV-Visible scan of supernatant of strain P_2d grown in 0.3% benzoate medium for 24 h (—), decolourized with 0.5 N HCl (---) and sodium-dithionite (—–)

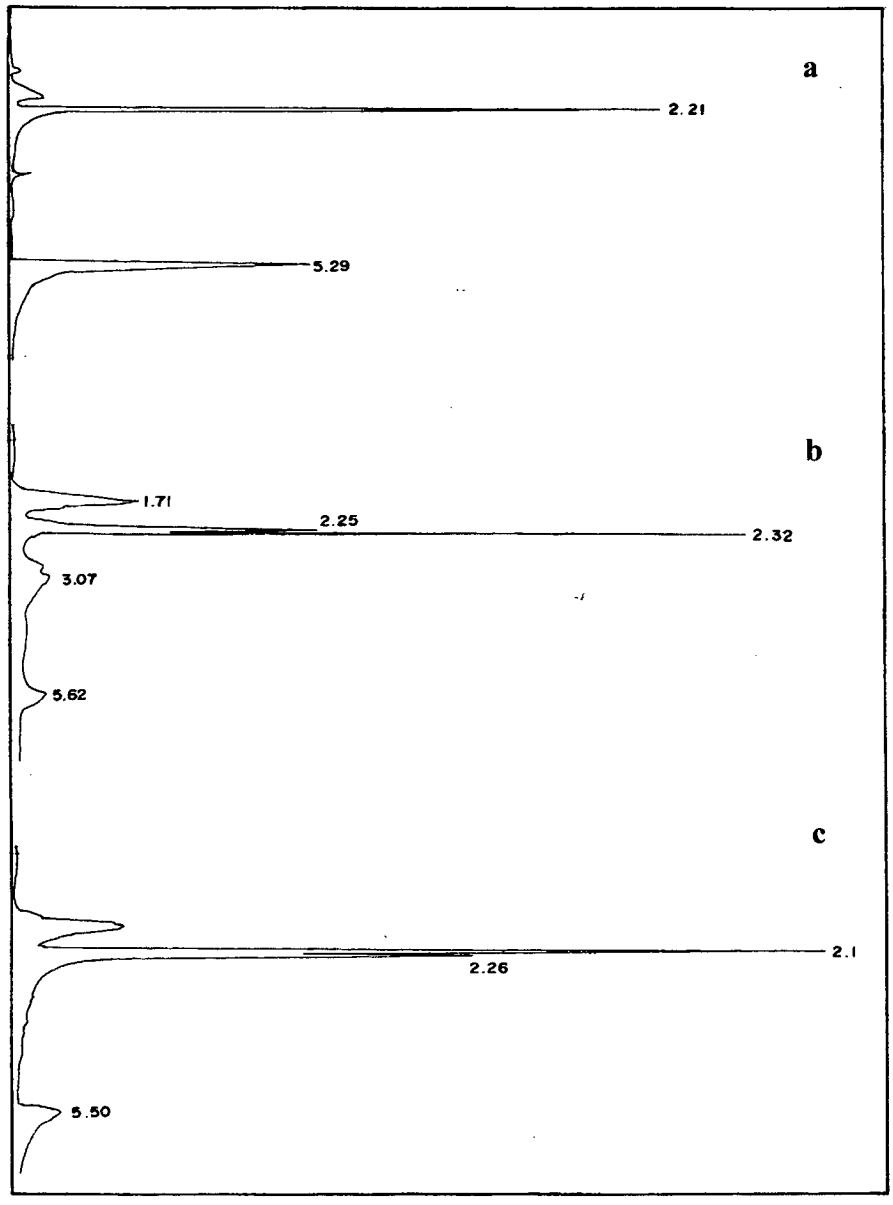


Fig.27: HPLC chromatogram of

a. ***ortho*-benzoquinone obtained from catechol**
b. **red supernatant of strain P_2d grown in 0.3% benzoate medium for 24 h**
c. ***ortho*-benzoquinone mixed with red supernatant**

The qualitative tests and HPLC profiles of red supernatant suggest the transformation product TPIII to be *ortho*-benzoquinone.

(ii) **Derivatization of TP III**: Strain P_2d cells grown in 0.3% benzoate, centrifuged and were incubated with 25 mM catechol and 1% (v/v) aniline. The reaction mixture yielded 16 mg of red precipitate after 3 h and 35 mg in 24 h (Table 3.5c). In contrast, control i.e. catechol and aniline incubated without culture, gave negligible amount of precipitate, 4 mg and 8 mg, respectively, after 3 and 24 h. The melting point of 190°C of the purified aniline derivative and infra-red spectrum (Fig.28) confirmed it to be 4,5-dianilino-*o*-benzoquinone. Above results confirmed that red product formed in culture supernatant, TP III is *ortho*-benzoquinone.

(iii) **Response of cells to *ortho*-benzoquinone**: *ortho*-benzoquinone when used as carbon source did not support growth of strain P_2d. So also, no oxygen uptake was shown by cells grown in

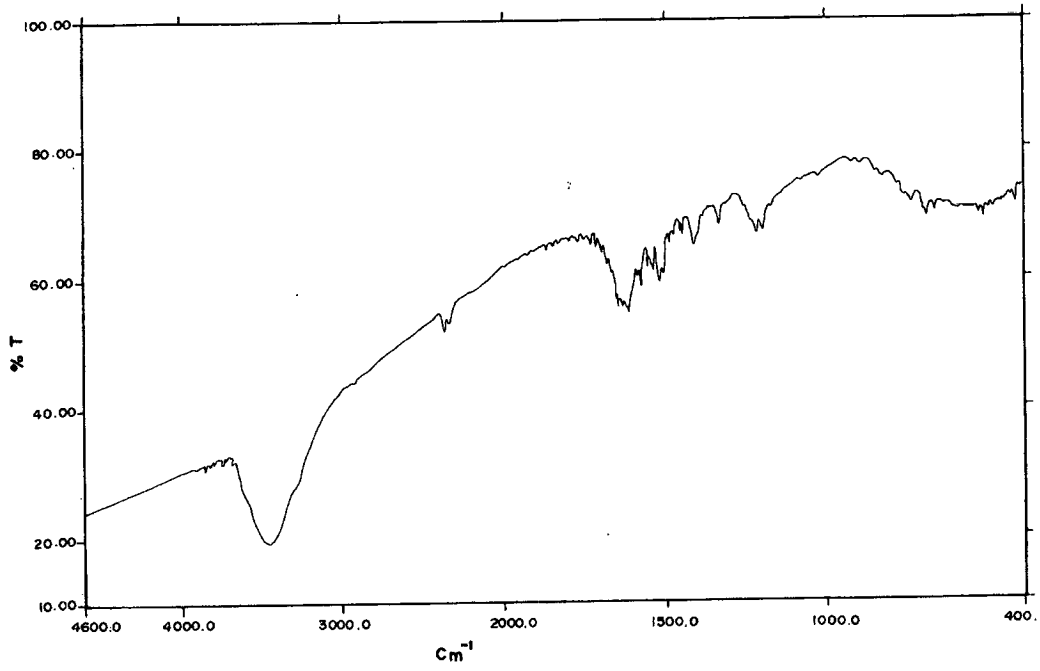


Fig.28: Infra-red spectrum of 4,5-dianilino-*o*-benzoquinone

benzoate with *ortho*-benzoquinone, showing that *ortho*-benzoquinone was not used for growth.

VI. Metabolism of tyrosine by strain P_2d:

During growth of culture in benzoate, red colouration was obtained in the growth medium (Fig.10). A similar colour reaction was obtained when cells were grown on tyrosine where pink to brown to black colours were formed (Fig.29). Efforts were therefore made to identify the transformation products of tyrosine, responsible for various colouration.

a. Growth of strain P_2d with 0.2% Tyrosine: Strain P_2d grew well at upto 5% tyrosine concentration. With 0.2%, the medium was white in colour at 0 h. Turbidity of 0.42 at 600 nm (Fig 30), initially present in the medium was due to insoluble tyrosine (1040mg/L) (Fig. 31). On incubation, however an increase in absorbance of 0.6 was seen due to turbidity of cells. At the end of 15 h, pink colour appeared and crystals of tyrosine started disappearing. Turbidity was seen to decrease after 24 h of growth with simultaneous decrease in tyrosine concentration to 650 mg/L.

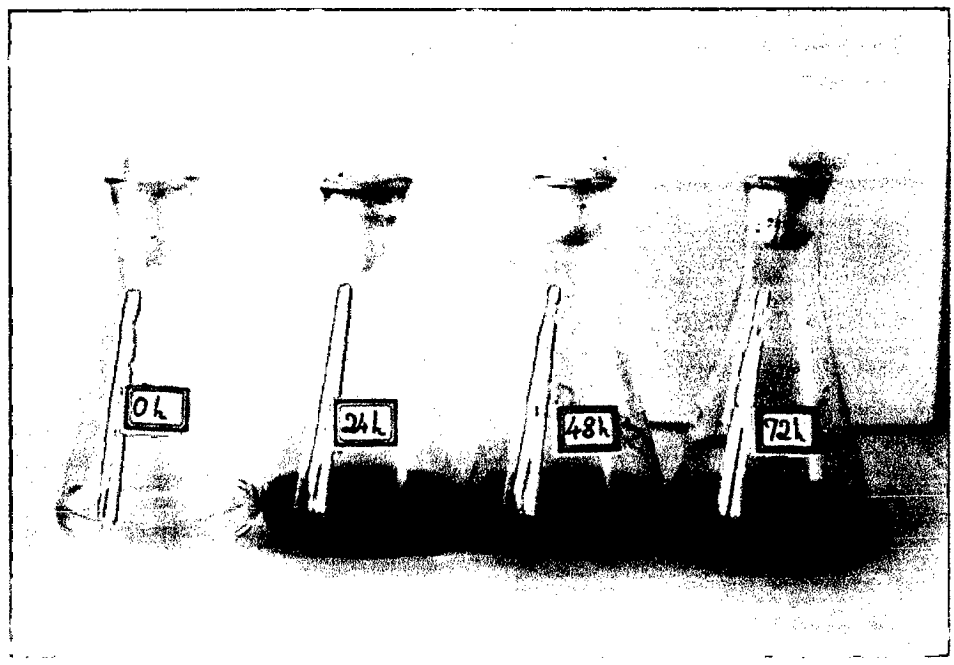


Fig. 29: Colour formation by *Pseudomonas mendocina* P_2d during growth in 0.2% tyrosine medium for 0, 24, 48 and 72 h

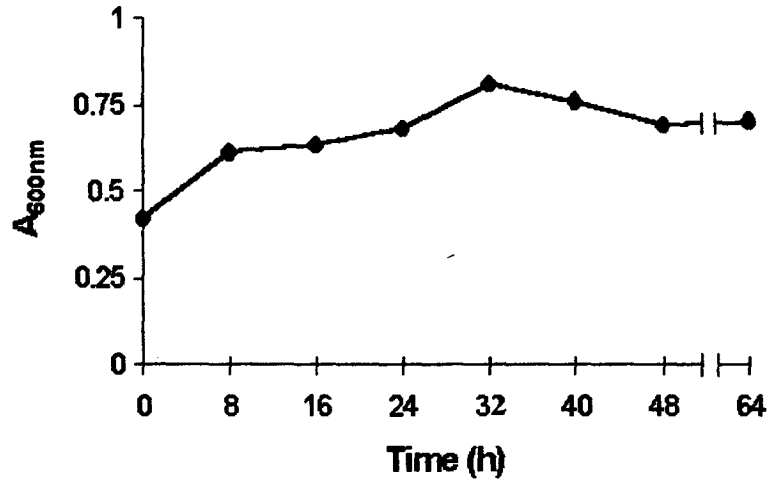


Fig. 30: Growth of *Pseudomonas mendocina* P_2d in 0.2% tyrosine medium in terms of turbidity at 600 nm

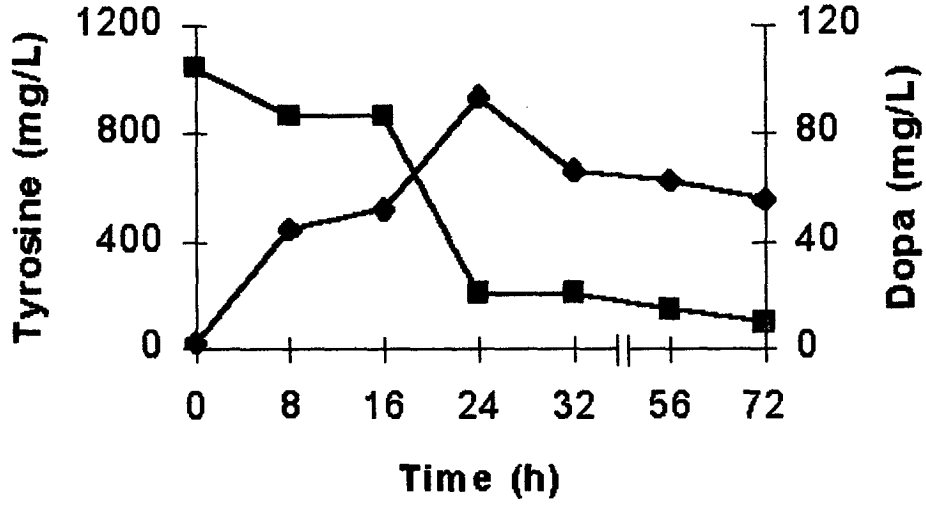


Fig. 31: Estimation of tyrosine (■)and dopa (◆)at different time intervals, during growth of *Pseudomonas mendocina* P_2d in 0.3% tyrosine medium

The initial turbidity in the medium observed because of tyrosine crystals was substituted by culture turbidity and therefore no significant change in absorbance is seen (Fig. 30). Absorbance of ~0.81, observed at 32 h was solely due to the bacterial turbidity. On further incubation the tyrosine content was reduced further until 72 h.

b. Formation of dopa: During growth formation and increase in concentration, if any, of dihydroxyphenyl alanine (Dopa), a known transformation product of tyrosine was estimated colorimetrically. Dopa concentration was found to increase, highest concentration of 94mg/L being detected at 24 h of growth (Fig. 31), followed by a decline.

c. Tests for quinone formation during tyrosine metabolism: The pink supernatant formed after 24 h growth of P_2d turns brown colour on further incubation. This solution was tested for presence of quinonoid compounds as per the tests given in Table 3.6a. Addition of acid leads to decolourisation followed by reappearance of colour on addition of alkali similarly, colour disappears on

addition of sodium-dithionite, hydrogen-peroxide and sodium-borohydride. The tests thus indicate the pink coloured product to be a quinone.

d. Identification of pigment formed from tyrosine:

After incubation of 40 h, medium turned light brown with intensity of colour increasing with incubation. With higher concentrations of tyrosine, darker colours were formed but culture took longer time to grow.

The characteristics of the brown / black product synthesized during growth in L-tyrosine by strain P_2d are summarized in Table 3.6b. The black supernatant from the culture when diluted with water gave black coloured particulate precipitate, indicate its insolubility in water. Addition of 0.1N NaOH led to dissolution of the precipitate. All these properties/characteristics of the black product are similar to those reported for melanin.

Table 3.6a: Qualitative tests for quinones with pink culture supernatant of strain P_2d grown in 0.2% tyrosine for 24 h

	Test	Observation
(1a)	Pink supernatant + 0.5 N HCl (pH2)	Pink →colourless
(1b)	1a + 0.5 N NaOH	Colourless→ pink
(2a)	Pink supernatant + sodium-dithionite	Pink → colourless
(2b)	2a + aerate	Colourless → Pink
(3a)	Pink supernatant + sodium-borohydride	Pink → colourless
(4)	Pink supernatnat + H_2O_2	Pink → colourless

Table 3.6b: Characteristics of the pigment formed during growth of strain P_2d in 0.2% tyrosine medium for 48 h

	Test	Property
1	Colour	Black
2	Solubility in water	Insoluble
3	Appearance in water	Particulate
4	Precipitate from (3)+ 0.1 N NaOH	Dissolution
5	HCl to (4)	Precipitate
6	Black supernatant + 5mM $FeCl_3$	Precipitate

e. Separation degradation products of tyrosine by HPLC:

The coloured supernatants formed at different time intervals during growth of strain P_2d in tyrosine medium, were analyzed by HPLC in the visible range (410 nm). However distinct detectable peaks were not obtained. Therefore, HPLC was carriedout at 270 nm.

Uninoculated 0 h sample of 0.2% tyrosine medium, as well as standard tyrosine (Himedia, Sigma) showed two peaks with retention time of 1.5 and 2.23 min (Fig. 32a), peak at 1.5 min being of higher intensity. The 24 h pink supernatant showed 2 additional minor peaks, which were not detected in HPLC of 48 h and 72 h old samples. Instead, newer peaks were detected in these samples (Fig. 32b, c, d).

VII. Characterisation of exopolysaccharide (EPS) formed during growth of strain P_2d in benzoate medium

During studies on aromatic degradation, it was noticed that the P_2d cells, centrifuged and resuspended to an absorbance > 3 and

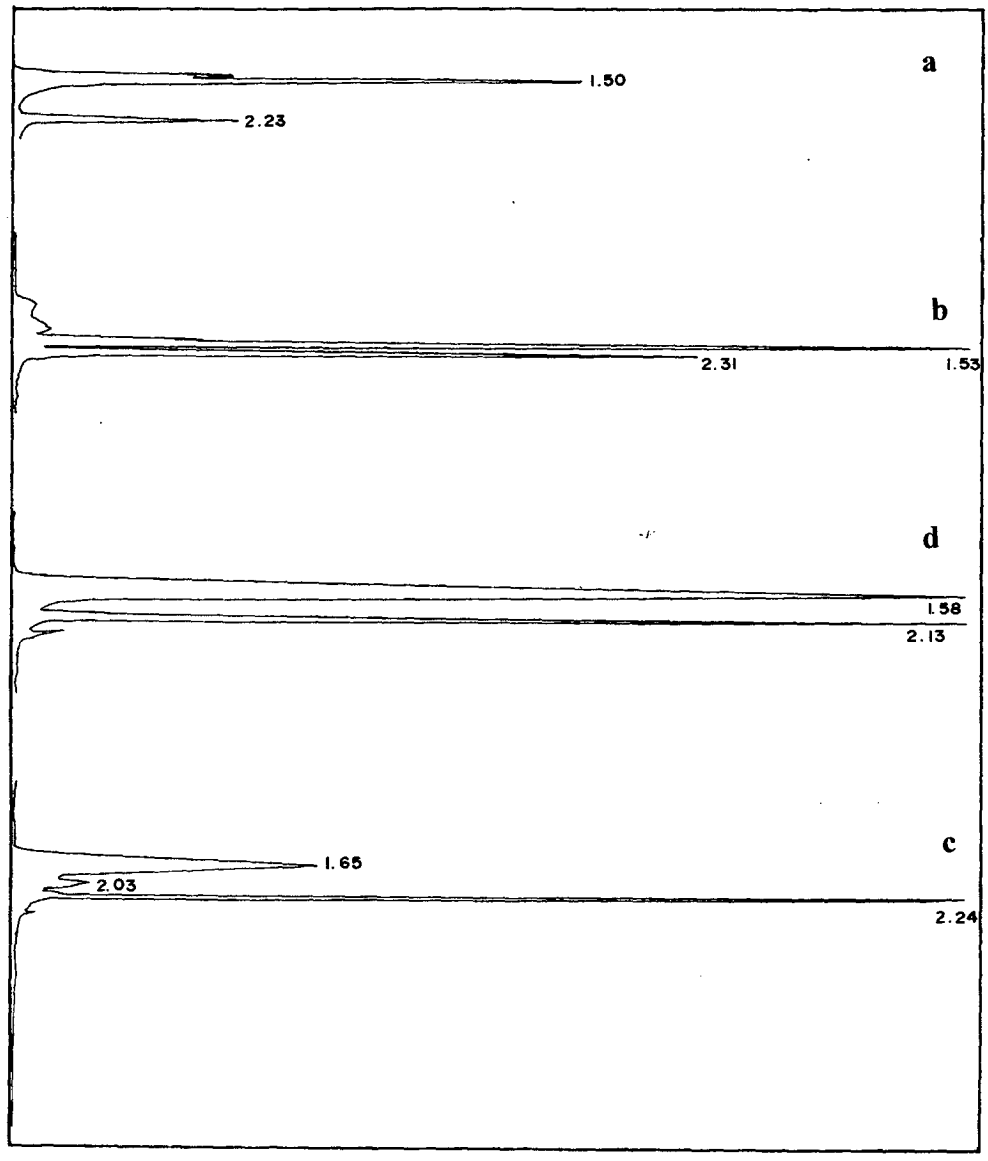


Fig. 32: HPLC chromatogram of (a) standard tyrosine and those of supernatants of culture grown in 0.2% tyrosine medium for (b) 24 h (pink); (c) 48 h (brown); (d) 72 h (black)

stored in ice for oxygen uptake and enzyme assays, became viscous. Difficulties were faced due to this viscosity and the culture could not be pipetted out either by glass/micropipette. The viscous mass could not be redispersed or resuspended by vortexing to form an even suspension. It was therefore of interest to study the nature of and the factors causing this viscosity in cold.

a. **Microscopic examination:** Strain P_2d showed the formation of capsules during growth in benzoate medium (Fig. 33). Presence of capsules suggested the possibility of capsular EPS being responsible for the viscosity of cells. EPS staining by congo-red method showed of cells incubated at R.T. and in ice, is depicted in Fig.34a & b. Ice incubated cells were seen as clumps with the EPS, stained red, masking the appearance of the individual cells. In contrast, the R.T. incubated cells were present as individual cells, spread evenly throughout the microscopic field. Electron micrographs of cells incubated at R.T. (Fig.35a) and ice (Fig.35b) also confirmed the light microscopy observations.

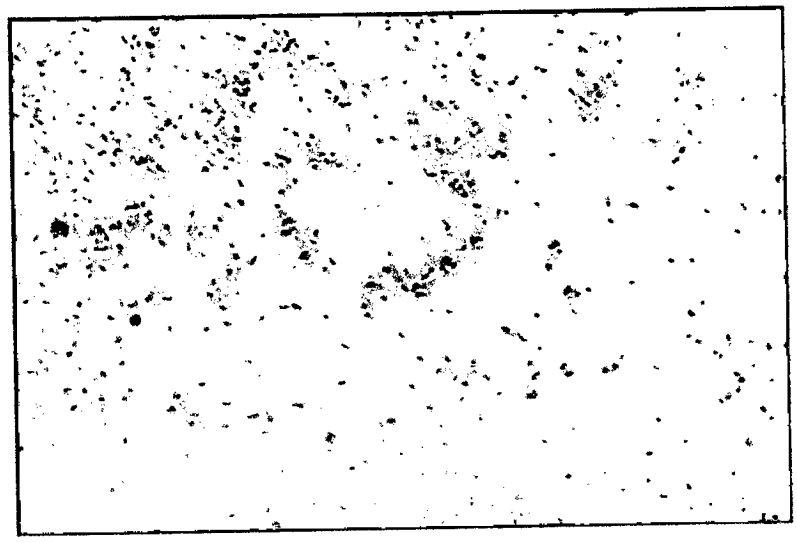


Fig. 33: ***Pseudomonas mendocina*** **P_2d with capsules under oil immersion (1000x)**

a

b

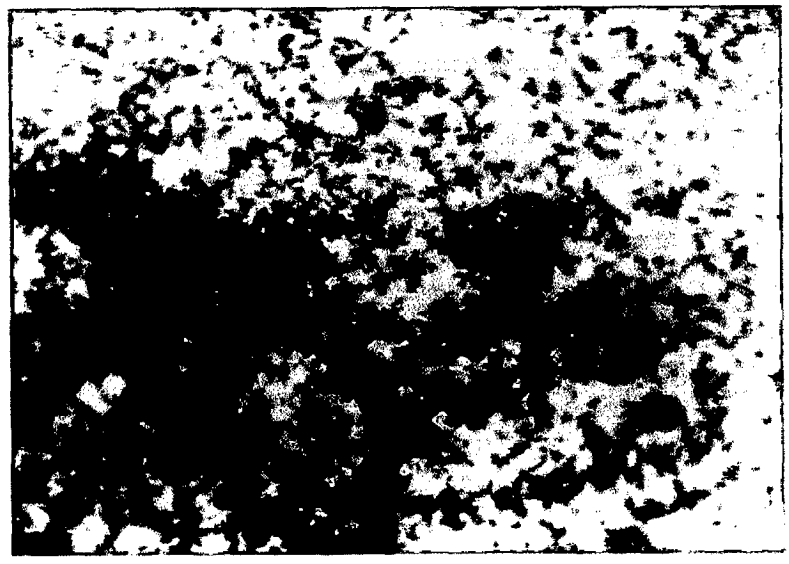


Fig.34: *Pseudomonas mendocina* P_2d EPS stained by congo-red method; Cells incubated at (a)R.T. cells and (b) in ice for 2 h

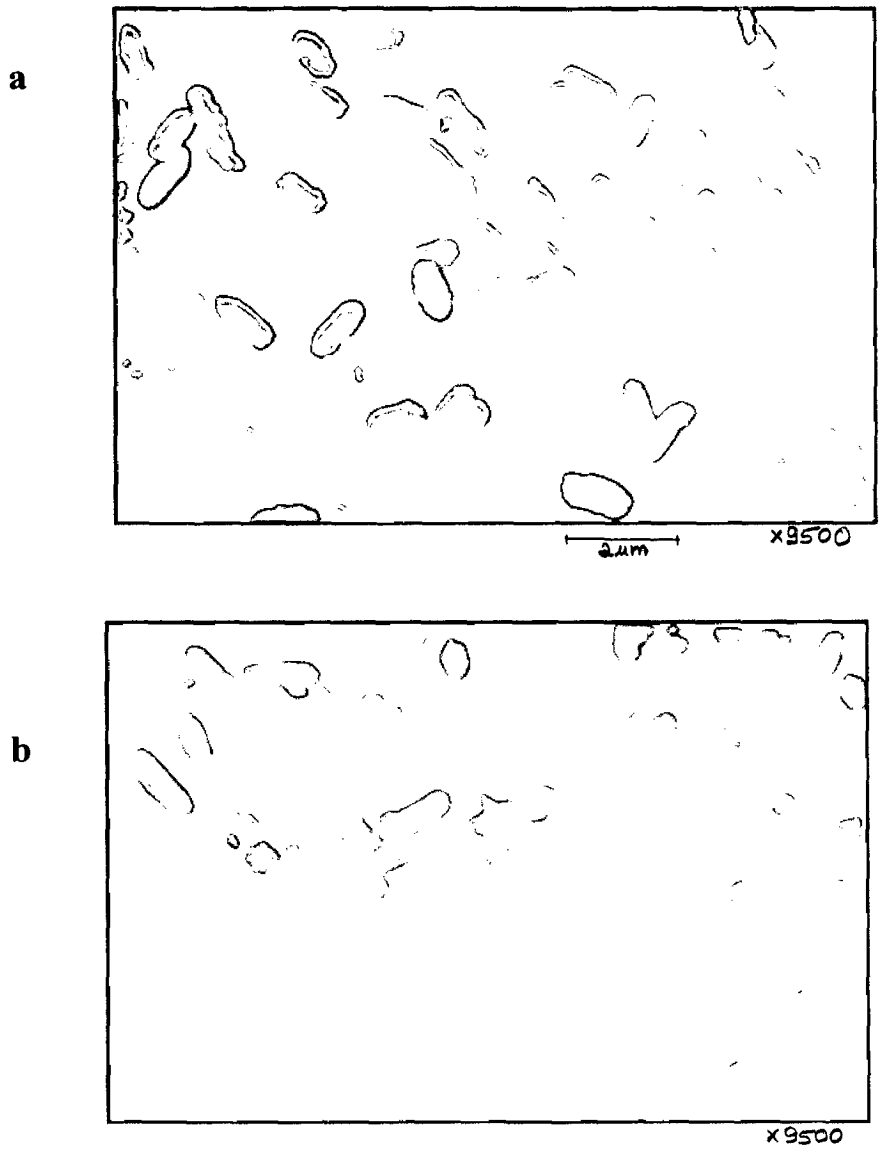


Fig.35: Scanning electron micrograph of *Pseudomonas mendocina* P_2d cell incubated (a) at R.T. and (b) in ice

b. Factors affecting viscosity formation: The effect of various physical and chemical parameters on viscosity formation and EPS extraction was studied. Culture grown in 0.3% benzoate medium for 24 h was incubated in ice and centrifuged. The pellet was checked for viscous mass formation. EPS was extracted in deionised D/W and precipitated using 3 volumes of ethanol.

(i) **Physical parameters**:

- ***Temperature of incubation***

P_2d cells grown in benzoate medium and incubated in ice showed a ten fold higher EPS yield than the R.T. incubated controls. Extraction of EPS from ice incubated cells under static conditions gave 48.0 mg while that under stirrer conditions gave 592.9mg. In contrast, R.T. incubated cells had 63.0 mg of EPS extracted under stirrer conditions and 4.4 mg under static condition. The viscous mass obtained by incubation in ice, followed by centrifugation on heating at higher temperature of 60°C and above for 10 min showed further increase in the viscosity.

Thus, incubation of cells in cold followed by extraction of EPS in deionised D/W under stirred condition resulted in maximum EPS yield. Incubation of cells in cold/ ice after EPS extraction, did not yield any more EPS.

- ***Time of incubation***:

Cells kept on ice became viscous. It was seen that a minimum period of incubation of 2 h was required to form the viscosity.

(ii) Chemicals:

Addition of various chemical agents such as EDTA, 10% SDS, 4 N NaCl, 0.1N HCl, *etc.*, did not help in decreasing the viscous nature. Addition of 2 N NaOH, however resulted in the formation of a clear suspension with concomitant loss of viability of cells.

c. Factors influencing EPS formation:

Effect of various factors was studied in terms of biomass, calculated as wet weight and EPS in terms of sugar concentration, as assayed by phenol-sulphuric acid method, of cells incubated in ice for 2 h.

(i) **Age of the culture:** Biomass and EPS formation of culture grown in 0.3% benzoate medium was studied at various time intervals. EPS was not detected below 15 h of growth. On centrifugation, viscous pellet was formed with 15 h old culture onwards. Whereas, young culture did not form a viscous mass. Thus, supporting the quantitative EPS results. An increase in sugar concentration was seen from 15 h till 36 h and further incubation resulted in a decrease (Fig.36a). The concentration remained static after 48 h. Biomass of cells also increased till 36 h of growth and then reduced.

(ii) **Effect of carbon source on EPS formation:** Culture grown in MM supplemented with various sugars as carbon source, incubated on ice and centrifuged did not show the formation of viscous mass. No EPS was obtained from culture grown in fructose, mannose, galactose and sucrose. However, 4 mg of EPS could be extracted from glucose grown cells as compared to 592.9 mg from benzoate grown cells.

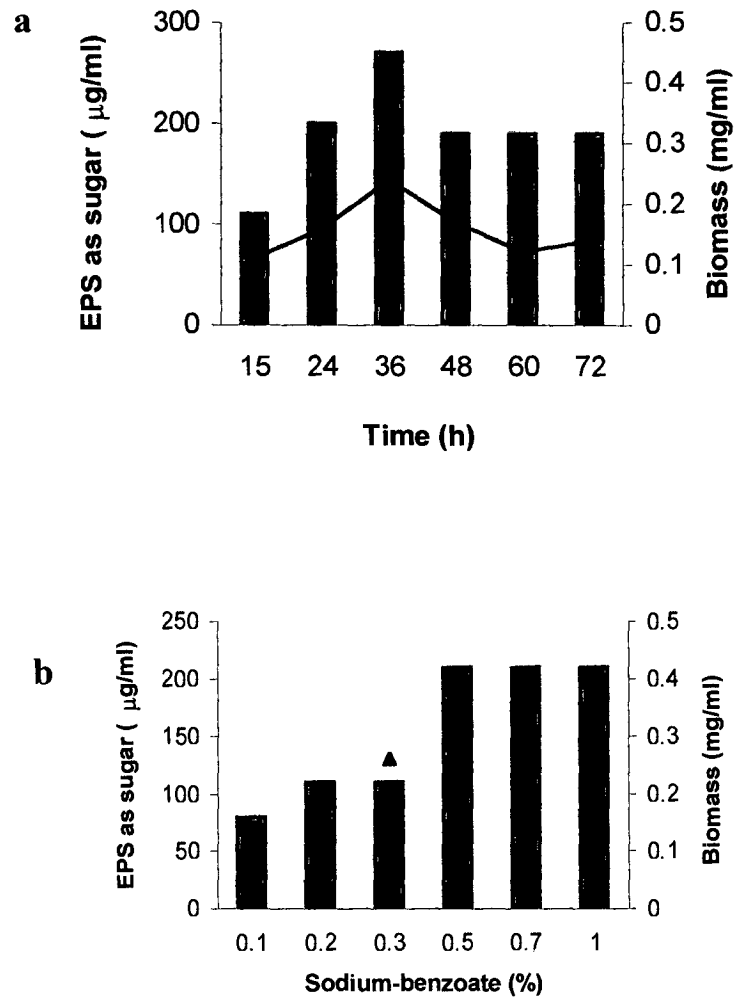


Fig. 36: Biomass (▲)and EPS (■) yield in strain P_2d cells grown at (a) different time intervals during growth in 0.3% benzoate medium
(b) varying benzoate concentration

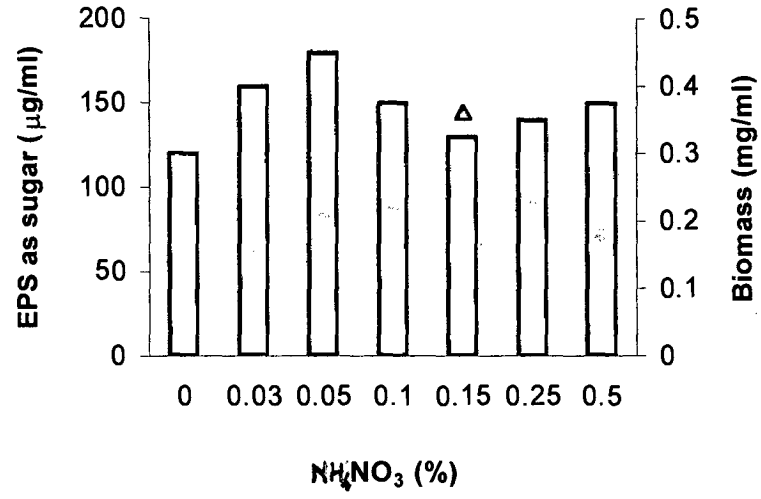
*Effect of varying benzoate concentration (*Fig. 36b*):* The sugar concentration of EPS was seen to increase with increasing benzoate concentration in the medium. Sugar concentration as well as biomass were maximum at 0.5% benzoate concentration and with higher concentration, the sugar level remained static though less biomass was obtained.

(iii). **Effect of nitrogen concentration on EPS formation:** NH_4NO_3 (0.1%) is the nitrogen source in the MM. The effect of varying nitrogen concentration in growth medium on formation of EPS by P_2d cells was studied (Fig. 36c). With increasing nitrogen concentration from 0 to 0.15% (w/v), the yield of biomass increased substantially, further increase however led to the decline in the biomass. EPS formation showed an initial increase from 0 to 0.05% and was invariant with further increase in NH_4NO_3.

(iv). **Effect of $CaCl_2$ concentration on EPS formation:** Presence of metal ions induces EPS formation.

As per Fig. 36d, maximum sugar formation was obtained at 0.08% concentration further increase in the $CaCl_2$ concentration

c



d

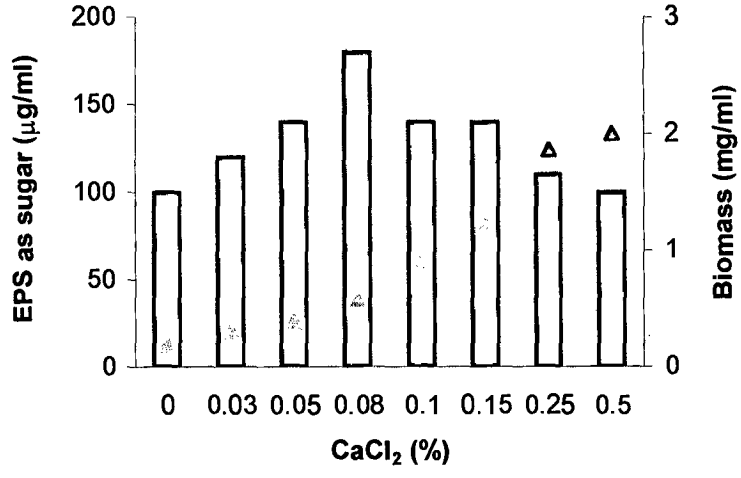


Fig. 36: Biomass (△) and EPS (□) yield in strain P_2d cells grown in 0.3% benzoate medium with varying
(c) nitrogen concentration;
(d) calcium-chloride concentration

resulted in a lowered sugar level. Biomass was seen to increase steadily with increasing $CaCl_2$ concentration.

From the above results, is seen that the optimal conditions for maximum yield of EPS and biomass are not necessarily the same and these vary with individual physico-chemical factors.

d. Emulsifying activity of EPS: The emulsifying activity of the EPS obtained from ice incubated cells was determined by BATH assay. In control, absorbance of the mixture of hexadecane in water, on vortexing is zero, as the organic solvent separates out rapidly from water. When 100µl EPS was added to the mixture of solvents, initial absorbance of 0.27 falls slowly to 0.14 with an emulsifying activity of 51.8% after 30 min and this emulsion is retained even after 1 h with absorbance of 0.13 i.e. 48% of emulsifying activity.

e. Whole cell protein profile of strain P_2d: Ice incubation gave higher viscosity to the cells than R.T. Since viscosity is caused by the EPS, it was of interest to find whether EPS is formed during ice incubation, with formation of new proteins. R.T. and ice incubated cells of strain P_2d after treating with sample buffer were assayed

for protein content by Folin-Lowry's method. The treated samples on SDS-PAGE showed the presence of 46 bands of identical molecular weights (Fig.37).

f. **Dry weight of EPS:** EPS extracted from R.T. and ice incubated cells, had a moisture content of 60 and 97.2%, respectively (Table 3.7) however their dry weights were almost the same i.e 25.44 and 25.41 mg respectively. Dry EPS on addition of water formed a suspension. On cold incubation, however, it did not turn viscous. In the presence of cells and cold incubation, the suspension turned viscous.

g. **GC analysis of the EPS:** Lyophilised EPS was hydrolysed and converted to alditol acetate derivatives. This on analysis by gas chromatography (Fig. 38a,b) showed presence of Rhamnose (50.79%), fucose (3.33%), glucose (7.23%), ribose (6.53%), arabinose (0.76%) and mannose (19.21%). Rhamnose and mannose were the major components of the EPS which is a heteropolysaccharide made up of pentoses and hexoses. Three minor peaks obtained in the chromatogram could not be identified.

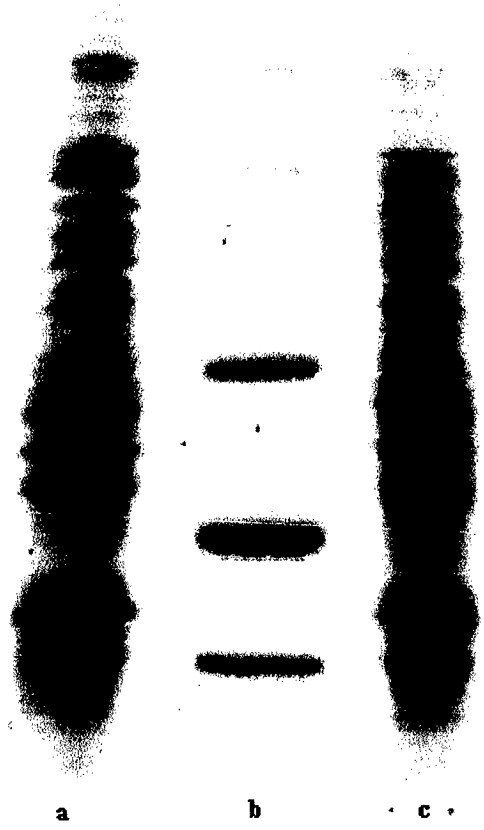


Fig.37: SDS-PAGE profile of whole cell proteins of (a) R.T. incubated cells; (b) molecular weight marker, 97, 68, 43, 29, 14 kD proteins; (c) Ice incubated cells

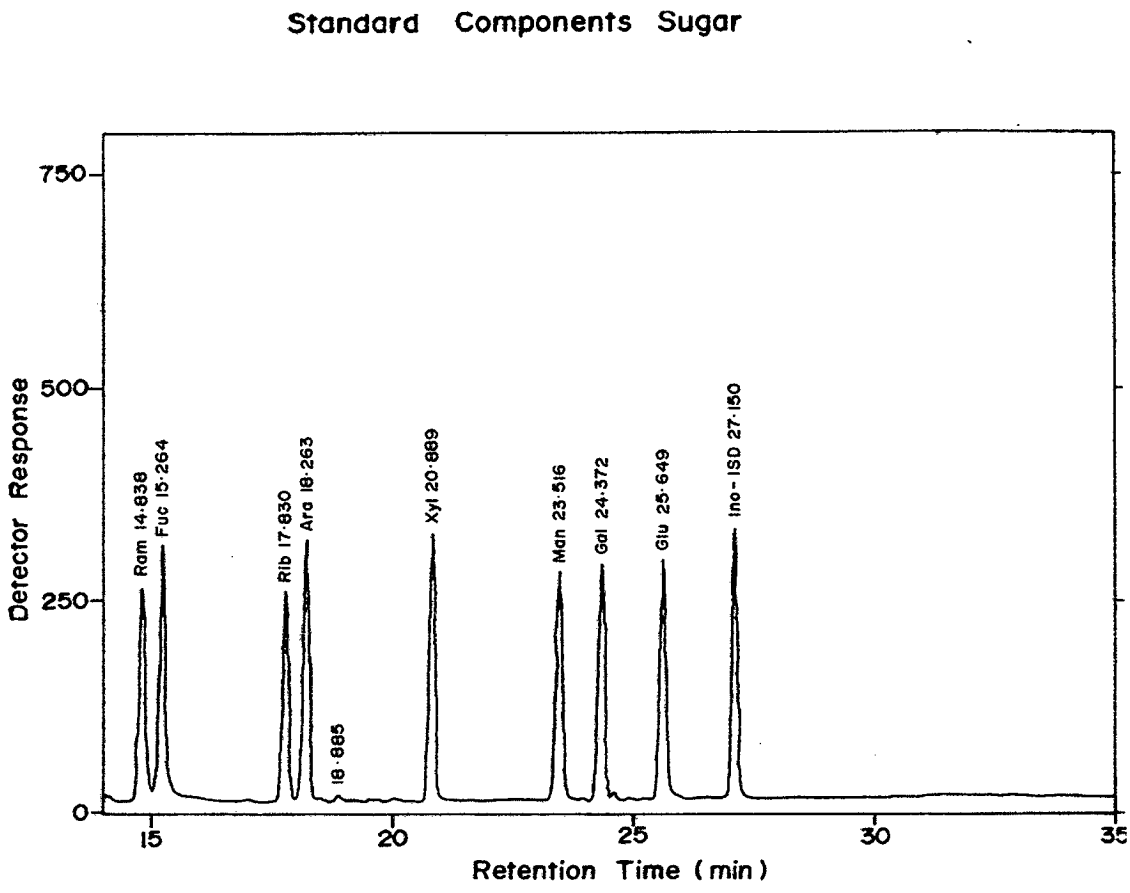


Fig. 38a: Gas chromatogram of standard sugars

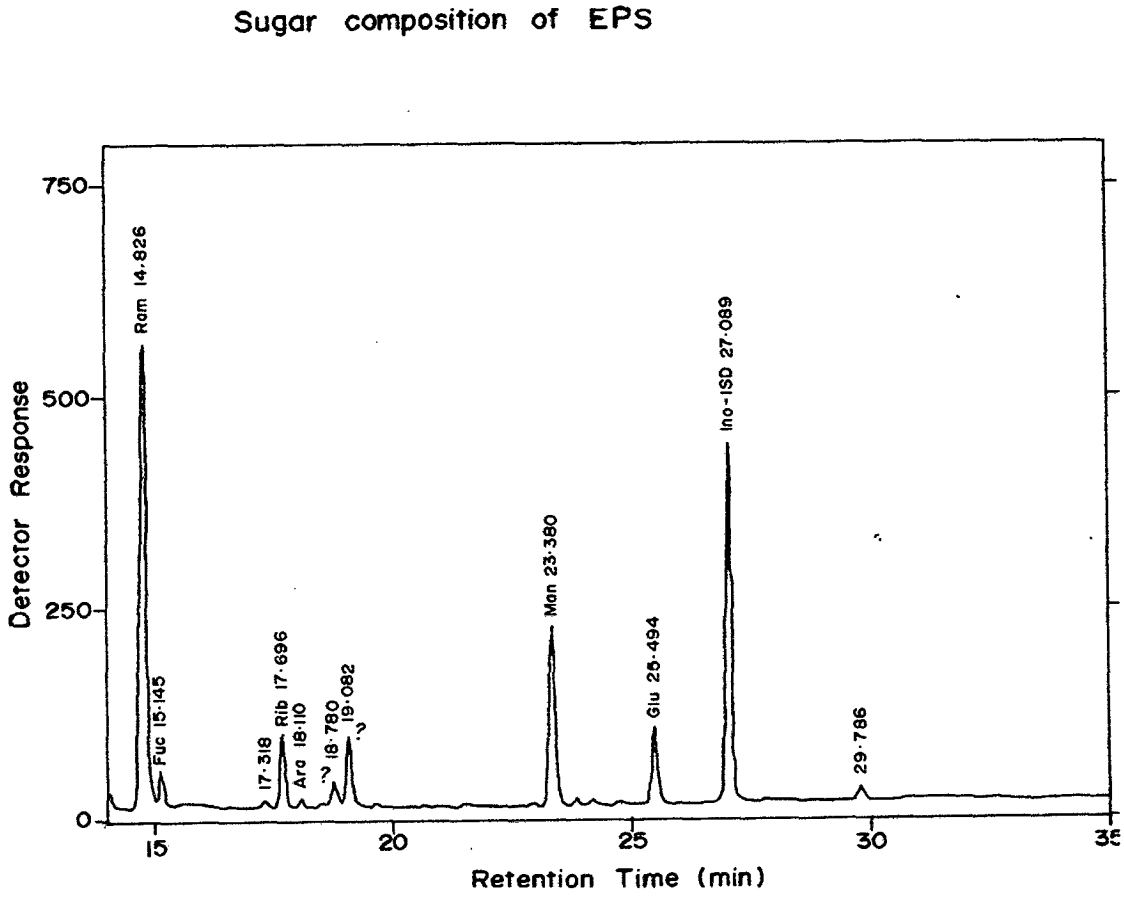


Fig. 38b: Gas chromatogram of hydrolysed EPS of *Pseudomonas mendocina* P_2d

Table 3.7: Wet and dry weights of EPS obtained from *Pseudomonas mendocina* P_2d

	R.T. incubation	Ice incubation
Weight of EPS before drying	15.1 mg	70.0 mg
Weight of EPS after drying	6.0 mg	3.0 mg
∴ Moisture content	60%	97.2%
∴ Dry wt./100ml medium	25.44mg	25.41mg

DISCUSSION

Constant presence of recalcitrant xenobiotics in effluents and treatment plants develop the microflora, which can withstand stress conditions and mineralize the polluting compounds. Pollution caused by aromatic and heterocyclic compounds can be controlled by use of such microorganisms (24). In the present work, an industrial effluent discharge site and areas surrounding the oil recovery stations such as Bombay High, were selected as sampling sites to get aromatic degraders.

Oil recovery operations lead to oil spills and leakage. Thus, the seawater surrounding the oil rigs is constantly exposed to pollution. The seawater samples collected from various oil recovery stations show a predominance of aromatic degraders (Table 3.1a). Microorganisms exposed to hydrocarbon pollutants act on it with the help of atmospheric oxygen and bring about degradation (223, 224). The surface water is thus a major site for aromatic degradation. Seawater isolates and strain P_2d, isolated from effluent sample, could grow luxuriantly in upto 1% sodium-benzoate medium. This shows the adaptability of the isolates to aromatic compounds in their natural environments and also their ability to withstand toxic levels of sodium benzoate. Sodium benzoate is used as food preservative; fungicidal at 0.05 to 0.1% concentration and bactericidal at 0.01 to 0.02% concentration (225). Hence growth in 1%

benzoate is an unusual and remarkable behaviour, shown by the isolates (Table 3.1b).

Cultures e, f, i and P_2d showed array of colours in benzoate medium during growth. Colouration in the medium may be due to formation and accumulation of coloured intermediates. Strain P_2d was selected for further studies as it showed the presence of both the ring cleavage pathways *i.e. ortho* and *meta* to be operative and formed colonies with yellow halo on benzoate agar turning orange-red. Benzoate, catechol, protocatechuate are generally metabolized *via* the *ortho* pathway while phenol, cresol, polycyclic hydrocarbons are reported to be metabolized *via* the *meta* pathway (23, 24, 63). Thus, strain P_2d was chosen since it was a promising culture with an ability to degrade a wide range of aromatic compounds.

Cells of strain P_2d are gram-negative, motile, short rods giving circular, mucoid colonies on nutrient agar medium. All biochemical tests indicated the culture to be a *Pseudomonas* species (Table 3.2a). Absence of nitrites in 36 h denitrification tests, apparently due to its rapid conversion to atmospheric nitrogen yielded initially a negative

nitrate-reduction test. Subsequent tests carried out after 6 h intervals however confirmed the rapid conversion of nitrates to nitrites and then to molecular nitrogen, allowing the placement of the culture in group I, non-fluorescent *Pseudomonas* (226) and further confirming its identity as *Pseudomonas mendocina* (211) designated as strain P_2d.

Until recently, the bacteria were classified based on Gram character, morphology, cultural and biochemical characteristics. Interest in evolutionary relationships between various microorganisms and also with other biological species led to search for macromolecules such as ribosomal RNA, cytochrome c-oxidase, for which the information in the form of base sequences in DNA is conserved over the period of evolution. Amongst the ribosomal RNA, the 5sRNA could not be used effectively because of their small size (227). The classification is presently being based on 16sRNA homology. As per the available data *Pseudomonas mendocina* is placed in the genus *Pseudomonas* under Phylum *Gammaproteobacteria*; Order *Pseudomonadales*; Family *Pseudomonadaceae*. Genera *Azotobacter, Rhizobacter, Azomonas, etc.* are also placed in the same family (228).

Pseudomonas species are known to be very versatile in their degradative abilities (28, 63, 66, 75, 96, 229). Monoaromatic, polyaromatic hydrocarbons as well as chlorinated and nitro-compounds could be utilized by strain P_2d (Table 3.2b). Scanty growth obtained with toluene, phenol, *p*-nitrophenol, 3-hydroxybenzoate, resorcinol, *o*-chlorophenol and naphthalene, which may have been due to the toxicity of these compounds at 0.1% concentration or the inability of the culture to utilize these compounds. Although the culture failed to show good growth on some of these substrates, benzoate grown cells showed high oxygen uptake rate for *p*-nitrophenol and an uptake of 1.87 units for toluene. Growth in the presence of benzoate must be inducing enzymes for metabolism of *p*-nitrophenol and toluene. The medium colour with *p*-nitrophenol was initially yellow, and turned slightly darker on further incubation. A number of reports on formation of *p*-benzoquinone from *p*-nitrophenol *via p*-nitrocatechol are available (68, 69, 70, 115). *p*-benzoquinone being yellow in colour (230) may be formed in the culture medium by oxidation. Marr, *et.al.* (87) reported utilization of toluene at concentration of 0.2%. Phenol is very toxic and used at concentration of 1 mM to 5 mM (15, 63, 66, 96, 231).

Strain P_2d grew very well with some substrates with no colouration in the medium. Utilization of the aromatic substrate *via ortho* or gentisate pathways do not yield any coloured intermediates (23, 40, 53). It is also possible that coloured intermediates were formed in too small quantities to be accumulated and detected visually. However, in the case of protocatechuate, higher concentration of even upto 0.5% failed to give colouration indicating operation of pathway that does not yield coloured metabolites.

Benzoate grown culture does not show oxygen uptake with *o*-chlorophenol, xylene, nitrobenzene, salicylate, tryptophan, phthalate, biphenyl, naphthalene, anthracene and phenanthrene, though culture showed growth in their presence (Table 3.2c), indicating the need for induction of specific enzymes. The enzymes induced during growth on benzoate may not be operative for metabolism of these substrates. For example, the chloro- and nitro- groups in *o*-chlorophenol and nitrobenzene may require to be removed enzymatically and such enzymes may not be formed during growth on benzoate. Chlorinated compounds are preferably metabolized *via* the *ortho* pathway (16, 36, 80, 81, 232); although in *Azotobacter* species GP1 (86), the *o*-chlorophenol is metabolized *via meta* pathway while in *Arthrobacter*

ureofacians CPR 706, a hydroquinone pathway is operative (82). Benzoate grown cells showed uptake for *p*-nitrophenol but not for nitrobenzene though both contain the nitro group. The position of the nitro group and the hydroxyl group are thus important in metabolic reactions. The nitro group is reported to be converted into amino group and removed after *meta* ring cleavage (74).

Strain P_2d grew well in the presence of protocatechuate, *p*-hydroxybenzoate and salicylate. Benzoate grown culture showed an oxygen uptake of 5.8 and 6.6 $\times 10^{-6}$ nmoles/mg dry weight, with protocatechuate and *p*-hydroxybenzoate, respectively (Table 3.2c). Johnson and Stanier (233) reported the metabolism of *p*-hydroxybenzoate *via ortho* pathway by *Alcaligenes eutrophus*. So also, in the case of *Roseobacter* strains and *Pseudomonas putida*, protocatechuate 3,4-dioxygenase activity for *p*-hydroxybenzoate catabolism was seen (31, 34). A *meta* cleavage *via* formation of protocatechuate in *Bacillus* species (234) while in *Trichosporum cutaneum* WYZ-2, formation of protocatechuate and hydroxyquinone has been reported (235).

Biphenyl, naphthalene, anthracene, phenanthrene supported growth of strain P_2d. Culture grown in benzoate however showed no oxygen

uptake with these substrates. Thus, the enzymes for their metabolism need to be induced and are not formed by the culture during growth on benzoate since these enzymes belong to the upper pathway (236, 237). Numerous reports are available on the mineralization of these compounds (2, 5, 8, 17, 29, 238, 239). Anthracene and phenanthrene are metabolized *via* naphthoic acid by *Pseudomonas fluorescens* 5R strain (240). Anthracene was metabolized *via* benzo-coumarin and anthraquinone formation while phenanthrene *via* 2,2'diphenolic acid, naphthoic acid and phthalic acid by *Mycobacterium* sp. strain PYR-1 (239). Yogambal and Karegoudar (241) reported naphthalene and anthracene metabolism by gentisate pathway and phenanthrene *via* protocatechuate formation by *Aspergillus niger*.

Catabolism of biphenyl, a fungicide, is reported with a few fungi such as *Paecilomyces lilacinus* (242) and in a few bacteria such as *Beijerinkia* sp., *Pseudomonas* sp., *Nocardia* sp. (243, 244, 245, 246). Tryptophan is metabolized *via* L-kynurenine to anthranilate and this sequence of reaction is characteristic of many *Pseudomonas* sp. (233). Anthranilate is further metabolized to catechol, a central key metabolite. During metabolism of tryptophan by strain P_2d, no colouration was

obtained. Cells grown on benzoate showed no oxygen uptake with tryptophan as the enzymes for tryptophan metabolism may need to be induced .

During growth in benzoate 0.2% and higher concentrations, yellow colour was formed initially followed by red colour. Benzoate is reported to be metabolized generally *via ortho* pathway although there are reports of *meta* cleavage (38, 247) and that of soil pseudomonads degrading benzoate *via p*-hydroxybenzoate formation (64, 232, 248).

Pseudomonas mendocina P_2d grown in the presence of all aromatic compounds as well as glucose and nutrient broth, showed presence of *ortho* as well as *meta* ring cleavage pathways, with catechol as substrate using modified Rothera's test. This indicates both pathways to be constitutively present in strain P_2d. This was further confirmed by the enzyme assays. *Meta* enzymes in *Pseudomonas putida* are reported to be constitutive (249). Presence of both pathways simultaneously in single culture has been reported earlier (59, 211, 250, 251, 252).

Catechol 2,3-dioxygenase involved in the *meta* ring cleavage was found to be very active in the cells as well as cfe of cells, grown in the presence of benzoate, glucose, tyrosine, protocatechuate, *etc*. Benzoate grown cells showed much higher oxygen uptake for catechol (189.4 x 10^{-7} nmoles/mg), than for other compounds including benzoate (40.15 x 10^{-6} nmoles/mg) as well as glucose (29.32 x 10^{-6} nmoles/mg) (Table 3.2c). The former reaction mixture with catechol turned yellow immediately, indicating the high specificity of the enzyme for catechol. Catechol 2, 3-dioxygenase activity with cfe was lower as compared to whole cells, possibly due to loss during extraction.

The *ortho* enzyme, catechol 1,2-dioxygenase, though functional, its activity was low in the cells possibly due to its activity masked by high activity of *meta* enzyme, catechol 2,3-dioxygenase (Table 3.4a). The *ortho* pathway enzymes are reported to be induced by cis, cis-muconate (211, 213), however in strain P_2d, these enzymes seem to be constitutively present. Catechol 1,2-dioxygenase activity was higher with mercaptoethanol treated cells in which no yellow colour was formed, indicating inactivation of catechol 2,3-dioxygenase. Heating reduced the catechol 1,2-dioxygenase activity corroborating earlier reports (20).

Strain P_2d cells showed activities of protocatechuate 3,4-dioxygenase *ortho* cleaving pathway enzyme, of 0.18 and 0.1 units with protocatechuate and *p*-hydroxybenzoate, respectively. Protocatechuate metabolism can proceed via *ortho,* proximal *meta* and distal *meta* pathways (34, 47, 51). The *ortho* enzyme, protocatechuate 3,4-dioxygenase, is chromosomally encoded and is found in bacteria and fungi (34, 233). The proximal *meta* enzyme, protocatechuate 2,3-dioxygenase, has been reported in *Bacillus macerans* (44, 45), while protocatechuate 4,5-dioxygenase, the distal *meta* enzyme, is reported in *Pseudomonas paucimobilis* (47, 48).

Salicylate is generally degraded via gentisate pathway (54, 96, 97, 236) while in *Pseudomonas putida* mt-2, *meta* pathway is operative (238). In the present work, gentisate 1,2-dioxygenase was not detected during growth on gentisate, salicylate or naphthalene. Surprisingly, cells grown on benzoate and induced with salicylate, also showed protocatechuate 3,4-dioxygenase activity. Salicylate metabolism by conversion through protocatechuate cannot be explained with available data.

The catechol 1,2-dioxygenase, is possibly under specific regulation, and hence non-functional and is substituted immediately by another

ortho enzyme namely protocatechuate 3,4-dioxygenase, in the presence of certain substrates such as protocatechuate, *p*-hydroxybenzoate and salicylate. The catechol 1,2-dioxygenase enzyme constitutively present, is still not effectively active with any of these substrates.

Cells of strain P_2d grown in tyrosine showed tyrosinase activity, which is absent in culture grown in the other substrates except benzoate and glucose, showing benzoate and glucose cells to be prone to induction. Tyrosinase belongs to polyphenol-oxidase or catechol-oxidase groups that catalyze the conversion of aromatic compounds to *ortho*-quinones by hydroxylation and oxidation reaction (30, 106).

Tyrosinase or catechol-oxidase oxidizes various diphenolic and triphenolic compounds to *ortho*-quinones, thereby producing varied colours (Table 3.2b). Strain P_2d grew in the presence of most of these compounds with colour formation. The enzyme activity was inhibited by copper chelating compounds such as mercaptoethanol, sodium-azide, cysteine, *etc.* (30). The red/pink colour formation during growth in benzoate/tyrosine medium was inhibited in the presence of mercaptoethanol, cysteine, sodium-bisulfide, sodium-metabisulfide and sodium azide. Enzyme activity in grown cells was also affected by the inhibitors in a similar way. Initial yellow colouration in benzoate

medium due to HMS as seen in control, was not affected by the presence of these inhibitors, but inhibited the quinone forming enzyme. Ascorbic acid and thiourea concentrations were probably low and hence no inhibition was observed (Table 3.4b).

KCN (respiratory inhibitor) inhibited yellow as well as red colour formation in benzoate showing that the *meta* ring cleavage enzyme and catechol-oxidase, both to be inactivated. KCN being toxic, affects the cells as well as the enzymes, both of which require oxygen for activity. The different colours formed with benzoate, catechol and tyrosine as well as with other diphenolic and triphenolic compounds (Table 3.2b), can be attributed to the activity of enzyme catechol-oxidase or tyrosinase.

Sodium-benzoate is known to display toxicity against microorganisms (225) and is used in limited concentration of 4 mM- 10 mM for growth in most of the studies (65, 231, 253) while strain P_2d could grow in much higher concentration upto 70 mM. It is seen that the cells show a fairly rapid growth at 0.1-0.3% benzoate. At higher concentration of 0.5, 0.7 and 1%, the cells do grow but after an extended lag. Formation of different colours at different concentration may be

due to accumulation of coloured intermediates in varying concentrations. Culture does not show pigmentation when grown in nutrient broth, glucose medium and most of the aromatic compounds other than benzoate, catechol and tyrosine. Hence pigment formation is not an inherent feature of the culture but the coloured product formation is substrate dependent.

The yellow colour formed at 0.1% concentration of benzoate is retained through the incubation period of 48 h. At higher concentration of above 0.2% of benzoate, the medium become initially yellow, turning orange in colour on further incubation. The yellow colour may be due to the accumulation of a transformation product of benzoate metabolism such as HMS (50, 52, 250) and a quinonoid compound, *p*-benzoquinone (103).

The absorption maxima at 375 nm seen in the yellow supernatant and that of the yellow spot of Rf value 0.16, eluted from TLC of supernatant, indicates the yellow coloured product, TP II to be HMS. The identity was further confirmed by comparing UV-Visible scan with HMS obtained from standard culture *Pseudomonas cepacia* AC1100 (212) by Bayley and Dayley's method (213). Growth, oxygen uptake in

the presence of HMS and a very high catechol 2,3-dioxygenase activity with catechol in the culture as described earlier (Table 3.2c) confirm HMS to be an intermediate in the benzoate breakdown.

Catechol is formed in the culture broth from benzoate as detected by the white precipitate with lead acetate and black spot on TLC of unacidified culture extracts. Spectrophotometric and colorimetric methods were unsuccessful for catechol detection, possibly due to the interference with other degradation products. Conversion of chlorophenol to catechol to yellow coloured HMS is reported in *Azotobacter* sp GP (75). Formation and utilization of catechol is seen clearly in Fig.14. Catechol at concentration below 10 mM is converted enzymatically to HMS.

With 10- 25 mM concentration of catechol, yellow colour formation was followed by red colour. This is similar to colouration in benzoate growth medium. Accumulation of more than one coloured intermediates may be responsible for the array of colours from red to wine-red, as seen at higher concentrations of benzoate. UV-Visible spectrum of red supernatant of culture incubated with catechol shows peaks at 210, 270, 360 and 375 nm; 210 and 270 nm being characteristic

peaks for catechol, 375 nm for HMS. TLC profile of acid extracted mixture shows 3 spots; yellow (TP II), corresponding to HMS and red (TP III) before iodine treatment and the 3rd spot of Rf value 0.91, corresponding to catechol. HPLC results strengthen the above findings. Catechol is thus an important intermediate formed from benzoate which gets converted to HMS and then to the red product (TP III).

Decolourization by sulphuric acid/sodium dithionite followed by appearance of colour by alkali/aeration, shows that the red colour formed in 12-14 h old culture supernatant to be quinonoid compound. Sodium-dithionite and sodium-borohydride reduce the coloured quinone to a leuco compound (103). Addition of 2% potassium iodide results in brown colouration due to release of iodine by quinone (219, 254). All these tests were supported by UV-Visible scans and HPLC analysis wherein all absorption peaks responsible for colouration disappear with disapperance of colour. Thus, the red product formed during benzoate and catechol metabolism is a quinone.

Quinones are known to impart varied colours from yellow to black (100, 101). The formation of quinones during aromatic degradation has been reported in bacteria and fungi (88, 113, 114, 115).

The aniline derivative of the red quinonoid compound had a melting point of 190-193°C and dissolved in H_2SO_4 forming violet-blue colour. The infra-red spectrum of the derivative showed stretching at 1625-1650cm^{-1} and 3400-3500 cm^{-1} which showed the presence of C=O and NH groups (255), thus confirming the product to be 4,5-dianilino-*o*-benzoquinone (105, 112). Formation of *p*-quinones as intermediates in degradation of various aromatic compounds such as *p*-nitrophenol and 2,4,5-trichlorophenoxy-acetic acid giving 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone have been reported (69, 70, 88, 115). However, *o*-benzoquinone formation is not as widely reported (105, 112, 113). Formation of quinone from catechol enzymatically as well as chemically has been described earlier (86, 101, 111, 256, 257, 258, 259).

Thus, aqueous catechol solution exposed to air gives pink, brown and black colouration, attributed to autooxidation of catechol. Amount of *ortho*-quinone assayed from such samples is negligibly small. In the

presence of strain P_2d cells however, a four-fold more increased amount of derivative was formed confirming the role of bacterial cells in quinone formation.

The red colour is retained in the culture medium over several days without fading, the quinone is thus not being utilized by the culture. Furthermore, *o*-benzoquinone used as sole carbon source did not support the growth of the organism. So also, culture showed no oxygen uptake with this substrate. This confirms that *o*-benzoquinone is formed as a byproduct unlike most of the reported quinones especially *p*-benzoquinone which is formed as intermediate during aromatic degradation (69, 70, 88, 115, 260).

HPLC analysis carried out with acetonitrile: water (80:20) supported the observation. This mobile phase has been used earlier for separation of phenol degradation products (15). Methanol: water: acetic acid have also been used by some workers (80, 261). HPLC peak corresponding to pure *o*-benzoquinone present in the culture supernatant grown in 0.3% benzoate was conspicuously absent in the culture supernatant of 0.1% benzoate as well as the decolourized supernatant samples.

In the presence of 2,2'bipyridyl, a known inhibitor of *meta* ring cleavage (16, 46), growth was slow with a long lag. Colour change from original dark-pink due to bipyridyl to wine-red was observed. Bipyridyl being inhibitor of ring cleavage, the catechol formed is apparently prohibited from forming HMS and hence no yellow colouration is visible. However, UV-Visible spectrum shows a low intensity peak of HMS at 375 nm, which is ten times more in the absence of bipyridyl. It is possible that very low concentration of HMS increased the lag phase and did not apparently impart yellow colour to the medium.

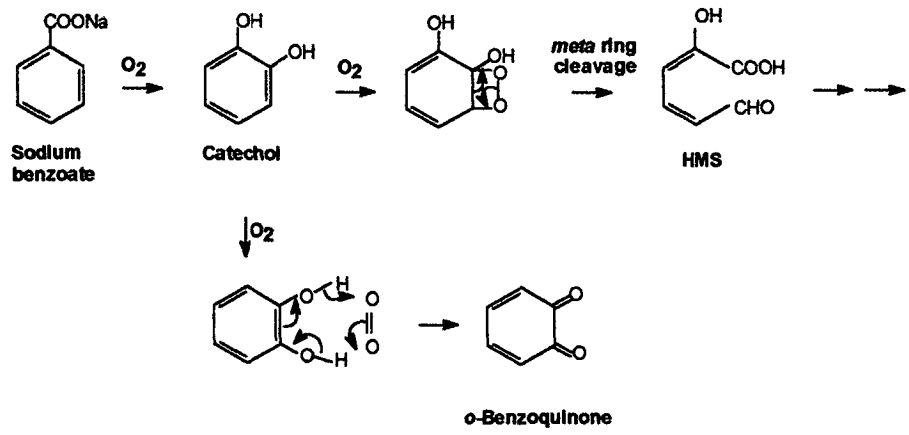
Most of the catechol is thus channeled into quinone formation which is unutilized by the culture. This may be a detoxification measure adapted by strain P_2d. Thus the culture is not affected by benzoate and catechol toxicity. Catechol estimated during extraction procedure shows 52.9% of catechol to be converted to HMS while 20% of it is converted to *o*-benzoquinone. Blocking the *meta* pathway thus inhibited the HMS formation, though quinone formation continued, suggesting the HMS and quinone formation pathways to be separate, linked *via* catechol, the central metabolite.

Growth was slow and no red colouration obtained at 37°C. Culture tolerants a wide pH range and grows in 0.3% benzoate medium. Absence of red colouration at pH below 6.5 may be either due to quinone not being formed or decolourization of the quinone because of acidic pH. Yellow colour of HMS is also decolourized under more acidity pH of 5.0 (13, 52). The culture was originally isolated from alkaline source, and could grow upto the pH 11, forming both HMS and quinone. Quinone formation is basically an oxidative process. Under moderate to very high aeration conditions such as at 100 and 150 rpm, good colouration was seen in culture medium. Lower aeration at 75 rpm and static conditions, affected the quinone formation and red colour was subdued. Based on these observations, catechol is proposed to be the central metabolite in benzoate metabolism that links the two pathways, namely, *meta* cleavage and *o*-benzoquinone formation, both operating simultaneously during benzoate degradation (Fig. 39) in *Pseudomonas mendocina* strain P_2d.

Culture utilizes tyrosine, as seen from the fall in tyrosine concentration from 1040 to 100 mg/L (Fig. 31), with the formation of pink to brown to reddish-black colouration in the medium. However, no

significant change in turbidity is seen (Fig. 30) as the medium is fairly turbidity to start with, because of the insoluble tyrosine. Dopa is formed from tyrosine as detected by colorimetric method. Culture shows very scanty growth with dopa (Table 3.2b) and hence dopa may not be an intermediate in metabolism but formed by oxidation of tyrosine. Dopa is converted to dopa-quinone confirmed by tests (Table 3.6a) rendering the medium pink in colour which further polymerizes to give melanin, the reddish-black pigment, confirmed by various tests (Table 3.6b) (123). Three types of melanin are known as described in introduction. The melanin formed by strain P_2d is reddish-black in colour and appears to be eumelanin formed from tyrosine (134, 137).

The pathway of tyrosine→ dopa→ dopaquinone→ dopachrome → melanin is widely reported in mushrooms, fungi, bacteria and in higher organisms including man (30, 106, 110, 134, 137). Melanin plays a number of roles such as in protection of fungi from bacterial enzyme, radiation effects in man, *etc.* (132, 133, 134, 135, 136, 250). Melanin is not utilized by strain P_2d and shows negligible to or poor growth in dopa. It therefore appears that dopa and melanin are formed in low concentration as these are not the intermediates of the major metabolic pathway in which tyrosine is utilized as carbon source.



o-Benzoquinone

Fig. 39: Proposed pathway for benzoate metabolism by *Pseudomonas mendocina* P_2d

Tyrosine is reported to be metabolized *via* varied pathways such as homogentisate, protocatechuate and homoprotocatechuate pathways (92, 93, 94). Homogentisate forms gentisate and is metabolized via gentisate pathway (92). However, gentisate 1,2-dioxygenase was absent in strain P_2d. The homoprotocatechuate leads to protocatechuate formation, which is further metabolized by any of the three pathways (34, 44, 47). Surprisingly, protocatechuate 3,4-dioxygenase, protocatechuate 4,5-dioxygenase and protocatechuate 2,3-dioxygenase could not be detected in tyrosine grown culture, although the *ortho* cleavage enzyme, protocatechuate 3,4-dioxygenase is induced in protocatechuate grown cells.

HPLC analysis initially showed two peaks with retention time of 1.5 and 2.2 min with the tyrosine, even of analytical grade. On incubation with cells the intensity of peak at 1.5 min is reduced and appears to be the peak corresponding to tyrosine. Whereas the peak at 2.2 min is intensified during incubation and may be ascribed to the transformation products, possibly dopa, although the identity is not confirmed. With the incubation of cells further more peaks at 2.5 and 1.7 min are also noticed, indicating the formation of newer metabolites.

o-Benzoquinone and melanin, are not utilized by strain P_2d and are dead-end products of cells. Strain P_2d cells must have acquired genes for quinone during adaptation to effluent. The role of these products/ enzymes cannot be explained presently but it appears that the oxidation reaction leading to quinone formation, apparently plays a role as an energy yielding process.

During studies with culture grown in benzoate medium, many difficulties were faced. The culture suspension when prepared and stored in ice for oxygen uptake analysis, enzyme assays, *etc.*, formed a viscous suspension, which could not be pipetted out. As a result, the culture had to be stored at R.T. and each time fresh culture suspension had to be prepared. Centrifugation of culture broth kept in refrigerator or below R.T., caused formation of viscous mass.

The studies were therefore undertaken on the factors causing viscosity. When stored at low temperature, P_2d cells clump together as seen microscopically after EPS staining (Fig. 34) and in SEM micrographs (Fig. 35). Gel forming EPS has been demonstrated in

mutant strains of *Alcaligenes faecalis* var *mycogens* (237). Water binding property of capsular EPS from *Streptococcus thermophilus* MRIC finds its application in moisture in cheese (181). In strain P_2d, EPS formation is observed after the 15 h of growth, corroborating earlier reports wherein EPS synthesis in bacterial strains such as *Myxococcus xanthus* (152) and *Pseudomonas* strain (182) has been reported after onset of exponential phase. The EPS is quite stable to heat treatment as well as to chemicals such as EDTA, SDS, *etc.*

EPS from the cells can be extracted by several methods depending upon the nature of culture and EPS. For example, from cyanobacteria (170) and *Pseudomonas* sp. (262), the EPS can be extracted very easily in deionized water, where as for capsular EPS extraction, buffer with NaCl is found to be a better extractant (263, 264). Organic solvents such as ethanol (180, 265) or acetone have also been used. In the present work, EPS of strain P_2d cells could be very easily extracted in deionised water, extracting under stirring condition giving about ten times higher EPS yield than under static condition. Much more EPS could be extracted from cold induced cells than R.T. incubated cells. It was therefore felt that the incubation at low temperature activates the EPS

forming enzymes. However, both the R.T. and cold incubated cells showing similar protein profile (Fig. 37), with no additional bands in ice incubated cells. Furthermore, cold incubation of cells, after the EPS is extracted, do not yield more EPS during subsequent extractions.

Nitrogen and $CaCl_2$ in the medium affects the EPS yield. Calcium ions stimulate polysaccharide production. Other divalent ions such as Sr^{+2}, Mg^{+2}, Li^{+2} are known to be less effective (152). Growth on sugars results in production of carbohydrate rich EPS in many bacteria (266) e.g. viscous EPS is formed by *Sphingomonas paucimobilis* GS-1 and in a fluorescent *Pseudomonas* sp. (267) during growth on sucrose (199, 268, 269, 270). Glucose is the most common carbon source for EPS production (58, 180, 181, 196, 271, 272, 273, 274, 275). Glucose was the most efficient carbon source followed by sucrose in EPS production by *Lactobacillus casei* CG11 (266). *Pseudomonas mendocina* forms high amounts of alginate under nitrogen limiting conditions in the presence of glucose (276). *Anabaena cylindrica* synthesized EPS during growth on acetate (277). Other than sugars, yeast extract also is used carbon source for EPS formation (152, 181). Surprisingly, *Pseudomonas mendocina* P_2d failed to produce appreciable amount of EPS when

grown in glucose, fructose, mannose, galactose and sucrose. However, when grown in benzoate medium, 592.9 mg EPS per 100 ml culture broth was obtained. Benzoate being a toxic compound, formation of EPS may be a means of protecting cells from benzoate toxicity (225). Production of EPS for combating toxicity of substrates is reported with culture *Azotobacter vinelandii* during growth on phenolic acids (265).

EPS produced by strain P_2d is a heteropolysaccharide composed of hexoses (rhamnose, fucose and glucose) and pentoses (ribose, arabinose and mannose). Such EPS constituted of heteropolysaccharides are reported widely (181, 262, 272, 278). For example, EPS of *Streptococcus thermophilus* MR-IC is made of galactose, rhamnose and fucose (181); EPS of *Pseudomonas fluorescens* strain III3 contains glucose, glucosamine, rhamnose, fucose, arabinose and acetate, while an acidic EPS containing glucose, galactose, acetate and pyruvate is formed by fluorescent pseudomonads associated with mushrooms (262, 278). EPS from *Pseudomonas putida* and *Pseudomonas fluorescence* both contains glucose, galactose and pyruvate (272).

EPS of strain P_2d has a good emulsifying activity, retaining 51.8% and 48% of emulsion after 30 and 60 min, respectively as assayed by BATH test. *Arthrobacter* RAG1 had percentage emulsification of 28% and 25% at 30 and 60min, respectively (200). EPS apparently has capacity to absorb moisture during cold incubation, thereby affecting 10-fold increase in wet weight, and simultaneously increasing viscocity and gelling.

Pseudomonas mendocina P_2d, an isolate from industrial effluent, can grow on a wide range of aromatic compounds, forming an array of colours such as yellow, orange, pink, wine-red, brown and black. Formation of these colors in succession is ascribed to the presence of multiple enzymes involved in the metabolism of aromatic compounds, such as catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, tyrosinase in the culture. a complex array of multiple enzymes involved in degradation of aromatic compounds formed apparently by constant adaptation to effluent rich in aromatic compounds. Presence of *ortho*, *meta*, protocatechuate and

tyrosinase pathways, makes this culture a highly potential degrader, with wide range of substrate specificity.

This culture forms an EPS during growth on benzoate as a detoxification measure. The EPS gels on cold incubation, which is an interesting property attributed to its water retaining capacity.

SUMMARY

Aromatic compound degraders as colony forming units on 0.1% benzoate agar were isolated from an industrial effluent discharge site and areas surrounding the oil recovery stations of Bombay High. Seven isolates showed very good growth in upto 1% benzoate concentration. Strain P_2d, an industrial effluent isolate was used for the present study as it could grow luxuriantly in benzoate, forming wine-red colouration and showed the presence of both ring cleavage pathways namely, *ortho* and *meta.* The culture was identified as *Pseudomonas mendocina* strain P_2d based on its morphological, cultural and biochemical characteristics.

Pseudomonas mendocina strain P_2d is a versatile culture having ability to degrade a wide range of aromatic compounds including monoaromatic, polyaromatic, chlorinated and nitro-compounds. An array of enzymes namely, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase, tyrosinase/catechol-oxidase, are formed during dissimilation of the aromatic compounds.

Growth in the presence of benzoate results in formation of yellow to orange to red colouration. Transformation product I (TP I) formed a black

spot on TLC and white precipitate with lead acetate, confirming it to be catechol. Transformation product II (TP II) is identified as 2-hydroxymuconic semialdehyde (HMS), due to its yellow colour, absorption peak at 375 nm and comparison with standard HMS formed from culture *Pseudomonas cepacia* AC1100. Red colour formed during growth on benzoate and catechol is due to transformation product III (TP III). Qualitative tests showed this product to be a quinone. Melting point and infra-red spectrum of the derivative of quinone with aniline confirm its identity as *ortho*-benzoquinone. The red product, *ortho*-benzoquinone formed from benzoate is not used by *Pseudomonas mendocina* strain P_2d for growth and no oxygen uptake is seen. . Thus, benzoate is metabolized *via meta* ring cleavage pathway and catechol, HMS and *ortho*-benzoquinone are the intermediates of benzoate biodegradation. Catechol is the common intermediate for HMS and *ortho*-benzoquinone formation and the proposed pathway is shown in Fig. 39.

Growth of *Pseudomonas mendocina* strain P_2d in the presence of tyrosine results in pink to brown to reddish-black colouration. Products formed in the culture broth on the basis of qualitative test, were identified as dopaquinone and melanin. However, dopa and melanin are not utilized by

the culture, showing the presence of an alternative pathway operating simultaneously for utilization of tyrosine.

Ortho-benzoquinone, dopa and melanin are the transformation products formed enzymatically as by-products from benzoate and tyrosine.

Pseudomonas mendocina strain P_2d forms an exopolysaccharide during growth in benzoate. Surprisingly, in the presence of sugars, no exopolysaccharide is formed but is conspicuously present in benzoate grown cells. The exopolysaccharide gels on incubation in cold. Deionised D/W was effective in extracting the exopolysaccharide and 592.9 mg of exopolysaccharides/100 ml broth, was obtained from ice incubated cells. R.T. incubated cells gave ten times less exopolysaccharide yield. Exopolysaccharide of *Pseudomonas mendocina* strain P_2d had a good emulsifying activity.

The exopolysaccharide is a heteropolysaccharide containing rhamnose, fucose, glucose, ribose, arabinose and mannose. On cold incubation, the exopolysaccharide absorbs moisture and swells resulting in a increased wet weight and becomes viscous.

Pseudomonas mendocina strain P_2d, an industrial effluent isolate has multiple enzymes involved in the degradation of wide range of aromatic compounds and various coloured intermediates are formed during growth on these compounds. The culture forms an exopolysaccharide to possibly afford protection during growth of cells on benzoate. The exopolysaccharide, an heteropolysaccharide, has an interesting property of gelling under cold incubation.

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APPENDICES

Appendix A: Media composition

A.1. Mineral media (MM) double strength for 1 L: (279)

D/W	1000 ml
Ferrous-sulphate	0.12 g
Dipotassium-hydrogen-phosphate	12.60 g
Potassium-dihydrogen-phosphate	3.64 g
Ammonium-nitrate	2.0 g
Magnesium-sulphate	0.20 g
Manganese-sulphate	0.0012 g
Sodium-molybdate	0.0012 g
Calcium-chloride (dihydrate)	0.15 g

For use: 10 ml of double-strength media made to 20 ml with D/W and sterilized for 10 min at 120°C and 15 psi. For MMB-0.3%, 0.6 ml of sterile 10 % sodium-benzoate added to MM just before inoculating the culture.

For MMBA: 50 ml of double-strength MM and 50 ml of D/W containing 3 g of agar, were sterilized separately. After autoclaving, the contents mixed and required amount of sodium-benzoate (10%) added.

A. 2 Nutrient Broth

Peptone	10.0 g
Beef extract	3.0 g
Sodium-chloride	5.0 g
D/W	1000 ml

pH adjusted to 7.0 with 0.1 N NaOH
For nutrient agar, 2.0 g of agar added to 100 ml nutrient broth. Digested in water-bath and sterilized.

Appendix B:

Standardization of HPLC with respect to detector wavelength, mobile phases, flow-rates and sample size.

B. 1.

Mobile phase = methanol: water (85:15) λ=254 nm ; Flow-rate= 1 ml/min		
Amount of sample (µl)	Retention time (min)	Area (%)
20	1.25 1.75 2.05	28.1 23.5 48.4
15	1.25 1.76 2.00	28.75 18.75 52.50
10	1.23 1.75	74.4 25.6
5	1.22 1.66 1.97	50.4 12.5 37.1

B. 2.

Mobile phase = methanol: water (85:15) λ=254 nm ; Amount inoculated = 20 µl		
Flow-rate (ml/min)	Retention time (min)	Area (%)
0.5	2.45 3.45	78.60 21.40
0.75	1.65 2.31	77.60 22.40
1.0	1.25 1.76 2.00	28.75 18.75 52.50
1.5	1.12 1.54 1.77	29.70 22.00 48.30

B. 3.

Mobile phase = methanol: water (85:15) Flow-rate =1 ml/min ; Amount of sample =20 µl		
λ (nm)	Retention time (min)	Area (%)
230	1.59 1.91 2.24	9.10 49.60 41.30
254	1.25 1.75 2.05	28.10 23.50 48.40
412	1.28 1.75 1.96	59.98 5.13 34.89

B.4

Mobile phase = acetonitrite: water (80:20) Flow-rate =1 ml/min; Amount of sample =20 µl		
λ (nm)	Retention time (min)	Area (%)
254	1.26 1.64	7.35 92.65
412	1.32 1.47 1.71	7.87 3.66 88.46

B.5.

Mobile phase = acetonitrite: methanol (10:50) Flow-rate =1 ml/min; Amount of sample =20 µl		
λ (nm)	Retention time (min)	Area (%)
412	1.20 1.65 1.93	32.79 24.73 42.48

B. 6

Flow-rate =1 ml/min; Amount of sample =20 µl Mobile phase = acetonitrite: water λ = 270 nm		
Ratio of mobile phase	Retention time (min)	Area (%)
40:60	1.78 2.13 2.44 2.89	9.90 23.45 44.45 21.99
20:80	1.68 2.33 3.14 3.31	8.88 36.23 10.60 44.31
0:100	1.83 2.32 2.39 4.82 24.68	13.14 2.30 12.37 5.36 63.23

Appendix C: Reagents, buffers and stains

C.1: Reagents for protein separation by PAGE/SDS-PAGE

(i) Monomer Solution (30%T, 27% C)

Acrylamide	58.4 g
Bis-acrylamide	1.6 g
D/W	200 ml

Store at 4°C in dark conditions

(ii) Separating gel buffer (1.5 M, pH 8.8)

Tris	36.3 g
D/W	200 ml

Adjust pH to 8.8 with 1N HCl

(iii) Stacking gel buffer (0.5 M, pH 6.8)

Tris	3 g
D/W	50 ml

Adjust pH to 6.8 with 1N HCl

(iv) 10% SDS

Sodium dodecyl sulphate	1 g
D/W	10 ml

(v) 10% ammonium per sulphate (Initiator) (APS)

APS	0.5 g
D/W	5 ml

(vi) Dye solution

Bromophenol blue	0.1 g
Sucrose	50 g
D/W	100 ml

(vii) Tank buffer (0.025 M Tris, 0.192 M glycine, pH 8.3)

Tris	6 g
Glycine	28.8 g
SDS (10%)	20 ml
D/W	2 L

(viii) Sample buffer

Stacking gel buffer	1.25 ml
SDS (10%)	3 ml
Glycerol	1 ml
Mercaptoethanol	0.5 ml
D/W	4.75 ml

Gel preparation

Solution	**Separating Gel**		**Stacking gel**	
	Big gel	Mini gel	Big gel	Mini gel
Monomer solution	10 ml	2.5 ml	1.33 ml	0.66 ml
Separating gel buffer	7.5 ml	1.875 ml	-	-
Stacking gel buffer	-	-	2.5 ml	1.25 ml
SDS (10%)	0.3 ml	0.075 ml	0.1 ml	0.05 ml
APS (10%)	150 µl	37.5 µl	50 µl	25 µl
TEMED	10 µl	2.5 µl	5 µl	2.5 µl
D/W	12 ml	3 ml	6.1 ml	1.05 ml

C.2: Staining of PAGE/SDS-PAGE gels by Coomassie blue method

(i) Staining solution

Coomassie Brilliant Blue R-250	1 g
Methanol	500 ml
Acetic acid	100 ml
D/W	400 ml

(ii) Destaining solutions

	I	II	or	II
Methanol	500 ml	50 ml		-
Acetic acid	100 ml	70 ml		7 ml
D/W	400 ml	1000 ml		100 ml

C.3: Staining of PAGE/SDS-PAGE gels by silver staining method

(i) Fixative solution

Ethanol	25 ml
Acetic acid	10 ml
D/W	100 ml

(ii) Dithiothreitol (5 mg/ml)

Dithiothreitol	0.5 mg
D/W	100 ml

(iii) Silver nitrate solution (0.1%)

$AgNO_3$	0.1 g
D/W	100 ml

(iv) Formaldehyde solution in 3% Na_2CO_3

40% Formaldehyde	0.1 ml
Na_2CO_3	6 g
D/W	200 ml

Dissolve Na_2CO_3 in D/W and then add formaldehyde and mix.

(v) 2.3M Citric acid

Citric acid	24.15 g
D/W	50 ml

C.4: Manewal's stain

i) 1.5 ml phenol + 28.5 ml D/W
ii) 1.2 ml ferric-chloride + 4 ml D/W
iii) 2 ml acetic acid + 8 ml D/W
iv) 20 mg acid-fuchsin + 2 ml D/W
Mix all of above.

C.5: Reagents for Arnow's methods

i) Mercuric-sulphate reagent

Mercuric-sulphate	15 g
5 N Sulphuric acid	10 ml

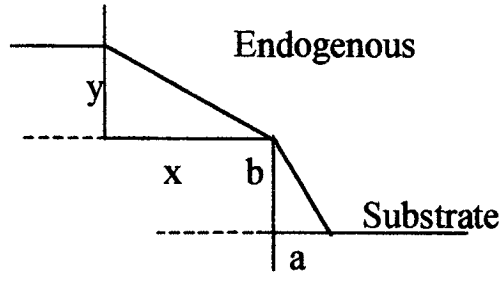
ii) Nitrite reagent

Sodium-nitrite	0.2 g
D/W	100 ml

iii) Sodium-molybdate reagent

Sodium-nitrite	10 g
Sodium-molybdate	10 g
D/W	100 ml

Appendix D: Calculations of oxygen uptake by cells



x, a = distance in 'mm'
y, b = % drop

0.1 mm $\rightarrow$1 sec

$\therefore$ x mm $\rightarrow$ x/0.1 sec

$\rightarrow$ 60 x/0.1 min = x_1 min

y % drop $\equiv$ x_1 min

y/x_1% $\equiv$ 1 min

$\therefore$ y/x_1 % drop per min is the oxygen consumed by cells endogenously

Similarly, for substrate,

b/ a_1% drop per min

We know that 100% drop $\equiv$ 0.36 μ moles of oxygen/min

$\therefore$ $(y/x_1 - b/a_1)$% $\equiv$ z μ moles/min

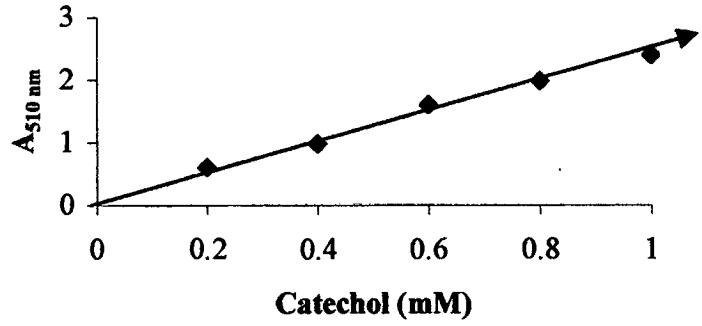
$\equiv$ z $x10^{-6}$ nmoles/min

$\therefore$ Oxygen uptake per mg dry weight of cells

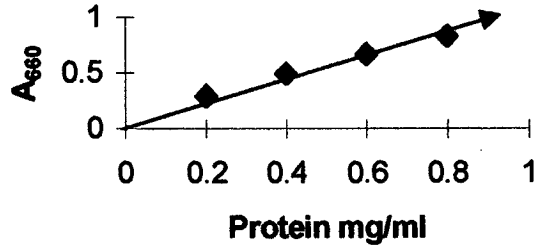
= $z/q\ x10^{-6}$ nmoles/min/mg dry weight

q = dry weight of 100μl of cells at 540nm.

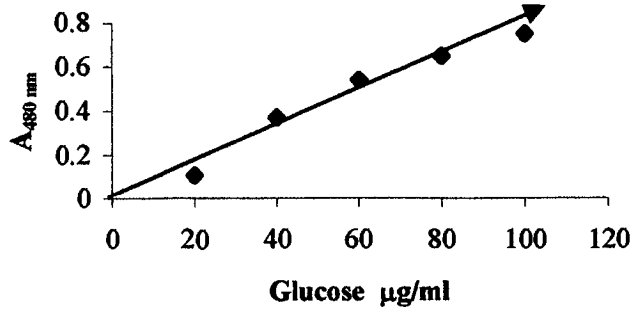
a



b

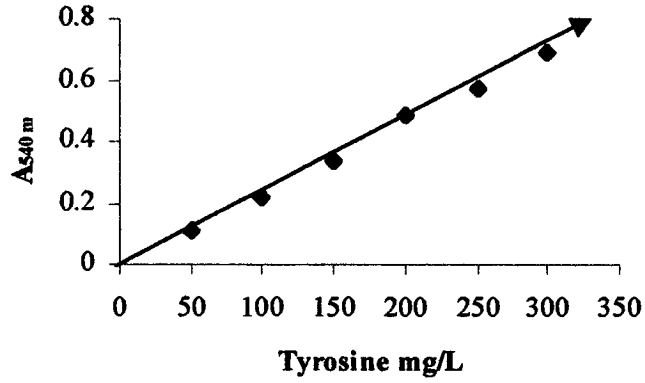


c

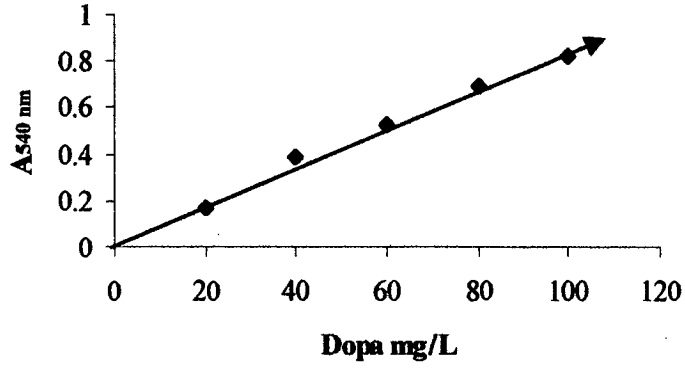


Standard graph of (a) Catechol; (b) Protein; (c) Glucose

d



e



Standard graph of (d) Tyrosine; (e) Dopa

Exopolysaccharides of *Pseudomonas mendocina* P_2d

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S. ROYAN, C. PARULEKAR AND S. MAVINKURVE. 1999. *Pseudomonas mendocina* P_2d cells grown at room temperature in sodium benzoate as sole source of carbon, followed by storage on ice, form a viscous pellet on centrifugation. Such viscosity is not produced by cells grown on glucose or any other carbohydrates. Viscosity was found to be associated with the extracellular polysaccharide (EPS) of cells and not released into the supernatant fluid. A combination of sodium dodecyl sulphate-citrate buffer and homogenization was effective in releasing the EPS. The EPS is a heteropolysaccharide, consisting of rhamnose, fucose, glucose, ribose, arabinose and mannose, which has good emulsifying activity.

INTRODUCTION

Microbial polysaccharides have found a wide range of applications in the food, pharmaceutical and petroleum industries and in medical fields and are widely accepted products of biotechnology (Sanford and Baird 1983; Sutherland 1998). A wide range of bacteria, from clinical and environmental habitats, is known to produce complex and diverse exopolysaccharides (EPS), occurring as capsular polysaccharides intimately associated with the cell surface or as slime polysaccharides, loosely associated with the cell. These are distinguished by the degree of cell association following centrifugation (Sutherland 1985; Whitfield 1988).

A wide range of chemical structures of homopolymeric or heteropolymeric type, made up of sugar and non-sugar components, is possible and the range of monosaccharide combinations, together with non-carbohydrate substituents and varied linkage types, makes the EPS an excellent agent and attributes diversity in bacteria (Keene and Lindberg 1983).

A bacterial culture P_2d identified as *Pseudomonas mendocina*, isolated from an industrial effluent, utilizes sodium benzoate as a source of carbon and forms a highly viscous mass on storage in ice or at low temperatures. The present paper reports on the factors influencing viscosity and on the isolation and characterization of the viscous material.

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MATERIALS AND METHODS

Bacterial strain and maintenance

Isolate P_2d, a Gram-negative bacterium isolated from an industrial effluent, was used for the study. Its morphological, cultural and biochemical characteristics revealed it to be a strain of *Ps. mendocina* (unpublished work). The culture was grown routinely in a mineral salts medium (MSM) with sodium benzoate at a final concentration of 0·3% (w/v) as the carbon source, at 28 ± 2 °C for 24 h on a shaker at 150 rev min^{-1}.

Isolation of exopolysaccharide from cells

Various methods (Domenico *et al.* 1989; Decho 1990; Adhikary 1998) were attempted for the effective isolation of EPS from the viscous pellet. The culture was grown in MSM with benzoate for 24 h at 28 °C, incubated on ice for 2 h and centrifuged at 6000 rev min^{-1} for 10 min. Exopolysaccharide from the viscous pellet was extracted in deionized distilled water by placing on a magnetic stirrer overnight at 4 °C. The supernatant fluid was analysed for EPS.

In other methods, sodium dodecyl sulphate (SDS)-citrate buffer (0·1%, 50 mmol l^{-1}) (Domenico *et al.* 1989), pH 4·5, or EDTA-NaCl buffer (10 mmol l^{-1}, 0·85%) (Decho 1990), 250 μl, was added to the cell pellet and the pellet homogenized using tissue homogenizer. The homogenate was heated at 42 °C (Domenico *et al.* 1989) or 60 °C (Decho 1990) for 3 min

and centrifuged at 4000 rev min^{-1} for 10 min. The pellet, after removal of the supernatant fluid, was homogenized again with the respective buffer and centrifuged. This step was repeated twice and all supernatant fluids obtained on centrifugation were pooled. Three volumes of cold ethanol were added to the supernatant fluid (Decho 1990; Cerning *et al.* 1994) and incubated overnight at 4 °C. The supernatant fluid was centrifuged at 4000 rev min^{-1} for 10 min. The precipitate obtained was analysed for EPS. Large-scale extraction of EPS for qualitative and chromatographic analysis was carried out using the SDS-citrate buffer method.

Quantitative analysis of exopolysaccharide

Exopolysaccharide extracted from supernatant fluids was lyophilized using Labconco lyophilizer (Kansas City, MO, USA). Lyophilized EPS was hydrolysed with 2 N HCl for 2 h at 100 °C in ampoules flushed with N_2 before sealing (Read and Costerton 1987). The EPS was assayed for total carbohydrates (Dubois *et al.* 1956), uronic acid (Dische 1947), methyl pentoses (Dische and Shettles 1948) and inorganic phosphate (Jayaraman 1981) using standard methods.

Factors affecting viscosity

The effect of various factors, such as temperature and time of incubation, age of the culture, substrates, chemicals, surfactants and heat, on the formation of viscosity was studied.

Emulsifying activity of exopolysaccharide

The emulsifying activity of EPS was assayed by the modified method (Rosenberg *et al.* 1979). Lyophilized EPS (0·5 mg) was dissolved in 0·5 ml deionized water by heating at 100 °C for about 15–20 min and allowed to cool to room temperature (28 ± 2 °C). The volume was then made up to 2 ml using phosphate-buffered saline (PBS). The sample was vortexed for 1 min after the addition of 0·5 ml hexadecane. The absorbance at 540 nm was read immediately before and after vortexing (A_0). The fall in absorbance was recorded after incubation at room temperature for 30 and 60 min (A_t). A control was run simultaneously with 2 ml PBS and 0·5 ml hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for time t: $A_t/A_0 \times 100$.

Analytical gas chromatography

Alditol acetates derivatives (Bhosle *et al.* 1995) of sugars released by hydrolysis from lyophilized EPS were analysed by gas chromatography (GC; Chrompack CP 9002 on Sil-88; Middleburg, The Netherlands (25 m, i.d. 0·32 mm, d_s 0·12)) using a flame ionization detector, with inositol (100 μg) as internal standard.

RESULTS AND DISCUSSION

The slime polysaccharides bound loosely to the cell surface are released into the medium on centrifugation (Sutherland 1985; Whitfield 1988). However, the EPS, which are tightly bound to the cell surface, require a more severe extraction process. *Pseudomonas mendocina* P_2d grown in MSM with benzoate as carbon source followed by storage on ice, gave a highly viscous cell pellet on centrifugation. The supernatant fluid was devoid of any viscosity. The viscosity-causing factor therefore seems to be tightly bound to the cells.

The use of EDTA is reported to give minimal cell lysis with efficient extraction of EPS fairly free from cellular components (Decho 1990). In the case of *Ps. mendocina* P_2d, however, extraction with EDTA-NaCl buffer was not as effective as that with cationic detergent SDS-citrate buffer. The latter buffer is reported to protect the cells from cell disruption during the extraction procedure (Domenico *et al.* 1989). By using this method, 919 mg (wet weight) EPS was obtained from 1000 ml culture with a yield of 0·9% (w/w). This method was followed routinely.

Factors affecting the viscosity

Pseudomonas mendocina P_2d incubated at the optimal growth temperature of 28 ± 2 °C, after centrifugation, yielded a pellet of cells devoid of any viscosity and the addition of cold ethanol to supernatant fluids did not yield any precipitate. However, the same cells incubated on ice and centrifuged yielded a highly viscous pellet, the viscous nature of which is thought to be due to the gelling of the EPS produced by the culture. Cold temperature incubation for a minimum period of 2 h is necessary for the formation of viscosity (Table 1). Young to exponentially growing cells of less than 15 h do not produce viscosity, corroborating earlier observations in bacterial strains of *Myxococcus xanthus* and *Pseudomonas* (Williams and Wimpeny 1977).

The carbon source used for growth determines both the quality and quantity of polysaccharide formation (Bryan *et al.* 1986; Kojic *et al.* 1992; Cerning *et al.* 1994; Meningitsu *et al.* 1994; Nourani *et al.* 1998). Sugar nucleotides play an important role in the EPS synthesis as activated precursors (Fett *et al.* 1995). Carbohydrate sugars, such as glucose, lactose, galactose and sucrose, are preferred carbon sources for the production of EPS. With glucose as a carbon source, *Ps. putida* and *Ps. fluorescens* synthesized an EPS composed of glucose, galactose and pyruvate (Read and Costerton 1987). *Sphingomonas paucimobilis* GS1 produces a highly viscous EPS composed of glucose, glucuronic acid, galacturonic acid and acetate using sucrose as a substrate (Ashtaputre and Shah

Table 1 Factors influencing the viscosity of the cell pellet of *Pseudomonas mendocina* P_2d

	Growth substrates		24-h-old benzoate-grown culture incubated at	
	Benzoate (0·3%)	Carbohydrate ψ (0·4%)	RT	On ice
Period (h)	Viscosity*		Viscosity	
0	–	–	–	–
2	–	–	–	++
8	–	–	–	++
15	++	–	–	++
24	++	–	–	++
48	++	–	–	+

* Cultures grown for varying periods were placed on ice for 2 h, centrifuged and the pellet observed for viscosity visually.
ψ Carbohydrates used: glucose, mannose, galactose, fructose and sucrose.
++, Highly viscous; +, slightly viscous; –, no viscosity.
RT, Room temperature.

1995a, 1995b). Surprisingly, strain P_2d cells grown on various sugars, such as glucose, mannose, fructose, galactose and sucrose, failed to show any viscosity, unlike benzoate-grown cells (Table 1). Benzoate is known to be toxic at concentrations higher than 0·03% (Silliker *et al.* 1980). The EPS is apparently formed, therefore, as a protective measure against the toxic effects of benzoate.

Surfactants such as Triton-X-100, Tween 80 and sodium deoxycholate added during the growth of the culture did not inhibit the formation of viscosity (Silman *et al.* 1990), nor did they have any effect on the dispersal of viscous pellets. NaOH (2 N) was very effective in solubilizing the viscous material at the same time as lysing the cells. Heating alone at 45/60 °C was not effective in dispersal of the ice-incubated cell pellets. At 100 °C viscous material was, however, charred.

Emulsifying activity

The emulsifying activity of EPS is determined by its strength in retaining the emulsion of the hydrocarbon in water. Generally the emulsion breaks rapidly within an initial incubation of 30 min. The absorbance reading after 30 and 60 min gives a fairly good indication of the stability of the emulsion. The EPS from P_2d retained 61·9% and 57·14% of the emulsion after 30 and 60 min, respectively (Table 2). The stability of the emulsion by EPS from P_2d is comparable to those reported earlier (Rosenberg *et al.* 1979).

Table 2 Emulsifying activity of exopolysaccharide (EPS)

Incubation (min)	Control	Sample*	% Emulsifying activity
0	0·00	0·21	100·0
30	0·00	0·13	61·90
60	0·00	0·12	57·14

* Hexadecane, 0·5 ml, was added to 0·5 ml EPS (1 mg ml^{-1}) diluted to 2 ml with phosphate-buffered saline (PBS), vortexed for 1 min and the absorbance monitored at 540 nm. A control was run with 2 ml PBS without EPS.

Composition of exopolysaccharide

Heteropolysaccharides containing multiple sugars have been reported in different bacteria, such as the saprophytic bacterium *Ps. fluorescens* strain III 3 which was found to contain glucose, glucosamine, rhamnose, fucose, arabinose and acetate and the plant pathogen *Ps. andropogonis* which produces an acidic EPS containing glucose, glucuronic acid, mannose, rhamnose and galactose (Fishman *et al.* 1997). The sugar composition of the EPS, analysed using GC (Fig. 1), shows the presence of hexoses (rhamnose, 50·79%; fucose, 3·33% and glucose, 7·23%) and pentoses (ribose, 6·53%; arabinose, 0·76% and mannose, 19·21%). Two minor peaks in the chromatogram could not be identified.

Pseudomonas mendocina P_2d is a promising bacterial culture in the production of EPS with a distinctive property to gel at cold temperature.

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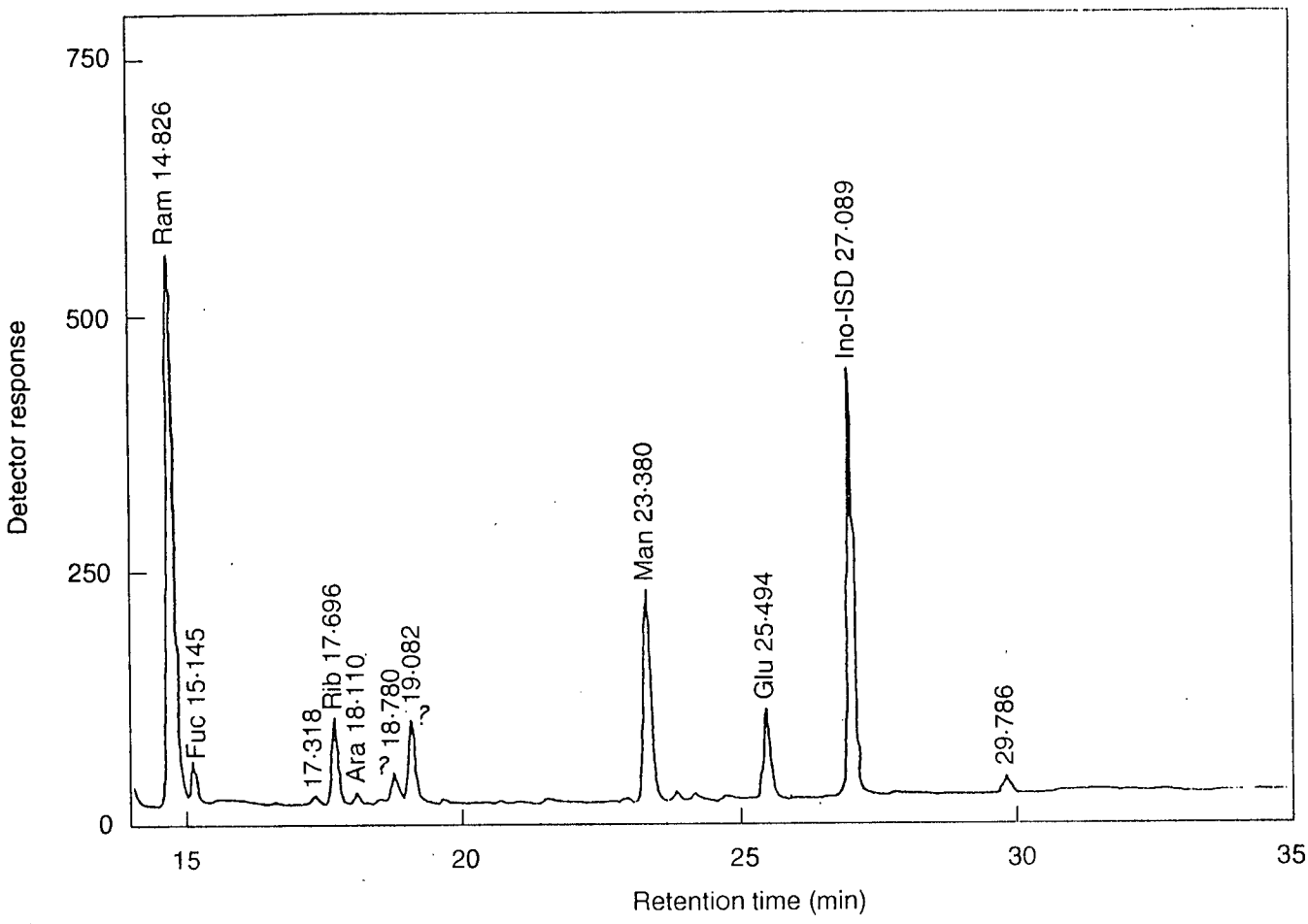

Fig. 1 Gas chromatogram of aldital acetate derivative of hydrolysed exopolysaccharide from *Pseudomonas mendocina* P_2d

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