ACTIVATION AND EXPRESSION OF SILENT GENES FROM *Pseudomonas cepacia* AC1100

Thesis submitted to the
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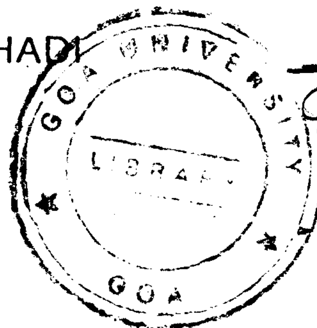
DOCTOR OF PHILOSOPHY
in
MARINE BIOTECHNOLOGY

by
S.C. GHADI



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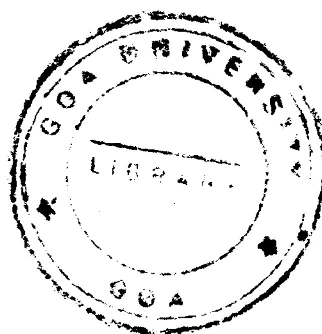
DEDICATED TO MY PARENTS
AND MY BROTHER ....

CERTIFICATE

This is to certify that the thesis entitled " ACTIVATION AND EXPRESSION OF SILENT GENES FROM *Pseudomonas cepacia* AC1100 " submitted by Shri. S. C. Ghadi for the award of the degree of Doctor of Philosophy in Marine Biotechnology is based on the results of investigations carried out by the candidate under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma of any University or Institute. The material obtained from other sources has been duly acknowledged in the thesis.



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STATEMENT

I hereby state that this thesis for the Ph.D degree on " Activation and expression of silent genes from Pseudomonas cepacia AC1100" is my original contribution and that the thesis and any part thereof has not been previously submitted for the award of any degree / diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive study of its kind from this area.The literature pertaining to the problem investigated has been duly cited. Facilities availed from other sources are duly acknowledged.*

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Sanjeev C. Ghadi

List of abbreviations

2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
Phe^+	phenol degrading ability
2,4-D	2,4-dichlorophenoxyacetic acid
CB	chlorobenzoate
MB	methylbenzoate
TCP	trichlorophenol
EB	ethylbenzoate
DMB	dimethylbenzoate
C23O	catechol 2,3-dioxygenase
C12O	catechol 1,2-dioxygenase
MP	methylphenol
CHQ	chlorohydroxyquinone
α-HMS	α-hydroxymuconic-semialdehyde
HMSH	Hydroxymuconic-semialdehyde hydrolase
HMSD	Hydroxymuconic-semialdehyde dehydrogenase
PH	Phenol hydroxylase
OD	optical density
kan^r	Kanamycin resistance
tet^r	Tetracycline resistance
CoA	Coenzyme A
TLC	Thin layer chromatography
kb	kilobase
UV	ultraviolet
bp	basepair

rpm	revolutions per minute
μ	Specific growth rate
D	dilution rate
BSM	Basal salt medium
LSM	Low salt medium

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Microorganisms play a key role in the biogeochemical cycles, contributing to recycling of carbon, nitrogen and sulphur in the environment. Though photooxidation and other abiotic mechanisms also play a minor role in the transformation of chemicals, few of them, in reality can be converted to inorganic products (Alexander, 1981). Microorganisms have evolved requisite enzyme systems for degradation of biogenic compounds (compounds naturally occurring) which have been present for millions of years in the biosphere by the process of diagenesis. Many of these chemicals bear little structural relationships to the biological products from which they had been derived. Soil and young sediments have been found to contain a staggering array of chemicals as complex as substituted polycyclic aromatic hydrocarbons (Blumer and Youngblood, 1975). These chemicals might have been derived from natural sources such as vegetation, forest fires or volcanic eruptions. Many of the chemicals exploited in the recent times by various industries were closely related to biogenic compounds. Hence it was not surprising that these chemicals when released into the environment could be degraded by microorganisms or eventually became biodegradable after adaptation.

The advent of modernization and industrialization has resulted in use of new synthetic and highly substituted organic compounds. Their sudden release into the environment on the present evolutionary time scale made them "strangers" to the present biosphere (xenobiotic). Due to their instant appearance in the environment these chemicals posed a major problem. They could not easily be degraded by microbial systems due to their foreignness and their resultant accumulation led to deleterious effects on living systems.

Current technologies such as excavation and incineration for cleaning up of hazardous wastes are often expensive, inappropriate for the site or ineffective in handling complex mixture of pollutants. Also, these disposal methods lead to secondary pollutions. Hence biological treatments using biosystems such as bacteria or fungi are preferred as they are less expensive, more natural and non-polluting. Technologies have been devised to provide cost-effective alternatives for cleaning up complex mixtures of pollutants found at important contaminated sites.

Biological treatment of waste material can be more promising by understanding the physiological and genetic basis of biodegradation. Improved strains with novel biodegradative ability can be constructed by transferring appropriate genes.

The most attractive option in favour of biodegradation is definitely the advantage of it being used _in situ_, unlike other waste treatment processes. Biodegradation process characterization in reactors and fields can help environmentalists to tailor the technology to clean up the pollutants at specific sites and in specific media (e.g., contaminated aquifers, waste lagoons, contaminated soils). Similarly, the rate of degradative activity by microorganisms can be increased by adding nutrients or other amendments like introduction of some other microorganism to supplement the existing microbial community.

It is not surprising that a vast interest in biodegradation has been shown by scientists from various fields. The most promising application of the biosystem programme was the clean up of portions of the shoreline

of Prince William Sand, Alaska after the March 1989 Exxon Valdez tanker accident. Further reports of microbial degradation of creosote in Conroe, Texas and bioremediation of three million gallons of acrylic acid and butyl acrylate from a railroad spill show promising opportunities offerred by biological systems in waste treatment .

Review of Literature

I. Biodegradation of Aromatic Compounds

Many microorganisms capable of degrading aromatic and heterocyclic compounds have been isolated and identified. Eubacteria, yeast, higher fungi, photosynthetic bacteria and even algae have been found to be capable of degrading aromatic compounds (Keishlich, 1976; Semple and Cain, 1995).

Although majority of laboratory-based studies on biodegradation have focussed on metabolism of single compounds by pure cultures, in reality, a complex array

of compounds are present at the polluted site, thus requiring the presence of a microbial consortium. Continuous culture techniques have helped in analysis of mixed cultures/mixed substrate systems with respect to biodegradation (Yang and Humphrey, 1975; Donaldson _et al_, 1984; Donaldson _et al_, 1987; Satsangee and Ghosh, 1990).

a) Aerobic attack on the aromatic rings :

Aromatic compounds can be either totally or partially degraded by microorganisms depending on the number of rings and the type of substituent groups present. Microbial attack on organic compounds involves various steps which has been extensively reviewed by Dagley (1987). Biodegradation by a microorganism is initiated with the entry of organic compound into the cell and evidence points to the existence of transport mechanisms (Higgins and Mandelstom, 1972; Cook and Fewson, 1972; Thayer and Wheelis, 1976).

Entry of aromatic compounds into the cell is followed by transformation of side chains and modification of substituents before ring cleavage. Most of the microorganisms convert benzenoid compounds into catechol, protocatechuate or gentisic acid which are the central metabolites in biodegradation. Similar pathways have been reported for degradation of toluene by P. putida and P. aeruginosa. Larger alkyl side chains may either remain intact during formation of catechol or the terminal methyl group may undergo oxidation to form carboxylic acids. A number of alkyl benzenes or alkyl benzene sulfonates are oxidized in this manner.

Numerous pesticides and some industrial pollutants such as phthalic acid esters contain ester amide or nitrile moieties which may undergo hydrolysis to corresponding acid and an alcohol or amine . The incorporation of a molecule of water into a substrate (hydrolysis) is catalyzed by a large group of commonly occuring enzymes including esterases, amidases, nitrilases, phosphatases and chitinases.

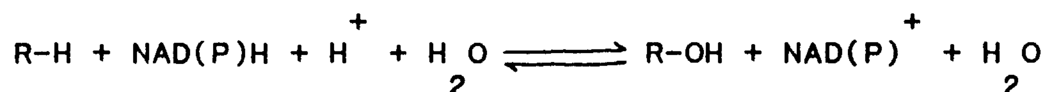
The presence of nitro, amino, halogen and sulfonic acid substituent is known to render benzenoid

compounds more resistant to microbial degradation (xenobiotic). The nitro groups are frequently reduced to amines via nitroso- and hydroxylamino- intermediates. These are occasionally found as by-products rather than obligatory intermediates. The hydroxyl group replaces the nitro substituents with concomitant formation of nitrite (Chapman, 1972; Spanggord et al., 1991; Valli et al., 1972). Groenewegen et al, (1992) have reported degradation of 4-nitro benzoate via 4-nitrosobenzoate and 4-hydroxylamino benzoate in Comamonas acidoorans NBA-10 The nitro group can also remain unchanged resulting in 4-nitrocatechol from 4- nitro phenol as seen in Pseudomonas sp. ATCC 29358 (Sudhakar et al, 1978).

Halogenated organics are widely used as herbicides, plastics, solvent and degreasers. Various micro-organisms have been isolated with the ability to biodegrade halogenated compounds, specifically the chlorinated hydrocarbons by aerobic and anaerobic mechanisms as reviewed by Chaudhary and Chapalamadugu

b) Oxygenases initiate biodegradation :

Monooxygenases and dioxygenases containing a variety of cofactors such as hemes, flavins, pterins, copper, manganese and non heme iron are mainly responsible for initial oxidative attack on biochemically inert organic compounds in bacteria. Monooxygenases are involved in hydroxylation of organic molecules using reducing power of NADH or NADPH, as seen by the equation below:



$$R\text{-}H + NAD(P)H + H^{+} + H_2O \rightleftharpoons R\text{-}OH + NAD(P)^{+} + H_2O$$

All monooxygenases are specific for their aromatic substrates.

Dioxygenases are responsible for the fixation of oxygen directly into organic compounds. Dioxygenases bring about cleavage of benzene rings by inserting both atoms of molecular oxygen (Dagley, 1975 a,b), provided that two hydroxyl groups are placed *ortho* or *para* to each other. Thus all aromatic compounds are transformed into *ortho* or *para* dihydroxybenzenes before the action of dioxygenases.

Converging metabolic pathways are used to transform the substrates into either di or trihydric phenolic key intermediates most of which are catechol, protecatechuic acid, homogentisic acid, homoproto catechuic acid, gentisic acid or gallic acid (Dagley, 1978 a,b,c).

Catechol, protecatechuate and gentisic acid can be formed from a variety of aromatic compounds as depicted in Figure 1.1, 1.2 and 1.3. Trihydric phenol may result from the bacterial oxidation of meta dihydric phenols such as orcinol, resorcinol or thymol (Chapman, 1972).

c) Aromatic ring cleavage pathways

Ring cleavage is achieved by reactions in which both atoms of molecular oxygen are introduced into the substrate molecule by dioxygenases. The mode of ring fission strongly depends on the structure of the 1,2 dihydric phenol, on the bacterial species and on the substrate used for growth. Hence, a catechol 1,2-dioxygenase is induced in *Moraxella woffi* grown with

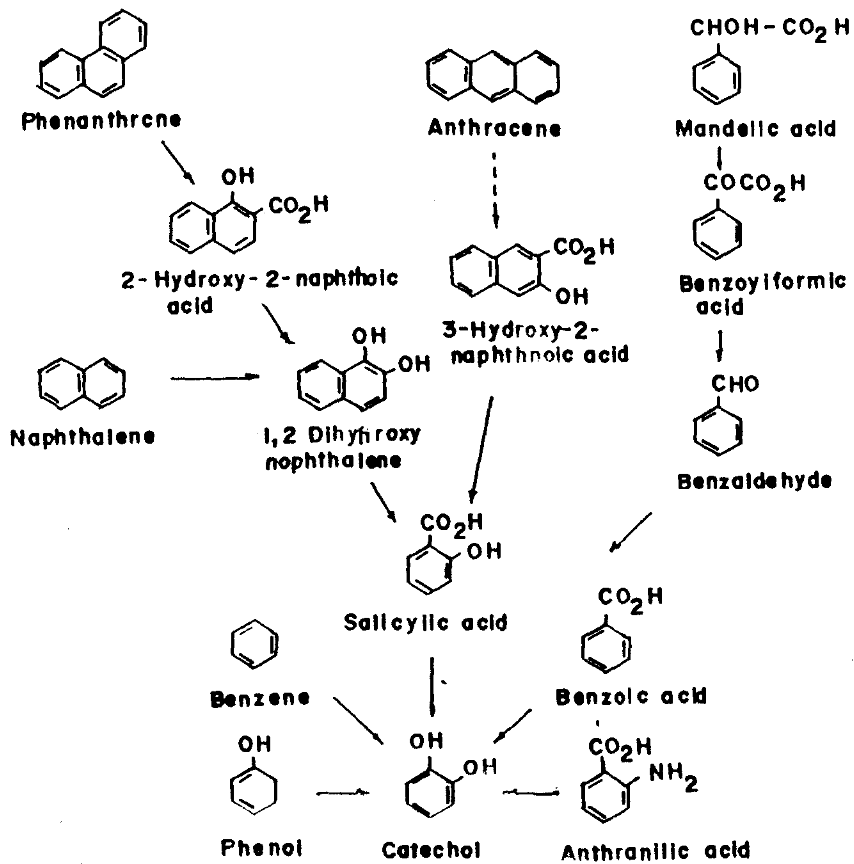


Fig.1.1 Catechol as central metabolite in the bacterial degradation of benzenoid compounds (adapted from Chapman, 1972).

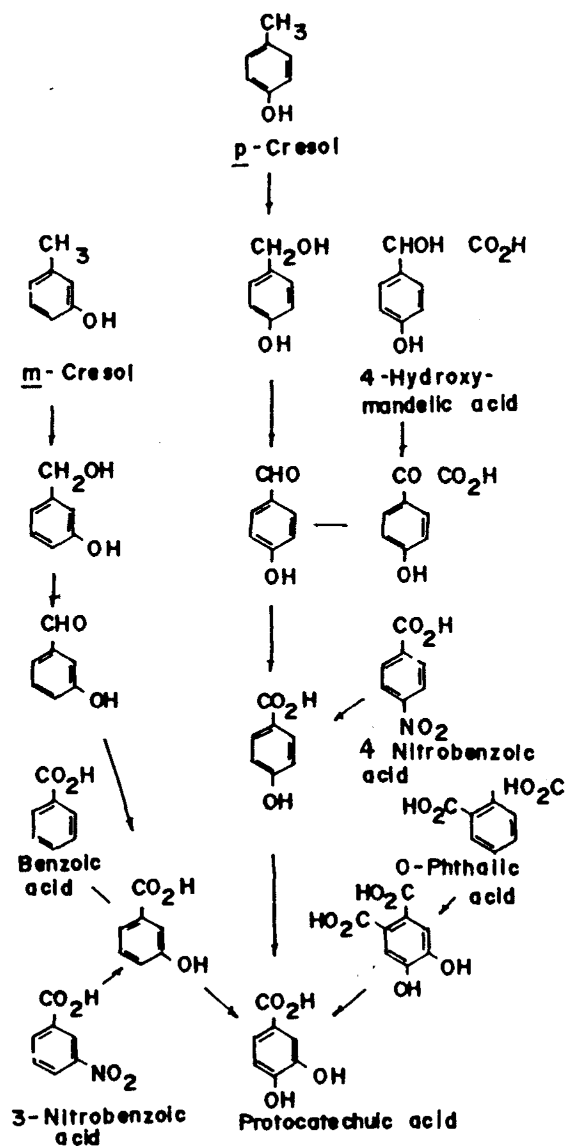


Fig.1.2 Protocatechuate as a central metabolite in the bacterial degradation of benzenoid compounds (adapted from Chapman ,1972).

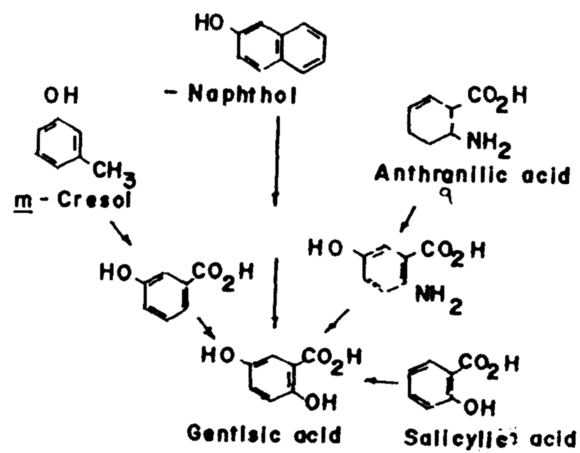


Fig.1.3 Gentisic acid as a central metabolite in the bacterial degradation of benzenoid compounds (adapted from Chapman, 1972).

benzoate whereas catechol 2,3-dioxygenase is induced when the same organism is grown in presence of naphthalene (Chapman, 1972). Similarly the kind of substituents on the benzene nucleus influences the method of ring fission. When Nocardia sp. DSM 43251 was grown with dimethyl/methoxy/methylthio-phenols, catechol 2,3-dioxygenase was induced. However, when the same organism was grown with unsubstituted or substituted phenols, enzymes of 1,2 cleavage pathway of catechol were induced (Engelhardt et al., 1979).

In case of ortho-dihydric phenols such as catechol, protocatechuic acid and homoprotocatechuate, intradiol ortho cleavage (reaction a and c in Fig. 1.4) or extradiol meta cleavage (reactions b,d,e,f in Fig. 1.4) may occur. Cleavage of catechol by catechol 1,2 dioxygenase and of protocatechuic acid by protocatechuate 3,4 dioxygenase leads to cis,cis-muconate or its 3-carboxy derivative. Alternatively the product of extradiol mode of ring fission by catechol 2,3-dioxygenase or protocatechuate 4,5- dioxygenase leads to formation of 2-hydroxymuconic semialdehyde and 2-hydroxy-4-carboxymuconic-semialdehyde. The para-

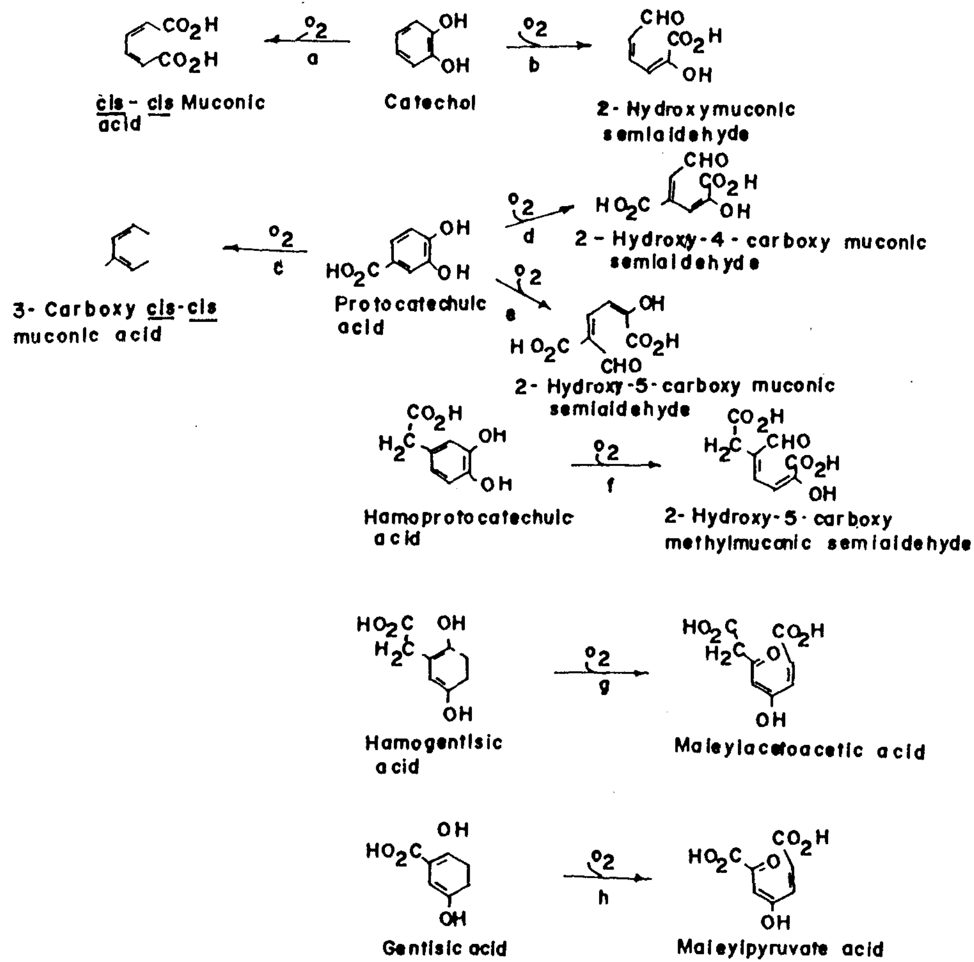


Fig.1.4 Enzymatic reactions involved in cleavage of Dihydroxybenzenes (adapted from Fewson, 1981).

dihydric phenols gentisic and homogentisic acid (reactions h and g in Fig. 1.4) are split by gentisate 1,2-dioxygenase and homogentisate 1,2-dioxygenase forming maleyl-pyruvic and maleyl-acetoacetic acid .

After ring fission, the resulting open chemical structures enter the main channels of metabolism such as tricarboxylic acid cycle. The reactions include hydrations, hydrolyses and aldolase fissions. The products of intradiol cleavage of catechol and protocatechuic acid are converted to succinic acid and acetyl-coA by 3-oxoadipate pathway .

The final products of extradiol fission of dihydric phenols are pyruvic acid and fumaric acid or closely related compounds such as acetaldehyde or aceto acetic acid (Fig. 1.5). Protocatechuate, however, when metabolized via meta fission pathway gives rise to pyruvate and oxaloacetate (Fig. 1.6). The final product formed from homogentisic acid is fumaric acid and acetoacetic acid, whereas gentisic acid forms fumaric acid and pyruvate in the meta-cleavage pathway.

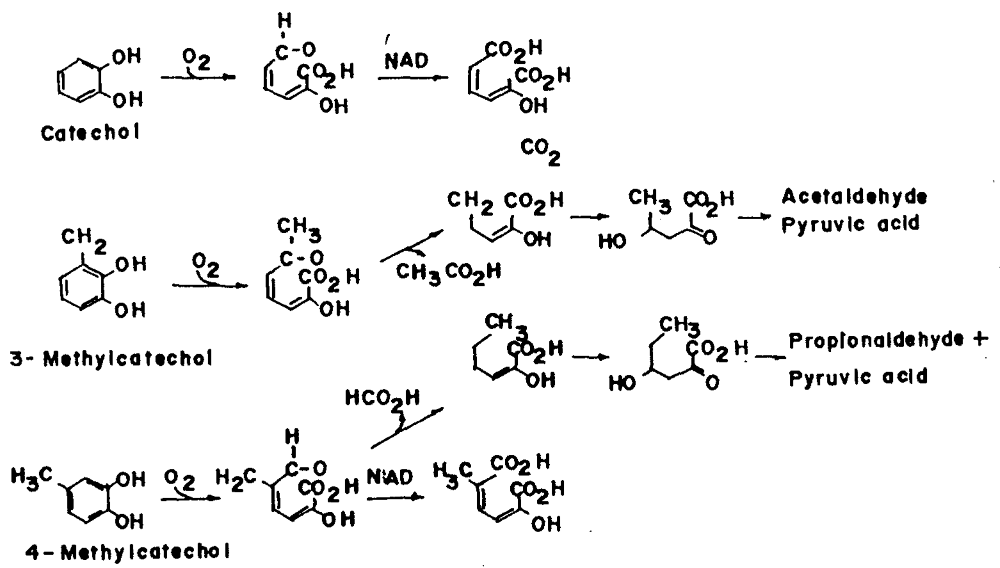


Fig.1.5 The meta-fission pathways of catechol and its homologs in bacteria (adapted from Chapman, 1972).

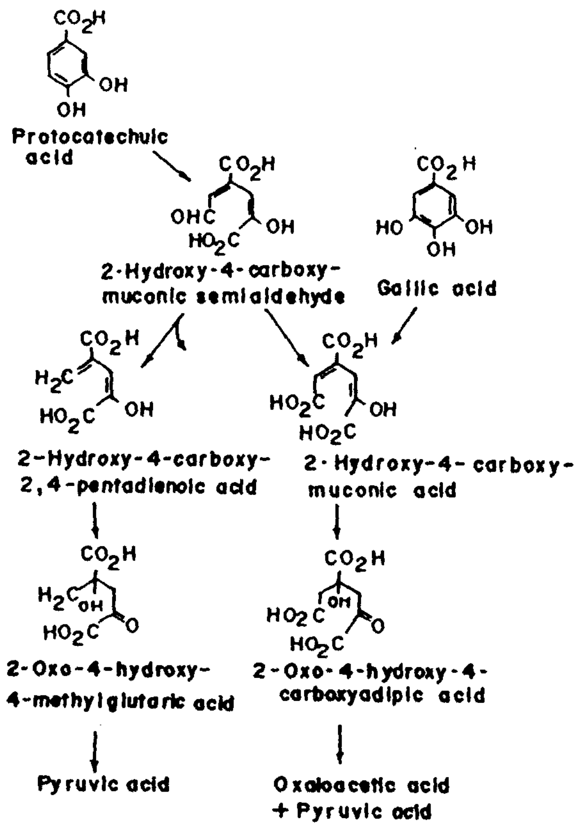


Fig.1.6 The _meta_-fission pathways of protocatechuate and gallic acid (adapted from Chapman, 1972).

d) Co-oxidation :

Co-oxidation as defined by Foster (1962) is the oxidation of non-growth substrate in the obligate presence of a growth substrate or another transformable compound . Employing this principle with *Pseudomonas methanica* growing at the expense of methane, a series of homologous oxidation products was obtained from co substrate gases : from ethane, ethanol, acetaldehyde and acetic acid were produced, from propane, n-propanol, propionic acid and acetone, from n-butane, n-butanol, n-butyric acid and n-butanone (Foster, 1962). The potential role of co-oxidative process in biodegradation of recalcitrant molecules has received considerable attention. Recalcitrant molecules are resistant to biodegradation, but do disappear from the environment at a slower rate. This persistence is due to inability of a micro-organism to assimilate these compounds. However, failure of a single microbial species to utilize recalcitrant molecule is overcome by a community of microbes of differing specificities, existing in nature. The initial attack via co-oxidation on a recalcitrant molecule in an environmental niche is a coincidental attack on the recalcitrant compound, that

is probably of little consequence to the microorganism. Neither energy nor carbon for biosynthesis results from this oxidation and it is probable that the reaction occurs at some expense of energy from microbes.

Recalcitrant molecules which yield products of co-oxidation that are structurally similar to naturally occuring metabolites disappear more quickly from the environment than those that do not. Thus compounds like dalapone and lindane which are rarely utilized by a single microbial species are much less persistent in environment. This is probably because dehalogenation of dalapone yields a molecule structurally similar to inositol.

Co-oxidation is a vital process in pesticide degradation and is well reviewed by Horvath (1972). Co-oxidation is also involved in the biodegradation of some hydrocarbons as suggested from studies presented by Beam and Perry (1973). Also initial oxidative process of pristine cycloparaffin molecule occurs _via_ co-oxidation (with hydrocarbon as co-substrate) and that oxygenated product is utilized by another microbial species (Beam and Perry, 1974; de Klerk and Linden, 1974).

II. Molecular Mechanisms of Genetic Adaptation and Accelerated evolution to Degrade Xenobiotic Compounds

Microorganisms play a major role in the breakdown and mineralisation of organic pollutants (Alexander, 1981). Though biodegradation is an attractive option over other technologies which rely on physical or chemical processes for the destruction of contaminants, the kinetics of biodegradation process is much slower than desired from public health or environmental considerations. Numerous field tests and laboratory experiments using microorganisms have shown varying degrees of success depending on the chemical contaminants and environmental considerations. The slow biodegradation of pollutants in natural environments may be due to unfavourable physiochemical condition such as temperature, pH, redox potential, salinity, oxygen concentration or may be affected by the availability of other nutrients, accessibility of substrate (solubility, dissociation from adsorbed material) or predation (Goldstein, 1985; Swindoll, 1988). Also, the low biodegradability may be due to incapacity of microorganisms present in the natural environment to

effectively metabolize pollutants with uncommon chemical structure or properties (xenobiotics).

Microbial communities exposed to xenobiotics often could adapt to these chemicals. An increasing knowledge of molecular events which lead to adaptation of microbial communities for degradation of xenobiotics may provide a better insight into the metabolic capacities of microorganisms and may reveal the underlying principles of metabolic diversification in bacteria.

Adaptation can occur both in mixed microbial communities and in individual microbial lineages. When microbial communities are exposed for the first time to a new compound, virtually no biodegradation occurs initially. However, after a period ranging from hours to months, mineralization starts.

Different molecular and biochemical processes may explain such an adaptive response:

1. Induction of specific enzymes in members of the community resulting in an increase in observed degradative capacity of the microbial community (Shimp and Pfaender, 1987; Spain and Vanveld, 1983).

2. Growth of a specific subet al.population of a microbial community to take up and metabolize the substitute (Aelion, 1987; Barkay, 1987; Barkay and Pritchard, 1988).

3. Selection of mutants which acquire altered enzymes, specifying novel metabolic activities and not present at the onset of exposure of the community to the introduced compounds (Spain and Vanveld, 1983; Barkay and Pritchard, 1988). This process requires a larger adaptation time than the other two processes and may possibly be responsible for mineralisation of xenobiotic compounds (Aelian et al., 1987; Sander et al., 1981, Spain and Nishino, 1987; Meer et al., 1987).

Though catabolic gene clusters are diversely organised, enzymes of aromatic pathway show a lot of similarities as reviewed by Meer et al., (1992). This indicates the possibility of combining several different

gene clusters (*e.g*. modified *ortho* cleavage pathway genes, *meta* cleavage pathway genes, dioxygenases and dihydrodiol dehydrogenase genes) in the form of modules to which other peripheral genes may be added by processes like gene transfer, mutational drift and genetic recombination and transposition. Although these mechanisms are difficult to prove experimentally, the existence of homologous genes between different organisms might clarify the evolutionary mechanism of catabolic pathways.

a)*Gene transfers:*

The homology observed in different aromatic pathway genes indicates the occurrence of extensive horizontal gene transfer during the evolutionary process. Genetic interaction among microbes occurs frequently by conjugation in plasmid replicons, transductions (Saye *et al.*, 1990; You *et al.*, 1991) and transformations (Lorenz *et al.*, 1988, Lorenz *et al.*, 1991). Self transmissible plasmids carrying genes for the degradation of aromatic or of other organic compouds are known (Chakrabarty, *et al.*, 1978; Chaudhary and

Chapalamadugu, 1991; Frantz and Chakrabarty, 1986; Haryama and Don, 1985; Sayler et al., 1989). TOL, NAH and SAL plasmids revealed strong DNA homologies amongst each other especially in regions both inside and outside the catabolic gene clusters (Assinder and William, 1990; Duggleby et al., 1977; Frantz and Chakrabarty, 1986; Lehrbach et al., 1983). Similarly catabolic plasmids pJP4, pAC25, pSS50 and pBRC60 carrying genes for chloroaromatic degradation have a strong homologous plasmid backbone that determines replication and transfer function (Burlage et al., 1990). The chlorobenzene plasmid pP51 was also homologuous to pJP4 outside the regions of catabolic genes (Meer, unpublished results). These observations suggest that a few common self transmissible ancestor replicons may have been involved in the acquisition and spread of different catabolic modules.

b)Point mutation

Single site mutations can result in altered substrate specificities of enzymes or effector specificities. The substrate range of catechol 2,3-dioxygenase encoded by TOL plasmid pWWO has been

extended to 4- ethylcatechol by single substitutions of amino acid (Ramos _et al._, 1987). Similarly the substrate specificity of Xylene Monoxygenase encoded by _xyl_ M,A,was extended to _p_-ethyltoluene which is not a natural substrate (Abril _et al._ 1986). The specificity of XylS regulatory protein was modified by mutagenesis resulting in recognition of 4-ethylbenzoate, salicylate, 3,5-, 2,5- and 2,6- dichlorobenzoate as effector molecules (Ramos _et al._, 1986). Also, XylS mutants capable of activating the _meta_ cleavage pathway genes in the absence of its effector were also isolated (Zhou _et al._, 1990).

Single site mutations are believed to arise continuously and at random as a result of errors in DNA replication or repair.

d) _DNA rearrangements by Recombination and Transposition_:

The order of genes encoding the cleavage pathways of a _Acinetobacter calcoaceticus_ and _P. putida_ differ from one another (Ornston _et al._, 1990) and from other

organisms. (Perkins *et al.*, 1990; Frantz and Chakrabarty, 1986). Gene rearrangements have been observed between different operons for TOL modified *ortho* pathways. Further, gene orders of part of *meta* cleavage operons in TOL and NAH plasmid are identical but differ in downstream genes (Haryama and Rekik, 1990; Haryama *et al.*, 1987).

e)*Gene duplication*:

Gene duplications have been considered an important mechanism for evolution of microorganisms. The extra copy of gene is free of selective constraints and can thus diverge faster by accumulating mutations leading to full inactivation and making this gene copy silent. Reactivation of silent genes could occur through action of insertion element. Studies with TOL-type plasmids have shown upper and lower pathway operons as well as *xyl*S and *xyl*R regulatory genes to switch positions, get inverted or increase their copy numbers (Osborne *et al.*, 1988; Chatfield and Williams, 1986). Catechol 2,3-dioxygenase and catechol 1,2- dioxygenase have also been observed to be duplicated (Kell *et al.*, 1985; Nakai *et*

al., 1990). Plasmid pJP4 encoding 2,4-dichloro phenoxyacetic acid appears to have undergone several gene duplications (Perkins et al., 1990; Don et al. 1985).

e)Transposition:

Insertion elements have been shown to play an important role in rearrangement of DNA fragments, in gene transfer and in activation or inactivation of silent genes. The TOL catabolic operons are part of a large transposable element Tn-4651 which is part of an even larger mobile element Tn-4653 (Tsuda and Lino, 1988). Even plasmid NAH7, contains catabolic genes residing on Tn-3 type element Tn-4655 (Tsuda and Lino, 1990). P. cepacia 249 is known to possess 9 different insertion elements present in 1 to 13 copies in its DNA genome and are resonsible for catabolic potential of this strain (Lessie et al., 1990). The chlorobenzene dioxygenase genes of Pseudomonas sp. strain P51 were shown to be flanked by 2 copies of iso-insertion elements IS-1066 and IS-1067 (Meer et al., 1991).

III Acceleration of evolution

The evolutionary process, leading to novel catabolic pathways, can be extremely slow, especially when the acquisition of multiple catalytic activity is necessary. Hence evolution of new metabolic activities in the laboratory may be helpful because the frequency of type of genetic events needed, can be carefully controlled and selective conditions can be optimized.

Accelerated evolution of metabolic pathways can be achieved in laboratory using three experimental approaches:

1. Long term chemostat selection with progressive replacement of a mineralizable substrate by a recalcitrant analog (Dorn *et al.*, 1974).

2. *In vivo* gene transfers, in which genes of critical enzymes from one organism is recruited into a pathway of another organism through experiments involving natural genetic transfer processes such as transduction, transformation or conjugation (Reineke and Knackmuss, 1979). This approach is possible as

most of catabolic pathways are located on transmissible plasmids or transposons (Haryama and Don, 1985).

3. In vitro evolution in which cloned and well characterized genes are selectively transferred into an organism in order to evolve a new pathway (Lehrbach et al., 1984; Ramos et al., 1987).

a) Horizontal and vertical expansion of catabolic pathways

The 3-chloro benzoate catabolic pathway of Pseudomonas sp. B13 involves transformation of 3-chlorobenzoate to 3-chlorocatechol which is ortho cleaved. The first enzyme of the B13 pathway, benzoate 1,2-dioxygenase has a narrow substrate specificity and hence cannot transform 4-chlorobenzoate or chlorosalicylates (Reineke and Knackmuss, 1979). Recruitment of the genes coding for the relaxed substrate specificity enzymes toluate 1,2-dioxygenase (TO) and dihydroxycyclohexadiene carboxylate dehydrogenase from TOL plasmid along with regulatory

regions allows B13 to degrade 4-chloro-benzoate (Fig. 1.7A). Recruitment of the gene of relaxed substrate specificity, enzyme salicylate hydroxylase (SH) of the NAH7 plasmid which specified pathway for the degradation of salicylate permits strain B13 now to degrade and grow on 3-, 4-, and 5-chloro salicylates (Fig. 1.7 B).

b) Expansion of the substrate range of a pathway by mutation:

The inability of strain B13 to utilize 3,5-dichlorobenzoate was due to inability to activate XylS regulator. (Ramos *et al.*, 1986). Isolation of XylS352 mutant with the ability to get activated by 3,5 DCB could be obtained which when introduced into B13, allowed the bacterium to grow at the expense of 3,5 DCB (Ramos *et al.*, 1986).

c) Sequential restructuring of catabolic pathways:

The pWWO plasmid encoded *meta*-cleavage pathway can catabolize benzoate, 3- and 4 MB, 3,4 DMB , 3EB but not

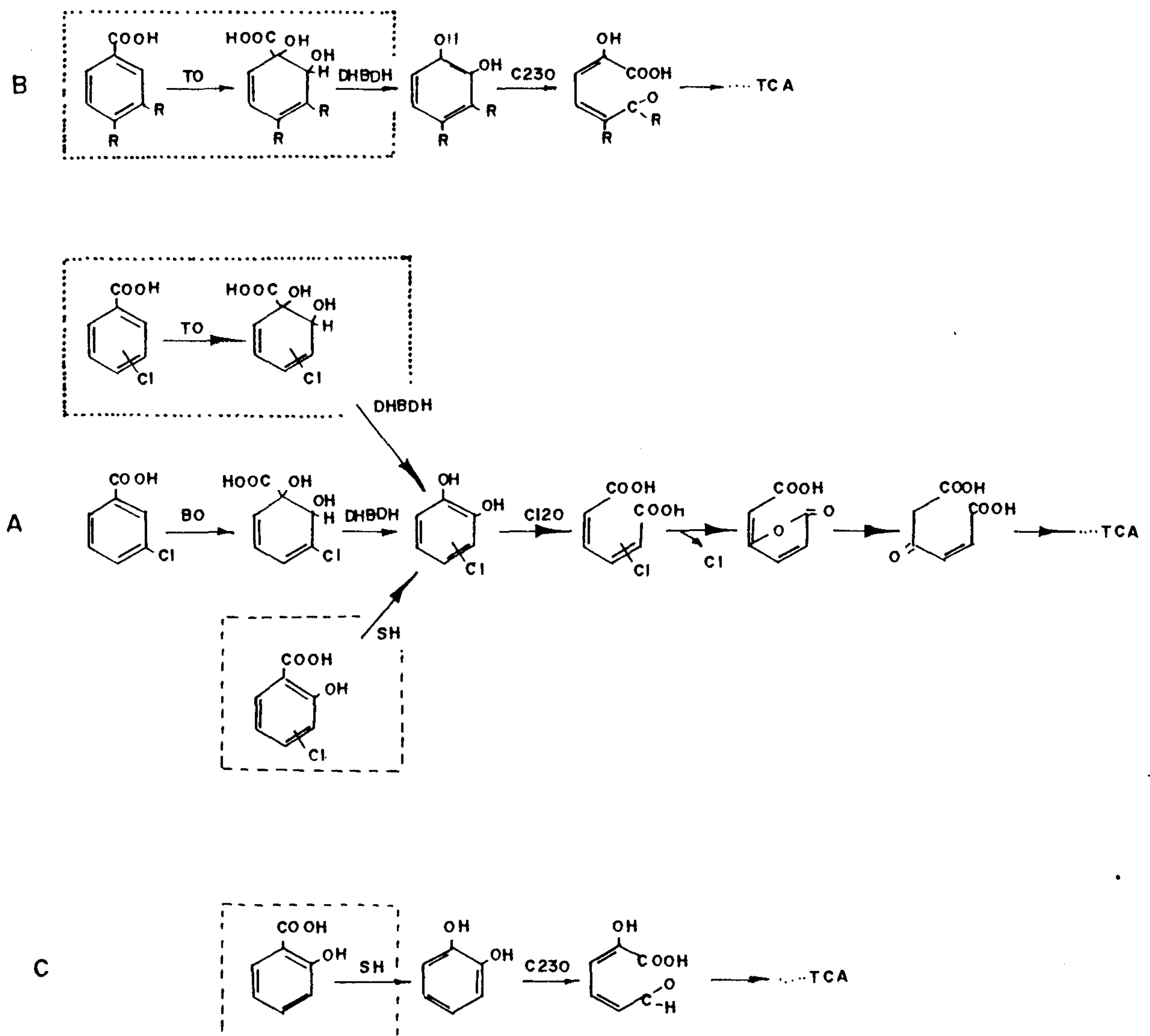


Fig.1.7 Horizontal and Vertical expansion of a catabolic pathway. Pseudomonas B13 (A) transforms 3-chlorobenzoate to 3-chlorocatechol, which is ortho cleaved, converted to 3- oxoadipate and subsequently channelized into the TCA cycle. Recruitment of gene coding for relaxed substrate specificity enzyme toluate 1,2-dioxygenases (TO) and dihydroxy-cyclohexadiene carboxylate dehydrogenases (DHBDH) from the TOL-plasmid (B) allows B13 to degrade 4-chlorobenzoate. Recruitment of gene of relaxed substrate specificity enzyme salicylate hydroxylase (SH) of the NAH7 plasmid permits B13 to degrade and grow on 3-,3-, and 5-chlorosalicylates (adapted from Lehrbach et al 1984).

4EB. 4EB was unable to activate XylS protein regulator. Isolation of a mutant XylS resulted in activation by 4EB and induction of all catabolic enzymes. However 4EB was metabolised only as far as 4-ethyl catechol as it is a suicidal substrate for enzyme C23O. The isolation of mutant C23O enzyme resitant to inactivation by 4 ethyl-catechol eliminates final metabolic block and permits complete degradation of 4EB through _meta_ pathway (Ramos _et al._, 1987).

d) _Patchwork assembly of enzymes_:

A mixture of chloro and methyl substituted phenol and benzoate are difficult to be degraded by a single micro-organism though it might possess both _ortho_ and _meta_ cleavage pathway. Catechols and chloro catechols are subjected to _ortho_ fission whereas methyl catechol undergoes _meta_ fission. Thus, only one fission pathway is usually functional at a given moment. The non productive routing of catechol cleavage products, during simultaneous metabolism of chloro and methyl substituted aromatics leads to dead end products resulting in death of the microorganism.

Rojo *et al.* (1987) have constructed a hybrid pathway in strain B13 for simultaneous degradation of chloro and methyl aromatics. Transfer of DNA fragments having toluate 1,2-dioxygenase and dihydroxy cyclohexadiene carboxylate dehydrogenase along with xylS regulator, expression of mutated gene of phenol hydroxylase in B13 followed by recruitment of a 4 methyl 2-enelactone isomerase from *Alclaigenes eutrophus* in B13 allows the strain to utilize 3CB, 4CB, 4MB, 4CP and 4MP without any dead end metabolite (Fig. 1.8).

IV Biodegradation of 2,4,5- T by **P. cepacia** AC1100

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is an extensively used herbicide for control of weeds. 2,4,5-T was also used as a component in agent orange in Vietnam for defoliation, control of poison ivy and poison oak. The persistence of 2,4,5-T in soil due to its slow degradation, by co-oxidative metabolism, created toxological problem. Few years back, there was no known microorganism capable of utilizing 2,4,5-T as sole source of carbon and energy.

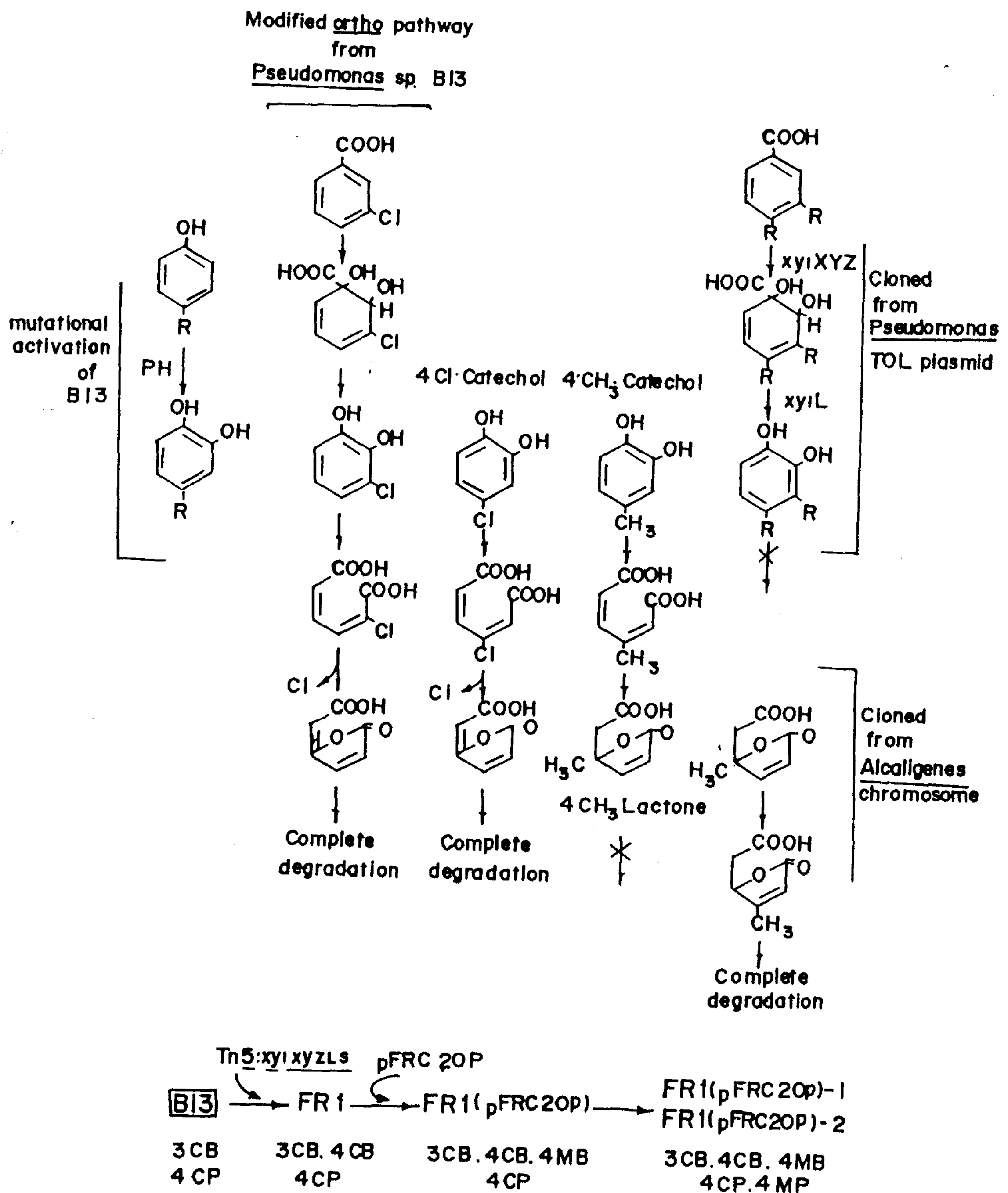


Fig1.8 Constructed hybrid pathway for the simultaneous degradation of chloro- and methylaromatics. Recruitment of TOL-plasmid gene encoding toluate 1,2-dioxygenases (xylXYZ) and dihydroxycyclohexadiene carboxylate dehydrogenases (xylL); mutational modification of expression of phenol hydroxylase gene of B13 and 4-methyl-2-enelactone isomerase from Alcaligenes eutrophus allows B13 to degrade 3CB, 4CB,4MB,4CP, and 4MP (adapted from Rojo et al., 1987).

a) Plasmid Assisted Molecular Breeding - Strategy for deliberate facilitated evolution:

Kellogg et al. (1981) developed a procedure for enriching 2,4,5-T utilizing microorganisms in a chemostat. The process involved mixing of samples from various waste-dumping sites along with laboratory bacterial strains possessing variety of catabolic plasmids in a chemostat. Continuous flow of media for 8 to 10 months with gradient increase in 2,4,5-T concentration, resulted in develoment of strain Pseudomonas cepacia AC1100 with a unique ability to utilize 2,4,5-T as sole source of carbon and energy (Kilbane et al., 1982). P. Cepacia AC1100 degraded 97% of 2,4,5-T with 100% release of chloride from substrate.

b) Oxidation of 2,4,5-T and Other chloro phenols by strain AC1100:

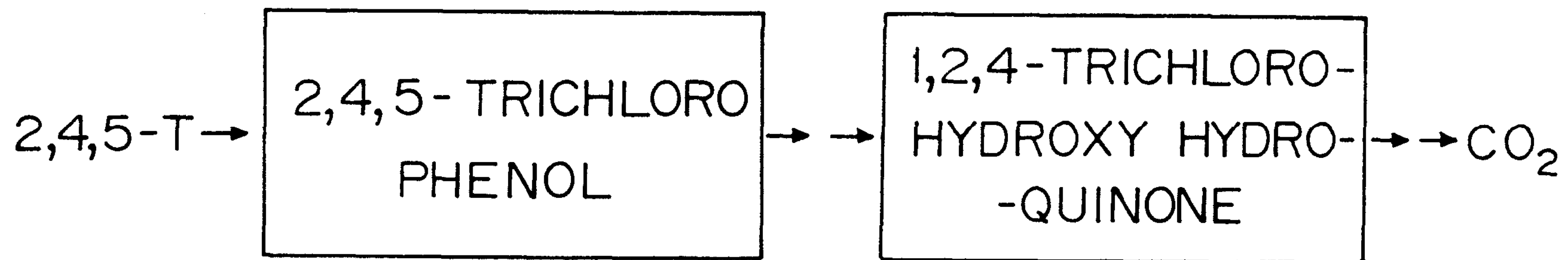
Many of the chlorinated compounds like 2,4,5; 2,3,5 trichlorophenol; 2,4,dichlorophenol; 3,4 dichlorophenol, tetrachlorophenol and pentachlorophenol were readily oxidatively dechlorinated by resting cells of strain

AC1100 (Karns _et al._, 1983).

c) _Biochemistry of 2,4,5-T degradation by strain AC1100_:

The 2,4,5-T degradative pathway (Karns _et al._, 1983; Chapman _et al._, 1987) has not been elucidated completely except for the identification of two intermediates, 2,4,5-trichlorophenol and chlorohydroxyquinone (ChQ) (Fig. 1.9). The first hydroxylation occurs in the _para_ position of the phenol regardless of whether this position is replaced by a chlorine substituent (Sangodkar _et al._, 1988). The _para_ hydroxylase enzyme is a flavin containing enzyme (Tomasi _et al._, 1995). The 2,4,5-trichlorophenol formed is further hydroxylated to form 2,5-dichlorohydroxyquinone (DCHQ) and 5-chloro-2-hydroxy-1,4-benzoquinone (Sangodkar _et al._, 1988). Recent studies with strain AC1100 indicate the possibility of quinone being the immediate intermediate for second hydroxylation (Tomasi _et al._, 1995) during the metabolism of polychlorinated phenols in strain AC1100.

BIODEGRADATION BY AC 1100



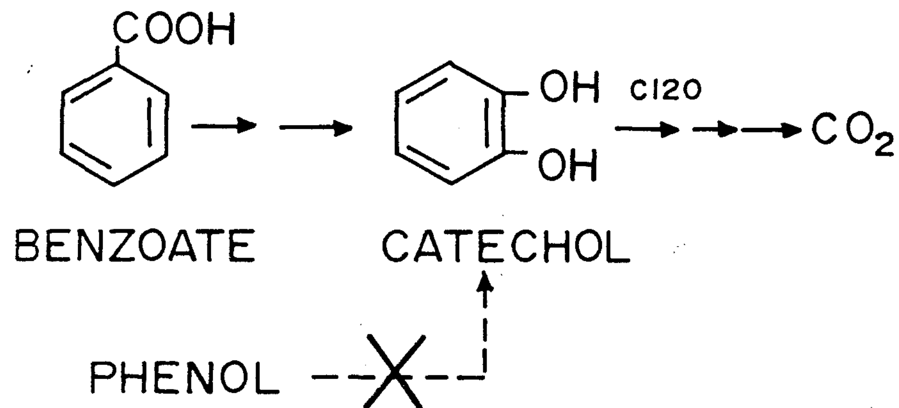


Fig.1.9 Metabolism of 2,4,5-T in P.cepacia AC1100.

d) Cloning of genes involved in 2,4,5-T degradation from strain AC1100:

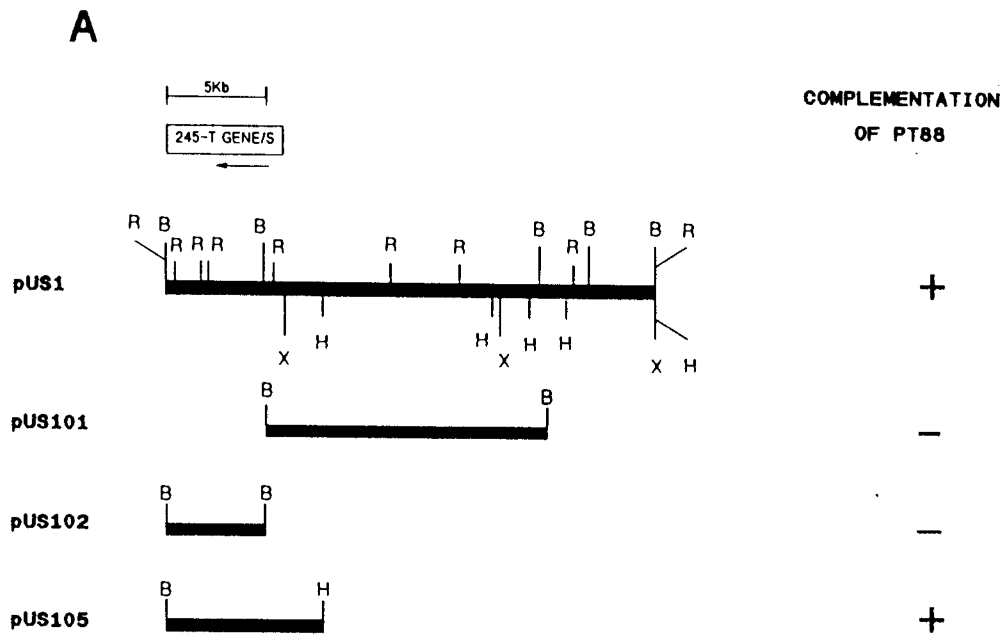
Transposon insertion mutagenesis with Tn5 was used to generate AC1100 mutants blocked in 2,4,5-T degradation. Strain PT88, a Tn5 mutant, when grown on glucose in presence of 2,4,5-T accumulated a bright red compound in the medium, indicating blockage in 2,4,5-T pathway. Analysis of the intermediates, indicated the presence of TCP, DCHQ and CHQ. Mutant PT88 evidently had a lesion in a gene(s) whose product is required for further metabolism of CHQ. Strain PT88 was used to identify genes encoding 2,4,5-T degradation.

A cosmid clone bank of AC1100 genome was mobilized in to mutant strain PT88. One of the cosmid clones containing 25kb chromosomal fragment referred to as pUS1 complemented the mutation in PT88 to 2,4,5-T^{+} (Sangodkar et al., 1988). This segment harbors the genes for metabolism of CHQ and has been designated as chq. The specific region of DNA required for restoration of CHQ^{+} phenotype was mapped and further defined to a 4.3 kb segment, cloned in pUS1029 (Sangodkar et al., 1988). A 290 bp fragment encoding putative regulatory sequences

of chq gene was also located (Sangodkar et al., 1988) (Fig. 1.10).

Similarly a 8.9 kb DNA fragment from AC1100 containing 2,4,5-T oxidation gene was able to complement, 2,4,5-T degrading ability in RHC22, an AC1100 mutant defective in its ability to utilize 2,4,5-T (Haugland et al., 1991). A 2.7 kb fragment from one of the construct pRHC89 which complements 2,4,5-T degrading ability was subcloned in pCD206. On electroporation to P. aeruginosa PA01 cells and incubation in presence of 2,4,5-T, TCP was maximally produced (Danganan et al., 1994).

This 2.7 kb fragment encodes 2 polypeptide of 51 and 18 kda respectively. Nucleotide sequence analysis of the DNA fragment reveals the presence of the genes tftA1 and tftA2 (encoding for 51 and 18 kda respectively). TftA1 and TftA2 show strong amino acid homology to BenA and BenB from the benzoate 1,2 dioxygenase of Acinetobacter calcoaceticus, as well as to XylX and XylY from toluate 1,2 dioxygenase of Pseudomonas putida. The 2.7 kb fragment transferred in pA01 could convert not only 2,4,5-T to 2,4,5- TCP but



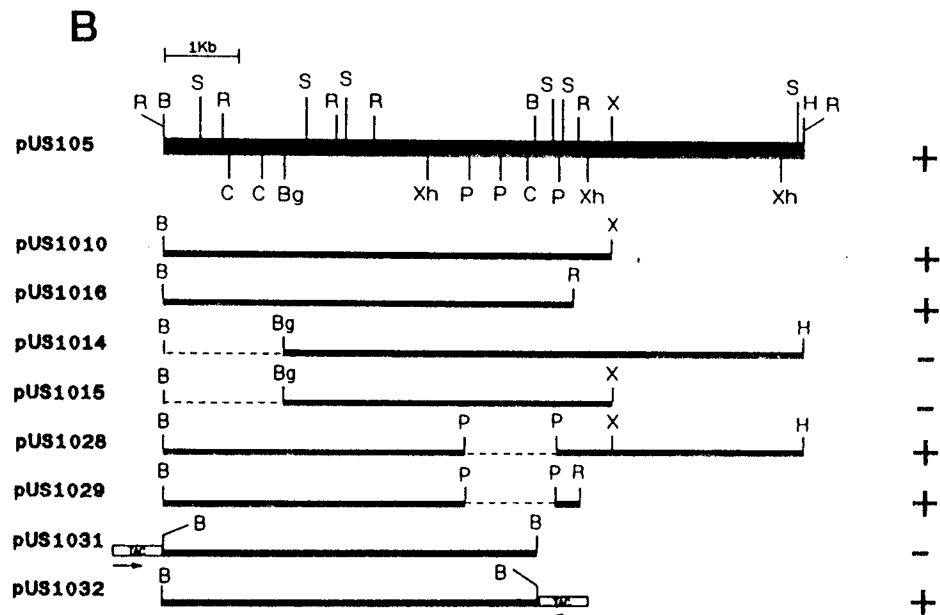


Fig.1.10 Complementation of clones from genomic library of strain AC1100 for 2,4,5-T degradation in deletion mutant PT88 (adapted from Sangodkar et al., 1988).

also 2,4 dichlorophenoxyacetic acid to 2,4 dichlorophenol and phenoxyacetate to phenol (Danganan *et al.*, 1994).

f) Repeated sequences in strain AC1100:

Repeated sequences have been discovered in many prokaryotic systems including gram negative and gram positive eubacteria as well as archaebacteria (Sapienza *et al.*, 1982; Fishman *et al.*, 1985).

Analysis of DNA flanking the Tn-5 insertion in PT88, a genetic element was located, which was repeated several times on the parental AC1100 genome. The nucleotide sequence of this repeat element designated RS1100, was found to share characteristics of insertion sequence elements (IS elements). RS1100 is 1477 bp long with 38-39 bp inserted repeats flanked by 8 bp direct repeats and contains a single large open reading frame that has structural similarity to the Mu and other transposases (Tomasie *et al.*, 1989).

Repeated sequences have been implicated as sites for homologous recombination between bacterial plasmids and chromosomes (Barsomian and Lessie, 1986; Szabo and Mills, 1984); deletion formation (Kaluza et al., 1985) and genetic rearrangements leading to evolution of new enzymes for the degradation of novel synthetic compounds (Negoro et al., 1983).

Haugland et al. (1990) showed the ability of RS1100 to transpose from the AC1100 genome on to the plasmid pKT240 and is able to activate the promoter-less aphC gene of the vector, thereby conferring streptomycin resistance in both P. cepacia and E. coli. Also, RS1100 is known to mediate transfer of a fragment from AC1100 chromosomol DNA on to a plasmid pKT240. A newly identified 340 bp repeat sequence was also identified from strain AC1100. RS1100 has been redesignated as IS931 and 3400 bp repeat sequence has been designated as IS932.

Gaffney and Lessie (1987) described a P. cepacia Strain 249 in which 12 IS elements were found, at least some of which were characterized as transposing at high frequency causing a number of chromosomal and plasmid

rearrangement and several auxotrophic mutants. Thus the insertion sequences in AC1100 mediating similar type of changes is not a remote possibility. Since 2,4,5-T degradative genes are chromosomally encoded, insertion sequences might have a major role in loss of 2,4,5-T phenotype in 50% of population when grown on non selective media (Kilbane *et al.*, 1982).

Unlike other insertion sequences IS931, occupies only one-third of AC1100 chromosome (Coco *et al.*, 1990). This might be due to target-specificity requirement by IS931 which the majority of *P. cepacia* chromosome does not fulfill. This is confirmed by the spontaneous auxotrophic mutations in AC1100 obtained at a frequency of less than 10^{-6} even though IS931 mediated chromosome deletions were frequently observed (Coco *et al.*, 1990).

g) Ancestral origin of 2,4,5-T genes and RS1100 from AC1100:

RS1100 and *chq* locus from strain AC1100 were used as probe to check its origin by hybridization with DNA from various *Pseudomonas* strain. Neither RS1100 nor *chq*

probes hybridized with any of the DNA from *Pseudomonas* including *P. cepacia* indicating that the ancestor genes of *chq* locus or RS1100 sequences may have diverged widely or may have been recruited from one or more taxonomically distant sources (Sangodkar *et al.*, 1988).

h) Application of strain AC1100 in degradation of 2,4,5-T from soil

Strain AC1100 degrades and grows in the presence of 2,4,5-T in soil. At optimum temperature of 30 ^{o}C and moisture content of 15 to 50% (w/v), strain AC1100 degraded 95% of 2,4,5-T at high concentration (1mg/g of soil) within 1 week (Chatterjee *et al.*, 1982). Strain AC1100 was highly dependent on the presence of 2,4,5-T in soil. In absence of 2,4,5-T in soil the titre of strain AC1100 fell precipitously. However addition of 2,4,5-T in soil caused a rapid rise in the titre of strain AC1100, with concomitant loss of 2,4,5-T from soil by 7 days (Chatterjee *et al.*, 1982; Kilbane *et al.*, 1983). Repeated application of AC1100 even allowed more than 90% removal of 2,4,5-T within 6 weeks from heavily contaminated soil containing as much as 20,000 ppm 2,4,5-T. Microbial removal of 2,4,5-T allowed the

soil to support growth of plants sensitive to low concentration of 2,4,5-T (Kilbane _et al._, 1983). Strain _P. cepacia_ AC1100 therefore appears to be constantly adapting to accomodate the utilization of substrates by rearranging the genetic makeup.

V) Phenol biodegradation: status overview

Phenol, (IUPAC Systemic name: Hydroxybenzene) locally known as carbolic acid or phenylic acid was first isolated from coal-tar in the 1830s. Coal-tar was the only source of phenol until the first world war, when sulfonation of benzene and hydrolysis of the sulfonate led to the production of first synthetic phenol (Considine, 1974; Thurman, 1982). Phenol production by the chlorobenzene process and toluene-based process have been of major importance in phenol production in the past and are still being used (Thurman, 1982). However, phenol production from cumene (isopropylbenzene) is the commonly used method worldwide due to its high yield and economy (Thurman, 1982). The annual production of phenol worldwide has been steadily increasing. USA has been the major producer of phenol

(Anon, 1987). In 1984, India produced 20 thousand tonnes of phenol, which was a mere one-hundredth of that produced in USA.

Phenol is the basic parent compound from which a number of commercially important materials are made, including phenolic resins, bisphenolA and caprolactam (IARC, 1987) as well as chlorophenols such as pentachlorophenols (IARC, 1987). Phenolic resins are used as adhesives in plywood and particle board, as binders for fibre glass, mineral wool and other insulating products, for impregnating and laminating wood and plastic agents and as moulding compounds and foundry resins. Phenol is also converted to alkyl phenols which are used as surface active agents, emulsifiers, antioxidants and lubricating oil additives and to make plasticizers, resins and synthetic lubricants. Medical uses of phenol include incorporation into lotions, salves and ointments. It is also used in disinfectants and antiseptics, paints and varnish remover, rubber, ink, perfumes, soaps, paints and illuminating gases.

a) Regulatory and status guidelines

Phenolic wastes cause serious repercussions, affecting both flora and fauna present near the residing bodies. According to Indian Safety Standards, the industrial effluent should not contain more than 1 mg/l of phenol (Ministry of Welfare, 1994). Also phenol concentration should not exceed the limit of 0.005 mg/l in drinking water (Ministry of Welfare, 1994). Environmental Protection Agency (USA) has estimated that phenol concentration below 3.5 mg/l may prevent most toxic effects to sea water and fresh water aquatic species (EPA, 1979). Occupational exposure limit for phenol in India is limited to 38 mg/m^3 (IARC, 1989).

b) Sources of phenolic wastes

Phenol is a constitutent of coal-tar and is formed during the natural decomposition of organic materials (Cleland and Kingsburg, 1977). They enter coastal waters through natural decomposition of attached algae and phytoplankton. (Riley and Chester, 1971; Burkema *et al.*, 1979; Carron and Afghan, 1989). Lignins, phenolics etc. are naturally released by plants (Glick and

Pasternak, 1994). Besides natural leaching of phenol into environment, phenol forms one of the major pollutants in the waste which enters the environment as a result of natural, domestic, industrial and agricultural activities. Phenol is one of the important components in the waste material/effluent released from pesticides, pharmaceuticals, dye leather, petroleum refineries, fertilizers, cement, ceramics, fibre glass units, pulps and paper mills, gas and coke industries, polymers resin product plants and smelting and connected metallurgical operations. (Atlas, 1981, 1984, 1995; Cairns and Scheier, 1978; Garland, 1972; Leahy and Colwell, 1970). Various oil spills and accidents such as Torrey Canyon in 1967, oil well blowouts such as Ekotisk in 1977 and the Gulf War disaster in 1991 have resulted in release of large amounts of hydrocarbon, of which phenol forms a major part of aromatic fraction (Atlas, 1995).

Average phenol concentration in liquid waste from different processes is listed in Table-1.1.

Sewage introduces about 0.03 to 20 mg/l of phenol whereas pesticidal residues contributes 0.5 mg/l of

Table 1.1 Avearge phenol concentration in liquid waste from different process (Mahajan, 1993)

Process	Phenol concentration mg/l
Metallurgical coke	
(a) Spent liquor after phenol recovery	900-1000
(b) Coke-oven effluent	35-250
Coal carbonization	
(a) Low-temperature carbonization	1000-8000
(b) High temperature carbonization	800-1000
Oil refining	2000
Phenol-formaldehyde resin manufacture	800-2000

phenol (Garland, 1972). Thus 4/5th of total introduced is by human activities and 1/5th by natural processes like run off waters, volcanoes etc (Cairns and Scheier, 1978).

c) Occurrence of phenol in environment

i) Occupational exposure: Workers are potentially exposed to phenol, especially in the industries mentioned above. Airborne phenol concentrations in sampled areas ranged from non detectable to 12.5 mg/m^3 in a bakelite factory in Japan. Exposure levels of 5.88 mg/m^3 have been reported for employees in the USSR who quenched coke with waste water containing 0.3 to 0.8 mg/l phenol (IARC, 1989).

ii) Air: Phenol was detected in urban air (0.55 - 1.0 ppb) in exhaust from cars (0.23 - 0.32 ppm) and in tobacco smoke (312 - 436 mg/cigarette) collected in Osaka, Japan (Kuwata et al., 1980).

iii) Water and Sediment : Phenols may occur in domestic and industrial waste waters, natural waters and potable water supplies. Effluents containing phenol when

released in coastal waters or rivers, generally get diluted multifold resulting in phenol concentration dropping below 100 mg/l. Chlorination of water containing phenols may produce chlorophenols giving the water an objectionable smell and taste. In India, phenol concentration in river or sea water depended on whether the receiving bodies received any domestic or industrial waste. Moderately high concentrations of 18.7 , 13.6, 12.1 and 13.1 mg/l have been observed at Vereval Hazira, Bassein and Bombay respectively presumably because of large quantities of domestic and industrial waste being disposed off (Kadam and Bhangale, 1993; NIO, 1992). Phenol concentration of 7.6 - 13.6 mg/l observed at Ratnagiri could possibly be due to decomposition of algal matter, that is abundantly present in the region (NIO, 1979). At lowly polluted areas, like Porbunder, Phenol concentration was around 5 mg/litre whereas at unpolluted areas like Okha, Diu, Daman and Murud, phenol could not be detected (Kadam and Bhangale, 1993).

iv) Soils and plants : Phenol has been reported to biodegrade completely in soil within two to five days (Baker and Mayfield, 1980). High concentration of

phenol may destroy the degrading bacterial population and leach to ground water (Baker and Mayfield, 1980).

v) Food: Phenol has been found to taint the taste of fish and other organisms, when present at concentrations of 1- 25 mg/litre in marine environment (Verschueren, 1983). It has been detected in smoked summer sausage (7 mg/kg) and in smoked pork belly (28.6 mg/kg) (US EPA, 1980).

d) Effect of phenol on biological system

The effect of phenol on biological organisms has been reviewed in IARC MONOGRAPHS, 1989. At lower doses, phenol induced leukaemia in male rats. Laboratory studies have also indicated the ability of phenol to act as a promoter, when tested in the two stage mouse skin model. In one control study on workers employed in wood industries, exposure to phenol resulted in higher incidences of mouth and respiratory tract tumors. Phenol is known to induce mutation in cultured animal cells, but not DNA damage. Phenol caused irritation, dermatitis, liver and kidney toxicity when tested on

experimental animals.

e) Effect of phenol on aquatic life

LC50 (24 hr) of less than 0.42 ppm for plankton and invertebrate larvae and 0.6 ppm for crustaceans was observed when tested with phenol (Cairns and Scheier, 1978). On an average, phenol is toxic to fish at concentrations higher than 5mg/l. 0.5 to 10 ppm of phenol have been found to damage the organs of chemical sense of fish. Respiratory difficulty, loss of equillibrium and reduced fertilization rates have also been observed.

f) Analysis of phenol

Phenol present in air may be collected by drawing the air through a 0.1 M solution of sodium hydroxide and determined by reversed phase HPLC after derivitizaton with p-nitrobenzene diazonium tetrafluoroborates (Kuwata et al., 1980). It can also be measured by gas chromotography with flame ionization detection method.

Phenol can be determined in water samples spectrophotometrically by 4-aminoantipyrene method. Environmental samples can also be analyzed by gas chromatography or mass spectrometry using either packed or capillary columns.

g) Microorganisms involved in phenol degradation

The presence of phenol in environment both from natural and man made sources have resulted in evolution of diverse organisms with the capability to utilize toxic aromatic compound like phenol as carbon source. Amongst the bacteria, *Pseudomonas* species have been found to exhibit the highest metabolic versatality especially, *Pseudomonas cepacia* group (Stanier, 1966). Literature survey on phenol biodegradation reveals the isolation of different microorganism from soil, sewage waters, and aquatic waters, with capability to degrade phenol (refer Table-1.2). Microorganisms were capable of degrading phenol either aerobically or anaerobically (Table-1.2).

h) Biochemical pathway for phenol degradation

Considerable work has been done on elucidating the pathway of phenol biodegradation in various microorganisms. Though phenol is degraded aerobically in majority of the microorganisms, certain mixed consortium and pure bacterial isolates have also been reported (see Table-1.2) to degrade phenol anerobically. All the microorganisms are known to metabolize phenol aerobically *via* a common central intermediate catechol. Though the conversion of phenol to catechol is predictable in all phenol degraders, further metabolism of catechol by ring fission depends on the genetic constitutent of phenol degraders.

Catechol, substitute for ring fission dioxygenases undergoes cleavage by incorporation of oxygen atom in the aromatic ring. The pathways observed are dictated by the site of cleavage of aromatic nucleus. *Meta* cleavage pathway involves cleavage at 2,3 position by catechol, 2,3 dioxygenase leading to formation of 2-hydroxymuconic semialdehyde which can be metabolized *via* NAD^+ dependent dehydrogenase or NAD^+ independent

hydrolase pathway, each forming a 4-oxalocrotonate as common intermediate, which is then finally metabolized to acetaldehyde. Ortho cleavage pathway involves cleavage at 1,2 position by catechol 1,2 dioxygenase leading to formation of cis, cis-muconic acid, which is further metabolized by the associated enzymes of B ketoadipate pathway (Fig. 1.11).

The initial conversion of phenol to a diphenolic intermediate, catechol, mediated by phenol hydroxylase enzymes in all phenol degraders is a universally observed phenomena. However, further degradation of catechol in phenol degraders can take place via ortho cleavage or meta cleavage. Phenol is degraded via meta cleavage pathway in Pseudomonas putida NCIB 10105. P. picketti PK01; Alcaligenes eutrophus JMP134; Alcaligenes eutrophus strain 345; B. stearothermophilus PH24; B. sterothermophilus FDTP-3; B. stearothermophilus BR219; Pseudomonas cepacia G4 and Ochromonas danica (Janke and Fritsche, 1981; Shields et al., 1991; Feist and Hegeman, 1969; Frey et al., 1983; Buswell , 1974; Pieper et al., 1989; Semple and Cain, 1995; Hughes et al., 1984; Enhardt and Rehm, 1989; Dorg, 1992; Bartilson et al., 1990; Kivisaar et al., 1989; Kukor and Olsen, 1991).

TABLE 1.2: Micro-organisms involved in phenol biodegradation

Micro-organisms	Reference
Aerobic	
1. Pseudomonas cepacia G4	Nelson et al., 1987
2. Pseudomonas putida H	Janke et al., 1981
3. Pseudomonas picketti PK01	Kukor and Olsen, 1990
4. Pseudomonas putida F	Spain and Gibson, 1988
5. Pseudomonas CF600	Frey et al., 1983
6. Pseudomonas sp EST-1001	Kivisaar et al., 1989
7. Pseudomonas putida P8	Bettmann and Rehm, 1984
8. Pseudomonas putida EKII	Hinterregger et al., 1992
9. Pseudomonas putida ATCC 17484	Stanier et al. 1966
10. Pseudomonas putida NCIB 10015	Dagley and Gibson, 1965
11. Pseudomonas putida ATCC 11172	Molin and Ternstrom, 1982
12. Rhodococcus sp P1	Hensel and Straube, 1984
13. Bacillus stearothermophilus BR219	Gurujeyalakshmi and Oriel,1989
14. Bacillus stearothermophilus PH24	Buswell, 1974
15. Bacillus stearothermophilusFDTP3	Dong et al., 1992
16. Alcaligenes eutrophus JMP 134	Pieper et al., 1989
17. Alcaligenes sp A72	Schwein and Schimidt, 1982
18. Alcaligenes eutrophus 345	Hughes et al., 1984
19. Streptomyces setonii 75vi2	Antai and Crawford, 1983
20. Yeast strain	Hashimito, 1970
21. Candida tropicalis	Neujahr et al., 1974
22. Rhodotorula rubra	Hiryama et al., 1991
23. Trichosporon cutaneum	Neujhar and Varga, 1970
24. Aureobasidium pullulans No. 14	Itoh et al., 1980
25. Ochromonas danica	Semple and Cain, 1995
Anaerobic	
26. Phenol degradating consortium	Genthner et al., 1989
27. Acclimated mixed culture	Satsangee and Ghosh, 1990
28. Cosortium of 6 different microbes	Bisaillon et al., 1991
29. Iron reducing organism GS-15	Lowley and Loneryan, 1990
30. Methanogenic Consortium	Dwyer et al., 1986
31. Pseudomonas Strain K172	Tschech and Fuchs, 1987
32. Desulfobacterium phenolicum sp	Bak and Widdel, 1986
33. Pseudomonas sp & spirillum sp	Bakker, 1977
34. Methanospirillum hungatei Desulfovibrio Mixed Consortium of long non motile rod and short motile rod bacterial cells	Knoll and Winter, 1989

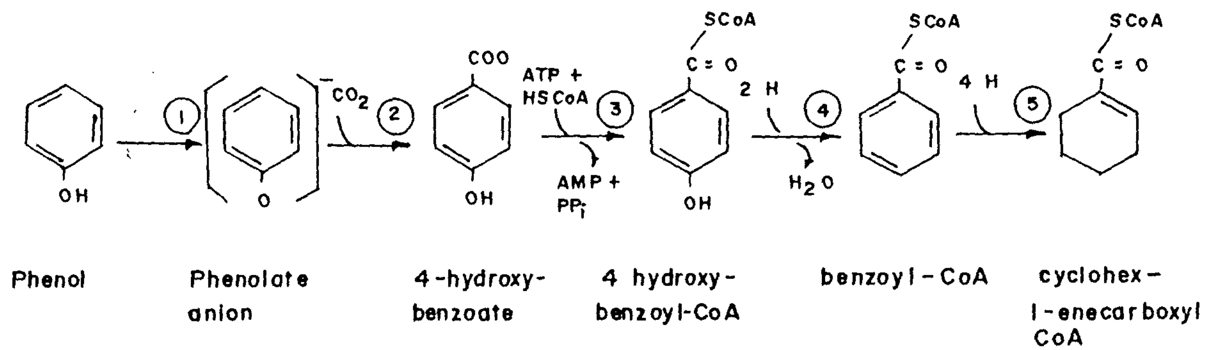


Fig.1.12 Anaerobic phenol metabolism in denitrifying Pseudomonas strain K172 . (1) and (2) phenol carboxylase; (3) 4-hydroxybenzoate-CoA ligase; (4) 4-hydroxybenzoate-CoA reductase; (5) benzoyl CoA reductase (adapted from Lack and Fuchs, 1992).

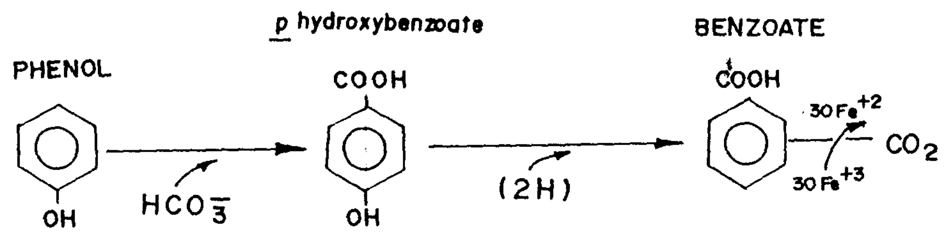


Fig.1.13 Oxidation of phenol coupled to Fe(III) reduction (adapted from Loveley and Lonergan, 1990).

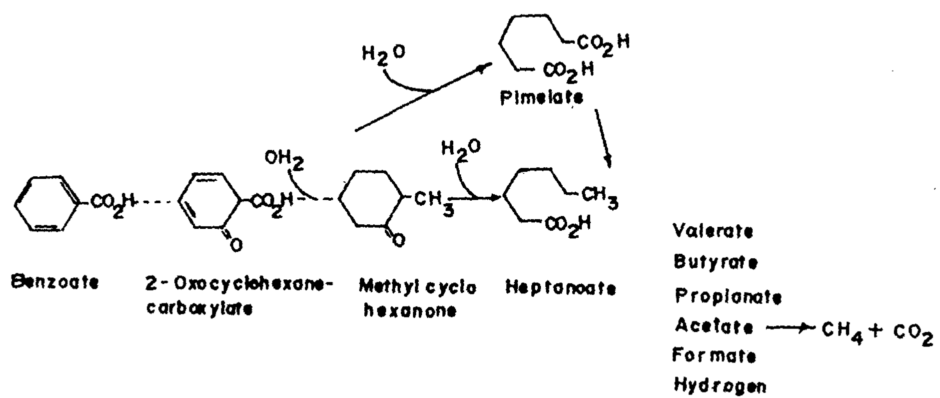


Fig.1.14 Probable pathway of benzoate degradation by adapted bacterial consortia (adapted from Evans and Fuchs, 1988).

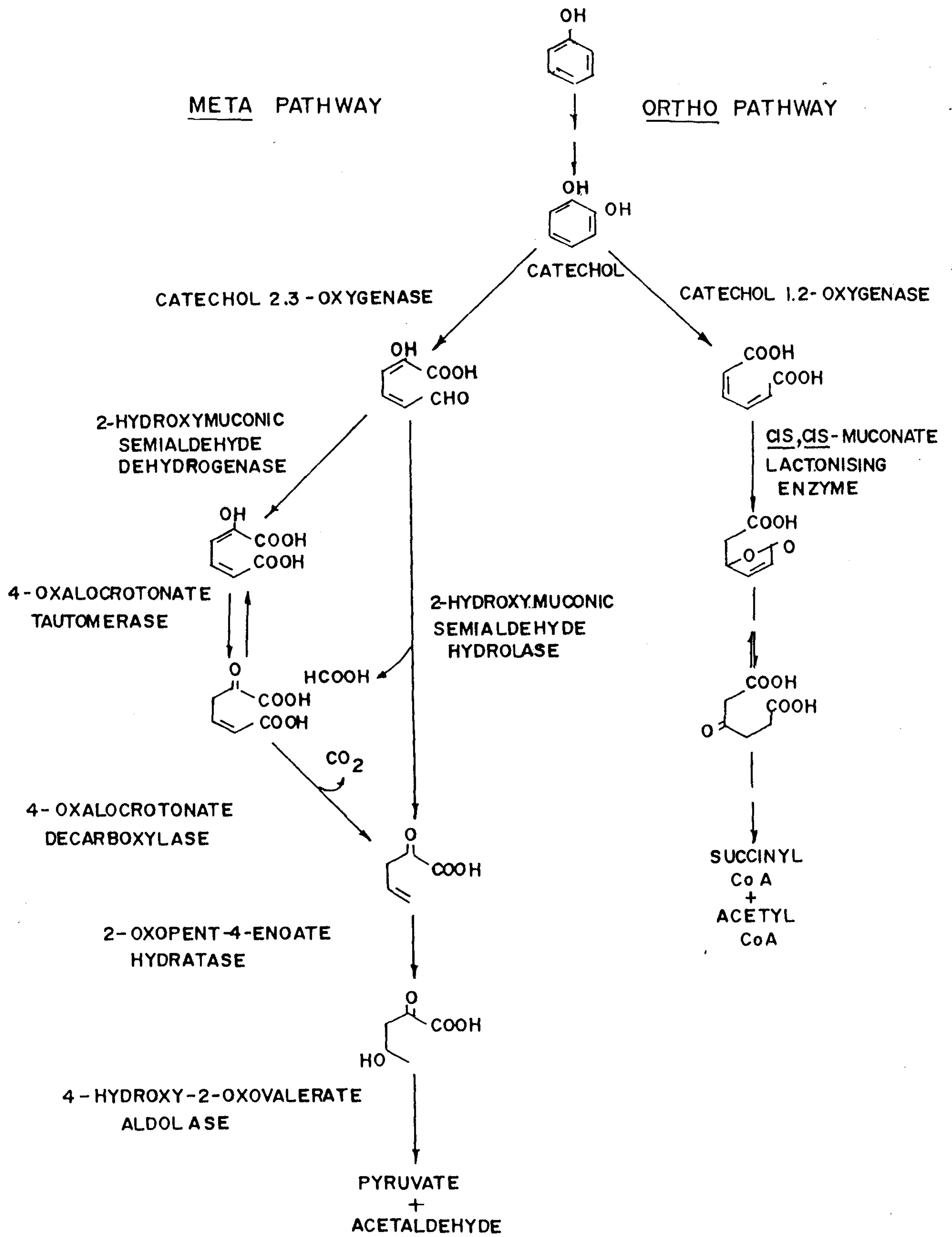


Fig.1.11 Metabolism of phenol in bacteria (adapted from Dagley, 1972).

Phenol is also degraded *via ortho* cleavage pathway system in *P. putida* H; *Pseudomonas* sp. strain EST 1001; *P. putida* EKII, *Alcaligenes* sp. A72, *Trichosporon cutaneum*; *Rhodotorula rubra*; *Streptomyces setonii* 75vi2 and *Rhodococcus* sp. (Neujahr and Varga, 1970; Janke *et al.*, 1981; Kivisaar *et al.*, 1989; Hinteregger *et al.*, 1992; Schwein and Schimidt, 1982; Hiryama *et al.*, 1991; Antai and Crawford, 1983; Hensel and Straube, 1984).

Anaerobic degradation of phenol by methanogenic consortium or by pure cultures involves carboxylation of phenolic ring to form *p*-hydroxybenzoate. This is further converted to benzoate, which is degraded further anerobically. Figure 1.12 and 1.13 shows the conversion of phenol to benzoate in anerobic cultures. The probable pathways for the degradation of benzoate by bacterial consortia is given in Figure 1.14.

Regulation of phenol degradation has been widely studied. Phenol, cresol or its related analogue itself acts as inducer of hydroxylase and *meta* pathway enzymes (Janke *et al.*, 1981; Dagley *et al.*, 1960). In microorganisms with both *ortho* and *meta* cleavage

capability, the phenotypic expression of the alternate pathway is determined by the chemical nature of the aromatic catechol precursor with which the organism are grown. In _Alcaligenes eutrophus_ JMP134, phenol and methyl phenol induced only the _meta_ pathway, whereas 2,4 dichlorophenol and 4-chloro, 2-methyl phenol did not, and they acted as inducers of _ortho_ pathway only. This efficient regulation prevents chloro aromatics from being misrouted into the unproductive _meta_ cleavage pathway (Pieper _et al._, 1989). Similarly the mutually exclusive occurrence of the _meta_ and _ortho_ pathway enzymes in phenol and benzoate grown cells of _P. putida_ was also explained on the basis of differences in the mode of regulation of these two pathways. The _meta_ pathway enzymes were inducible by the primary substrates, phenol, cresols or analogues where as enzymes of the _ortho_ pathway were inducible only by _cis,cis-_ muconate produced from catechol by 1,2 oxygenase mediated cleavage. Thus the _meta_ pathway serves as a general mechanism for catabolism of various alkyl derivatives of catechol derived from substituted phenolic compounds. The _ortho_ pathway is more specific and serves primarily in the catabolism of precursors of catechol and catechol itself (Feist and Hageman, 1969).

i) Utilization of various aromatic compounds as carbon source for growth in phenol utilizing strains:

The occurence of isofunctional enzymes for the degradation of same or structurally related compounds is not uncommon in biodegradative pathways (Chapman, 1972; William and Murray, 1974). Many of the phenol degrading strains are capable of degrading or oxidizing analogues of phenol. In *P. putida* strain 144 twenty three analogues were screened for the ability to induce enzymes of *ortho* and *meta* pathway. Compounds like 2 ethyl- and 3-ethyl phenol, allyl phenol, and 2 methoxy - 4 methyl phenol all induce *meta* pathway enzyme. These substituted phenols were metabolized to varying extents in reactions mediated by induced enzyme. Ethylphenol supported some growth of *P.putida*, whereas allylphenol and 2-methoxy, 4-methyl phenol did not support growth but were hydroxylated and cleaved by the enzymes they induced. However, halogenated phenols and hydroxymethyl pyridines did not support growth or induce any enzymes. Benzylalcohol and 2,6-dimethylphenol were found to be non metabolizable inducers of *meta* pathway enzyme (Feist and Hageman, 1969).

P. putida strain EKII strain pregrown in phenol showed whole cell hydroxylating activity for cresols, chlorophenols, 3,4-dimethylphenols and 4-chloro, m-cresol (Hinteregger et al., 1992). Similarly oxygen uptake studies with phenol grown P. putida showed oxidation with analogues like cresol (Pohl and Fritsche, 1981).

Resting cells of B. stearothermophilus PH24 could oxidize phenol and o/m/p cresol (Buswell, 1975). Hughes et al. (1984) found the presence of isofunctional enzymes in the degradation of phenol, toluate and p-cresol in Alcaligenes eutrophus. Their results indicated the hydroxylation of phenol and cresol to be mediated by the same phenol hydroxylase enzyme.

Phenol hydroxylase enzyme from Rhodococcus sp. was characterised for substrate specificity. The polyhydric phenols like resorcinol, quinol, pyrogallol and cresols were oxidized at higher rate than phenol. Chloro phenols were oxidized at a slight lower rate than phenol (Straube, 1987).

Gaal and Neujahr (1981) showed the ability of phenol hydroxylase from Trichosporon cutaneum to

oxidize resorcinol, cresol and fluorophenols besides phenols. Also their results indicated that cells grown in resorcinol contain enzymes that participated in the degradation of phenol and *vice versa* .

j) Genetics of phenol biodegradation

Herrmann *et al.*, (1987) have reported involvement of plasmid pPGH1 in the phenol biodegradation in *Pseudomonas putida* strain H. Plasmid pPGH1 was 200-220 kb and encoded for enzymes involved in the catabolism of phenol or cresol through *meta* cleavage pathway. The plasmid pPGH1 was non-conjugative but could be mobilized by RP1 or R68.45 (Herrmann *et al.*, 1987; Hermann *et al.*, 1988). Kivisaar *et al.* (1989) have reported a multiplasmid system responsible for the degradation of phenol, salicylate and *m.*toluate in *Pseudomonas* strain EST1001. The plasmid DNA was found to undergo structural rearrangement leading to phe^-, Sal^- and *m*Tol^- segregants which could again revert back to *wt*.

Plasmid pVI15 an, IncP-2 degradative mega plasmid confers the ability to utilize phenol and 3,4 dimethyl phenol in *Pseudomonas* CF600 *via meta* cleavage pathway. A 19 kb contiguous fragment encoding all catabolic genes

of the pathway was identified. The genes were clustered and nucleotide sequences of dmp KLMNOPBC and D encoding the first 4 biochemical steps of pathway have been determined (Shingler et al., 1992). The fifteen genes in the order of dmp KLMNOPQBCDEFGHI lie in a single operon with intergenic spacing (0-70 nucleotides).

The gene order of phenol/3,4 dmp meta pathway of pVI150 was same as that of pWWO and NAH17. Further a considerable homology has been observed between C23O encoding genes (80% at nucleotide level and 85% at amino acid level) of pVI150 with pWWO indicating a common origin (Bartilson et al., 1990; Bartilson and Shingler, 1989).

Phenol hydroxylase of Pseudomonas CF600 has been well characterized and found to comprise of 5 polypeptides, P1 to P5 (Powlowski and Shingler, 1990). As a general rule, mono-hydroxylated ring structures such as phenol are oxygenated by single component flavoprotein monoxygenases (Ballon, 1982). This was the case as observed in P. pickettii (Kukor and Olsen, 1992). Pseudomonas EST 1001 (Nurk et al., 1991) and yeast Trichosporon cutaneum (Kalin et al., 1992). The

2,4-dichlorophenol hydroxylase of Acinetobacter and Alcaligenes species have also been shown to be flavoproteins, although these enzymes shows no activity with unsubstituted phenol (Beadle and Smith, 1982; Liu and Chapman, 1984). However, studies of phenol hydroxylase in Bacillus species suggest that it may not be a single component flavoprotein (Gurujeyalakshmi and Oriel, 1989). Nordlund et al. (1993) isolated ten phenol utilizing marine isolates from eight sea water samples. Five out of ten phenol utilizing marine bacteria and the archetypal phenol degrader Pseudomonas U all posess DNA highly homologous to each of the components of phenol hydroxylase from strain CF600. The homology extended even to the meta cleavage pathway genes.

Shingler et al. (1993) have characterized the regulatory elements of catabolic plasmid pVI150 from Pseudomonas sp. strain CF600. A 67 kDa dmpR gene product was needed for the activation of transcript from the dmp operon promoter.

Dong et al. (1992) isolated two genes from B. stearothermophilus coding for phenol hydroxylase and catechol 2,3 -dioxygenase respectively. Kukor and Olsen

(1990, 1991) observed that the meta pathway genes from Pseudomonas pickettii were organised in to a single operon. A 1.3 kb PstI fragment designated tbuS was found to act as a repressor as well as activator.

k) Degradation of phenol in continuous cultures

Initial work by Wale et al. (1959) and Evans and Kite (1961) showed the possibility of using microorganism in continuous culture to degrade phenolic waste. Wase and Hough (1966) showed the ability of Debaryomyces subglobosus (NCYC459) to degrade phenol in continuous culture. Yang and Humphrey (1975) studied the degradation of phenol by pure and mixed cultures of Pseudomonas putida in batch and continuous culture. They succeeded in achieving phenol removal from waste water down to levels of 1-2 ppm in a single stage system.

Hutchinson and Robinson (1988) studied the kinetics of simultaneous batch degradatiion of p-cresol and phenol by P. putida. A model was developed which was able to successfully predict phenol and p-cresol catabolism during batch growth.

Biofilms are frequently utilized in aerobic waste water treatment (Atkinson and Knights, 1975). Molin and Nilsson could achieve a maximum phenol degradation rate from 0.23 g/l per h (without biofilm) to 0.72 g/l per h at highest biofilm level (55 m^2 of biofilm surface per ml of reactor volume). The continuous cultures with biofilms could tolerate a higher phenol concentration of medium (3.0 g/l) than the non biofilm system (2.5 g/l).

Pawlosky (1973) observed stability of a continuous culture utilizing inhibitory substrate such as phenol could be improved by increasing the degree of wall growth. Takahashi et al. (1981) observed that Aureobasidium pullulans No. 14 could remove phenol at the rate of 50 mg/l/reactor volume, when the culture was adhered to fibrous asbestos.

Pseudomonas sp and P. putida P8 were immobilized in alginate and polyacrylamide hydrazide and could degrade phenol at much higher concentrations than observed in abscence of immobilisation (Bettmann and Rehm 1984, 1985). Donaldson et al. (1984) observed higher phenol degradation rates with commercially available culture systems namely Bichem 1001 and 1002

and Phenobac.

Aim and Scope of the Work

The foregoing review indicates that microorganisms play a major role in the breakdown and mineralization of organic compounds released into the environment. Strains of P.cepacia in particular, are known to utilize a greater number of organic compounds than any other bacteria (Stanier et al, 1966). The unique manner in which novel catabolic pathways have evolved in versatile organisms such as Pseudomonas has been attributed to deregulation or activation of cryptic genes, relaxed substrate specificity of enzymes and molecular mechanisms that include recruitment and expression of foreign genes (Coco et al, 1990; Meer et al, 1992). These mechanisms are ongoing in nature and as microorganisms are exposed to new compounds, genetic flux in the microbial community leads to adaptation of a specific microorganism which has by then evolved the capacity to degrade new compound.

The evolutionary process occurring in nature is extremely slow. Evolution of new metabolic activities

in the laboratory may therefore be helpful as the frequency and type of genetic events needed (for e.g., mutation, alteration of gene expression or gene transfer) can be carefully controlled and selective conditions can be optimized.

Pseudomonas cepacia AC1100 is one such organism which has been evolved by Plasmid Assisted Molecular Breeding (Kellogg *et al*, 1981). It was developed in a chemostat under strong selective pressure to utilize 2,4,5-T (Kellogg *et al*, 1981). Strain AC1100, besides using 2,4,5-T, has a capability to metabolize benzoate.

Studies involving detoxification of 2,4,5-T from soil by strain AC1100 have revealed its ability to remove 99% of 2,4,5-T present, thus promoting the growth of plants which were hitherto sensitive to even low concentrations of 2,4,5-T (Kilbane *et al*, 1983; Chatterjee *et al*, 1982).

Strain AC1100, although capable of utilizing 2,4,5-T or TCP as the sole carbon source, does not utilize non- chlorinated analogues such as phenoxyacetic acid and phenol (Karns *et al*.1983). Danganan *et al*.

(1994) have cloned the gene encoding 2,4,5-T oxygenase which has the ability to form TCP, DCP and phenol from 2,4,5-T, 2,4-D and phenoxyacetate respectively. Thus, although phenoxyacetate was being converted to phenol by the oxygenase enzyme, the failure of strain AC1100 to metabolize phenol further could be attributed to the absence of any functional gene(s) for the pathway of phenol metabolism.

Biological treatment of waste water and contaminated soils involves degradation of substrate mixtures rather than single compounds. Besides polychlorinated phenols, various monoisomers of chlorophenols are present in the environment with phenol forming a predominant compound of such wastes.

The aim of the present study was to expand the catabolic trait of strain AC1100, making it capable of utilizing phenol besides 2,4,5-T, and to study the potential of the resultant strain in treatment of liquid waste containing phenol by continuous culture method. Also, by simulating low nutrient like prevalent in rivers and estuaries, the efficacy of the emerged strain in degrading phenol could be determined.

The emergence of a phenol degrading strain from Pseudomonas cepacia AC1100 could be brought about by mutating the existing TCP hydroxylyzing enzyme to one with a more relaxed substrate specificity so that it could use phenol as a substrate. Thus phenol could be channelized *via* the existing 2,4,5-T pathway (horizontal expansion). Alternatively, phenol hydroxylase gene could be transferred to Pseudomonas cepacia AC1100, enabling strain AC1100 to convert phenol to catechol, which could then be channelized through the existing ortho pathway in strain AC1100 (vertical expansion).

P. cepacia AC1100 is a unique organism obtained by deliberate facilitated evolution (Kellogg, et al, 1981). Its uniqueness lies in the presence of a set of insertion elements in one-third of the AC1100 chromosome (Coco *et al*. 1990), which are unique to Pseudomonas and are known for gene activation (Haugland *et al*, 1990). Further, the 2,4,5-T genes recruited in this organism are unique only to strain AC1100 (Haugland *et al*, 1990).

The emergence of a phenol utilizing strain from AC1100 *via* mutation or gene transfer would indeed be novel as it may lead to phenol utilization through the

existing pathway. Also, insertion sequences having a possible role in mutation or activation of cryptic genes *in situ* or during transfer could also be studied. Characterization of this molecular event and the pathway leading to phenol utilization in the newly emerged strain would certainly help us to understand the underlying principles in creating novel microorganisms with unique degradative capabilities. This would be possible especially in strain AC1100 which in itself contains genes possibly recruited from taxonomically distant sources during forced evolution under selective pressure.

The newly emerged phenol-utilizing strain could be further checked for its potential to degrade phenol in continuous culture. Earlier work on phenol degradation has been carried out with microorganisms which have been obtained from the polluted site itself (Chapter I). Our work on an engineered microorganism would explore the feasibility of such laboratory-evolved microorganisms in actual degradation, thus enabling us to determine its efficacy in field test conditions as done for strain AC1100.

CHAPTER II

DEVELOPMENT OF PHENOL DEGRADING STRAIN PAA FROM P.CEPACIA AC1100

A review of literature on phenol biodegradation reveals the presence of diverse microorganisms in terrestrial and aquatic environment, which have evolved so as to possess unique capabilities to breakdown toxic organic compound such as phenol. Phenol has been prevalent in environment in very low quantities, mostly from biological origin, either from plants, animals or microbial activity. However the advent of industrialization has resulted in making less abundant organic compound like phenol as one of the major pollutant in the environment. The ability of micro-organisms to degrade phenol *via* ring fission has been well studied and both aerobic as well as anaerobic processes have been identified.

With advent of genetic manipulation the era of expanding genetic trait of microorganism to accomodate diverse biodegradative capabilities in a single micro-organism were developed. Existing pathways were made to accomodate new/related substrates by either recruiting new genes or by mutagenizing the existing genes to accomodate related substrates.

Materials and Methods :

Chemicals :

2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was purchased from BDH, Germany. Phenoxyacetic acid was obtained from Sigma, where as phenol (molecular biology grade) was purchased form SRL SISCO Research Laboratories. p-toluidine was obtained from Pune Chemical Laboratories. Catechol was purchased from Emerck, Darmstdat. p-Nitro-phenoxyacetic acid was a gift from Drs. P.J. Chapman and S.K. Paknikar. Chemicals used for preparation of media and buffers were of LR or AR grade and purchased from HiMedia, Qualigens or SRL Sisco Ltd. Nitrilotriacetic acid was purchased from Wilson Laboratories, Bombay. Chemicals used for TLC were obtained from SRL Sisco Ltd.

Microorganisms:

Strain *Pseudomonas cepacia* AC1100 and PT88 (a deletion mutant of strain AC1100) were obtained from Dr. A. M. Chakrabarty, University of Illinois, Chicago.

Table 2.1 : Bacterial Strains Used

Strains	Relevant Properties	Source/Reference
P. cepacia		
1) AC1100	2,4,5 $-T^{+}$, $Benz^{+}$ TCP^{+}	Dr. A. M. Chakraborty University of Illinois, USA.
2) PT88	AC1100::Tn5; *chq*$^{-}$ 2,4,5 $-T^{-}$ Km^{r}, $Benz^{+}$ (Tn-5 induced deletion mutant of AC1100).	Dr. A. M. Chakraborty, University of Illinois, USA.
3) PAA	2,4,5-T^{+}, $Benz^{+}$ phe^{+} (spontaneous mutant of strain AC1100).	Present study.

P. cepacia AC1100 is a wt strain encoding genes for the degradation of 2,4,5-T and benzoate (Kilbane et al.., 1982). P. cepacia PT88 is a Tn-5 induced mutant of strain AC1100, which has a Kanamycin resistant gene of Tn-5 as well as deletion of a part of genes encoding 2,4,5-T degradation, but however still can utilize benzoate. P. cepacia PAA is a spontaneously generated mutant from wt AC1100 obtained during the present study and unlike strain AC1100, is capable of utilizing phenoxyacetic acid and phenol as sole source of carbon and energy.

Table 2.1 gives the genotypes of the P. cepacia strains used in the following studies.

Culture media and Growth of microorganisms

P. cepacia cultures were routinely grown in Basal Salt Medium (BSM, Appendix I) with respective carbon substrate. Glucose was used at a concentration of 0.2%. Phenoxyacetic acid and 2,4,5-T were used at a concentration of 0.08% (w/v); where as phenol was used at a concentration of 1 mM (0.01% w/v). Benzoate was

added to a final concentration of 1 mM. Agar was used at a concentration of 1.7% to prepare BSM agar plates. *Pseudomonas* cultures were routinely grown in BSM at 30 ^{o}C on orbital shaker at 150 rpm with glucose, phenol or benzoate as carbon substrate. For routine maintainence, the cultures were maintained on BSM agar plates with respective carbon substrates.

Isolation of metabolically altered mutants :

P. cepacia AC1100 and PT88 was grown in 100 ml of BSM with 0.2% glucose as carbon substrate for 48 h. The cultures were centrifuged down at 8000 rpm for 10 minutes at 4 ^{o}C and cell pellet was washed twice in 0.85 % KCl. The cell pellet were resuspended in 10 ml of 50 mM phosphate buffer. 0.1 ml of cell suspension was then spread plated on BSM agar plates containing either 0.08% phenoxyacetic acid or 0.01% phenol as sole source of carbon and energy. Viable count of strain AC1100 was obtained by plating 0.1 ml of cell suspension on BSM glucose agar plates. The plates were sealed with parafilm and then incubated at 30 ^{o}C for 3 weeks. Alternatively *wt* AC1100 and PT88 cell suspension was

alsopatched on BSM phenoxyacetic acids or phenol plates by a sterile inoculum loop and incubated as mentioned above.

Phenol utilization by strain PAA

Phenol has a characteristic absorption maxima at 270 nm. Strain PAA cells pregrown in 25 ml of BSM containing 0.1% glucose and 1 mM phenol was centrifuged and washed twice with 0.85% KCl. The cell pellet was finally resuspended in 1 ml of phosphate buffer containing 1 mM phenol. Spectral characteristics of the buffer containing 1 mM phenol was checked after 6 hours on 1201 Spectronic Spectrophotometer, before and after addition of PAA cells.

Similarly, PAA cells pregrown on BSM phenol plates were inoculated in 50 ml of BSM containing benzoate, phenol or glucose as carbon substrates and kept on orbital shaker at 150 rpm. 1 ml samples were asceptically taken out after 24 and 48 h. respectively from each flask, centrifuged at 12,000 g in a microfuge and the supernatant was scanned from 240 to 400 nm

using Spectronic 1201 spectrophotometer. Culture supernatant (24 h) of strain PAA grown in BSM containing 0.2% glucose was used as control during the scan.

Detection of intermediates during phenol metabolism in strain PAA :

a) Catechol - *P. cepacia* PAA was grown on BSM glucose agar plates containing 1.5mM $FeCl_3$ and 1 mM phenol. Prior to plating of PAA culture on the plates , 40 µl of 1 mM *p*-toluidine was spread plated as described by Parke (1992). The plates were incubated at 30 ^{o}C after sealing with parafilm and observed after 2-3 weeks.

α-α'-bipyridyl is known to inhibit catechol 2,3 dioxygenase (Nozaki 1970). Hence PAA cells pregrown in 100 ml of BSM medium containing 0.2% glucose for 36 hrs. were washed twice with 0.85% KCl and then resuspended in fresh sterile BSM medium containing 1 mM phenol with and without bipyridyl (1 mM) to a final absorbance of 1 OD at 560 nm. After 8 hrs of incubation at 30 ^{o}C on shaker at 150 rpm, a fraction of the culture

supernatant was analyzed by thin layer chromatography using benzene-methanol-acetic acid (45:8:4) as solvent system and using silica gel-G as supporting matrix. The relevant spots were identified by exposing the plates to iodine vapours. Phenol spots were identified by spraying a freshly prepared mixture of 1% potassium ferricyanide and 2% ferric chloride. Phenol spots could be further intensified by spraying 2 N HCl. Authentic phenol and catechol was chromatographed on the same plate. Rf values were calculated for unknown spots and compared with Rf values of authentic controls.

b) α-Hydroxymuconic semialdehyde-. PAA cells grown on BSM agar plates containing phenol as carbon source, were sprayed with 10 mM catechol to check for the formation of yellow colour intermediate, α-hydroxymuconic semialdehyde (HMS).

Similarly 100 ml of cell free extracts prepared from PAA cells pregrown in BSM containing 1 mM phenol (refer preparation of cell free extract) was incubated with 100 µl of 10 mM catechol in a final volume of 3 ml of 50 mM phosphate buffer. Absorbance at 375 nm was measured at different time intervals.

c) γ- Oxalocrotonate- PAA cells pregrown in BSM phenol were sonicated to prepare cell free extract as mentioned below. For detection of γ-oxalocrotonate from α-HMS, reaction mixture contained 50 μm Na_2 EDTA, 1 μm of NADH,100 nm of α-HMS and 200 μl of cell free clarified extract obtained at 35,000 g at 4 °C. Clarified extracts containing high level of NADH oxidase were used to avoid interference due to the formation of NADH. The reaction mixture was incubated, till all the semialdehyde had disappeared. This reaction mixture was used for baseline correction before scanning. Oxalocrotonate formed was later detected by absorbance at 350 nm after adding 1 drop of 10 M NaOH (pH 12) to the same reaction mixture as mentioned by Buswell (1974).

Preparation of resting whole cells :

Exponentially growing cells of *Pseudomonas* strains grown in 100 ml of BSM medium containing 0.1% glucose and respective carbon substrates were incubated at 30 °C on orbital shaker at 150 rpm. The cells were centrifuged at 8000 rpm for 10 minutes at 4 °C. The cell

pellet was washed twice in 0.85% KCl and later resuspended in ice cold phosphate buffer (50mM,pH 7) to a final absorbance of 5 OD units at 560 nm. The cell suspension was kept at 4 ^{o}C till further use.

Preparation of cell free extracts :

Exponentially growing cells of *Pseudomonas* strains (250 ml) were harvested by centrifuging at 8000 rpm for 10 minutes at 4 ^{o}C. The cell pellet was washed twice with 0.85% KCl and resuspended in 3 ml of ice cold 50 mM phosphate buffer (pH 7) containing 10% acetone (v/v). The cell suspension was sonicated at 4 ^{o}C by a Vibra cell sonicator (Sonic & Material Inc. Connecticut, USA) at 20 seconds interval for 6 minutes using microtip probes. The sonicated suspension was centrifuged at 12,000 rpm at 4 ^{o}C for 30 minutes. The supernatent was used for enzyme assays and detection of intermediates during metabolism of phenol, by strain PAA.

Determination of protein concentration :

Protein concentration was determined by Biuret method with Bovine serum albumin as standard protein.

Enzyme Assays :

a) Phenoxyacetic acid monooxygenase : Strain AC1100 , PT88 and PAA were grown in 100 ml of BSM medium containing 0.1% glucose and 0.08% 2,4,5-T as carbon sources. Exponentially growing cells were obtained by centrifuging at 8000 rpm for 10 minutes at $4^{o}C$. The cell pellet was washed with 0.85% KCl and resuspended in 50 mM phosphate buffer to a final absorbance of 0.5 OD at 560 nm. 1 ml of the cell suspension was incubated at $30^{o}C$ with 2 mM*p*-nitrophenoxyacetic.The amount of *p*-nitrophenol formed in the culture supernatant was measured at 410 nm. Monoxygenase activity was expressed as nmole of *p*-nitrophenol formed per minute per mg dry weight of cells. Extinction coefficient of *p*-nitrophenol was found to be 1.05×10^{-4} M.

b) Phenol hydroxylase- Phenol hydroxylase activity was determined by measuring the oxygen uptake by the resting

whole cells in presence of phenol using Gilson 5\6 Oxygraph . The resting whole cells pregrown in BSM containing 0.1% glucose and 0.01% phenol were prepared as mentioned previously. 100 µl of resting cells was added to 1.6 ml of preaerated 50 mM phosphate buffer in the micro-cuvette containing the platinum electrode. Endogenous oxygen consumption of resting whole cell was measured in absence of carbon substrate. Later phenol was added to microcuvette at a final concentration of 0.005% to 0.01% The oxygen uptake after addition of phenol was subtracted from endogenous oxygen consumption. The activity was expressed as nmole of oxygen consumed per minute per mg dry weight of cells. An OD of 0.5 at 560 nm corresponded to 0.2 mg dry weight cells.

c) Catechol 2,3 dioxygenase- Catechol 2,3-dioxygenase was assayed by measuring the increase in absorbance at 375 nm due to formation of α-hydroxymuconic semialdehyde which is yellow in colour. The assay system in a final volume of 3 ml contains 100 µl of 10 mM catechol, 3.7 ml of 50 mM phosphate acetone buffer and 100 µl of cell free extract (Nozaki, 1970). The specific activity was expressed as u/mg of protein. one

unit of enzyme corresponds to an optical density increase of 14.7 per minute (Nozaki, 1970).

d. <u>Catechol 1,2-dioxygenase</u> - Catechol 1,2-dioxgenase activity was determined by measuring the increase in absorbance at 260 nm due to formation of <u>cis,cis</u>-muconic acid. The assay system in a final volume of 3 ml contains 2.7 ml of 50 mM phosphate buffer, 60 μl of 10 mM catechol, 300 μl of crude extract and 3 μl of 5 mM EDTA. The specific activity was expressed as U/mg of protein. One unit of enzyme corresponds to optical density increase of 5.6 per minute (Feist and Hageman 1969). In mutant PAA, catechol 1,2-dioxygenase was measured after incubating the protein extract with 25 mM mercaptoethanol for 30 minutes where 80% of the catechol 2,3-dioxygenase activity was lost. Catechol 1,2-dioxygenase activity was stable and no change in the activity was found as reported elsewhere (Dorn and Knackmuss, 1978).

e) <u>Hydroxymuconic-semialdehyde hydrolase (HMSH)</u>- Hydroxymuconic semialdehyde hydrolase was determined by measuring the rate of decrease in absorbance at 375 nm as the strongly absorbing substrate was converted to non

absorbing product as mentioned by Feist and Hageman (1969). All rates reported, were measured in the absence of added NAD^+. In a final volume of 3 ml of buffer, 100 µl of crude extract was added and hydroxymuconic semialdehyde was added to a final concentration of 2.5 µm. The reaction was monitored by decrease in absorbance at 375 nm.

f) Hydroxymuconic-semialdehyde dehydrogenase (HMSD)

Hydroxymuconic semialdehyde dehydrogenase was assayed by measuring the rate of decrease in absorbance at 375 nm using similar reaction mixture as above but in presence of 0.10 µm NAD^+.

Preparation of a-hydroxymuconic semialdehyde (HMS) :

The substrate a-HMS was prepared by preheating the crude extract at 53 ^{o}C for 10 minutes and incubating the crude extract with 10 mM catechol in 50 mM phosphate buffer, till the formation of HMS stopped (no further increase in absorbance at 375 nm). The incubation reaction mixture was extracted twice with equal volume

of diethyl ether to remove catechol. The aqueous layer was acidified (pH 2), centrifuged to remove precipitated protein and reextracted with diethyl ether to remove HMS from aqueous mixture. The ether layer containing HMS was later extracted with a suitable volume of 50 mM phosphate buffer and used immediately for assay of HMSD and HMSH.

Results

Development of spontaneous mutant utilizing phenoxyacetic acid and phenol from strain AC1100.:

P. cepacia AC1100 besides utilizing 2,4,5-T has a capability to metabolize benzoate. However, it does not utilize phenoxyacetic acid and phenol (Karns *et al.*, 1983). In order to explore the possibility of expanding the pre-existing catabolic trait of AC1100 strain, washed cell suspensions of AC1100 were densely patched\plated on BSM agar plates containing 0.08% phenoxyacetic acid as the sole source of carbon. After incubation of the culture plate at 30 ^{o}C for 3-4 weeks, few colonies emerged on the plates. The individual

colonies were picked up and purified by repeated streaking. All isolates were identical and exhibited the ability to grow on 1 mM phenol in addition to phenoxyacetic acid, 2,4,5-T and benzoate. Simultaneously when washed cell suspension of AC1100 were densely inoculated on BSM agar plates containing 0.01% phenol, similar type of spontaneous mutants emerged having capability to grow on phenol as well as phenoxyacetic acid. The emergence of these spontaneous mutants on phenoxyacetic acid or phenol plate was reproducible at a very low frequency of 1×10^{-8}. These spontaneous mutants were designated as P. cepacia strain PAA.

Washed cell suspension of P. cepacia mutant strain PT88 was also patched\plated on BSM agar plates containing 0.08% phenoxyacetic or 0.01% phenol. The plates were incubated at 30 °C for 7-8 weeks. However, PT88 did not generate any spontaneous mutants which could utilize phenoxyacetic or phenol.

Table 2.2 shows the comparison of various substrates utilized by wt AC1100 strain, deletion mutant PT88 and the newly emerged spontaneous mutant PAA.

Table 2.2 : Substrates Utilized by *P. cepacia* AC1100 & its mutant

Strains	2,4,5-T	Benzoate	Phenoxyacetate	Phenol
AC1100	+	+	-	-
PT88	-	+	-	-
PAA	+	+	+	+

\+ Growth - No Growth

Unlike its parent wt AC1100, mutant PAA could use phenol and phenoxyacetic acid in addition to 2,4,5-T and benzoate.

Intermediates of phenoxyaceticacid/phenol biodegradation in strain PAA

a) Detection of catechol

Unlike strain AC1100 and PT88, mutant PAA has a unique ability to metabolize phenoxyacetic acid and phenol. When PAA was patched on BSM plates containing 0.1% glucose, 0.01% phenol,*p*-toluidine and $FeCl_3$, a brown colour formation was observed around the colonies indicating the formation of catechol during utilization of phenol as shown in Fig. 2.1. strain AC1100 and PT88 were also patched as negative control and did not show the formation of brown colour. Catechol was used as positive control and showed dark brown colour formation as seen in Fig 2.1 .

Further confirmation of catechol as central intermediate during phenol metabolism in PAA was

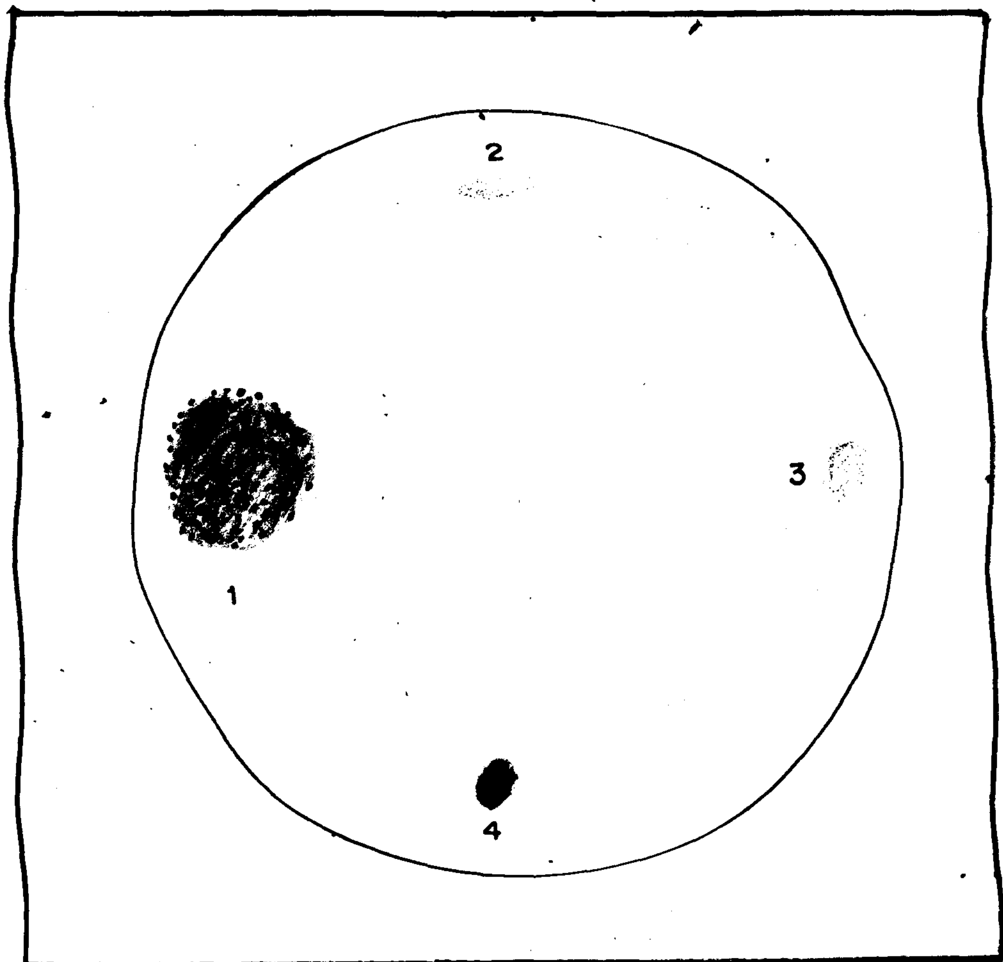


Fig.2.1 Amplification of catechol detection by incorporating p-toluidine in the medium. The petriplate was divided into 4 sectors and bacterial cells were inoculated as a patch. (1) PAA (2) PT88 (3) AC1100 (4) catechol.

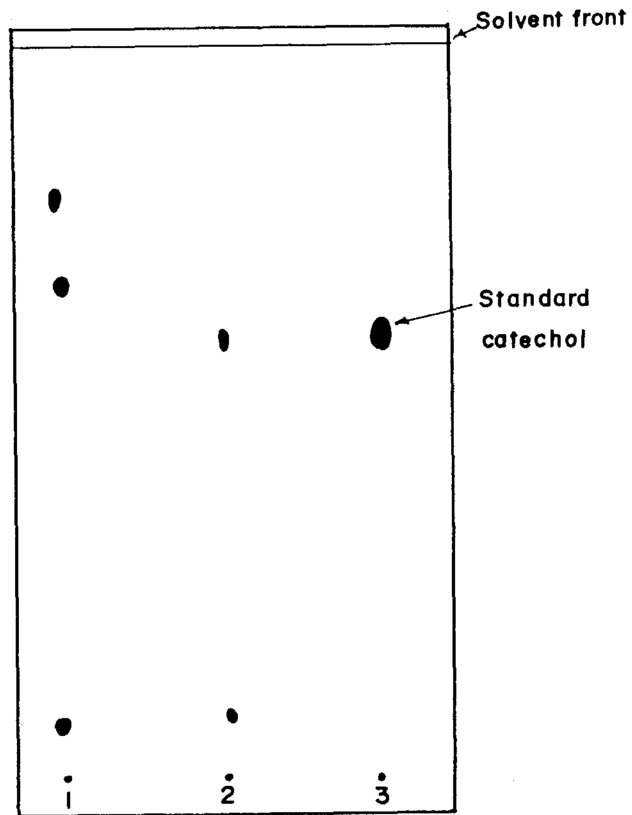


Fig.2.2 Detection of catechol by TLC. (1) culture supernatant of strain PAA grown in BSM phenol. (2) culture supernatant of strain PAA incubated in BSM containing phenol and p-toluidine. (3) authentic catechol.

obtained by Thin Layer Chromatography of culture suprnatant using benzene-methanol-acetic acid 45:8:4) as solvent system. Resting whole cells pregrown in glucose were incubated for 8 hours in 1 mM phenol with or without α,α'-bipyridyl. A fraction of the culture supernatant itself was analysed by TLC using authentic catechol as positive control and phenol as negative control. The PAA cells were found to accumulate a compound having an Rf value of 0.55 which co-migrated with the authentic catechol as seen in Fig 2.2. Spots specific to phenol could not be detected as such when TLC plates were exposed to iodine vapours, however could be detected when sprayed with Ferricyanide -Ferric chloride mixture. Phenol was completely metabolized in PAA grown cells and no spot could be detected at position comparable to authentic phenol. (Fig. 2.3)

b) Identification of HMS as one of the intermediates

PAA cells grown on BSM agar plates containing phenol as sole source of carbon, when sprayed with 10 mM catechol, colonies turned yellow indicating the formation of a-hydroxymuconic semialdehyde . Further,

cell free extracts when incubated with 10 mM catechol, reaction mixture depicted an increase in absorbance at 375 nm, as depicted in Fig 2.4.

d) Identification of γ-oxalocrotonate formation from HMS using cell free extract of strain PAA

γ-oxalocrotonate was detected as a peak having absorbance at 350 nm using clarified crude extract obtained from strain PAA as seen from Fig 2.5.

Growth of PAA on phenol as the sole source of carbon

Figure 2.6 depicts the depletion of 1 mM phenol as depicted by decrease in absorbance at 270 nm when incubated in presence of resting whole cells of PAA. Figure 2.7 depicts the UV-spectrophotometric scan of culture supernatent when PAA was grown in phenol, benzoate or glucose as carbon source. Culture supernatent was scanned after 24 hours and 48 hours for the detection of intermediates. Culture supernatant from Strain PAA grown in glucose did not show any

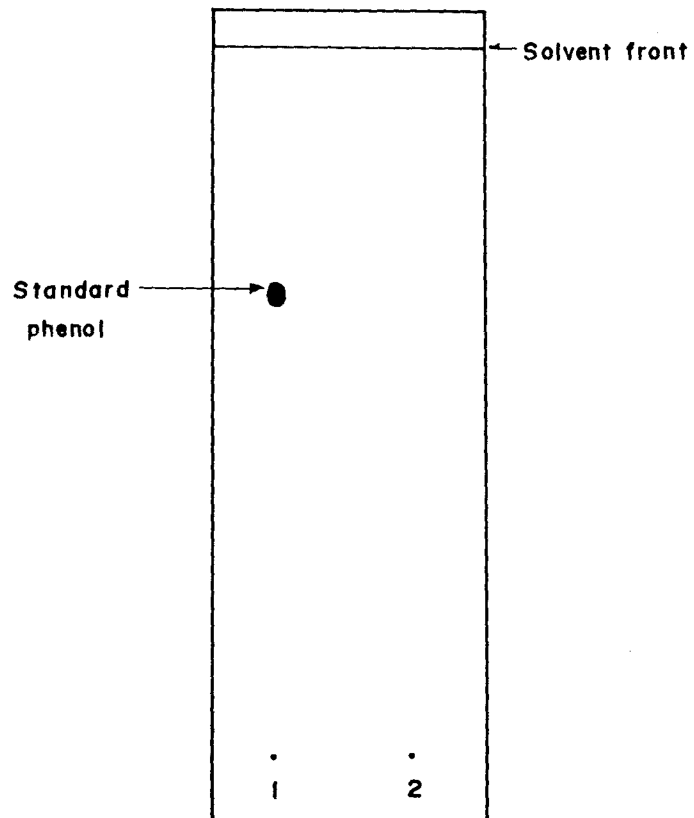


Fig.2.3 Phenol disappearance as observed by TLC. (1) authentic phenol. (2) culture supernatant of strain PAA grown on phenol.

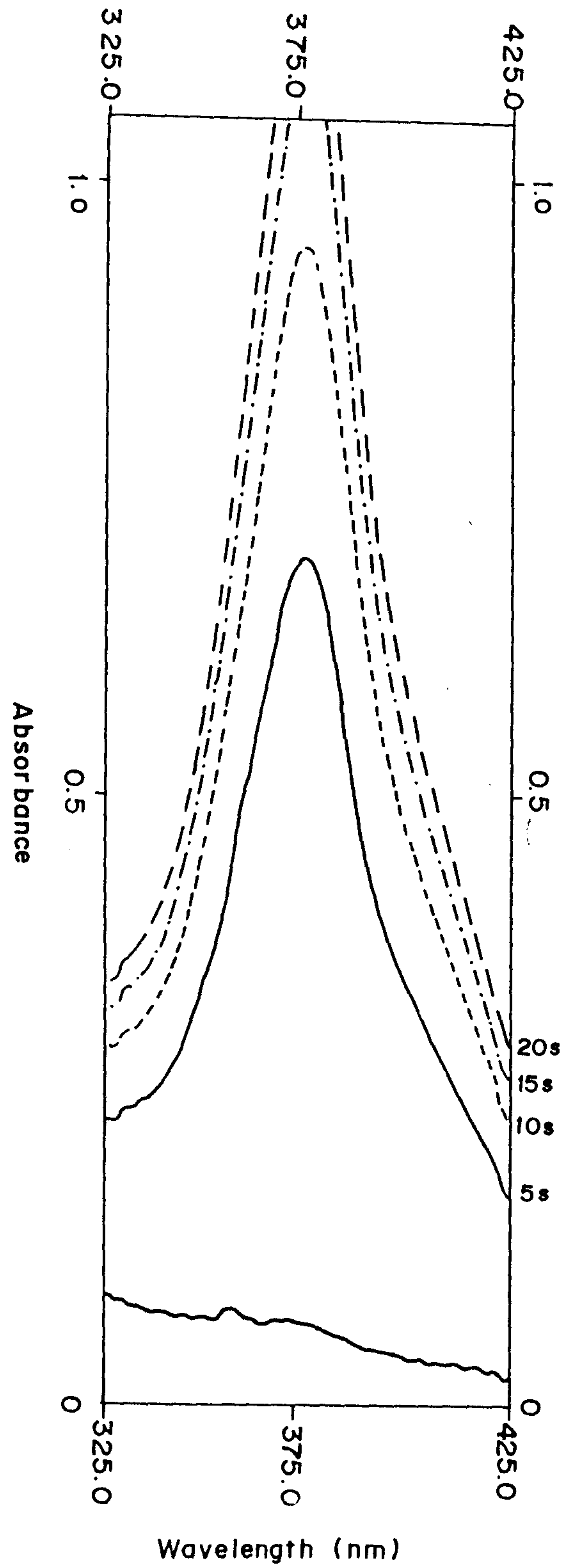


Fig. 2.4 Spectral scan at different time intervals in seconds for the detection of a-hydroxymuconic semialdehyde using cell free extract from strain PAA.

characteristic absorption peak [Fig. 2.7(1)]. When PAA cells were grown in phenol, culture supernatant scan after 24 h showed a characteristic peak at 270nm which is the absorption maxima for phenol. It also showed a peak at 278 nm which was presumed to be due to presence of catechol [Fig.2.7(2)]. However, when scan after 48 h, the peak with absorption maxima at 270 and 278 nm were not observed indicating complete mineralisation of phenol by strain PAA [Fig. 2.7(3)]. Culture supernatant of strain PAA grown in benzoate showed a characteristic peak at 375nm, 278nm, 264nm and 248nm [Fig. 2.7(4)]. However, after 48 hours, peak with absorption maxima at 278nm, 264nm and 248nm were still detected [Fig. 2.7(5)]. The peak at 375nm detected only during 24 hours scan was due to the formation of yellow colour HMS. Strain AC1100 when grown in benzoate did not show any yellow coloured intermediate formation. The peak having absorption maximum at 264 and 278nm indicate transient formation of cis,cis-muconic acid and catechol respectively. (Nakazawa & Nakazawa, 1970). The peak having absorption maxima at 248 nm however could not be identified.

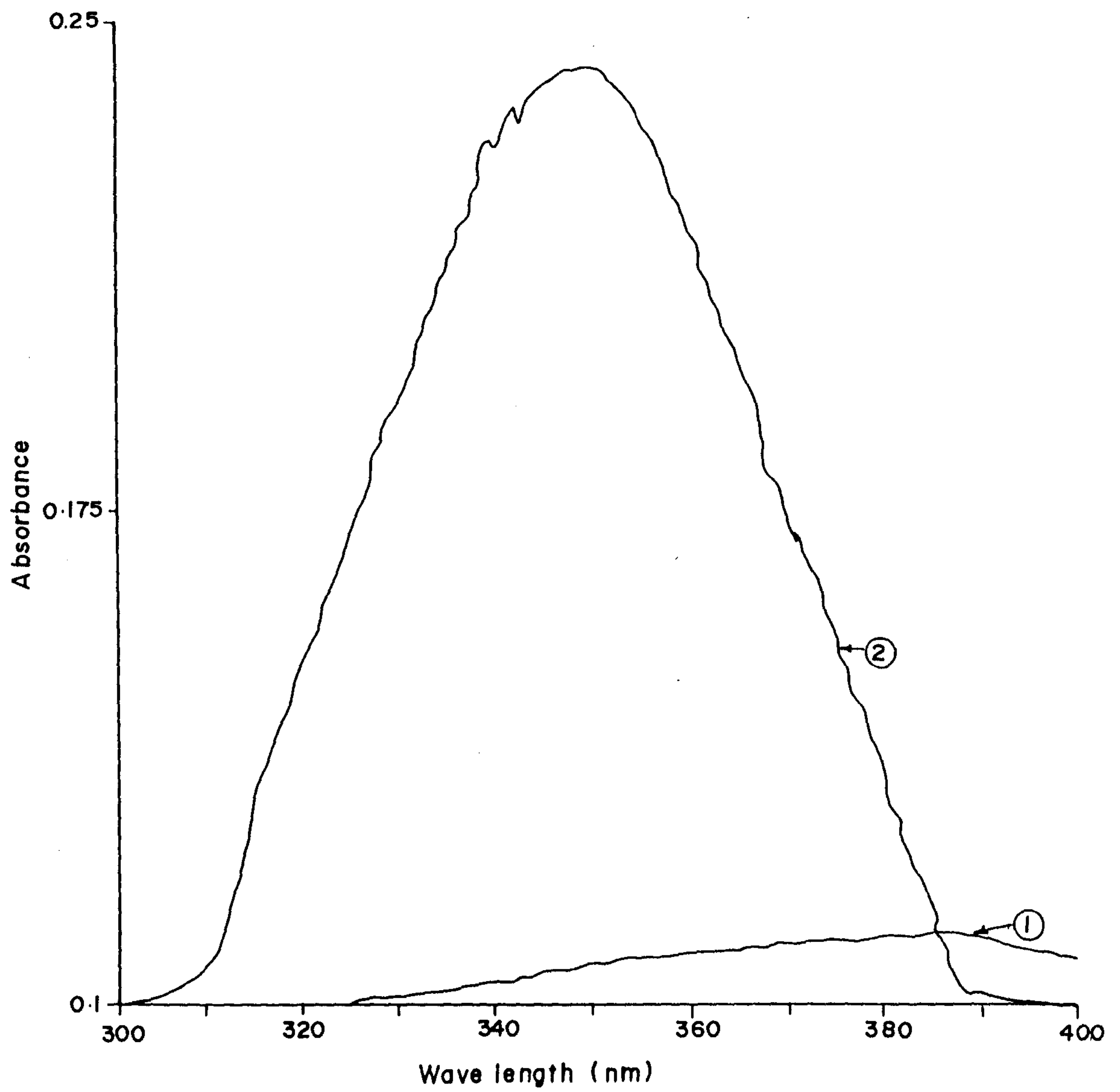


Fig.2.5 Detection of r-oxalocrotonate formation in strain PAA. (1) control without cell free extract. (2) after addition of cell free extract from strain PAA.

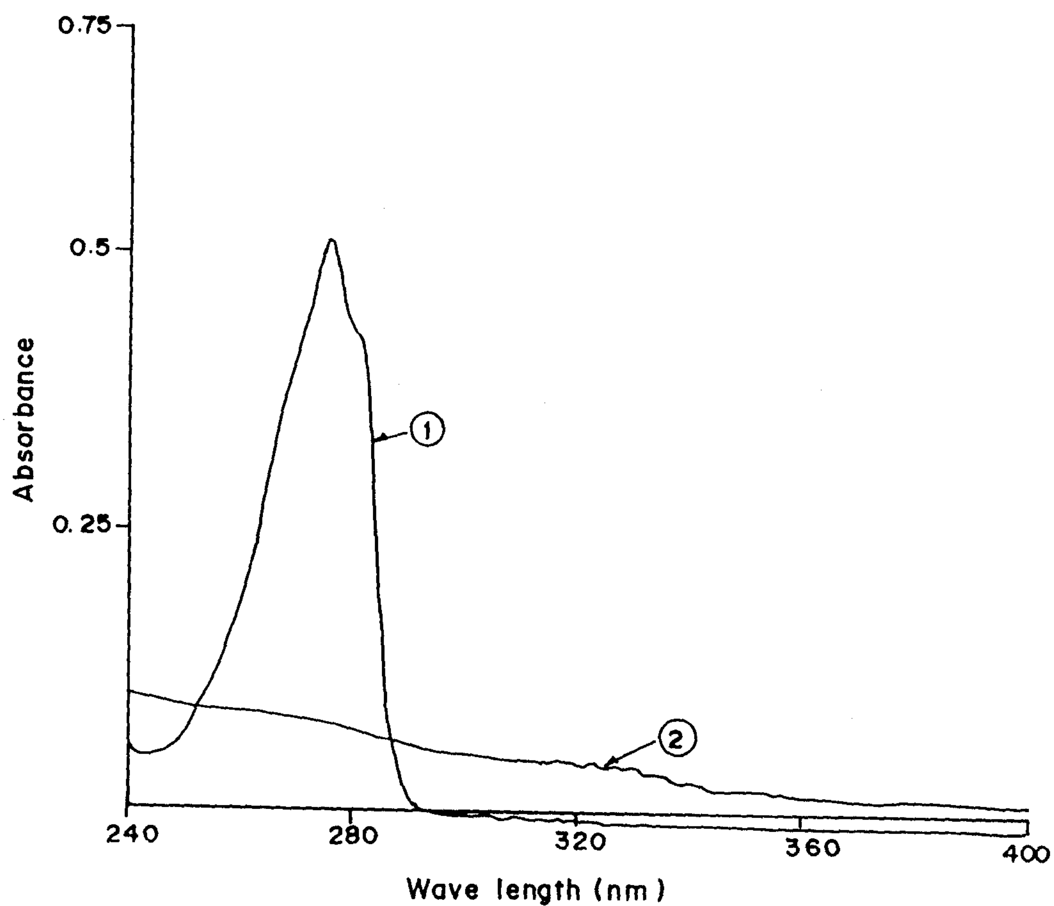


Fig.2.6 Phenol utilization by resting whole cells of strain PAA. (1) BSM medium with 1mM phenol as control. (2) 6 h. after addition of resting cells of strain PAA.

Oxidation of aromatic compounds by strain AC1100 and its mutants

Rates of oxidation of benzoate, phenol and catechol by resting cells of AC1100 and its mutant, pregrown in various growth substrates is as summarized in table 2.3. It can be clearly seen that strain AC1100 and its mutant PT88 do not have the ability to oxidize phenol, but they do metabolize benzoate and catechol via ortho-cleavage pathway. In fact, the addition of 0.005% phenol to resting cells of strain AC1100 and PT88 was found to be toxic and even the endogenous respiration was affected. P. cepacia PAA however, oxidize phenol. Washed cells of PAA pregrown on either glucose or on glucose plus phenol exhibited the ability to oxidize phenol without any lag. Further, when PAA was grown in glucose and benzoate, no appreciable change was detected in the ability to oxidize phenol. The ability to oxidize benzoate was inducible in strain AC1100, PT88 and PAA in presence of benzoate and glucose. Also the oxidation of catechol was inducible in AC1100 and PT88, however in strain PAA it was not inducible, even in the presence of primary aromatic substrates like benzoate or phenol as seen from table 2.3. Though, strain AC1100, PT88 and

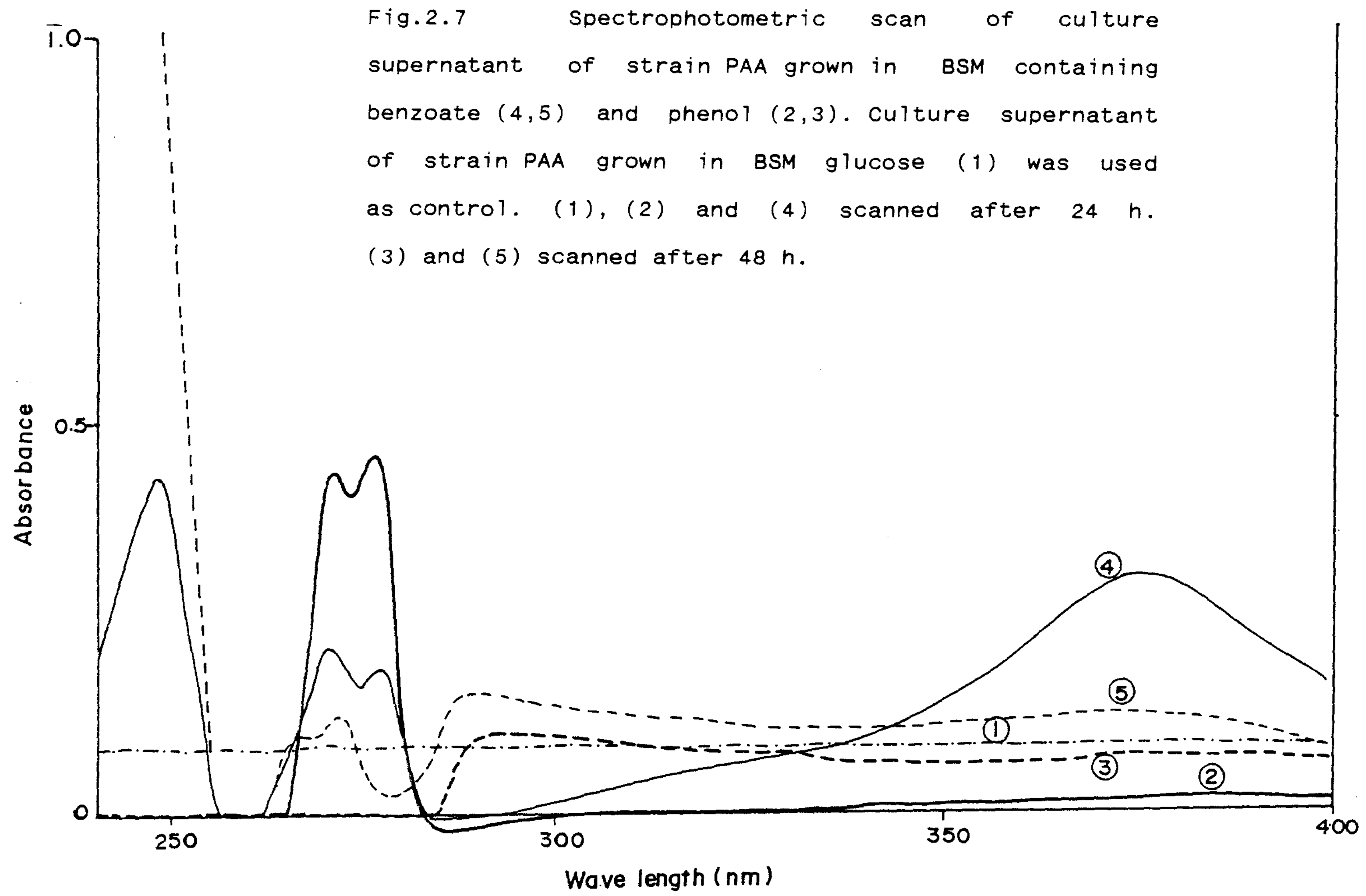


Fig.2.7 Spectrophotometric scan of culture supernatant of strain PAA grown in BSM containing benzoate (4,5) and phenol (2,3). Culture supernatant of strain PAA grown in BSM glucose (1) was used as control. (1), (2) and (4) scanned after 24 h. (3) and (5) scanned after 48 h.

PAA showed inducible benzoate and catechol oxidation activity in presence of benzoate and glucose together, the ability to oxidise phenol and catechol by PAA drastically decreased when phenol and glucose were used as growth substrates .

Enzymes involved in phenoxyacetic and phenol biodegradation in strain PAA.

Strain PAA has active phenoxyacetic acid monooxygenase similar to AC1100 and PT88 as seen from Table 2.4. However, unlike strains AC1100 and PT88, PAA could continue to grow in presence of phenoxyacetic acid.

Strain PAA has phenol hydroxylase activity, as evident from the oxygen uptake by washed cells in presence of phenol (Table 2.3). PAA cells also show catechol 2,3-dioxygenase activity, this being the first enzyme of *meta*-cleavage pathway. As observed from table 2.5, catechol 2,3-dioxygenase in PAA did not show any appreciable change whether PAA was grown in glucose,

Table 2.3 : Rates of oxidation of benzoate, phenol and catechol by resting cells of AC1100 and its mutants

Strains	Growth Substrate	O_2 uptake in nmole/min/mg cells		
		Benzoate	Phenol	Catechol
AC1100	Glucose	50.0	n.d.	13.0
	Glucose + Benzoate(1mM)	125.0	n.d.	20.5
PT88	Glucose	11.0	n.d.	25.0
	Glucose + Benzoate (1mM)	78.0	n.d.	45.0
PAA	Glucose	22.5	35.0	100.0
	Glucose + Benzoate (1mM)	46.5	29.0	63.5
	Glucose + Phenol (1mM)	22.5	21.0	60.0

n.d. - not detected.

Table 2.4 : Phenoxyacetic acid monooxygenase activity in P. cepacia strain AC1100 and its mutants

Strains	Phenoxyacetic acid monooxygenase (nmole of p-nitrophenol formed/min/mg dry weight of cells from p-nitrophenoxyacetic acid)
AC1100	2.13
PT88	2.30
PAA	1.75

Table 2.5 : Specific activities of enzymes involved in catechol assimilation in strain AC1100 and its mutants

Strains	Growth	Specific activities mU/mg			
		C12O	C23O	HMSH	HMSD
AC1100	Glucose	2.5	n.d.	n.d.	n.d.
	Glucose + Benzoate (1mM)	5.0	n.d.	n.d.	n.d.
PT88	Glucose	0.1	n.d.	n.d.	n.d.
	Glucose + Benzoate (1mM)	4.7	n.d.	n.d.	n.d.
PAA	Glucose	14	74	43	129
	Glucose + Benzoate (1mM)	6.6	90	26	117
	Glucose + Phenol (1mM)	4.5	77	17	71

n.d. - not detected

C12O - Catechol 1,2 dioxygenase; C23O - catechol 2,3 dioxygenase; HMSH - HMS hydrolase; HMSD - HMS dehydrogenase.

benzoate or phenol, unlike catechol 1,2 dioxygenase activity which was observed to decrease in presence of benzoate or phenol.

As seen from Table 2.5, strain PAA cells also showed hydroxy muconic-semialdehyde hydrolase (in absence of NAD^+) and hydroxymuconic semialdehyde dehydrogenase (in presence of added NAD^+) activity. Though C,12O activity was detected in strains AC1100, PT88 and PAA ; C23O, HMSH and HMSD activity were detected only in strain PAA . HMSH and HMSD activity was not inducible in PAA cells when grown in glucose alongwith benzoate or phenol.

Discussion

Pseudomonas cepacia AC1100 can utilize 2,4,5-T as the sole source of carbon and energy (Kilbane et al.., 1982). It was initially developed in a chemostat by Plasmid Assisted Molecular Breeding under strong selective pressure to utilize 2,4,5-T (Kellogg et al., 1981). The 2,4,5-T degradative pathway has not been elucidated completely except for the identification of

two intermediates , 2,4,5-trichlorophenol and chlorohydroxy-hydroquinone (Karns et al., 1983; Chapman et al., 1987).

Members of Pseudomonas group are known for their versatility in mineralizing a vast majority of natural and synthetic organic compounds (Stanier et al., 1966). The unique manner in which such novel catabolic pathways are evolved is often attributed to degradation or activation of cryptic genes, relaxed substrate specificity of enzymes and molecular mechanisms that include recruitment and expression of foreign genes (Coco et al., 1990; Meer et al., 1992).

Since one of the intermediates during 2,4,5-T utilization was found to be 2,4,5-trichlorophenol, efforts were directed to explore the possibility of expanding the pre-existing catabolic traits of Strain AC1100 by training the organism to utilize non chlorinated analogs such as phenoxyacetate and phenol, as sole carbon source. The principle for the generation of metabolically altered mutants was based on the fact that the enzymes can evolve to accomodate closely related analogues of organic substrates. This would be possible

by inducing mutation in the relevant genes of the micro-organism, when grown under stress/evolutionary pressure in the presence of same analogues. According to the evolutionary principles, the organisms which fail to adapt will be eliminated and only those organisms adapting to degrade new toxic compounds would survive and emerge. Such horizontal expansion of catabolic trait for microorganism has been already achieved (Ramos *et al.*, 1987, Ramos *et al.*, 1986). *Alcaligenes eutrophus* JMP134 utilizing 2,4-dichloro-, 4 chloro-2-methyl- and 2- methyl phenoxyacetic acid could not however utilize chlorinated phenoxyacetic acid. Spontaneous mutants of JMP134 gave rise to phenoxyacetic acid utilizing strain, which was possible because of the ability of phenoxyacetate to induce 2,4-D monoxygenase gene (Pieper *et al.*, 1989).

P. cepacia AC1100 when patched on BSM phenoxyacetic acid plates gave rise to spontaneous mutants which were capable of degrading phenoxyacetic acid besides 2,4,5-T. These mutants also could grow on phenol. Emergence of mutant from AC1100 on phenol plates were similarly capable of utilizing phenoxyacetic acids.

AC1100 cannot grow on phenoxyacetic acid and phenol (Karns _et al._, 1983). Initial cleavage of ether bonds in 2,4,5-T by monooxygenes generates 2,4,5-trichlorophenol in AC1100. Emergence of spontaneous mutants on exposure to phenol and their ability to grow on phenoxyacetic acid and _vice versa_ implies that an event of mutation in the existing silent genes of AC1100 responsible for phenol utilization has enabled the microorganism to accomodate phenoxyacetic utilization. Assuming that the initial attack on phenoxyacetic acid in PAA is mediated by same monooxygenase present in strain AC1100 (table 2.4), the immediate intermediate should be phenol. This is indeed the case as Danganan _et al._ (1994) have cloned the 2,4,5-T oxygenase gene which converted 2,4,5-T, 2,4- D and phenoxyacetate to TCP, DCP and phenol, respectively. Thus it is clear from the results that all spontaneous mutants emerging on phenoxyacetic plates also utilized phenol as the sole source of carbon because of activation of genes specifically involved in phenol utilization.

The genetic adaptation of _P. cepacia_ AC1100 which led to the development of mutant strain PAA can be

attributed either to relaxed substrate specificity of the enzymes involved in the 2,4,5-T degradation or to activation of a certain set of genes which were dormant in strain AC1100. Micro-organisms are known to degrade closely related compounds or analogues because of development of variants/mutants with relaxed substrate specificity (Ramos et al., 1986; Ramos et al., 1987). However, the failure of PT88 to give rise to any spontaneous mutant growing on phenol could be attributed to the fact that genes involved in phenol utilization might be present in the deleted region of the chromosomal DNA, which otherwise has been known to be absent in PT88 (Sangodkar et al., 1988). However, if phenol was indeed channelized via 2,4,5-T pathway due to mutation resulting in relaxed substrate specificity of enzymes, PT88 could also have given rise to spontaneous blocked mutants in phenol biodegradation which instead of chlorohydroxyquinone (CHQ) would have accumulated hydroxy benzoquinone, which would have been detected in the medium as a coloured compound on immediate autooxidation (Sangodkar et al., 1988).

Thus the emergence of spontaneous mutant PAA from AC1100, and not PT88, having the capability to degrade

phenoxyacetic acid and phenol suggests that cryptic genes for phenol biodegradation unrelated to 2,4,5-T degradation have undergone a mutation or rearrangement, so as to confer the capability of phenol utilization in strain PAA. Our results contrasts the observation of Pieper _et al._ (1989) who observed that non-chlorinated analogue of phenoxyacetic acid could be made to metabolise through the existing 2,4-D pathway in _Alcaligenes eutrophus_ by isolating spontaneous mutants which could be induced by phenoxyacetate unlike the wild type.

Strain AC1100 and its mutants, namely PT88 and PAA could utilize benzoate. AC1100 and PT88 utilize benzoate _via_ inducible _ortho_-cleavage pathway. Thus, though strain AC1100 converts phenoxyacetic acid to phenol by the 2,4,5-T monooxygenase enzyme (Danganan _et al._, 1994), the accumulation of phenol as a toxic product, results in the death of organism, as it could not be channelized by any of the known genes involved in 2,4,5-T degradation because of stringent substrate specificity of enzymes.

Thus, a single event of mutation resulting in activation of cryptic phenol hydroxylase gene in AC1100 would result in the emergence of mutant PAA, which now could utilize phenoxyacetic acid as well as phenol, preventing the accumulation of phenol by converting it to catechol. The resultant catechol so formed could converge into inherent ortho-cleavage pathway for benzoate metabolism present in PAA. However, it is known that phenol is generally metabolized by inducing functional genes of meta-cleavage pathway (Dagley, 1968). Though the results indicate the existence of ortho-cleavage pathway in AC1100 and its mutants, there is clear evidence of existence of meta-cleavage pathway only in phenoxyacetic acid utilizing mutant strain PAA. Enzyme studies also revealed that unlike in AC1100 and PT88, PAA contained a fully operational enzyme system namely phenol hydroxylase, C23O, HMSH and HMSD. At the same time, all meta-cleavage pathway enzymes were found to be quite active in PAA even in presence of functional ortho-cleavage pathway as is evident from the HMS formation in the cultures of PAA grown in presence of benzoate (Fig. 2.7).

Thus PAA metabolized phenoxyacetic acid and phenol via *meta*-cleavage pathway as evident from the detection of intermediates like catechol, HMS and γ-oxalocrotonate during phenol metabolism. When PAA was grown in benzoate though the benzoate oxidase activity was found inducible, C12O enzyme activity was not inducible in PAA. This could be attributed to the active catechol 2,3 dioxygenase activity which is maximally expressed in mutant PAA as a result of which most of the catechol is possibly being channelized via *meta*-cleavage pathway and is not available for catechol 1,2 dioxygenase action which is inducible only by its product, *cis,cis*-muconic acid (Bayley and Dagley, 1969). Similarly, the high activity of catechol 2,3 dioxygenase was more evident during the growth of PAA in presence of benzoate, when a yellow coloured product in the culture supernatent having a λ max of 375 nm was detected and which is the characteristic absorbance maxima of α-hydroxymuconic semialdehyde. This indicates the spill-over of catechol from benzoate into *meta* cleavage, which is found to be expressed in mutant PAA, preventing its entry in to *ortho* pathway and thus decreasing the inducibility by preventing the formation of *cis,cis*-muconic acid which is needed for induction of catechol 1,2-dioxygenase.

Though strain PAA, has an active HMS hydrolase activity, which was measured in absence of added NAD^+, cell free extract when incubated with catechol and added NAD^+ showed the presence of γ-oxalocrotonate as one of the intermediates confirming the fact that P. cepacia PAA has an active phenol degradative gene involved in phenol degradation via the meta-cleavage pathway. Fig2.8 shows the pathway proposed for the phenol biodegradation in mutant PAA.

The slight decrease in phenol hydroxylase activity as evident from the oxygraph studies might be due to catabolite repression phenomena, as mutant PAA was grown in phenol in presence of glucose for enzymatic studies. Catabolite repression on phenol hydroxylase activity is well established in phenol utilizing T. cutaneum. Glucose inhibited the induction of phenol oxidation by non proliferating cells and also inibited phenol oxidation by pre-induced cells.(Gaal and Neujahr, 1981). However, Pohl and Fritsche (1981) reported the degradation of phenol simultaneously with assimilation of glucose and pyruvate in P.putida

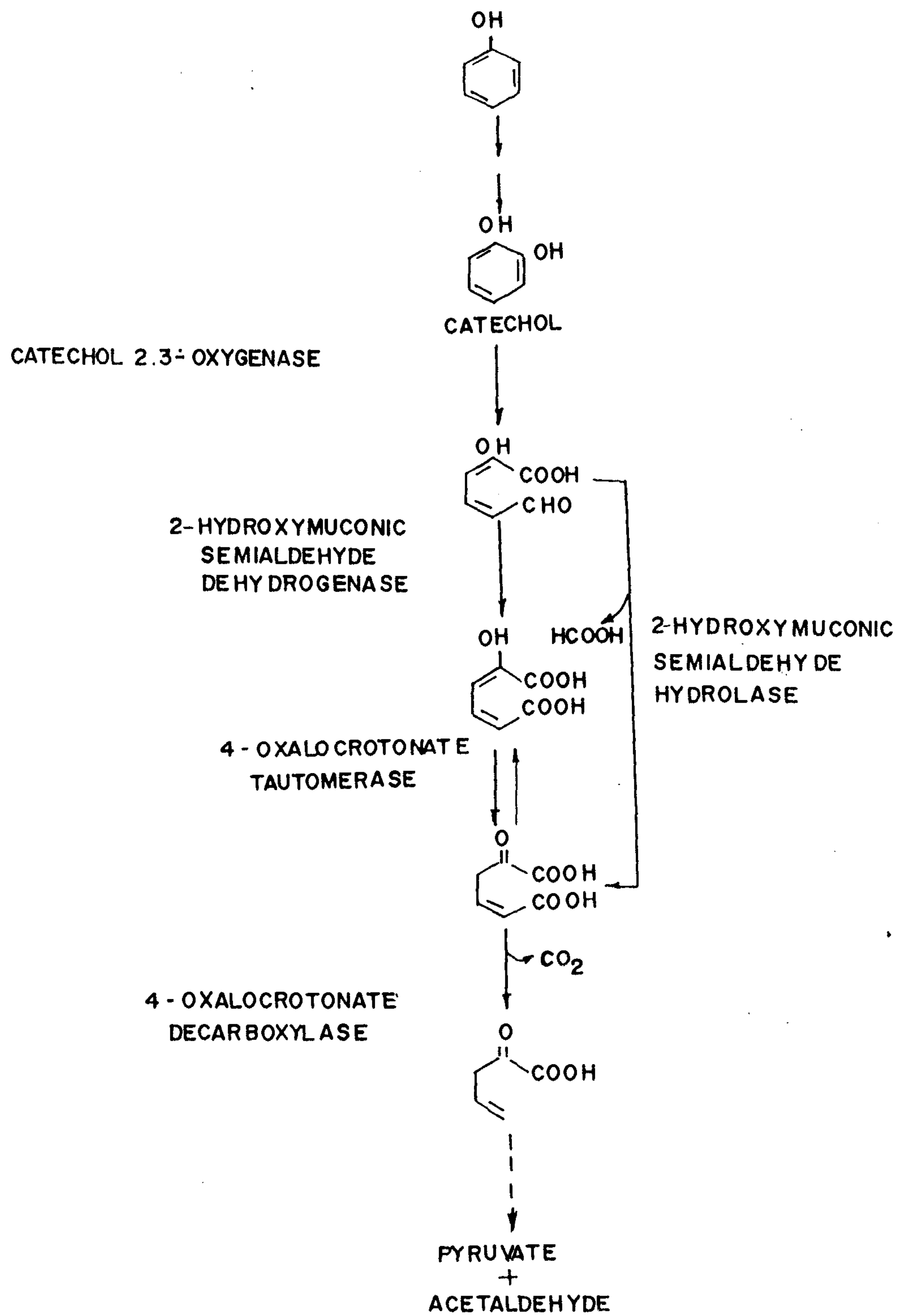


Fig.2.8 Metabolism of phenol in _P. cepacia_ PAA.

Thus the activation of cryptic genes in mutant PAA, conferring the ability of phenol utilization has been further studied to understand the molecular process involved in such activation and by trying to simulate the same molecular process in the cloned fragments from genomic library of AC1100 and characterizing it further to understand exact mode of activation of phenol degradation in strain PAA (results given in chapter V).

Chapter II reported the emergence of a strain PAA from *P. cepacia* AC1100 which had unique capability to utilize phenoxyacetic acid and phenol due to the activation of cryptic genes. Phenol and phenolic compounds are toxic pollutants and inhibitors of biodegradation and have been used as models to test the effect of inhibitory substrates on cellular metabolism and growth kinetics (Folsom *et al*. 1990; Hill and Robinson, 1975; Yang and Humphrey, 1975).

Kinetic models are of value in investigating both the capacity and stability of biological processes which utilize inhibitory substrates. Haldane's expression has been widely used to study inhibition of phenol degradation at high phenol concentrations (Folsom *et al*. 1990; Hill and Robinson, 1975).

We report here kinetics for degradation of phenol by intact cells and inhibitory substrate utilization. Besides, the ability of strain to degrade other analogues of phenol and related compounds has also been studied.

Materials and Methods :

Growth of strain PAA on phenol :

Strain PAA cells pregrown on BSM phenol plates were picked by a sterile inoculum loop and inoculated in 100 ml of BSM medium containing 1 mM phenol. The flask was incubated at 30 ^{o}C on an orbital shaker at 150 rpm. One ml of sample was collected aseptically from the flask at different time intervals. Cell concentration was determined spectrophotometrically at 560 nm using a Spectronic 1201 Spectrophotometer. A small fraction of the sample (10 µl to 100 µl) was used for determining the concentration of phenol remaining in the medium as detailed below.

Determination of phenol in the medium: Phenol concentration was determined spectrophotometrically at 500 nm using the aminoantipyrene method as described by Folsom *et al*. (1990). The phenol concentration was determined by taking 10 µl - 100 µl of the sample in a microfuge tube containing 50 µl of 2 N NH_4OH and 25 µl of 2% 4-aminoantipyrene. The tubes were closed and the

contents were mixed. 25 μl of 8% $K_3Fe(CN)_6$ was added . The contents were mixed again and the volume was made to 1 ml with distilled water. The tubes were centrifuged at 12,000 x g for 2 minutes. The absorbance of the supernatant was measured at 500 nm. Phenol concentrations were calculated by reference to a standard curve as mentioned below.

Phenol (Molecular biology grade) crystals were used to prepare a 1 M stock solution in distilled water. Phenol was added in different microfuge tubes, at concentrations ranging from 0.5 μg to 3 μg in a final volume of 1 ml. 50 μl of 2 N NH_4OH and 25 μl of 2% 4-aminoantipyrene were added and the contents were mixed. 25 μl of $K_3Fe(CN)_6$ was then added and the absorbance was measured at 500 nm and a graph of absorbance vs phenol concentration (μg/ml) was plotted. The concentrations used for standard assay were within the linear range of absorbance at 500 nm.

Degradation of various concentrations of phenol by strain PAA :

P. cepacia PAA was grown in BSM containing 1 mM phenol as the sole carbon source. To check for the capability of mutant P. cepacia PAA to degrade different concentrations of phenol, 0.5% (v/v) of inoculum was added to 100 ml of BSM containing 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 2.5 mM or 5 mM phenol as the sole carbon source and incubated at 30 ^{o}C on rotary shaker at 150 rpm. At defined intervals, 1 ml of sample was aseptically taken from each flask. Cell concentration was measured spectrophotometrically at 560 nm. Phenol concentration was determined by the modified aminoantipyrene method as described above.

Oxidation of various aromatic compounds by strain PAA.

P. cepacia PAA cells were pregrown in 100 ml of BSM media with 1 mM phenol as carbon source. Exponentially growing cells of Pseudomonas strains were harvested by centrifuging at 8000 rpm for 10 minutes at 4 ^{o}C. The pellets were washed in 0.85% KCl

and resuspended in ice-cold phosphate buffer (50 mM, pH 7) to give a final absorbance of 5 OD units at 560 nm. Rates of oxygen uptake by washed cells of PAA in presence of various aromatic substrates were measured on a Gilson 5/6 Oxygraph at 30 °C. 100 µl of cell suspension was added to 1.6 ml of oxygen-saturated phosphate buffer (50 mM, pH 7) and respective substrate added. Phenol, resorcinol, hydroxybenzoate *m*-cresol, aminophenol, nitrophenol, hydroxyquinone and other aromatic compounds were used as substrate at a concentration of 0.005%. Rates of oxygen uptake were corrected for the endogenous respiration of cells and expressed as µmole of oxygen /min/mg cells.

<u>Stoichiometry of phenol and catechol consumption by strain PAA</u> :

P. cepacia PAA cells were pregrown in BSM containing 0.1% glucose and 1 mM phenol or 1mM benzoate. Exponentially growing cells of PAA were harvested at 8000 rpm at 4 °C. The cell pellet was washed twice with 0.85% KCl and then resuspended in 50 mM phosphate buffer (50 mM, pH 7) to give a final absorbance of 5 OD units

at 560 nm. In a final volume of 1.6 ml, 100 μl of resting whole cells of PAA were used for oxygraph studies. After measuring endogenous respiration, the respective substrates were added and the oxygen consumption in presence of substrate was measured until it fell to endogenous levels. The corrected oxygen consumption was expressed as mole of oxygen consumed/mole of substrate.

Kinetics of Phenol degradation :

Rates at which P. cepacia PAA cells degraded phenol were determined by monitoring changes in phenol concentration using the modified colorimetric assay as mentioned before. P. cepacia PAA was grown in 500 ml of BSM containing 1 mM phenol at 30 ^{o}C on rotary shaker at 150 rpm. Exponentially growing cells were harvested by centrifuging at 8000 rpm for 10 minutes at 4 ^{o}C. The cell pellet was washed twice in 0.85% KCl and recentrifuged. The cell pellet was resuspended in 50 mM phosphate buffer, pH7 to give a final absorbance of 0.5 unit at 560 nm.

In the phenol disappearance assay, phenol was added to 10 ml of resting whole cells (in duplicate), to give a final concentration ranging from 10 to 500 µm. At defined intervals, 1 ml of sample was transferred from each tube to microcentrifuge tubes and phenol concentration was estimated by the modified aminoantipyrene method at 500 nm. Phenol disappearance rates were calculated and reported as nanomole per minute per milligram cells. The rates were calculated from six determinations over a 10 minute time period. These values were then plotted against initial phenol concentration. The K_s and V_{max} values were estimated from the Lineweaver-Burk plot and used to modify the hyperbolic curve. Inhibition of phenol degradation at high phenol concentration was modelled by a Haldane's expression which incorporates a second order inhibitory term K_{si} into the Michaelis Menten 's expression. K_s is the Michaelis constant for cellular kinetics and is analogous to K_m for enzymatic reactions. [S] is the substrate concentration and v_o is the initial reaction rate (Folsom et al. 1990).

$$v_o = \frac{Vmax[S]}{[S] + \frac{1}{K_{Si}}[S] + Ks}$$

Oxygen uptake at various concentrations of phenol by resting cells of strain PAA

Strain PAA cells were pregrown in BSM containing 1 mM phenol. Resting cells were prepared as mentioned in Chapter II. Oxygen uptake was studied at various concentrations ranging from 0.005% to 0.2% of phenol.

Determination of Km of Catechol 2,3-dioxygenase from strain PAA :

P. cepacia PAA was grown in 250 ml of BSM containing 0.1% glucose and supplemented with 1 mM phenol. Exponentially growing cells were harvested at 8000 rpm for 10 minutes at 4 °C. The cell pellet was washed twice with 0.85% KCl and later resuspended in 3 ml of 50 mM phosphate buffer(pH7) containing 10% acetone. The cell suspension was sonicated at 4 °C for 5 minutes using a Vibra cell sonicator. Cell free extract was obtained by centrifuging at 10,000 rpm for 10 minutes at 4 °C. The supernatant was used for the assay

of catechol 2,3-dioxygenase, which was carried out as mentioned in chapter I. Initial reaction rate (v_o) was determined at different substrate concentrations. K_m of catechol 2,3-dioxygenase was determined using the Lineweaver-Burk plot.

Determination of biomass concentration

Biomass concentration was determined indirectly using absorption spectroscopy at 560 nm. The absorbance and dry weight of the *Pseudomonas* strain PAA was determined at various cell densities and plotted. A linear relationship was observed. The biomass concentration (x) in mg/ml was given by the following relation x= 0.4 (OD at 560 nm).

Results

Degradation of phenol by strain PAA in batch cultures:

The spontaneous mutant *P. cepacia* PAA formed on phenoxyacetic/phenol plates from *P. cepacia* AC1100 (Chapter II) was grown in BSM medium at pH 7 in presence of 1 mM phenol as the sole source of carbon. Degradation of phenol as measured spectrophotometrically by the 4-aminoantipyrene method was well correlated with growth of strain PAA as measured by increase in absorbance at 560 nm (Fig. 3.1). Phenol-pregrown PAA cells inoculated in 100 ml of BSM medium at an initial cell mass of 0.004 mg dry wt resulted in a yield of 0.104 mg dry wt/ml, after incubation for 31 h. Figure 3.2 depicts the optical density at 560 nm plotted against time, indicating a linear growth. The specific growth rate monitored was 0.13 h^{-1}. The phenol degradation corresponded to 4.66 μM/h. Phenol was found to be totally degraded from 100 ppm to 1.5 ppm within 35 hours.

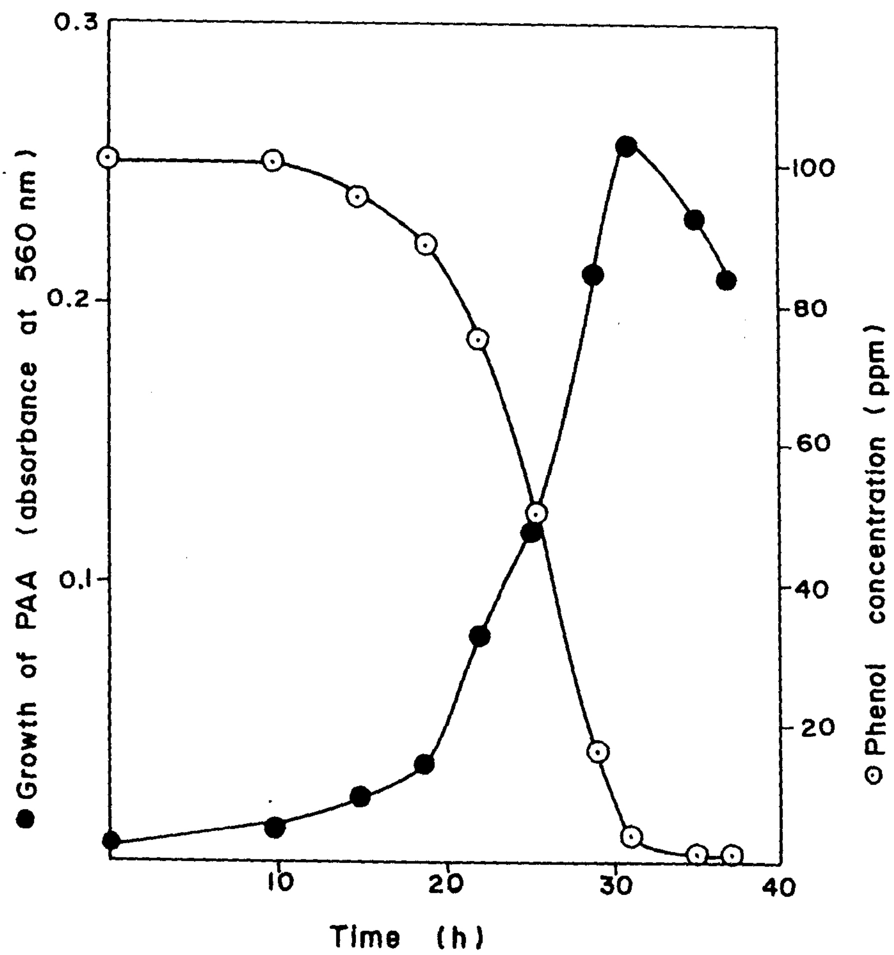


Fig.3.1 Batch culture of <u>P.cepacia</u> PAA in BSM containing 1mM phenol. ●-growth of PAA; ⊙-phenol remaining in the medium.

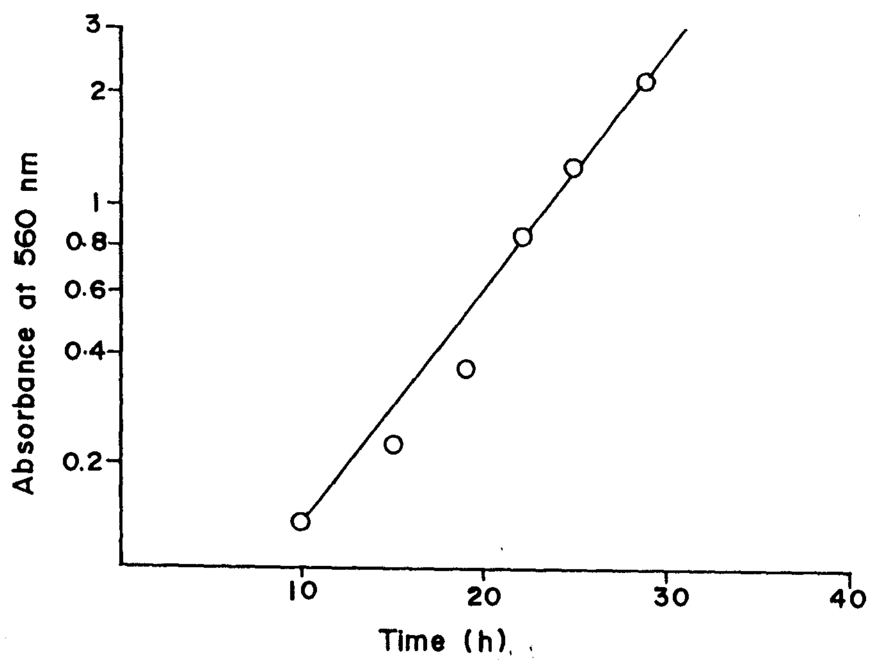


Fig.3.2 Semilogarithmic plot of optical density at 560 nm *verses* time for batch growth of PAA on 1 mM Phenol.

Degradation at various phenol concentrations by strain PAA.

Strain P. cepacia PAA was able to degrade phenol completely up to a concentration of 5 mM. Increasing phenol concentration resulted in increased cell mass of strain PAA, with simultaneous increase in lag period (Table 3.1).

Further, even at a high concentration of 5 mM, phenol could be completely degraded to a final concentration of 2 ppm. Figure 3.4 depicts the batch degradation of phenol at various concentrations by P. cepacia PAA. Table 3.1 indicates the lag period, cell mass, phenol degradation rate and specific growth rate of strain PAA at various concentrations of phenol. Figure 3.3 clearly shows the growth of P. cepacia PAA at various concentrations of phenol. There was a lag of about 13 hours at phenol concentration up to 1 mM, whereas above concentration of 1 mM phenol, the lag period was increased up to 38 hours, after which growth was exponential correlating with decrease in phenol from the medium. The increase in PAA cells as measured by absorbance at 560 nm correlated with increase in cell

Table 3.1 : Batch degradation at various concentration of phenol by strain PAA.

Initial Phenol concentration (mM)	Lag period (h)	Growth rate h^{-1}	Degradation rate.(mg/h)	Yield mg %
0.1	8	0.115	0.85	2.4
0.25	9	0.111	1.6	3.24
0.5	12	0.123	4.0	7.36
1.0	13	0.125	5.33	9.44
2.5	26	0.120	7.5	22.12
5.0	38	0.100	16.66	37.04

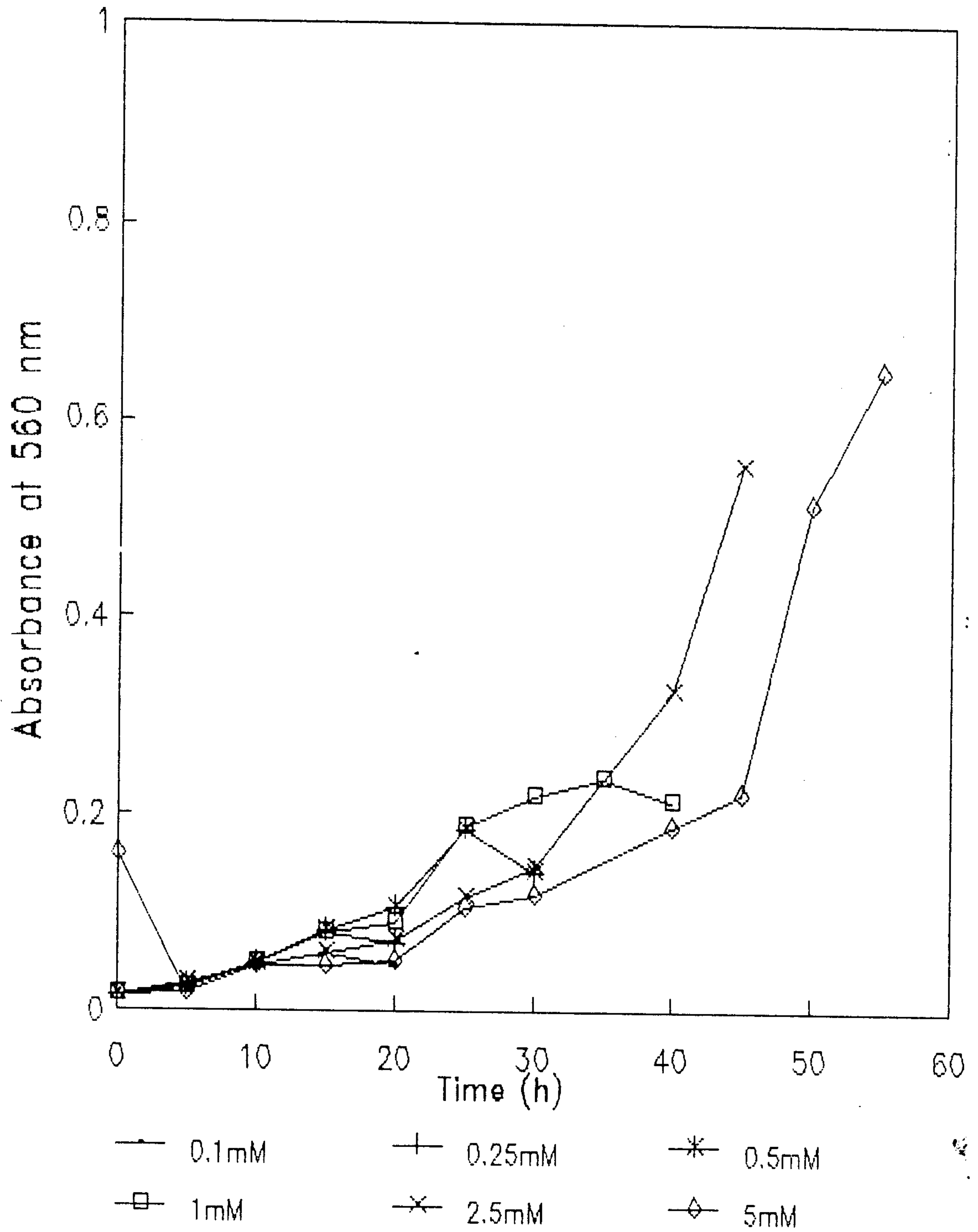


Fig.3.3 *Growth of PAA* at various concentrations of phenol.

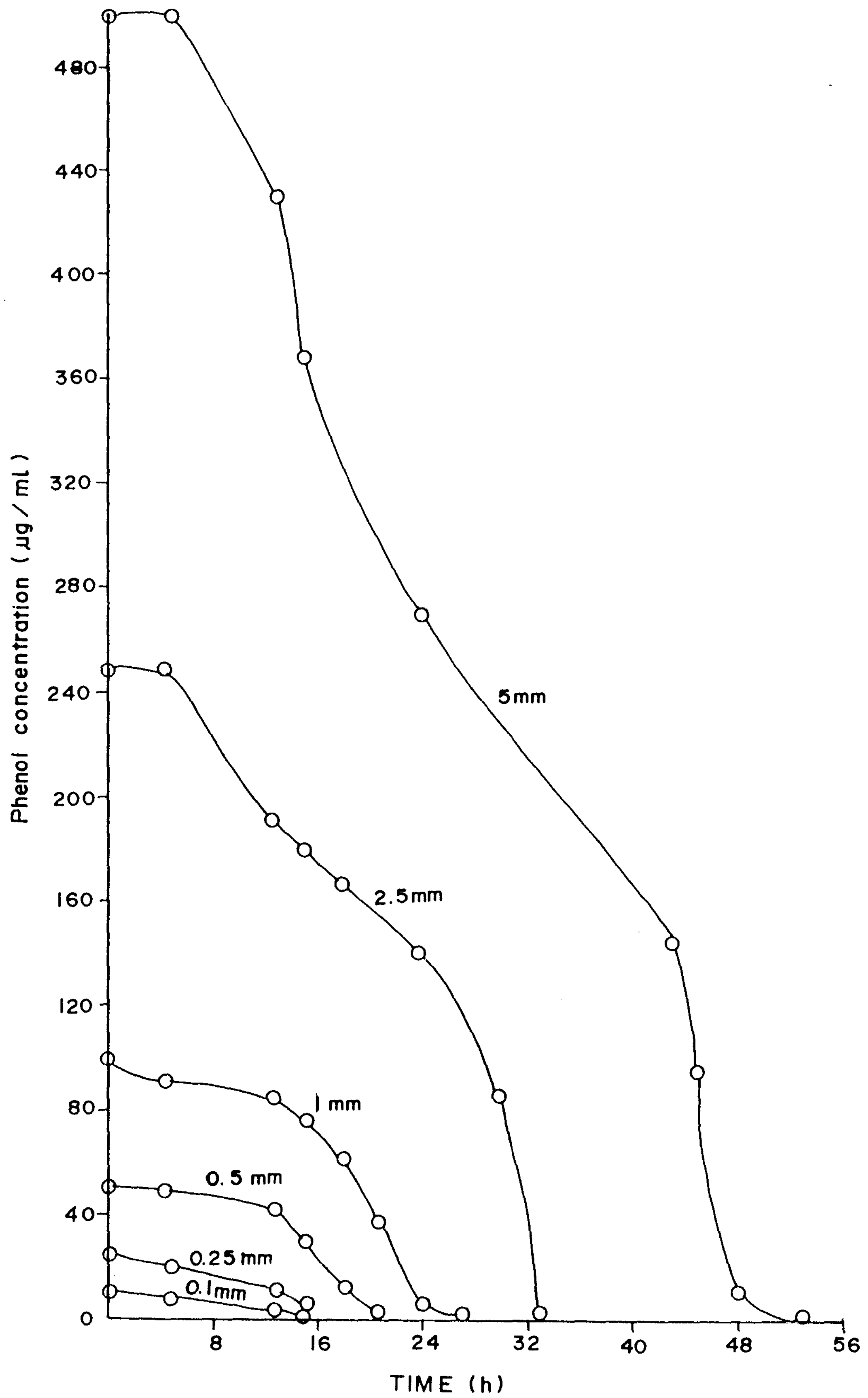


Fig.3.4 Biodegradation by strain PAA at various concentration of phenol.

mass which has been expressed as mg%. Figures 3.3 and 3.4 have been replotted as final absorbance _versus_ different substrate concentrations (Fig. 3.5). A linear relationship was observed in the above case.

Oxidation of aromatic compounds by resting cells of strain PAA

The results of oxygen uptake by strain PAA with phenol and other related compounds are shown in Table 3.2. It can be seen that many of the phenol-related compounds like resorcinol, cresol and hydroxybenzoate were oxidized, although the rate of oxidation was less than the phenol oxidation rate. Although PAA was obtained as a spontaneous mutant from AC1100 and had the capability to grow on 2,4,5-trichlorophenoxyacetic acid, it did not show any oxidation when exposed to 2,4-dichlorophenol or any of the monochlorophenols. However, other substituted phenols such as nitrophenol and amninophenols could be oxidized at variable rates (Table 3.2). Similarly, besides catechol, certain other diphenols like resorcinol and hydroxyquinol could also be readily oxidized by mutant PAA. Strain AC1100 did not show any oxidation of phenol or phenol-related

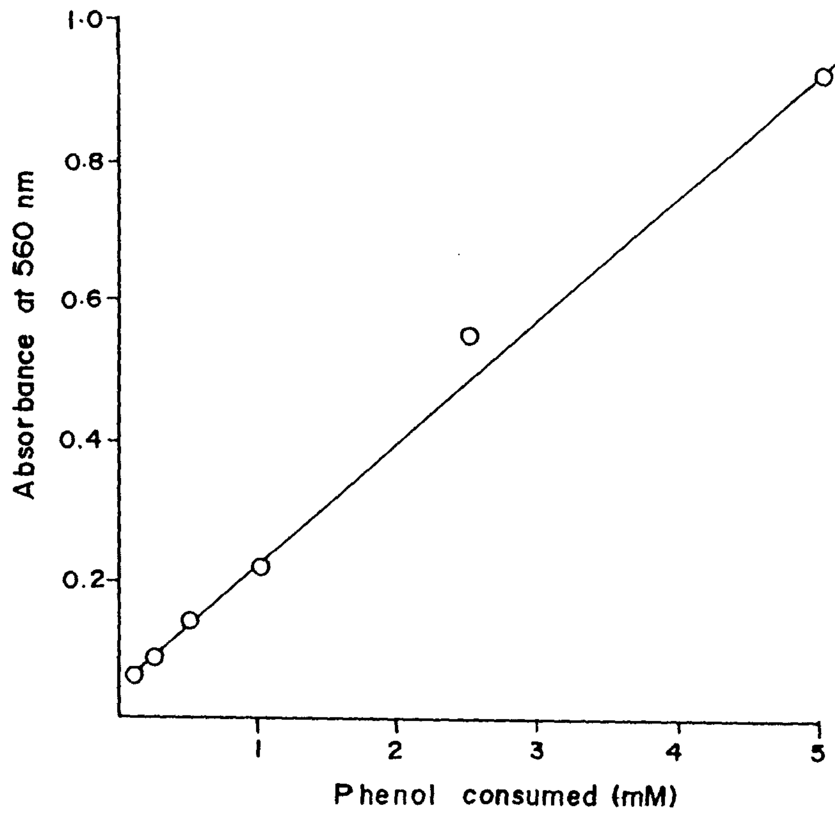


Fig.3.5 Optical density at 560 nm *versus* total substrate consumed for the batch growth of PAA.

Table 3.2 : Oxidation of various aromatic compounds by resting cells of Strain PAA

Substrates	O_2 uptake µmole/min/mg.cells	Relative activity %
Phenol	0.077	100
Hydroxybenzoate	0.013	16.8
Resorcinol	0.038	49.3
_m_-cresol	0.038	49.3
4-nitrophenol	0.032	41.5
2-nitrophenol	0.0153	19.8
_o_-aminophenol	0.0294	38.2
_p_-aminophenol	0.0073	9.47
Hydroquinone	0.0365	47.4
_o/m/p_chlorophenol	< 0	--
2,4-D	< 0	--
2,4,5-TCP	< 0	--

compounds, except for the chlorinated compounds.

Stoichiometry of oxygen uptake by strain PAA :

Table 3.3 depicts the stoichiometry of oxygen uptake by strain PAA in response to phenol or catechol as substrate. Resting PAA cells pregrown in phenol or benzoate showed consumption of one mole of oxygen per mole of catechol. However, such resting cells of PAA showed consumption of only 0.5 mole of oxygen per mole of phenol.

Characterization of phenol degradation rates :

Changes in phenol degradation rates were monitored spectrophotometrically as mentioned previously. Phenol was used as primary growth substrate and addition of phenol did not show any appreciable change in pH.

Kinetic parameters for phenol degradation by PAA cells were determined by measuring phenol disappearance rates. Phenol disappearance exhibited first order rate up to 75 μM. Above 100 μM, phenol disappearance rate

Table 3.3 : Stoichiometry of phenol and catechol oxidation by resting cells of strain PAA

Substrates	mole O_2 consumed/mole of substrate
Phenol (Phenol induced)	0.495
Catechol (Phenol induced)	1.13
Catechol (Benzoate induced)	1.08

dropped significantly (Fig. 3.6).

Since intact whole cell organisms were used instead of purified enzymes, the term K_s was employed instead of K_m. Figure 3.7 indicates the Lineweaver-Burk plot for the same. The K_s and V_{max} values as obtained from Figure 3.7 were 41.73 μM and 11.38 nmole/min/mg cells, respectively.

Inhibition of phenol degradation at higher phenol concentrations was modelled by Haldane's expression which incorporates a second order inhibitory term, K_{si} into Michaelis - Menten expression as mentioned previously. K_{si} value was found to be 175 μM (Fig. 3.8).

The hyperbolic curve (----) redrawn with K_s and V_{max} values estimated from the Lineweaver-Burk plot is shown in Figure 3.6.

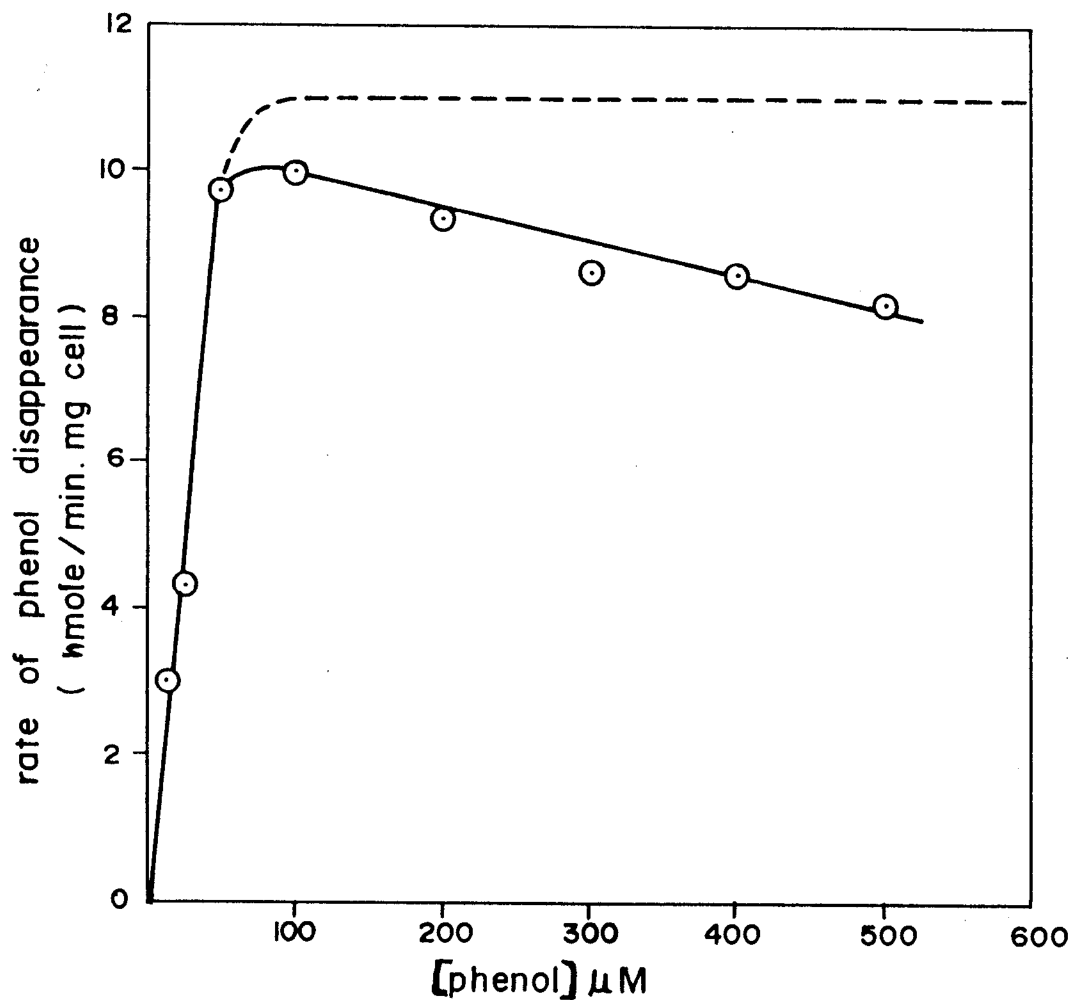


Fig.3.6 Phenol degradation kinetics. Phenol disappearance rate, determined in duplicate, were plotted against initial phenol concentration. The hyperbolic curve (-------) was calculated from K_s and V_{max} values estimated from Lineweaver- Burk plot (see Fig.3.7).

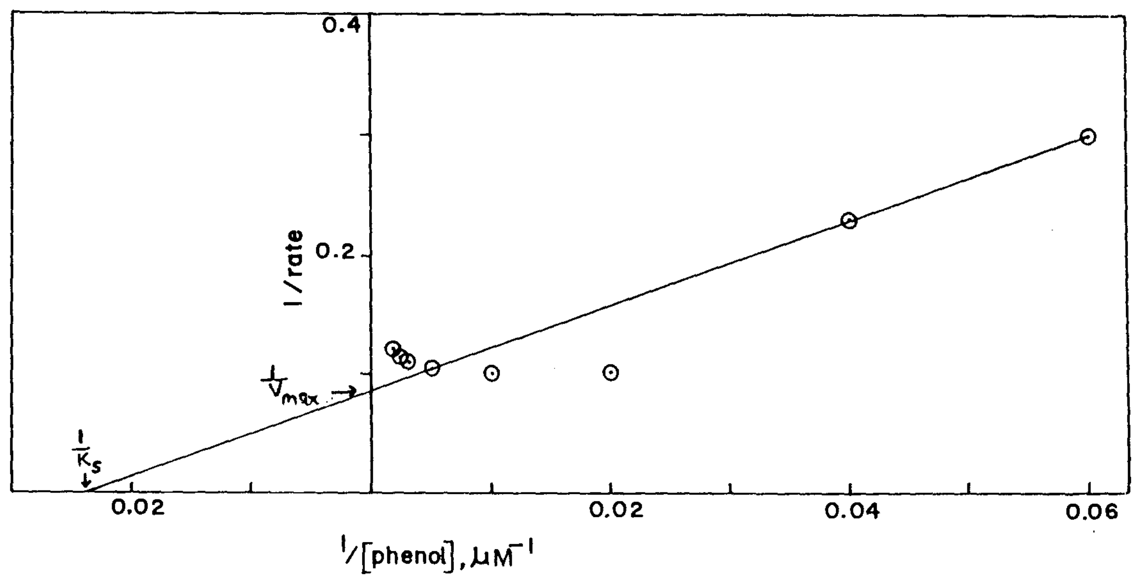


Fig.3.7 Lineweaver -Burk plot to determine k_s and V_{max} for phenol degradation by whole cells of PAA.

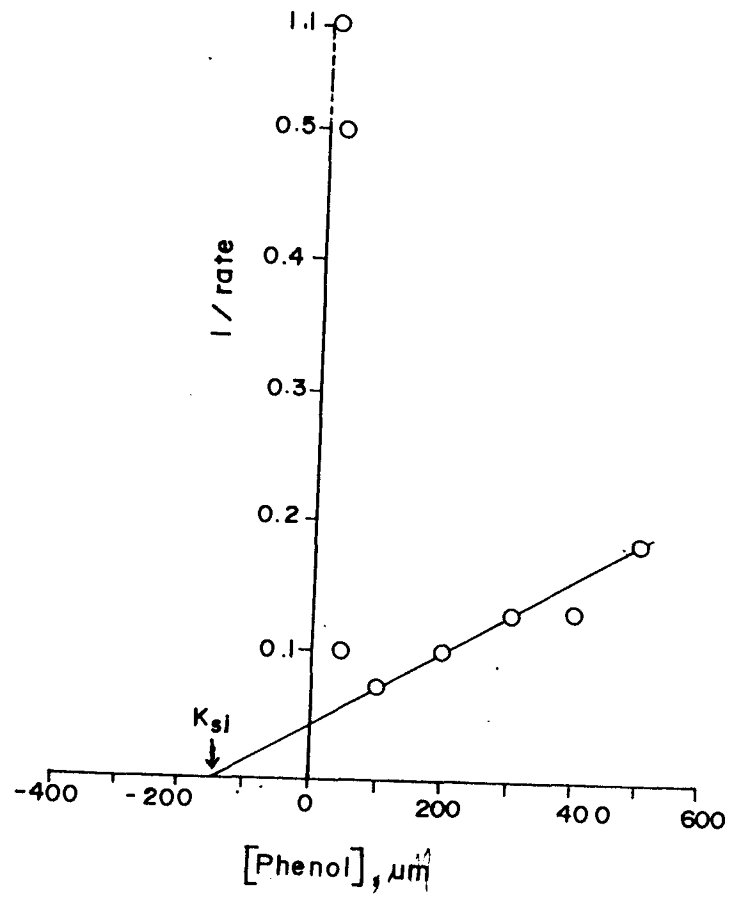


Fig.3.8 Determination of ks_i for phenol degradation by Haldane's plot.

Characteristics of catechol 2,3-dioxygenase in cell free extract of strain PAA :

Activity of catechol 2,3-dioxygenase was assayed using cell free extract from strain PAA. Initial reaction rates were determined at low substrate concentrations. Figure 3.9 gives the Lineweaver—Burk plot of 1/[S] *versus* $1/v_o$. K_m of C23O from PAA was 1.4×10^{-6} M and V_{max} was 0.133 μmole/min/mg protein.

Tolerance of maximum concentration of phenol by strain PAA.

Table 3.4 depicts the tolerance of phenol by strain PAA as measured by its oxygen uptake. Strain PAA oxidized phenol up to 0.15% without showing any inhibition in oxygen uptake. However the ability to oxidize phenol decreased rapidly when phenol was provided as substrate at concentrations above 0.15% and proved inhibitory over 0.2%.

Table 3.4 : Phenol Tolerance by resting whole cells of Strain PAA

Phenol %	Oxygen uptake nM/min/mg cells
0.05	21.76
0.1	19.6
0.15	21.25
0.16	7.48
0.18	4.93
0.2	< 0

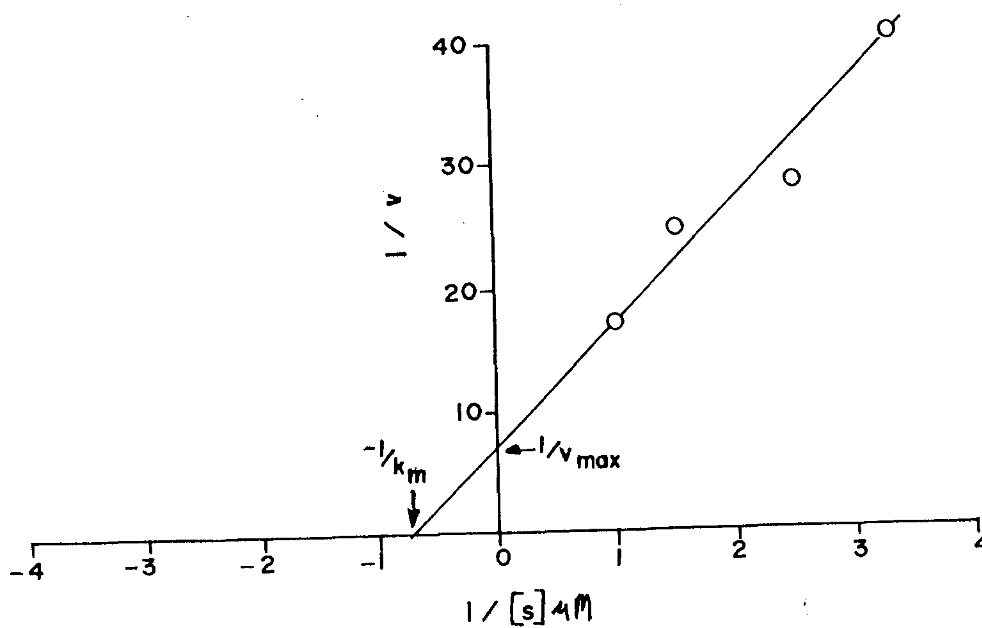


Fig.3.9 Lineweaver-Burk plot for k_m determination of C23O from PAA.

DISCUSSION

P. cepacia PAA was obtained as a spontaneous mutant by forcing strain AC1100 to grow on phenoxyacetic acid or phenol. The mutant strain PAA could grow on phenol and phenoxyacetic acid, unlike the *wt* AC1100. Chapter II clearly indicates the utilization of phenoxyacetic acid and phenol through *meta*-cleavage, independent of the 2,4,5-T biodegradative pathway.

This chapter covers studies to ascertain total mineralization of phenol and its degradation kinetics.

The presence of an active enzyme machinery in strain PAA, as mentioned in chapter II seems to be responsible for complete degradation of phenol as evident from batch culture experiments. Strain PAA provided with phenol as sole source of carbon and energy showed increase in cell biomass with simultaneous loss of phenol from medium. The absence of an active enzyme machinery from strain AC1100 and PT88 as mentioned in Chapter II, results in their inability to utilize phenol as carbon source. The PAA culture was inoculated from

phenol plates, as a result of which a lag of 13 h was observed at 1mM concentration. This long lag was not due to substrate inhibition, but solely due to low concentration of inoculum. Complete degradation of phenol was observed within 30 h, with exponential decrease of phenol from the initial 100 ppm to 2 ppm. There was decrease in cell mass after the phenol concentration in the medium decreased to 2 ppm, presumably due to cell death and lysis. As seen from Figure 3.2, there is a linear increase in absorbance at 560 nm from 10 hrs onwards and the specific rate was estimated to be 0.13 h^{-1}. The specific growth rates reported for Pseudomonas putida during simultaneous batch degradation of phenol and cresol were 0.388 and 0.340 h^{-1} respectively (Hutchinson and Robinson, 1988). P. putida EKII was also shown to degrade phenol upto 8.5 mM and the maximal growth rate was 0.16h^{-1} (Hinteregger et al. 1992).

Most of the earlier reports on phenol degrading bacteria as mentioned in the literature review (Chapter I) indicate that they have been isolated from environmental sites which had been exposed to phenol-like pollutants in the past. Some of these cultures

could degrade phenol anaerobically at a higher rate after being acclimatized with phenol. We report here the isolation of a phenol-degrading mutant of parent strain P. cepacia AC1100 which itself did not have the capability to utilize phenol because of non-expression of the genes specifying the requisite pathway for phenol degradation. The unique mutant not only shows enzyme machinery involved in phenol metabolism, but also degrades it completely, clearly providing the evidence of genes which were hitherto silent in wt AC1100 strain.

The mutant strain PAA showed no substrate inhibition upto 5 mM, except for the increase in lag period. Figure 3.3 clearly indicates the linear increase of cell mass at various substrate concentrations up to 500 mg/litre. Such linear increase would not have been observed if substrate inhibition or the effect of saturation constant were important. P. putida also showed a similiar type of linear relationship up to 160 mg/litre (Hutchinson and Robinson, 1988). Also, a slight decrease in specific growth rate was observed when PAA was grown up to 500 mg/l. However Yang and Humphrey (1975) observed a rapid decrease in specific growth rate as concentration

of phenol was increased to 500 mg/l.

The biomass yield factor as calculated from Figure 3.5 is 1.81×10^{-3} in units of optical density increase per mg of substrate consumed. The yield factor was converted to units of g biomass produced per g substrate consumed using the proportionality constant of 400 (mg/litre at OD 560 nm) which resulted in a value of 0.72 g biomass produced per g substrate consumed.

As seen form Figure 3.3, the strain showed much higher growth at 5 mM, although the lag period increased to 38 h (Table 3.1). Unlike _P. putida_ and _T. cutaneum_ which showed substrate inhibition at a phenol concentration above 100 mg/litre, mutant strain PAA showed no such inhibition upto 500 mg/litre. However, there are several reports of microorganisms degrading phenol at higher concentration. Satsangee and Ghosh (1990) have observed an increase in phenol degradation rate by a continuous enrichment of microbial consortium in presence of phenol. Even Hinteregger _et al_. have isolated a phenol-degrading _P. putida_ EKII by selective enriching for higher degrading potential.

Strain PAA shows characteristic phenol hydroxylase activity as measured by oxygen uptake experiments (Chapter II). Phenol and phenol-related compounds like resorcinol, *m*-cresol and hydroxybenzoate could be oxidized by strain PAA without any lag. Other substituted phenols like nitrophenol and fluorophenol could also be oxidized by strain PAA without any lag. However, halosubstituted phenols such as *o/p/m*-chlorophenols and 2,4,5-trichlorophenol could not be oxidized by strain PAA, though strain AC1100 could dechlorinate and oxidized them (Karns *et al*., 1983). This indicates the possibility of either a single broad substrate specific phenol hydroxylase in strain PAA or the presence of several hydroxylating enzymes. Neujahr and Varga (1970) have demonstrated a broad substrate hydroxylase activity in yeast as a result of which it could oxidized substrates like resorcinol and quinol but the possibility of other hydroxylases responsible for oxidation of substituted phenol could not be ruled out . Hydroxyquinol also could be readily oxidized by strain PAA. *P. putida* also could readily oxidize phenol and cresol (Bayly and Wigmore, 1973). Gaal and Neujahr (1979) have reported the ability of *T. cutaneum* to grow on phenol and resorcinol. Cells grown on resorcinol

contained enzymes that participated in the degradation of phenol and *vice versa*. *B. stearothermophilus* showed similar rate of oxidation for cresol and phenol (Buswell, 1975). Straube (1987) characterized phenol hydroxylase from *Rhodococcus* sp. which showed broad substrate activity against resorcinol,cresol and aminophenol. Both *ortho* and *meta* cleavage activity has been demonstrated in strain PAA. Catechol 2,3-dioxygenase enzyme is known to have a narrow substrate specificity, whereas catechol 1,2-dioxygenase is known to accommodate substituted catechols. The oxidation of hydroxyquinol by strain PAA might be due to active catechol 1,2-dioxygenase present in strain PAA.

The most interesting feature comes from the stoichiometric studies of phenol and catechol oxidation in strain PAA. Strain PAA showed 1 mole of oxygen consumption for every mole of catechol consumed, irrespective of whether it was pregrown in phenol or benzoate. However, strain PAA pregrown in phenol showed less than 1 mole of oxygen consumption per mole of phenol as a result phenol hydroxylation is less efficient. This has been further confirmed by determining K_s and V_{max} for phenol utilization in

strain PAA.

It is interesting to consider the seemingly exclusive occurrence of *meta* pathway in PAA in view of the preexisting *ortho* cleavage pathway. Each pathway was inducible by its respective substrate *i.e.* phenol or benzoate. Though catechol 2,3 -dioxygenase was more active than catechol 1,2-dioxygenase, no change was observed when stoichiometric studies were carried out. The inability of strain PAA to metabolise chlorophenols was not surprising. Utilization of haloaromatics requires prevention of *meta* pathway as ring cleavage of chlorocatechol by catechol 2,3-dioxygenase leads to a dead end product (Bartels *et al*., 1984). The possibility that activation of *meta* cleavage pathway in strain PAA leading to loss of ability to metabolize chlorinated phenols could not be ruled out. Similarly, since chlorinated phenols are not being oxidized, degradation of phenol is indeed a phenol hydroxylase dependent activity independent of the enzymatic pathway involved in 2,4,5-T utilization.

The apparent K_s for phenol degradation by strain PAA was 41.73 µM. K_s values determined in several

Pseudomonas species ranged from 10 to 30 μM (Folsom, 1990; Hill and Robinson 1975; Molin and Nilsson, 1985; Yang and Humphrey, 1975). Inhibition of phenol degradation at higher phenol concentrations has been shown previously to be modelled best by use of a Haldane's function (Hill and Robinson, 1975). Earlier K_{si} values reported for *P. putida* and *T. cutaneum* ranged between 1 and 5 mM whereas for *P. cepacia*, G4, K_{si} value reported was 0.45 mM (Folsom *et al*. 1990). The K_{si} value of 175 μM for strain PAA is lower than those reported above. The apparent V_{max} for phenol degradation in strain PAA was 11.38 nmole/min/mg of cells. The V_{max} reported for *P. cepacia* G4 was 466 nmole/min/mg of protein, which is very high (Folsom *et al*. 1990). Because of these kinetic characteristics, phenol degradation in strain PAA was less efficient compared to catechol degradation and complete mineralization of phenol took 20 to 30 h, unlike strain G4, which mineralizes phenol at a much faster rate because of low K_s and high V_{max} values observed (Folsom *et al*., 1990).

Activity of Catechol 2,3-dioxygenase, the key enzyme of the *meta* cleavage pathway was observed in

strain PAA, unlike parent strain AC100 which shows no evidence of catechol 2,3-dioxygenase activity. K_m of catechol 2,3 dioxygenase from strain PAA was 1.4×10^{-6} M. K_m of catechol 2,3 dioxygenase from P. putida for catechol as substrate was reported to be 3×10^{-6} M (Nozaki, 1970; Nakai et al., 1983). The low K_m value as determined for C23O in strain PAA in crude extracts indicates a novel enzyme encoded by a novel gene which might have been recruited in strain AC1100 from a distant microbial species during Plasmid Assisted Molecular Breeding. Previous studies using known C23O gene probe have not shown any hybridization with strain AC1100 DNA even under low stringency (Sangodkar et al., 1988). Also, our preliminary studies of in-situ detection of C23O from strain PAA indicates differential migration on native polyacrylamide gel in comparison to archetypal P. putida .

In conclusion, strain PAA is a unique mutant having phenol degrading ability. Kinetic studies of phenol degradation indicate 2 important steps :

1) The conversion of phenol to catechol, which takes place at a much slower rate as seen from the long lag period even though the culture was pregrown in phenol.

Also the K_s value for phenol hydroxylase was observed to be 41.73 μM resulting in low substrate affinity.

2) Catechol was being depleted at a much faster rate as seen from K_m values which were found to be lower than that reported for P. putida. Further, the existence of two or more hydroxylases in PAA involved in oxidation of phenol-related compounds such as resorcinol and cresol is a possibility. The inability of PAA to metabolize any chlorinated phenol but still metabolize phenol indicates a pathway distinct from that of 2,4,5-T utilization. Simultaneous presence of C23O in strain PAA further confirms the utilization of phenol through meta cleavage pathway.

CHAPTER IV

POTENTIAL OF STRAIN PAA IN MINERALIZATION OF PHENOL BY CONTINUOUS CULTURE SYSTEM

There are several reports on bioremediation of phenol using continuous culture system (Wase & Hough, 1966; Yang and Humphrey, 1975; Donaldson _et al_. 1984; Enrhardt and Rehm, 1989; Federov _et al_. 1992; Ehrhardt and Rehm, 1985; Molin and Nilsson, 1985; Bettmann and Rehm, 1984 and Takahashi _et al_. 1981). Most of these phenol-utilizing micro-organisms were isolated from natural sites as mentioned in Chapter I . However, as reported in Chapters II and III, we have isolated a unique mutant from _P. cepacia_ AC1100 which was initially developed by Plasmid Assisted Molecular Breeding (Kellogg _et al_, 1981). Unlike _wt_ AC1100, mutant strain PAA has a unique capability to grow on phenoxyacetic acid and phenol. Characterization of enzymatic pathway of phenol metabolism in strain PAA revealed the activation of silent genes coding for phenol hydroxylase and the _meta_ cleavage pathway . Enzyme kinetic studies of phenol hydroxylase and catechol 2,3-dioxygenase (Chapter III) revealed a very active catechol 2,3-dioxygenase, metabolizing catechol at a much faster rate than the conversion of phenol to catechol by phenol hydroxylase.

Present chapter reports the study of microbial degradation of phenol by mutant P. cepacia PAA by using continuous culture system. Estuarine/riverine - like conditions have been simulated to determine the practical feasibility of using the culture in bioremediation of aquatic waste containing phenol, especially in rivers and estuaries.

Materials and Methods :

Micro-organism and growth conditions : P. cepacia PAA obtained as a spontaneous mutant from P. cepacia AC1100 was used to study microbial degradation of phenol in batch and continuous culture system with 1mM (100 ppm) of phenol as limiting substrate. P. cepacia PAA was maintained by periodic subculture on BSM agar plates containing 1 mM (100 ppm) phenol. The primary culture (inoculum) was prepared by transferring a loopful of stock culture to 50 ml of BSM medium containing 100 ppm of phenol as the sole source of carbon and energy. The culture was then incubated on a shaker at 150 rpm for 24 h at 30 ^{o}C. The above inoculum was used for the batch and continuous culture studies carried out on a New

Brunswick, 1.3 ℓ bench-top chemostat.

Growth Media :

Mutant strain PAA was grown in a defined minimal medium (BSM) as mentioned in Appendix I . Phenol was used as limiting substrate during the batch and continuous culture studies. To prevent the precipitation of Ca^{++} and Mg^{++}, the medium was prepared as two solutions A and B, which were separately steam sterilized. Solution A contained KH_2PO_4, K_2HPO_4 and NH_4Cl. It was adjusted to the desired pH with 1 M KOH before steam steriliation. After cooling to room temperature, sterile solution B which contained $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $MnSO_4$, $CaCl_2$, $ZnSO_4$ and $CoCl_2$ was added aseptically. Phenol stock (1 M) prepared in distilled water was added to the BSM medium at a final concentration of 100 ppm.

Measurement of Growth:

The bacterial cell concentration was determined by absorbance measurement at 560 nm using Spectronic 1201 spectrophotometer. Since the maximum optical density of PAA observed during batch culture experiment was around 0.25, where the Beer-Lambert's law holds true, the absorbance at 560 nm was directly determined without prior dilutions. The cell concentrations are reported in terms of units of optical density or dryweight (mg/ml), using the relation as mentioned in Chapter III.

Determination of Phenol:

Phenol was measured by a colorimetric method as mentioned in Chapter III (Materials and Methods). 1 ml samples were collected at defined time intervals aseptically from the fermentor, centrifuged at 12,000xg for 10 minutes to obtain the culture supernatant and the phenol remaining in the medium was assayed spectrophotometrically using the aminoantipyrene method. The phenol concentration remaining was estimated from a

precalibrated standard curve prepared for phenol.

Equipment :

Figure 4.1 is a schematic representation of the New Brunswick Bioflow^{R} 1.3. l chemostat used for the study of microbial degradation of phenol by batch and continuous culture.

Batch culture of strain PAA :

Batch culture experiments using strain PAA were conducted in a 1.3 l fermentor operated at a working volume of 1·0 l. The temperature of the culture was maintained at 30±2 ^{o}C. The aeration rate was 0.4 LPM and the agitation speed was 200 rpm. 50 ml of primary culture pregrown in BSM containing 100 ppm phenol was added as inoculum in the fermentor which had 950 ml of BSM medium containing 100 ppm phenol. At defined intervals, 1 ml of sample was collected aseptically in a screw - cap tube fitted to an outlet port. Cell concentration was determined spectrophotometrically at

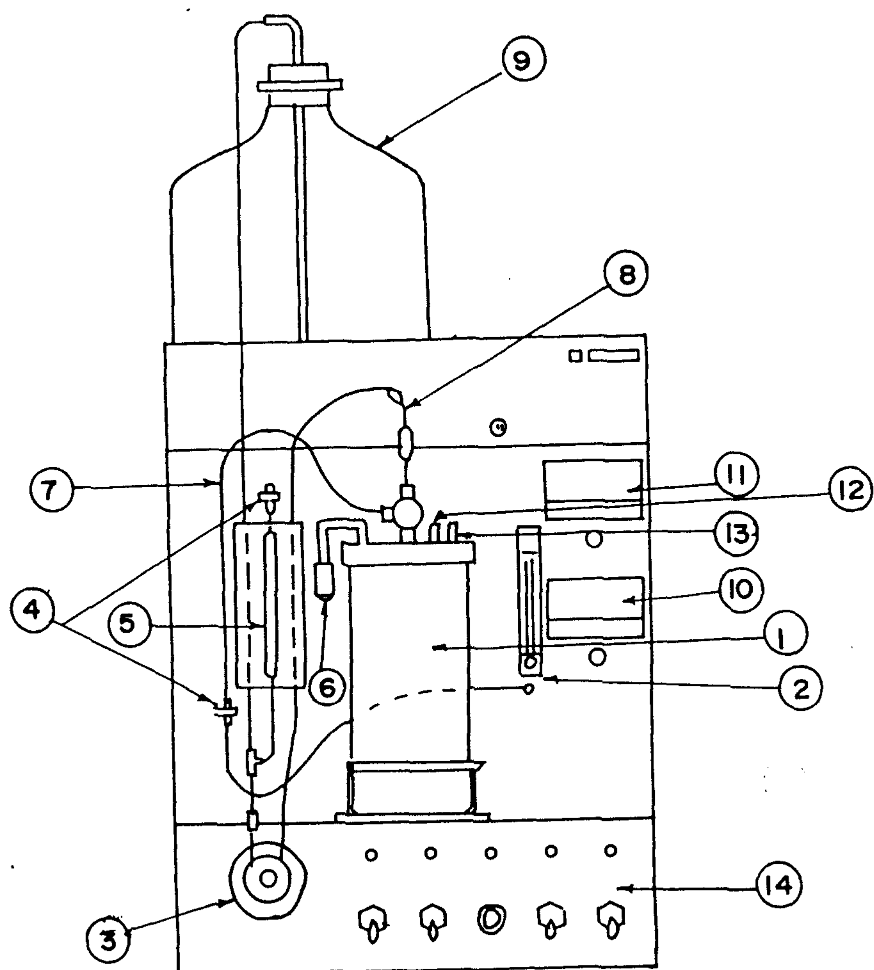


Fig.4.1 Schematic representation of BioFlow^{R} 1.3 litre benchtop chemostat. (1) culture vessel, (2) air flow meter, (3) pump motor, (4) filter, (5) flow rate tube, (6) sampling port, (7) air inlet line, (8) medium inlet, (9) medium reservoir, (10) pump speed control, (11) agitation speed control, (12) dissolved oxygen probe, (13) combined pH electrode and (14) control panel for the chemostat.

560 nm whereas phenol concentration was measured at 500 nm using the aminoantipyrene method. Throughout the experiment, pH of the medium was measured with steam sterilizable combined pH electrode. Dissolved oxygen was measured by an autoclavable dissolved oxygen probe. The batch experiments were carried out for 20-22 h, after which there was a decline in absorbance at 560 nm.

Continuous cultures of strain PAA :

In continuous culture experiments, cultivation conditions were similar to those of batch experiments as mentioned above. Batch culture was initiated by inoculating 50 ml of the primary inoculum prepared as mentioned above. The batch culture was continued until the absorbance at 560 nm was 0.22 OD units. Continuous medium flow was started by means of a peristaltic pump adjusted at various flow rates to give dilution rates ranging from 0.01 to 0.2 h^{-1}.

The temperature was maintained at 30±2 ^{o}C. The culture was agitated at 200 rpm and aeration was controlled at 0.4 LPM. The steady state was considered

established when the absorbance at 560 nm remained unchanged for a period corresponding to at least 48 h. At steady states, concentration of phenol remaining in the medium was determined by 4-aminoantipyrene method whereas cell mass (mg/l) was calculated on the basis of absorbance at 560 nm and using the relationship mentioned in Chapter III.

Batch and continuous phenol degradation by strain PAA in diluted media :

Batch and continuous phenol degradation using diluted media was conducted by diluting the BSM media with distilled water (LSM). The dilution of the BSM media ranged from 0 to 75 percent. Phenol used as carbon source was added at a final concentration of 100 ppm.

Shake flask experiments with 0 to 75% LSM and 100 ppm phenol as carbon source were used to establish batch cultures of PAA to study the effect of diluted media on phenol degradation and on microbial growth. Phenol concentration and cell growth measurements were as

mentioned in Chapter III.

The conditions for establishing continuous cultures were similar to those mentioned above except for the use of LSM for growth of strain PAA and also during continuous runs. The dilution of BSM which promoted effective growth during batch culture was chosen for continuous culture studies.

RESULTS

Phenol degradation by strain PAA in batch culture

Typical phenol batch culture data for growth of strain PAA at 30 °C and a pH of 7.4 using the 1.3 l benchtop fermentor are given in Figure 4.1. Increase in absorbance at 560 nm was concomitant with depletion of phenol from the medium. Though the inoculum was pregrown in phenol, there was an initial lag of 12 h, after which linear exponential growth was observed. Similarly, there was an exponential decrease in phenol from the medium around 22 h. The strain PAA metabolized over 98% of the phenol initially present after which

there was a decline in absorbance at 560 nm, indicating advent of death phase. Figure 4.2 shows the plot of cell absorbance *versus* time. The specific growth was 0.126 h^{-1}.

The ability to metabolize 100 ppm of phenol to less than 2 ppm of phenol was a characteristic possessed only by mutant PAA and not by wild type *P. cepacia* AC1100. As seen from Figure 4.2 there was no change in the pH of the medium after addition of 100 ppm phenol. Further, there was negligible change in pH even after the batch culture reached stationary phase (Fig. 4.2). The oxygen concentration in the medium decreased from 100% to 88% during the batch culture and stabilized thereafter.

Continuous phenol degradation by strain PAA in growth medium :

The continuous degradation of phenol by strain PAA was studied in BSM using 100 ppm phenol as the limiting substrate. The cultures were run with dilution rates ranging from 0.01 to 0.2 h^{-1}. Aeration rate of 0.4 LPM and agitation speed of 200 rpm were kept constant

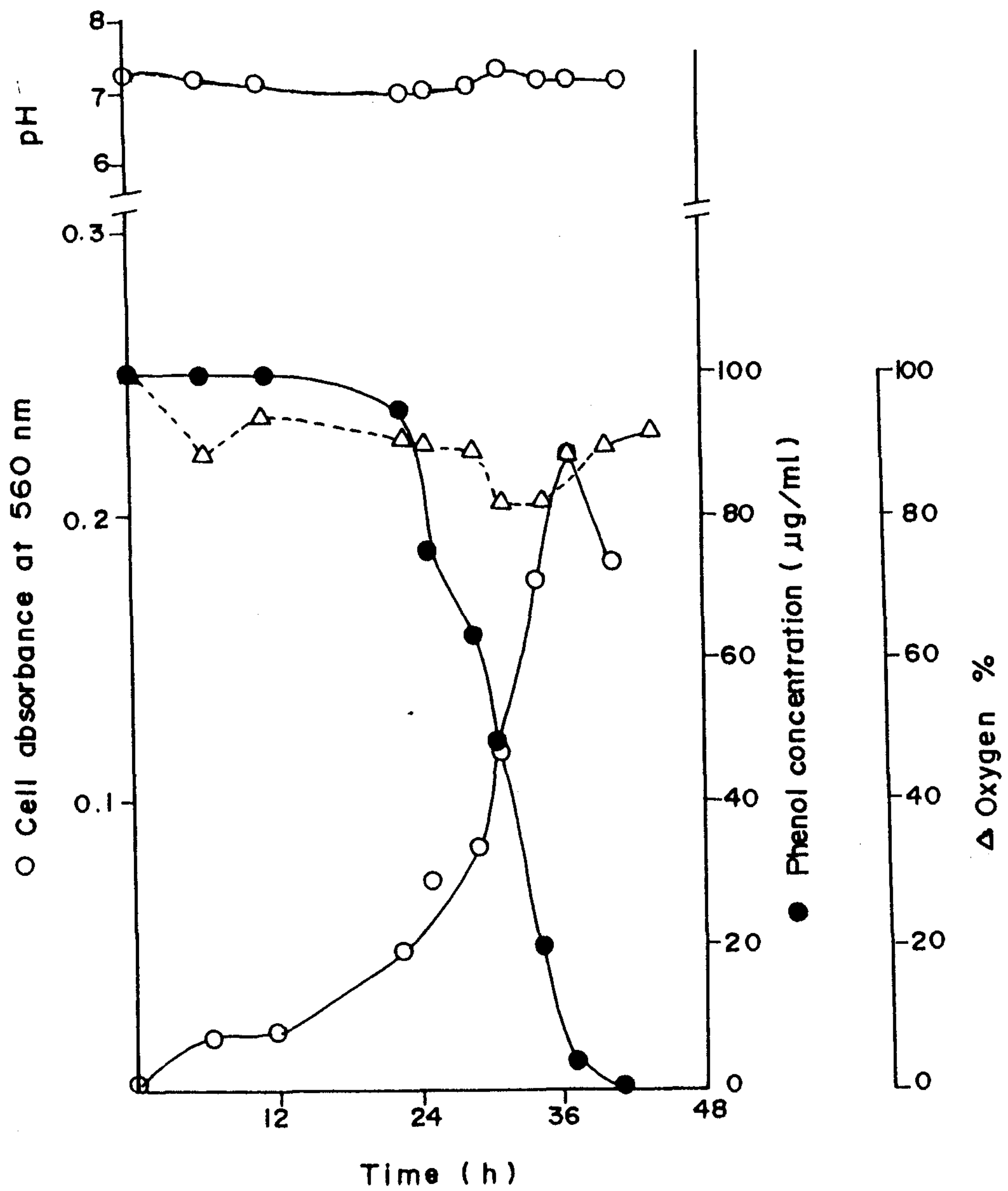


Fig.4.2 Batch culture of strain PAA at controlled phenol concentration of 100 ppm at 30 °C and pH 7 using bench-top fermentor.

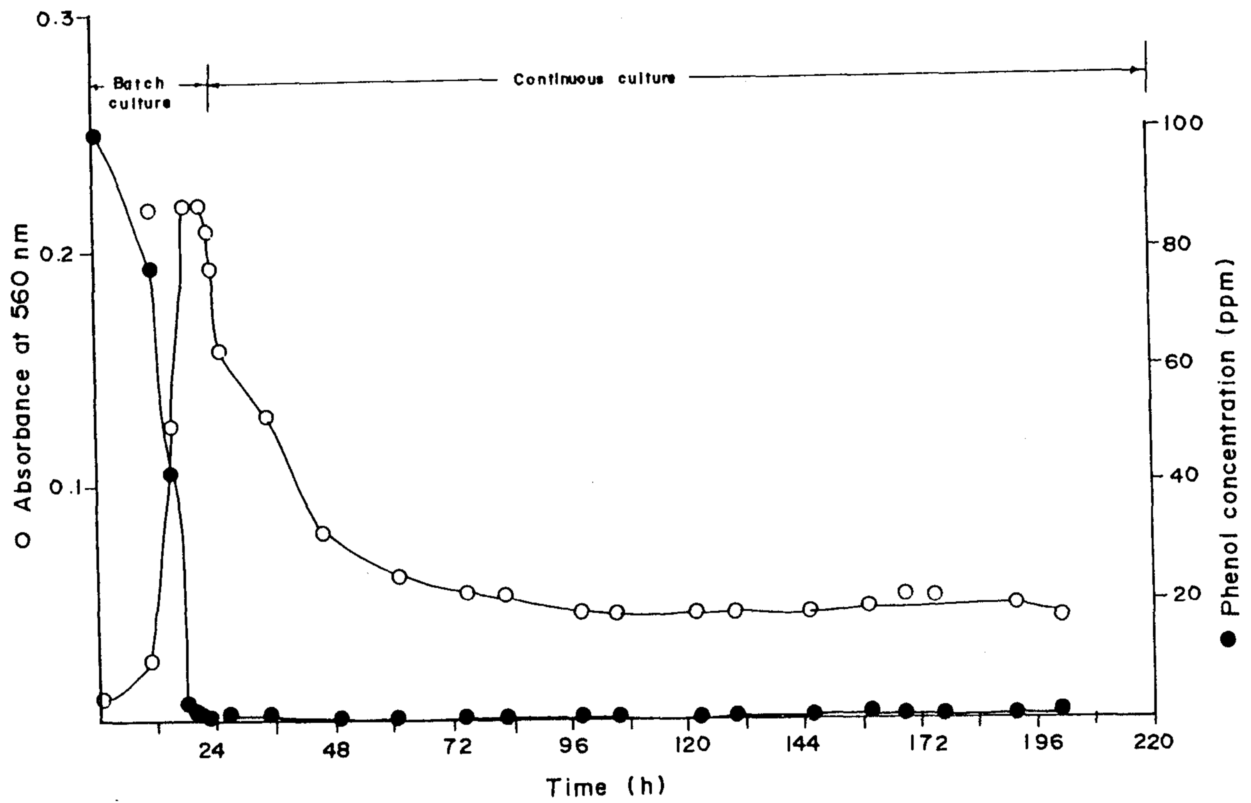


Fig. 4.3 Continuous culture of PAA at D=0.01 h^{-1}.

-Growth of PAA; ●- Phenol concentration.

throughout the continuous culture experiments. Since no change in pH was observed at any of the dilution rates, pH was not regulated artificially. Complete phenol degradation was observed at dilution rates from 0.01 to 0.175 h^{-1}. As seen in Figures 4.3, 4.4, 4.5, 4.6, 4.7 and 4.8, steady state could be achieved at various dilution rates. At steady states, cell mass reached a stable value as observed from constant absorbance at 560 nm. Constant and steady rate of phenol utilization from the medium was observed throughout the steady state. The phenol concentration remaining in the medium ranged from 0.6 μg/ml to 3.5 μg/ml (i.e., from 0.6 to 3.5 ppm) at various dilution rates. As seen in Figure 4.6, the average phenol concentration remaining in the medium was 0.70 μg/ml, i.e., less than 1 ppm of phenol. Similarly as seen in Figure 4.8, cell absorbance at 560 nm was maximum when compared with continuous runs at other dilution rates. However, the phenol remaining in the medium was 3.83 μg/ml.

When the dilution rate was 0.2 h^{-1}, exponential decrease in bacterial cell mass as observed by decreasing absorbance at 560 nm indicated wash-out (Fig. 4.9). Correspondingly, there was a gradual increase in

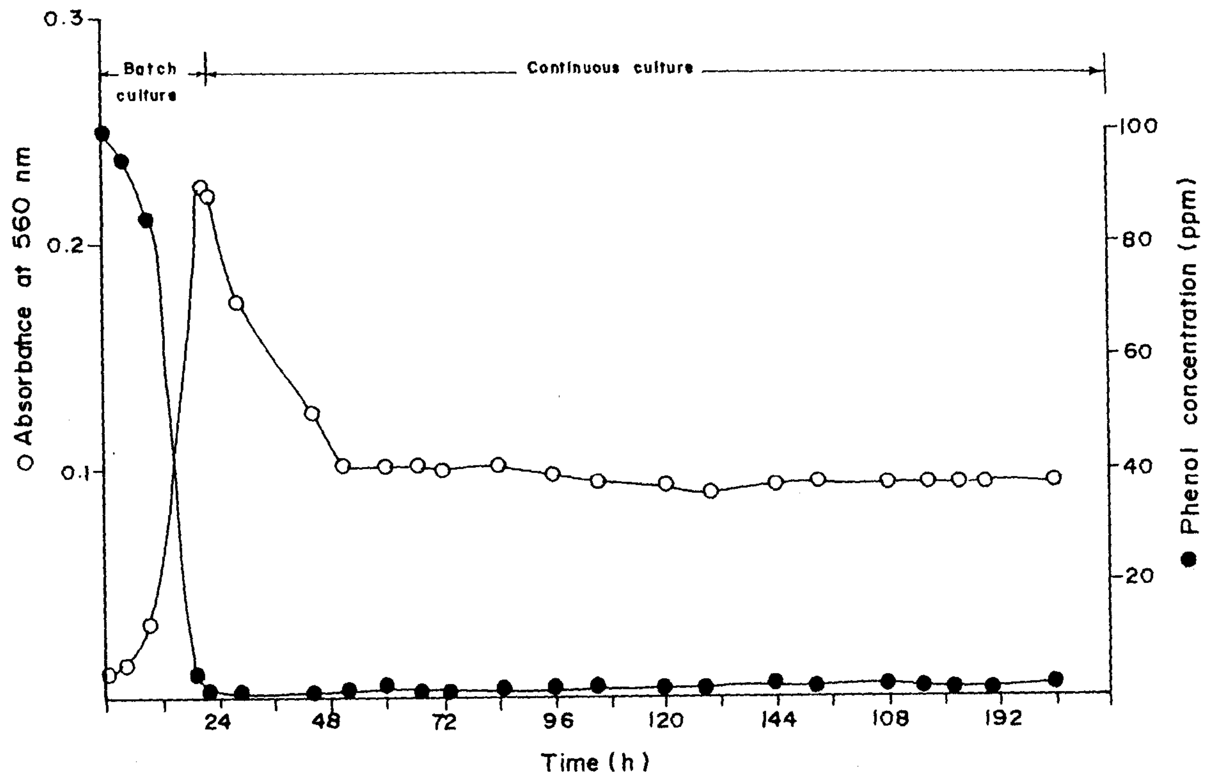


Fig. 4.4 Continuous culture of PAA at D=0.025 h^{-1}.

- Growth of PAA ; ●- Phenol concentration.

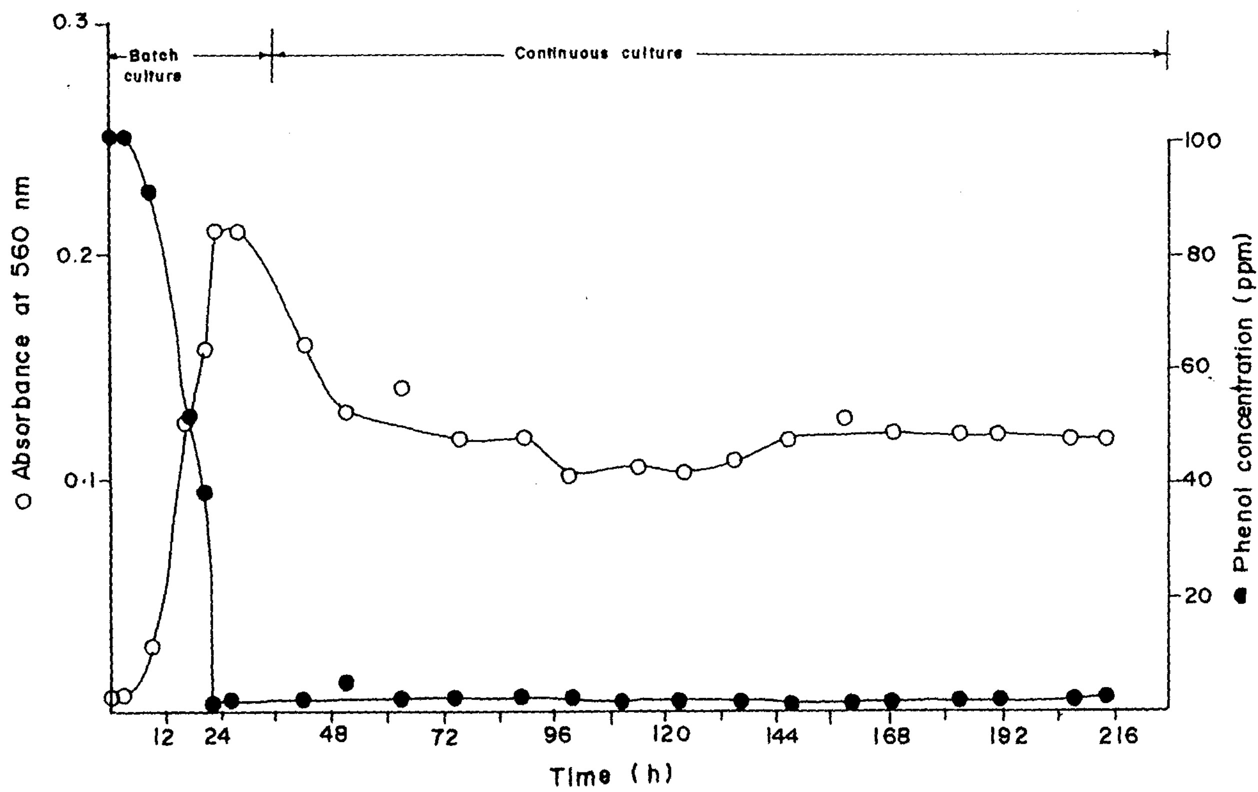


Fig.4.5 Continuous culture of PAA at D=0.05 h^{-1}.

O- Growth of PAA; ●- Phenol concentration.

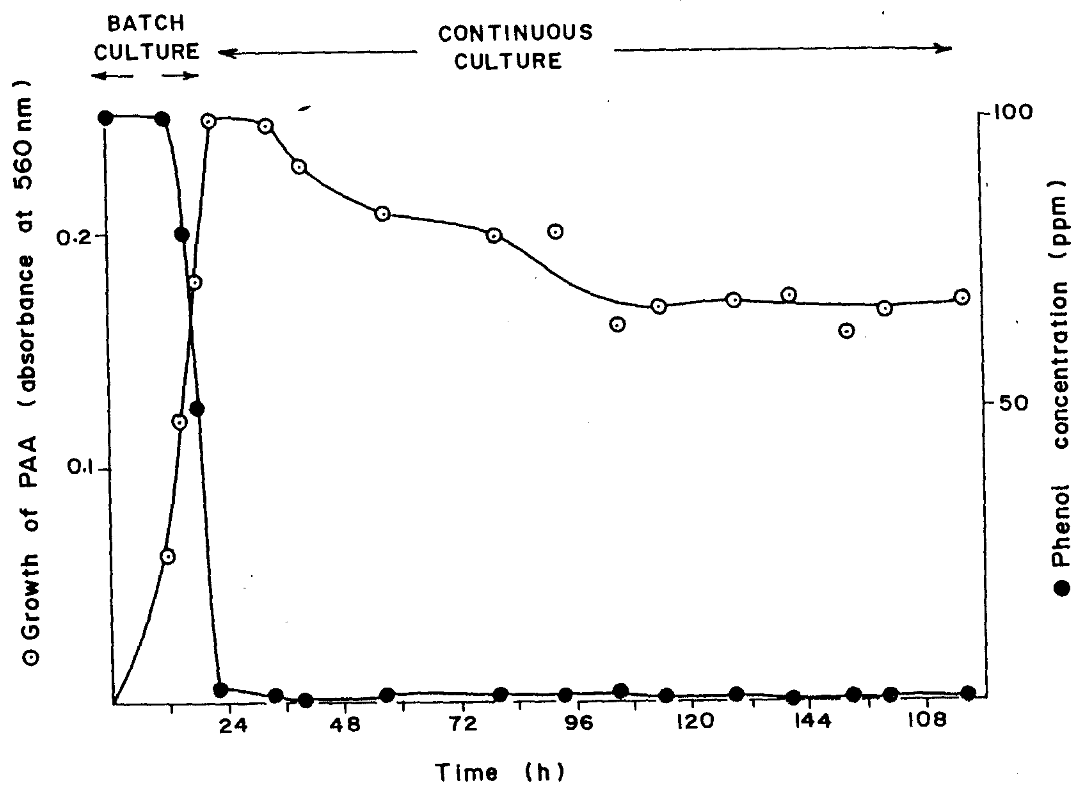


Fig.4.6 Continuous culture of PAA at D=0.1 h^{-1}.

O- Growth of PAA; ●- Phenol concentration.

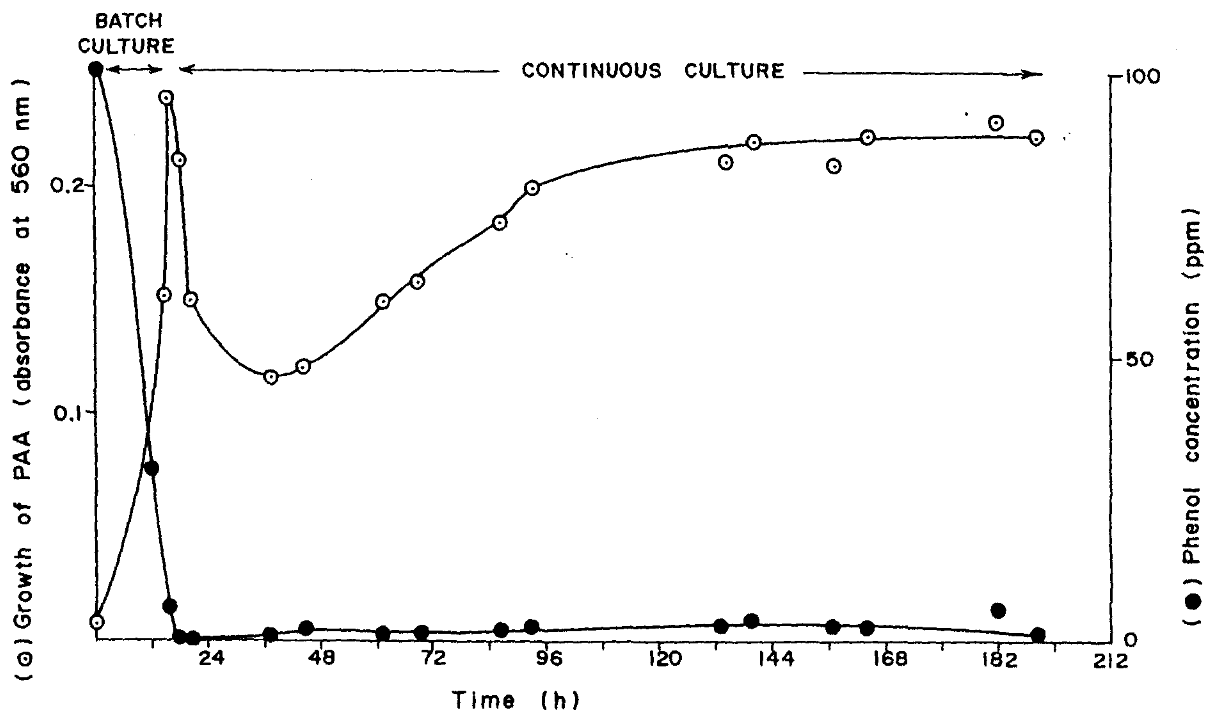


Fig.4.7 Continuous culture of PAA at D=0.15 h^{-1}.

O- Growth of PAA; ●- Phenol concentration.

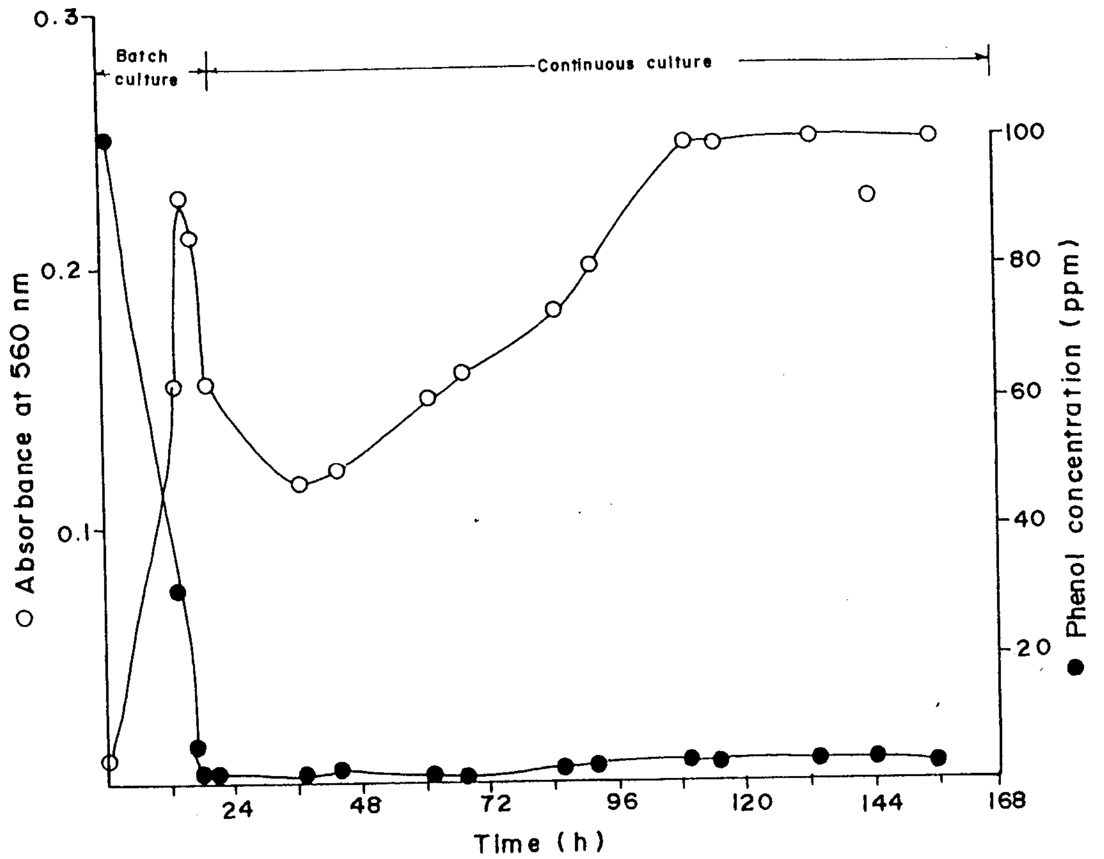


Fig.4.8 Continuous culture of PAA at D=0.175 h^{-1}.
O- Growth of PAA; ●- Phenol concentration.

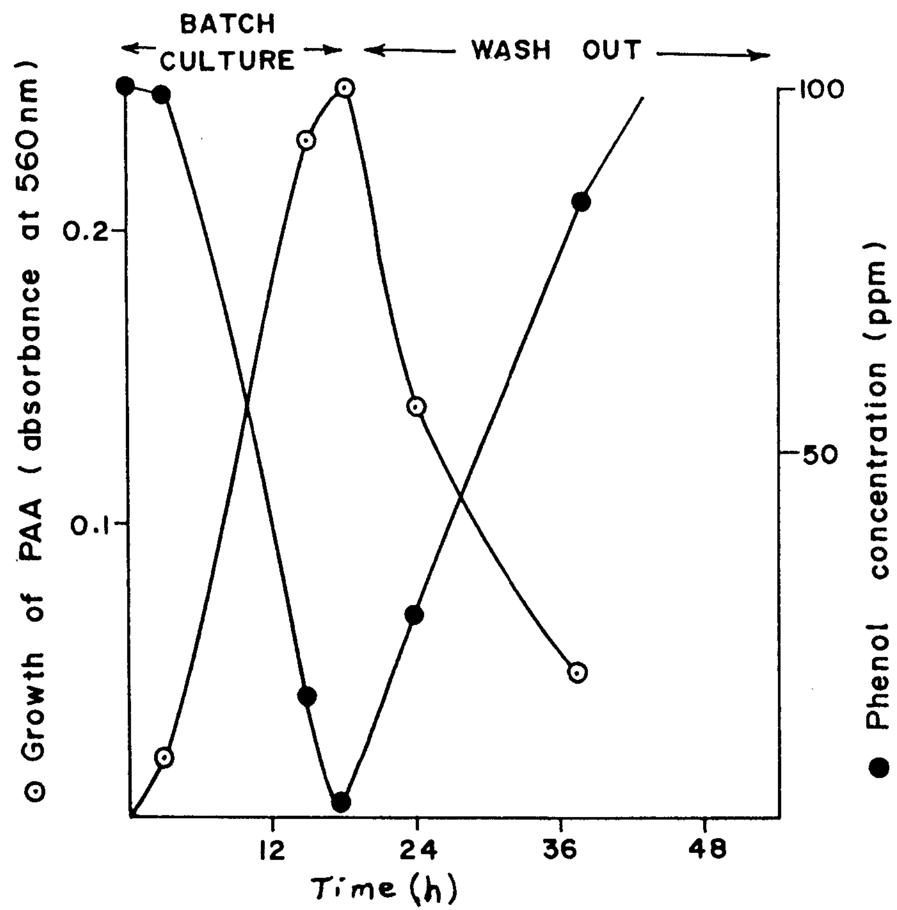


Fig.4.9 Wash-out of PAA at D=0.2 h^{-1}. ⊙- Bacterial cell mass; ●- Phenol concentration remaining.

the concentration of phenol until it reached 100 ppm (Fig 4.9). Even after prolonged continuous runs, cell concentration was at minimum, with high concentrations of phenol remaining in the effluent.

Figure 4.10 depicts the results of emergence of a mutant from strain PAA with high rate of phenol degradation. After the wash- out was observed at D= 0.2 h^{-1}, batch culture was resumed. After the culture system reached the stationary phase, continuous run at a dilution rate of 0.175h^{-1} was carried out, leading to partial wash - out. However, the phenol concentration which increased upto 38 µg/ml at D= 0.175 h^{-1} (Fig. 4.10) was later found to decrease to 2 µg/ml the moment the culture system stabilized. No wash-out was observed even after further increase in dilution rate upto 0.35 h^{-1} and a new steady state could be established. The phenol concentration even at such high dilution rate was less than 2 ppm (Fig. 4.10).

Figure 4.11 indicates the plot of cell absorbance *versus* phenol concentration remaining in effluent at steady state at various dilution rates. With increasing dilution rate, cell mass at steady state increased

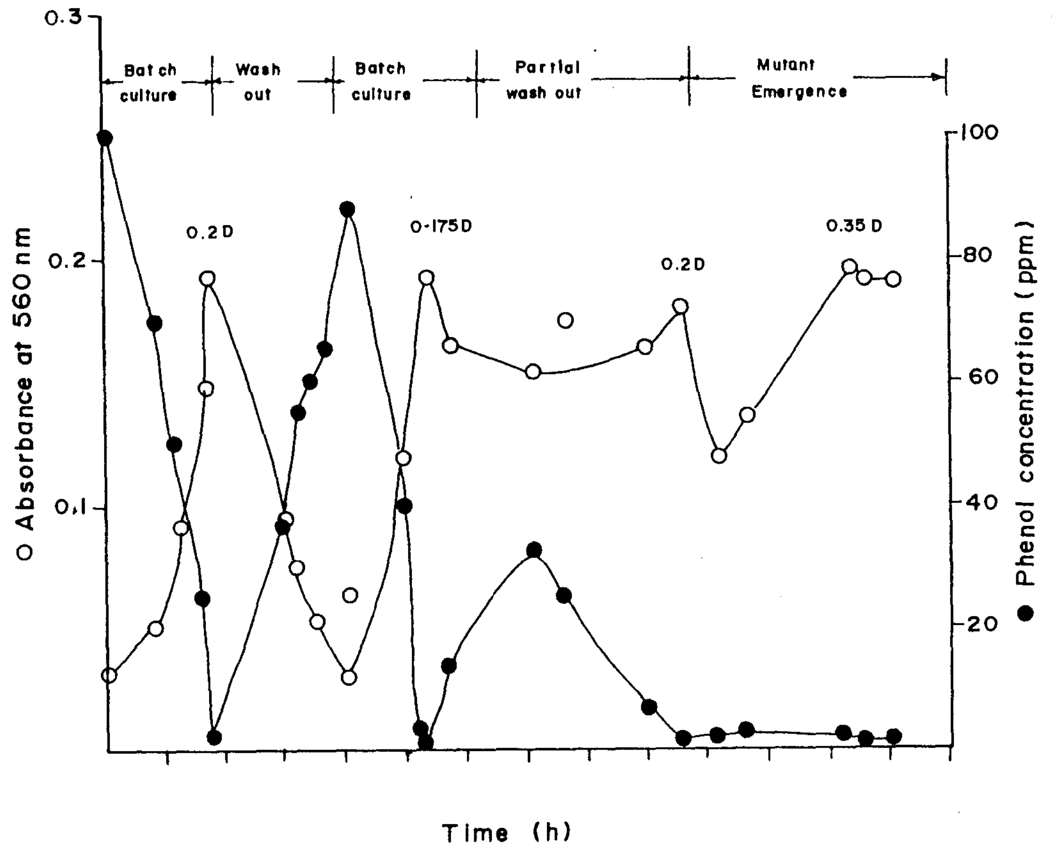


Fig.4.10 Emergence of variant from strain PAA during partial wash out at D=0.175 h^{-1}. After wash out at D= 0.2 h^{-1}, batch culture was resumed till stationary phase was reached. Dilution rate was adjusted to D=0.175 h^{-1}. Partial wash out was observed . Cells over came the pressure and the transient increase in phenol concentration observed previously came to minimum value. On further increase in dilution rate to 0.2 and 0.35 h^{-1} no wash-out was observed (C- Growth; ●-phenol remaining in the medium).

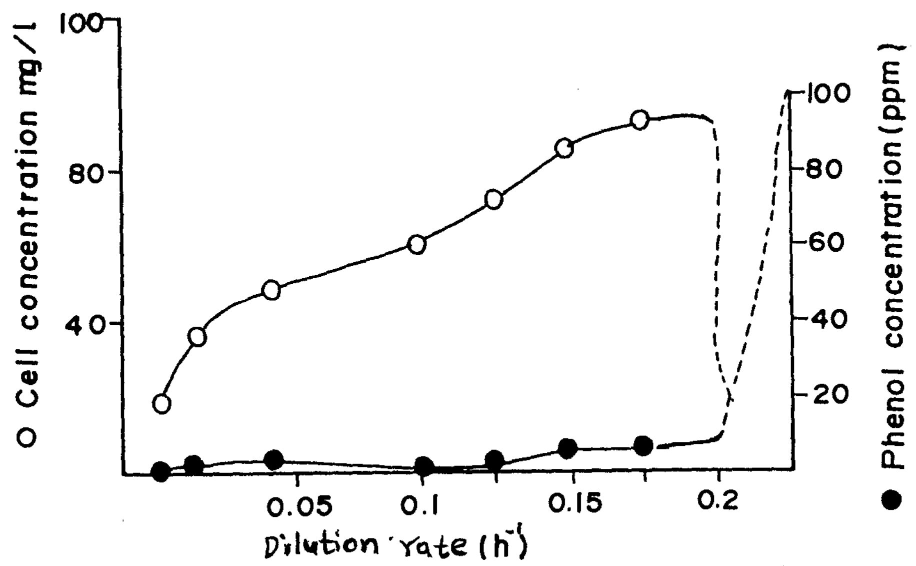


Fig.4.11 Steady state continuous culture results for strain PAA at 1mM phenol (O- cell mass and ●-phenol concentration at steady state).

till the dilution rate reached 0.175 h^{-1}, after which there was a decline, indicating a washout. Similarly, the phenol concentration remaining in the effluent was minimum, being as low as 1 ppm at D=0.1 h^{-1}, but increased at dilutions higher than 0.15 h^{-1} and was maximum at critical wash-out. The results of continuous run with strain PAA as seen in Figure 4.11 have been replotted in Figure 4.12. A yield of 1.39 mg cells/mg phenol and a maintenance requirement of 0.042 mg phenol/mg cell.h was observed for strain PAA.

<u>Degradation of phenol by PAA in low salts media</u> :

To simulate the environment found in estuaries or rivers, low salts medium (LSM) was used for growth of PAA. Batch cultures in LSM medium with 1 mM phenol as carbon source were carried out. Figure 4.13 shows the results of the batch experiment carried at various dilutions of BSM medium. As seen from the Figure 4.13 , increase in cell density was evident even in LSM equivalent to 25% of BSM and was comparable to control experiment carried out using normal BSM. The maximum absorbance at 560 nm could be reached in all the diluted

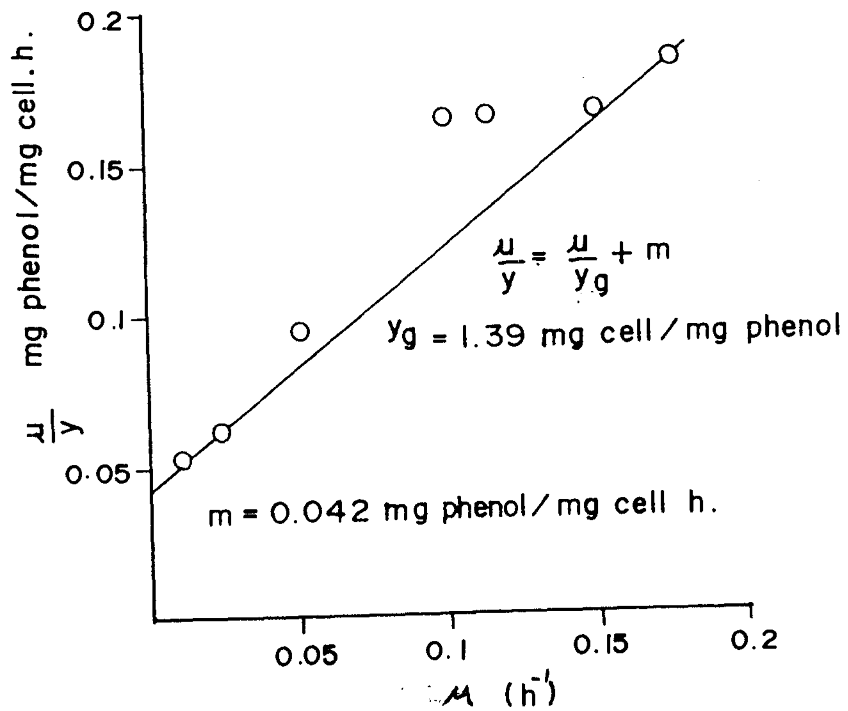


Fig.4.12 Relationship between specific growth rate and specific phenol degradation rate at steady state continuous culture.

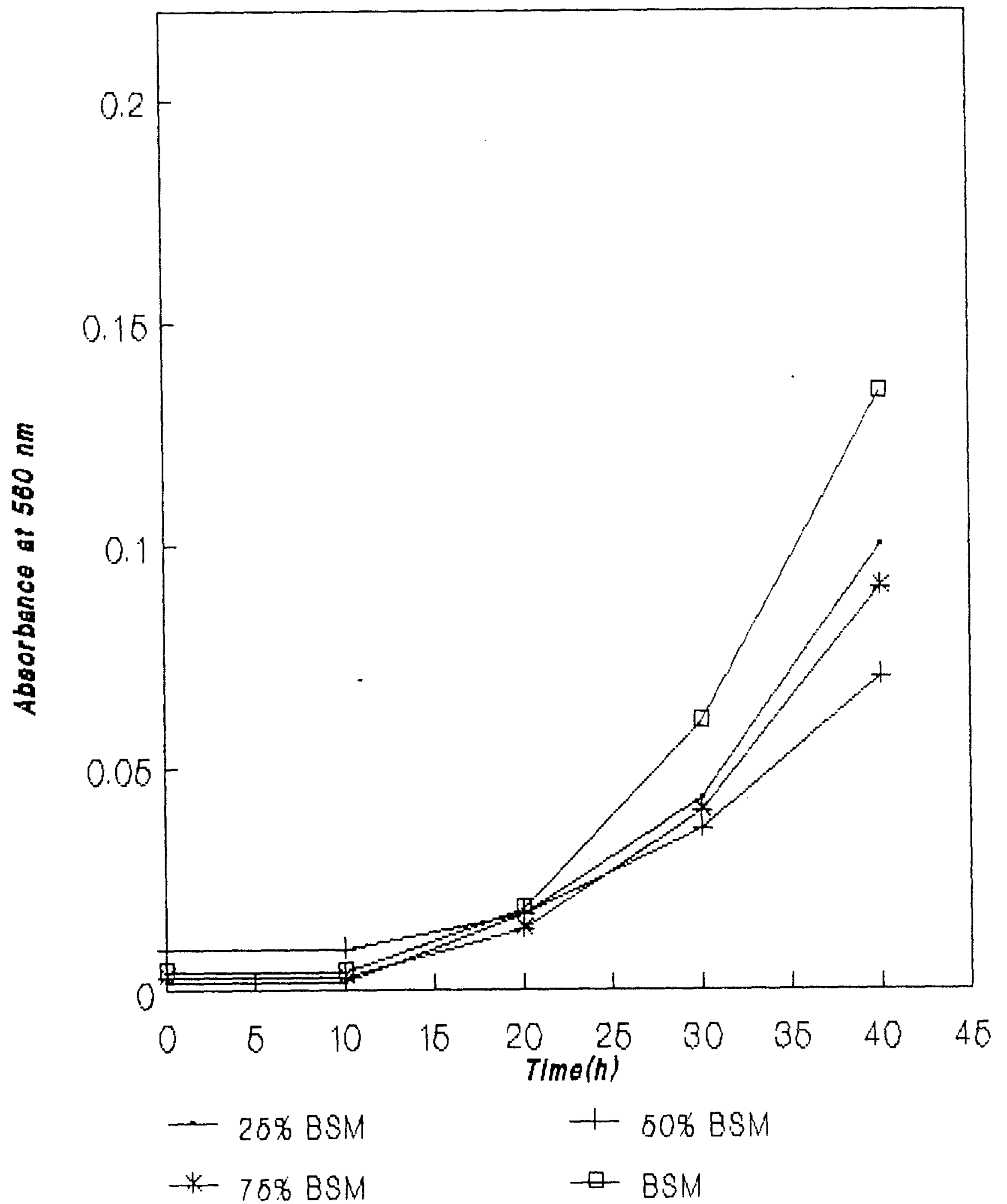


Fig.4.13 Batch culture of PAA in LSM containing 1mM phenol .

media and was similar to that seen in control (Fig. 4.13). Also, complete degradation of phenol from 100 ppm to 2 ppm was observed in all the cases.

Continuous phenol degradation by PAA in low salts media:

Continuous phenol degradation using 25% LSM was carried out at 0.125 h^{-1}. Steady state could be maintained even though diluted medium was used in continuous culture as seen in Figure 4.14. Cell concentration was constant during steady state and phenol concentration remaining in the medium was 1.5 ppm. These results show similarity to the continuous culture experiments carried out using control undiluted BSM medium. The cell concentration at steady state (D= 0.125 h^{-1}) using LSM for continuous culture (Fig. 4.14) was much higher than cell concentration at steady state (D= 0.15 h^{-1}) using BSM medium. However, the phenol remaining in the effluent was around 1.5 ppm at 0.125 h^{-1} (Fig. 4.14) whereas phenol concentration in the effluent at 0.15 h^{-1} using undiluted BSM media was 3.83 ppm (Fig 4.7).

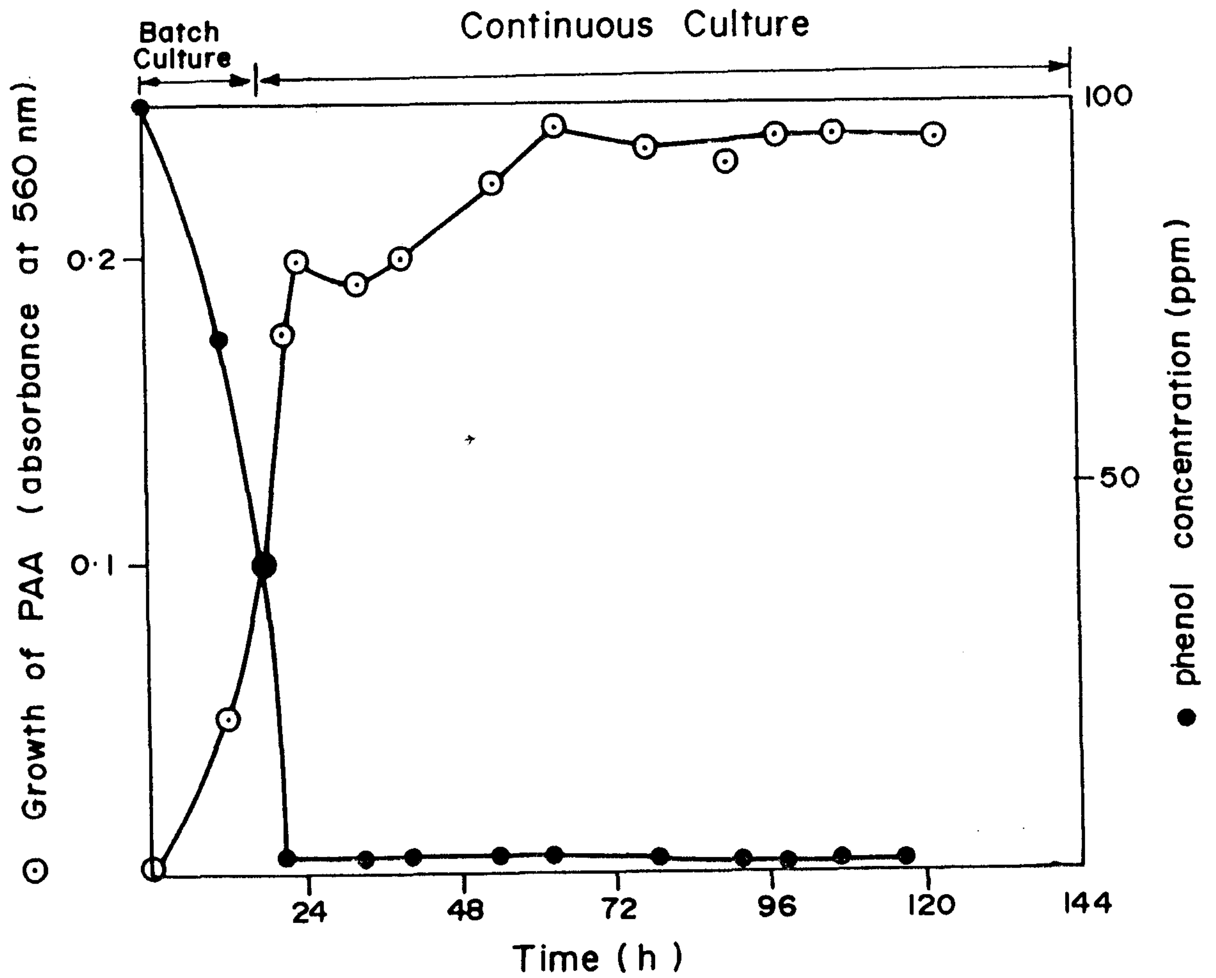


Fig.4.14 Continuous culture of strain PAA at D=0.125 h^{-1} using LSM containing 1 mM phenol. ⊙– cell growth; ●– phenol concentration.

DISCUSSION

P. cepacia PAA developed in our laboratory had the unique ability to grow on phenoxyacetic acid and phenol, besides utilizing 2,4,5-T and benzoate. Earlier studies on batch culture of strain PAA and its growth on phenol at concentrations upto 500 ppm indicated increase in cell mass with concomitant depletion of phenol from the medium (Chapter III, Fig.3.3 and 3.4). Hence, in order to check the practical feasibility of using strain PAA in bioremediation of aquatic wastes containing phenol, batch and continuous cultures of PAA were established with phenol as limiting substrate.

Discharge of industrial waste containing phenol is routed through nearby rivers and estuaries to finally reach the ocean. Phenol is present at various concentrations in the industrial waste depending on the type of industry and the processes involved. Liquid waste from oil refineries have phenol concentrations upto 2000 mg/l, whereas spent liquor from metallurgical coke processes contain about 1000 mg/l of phenol. Low temperature coal carbonization processes result in

liquid wastes containing 1000-8000 mg/l of phenol per annum (Mahajan, 1985).

However, the effluents released into rivers and estuaries get diluted several-fold leading to decrease in the overall concentration of a pollutant such as phenol. The dilution of river and estuarine water during rains further brings down the concentration of pollutants in aquatic system, whereas in summer the phenol content in water may increase.

Estuaries , in general, support maximum degradative capabilities by microorganisms leading to mineralization of phenol. While the overall salt concentrations in oceans is high, the estuaries display a gradient of salt concentrations. The effective phenol concentration in various rivers and estuaries have been well characterized. River Thames has phenolic substances at a concentration 0.11 mg/litre (0.1 ppm)and the mass flow is 0.12 ton /day whereas the concentration of phenolic substances in the waters of river Soar having a mass flow of 0.09 ton /day is 0.11 mg/litre.

In our investigation, 100 ppm (100mg/litre) was

chosen as the ideal phenol concentration for simulation studies in case of strain PAA. Though strain PAA shows complete degradation of phenol when grown in 500 ppm, a lag of almost 35 h was observed during our batch studies. Further, literature survey of phenol concentration present in rivers, effluents and sea indicates the concentration of phenol to be less than 100 ppm though the initial input contained phenol at a concentration ranging from 2500-8000 ppm indicating that dilution of pollutants in rivers and estuarine water plays a major role. Studies conducted by Kadam and Bhangle (1993) indicate presence of moderately high concentrations of 18.7, 13.6, 12.1 and 13.1 mg/l of phenol along the west coast at Vereval, Hazira, Bassein and Bombay, respectively. Further, phenol concentration of 7.6-13.6 mg/l was observed at Ratnagiri coast. Also along the coast of Okha, Daman and Murud , phenol could not be detected (Kadam and Bhangale,1993).

Batch culture studies of strain PAA indicates complete mineralization of phenol to 2 ppm. As seen in Figure 4.2 there was no change in pH even at the end of stationary phase Bettmann and Rehm (1984,1985) have reported decrease in pH caused during degradation of

phenol. However, with strain PAA, there was no change in pH during phenol degradation in batch culture and even during continuous culture. The fall in cell density at stationary phase was due to depleting levels of phenol used as limiting substrate (Fig.4.2). Only a slight change was observed in oxygen concentration during batch culture, unlike previously reported by Yang and Humphrey, (1975) who observed decrease in oxygen concentration, as a result of which oxygen transfer rather than phenol became a growth limiting factor.

Continuous culture studies revealed the establishment of steady state at various dilutions. At D = 0.1 h^{-1}, phenol concentration was less than 1 ppm unlike at other dilutions where phenol was present at a concentration of 1 to 4 ppm. Thus PAA degrades effectively at D= 0.1 h^{-1} resulting in complete conversion of 100 ppm of phenol to 0.8 ppm, which is within the prescribed limits suggested by the Environmental Protection Act (EPA). This gives a phenol degradation rate of 240 mg/litre/day. Table 4.1 shows the phenol degradation rates of PAA in continuous culture at various dilutions. The maximum degradation

Table.4.1 Influence of D on growth and phenol degradation rate in strain PAA

Dilution rate, h^{-1}	Cell mass mg/l	Phenol degradation rate, mg/l/day	Phenol concentration in effluent (µg/ml)
0.01	19.6	24	0.72
0.025	36.8	60	1.61
0.05	49.6	120	2.31
0.1	60	240	0.7
0.15	87.6	360	3.8
0.175	94	420	3.1

rate of 420 mg/litre/day was obtained at D= 0.175 h^{-1} resulting in 98% phenol degradation. A yield of 1.34 mg cells/mg phenol and a maintenance requirement of 0.042 mg phenol/mg cells.h was observed for strain PAA. Yang and Humphrey (1975) studied microbial degradation of phenol by pure and mixed cultures of <u>P. putida</u>. They obtained a yield of 0.85 mg cells/mg phenol and a maintenance requirement of 0.055 mg phenol/mg cell.h. Unlike continuous culture studies of strain PAA, where a wash-out at dilution rate of 0.2 h^{-1} was observed, for <u>P.putida</u> it was found to be around 0.33 - 0.4 h^{-1}.

The ability of yeast to degrade phenol by continuous culture has also been studied, although at a very low concentration of 12 μmol/ml. Phenol could be degraded at dilution rates between 0.1 and 0.3 h^{-1} (Wase & Hough, 1966).

There was no visible transient accumulation of yellow intermediate during batch and continuous culture of strain PAA unlike in <u>P.putida</u> where accumulation of α-HMS was observed during continuous culture at higher dilutions (Moline and Nilsson, 1985).

The possibility of obtaining mutants during continuous culture studies has been reported (Reuseer 1961; Thorne, 1968; Pirt and Carlow, 1958; Bartlett and Gerhardt, 1959). When continuous culture was carried out at D= 0.2 h^{-1}, there was a cell wash-out with increase in phenol concentration. Batch culture was resumed till it reached stationary phase. The culture system was subjected to medium flow at D=0.175 h^{-1}. However there was a partial wash-out unlike observed in the previous experiment (Fig 4.8). Phenol concentration increased to 38 ppm with slight decline in cell mass because of a partial washout. After a few more hours of continuous run, the cells overcame the pressure and cell absorbance at 560 nm increased with simultaneous decrease in phenol concentration . Further increase in dilution rate to 0.2 and 0.35h^{-1} led to no washout. Steady state could be achieved as observed from our preliminary experiment. This indicates the emergence of a mutant from strain PAA with unique capability to degrade phenol much more efficiently than strain PAA. This phenomenon could be observed only at the dilution rate of 0.175 h^{-1} and not when continuous culture was carried out at a dilution rate of 0.2 h^{-1} indicating 0.175 h^{-1} as μ_{max} for strain PAA, as mutants could be

selectively favoured either under strong selective pressure or at high maximum specific growth rate (Jannasch and Matles, 1970; Tempest, 1974). Wase and Hough (1966) had observed substantial deviations during continuous culture of yeast at higher dilution rate when phenol was used as limiting substrate. This was attributed more to the fact that the yeast population became heterogeneous during higher dilution rate resulting in clumping and some cells having a larger appearance. The latter cells were found to have lost their ability to metabolize phenol but were still able to utilize catechol.

Continuous culture of strain PAA was carried out with low salt media. River and estuarine waters are low in nutrients and elements. Batch studies using diluted BSM media mimic the nutrient conditions existing in estuaries to a large extent. Studies revealed that there was no variation in the growth and phenol degradation ability of strain PAA, when compared with its growth in normal BSM medium . No change in pH or oxygen concentration was observed. Continuous culture at D= 0.125 h^{-1} resulted in establishment of steady state with complete mineralization of phenol indicating

clearly the feasibility of using strain PAA *in situ* in bioremediation of phenol in river and estuarine waters.

In conclusion, this chapter reveals the ability of strain PAA to degrade phenol in batch and continuous culture and establishes the usefulness of strain PAA in mineralizing phenol in simulated estuarine conditions at a rate similar to that in growth medium (BSM).

CHAPTER V

MOLECULAR REARRANGEMENT RESPONSIBLE FOR EMERGENCE OF STRAIN PAA FROM *P.CEPACIA* AC1100

Chapter IV revealed the potential of mutant strain PAA in biodegradation of phenol by continuous cultures. The ability of mutant strain PAA to degrade phenol in LSM indicates the possibility of it being used in degradation of phenol from aquatic/estuarine water system.

The present chapter deals with molecular characterization of an event which resulted in the development of phenol-degrading mutant PAA from strain AC1100 as observed in Chapter II. Strain AC1100 was isolated by long term chemostat selection using Plasmid Assisted Molecular Breeding (Kellogg et al., 1981). Repeated sequences in AC1100 have known to activate genes by reallocation of genetic sequences and are suspected to have played a role in sequestering diverse aromatic degrading genes, like those involved in 2,4,5-T utilization, from other bacterial genomes (Tomasek et al., 1989; Haugland et al., 1990). The studies conducted in present chapter will help us to understand the molecular mechanism involved in the activation of phenol-degrading ability, which was otherwise silent in parent strain AC1100.

Materials & Methods

Chemicals

Agarose was purchased from Himedia Ltd. Restriction enzymes were obtained from Genei Chemical Co. Ltd., Bangalore. Cesium chloride was of analytical grade and was purchased from SRL SISCO. 2,4,5-T was obtained from Sigma. p-toluidine was obtained from Pune Chemical Co.Ltd, whereas Phenol crystals (molecular biology grade) were purchased from SRL SISCO Ltd. Dialysis tubing was obtained from Sigma. All other chemicals used for preparation of buffers and media were either of LR or AR grade and purchased from local suppliers.

Micro-organisms :

Strain PAA used in the experiments was obtained from *P. cepacia* AC1100 as mentioned in Chapter II. Strain AC1100, PT88 and *E.coli* AC80 were obtained from Dr. Anand Chakrabarty, University of Illinois, USA. PT88[pUS1], PT88[pUS105] and PT88[pSG1] were obtained

during the course of present study. The genotype of these strains are mentioned in Table 5.1.

Plasmids

Plasmid pUS1 and pUS105 were obtained from Dr. Anand Chakrabarty, USA. Plasmid pSG1 was obtained during the course of present studies. Characteristics of these plasmids are mentioned in Table 5.1.

Growth of micro-organisms:

P.cepacia AC1100 and its mutant strains PT88 and PAA were grown and maintained as mentioned in chapter II. E.coli AC80 was routinely grown in LB medium on rotary shaker at 150 rpm at 30±2 ^{o}C. E.coli AC80 transformants i.e., AC80 [pUS1]; AC80 [pUS105] and AC80 [pSG1] were grown in LB medium under selective pressure using tetracycline at a concentration of 15 µg/ml. AC80 transformants were routinely maintained in LB stabs at 4 ^{o}C. For inoculation purpose, AC80 transformants were cultured on LB agar plates containing 15 µg/ml

TABLE 5.1 : Bacterial strains and Plasmid characteristics

Strains/Plasmids	Relevant Properties	Source/Ref.
Strains		
1) PT88	AC1100::Tn5; chq^{-}, 2,4,5-T^{-}, Kan^{r}	Tomasek et al., 1986.
2)PT88[pUS1]	AC1100::Tn5; chq^{+} 2,4,5-T^{+}; kan^{r}; Phe^{-}; tet^{r}	Present study
3)PT88[pSG1]	AC1100::Tn5; chq^{+} 2,4,5-T^{+}; kan^{r}; Phe^{+}; tet^{r}	Present study
4)PT88[pUS105]	AC1100::Tn5; chq^{+} 2,4,5-T^{+}; kan^{r}; Phe^{-}; tet^{r}	Present study
Plasmids		
1)pRK2013	ColE1,mob^{+},tra^{+} Km^{r}	Dr.Anand Chakrabarty University of Illinois USA.
2)pUS1	pCP 13Ω(chq BamHI 25kb); Tet^{r} phe^{-}	Sangodkar et al., 1988
3)pUS105	pCP 13Ω(chq BamHI 8kb); Tet^{r} phe^{-}	Sangodkar et al.,1988
4)pSG1	pUS1 phe^{+}	Present study

chq - Chlorohydroxyhydroquinone, phe - phenol

tetracycline and later inoculated in LB medium containing tetracycline (15 μg/ml). P.cepacia PT88 was grown in BSM containing 0.2% glucose and 50 μg/ml kanamycin.

Triparental mating :

The donor plasmids pUS1 and pUS105 from E. coli AC80 were transferred to recipient P. cepacia PT88 with the help of mobilizer plasmid pRK2013 residing in E. coli AC80. The ratio of donor:helper:recipient was 10:1:1. E. coli AC80 strains containing pUS1 and pUS105 were grown in LB medium containing 15 μg/ml tetracycline for 15 h on rotary shaker at 150 rpm at 30 ^{o}C. Strain PT88 was grown for 36 h in BSM supplemented with 0.2% glucose and kanamycin (50 μg/ml) on a rotary shaker at 150 rpm at 30±2 ^{o}C. Cell suspension of donor, recipient and helper was filtered aseptically through a 0.22 μm filter. The filter paper was washed twice with 2 ml of sterile LB . The filter paper was incubated on LB agar plate at 30 ^{o}C for 24 h and was asceptically transferred to a sterile centrifuge tube. The cells on the filter paper were flushed with 0.85% KCl. The

cell suspension was centrifuged at 10,000 rpm for 10 minutes at 4 ^{o}C. The cell pellet was washed twice with 0.85% KCl and finally resuspended in 1 ml of 0.85% KCl. PT88 transconjugants were selected by plating the above mixture on BSM agar plates containing 75 µg/ml tetracycline, 50 ug/ml kanamycin, supplemented with 1mM phenol or 0.2% glucose as sole carbon source. Washed cells of strain PT88 were used as control by plating a concentrated cell suspension on BSM agar plates containing tetracycline, kanamycin and phenol . Viable cell count of recipient PT88 was obtained by serial dilutions and plating on BSM agar plates containing 0.2% glucose and kanamycin (50 µg/ml). Plasmid transfer frequency was calculated as the ratio of number of transconjugants obtained on BSM agar plates containing tetracycline (75 µg/ml), kanamycin (50 µg/ml) and glucose (0.2%) to viable cell count of recipient (PT88) cells.

Phe^{+} transconjugants obtained during triparental mating were checked for ability to grow on BSM agar plate supplemented with 0.2% glucose and kanamycin (50 µg/ml). Further, PT88 transconjugants growing on BSM agar plate with 1 mM phenol as carbon source under

selective pressure of tetracycline (75 μg/ml) and kanamycin (50 μg/ml) were then confirmed by streaking on BSM agar plates supplemented with 0.1% glucose and 0.08% 2,4,5-T.

Plasmid DNA isolation :

Plasmid DNA from PT88 transconjugants were isolated as described previously (Sangodkar et al. 1988). The plasmids were further purified by Cesium chloride-ethidium bromide density gradient ultracentrifugation as mentioned by Sambrook et al. (1989). Small scale plasmid isolation from E. coli strain AC80 was done by alkaline lysis method (Sambrook et al. 1989).

Transformation :

E.coli AC80 strains were rendered competent using a $CaCl_2$ treatment and transformation was carried out by heat shock treatment (Sambrook et al . 1989).

Detection of catechol by p-toluidine in PT88 transconjugants:

Chromogenic detection of catechol was done using *p*-toluidine as mentioned in Chapter II. BSM agar plates were prepared supplemented with 1 mM phenol. $FeCl_3$ was added aseptically to BSM after sterilization at a final concentration of 1.5 mM. PT88, PT88 pUS1 and PT88 pSG1 were patched on the 3 corners of BSM agar plate which was previously plated with 40 µl of 50 µg/ml *p*-toluidine. 10 µl of 0.01 mM catechol was also patched as positive control. The plate was sealed with parafilm and incubated at 30 ^{o}C for 2 weeks.

Growth of PT88 [pSG1] on phenol :

Colonies of PT88 [pSG1] transconjugants from BSM tetracycline, kanamycin phenol agar plates were inoculated in 100 ml of BSM solution containing 50 ug/ml kanamycin and 1 mM phenol as sole carbon source and incubated at 30 ^{o}C on rotary shaker (150 rpm). The cell growth was monitored at 560 nm and phenol concentration in the medium was determined by modified aminoantipyrene

method (Chapter III). 1 ml of the culture was taken from the flask aseptically at defined time intervals for the above measurements.

Oxygen uptake studies with resting cell suspension of PT88 [pSG1] and PT88 [pUS1] transconjugants:

Oxygen uptake by resting cell suspension of PT88, PT88 [pUS1] and PT88 [pSG1] were determined by Gilson 5/6 oxygraph equipped with a Clarke electrode. Endogenous oxygen uptake was determined before addition of phenol or catechol. Corrected oxygen uptake was expressed as nmole of O_2/min/mg cells. Phenol was used at a concentration of 0.01% whereas catechol was used at a concentration of 0.05%. Resting cells were prepared as mentioned in Chapter II. PT88 [pUS1] tranconjugant and PT88 were grown in BSM containing kanamycin (50 μg/ml), tetracycline (75 μg/ml) , glucose (0.2%) and BSM containing kanamycin (50 μg/ml), glucose (0.2%) respectively. PT88 [pSG1] was grown in BSM containing 0.1% glucose, 1 mM phenol and under selective pressure of 50 μg/ml kanamycin and 75 μg/ml tetracycline.

Restriction analysis of plasmid pUS1 and pSG1 :

Plasmid DNA obtained by alkaline lysis as per Sambrook *et al*.(1989) was digested with restriction enzymes. BamHI and EcoRI were used for restriction digestion of plasmids as per Sambrook *et al*.(1989). The samples were electrophorosed on 0.7% agarose gel as per Sambrook *et al.* (1989). λ DNA precut with Hind III was used as standard marker for determination of molecular weights of restricted DNA fragments.

Results

Manipulation of mutant strain P. cepacia PT88 to acquire phenol utilizing phenotype :

Strain PT88 is a deletion mutant of *P. cepacia* AC1100 that lacks the ability to degrade 2,4,5-T and 2,4,5-Trichlorophenol. As seen in Chapter II, no spontaneous mutants emerged from strain PT88 even on prolonged incubation in presence of phenol. The strategy to manipulate PT88 to utilize phenol, involved

transfer of plasmids carrying genomic regions of wt AC1100 by conjugation and complementing the deletion mutation to phe^{+} by forcing the transconjugants to grow in presence of phenol as done for the emergence of PAA from strain AC1100. The assumption was that plasmids maintained in PT88 will possibly be prone to similar genetic rearrangement as occurred in strain PAA. Plasmid [pUS1] and [pUS105] (Fig.5.1) carrying chq genes on pCP13 cosmid replicon were used and tetracycline resistant transconjugants were selected with glucose as carbon source. The transconjugants were designated as PT88 [pUS1] and PT88 [pUS105]. Forced adaptation of PT88 [pUS1] and PT88 [pUS105] to utilize phenol were carried out using two protocols.

In the first protocol transconjugants were picked from BSM tetracycline Kanamycin, glucose plates and patched by using a heavy inoculum of transconjugants on BSM agar plates containing phenol and incubated for 4 weeks to check for phenol utilizing colonies. In the second protocol, 0.1 ml of transconjugants after mating were plated and allowed to develop slowly on BSM agar medium with 1 mM phenol as sole source of carbon. Plasmid pUS1 and pUS105 could be transferred into PT88

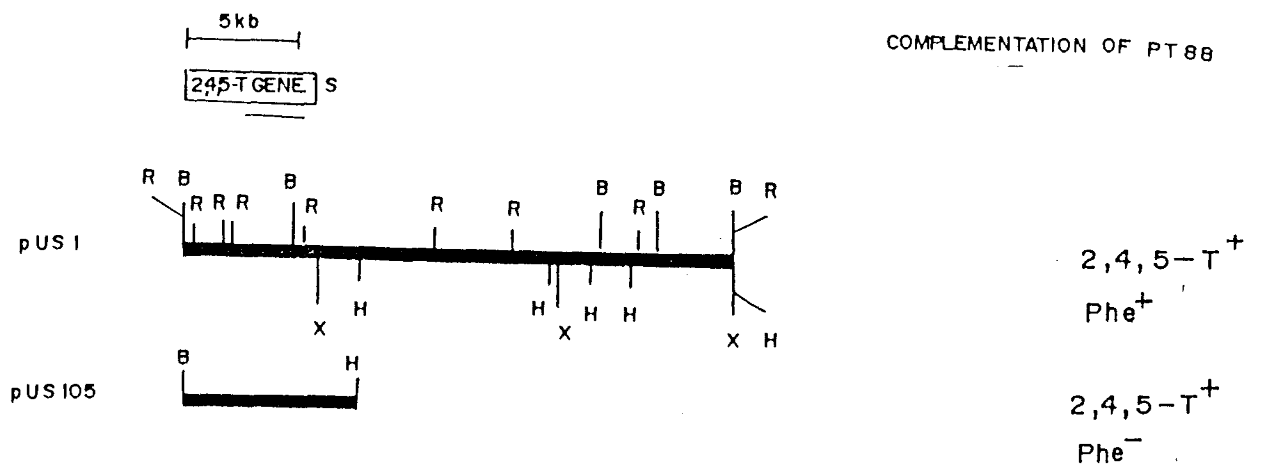


Fig.5.1 Restriction map of 25 kb insert of pUS1 and 8 kb insert of pUS105. Open box indicates the location of chq gene.

at a conjugation frequency of 5×10^{-5} and 9×10^{-5}. However, no immediate emergence of PT88 transconjugants either with pUS1 or pUS105 were observed, when cells were patched on phenol plates. Experiments using second protocol , wherein the mated mixture were plated direcly on phenol plates, few phenol utilizing (Phe^{+}) colonies from transconjugant PT88 [pUS1] were found to emerge after four weeks.. Further incubation of these plates for 2-3 days, resulted in increase in the colony size. The emergence of phenol utilizing transconjugants were observed at a low frequency of 4×10^{-8}. All the phenol utilizing transconjugants were tetracycline resistant indicating the presence of plasmid. The PT88 [pUS1] transconjugants growing on phenol were stable and could be propagated on phenol plates through successive cultures (Fig. 5.2).

No Phe^{+} colonies emerged on BSM agar phenol plates in case of transconjugants PT88 [pUS105] even on prolonged incubation. Few very minute colonies emerged, which when restreaked on phenol plates did not promote growth. Further, the cells of recipient, mutant PT88 alone when used as control did not give rise to any confluently growing phenol utilizing strain on BSM agar

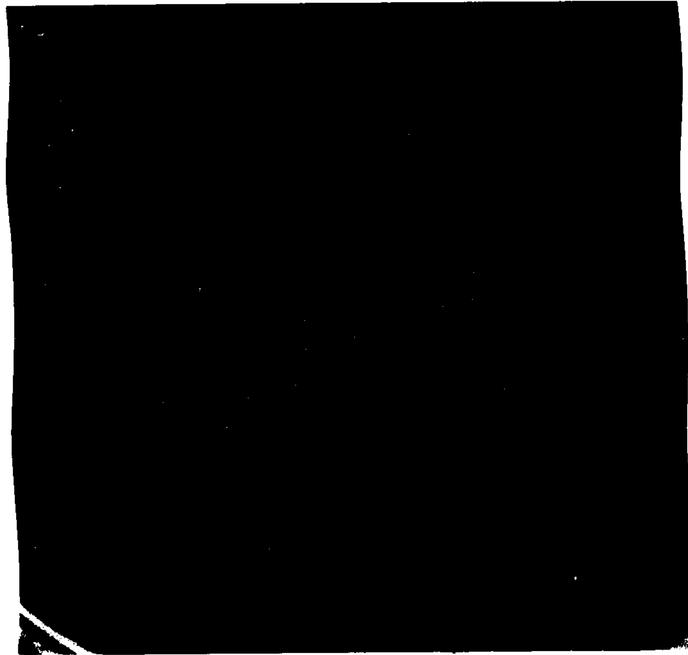


Fig.5.2 Growth of PT88 [pSG1] transconjugants in BSM containing tetracycline (75 μg/ml); Kanamycin (50 μg/ml) and phenol (1 mM).

plates containing kanamycin, tetracycline and phenol indicating that the Phe^{+} ability acquired by transconjugants PT88 [pUS1] was by virtue of presence of pUS1, which had presumably undergone a rearrangement .

Confirmation of mutation of pUS1 in PT88 :

One of Phe^{+}, PT88 [pUS1] transconjugant clone which was rapidly growing on phenol was grown in 500 ml of BSM containing tetracycline (75 µg/ml), glucose (0.1%) and phenol (1 mM) for 48 h. The plasmids from PT88 transconjugants was isolated and purified by Cesium chloride density gradient centrifugation. The plasmid pUS1 obtained from PT88 transconjugant growing on phenol was designated as pSG1. The plasmid preparation was used to transform the competent cells of *E. coli* AC80. The tetracycline resistant transformants of AC80 were obtained.

The resultant *E. coli* AC80 [pSG1] was now used as a donor strain to transfer the plasmid back into mutant strain PT88 by triparental mating. The PT88 transconjugants were selected on BSM agar plates

containing tetracycline, kanamycin and glucose as well as on BSM agar plates containing tetracycline, kanamycin and phenol. The plasmid transfer frequency was 1×10^{-5} as observed on BSM glucose plates containing tetracycline and kanamycin. Similarly the transconjugants selected on BSM phenol plates were obtained at a frequency of 4×10^{-5} which was 10^{3} fold higher than that obtained during previous transfer, confirming that the Phe^{+} phenotype acquired by PT88 transconjugant was due to mutation in pUS1. The rearranged plasmid was therefore designated as pSG1. Utilization of aromatic substrates by transconjugants PT88 [pUS1] and PT88 [pSG1], showed a characteristic bright red colour formation when streaked on BSM agar plates containing glucose and 2,4,5-T (Fig. 5.3). Similarly, PT88 which did not grow on 2,4,5-T, could utilize it as sole source of carbon, when its transconjugants carried either plasmids pUS1, pSG1 or pUS105. Further, like PT88, all its transconjugants showed kanamycin resistance and utilized benzoate as sole source of carbon.

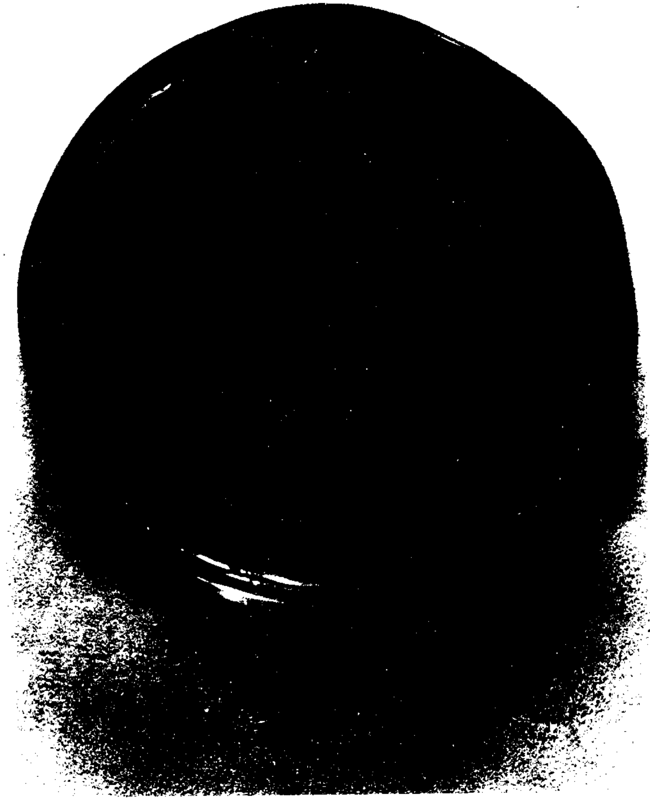


Fig.5.3 Ability of PT88 [pSG1] transconjugants to form red colour intermediate, 5-chloro,2-hydroxy-1,4-benzoquinone, when grown in presence of glucose and 2,4,5-T.

Kinetics of phenol utilization by PT88 [pSG1]:

Since amongst the transconjugants only PT88 [pSG1] could grow on phenol, pure colonies of PT88 [pSG1], pregrown on BSM agar plates containing tetracycline, kanamycin and phenol were picked and inoculated in 100 ml of BSM solution containing kanamycin and 1 mM phenol. Figure 5.4 shows the typical growth pattern of PT88 [pSG1]. There was a lag of almost 16 h before active growth ensued. There was exponential increase in cell mass as observed by increase in cell absorbance at 560 nm with simultaneous depletion of phenol from the medium as seen from Figure 5.4. Phenol was completely degraded from 100 ppm to a final concentration of 3 ppm. When PT88 [pUS1] and PT88 [pUS105] were used as control, no growth was observed in BSM kanamycin phenol, even after addition of high inoculum. Strain PAA was grown separately in BSM containing phenol, as control (Fig. 5.4).

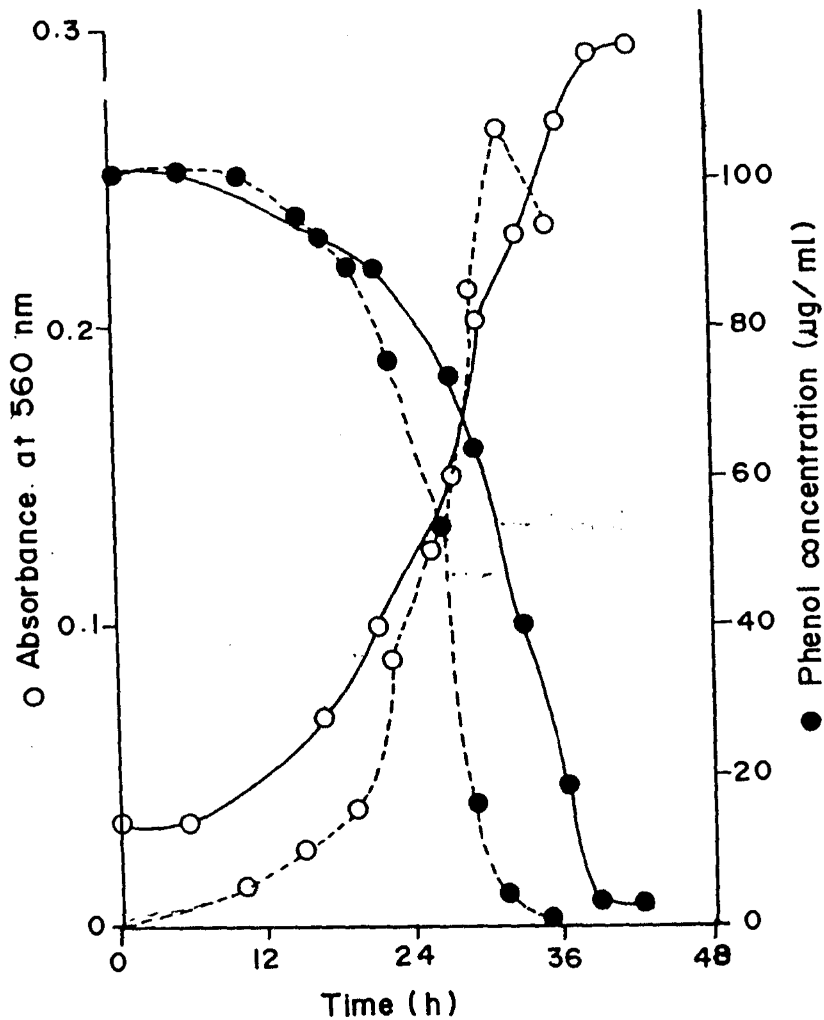


Fig.5.4 Batch culture of PT88 [pSG1] (———)and PAA (-----) in BSM containing 1 mM phenol. Strain PAA was grown in seperate flask for comparing the growth kinetics with PT88 [pSG1] transconjugants. O - Growth of PAA and PT88 [pSG1]; ●- phenol concentration.

Biochemistry of phenol utilization by PT88 [pSG1]:

a) Detection of catechol as an intermediate of phenol degradation :

To detect formation of catechol, a key intermediate of phenol, PT88 [pSG1] colonies were patched on BSM agar plates containing $FeCl_3$ and phenol as mentioned in Chapter II. *p*-toluidine was used for chromogenic detection. PT88 [pSG1] colonies became darkish brown in colour in presence of *p*-toluidine as seen in figure 5.5. Catechol used as control also showed dark brown spots. Strain PT88 and its transconjugants when used as control did not show any colour formation (Fig. 5.5) indicating that PT88 [pSG1] generated catechol from phenol. Colonies of PT88 [pSG1] when sprayed with catechol, however, did not show formation of any yellow colour, indicating distinct absence of α-hydroxymuconic semialdehyde in the pathway. Also, crude extracts prepared from transconjugants PT88 [pSG1] pregrown in BSM phenol did not show any C23O activity.

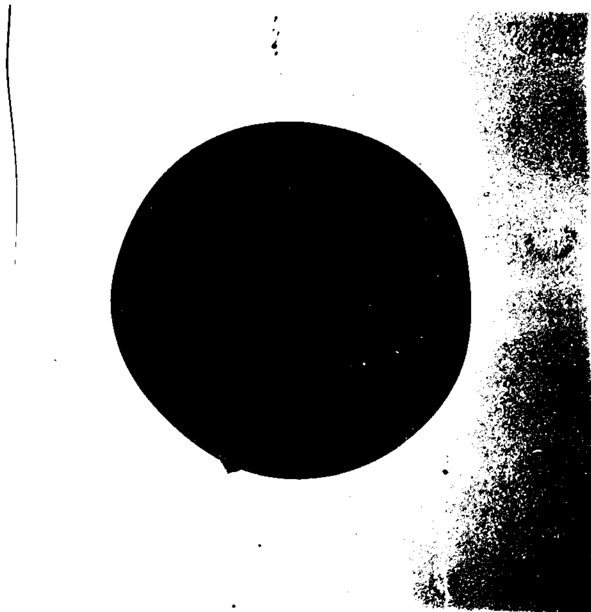


Fig.5.5 Amplification of catechol detection by incorporation of p-toluidine in the medium. The petriplate has been divided into 4 sectors and bacterial cells were inoculated as patch. (1) PT88; (2) PT88[pUS1]; (3) PT88[pSG1] and (4) catechol.

Detection of phenol degrading enzymes :

To detect phenol hydroxylase activity, washed cells of PT88 [pSG1] pregrown in phenol were incubated with phenol. Table 5.2 shows the results of oxygen uptake studies done using PT88 and its transconjugants in presence of phenol. It can be clearly seen that PT88 [pSG1] transconjugants indeed oxidized phenol without any lag. The oxygen uptake by PT88 [pSG1] transconjugants is nearly identical to that observed in case of PAA (Chapter II ,Table 2.3). In case of PT88 and its transconjugants PT88 [pUS1], oxidation of phenol was negligible as seen from Table 5.2. Strain PT88 and all other transconjugants were seen to oxidize catechol at the same rate.

Genetic analysis of PT88 [pSG1] :

After confirmation of the ability of PT88 [pSG1] transconjugants to grow on phenol, plasmids pUS1 and pSG1 were isolated from respective E.coli AC80 transformants and analyzed by gel electrophoresis. Figure 5.6 depicts the DNA profile.

Table 5.2 Oxygen uptake nmole O_2/min/mg cell

Strain	Phenol	Catechol
PT88 [pSG1]	18.05	34.51
PT88	< 1	28.7
PT88[pUS1]	0	10.2

Fig.5.6 Plasmid profile from _E.coli_ AC80 [pUS1] and AC80 [pSG1] on a 0.7% agarose gel. Lane 1. plasmid DNA from AC80 [pUS1]; Lane 2. plasmid DNA from AC80 [pSG1].

Figure 5.7 depicts the BamHI digest of plasmid pUS1 and using the standard molecular weight markers of λ DNA cut with Hind III, the molecular weight of pUS1 digested fragments were 21, 13.4, 5, 3.8 and 2.8 kb, respectively. The EcoRI digest of plasmid pUS1 resulted in 21, 6.5, 5.8, 4.3, 4, 3, 1.4, 0.56 and 0.55 kb, respectively (Fig. 5.8).

Figure.5.9 shows the profile of EcoRI digest of plasmid pSG1. Fragments with molecular weights 21, 3, 2.5 and 2.2 kb could be observed. Plasmid pSG1 when treated with BamHI showed only one linearised band.

DISCUSSION

In the previous studies, we have reported the evolution of mutant PAA from P. cepacia AC1100 by growing it under selective pressure in presence of phenoxyacetic acid and phenol. Enzymatic studies also revealed the presence of an active enzymatic machinery in strain PAA, which is independent of the 2,4,5-T pathway and is involved in phenol metabolism (Ghadi & Sangodkar, 1994). Surprisingly, strain AC1100 or PT88

Fig.5.7 Restriction analysis of pUS1. Lane 1. pUS1 plasmid DNA from AC80 digested with Bam HI. Lane 3. λ DNA digested with Hind III. Molecular weight of fragments from top to bottom : 23, 9.4, 6.5, 4.3, 2.3 and 2 kb.

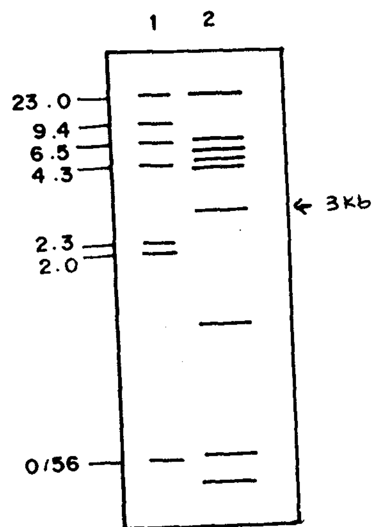


Fig.5.8 Restriction analysis of pUS1. Lane 1. λ DNA digested with Hind III. Molecular weight of fragments from top to bottom: 23, 9.4, 6.5, 4.3, 2.3 and 2 kb. Lane 2. plasmid pUS1 DNA digested with EcoR I.

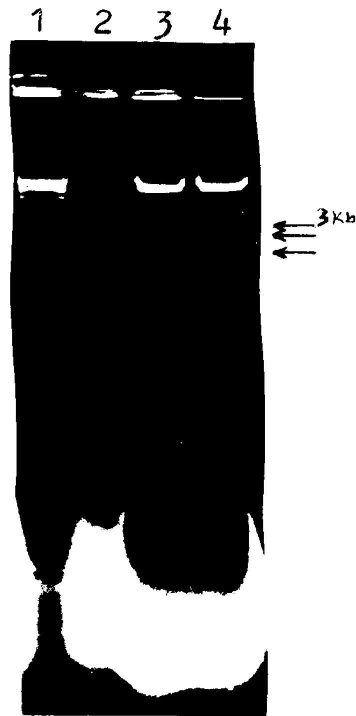


Fig.5.9 Restriction analysis of pSG1. Lane 1. λ DNA digested with Hind III. Molecular weight of fragments from top to bottom: 23, 9.4, 6.5, 4.3, 2.3 and 2 kb. Lane 2. Plasmid pUS1 digested with BamHI. Lane 3 and 4. plasmid pSG1 DNA from AC80 digested with EcoR I.

did not show the presence or any evidence of enzymes which are involved in phenol catabolism.

Members of *Pseudomonas* species, especially *P. cepacia* is the most biochemically versatile organism. The ability to evolve new pathways, as reviewed by Coco et al, (1990) and Meer *et al* ,(1992) has conferred *Pseudomonas* with the unique ability to mineralize vast majority of natural and synthetic organic compounds. *P. cepacia* AC1100 is a unique organism created and evolved by Plasmid Assisted Molecular Breeding in a chemostat (Kellogg *et al*., 1981). Strain AC1100 degrades 2,4,5-T *via* 2,4,5-trichlorophenol and chlorohydroxyquinone (Karns *et al.*, 1983; Chapman *et al*., 1987). The forced evolution of strain AC1100 to give rise to strain PAA (Chapter II) could be possible either by modifying key enzymes of the existing 2,4,5-T pathway to accommodate non-chlorinated phenol or by activation of silent or cryptic phenol degrading genes.

The presence of phenol hydroxylase and *meta*-pathway enzymes in PAA and its absence in strain AC1100 and PT88 indeed confirms the phenomenon of gene activation which occurs during selective pressure of AC1100 as mentioned

before (Chapter II). To characterize the molecular mechanism leading to emergence of PAA, the following studies were carried out.

Strain AC1100 harbors at least two plasmids (Ghosal et al., 1985), one of which shows considerable homology to 2,4-D degradative plasmid pJP4 (Don and Pemberton, 1983). A substantial number of aromatic catabolic pathways are plasmid encoded. There are few reports on catabolic plasmids responsible for phenol degradation (Shingler et al., 1989; Hermann et al., 1987 and Kivisaar et al., 1989). However, P. putida strain PP1-2 is known to degrade phenol by ortho cleavage pathway which is chromosomally encoded (Wong and Dunn, 1974). Hence strain PAA was conjugated with a Phe^- strain PT88. Our experiments indicate the possibility that the two plasmids present in AC1100 are not directly responsible for phenol degradation. Also continuous subculturing of strain PAA in glucose has revealed no loss in phenol degrading characteristics of strain PAA as observed by Hermann et al (1987) in case of P.putida. Thus the phenol degrading genes in strain PAA are chromosomally encoded.

The *meta*-cleavage pathway genes are generally plasmid encoded. A fraction of mixed cell suspension of strain PAA and PT88 was incubated for 24 hours for biparental mating. 0.1 ml of cell suspension was spread plated on BSM plated containing kanamycin (50 ug/ml) and 0.2% glucose, so as to obtain a dense growth. The colonies were spread with 10 mM catechol. No yellow colour colonies were observed when sprayed with catechol indicating the possibility of both phenol degrading and *meta*-cleavage pathway genes to be encoded chromosomally in strain PAA.

Further confirmation of phenol degrading genes being chromosomally encoded came interestingly by complemenation of 25 kb fragments from AC1100 which is known to complement 2,4,5-T degradation in PT88 (Sangodkar *et al*, 1988). During triparental mating, though plasmid transfer frequency was 5×10^{-5} as observed on BSM agar plates containing tetracycline, kanamycin and glucose, initially no transconjugants could be obtained on phenol. However, on prolonged incubation, few PT88 [pUS1] transconjugant colonies started emerging on phenol plates. The emergence of such phenol utilizing transconjugants from pUS1 was

observed at a very low frequency of 4×10^{-8}.

The situation observed during emergence of PT88 transconjugants growing on phenol is analogous to emergence of mutant strain PAA from AC1100, when the latter was grown under selective pressure in presence of phenol. The emergence of strain PAA from AC1100 took about 3 weeks and occured at a frequency of 1×10^{-8}. PT88 which has a deletion mutation did not give rise to phenol degrading variants. The similarity in both the experiments point out to the possibility that the molecular mechanism resulting in gene activation in PAA and PT88 [pSG1] seems analogous.

pSG1 plasmid was isolated from PT88 background and was transformed back into E. coli strain AC80. The resultant E coli AC80 [pSG1] was used as a donor strain to transfer pSG1 back in to strain PT88 by triparental mating. The results indicate that pSG1 could indeed generate phenol utilizing transconjugants at a frequency of 1×10^{-5}, which was comparable to plasmid transfer frequency and was 10^{3} fold higher than the frequency with which the Phe^{+} emerged from PT88 [pUS1] indicating that all the pSG1 plasmids transferred the Phe^{+}

phenotype to PT88 . Thus, unlike pUS1, pSG1 contains the rearranged or mutated fragment which is solely responsible for high number of Phe^{+} transconjugants.

The PT88 [pSG1] transconjugant was stable only when grown under selective pressure in presence of tetracycline and phenol. Strain PT88 is a deletion mutant of AC1100 (Tomasek et al., 1986) and is a blocked mutant for 2,4,5-T degradation. In presence of glucose and 2,4,5-T, it accumulated a bright red coloured intermediate (Sangodkar et al., 1988). This test was used to confirm the identity of transconjugants as PT88. All Phe^{+} transconjugants accumulated bright red compound which was less intense than strain PT88 because of complementation of chq mutation. pUS1 also complements 2,4,5-T mutation in PT88 by producing CHQ metabolizing enzymes (Sangodkar et al 1988).

It may be recalled from previous work of Sangodkar et al. (1988) that the 4.8 kb XbaI-BamHI fragment of pUS1028 containing CHQ metabolizing genes of 2,4,5-T pathway fails to hybridize with BamHI digested DNA from PT88 indicating deletion in this region and also possibly in the lower part of the pathway. Since pUS1

containing 25 kb fragment shows Phe^+ complementation after getting rearranged to pSG1, it clearly indicates that the fragment contains the genes responsible for initial conversion of phenol to catechol. The genes responsible for conversion of phenol to catechol is not related to 2,4,5-T degrading genes as pUS105 which exactly complements 2,4,5-T mutation in PT88 (Sangodkar et al, 1988) did not give rise to Phe^+ transconjugants. Also PT88 failed to give rise to any Phe^+ mutants indicating a possible deletion in genes responsible for conversion of phenol to catechol. The above observation confirm that initial part of phenol degradation is chromosomally encoded and the fragment responsible is present on plasmid pSG1.

The Phe^+ transconjugants obtained when sprayed with catechol, did not form yellow colour. Even crude extracts prepared by sonication of PT88 [pSG1] transconjugants pregrown with phenol as sole source of carbon did not show any catechol 2,3-dioxygenase activity. This suggests that the 25 kb fragment contains only the active phenol hydroxylase gene.

PT88 [pSG1] accumulates catechol as intermediate,

when grown in presence of phenol which interacts with *p*-toluidine to form a brown coloured intermediate (Fig. 5.5). Detection of catechol as an intermediate using *p*-toluidine has been widely used by Parke (1993). PT88 [pSG1] however, did not accumulate any brown colour intermediate as such when grown in presence of phenol (in absence of *p*-toluidine). Mutants defective in catechol metabolism, generally turn brown. The presence of complementation and absence of accumulation of brown colour intermediate indicates the channeling of catechol *via* *ortho* pathway which is preexisting in PT88. A similar phenomena has been observed in *Pseudomonas* CF600 which degrades phenol *via* *meta* cleavage pathway (Shingler *et al*. 1989). A Tn5 mutant of CF600 having lost the ability of metabolizing phenol could be complemented by 1.2 kb fragment which encoded for phenol hydroxylase. However, the catechol which was formed was channelized *via* the *ortho*-pathway which was chromosomally located in strain CF600 (Shingler *et al*., 1989).

Unlike pUS1, pUS105 fails to complement Phe^- PT88, we conclude that,the presence of 16 kb fragment to the right of CHQ metabolizing genes, is essential for

activation of phenol degrading genes. However, the exact role could not be determined. Also , failure to obtain any positive indication of meta- cleavage activity from pUS1 or pSG1 might indicate the possibility of existence of meta-cleavage pathway genes elsewhere in the genome.

Clustering of aromatic pathway genes on chromosome or plasmid have been well documented (Bartilson et al., 1990; Shingler et al., 1989; Kivisaar et al., 1989; Kukor and Olsen, 1991). However, our results differ from above. Strain AC1100 has been isolated by Plasmid Assisted Molecular Breeding in a chemostat by breeding a mixed population of bacteria from diverse polluted sites. The creation of P. cepacia AC1100 with 2,4,5-T degrading ability can be attributed to gene flux taking place amongst the microorganism. Many aromatic catabolic pathways are either plasmid encoded or found to be either wholly or partially encoded within large transposon (Meer et al., 1992). The potential degrading ability must have been built up piece meal by acquisition of preevolved metabolic modules either from Pseudomonas or non Pseudomonas ancestor. Hence strain AC1100 might possess a reservoir of genes, homologous or non homologous. Also , the genetic location of

catabolic pathway may be different.

The 2,4,5-T genes located on pUS1 are unique to P.cepacia AC1100, as none of the DNA from other Pseudomonas species showed any hybridization indicating the acquisition of 2,4,5-T degrading genes from non Pseudomonas anchestor (Tomasek et al., 1989).

The PT88 [pSG1] transconjugants indeed metabolize phenol as seen from batch culture experiments. Similarly oxygen uptake experiments indicate higher phenol oxidation rate by PT88 [pSG1] transconjugants, when compared to PT88 [pUS1] transconjugants. The values are comparable to oxidation rate of PAA pregrown in phenol (chapter II ,table 2.3).

EcoRI digestion of pSG1 resulted in fragments having molecular weight 21, 3, 2.5 and 2.2 kb respectively. By contrast, many fragments detected during digestion of pUS1 by EcoRI, could not be observed in pSG1. For instance, 6.5, 5.8, 4.3, 4, 1.4 were not represented in pSG1. Besides the vector arm of pCP13, only 3 kb-EcoRI fragment was observed to be common in both, pUS1 and pSG1. The presence of 2.5 and 2.2 kb in

pSG1 were additional and were not detected during EcoRI digest of pUS1. These observations clearly provide an evidence that the pSG1 has resulted after the rearrangement of pUS1.

A significant conclusion which can be drawn to explain the variation in restriction pattern of pSG1 is the molecular rearrangement of 25kb fragment in pUS1 (Phe^-) to pSG1 (Phe^+). This involves deletion of DNA from pUS1, during growth of PT88 [pUS1] transconjugants in presence of phenol.

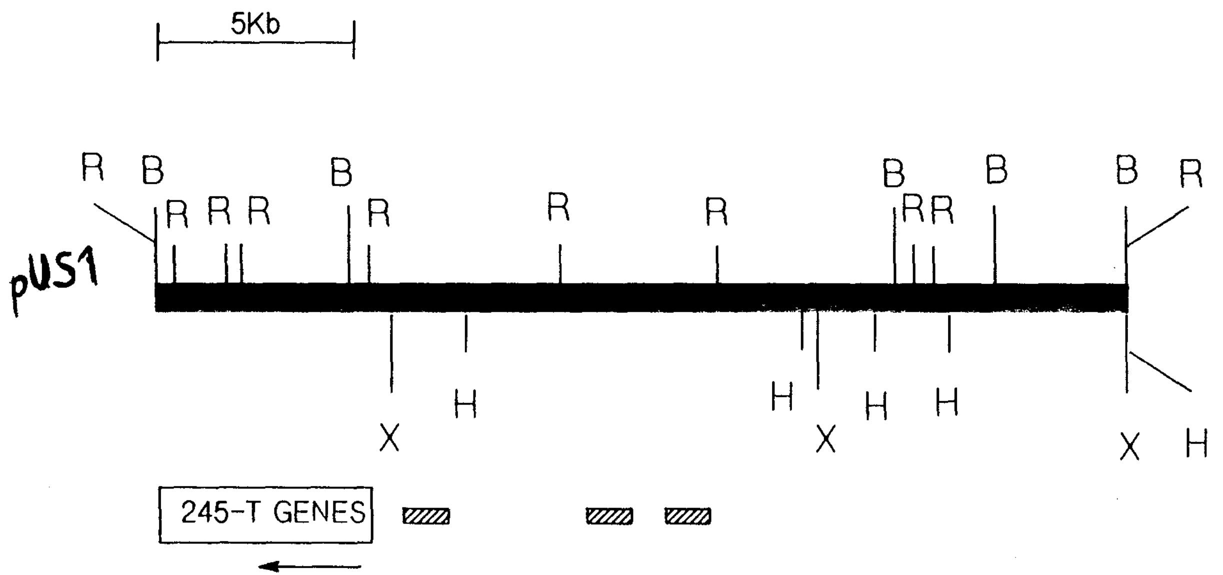
Strain AC1100 and PT88 is a reservoir of repeat sequences and both IS931 and IS932 have been found to be mostly clustered around 1300 kb or one third of chromosomal DNA. (Haugland et al. 1990; Coco et al. 1990). Also three copies of IS931 were mapped near 2,4,5-T genes encoding degradation of 5-chloro-1,2,4-trihydroxybenzene, an intermediate in the degradation of 2,4,5-T. (Tomasek et al. 1989).

IS931 is known to undergo transposition in AC1100 and can also serve to activate the expression of promoterless genes and mobilise adjacent DNA (Haugland

et al. 1989). Figure. 5.10 shows the relative position of three copies of IS931 sequences adjacent to chq genes in pUS1. Similarly pUS105, a subclone of pUS1 which complements 2,4,5-T mutation in PT88 also has a single copy of IS931 as seen in Figure 5.10 . (Tomasek et al, 1989).

The 3 kb-EcoRI fragment seen in restriction pattern of pUS1 and pUS105, has also been observed in pSG1 (Fig.5.9) However, this entire 3 kb fragment is not directly involved in 2,4,5-T utilization in pUS1 and pUS105 because pUS1028, a subclone of 25 kb fragment from pUS1, complemented 2,4,5-T mutation in PT88 though there was a internal deletion of 1.1 kb inside the region of 3 kb fragment. (Sangodkar et al. 1988). Thus, the presence of 3 kb-EcoRI fragment seems to be directly responsible for phenol utilization or activation. The presence of IS931 sequence just adjacent to the 3 kb-EcoRI fragment may have a very crucial role to play in rearrangement.

Earlier studies have indicated that 2,4,5-T genes and IS931 sequences have been recruited from taxonomically distant sources. (Tomasek et al. 1989).



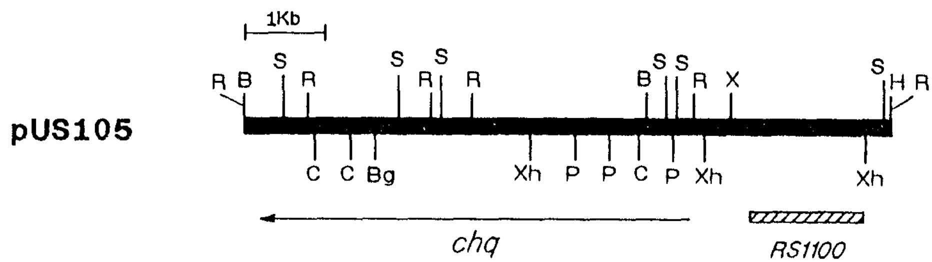


Fig.5.10 Relative position of RS1100 in pUS1 and pUS105. Open box shows the position of 2,4,5-T genes involved in metabolism of CHQ. Hatched boxes indicate the location of RS1100.

Thus the possibility of phenol degrading genes being recruited from distant microbial species cannot be ruled out. Sangodkar _et al_., (1988) found no hybridization of _xyl_E gene from _P. putida_, encoding C23O, with AC1100 DNA even under low stringency. However, strain PAA clearly shows the presence of C23O activity. This suggests that the genes of C23O which was silent in strain AC1100 is not homologous to that of _P. putida_.

Most of the aromatic pathway genes are clustered and strain AC1100 seems to have recruited genes bypassing the requirements for genetic relatedness, leading to gene acquisition from any sources during directed evolution. Danganan _et al_., (1994) have cloned the 2,4,5-T oxygenase gene which can convert phenoxy acetate to phenol, a suicidal substrate for strain AC1100 because of non utilization. However, the presence of silent gene(s), which is activated during the emergence of PAA indicate the possibility of 2,4,5-T and phenol degrading genes being recruited at the same time during directed evolution. However, it is possible that phenol degrading gene might have been inactivated by insertion sequences like IS931 which on selective pressure in presence of phenol, might transpose adjacent

DNA sequences ,especially the 16 kb fragment distal to <u>chq</u> gene(s) leading to gene activation resulting in phenol utilization.

CHAPTER VI

SUMMARY AND CONCLUSION

Our strategy for in vitro evolution to expand the catabolic trait of strain AC1100 has resulted in the emergence of a mutant strain PAA developed under strong selective pressure. Although *P. cepacia* AC1100 could give rise to mutant PAA when grown in presence of phenoxyacetate or phenol, strain PT88 (a deletion mutant of strain AC1100, 2,4,5-T$^-$) did not give rise to any spontaneous mutant under similar conditions, indicating that genes involved in phenol metabolism may indeed have been deleted along with the segment of 2,4,5-T genes.

The emergence of strain PAA from *P. cepacia* AC1100 occurred at a frequency of 1×10^{-8}. Unlike AC1100, strain PAA showed the presence of C23O activity. Oxidation studies with resting cells of strain AC1100 and its mutants, indicated that the ability to oxidise phenol was present only in strain PAA. Enzymatic studies indicate the presence of an active enzyme machinery (C23O, HMSD, HMSH) of the *meta* cleavage pathway solely in strain PAA. None of the *meta* cleavage pathway enzymes could be detected in AC1100 or PT88. It

is thus evident that strain PAA metabolizes phenol *via* the *meta* cleavage pathway independent of the 2,4,5-T pathway.

The emergence of a spontaneous mutant from strain PT88 growing on phenol would have resulted in a blocked mutant if phenol was indeed channelized *via* the existing 2,4,5-T pathway, due to relaxed substrate specificity of certain key enzymes. The blocked mutant would have accumulated hydroxyquinone, which could have been detected as a coloured intermediate. Failure to obtain any mutant of strain PT88 growing on phenol is indicative of a separate pathway for its utilization.

Intermediates such as catechol, HMS and γ-oxalocrotonate have been detected in strain PAA during phenol metabolism, thus confirming its degradation *via* *meta* cleavage, even though the *ortho* cleavage pathway preexisted in strains AC100 and PAA.

Batch culture experiments in flask and fermentor indicated the ability of strain PAA to completely mineralize phenol. Batch culture of strain PAA, up to 500 mg/l of phenol indicated increase in lag period.

The specific growth rate was not, however, affected. Similarly, a plot of final absorbance *versus* initial phenol concentration indicated a linear relationship. Kinetic studies on phenol biodegradation indicates a high K_s and low V_{max} resulting in a slow rate of phenol degradation in strain PAA. However, the degradation of catechol is faster, as observed by the low K_m value when compared to archtype *P. putida*.

Strain PAA, besides showing the ability to oxidize phenol, could oxidize related analogues or substituted phenolic compounds such as aminophenol and nitrophenol but not chlorophenol. The oxidation of substituted or analogues of phenol occurred at a lower rate. The presence of broad substrate-specific hydroxylase in strain PAA is a possibility.

Steady states could be achieved during continuous culture of strain PAA with phenol as the limiting substrate. At D = 0.1 h^{-1}, the phenol in the effluent was less than 1ppm. The wash out point was obtained at D = 0.2 h^{-1}. The μ_{max} for strain PAA was 0.175 h^{-1}, and therefore selection of a variant strain with ability to degrade phenol at a faster rate could be

achieved. No wash out was observed with this variant even at $D = 0.35\ h^{-1}$. Further, the growth of strain PAA in LSM was achieved without any effect on cell growth or phenol degradation. The ability of strain PAA to degrade phenol in batch and continuous culture indicates the feasibility of strain PAA for bioremediation of phenolic waste in estuaries and rivers where low nutrients and elements are present.

The emergence of PT88 [pSG1] transconjugants during conjugation of pUS1 into PT88 occurred at 5×10^{-8}, which was onethousand times less than the actual plasmid transfer frequency. This indicates rearrangement or mutation on the 25 kb fragment of pUS1 when forcefully maintained in the background of PT88 host on phenol. This is analogous to the molecular rearrangement occurring in strain AC1100 during emergence of strain PAA under selective pressure.

Batch culture of strain PT88 [pSG1] indicates complete mineralization of phenol. Catechol was detected as an intermediate during growth of PT88 [pSG1] on phenol. Unlike strains PT88 and PT88 [pUS1], PT88 [pSG1] could oxidize phenol. Interestingly,

although possessing Phe^{+} phenotype, PT88 [pSG1] was devoid of C23O enzyme activity.

Restriction analysis of plasmids pSG1 and pSU1 indicated that DNA fragments were missing in pSG1. A common 3 kb EcoRI fragment was found in both the plasmids. This fragment is presumed to be involved in phenol utilization. Although the common 3 kb fragment has been identified as part of the 2,4,5-T genes, internal deletion in this region did not affect the ability to complement 2,4,5-T mutation in PT88.

Since no C23O activity was observed in transconjugants PT88 [pSG1], the presence of catechol as intermediate detected during its growth on phenol and complementation indicates the possibility of the 3 kb fragment harbouring a phenol hydroxylase gene which would convert phenol to catechol. The catechol could then be channelized _via_ the preexisting _ortho_ pathway.

Insertion sequences IS931 (found close to 2,4,5-T genes) indeed have a major role in activation of phenol degrading gene(s). The activation proceeded only after loss of a DNA fragment distal to the 2,4,5-T genes

already identified. It is however not clear at this stage whether activation has been brought about by transposition of IS931 along with adjacent DNA sequences resulting in activation, or due to transposition of IS931 near the cryptic gene.

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* not referred to in original

APPENDIX I

Basal Salts Medium (BSM)

Stock A (10 X)

Dipotassium hydrogen phosphate	42.5 g
Sodium dihydrogen phosphate	10 g
Ammonium chloride	20 g

Adjust the pH to 7.0 and make up the volume to 1 l with distilled water.

Stock B (10X)

Nitrilotriacetic acid (disodium salt)	1.23 g
Magnesium sulphate	2.00 g
Ferrous sulphate	0.12 g
Manganous sulphate	0.03 g
Zinc sulphate	0.03 g
Calcium chloride (hydrated)	0.03 g
Cobaltous chloride	0.01 g

Make up the volume to 1000 ml with D/W

Mix stock A and Stock B to obtain a final concentration of 1X.

Carbon sources were added aseptically after sterilization.

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