BIODEGRADATION OF AROMATIC AMINES BY ALKALOPHILIC BACTERIA

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DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

by

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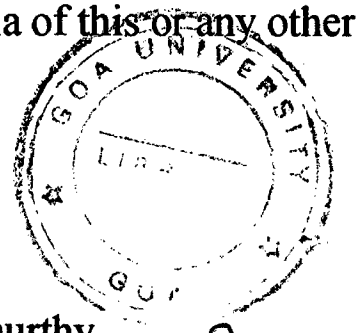
CERTIFICATE

This is to certify that *Mr. Kumar Naveen Krishnamurthy* has worked on the thesis entitled "BIODEGRADATION OF AROMATIC AMINES BY ALKALOPHILIC BACTERIA" under my guidance and supervision.

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



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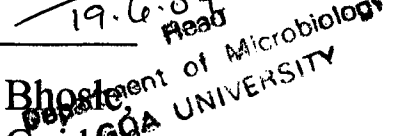

Dr. Saroj Bhosle,
Research Guide &
Head, Department of Microbiology ,
Goa University

STATEMENT

I hereby state that this thesis for the Ph.D. degree on "**Biodegradation of Aromatic Amines by Alkalophilic Bacteria**" is my original contribution and that the thesis and any part of it have 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 work of its kind from this area.

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I dedicate this work to my parents, because what I achieved till the date is only due to their love and blessings. They are my constant inspiration.

Naveen

Dedicated

to my

Parents.

ABBREVIATIONS

α	alpha
Abs	Absorbance
APS	Ammonium per sulfate
β	Beta
b.p.	Boiling point
BCA	Bicinchoninic Acid
°C	Degree celcius
cfu	Colony forming unit
D/W	Double distilled Water
EDTA	Ethylene diamine -
	- tetra acetic acid
EPS	Exopolymeric substances
FAME	Fatty Acid Methyl Esters
FP	Fluorescent product
Fig.	Figure
gm	Gram(s)
GC	Gas chromatography
Glu	Glucose
hr(s)	Hour(s)
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
IR	Infra Red
KDa	Kilo Dalton
Kbps	Kilo base pairs
KNO_3	Potassium nitrate
L	Litre
lbs	Pounds
M	Molar
mg	Milli gram(s)
MSM	Mineral Salt Medium
min(s)	Minute(s)
MIS	Microbial Identification System
ml	Milli litre
m	Meta
mg	Milligram
mM	Milli molar
MS	Mass spectrometry
μg	Microgram
μL	Microlitre
μM	Micromolar
μ	Micron
NaCl	Sodium chloride
Na^+	Sodium ion
NH_4NO_3	Ammonium nitrate
NH_4Cl	Ammonium chloride
NA	Nutrient Agar
NaOH	Sodium hydroxide
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NND	N,N-Dimethyl,1-Naphthylamine
O.D.	Optical Density
ONGC	Oil and natural gas commission
o	Ortho
p	Para
PAGE	Poly-acrylamide gel electrophoresis
PPYG	Polypeptone yeast extract glucose agar
PPY	Polypeptone yeast extract medium
	without glucose
Rf	Resolution factor
rpm	Revolutions per minute
RT	Room temperature
Rt	Retension time
SDS	Sodium dodecyl sulfate
sec.	Second(s)
sp.	Species
TEMED	Tetra methyl ethylene diamine
TLC	Thin layer chromatography
TP2	Transformed product 2
UV-Vis	Ultra violet - Visible
V	Volts
v/v	Volume / Volume
w/v	Weight / Volume
%	Percentage
λ	Lambda

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CHAPTER I

INTRODUCTION

Aromatic compounds constitute a major portion of organic compounds following the glucosyl ones in nature. Anthropogenic activities have also led to release of various other complex aromatic compounds into the environment through effluents from industries and unregulated use of pesticides and fertilizers. Such compounds alien to the environment are termed as xenobiotics.

The manufacture of xenobiotics involves the use of various substituted aromatic compounds of which aromatic amines are one such group that includes aniline - the simplest aromatic amine, chloroanilines, nitroanilines, aminobenzoates, polycyclic aromatic amines. Aromatic amines are widely used in the manufacture of drugs, dyes and pesticides; anilines are known to form coloured compounds on coupling with other aromatic compounds collectively known as azo dyes. These dyes are used in fabric and leather dying industries, food and drink, pharmaceutical, paper, plastics, lacquer, paints and wood staining (Combes and Haveland-Smith, 1982; Mainwright, 2007; Ardejani *et al.*, 2007). Substituted anilines are potential contaminants of aquatic environments because of their large-scale use as precursors in the industrial synthesis of pesticides, plastics, dyes, polyurethanes, rubber, drugs, photographic chemicals, varnishes, and pesticides (Struijs and Roger, 1989; Radulescu, *et al.*, 2006; Boon, *et al.*, 2000). Amines with hydroxyl groups, e.g. 4-Acetamidophenol is an extensively used analgesic/antipyretic worldwide. 4-Acetamidophenol and 4-aminophenol are also used in the manufacture of azodyes and photographic chemicals (Merck index). Diphenylamine (DPA) is the most commonly used stabilizer for nitrocellulose-containing explosives and propellants. As a result of nitration of DPA, the products *N*-nitroso-DPA, mononitro-DPAs, dinitro-DPAs and to

a minor extent trinitro-DPAs and nitro-*N*-nitroso-DPAs are formed. In some cases, 2-nitro-DPA is also used for stabilizing propellants and explosives (Drzyzga, *et al.*, 1996).

Anilines are used as raw materials in the manufacture of pesticides belonging to the class herbicides and fungicides such as phenylurea, phenylcarbamate, and acylanilide (Engelhardt, *et al.*, 1977). The most extensively used aniline based products include azo dyes and pesticides. Azo dyes are also formed on coupling of anilines and chloroanilines liberated from pesticides. Anilines are therefore added in to the environment from various sources, the direct sources involve their addition as free anilines from the raw materials through effluents from chemical industries, the chemical or biological breakdown of aminophenols, chloroanilines, etc.

Chung, *et al.*, (1997), has reported in his review that aromatic amines are carcinogens and vary in their carcinogenic potency. Biodegradation of such aromatic amines is therefore of considerable importance to protect the ecosystems and the management of industrial effluents prior to disposal in the environment.

Biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants to a less harmful, non-hazardous substance, which are then integrated into natural biogeochemical cycles. The intensity of biodegradation is influenced by several factors, such as nutrients, oxygen, pH value, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics and the pollution history of the contaminated environment. The parameters of effluents, wastewaters and environmental conditions such as low or high temperatures, acidic or alkaline pH, high salt concentration or high pressure

require the degradation to be carried out under such stress conditions. Extremophilic microorganisms adapted to grow and thrive under such adverse conditions and capable of biodegradation are therefore ideal candidates for the biological treatment of effluents in extreme habitats. Further, those adapted to more than one extreme condition offers a special potential for the biological decontamination of habitats where various different extreme conditions prevail simultaneously. The increasing number of patents indicates that there is a growing interest in the commercial application of extremophilic hydrocarbon degraders for the biological, environmentally friendly treatment of polluted wastewater or soil. The presence of extremophiles in polluted extreme habitats which are adapted to and able to metabolize a wide range of aromatic compounds indicate their usefulness for bioremediation, however, their full potential is yet to be exploited.

Reports are available on the degradation of aniline or aromatic amine, which is largely used in industrial manufacturing process. Such degradation has been reported at neutral condition by oxidative deamination and degradation involving enzyme dioxygenases.

Many of the effluents with aromatic amines from the industries are found to be having pH ranging from 10.0 to 13.0. It is important to develop a system which would help such effluents to be treated at alkaline pH. It was therefore proposed to study the degradation of aromatic amines under alkaline condition so as to understand the mechanisms that could be involved in such transformations using either alkalophiles or alkalotolerant bacteria.

Although large number of reports are available (Ajithkumar and Kunhi, 2000; Annwieler, *et al.*, 2002; Bhat, *et al.*, 1998; Cartwright and Cain, 1958; Harwood and Gibson, 1997; Hebes and Schwall, 1987; Konopka, *et al.*,) which have shown that number of bacteria can degrade organic compounds at neutral pH, however; very little information is available on the degradation of such compounds by bacteria which can survive and grow at an alkaline pH. For example phenol biodegradation under alkaline conditions has been demonstrated (Sarnaik and Kanekar, 1995) using *Pseudomonas* sp.

Alkalophilic microorganisms are defined as organisms that have their optimum growth rate at least 2 pH units above neutrality, while alkalotolerant bacteria are able to grow or survive at pH values ranging 7-9. Since aniline is one of the most potent reported pollutants, it was envisaged to take this as a model system for the undertaking of mechanisms and biotransformation procedure. With this aim, the study was directed as given below:

1) Isolation, characterization and identification of bacteria growing at alkaline pH (10.5) and their ability to tolerate aromatic amines.
2) Response of potential isolates to aniline and N,N-Dimethyl,1-Naphthylamine (NND) and to an alkaline effluent from a pesticide industry.
3) Characteristics of the potent isolates during growth in the presence of aniline.
4) Isolation, purification and identification of the product formed during the growth in the presence of aniline.

CHAPTER II

LITERATURE SURVEY

Nutrients play a vital role in the maintenance and functioning of an ecosystem. Homeostasis of an ecosystem is maintained by recycling of organic matter within the ecological niche brought about by the microorganisms present within the system. Microorganisms, ubiquitous in nature, are the sole entities that bring about biodegradation a process in which complex organic compounds are broken down to simple utilizable compounds which are further recycled through biogeochemical cycles (Pelczar *et al.*, 1993).

2.1. SOURCES OF AROMATIC COMPOUNDS

2.1.1. *Natural compounds*

The major contribution to the input of the organic compounds is plant based, which includes cellulose, hemicellulose and lignin which are glucosyl based except for lignin which has an aromatic backbone structure. The benzene ring is the next widely distributed structures in nature after the glucosyl residue (Diaz *et al.*, 2001). Other aromatic compounds present in nature include aromatic amino acids (tyrosine, tryptophan, phenylalanine), alkaloids (nicotine, quinine, cocaine), hormones (epinephrine, acetylcholine), vitamins (thiamine, biotin), steroids (Flavanoids, quinones), pigments (chlorophyll), nucleic acids, etc. Commonly found aromatic compounds at the plant roots include benzoate, phenols, 1-carvone, cymene, limolene (Hegde and Fletcher, 1996; Gilbert and Crowley, 1997). All these compounds belong to the aromatic homocyclic, heterocyclic and polycyclic ring structures. Lignin being aromatic based polymer is the major contributor of aromatic compounds comprising up to 25% of the land based biomass on earth (Diaz *et al.*, 2001) and its recycling along with other plant-derived aromatic compounds is vital for maintaining the Earth's carbon cycle.

Some of the resistant aromatic compounds include tannins, a plant based polyhydroxy aromatic compound. After lignin, these are the second most abundant group of plant phenolics. The presence of a number of phenolic hydroxyl groups enables them to form large complexes, mainly with proteins, and to a lesser extent with other macromolecules like cellulose and pectin (Bhat *et al.*, 1998). Other naturally found aromatic compounds include crude oil which comprises of benzenes, toluenes, ethylbenzenes, xylenes (BTEX), polyaromatic hydrocarbons and resins. Though natural and being secluded, their introduction to the outer world has been due to anthropogenic activities and since then has gained a lot of importance due to their competitive involvement for degradation.

2.1.2. *Anthropogenic compounds*

Since industrialization, aromatic compounds are produced in large amounts and are released into the environment by human activities. Various industries from which the pollutants are released includes 1) chemical and pharmaceutical industries that produce a variety of synthetic compounds and polymers, 2) paper and pulp bleaching industries, 3) coal and petroleum industry 4) agricultural practices where pesticides are extensively used (Díaz, 2004). In 1988 the US Environmental Protection Agency listed a number of chemicals as priority pollutants which included pesticides, halogenated aliphatics, nitroaromatics, chloroaromatics, polychlorinated biphenyls, phthalate esters, polycyclic aromatic hydrocarbons and nitrosamines (Fewson, 1988). Certain structural elements such as halo or nitro-substituents are rare in naturally occurring compounds. Fluoro- compounds particularly perfluoroalkyl, sulfo and azo groups, which are structural features of technically relevant commodity

chemicals, are practically unknown amongst natural products and can be considered as real xenophors (Reiger, *et al.*, 2002). Gribble, (2003), has reviewed the presence of various organohalogenic compounds in nature produced by diverse species such as marine plants, marine sponges, bacteria and fungi, plants, algae, lichens, terrestrial plants, animals and humans. Other abiogenic methods of introduction of these compounds include biomass fires, volcanoes, and other geothermal processes. Nitroaromatic compounds are used worldwide as explosives, pesticides, and as precursors for the manufacture of many products, including dyes, pharmaceuticals, and plastics. These compounds do not only come from man-made sources but are also formed by some natural processes, such as photochemical reactions in the atmosphere. Nitroaromatic compounds are well known as toxins; some are mutagenic and/or carcinogenic and others are uncouplers of cellular phosphorylation reactions (Crawford, 1995).

Xenobiotic compounds (organohalogens such as polychlorobiphenyls (PCB) Dichloro-Diphenyl Trichloroethane (DDT), are recalcitrants and their lipophilic property enhances their bioaccumulation and biomagnification (Vettery, 2002; Gray, 2002; Goerke *et al.*, 2004; Richter and Nagel 2006; Nfon and Cousins, 2006). Though the concentration of xenobiotics is present in sub-lethal levels, their long-term exposure causes significant damage to marine populations and may be carcinogenic, mutagenic or teratogenic. Animals such as seals, bald eagle, and seabirds showed disrupted hormonal cycle, leading to reproductive dysfunction such as reduction in fertility, hatch rate, alternation of sex behavior and viability of offspring (Crews *et al.*, 1995). Due to long environmental and biological half lives, recovery from the effects of many xenobiotic compounds is expected to be slow. Indeed, it has been shown that

more than 15 yrs are required to remove the negative effects of DDT on reproduction of white tail eagles (*Haliaeetus albicilla*) in the Baltics, and another 10 years for the population to recover. Likewise, long recovery times have been reported for harbour, grey and ringed seals in the Baltic. The grey seal (*Halichoerus grypus*) population in the northern Baltic has shown marked increase since the ban of DDT and PCB in the Baltic region (Wu, 1999).

Degradations of such compounds are brought by microorganisms occur either through metabolic or cometabolic processes.

2.2. METABOLISM / COMETABOLISM (Grady, 1985)

Microorganisms can use aromatic compounds as a source of carbon and energy or they may be biotransformed with reduction of toxicity or to an inactive form. Such metabolism normally occurs in the presence of additional carbon sources, which supports the growth of the organism and simultaneously degrade them. Thus is the biotransformation of the additional aromatic compounds present in the medium. In some metabolic cases the organic compound is similar to a substrate and therefore gets metabolized by a mechanism called gratuitous.

With various pathways present in nature to bring about degradation of aromatic compounds, many microorganisms utilize these compounds as sole source of carbon based on the activity of enzymes. Xenobiotic compounds, due to their complexity and uniqueness, tend to remain in the environment for long periods of time. These compounds are called persistent if they are biologically degraded at a

2004), is widely distributed in nature is not utilized under anaerobic condition in the absence of nitrates. Kesseru *et al.*, (2005), has shown the dependence of the anaerobic bacteria *Pseudomonas butanovorans* to use salicyclate as electron donor for nitrate reduction. This was not achieved in the presence of sulfates or phosphates even at high concentrations. Glucose abundantly present in nature also proves to play an important role in the degradation of certain xenobiotic compounds. Tharakan and Gordon, (1999), showed that Trinitrotoluene (TNT) a chemical used in explosives has been placed as priority chemical list by the US government, can be significantly removed in the presence of glucose by bacteria. Bacteria isolated from the TNT contaminated soil could bring about 100% transformation, which was only partially removed in the absence of glucose to about 38% of the initial concentration. Glucose also facilitated a *Pseudomonas* sp. (Ziagova and Liakopolou, 2007), in the degradation of 1,2-Dichlorobenzene a known xenobiotic without which it hardly grew. Raymond and Alexander, (1971), has shown that m-nitrophenol resistant bacteria utilized it only in the presence of p-nirophenol, which was used as a source of carbon and energy. Benzopyrene, one of the polyaromatic hydrocarbons (PAH) was seen to be removed when *Sphingomonas* JAR02 was incubated with benzopyrene in the presence of root products. The cometabolism of the benzopyrene was facilitated during the utilization of other aromatic compounds present (Rentz *et al.*, 2005). Van Herwijnen *et al.* (2003), indicated that the isolate *Sphingomonas* LB216 could cometabolise various PAHs such as phenanthrene, fluoranthene, anthracene, dibenzothiophene only when initially grown in the presence of fluorene.

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Cometabolism and gratuitous metabolism thus play a very important role to bring about biotransformation of various xenobiotic compounds which otherwise would only be persistent or recalcitrant.

In nature, microbial biotransformation and metabolism of aromatic rings is found to occur by cleavage of the aromatic nucleus via catechol by different pathways as elucidated.

2.3. AROMATIC RING CLEAVAGE PATHWAYS

The most abundant aromatic nucleus encountered in the environment is that of benzene, most stable but enzyme labile. For an enzyme to cleave the benzene ring, it is a prerequisite to add two molecules of oxygen in to the ring to convert it to a dihydrodiol product. Aromatic acids such as benzoates, biphenyls, etc. required the addition of two oxygen atoms in the ring, while, monohydroxylated aromatic compounds such as phenols, hydroxybenzoates required only one oxygen atom to be added in the ring.

The fission of the aromatic ring takes place by two major mechanisms. An intradiol fission wherein the bond between the two vicinal hydroxyl groups is broken known as the ortho-cleavage pathway or by extradiol fission where the cleavage takes place adjacent to either of the hydroxyl groups called meta-cleavage pathway. A third kind of ring cleavage is seen where the p-hydroxydiol compounds are broken is called the gentisate pathway. The breakdown is not ortho due to para- positioned hydroxyl group, but is similar to meta-cleavage.

Most of the aromatic compounds that undergo biodegradation converge to catechol (Cerdan *et al.*, 1994; Ngai *et al.*, 1990) (fig. 2.1), protocatechuate (Noda *et al.*, 1990) (Fig. 2.2), gentisate (Tomasek and Crawford, 1986; Stolz *et al.*, 1992) or homogentisate (Hagedorn and Chapman, 1985; Fernandez-Canon and Penalva, 1998) (Fig. 2.3) or their derivatives.

2.4. CLEAVAGE PATHWAYS OF SUBSTITUTED AROMATIC COMPOUNDS

In the environment large number of compounds are seen with different molecular structures, some of these compounds have a basic aromatic ring with substitutions which are hydrogenated, nitrated, chlorinated or have heterocyclic ring with the incorporation of either oxygen, nitrogen or sulphur in the aromatic ring. Many of such compounds are known to be biotransformed by a wide variety of microorganisms. However, degradation of nitrogen containing monocyclic aromatic compounds are explained herewith.

2.4.1. *Nitroaromatic compounds*

Nitroaromatic compounds constitute a major class of widely distributed environmental contaminants. Compounds like nitrobenzene, nitrotoluenes, nitrophenols, nitrobenzoates and nitrate esters are of considerable industrial importance. They are frequently used as pesticides, explosives, dyes, and in the manufacture of polymers and pharmaceuticals. Many nitroaromatic compounds and their conversion products have been shown to have toxic or mutagenic properties. Most of them are biodegradable in nature by various microorganisms. However, most

Fig. 2.1: Ortho- and meta- cleavage of Catechol

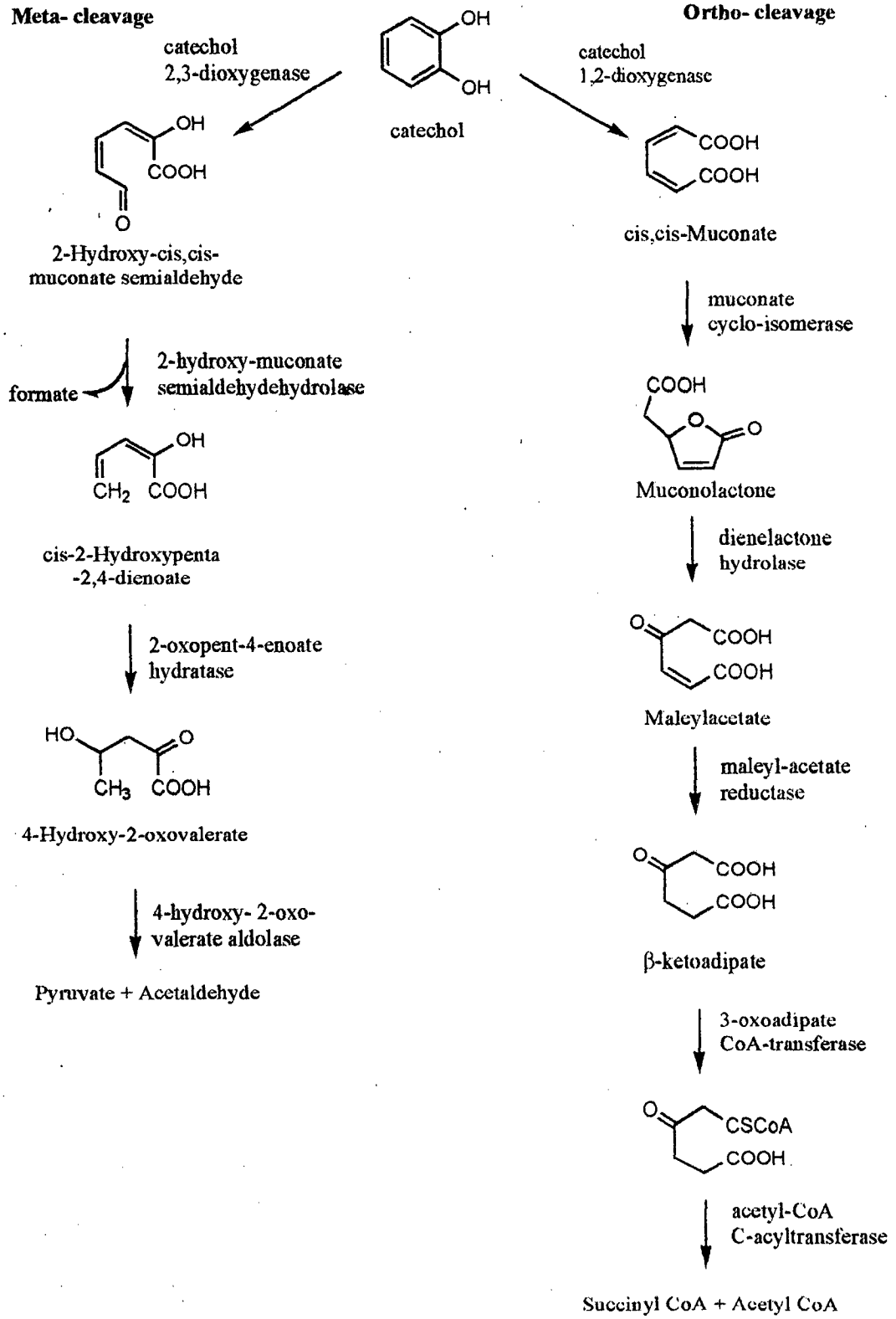


Fig. 2.2: Ortho- and meta- cleavage of protocatachuate

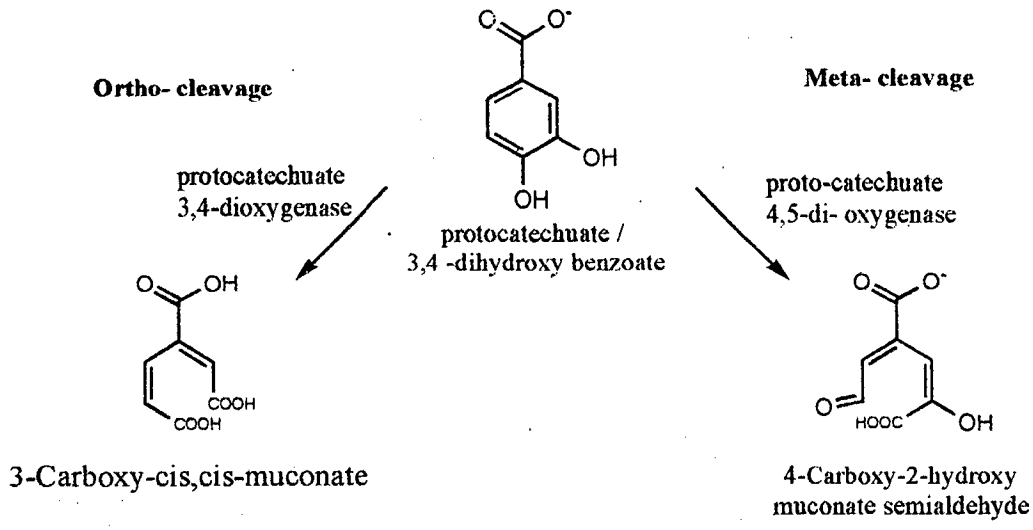
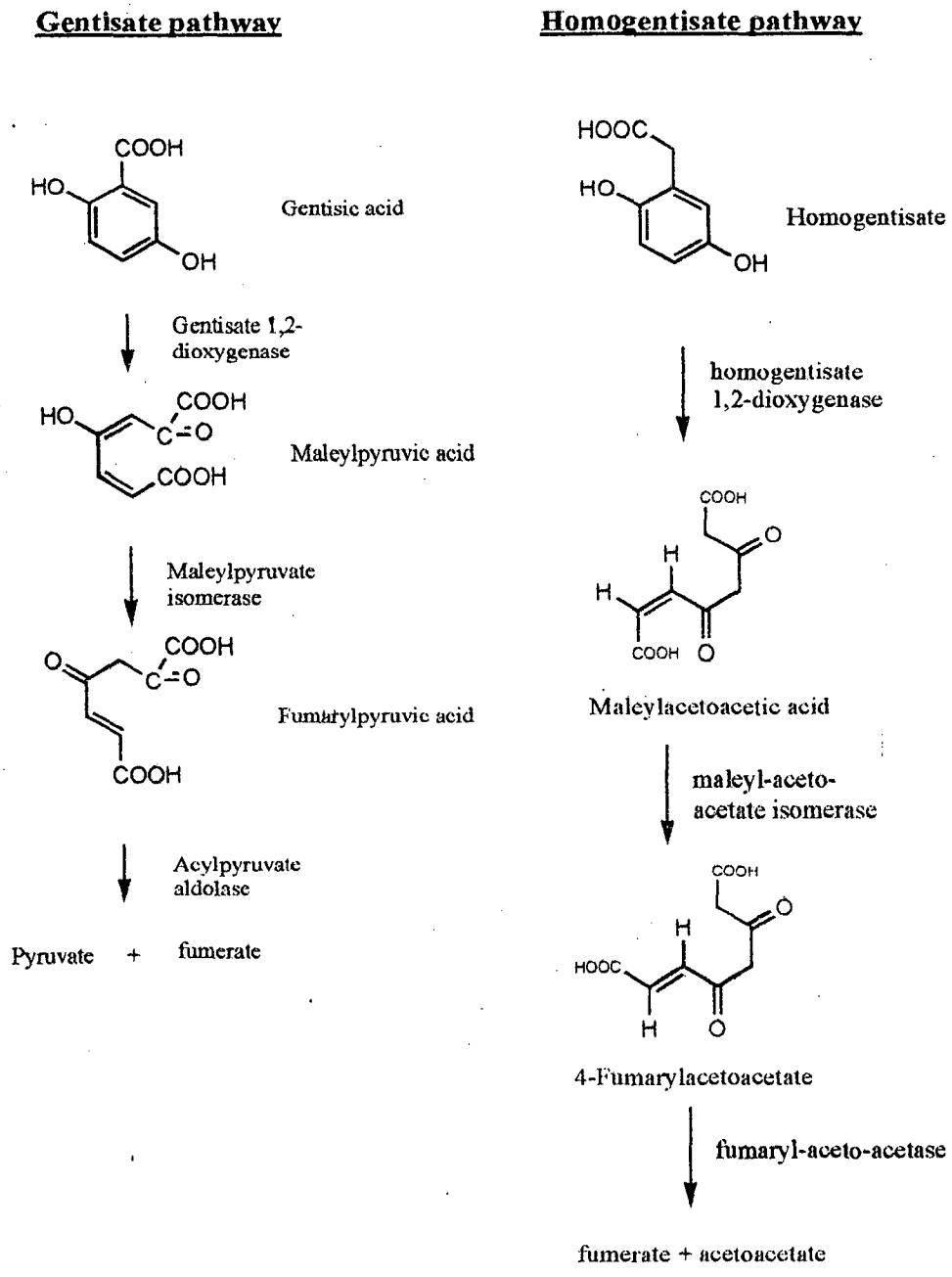


Fig. 2.3: Gentisate/homogentisate pathway



contaminated environments have combinations of nitroaromatic compounds present, which complicates the bioremediation efforts (Ye *et al.*, 2004).

Bacteria appears to have evolved four main strategies to address the nitro-group under aerobic conditions (Nishino *et al.*, 2000): (a) dioxygenation of the nitroaromatic ring, with release of the nitro-group as nitrite and production of dihydroxy intermediates, (b) monoxygenation to epoxides, (c) formation of a Hydride-Meisenheimer complex and (d) partial reduction of the nitro-group, formation of hydroxylaminobenzene derivatives and ammonia release, followed by rearrangement of the hydroxylaminobenzene to the corresponding catechol and elimination of another ammonia molecule.

The aromatic π electron nucleophilic mechanism with the additional nitro ($-NO_2$) electron withdrawing property protects nitroaromatics from initial attack by oxygenases but is favourable for reductive attack (Rieger & Knackmuss, 1995). On the other hand, anaerobic reductive attack produces the aromatic amine ($-NH_2$), an electron-donating group which represents a barrier to further attack by anaerobes (McCormick *et al.*, 1976). Thus, nitroaromatics often either persist or become amino end products in the environment.

2.4.1.1. Nitrobenzene

Nitrobenzene is the simplest of all aromatic nitrates used in the manufacture of rubber, drugs, dyes, pesticides, lubricating oils, etc.

The most common widespread method of degradation of these compounds is either by partial reduction of the nitro group or by dioxygenase pathway (Ye, 2004).

Nishino *et al.*, (2000), reported the pathway followed by *Pseudomonas pseudoalcaligenes* (Fig.2.4) through partial reduction of nitrobenzene by enzyme nitrobenzene reductase to give nitrosobenzene which is further reduced by reductase to give hydroxylaminobenzene. Enzyme mutase rearranges the hydroxylamino group to amine and hydroxyl at subsequent positions on the ring. Somerville (1995), has reported that *Pseudomonas putida* carried out these steps followed by ring cleavage brought about by aminophenol dioxygenase. Intermediates 2-amino muconate and 2-amino-2,4-penteneoate was found to undergo deaminase reaction to form 2-oxo-3-hexene -1,6-dioate and 2-oxo-4-penteneoate (He *et al.*, 1997), which are intermediates of the m-cleavage of catechol pathway. The nitrobenzene dioxygenase enzyme produced by *Comamonas* sp. yielded catechol by the loss of nitro group (Nishino *et al.*, 1995). Catechol breakdown leads to form metabolites which are utilized in the TCA.

Nitrobenzene can be converted to aniline under anaerobic condition or can be reduced to aniline under aerobic condition as shown by the reaction steps via hydroxylaminobenzene.

2.4.1.2. Nitrophenol

Degradation o, m & p, forms of nitrophenols are discussed here. 2-nitrophenol has shown to have the simplest degradation pathway in which the nitro group is removed by the action of 2-nitrophenol 1,2-dioxygenase giving catechol. Catechol proceeds towards the ring fission and intermediates utilized in TCA (follow downstream pathway from catechol in fig.2.5 - degradation of nitrobenzene) and the

Fig. 2.4: Degradation pathway nitrobenzene

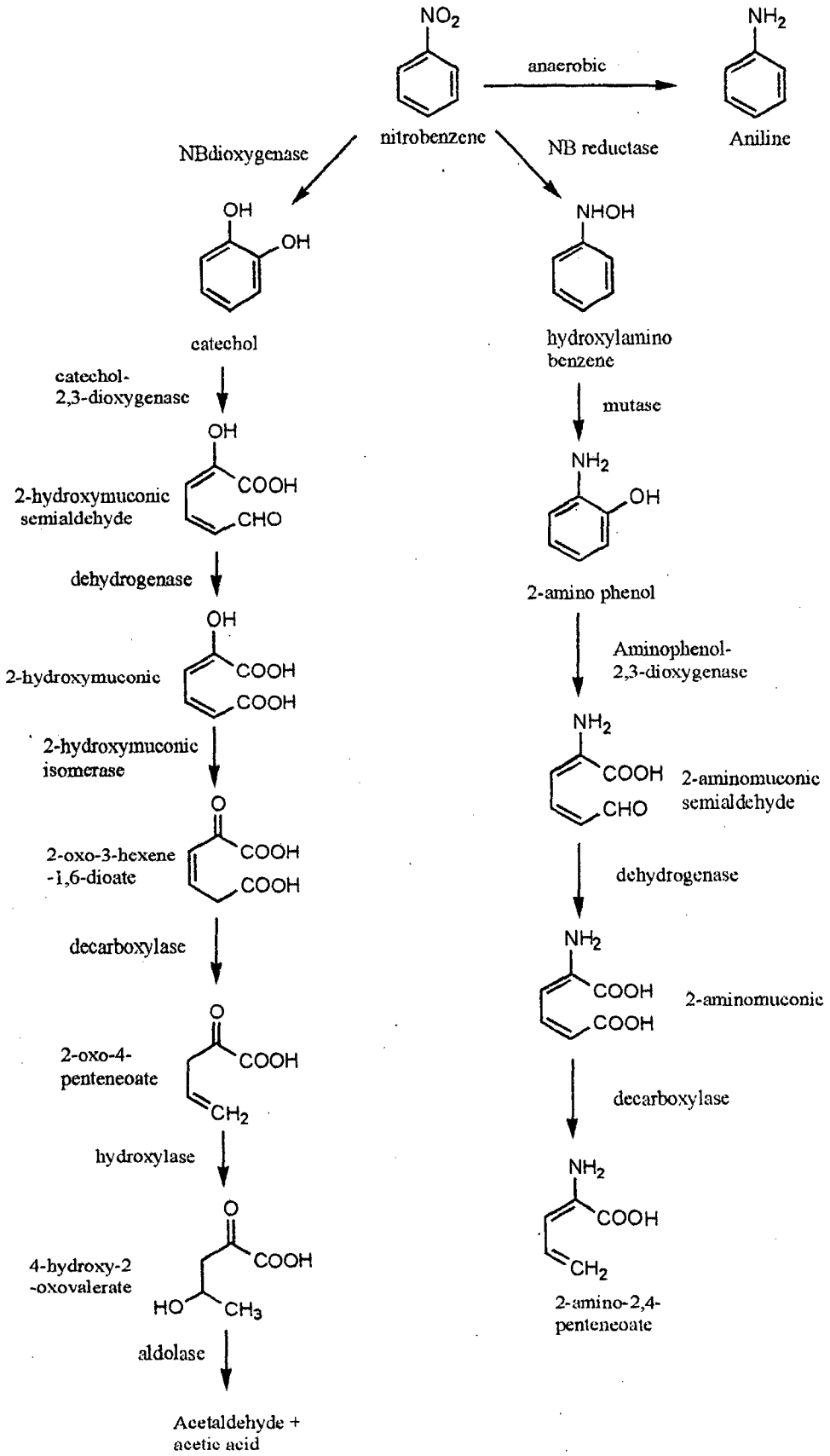
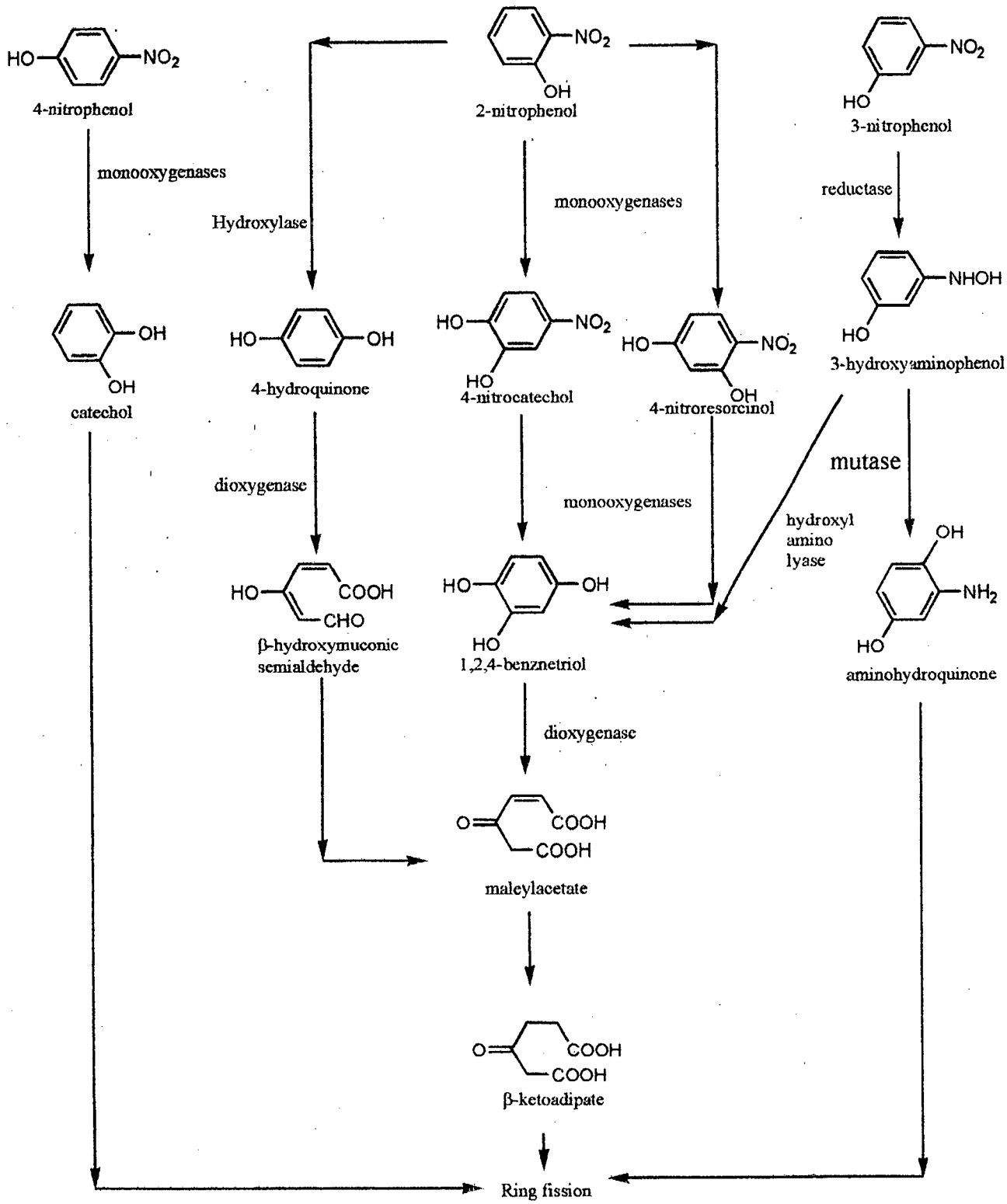


Fig. 2.5: Degradation pathway of nitrophenols



organism that followed such a pathway reported by Zeyer *et al.* (1985), was *Pseudomonas putida*.

3-nitrophenol was seen to have broken down by two other pathways by different organisms (studies carried out by different authors). *P. putida* B2 partially reduced 3-nitrophenol to 3-hydroxyamino phenol followed by an addition of two hydroxyl groups, a reaction catalysed by 3-hydroxyamino phenol-3,4-dioxygenase to give 1,2,4-benzenetriol (Meulenberg, 1996). Organism *Ralstonia eutropha* transformed 3-hydroxyamino phenol to amino hydroquinone by the enzyme 3-hydroxyamino phenol mutase (Schenzle, 1997). Further reactions cleave amino hydroquinone which is then utilized for cellular purposes.

Three different pathways were found to follow during the breakdown of 4-nitrophenol. Bacterial species such as *Pseudomonas* (Chauhan *et al.*, 2000), *Moraxella* (Spain *et al.*, 1991), *Bacillus* (Kadiyala, 1998), *Arthrobacter* (Hanne *et al.*, 1993) were found to follow different pathways to transform 4-nitrophenol. The major route being their conversion to 4-hydroquinone followed by ring cleavage to give β-hydroxymuconic semialdehyde. Enzyme monoxygenase catalysed the reaction of hydroxylating at 2- and 3- positions of 4-nitrophenol to give catechol and resorcinol respectively in species *Bacillus* and *Arthrobacter*. Further hydroxylation and removal of nitro group gave 1,2,4-benznetriol which is ultimately cleaved by dioxygenase to give linear compounds easily utilizable by bacteria.

2.4.1.3. Nitrotoluene degradation

Mono and di-Nitrotoluenes contain single or two nitro groups in benzene ring with methyl group as a parent compound. Degradation of the mono-nitrotoluenes has

been shown to follow three pathways (Fig.2.6). Parales, *et al.* (1998), proposed that *Pseudomonas* species converted 2-nitrotoluene to 2-methyl catechol which proceeds with ring cleavage. In case of 4-nitrocatechol, two pathways were seen to have followed; *Pseudomonas* strain TW3 and 4NT (James *et al.*, 1998) followed an initial hydroxylation of the methyl group by hydroxylases then further oxidizing until 4-nitrobenzoate was formed. Further, partial reduction of the nitro group lead to conversion of 4-hydroxyaminobenzoate to 3,4-dihydroxycatechol by dioxygenases, which was further cleaved by enzyme dioxygenases. The other pathway shown by *Mycobacterium* HL4NT-1, includes partial reduction of nitro group not altering the methyl group. Enzyme reductase catalyzed the conversion of nitrotoluene to hydroxylaminotoluene to aminocresol by mutase followed by aromatic ring cleavage by dioxygenases.

Dinitrotoluene (2,4-Dinitrotoluene and 2,6-Dinitrotoluene) are found to follow different modes of breakdown (Fig. 2.7). Breakdown of 2,4-Dinitrotoluene by *Pseudomonas* was studied by Suen *et al.* (1993). Reaction flows from denitrification by dioxygenases to form 4-methyl-5-nitro-benzene-1,2-diol, followed by monooxygenases converting this compound to a quinone by replacing the remaining nitro group with oxygen, which gives methyl-benzenetriol. The benzenetriol becomes available for ring cleavage by dioxygenases. 2,6-Dinitrotoluene is also initially attacked by dioxygenases replacing a nitro group with two vicinal hydroxyl groups to give 3-methyl-4nitrocatechol. The dihydroxy compound becomes vulnerable to the dioxygenase enzyme attack. This pathway was proposed by Nishino *et al.*, (2000) in organisms *Burkholderia cepacia* and *Hydrogenophage paleronii*.

Fig. 2.6: Degradation pathway of Nitrotoluenes

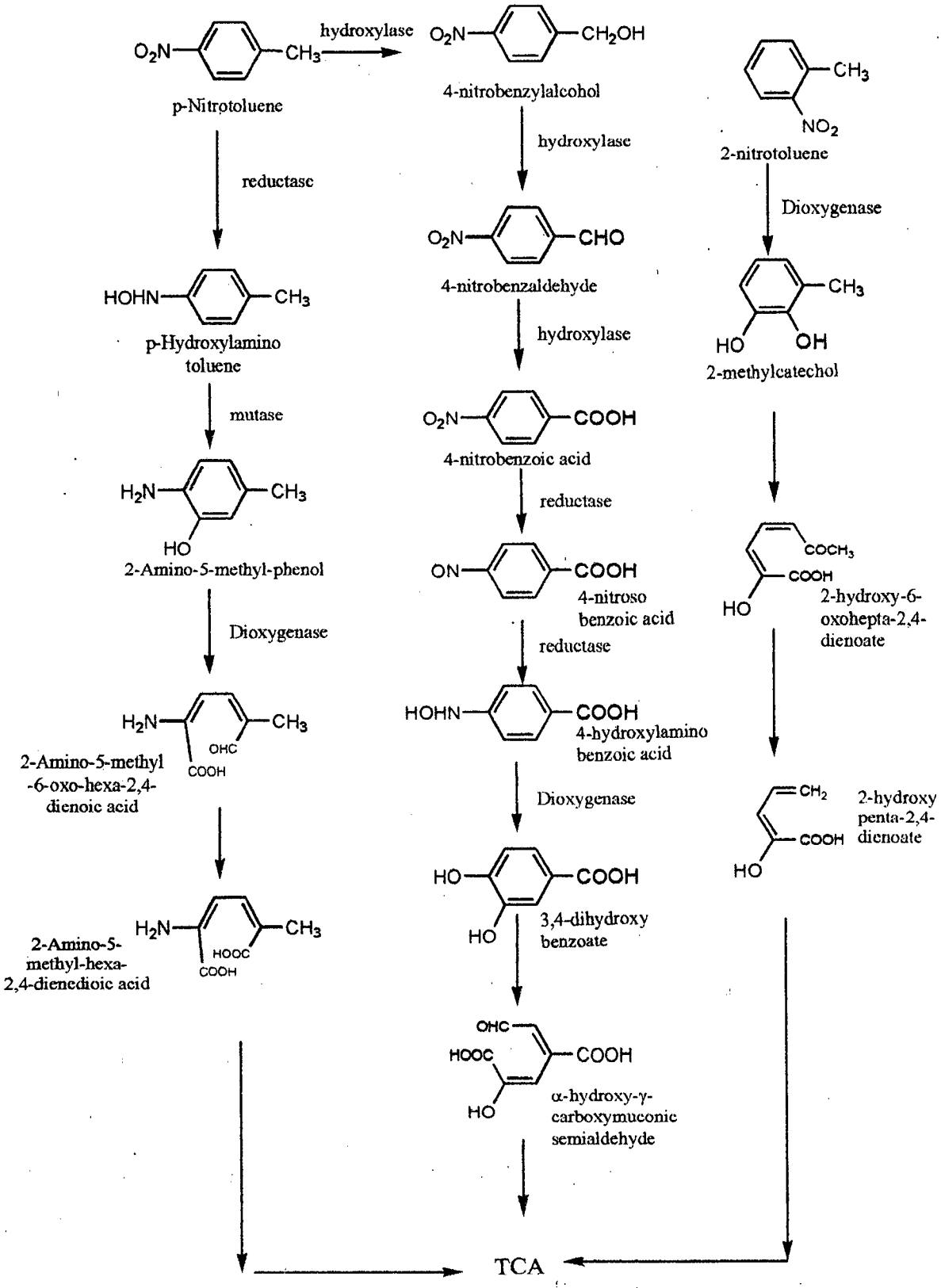
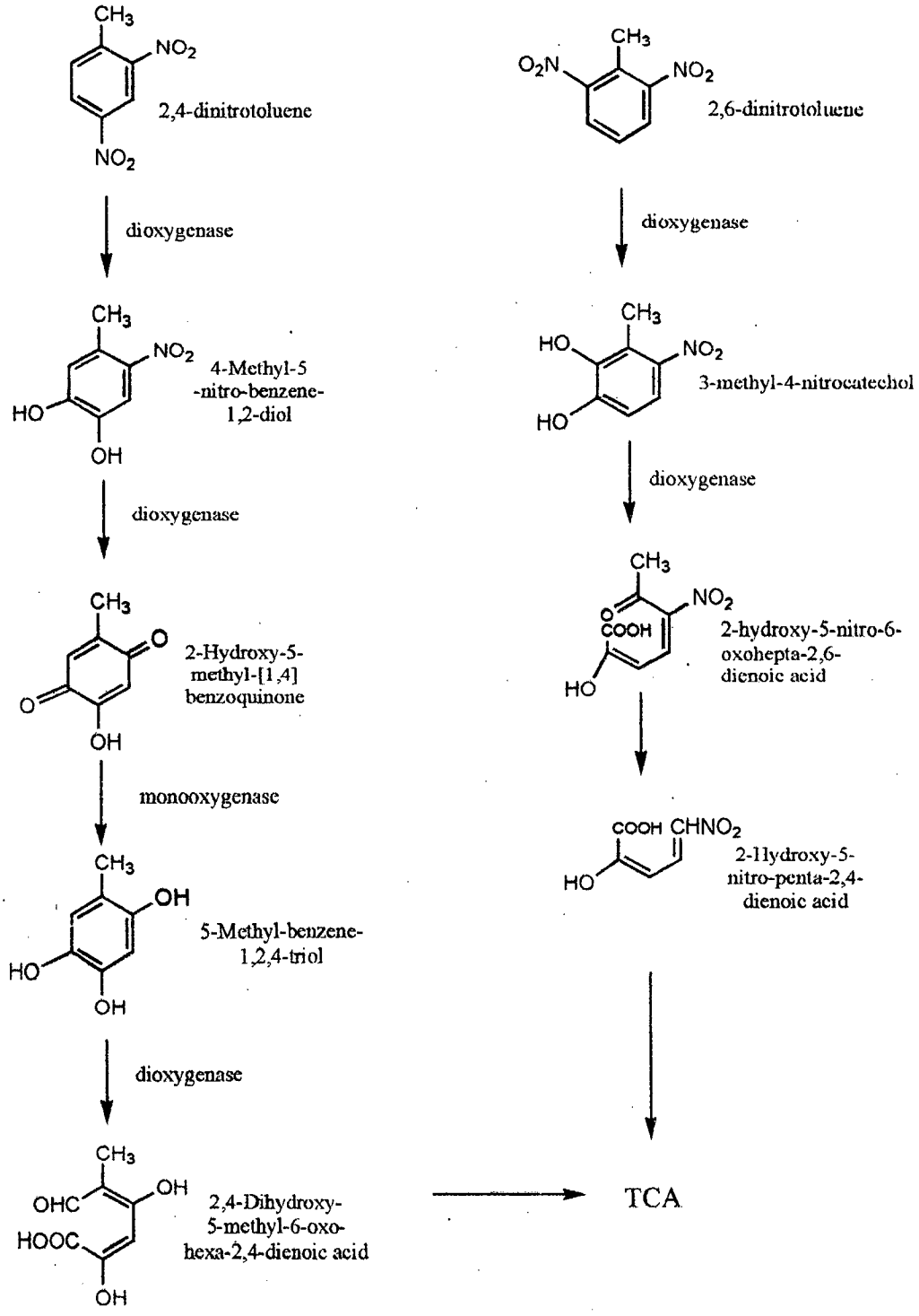


Fig. 2.7: Degradation pathway of Dinitrotoluenes



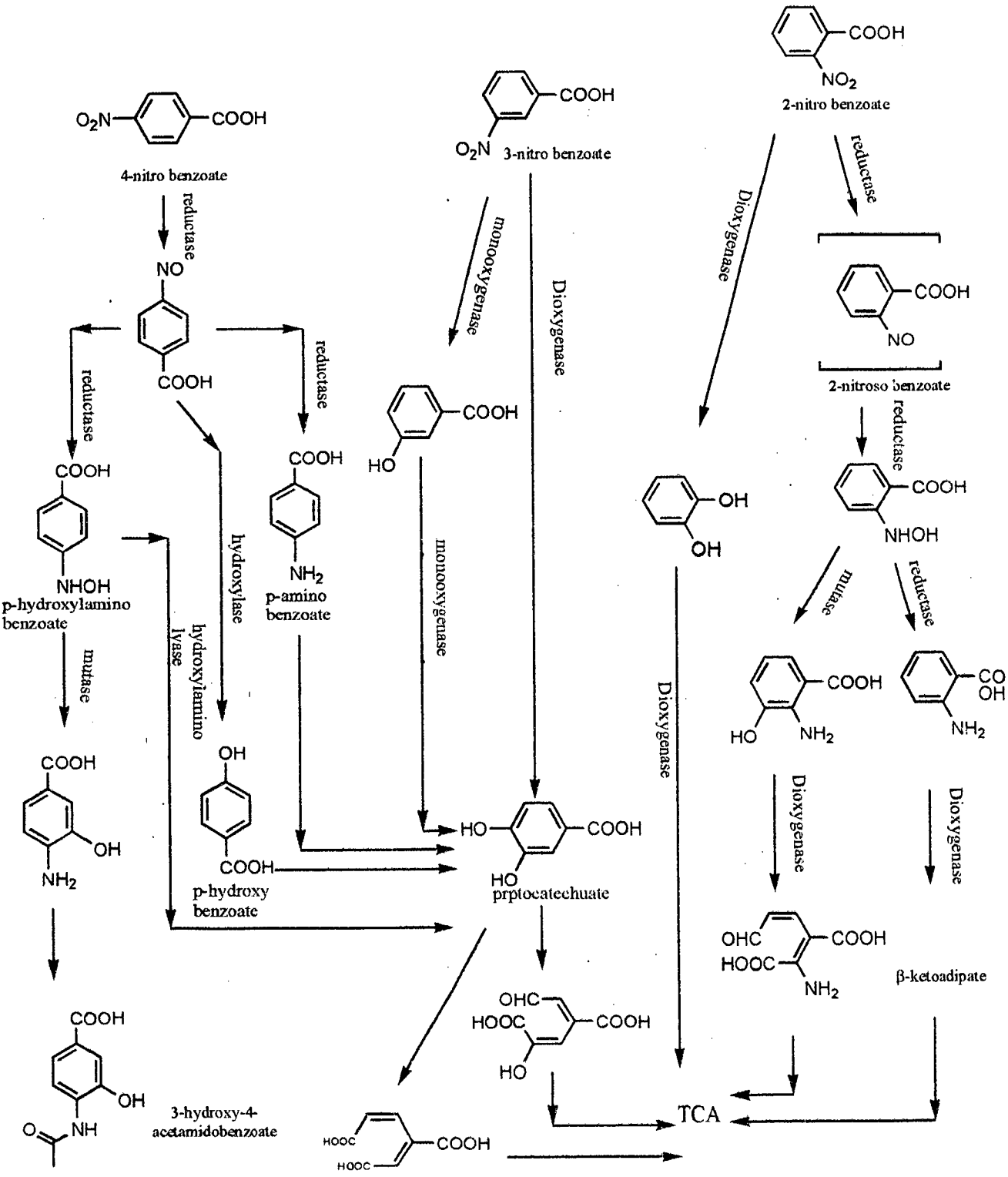
2.4.1.4. Nitrobenzoate degradation

Three different nitrobenzoates are found based on the placement of the nitro groups on the parent benzoate (Fig.2.8). 2-nitrobenzene is converted to 2-hydroxylaminobenzoate by undergoing a reductive degradation in aerobic condition (Durham, 1958; Chauhan *et al.*, 2000; Hasegawa *et al.*, 2000). *Pseudomonas fluorescens* further carried out transformation to produce 3-hydroxy-2-anthranilate catalyzed by enzyme mutase, which followed a breakdown reaction by dioxygenases. In *Athrobacter protophormiae*, further reductive reaction was carried by converting hydroxylamino group to form 2-anthranilic acid. Anthranilate dioxygenase released the amino group from the ring which made the compound susceptible to ring cleavage.

Formation of protocatechuate from 3-nitrobenzene was catalyzed by dioxygenase in *Pseudomonas* or it may be sequentially hydroxylated as observed in *Nocardia* species (Cartwright *et al.*, 1958) to give 3-hydroxybenzoate before the formation of protocatechuate. Protocatechuate was then available for ring cleavage.

4-nitrobenzene was partially reduced to nitrosobenzene before further transformation could be carried out. Various pathways were observed to have followed from this compound. *Comamonas acidovorans, P. picketii* and *P. putida* were seen to partially reduce nitrosobenzene to give hydroxylaminobenzoate which was then converted to protocatechuate by releasing amino group by the enzyme hydroxylaminolyase. *Burkholderia* and *Ralstonia* partially converted hydroxylaminobenzoate to protocatechuate but the major conversion was to 2-hydroxy-p-aminobenzoate and 3-hydroxy-4-acetoamidobenzene formed the action of mutase on 2-hydroxy-p-aminobenzoate.

Fig. 2.8: Degradation pathway of nitrobenzoates



Complete reduction of the nitroso group of the 4-nitrosobenzoate gave p-anthranilic acid which was converted by enzyme p-anthranillic aminolyase to give protocatechuate. Protocatechuate is then normally broken down by various organisms for their source of carbon and energy.

2.4.2. *Degradation of anilines*

Anilines are usually easily metabolisable and are utilized by various soil borne micro-organisms. But it has been revealed that with addition of a substituent group to the aromatic ring, its susceptibility to bacterial degradation reduces. Paris *et al.* (1987), has shown that aniline is the most easily metabolisable compound but insertion of methyl, chloride, bromo, methoxy, nitro or cyano groups in the aromatic ring increases its resistance to degradation by micro-organisms. The rate of transformation of these compounds decreased in the order aniline > 3-bromoaniline > 3-chloroaniline > 3-methylaniline > 3-methoxyaniline > 3-nitroaniline > 3-cyanoaniline. Other simple forms of anilines include aminophenols, chloroaminophenols, aminobenzoates and chloroanilines.

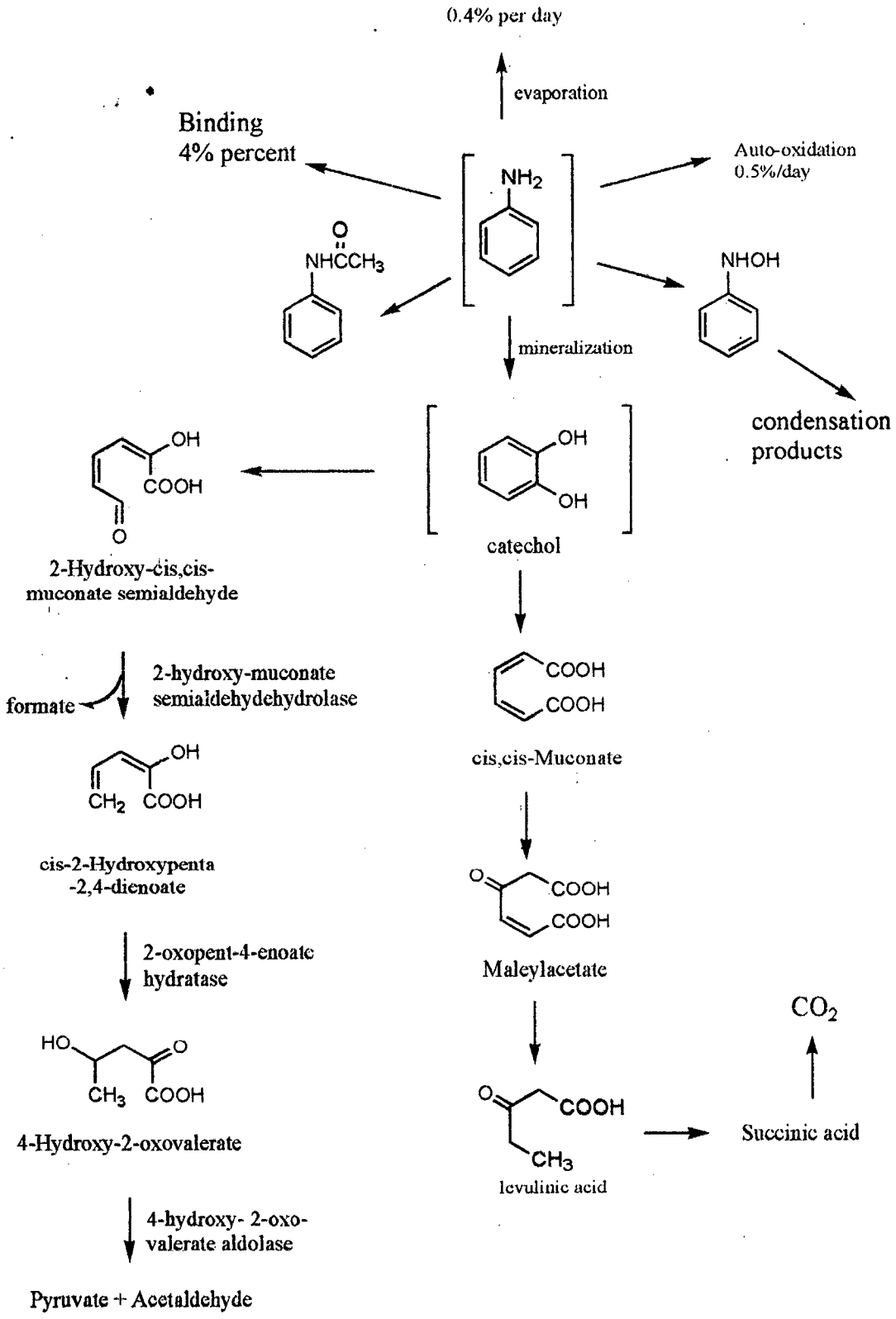
Besides their biodegradability, aromatic amines are also gaining importance for their carcinogenic properties. 80 different aromatic amines were tested for their mutagenecity by carrying out AMES test involving various strains of *Salmonella typhimurium* (Chung *et al.*, 1997). A transformed product of an azo dye, an aromatic amine p-phenylenediamine, which is extensively used in hair dyes, was found to be the most potent carcinogen. According to Chung, Crebelli *et al.* (1989), has reported that p-phenylenediamine is not a direct or weak carcinogen but gets transformed to a potent carcinogen when activated by the action of enzymes within a biological entity.

Many aromatic amines have been found to be candidates of potent mutagens on enzymatic induction and the main criterion for the aromatic amines to be carcinogenic is based on their ability to form nitrenium ion (Wild, 1990). Chung concluded from his study that diamino aromatic compounds with distal placement of amino groups showed highest mutagenicity. Such compounds with other groups such as nitro or large alkyl groups placed at its vicinity were found to be less mutagenic. Compounds such as aniline, m and p-aminophenol, were found to be non toxic but in late 19th century, carcinogenicity in workers (urinary bladder cancer) at a dyestuff industry was related to aromatic amine toxicity. Further, Weisburger (2002), reported that 2-aminofluorene was tested to show a positive reaction towards carcinogenicity in mice after activation by cytochrome P450 present in its liver. Aromatic amines therefore have been gaining importance as the intermediates formed during the transformation of complex aromatic amino compounds could be a potent carcinogen.

Several workers have reported the degradation of aniline by various organisms (Anson *et al.*, 1984; Peres *et al.*, 1998; Vijay Shanker *et al.*, 2006; Liu *et al.*, 2002). Catechol has been reported as most common intermediate during the degradation of aniline, further breakdown of catechol could proceed the catechol-1,2 or catechol,2-3 dioxygenase pathway for complete mineralization. Lyons *et al.*, (1984) proposed various interactions of aniline in the environment, which explained its degradation and polymerization (Fig. 2.9) and has reported the aniline transformation via catechol, 1,2-dioxygenase.

Degradation of 2-aminophenol has been reported in *Pseudomonas putida* HS12 by Park *et al.*, (2001); and in *Pseudomonas* sp. AP-3 by Takenaka *et al.*, (1998), wherein, the dioxygenases cleaves the aromatic ring via ortho-cleavage of catechol

Fig. 2.9: Interactions of aniline in the environment



with amino group still present on the ring, to give 2-aminomuconic semialdehyde, Enzyme deaminase replaces the amino group with hydroxyl group to give 2-oxocrotonic acid. Zhao *et al.*, (2000), reported the transformation of 2-aminophenol and 4-aminophenol by *P. putida* 2NP8 to respective intermediates 2- and 4-iminoquinone which were further converted to quinones (Fig. 2.10). Further downstream pathway followed the catechol or hydroquinone ring cleavage.

Degradation of 2- and 4- aminobenzoic acids follow two different pathways; 2-aminobenzoic acid could be transformed to 2,3-dihydroxy benzoic acid brought about by anthranilate 3-monooxygenase seen in *Pseudomonas* sp. (Tanuichi *et al.*, 1964) or could be converted to catechol by anthranilate 1,2-dioxygenase in a fungus *Aspergillus niger* (Kamath *et al.*, 1990). P-aminobenzoate, similarly can be converted to p-aminophenol by 4-aminobenzoate hydroxylase in fungus *Agaricus bisporus* (Tsuji *et al.*, 1986), or converted to 3,4 dihydroxy benzoate by 4-aminobenzoate 3,4-dioxygenase (Fig. 2.11). The catechol and hydroquinone formed are common intermediates and is used by a variety of micro-organisms.

p-Chloroaniline is degraded by *Moraxella* sp. following the pathway shown in figure 2.12, as reported by Zeyer *et al.*, (1985) via ortho-cleavage. Meta-cleavage pathway of m-chloroaniline is reported to have followed by *Comamonas testosterone* (Boon *et al.*, 2000) via 2,3-dioxygenase pathway. Organism *P. putida* GJ31, able to degrade 3-chlorocatechol was found to follow the proximal 2,3-dioxygenase (Mars *et al.*, 1997; Kaschabek *et al.*, 1998) or distal 1,6-dioxygenase (Kaschabek *et al.*, 1998) (Fig. 2.13).

Fig. 2.10: Degradation pathway of aminophenols

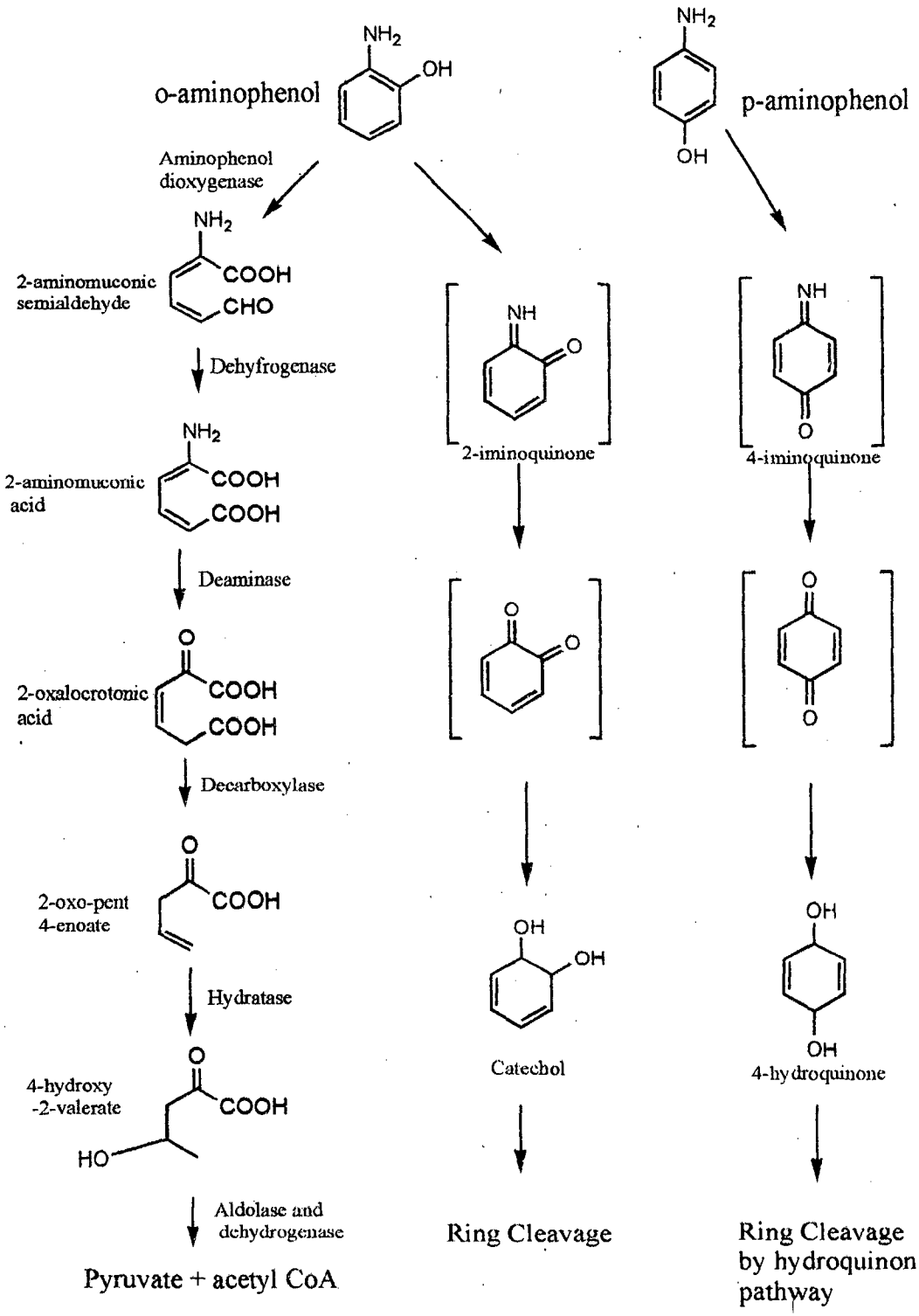


Fig. 2.11: Degradation pathway of aminobenzoates

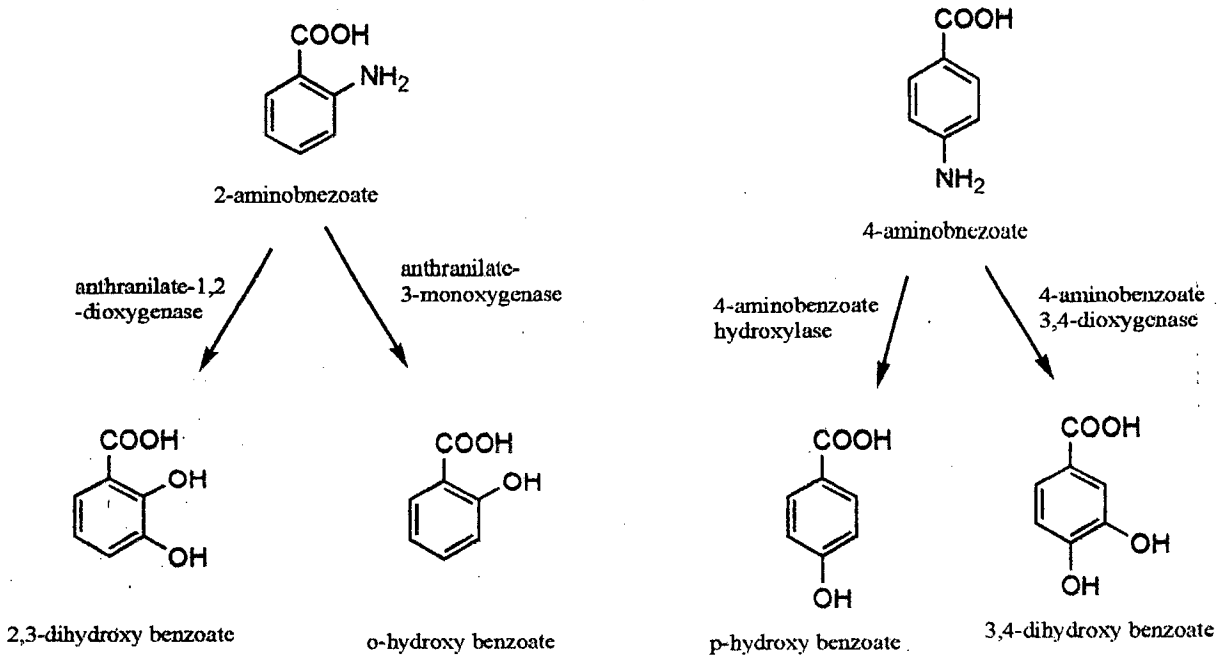


Fig.2.12: Degradation pathway of p-chloroaniline

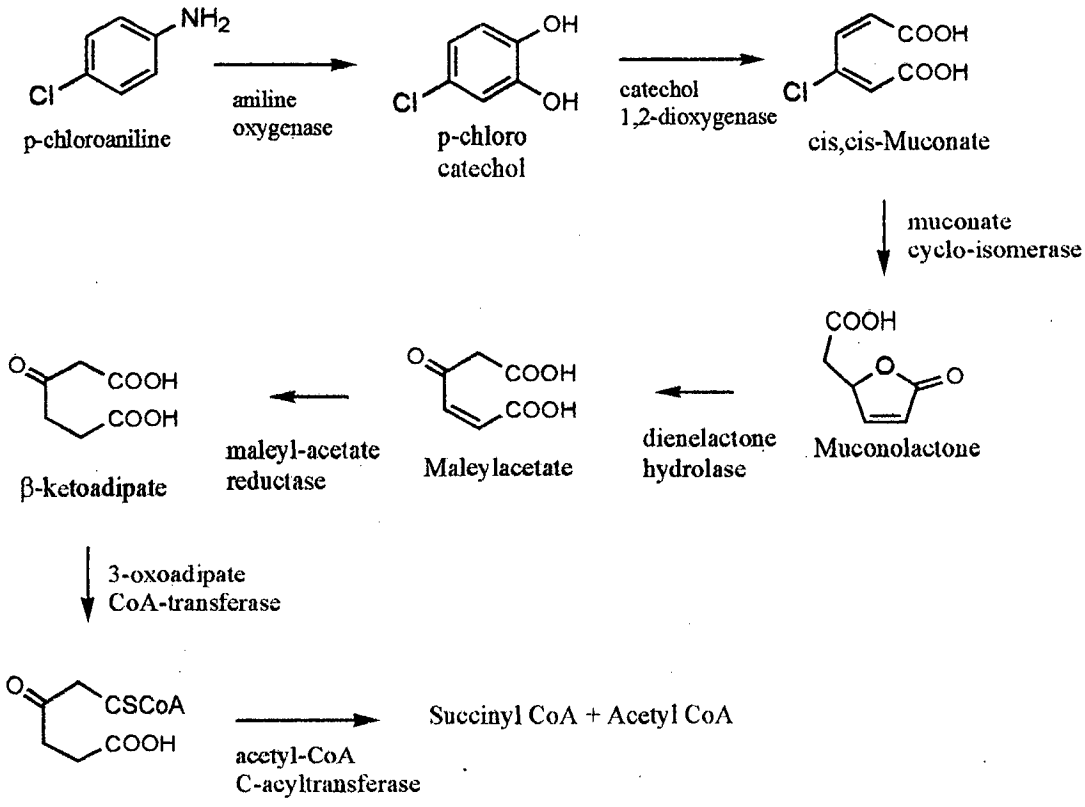
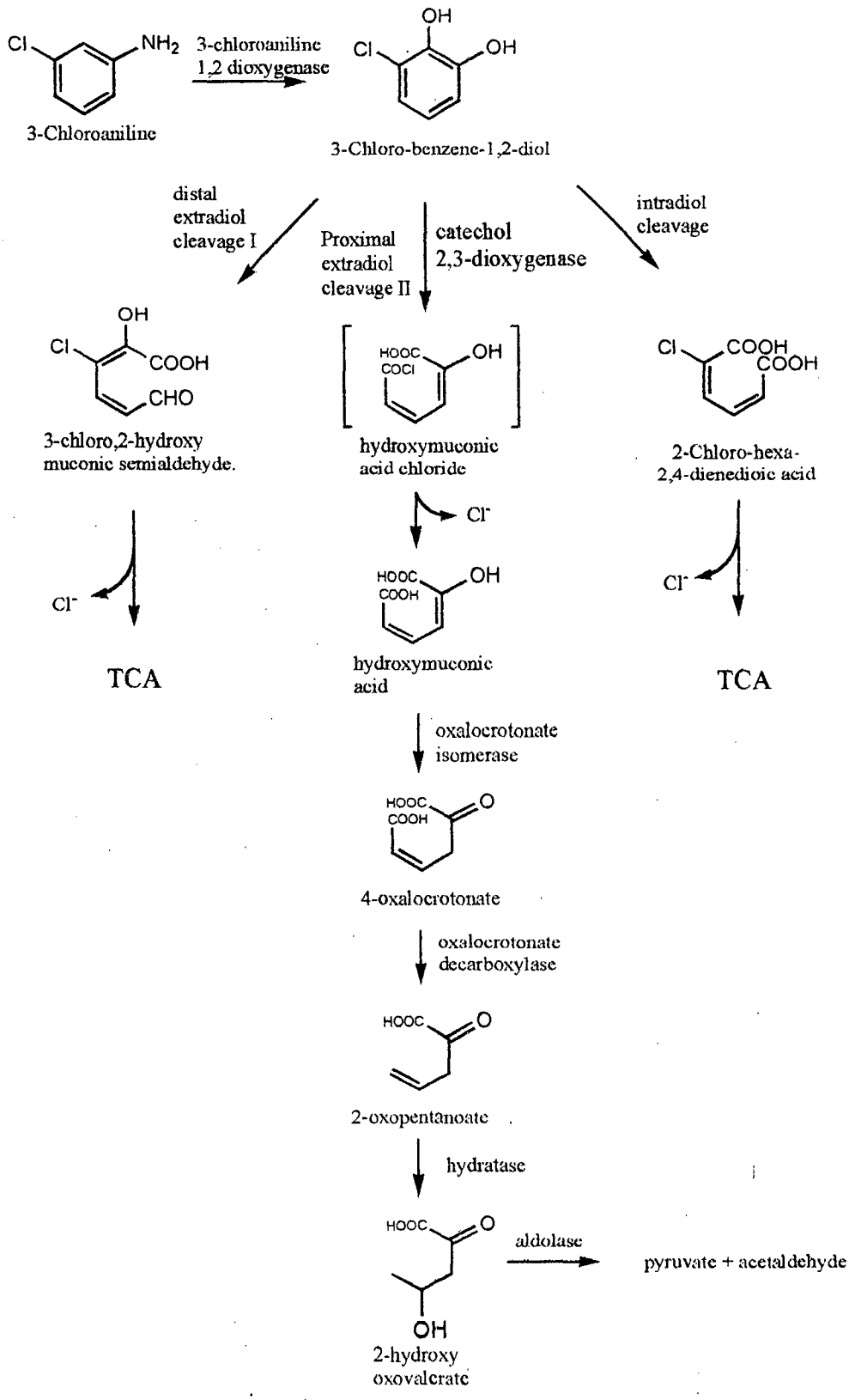


Fig. 2.13: Degradation pathway of m-chloroaniline



2.5. ENZYMES

2.5.1. *Oxygenases*

Under aerobic condition, bacteria use oxygen not only as a terminal electron acceptor but also to activate the aromatic compound for its breakdown and utilization. Various enzymes produced by them catalyze the addition of oxygen to the aromatic ring. These are widely called oxygenases. The difference between enzymes oxidases and oxygenases is positional, non-specific addition of oxygen on the aromatic ring, thus it would be difficult to predict the product formed, whereas the oxygenases add oxygen atom at a fixed specific position in the ring.

Oxygenases can be classified based on the co-enzyme requirement or the nature of the oxidizing substrate and the reaction products formed. Figure 2.14 shows the classification of various oxidizing enzymes (http://us.expasy.org).

2.5.1.1. Monooxygenaes

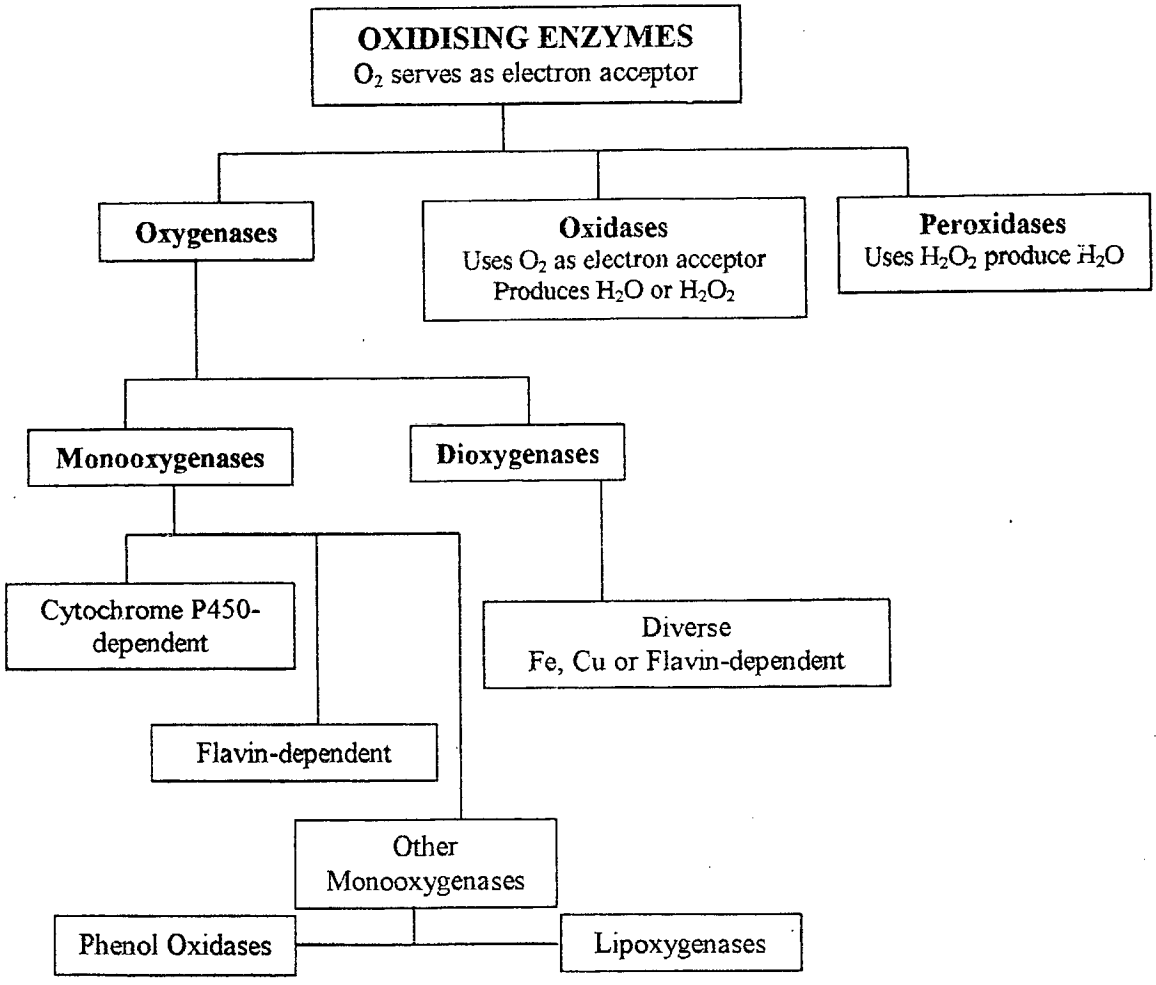
Monooxygenases catalyzes the reaction of addition of one oxygen atom in to the aromatic ring. Based on the co-factors required for their recycling into active form, there are four kinds. 1) NADH of NADPH dependent monooxygenases, 2) Cytochrome P450 which involves an Fe(III) substrate, 3) Flavin-dependent monooxygenases and 4) hydroxylases.

Reaction mechanism

The reactions carried out by these redox enzymes are simple. In case of NADH of FADH dependent monooxygenases, the enzyme-substrate complex formed uses molecular oxygen to oxidize the aromatic ring with a single oxygen atom while, the other atom is reduced with the co-factor forming water.

Substrate + donor-H + O_2 + H^+ → Substrate-O + donor + H_2O

Fig. 2.14: Classification of various oxidizing enzymes



Various reactions are catalyzed by the monoxygenases in transformation processes; styrene monooxygenases oxidizes the ethene bond present on the side chain of the benzene ring to form an epoxide called styrene oxide (Panke, 1999). Wubbolt *et al.* (1994), has studied the function of xylene monoxygenases on the conversion of toluene, xylene (Fig. 2.15) and certain non specific substrate such as ethylbenzene to convert it to styrene oxide (Fig. 2.16a). Polyaromatic compounds

Fig. 2.15: Action of monooxygenases on toluene and xylenes

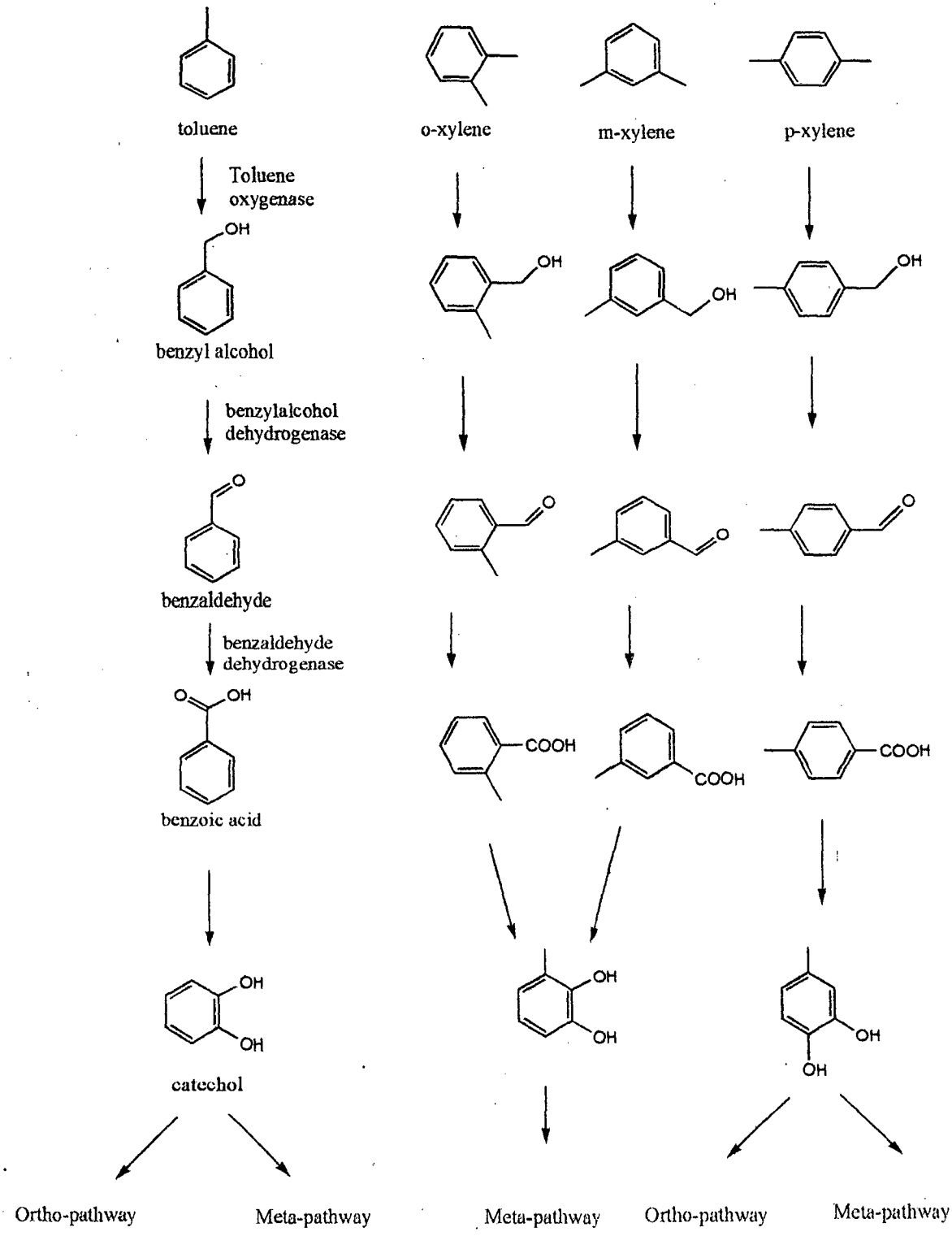
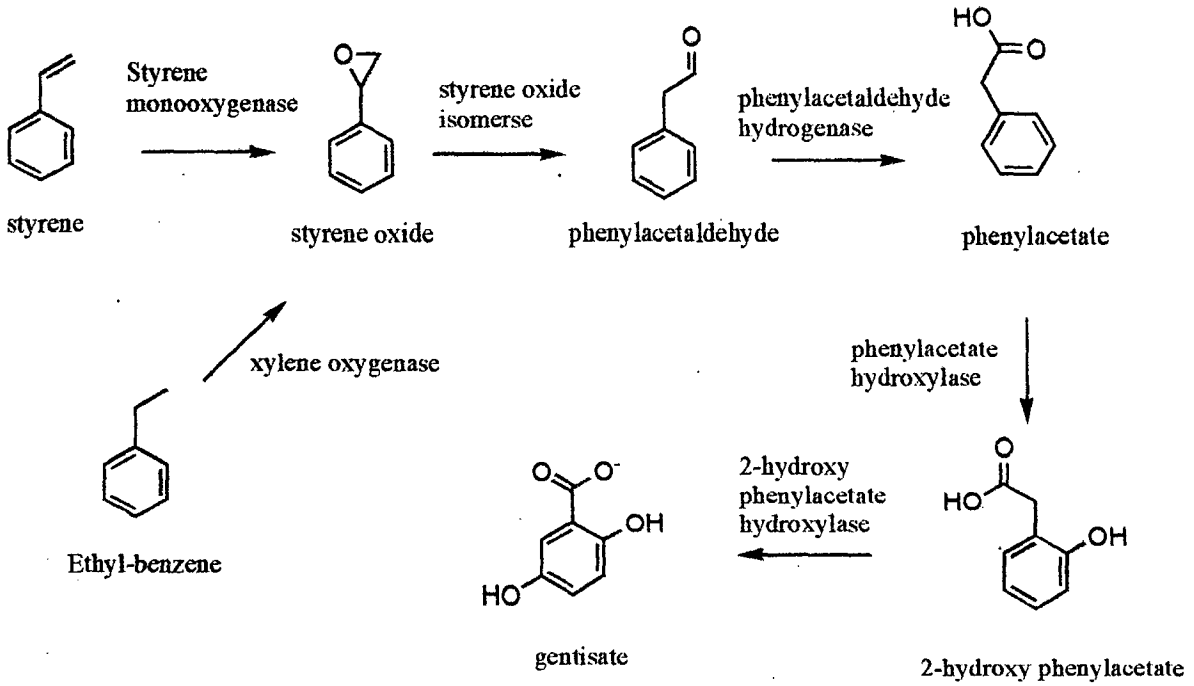
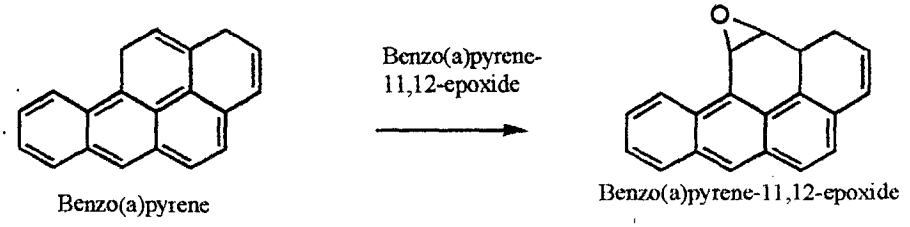


Fig. 2.16: Styrene / ethylbenzene and benzo(a)pyrene oxidation by enzyme monooxygenase

a Styrene / ethylbenzene oxidation



b benzo(a)pyrene oxidation



such as benzopyrene found in abundance in crude oil is affected by the enzyme monooxygenase (benzo(a)pyrene 11,12-epoxidase) and is converted to an epoxide (Moody *et al.,* 2004) (Fig. 2.16b). The epoxide formed is found to be toxic to humans and the environment. Further hydroxylation of epoxide leads to the formation of hydroxyl groups increasing its susceptibility towards ring cleavage. These are the reactions brought about by NADH dependent monooxygenase. Monooxygenases can also help in catalyzing various ringed ketone. One such example is conversion of cyclohexanone to 1-oxa-2-oxocycloheptane (Mihovilovic *et al.*, 2001) (Fig.2.17).

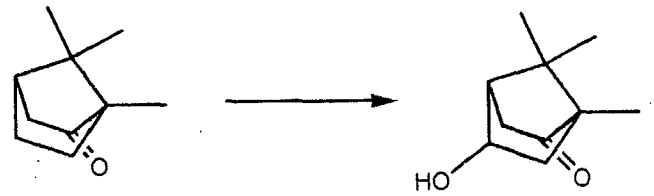
Buhler *et al.* (2002), has studied the catalyzation of the non-heme dependent monooxygenase enzyme on the hydroxylation of a wide range of benzyl compounds which includes toluene and xylenes and substituted toluenes and xylenes. These compounds were converted to corresponding benzoyl alcohols. 2-Hydroxyl biphenyl was converted to 2,3-dihydroxybiphenyls by enzyme 2-Hydroxylbiphenyl 3-monoxygenase (Suske, 1997).

a) Cytochrome P450

Cytochrome P450 exists in most living creatures, including animals, plants, and microorganisms, and plays an extremely important role in metabolism. These enzymes are used to detoxify the chemicals by addition of a single oxygen atom in the aromatic moiety. The mode of action of P450 is shown in fig 2.18 (Hata *et al.*, 2005). The monooxygenation reaction by P450 is initiated by the substrate binding to ferric P450 (Fig.2.18(1)). When an electron (1^{st} e^-) is introduced into substrate-bound P450, the heme iron converts into the reduced form, Fe^{2+} (2), and then an O_2 molecule is incorporated in the heme pocket (3). When another electron (2nd e^-) is introduced (4),

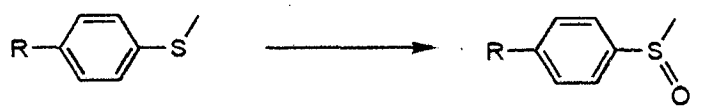
Fig. 2.17: Monooxygenation by cytochrome P450 and enzyme monooxygenase

Cytochrome P450



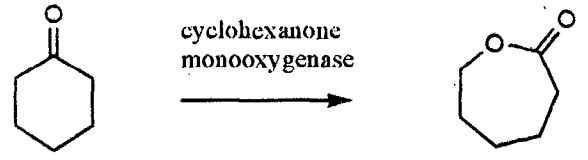
camphor — 5-hydroxy camphor

terpineol — hydroxylated terpneol



thioanisole — Methanesulfinyl benzene sulfoxide

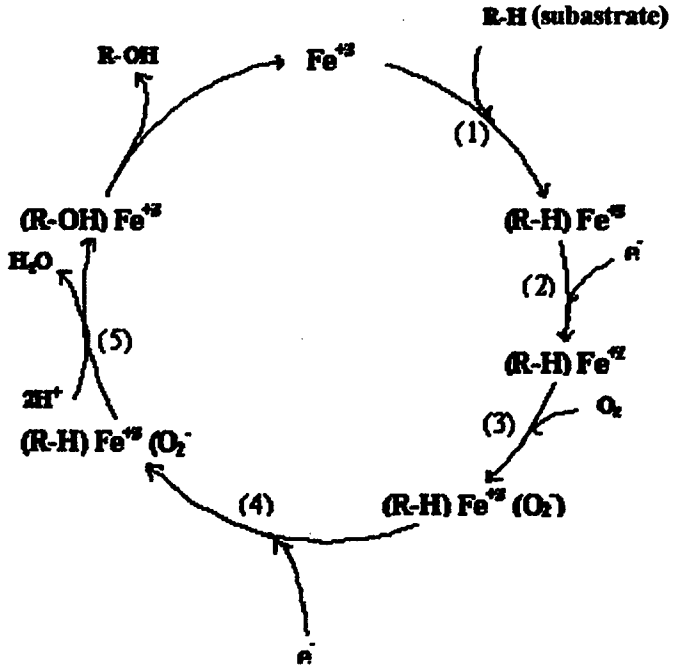
Monooxygenase



cyclohexanone — 1-oxa-2-oxocycloheptane

the O_2 molecule becomes very reactive and the substrate will be oxygenated by an insertion of one O atom into the R–H bond (5). Hence, O–O bond cleavage should occur after the introduction of the 2nd e^-. The intermediates are, however, not able to be observed in experiments because this reaction proceeds very quickly.

Fig. 2.18: The monooxygenase cycle by cytochrome P450.

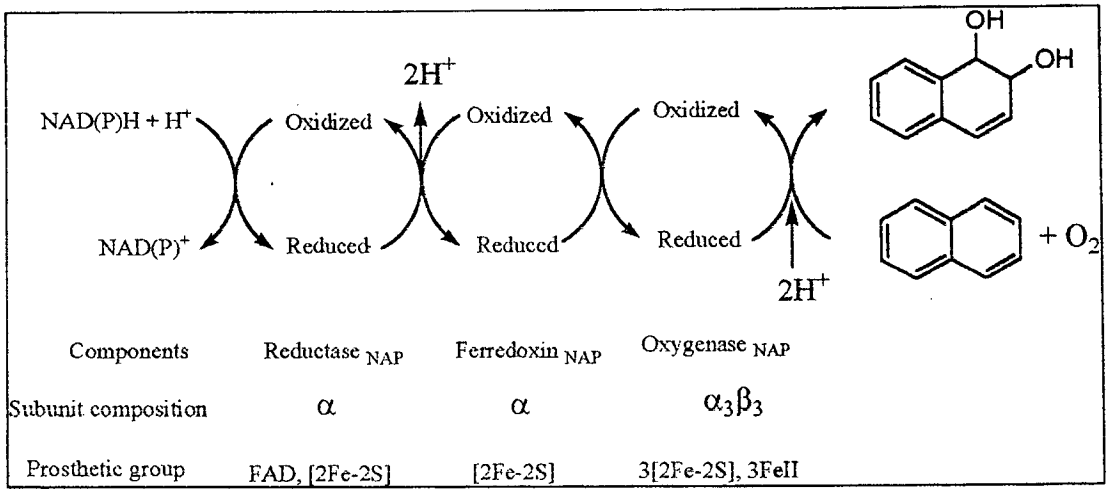


Fruetel *et al,*, (1994) has studied some of the reactions carried out specifically by the isolated enzyme cytochrome P450, they include the oxidation of camphor (terpene) to hydroxylated camphor, α-terpineol to hydroxyl terpineol; thioanisoles to sulfoxides, styrenes to epoxides (Fig.2.17).

2.5.1.2. Dioxygenases

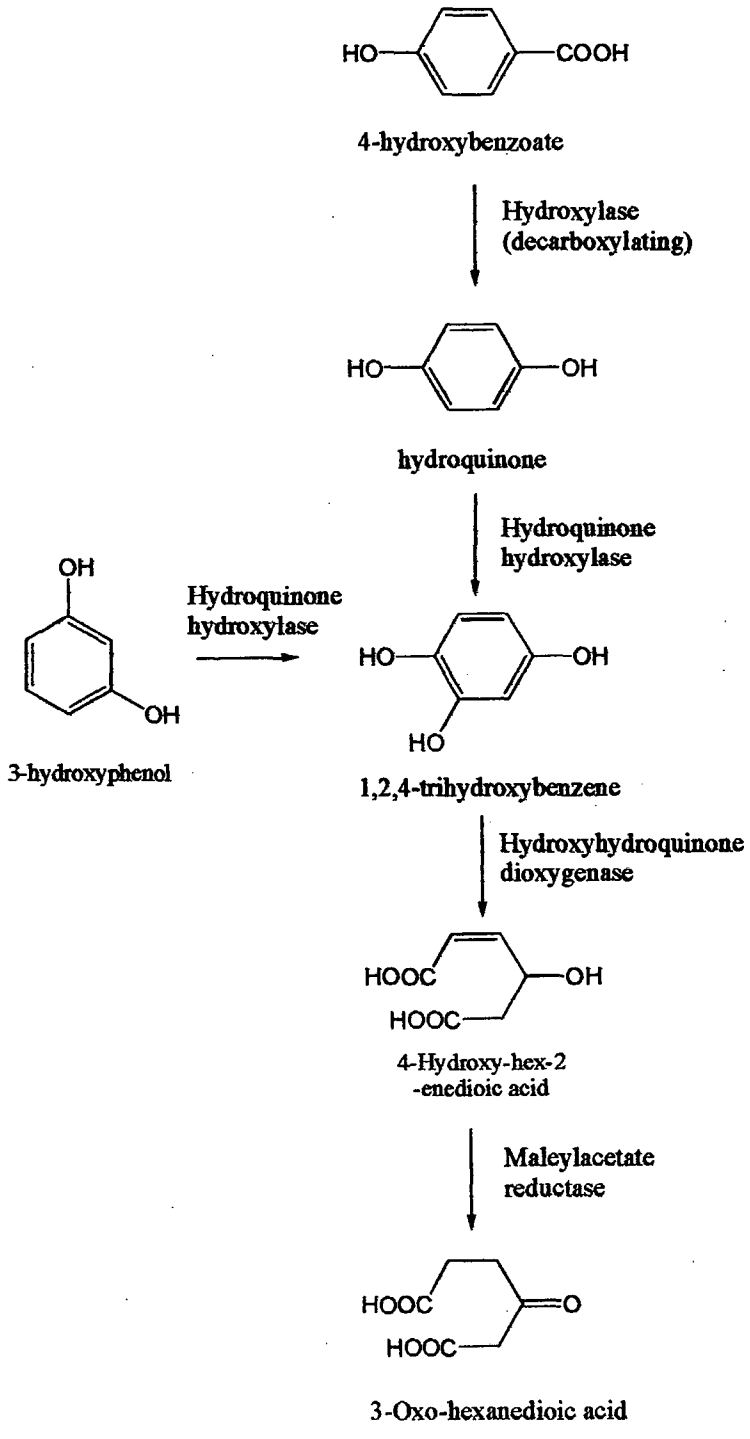
Dioxygenases is oxygen incorporating enzyme which is Fe-S dependent and heme non-dependent. This enzyme catalyzes an addition reaction of two oxygen atoms into the aromatic ring at vicinal positions. One of the well studied dioxygenase enzymes is Naphthalene dioxygenase (Gibson and Parales, 2000). The mechanism of the enzyme is shown in (Fig.2.19).

Fig 2.19: Mechanism of the naphthalene dioxygenase enzyme



Dioxygenases catalyzes the oxygen addition reaction to many aromatic compounds followed by ring cleavage. One of the most common intermediates is catechol, a simplest aromatic diols which is susceptible for ring cleavage. Ring cleavage usually takes place around the two hydroxyl groups. The cleavage in between the two hydroxyl groups (intradiol) is called ortho-cleavage (Cerdan *et al.*, 1994) while cleavage adjacent to the hydroxyl group is called (extradiol) meta-cleavage (Ngai *et al.*, 1990) (Fig.2.1), such a ring cleavage reactions is seen in hydroquinone pathway (Eppink *et al.*, 2000) (Fig.2.20), benzopyrene degradation pathway

Fig. 2.20: Degradation pathway of 4-hydroxybenzoate/m-Hydroxy phenol



(Moody *et al.*, 2004) (Fig. 2.21), 3,4-hydroxyphenylacetate 2,3-dioxygenase (Arias-Barrau *et al.*, 2004) (Fig. 2.22). Dioxygenase also catalyzes reaction where in the oxygen atoms are added on the side chain as in the case of conversion of tryptophan to N-formyl kyurenine (Colabroy and Begley, 2005) (Fig. 2.23) cleavage reaction catalyzed by 3-hydroxy anthranilate 3,4-dioxygenase (Fig. 2.8). Dioxygenase enzyme action of addition of the oxygen atoms on naphthalene to give 1,2-dihydroxy naphthalene (Barnsley, 1976) (Fig. 2.24) are some of the reactions catalyzed by this enzyme.

2.5.1.3. Hydroxylases

Enzyme hydroxylases catalyze a reaction wherein it replaces a hydrogen atom of the aromatic ring with a hydroxyl group. These enzymes are found to be membrane-bound multiprotein complexes (Holland and Weber, 2000). Since the activity of the protein is highest in its bound state, the mechanism of the enzyme has not yet known as it could not be isolated.

Some of the reactions catalyzed by this enzyme include hydroxylation of 4-hydroxybenzoate wherein decarboxylation followed by hydroxylation of the benzene ring forms hydroquinone, further hydroxylation of hydroquinone or 1,3-dihydroxybenzene forms 1,2,4-trihydrobenzene (Eppink *et al*, 2000) (Fig.2.20). Arias-Barrau *et al.*, (2004), has shown the hydroxylation of phenylalanine to give tyrosine (Fig. 2.22). Phenylalanine and tyrosine are both amino acids. Suzuki *et al.*, (1991), has shown the conversion of Salicyclaldehyde to catechol by salicylate hydroxylase (Fig. 2.24).

The reactions carried out by cytochrome P450, monooxygenases, hydroxylases explains the addition of a single oxygen atom in to the ring which could

Fig. 2.21: Degradation pathway of benzo(a)pyrene

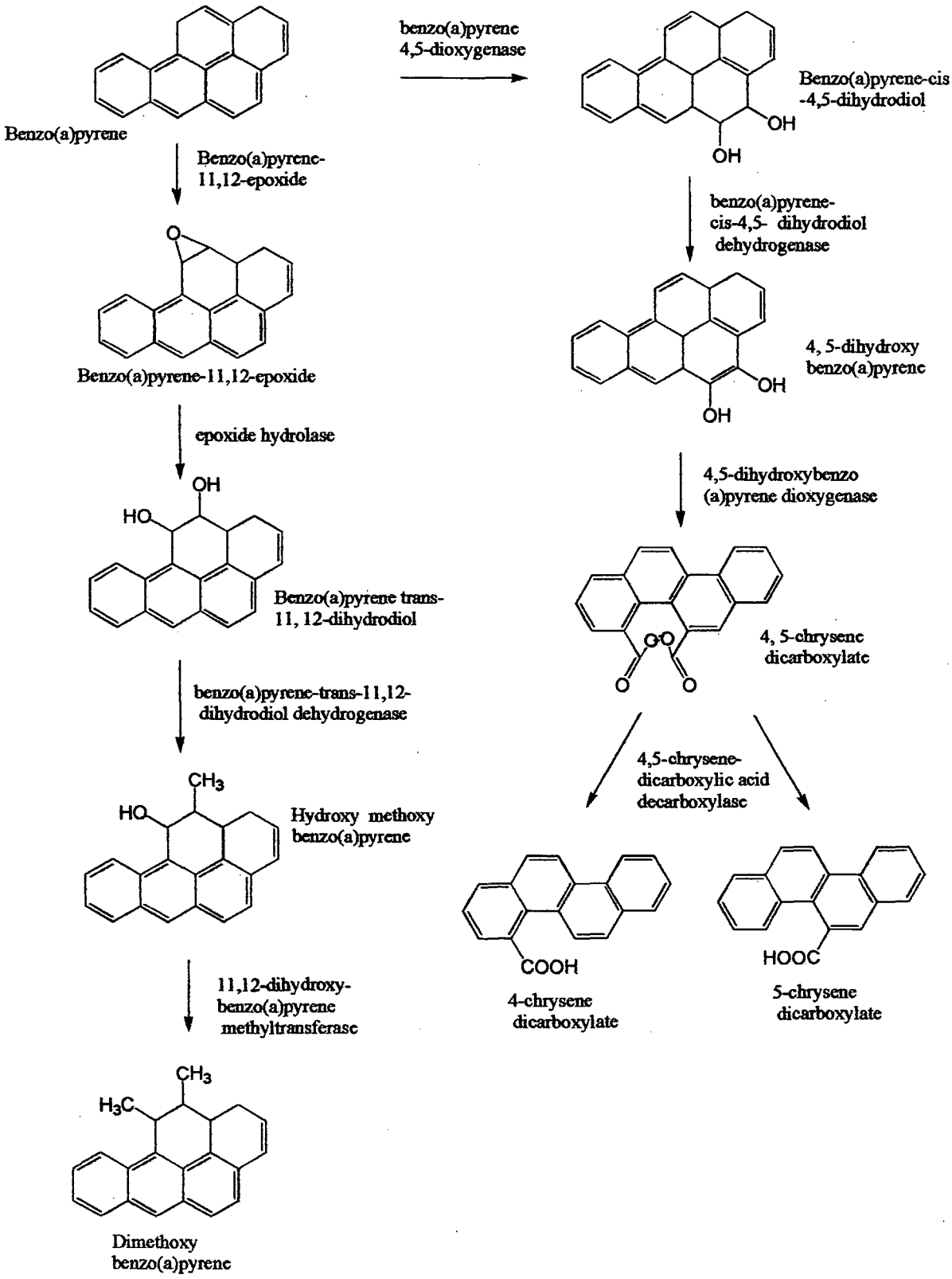


Fig. 2.22: Degradation pathway of Phenylalanine/Tyrosine

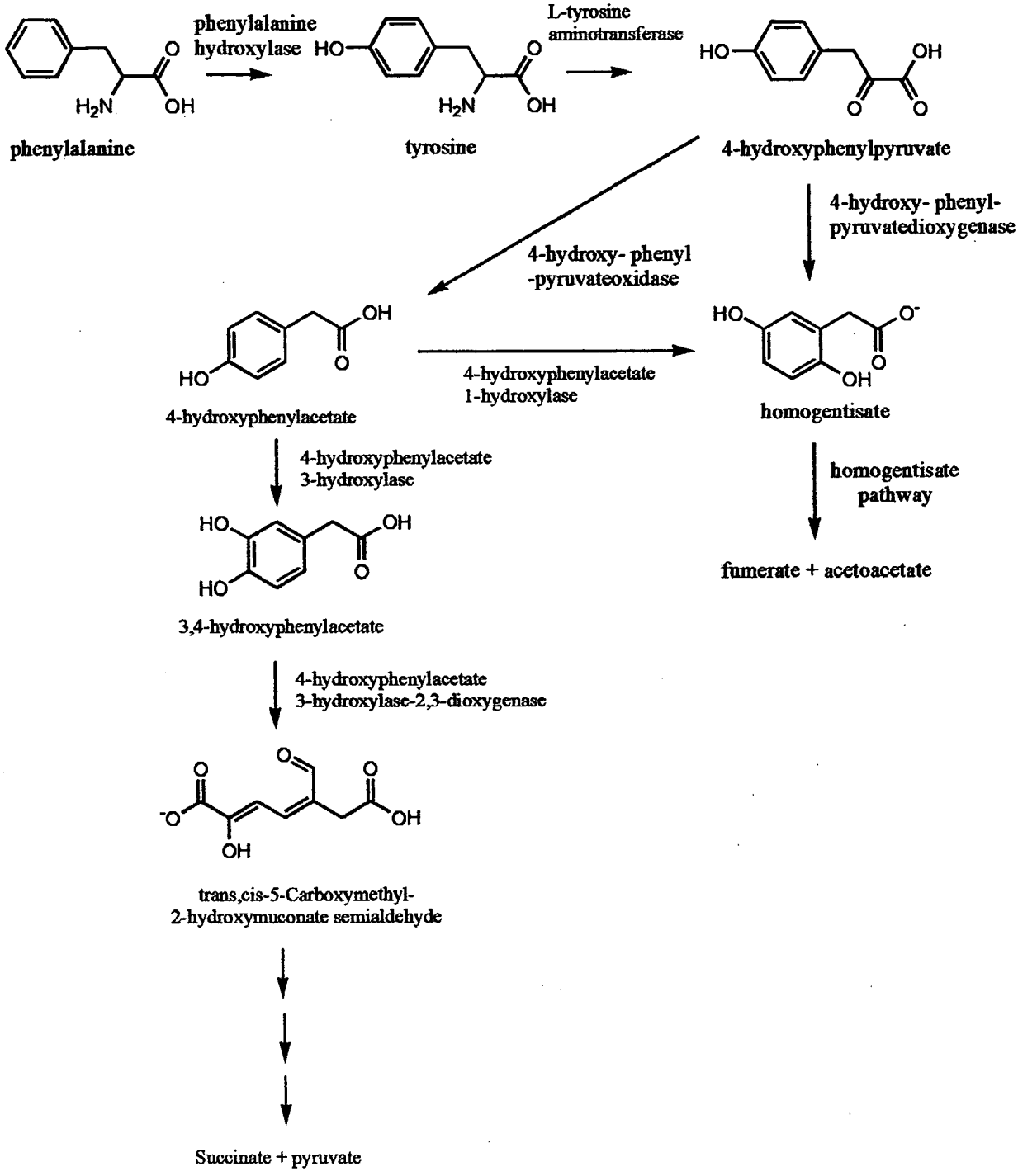


Fig. 2.23: Degradation pathway of Tryptophan

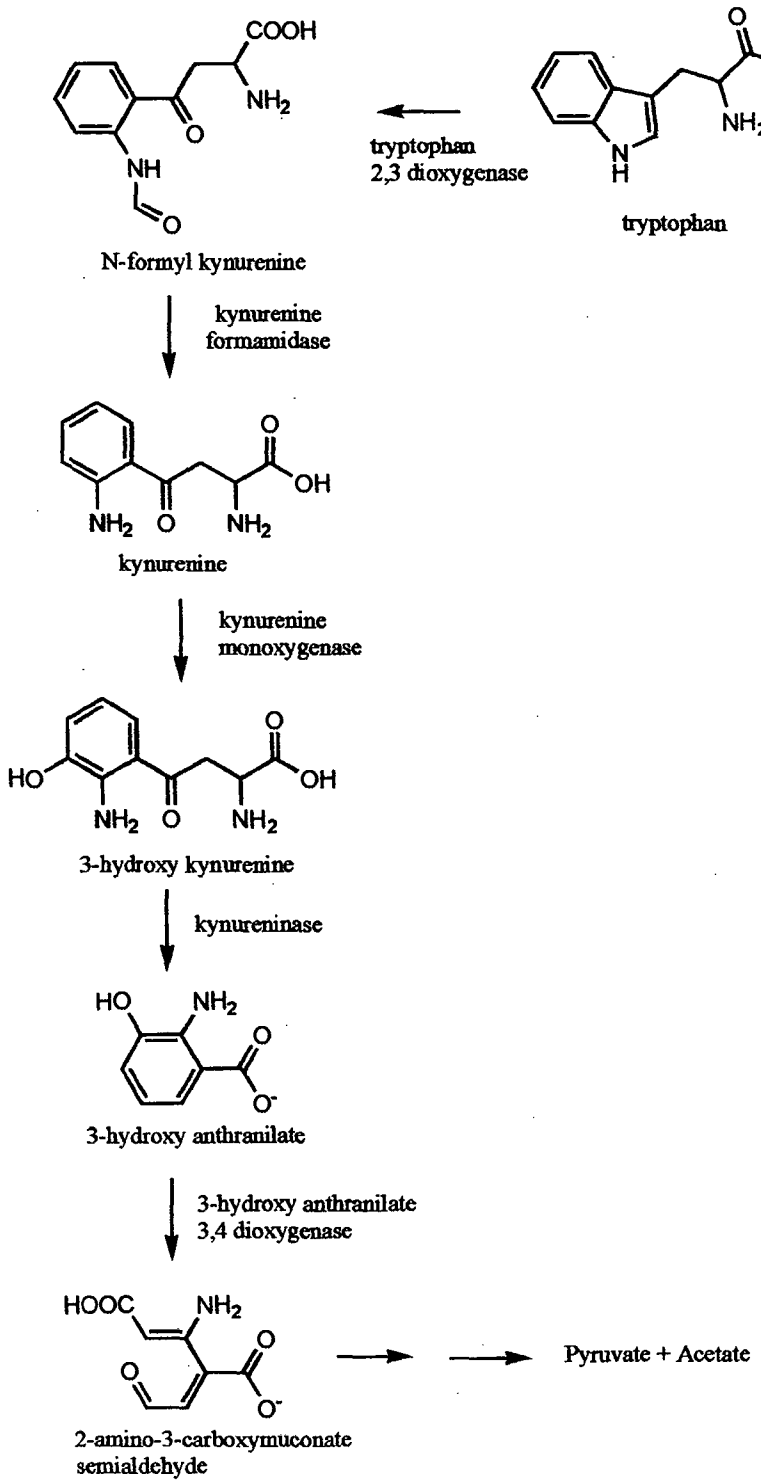
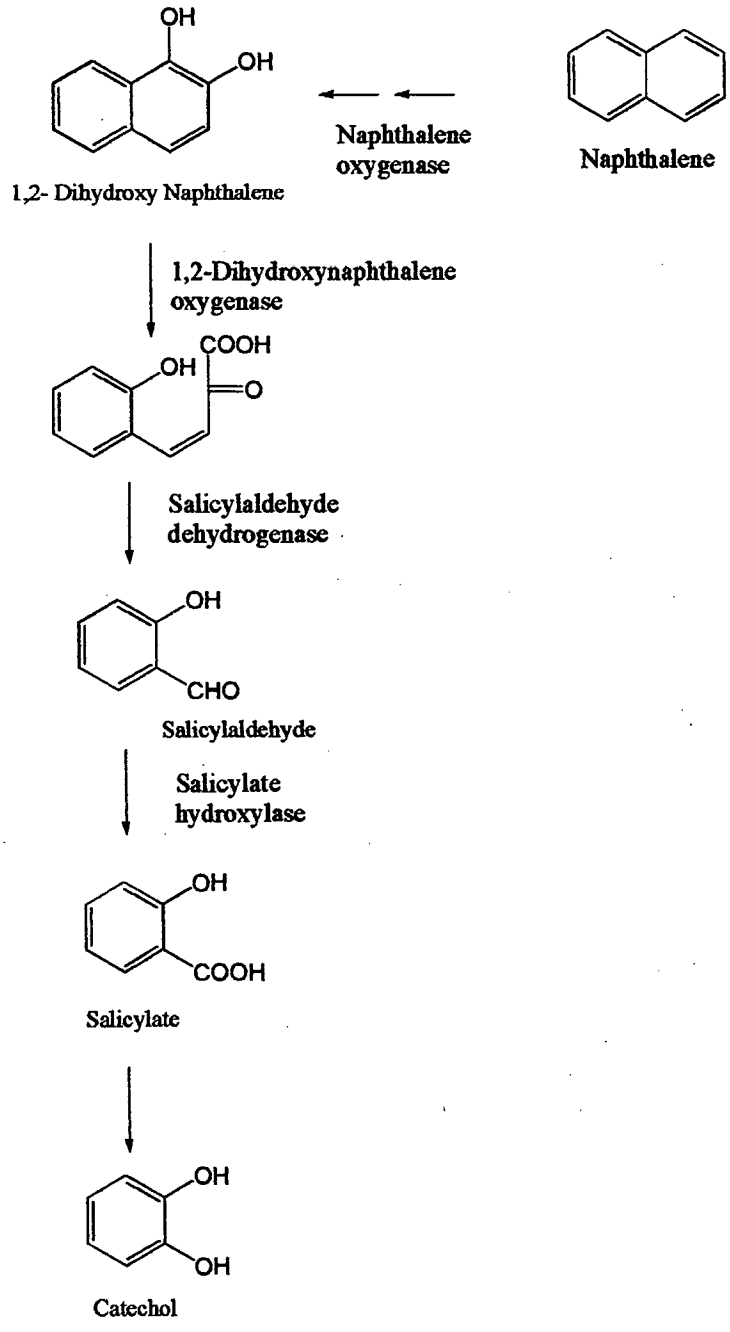


Fig. 2.24: Degradation pathway of Nathphalene



take place anywhere in the pathway both in biodegradative as well as during synthesis. The reactions referred in this section explains the hydroxylation during various degradative pathways. Hydroxylation of 2-hydroxyphenylacetate during styrene degradation (Panke *et al.*, 1999) (Fig. 2.16), kynurenine to 3-hydroxy kynurenine (Colabroy and Begley, 2005) (Fig. 2.23), conversion of 4-hydroxyphenylacetate to 3,4-hydroxyphenylacetate by 4-hydroxyphenylacetate 3-hydroxylase (Arias-Barrau *et al.*, 2004) (Fig. 2.22) are examples of monooxygenation of aromatic compounds.

The incorporation of the oxygen atom in the aromatic ring plays an important role in the degradation as it is this hydroxylation that makes it susceptible to microbial attack. The introduction of two oxygen atoms makes it ready for the ring cleavage (explained further) to give an aliphatic compound especially an acid or aldehyde which is easily utilized by various organisms.

2.5.1.5. Laccases

These oxidases are included in the copper containing enzyme category. These are involved in catalytic oxidation of diphenols and the major difference between the two enzymes is that laccase has the ability to oxidize both o- and p-diphenols, where as the enzyme phenol oxidase catalyzes reactions only with o-diphenols (Burton, 2003).

Laccases are blue multi-copper oxidases with four copper ions that are coordinated with 3 redox sites. These have a molecular weight between 40000 to 140000 and are often produced as highly glycosylated derivates, where the carbohydrate moieties increase their hydrophilicity and thus stabilize them in their extracellular role.

Reaction mechanism

Laccases initially reacts with the substrate forming a complex which then reacts with molecular oxygen within the enzyme wherein, an intramolecular electron transfer takes place leading to oxygen reduction. With the transfer of oxygen atom, the product is released from the enzyme with liberation of water.

The substrates for the enzyme laccase includes methoxy phenol, phenols, o- and p-diphenols, aminophenols, polyphenols, polyamines and lignin related molecules. Demethylation reactions of lignin, ethoxy phenol acids and methoxy aromatics, benzylamine conversion to benzaldehyde (Fig. 2.25), various polymerization and co-polymerization of lignin molecules with phenols and acrylamide are also catalyzed by laccases.

These enzymes in the presence of certain chemicals called mediators can catalyze various reactions that are chemically not feasible. Mediators (hydroxybenzotriazole (HBT); 2,2'-azinobis-(3-ethylbenzylthiozoline-6-sulphonate (ABTS); 3-hydroxy-anthranilic acid (HAA)) are compounds that can undergo redox reactions and in the process carry out reactions with high redox potential. Some of these reactions involve the transformation of toluene and dimethoxytoluene to their respective benzaldehydes.

Laccases also bring about coupling reactions using free radicals (Chignell, 1985) where the simple low molecular weight aromatic compound is converted to a high molecular weight compound. Isouegenol is converted to dimeric products (Baminger *et al*, 2001) and dimerization of substituted imidazole (Shuttleworth and Bollag, 1986) (Fig.2.26a & b).

Fig. 2.25: Examples of typical laccase catalysed reactions

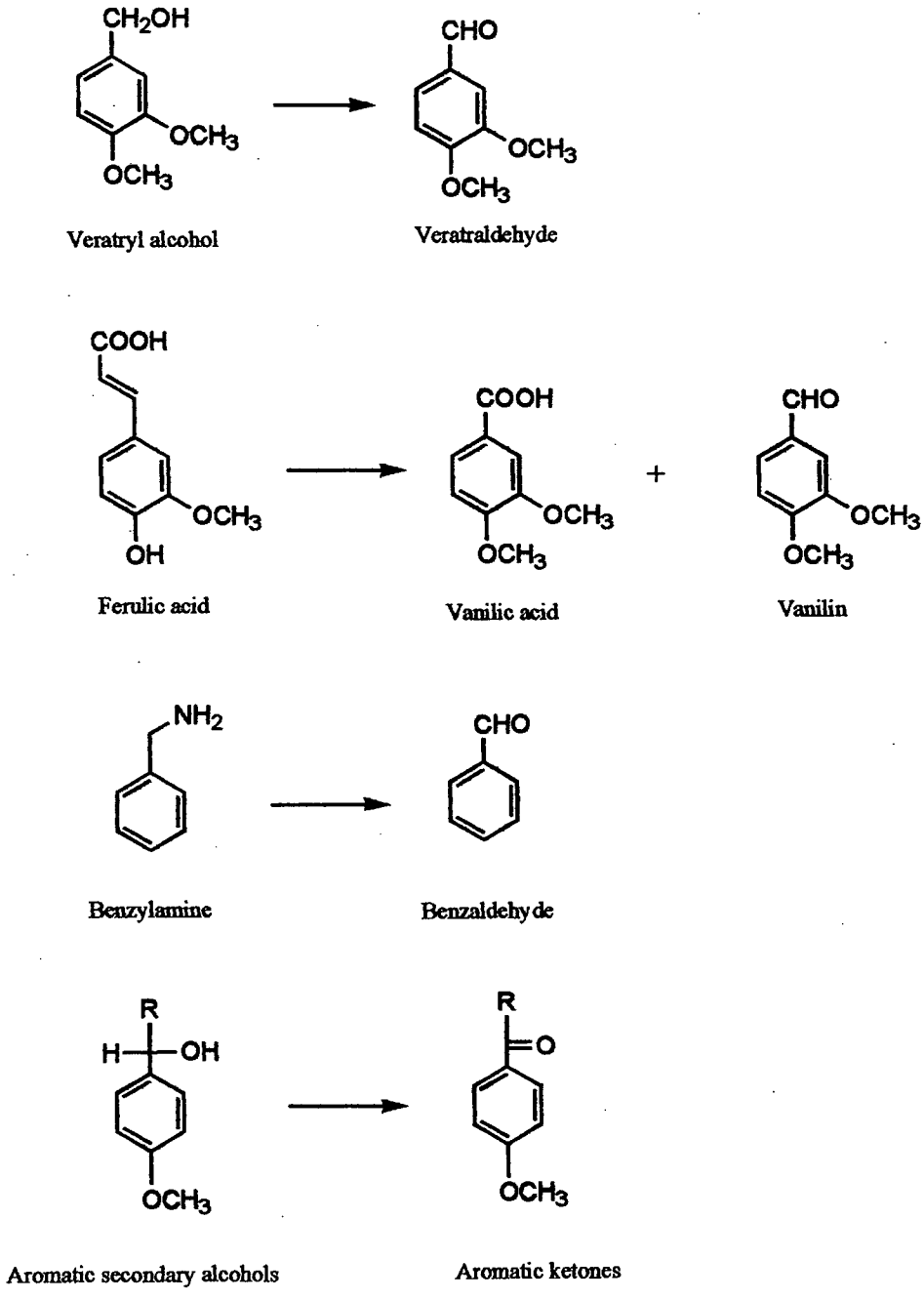
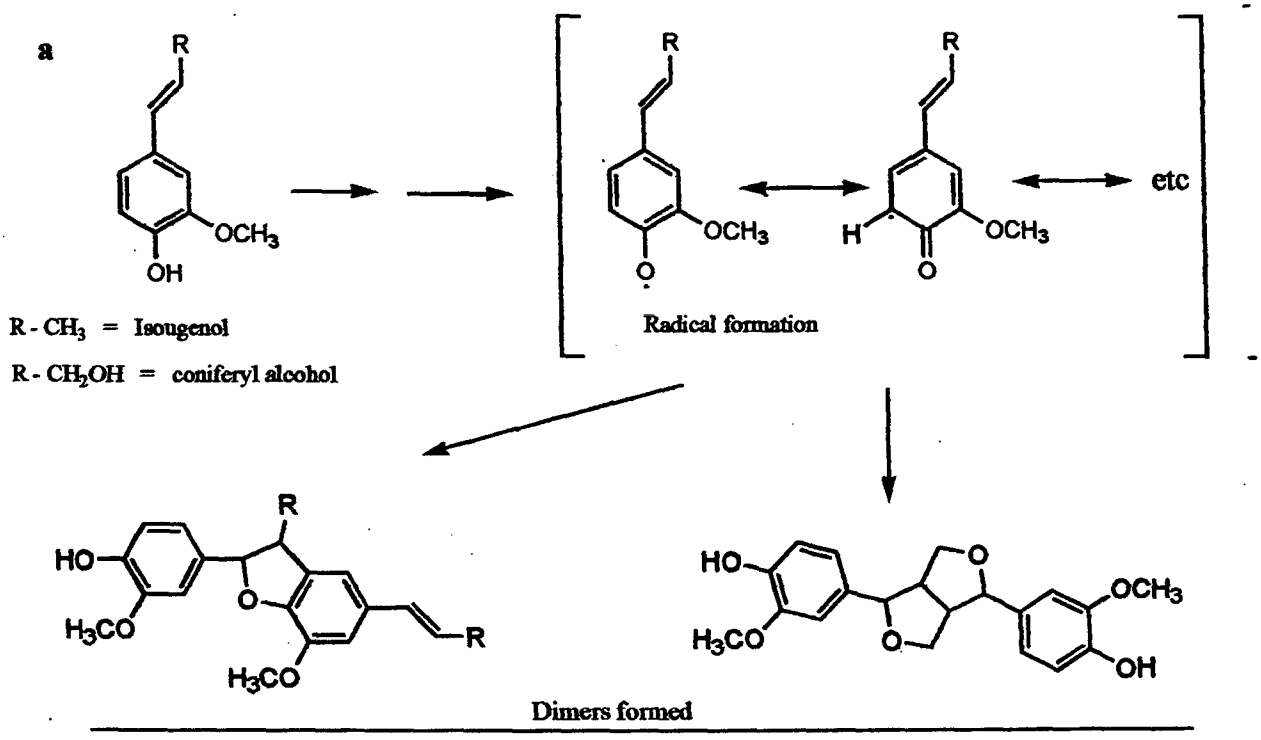
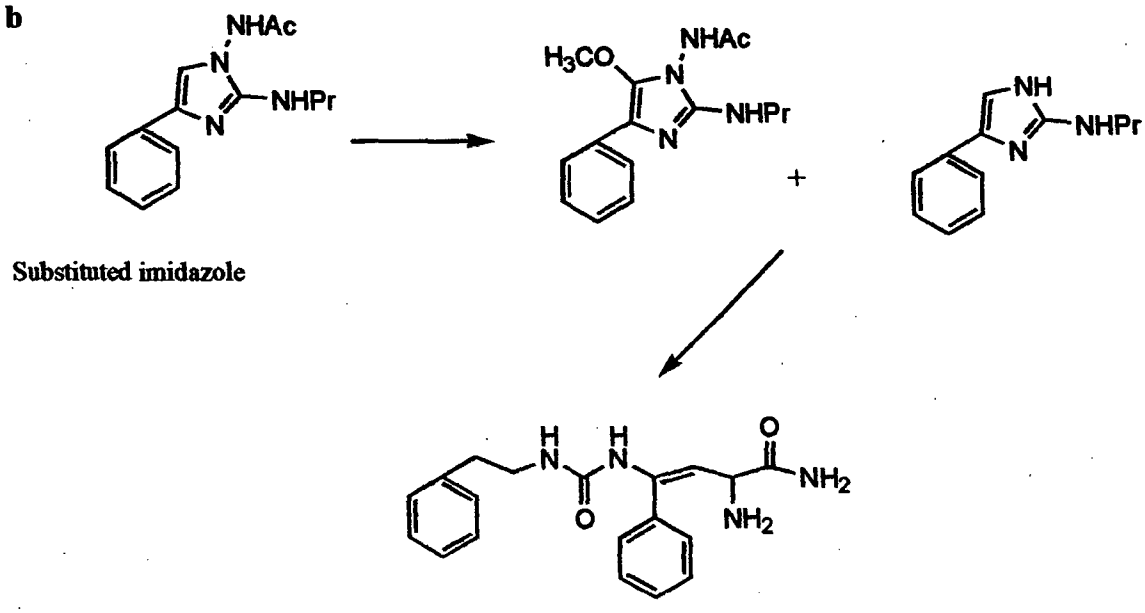


Fig. 2.26: Polymerization reactions by laccases





2.5.1.6. Polyphenol oxidases

Polyphenol oxidases are also called enzyme tyrosinases as they hydroxylate tyrosine with a hydroxyl group. They contain 2 copper ions at one reaction site in each functional unit responsible for binding of both the molecular oxygen and the substrate. The substrates are always phenol or dihyroxyphenol.

Phenol oxidases catalyses two reactions: 1) the hydroxylation of phenol to catechol the enzyme is also called cresolase and 2) oxidation of catechols to quinones the enzyme is called catecholase. There could be an extension of the reaction wherein the quinones could polymerize to form melanin or give a polyphenolic compound.

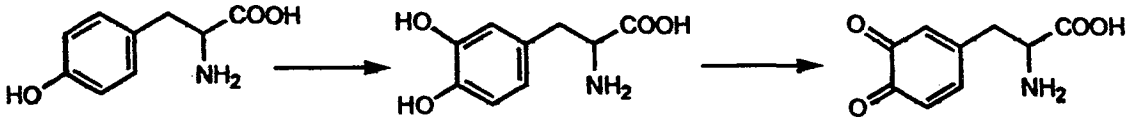
The reaction mechanism proceeds in the following pathway; 1) the phenol (substrate) is converted to a phenoxy radical, 2) Addition of the oxygen atom from molecular oxygen. This oxygen is destabilized by polarization in the enzymes di-copper active site, 3) oxidation reaction to convert catechol to quinone.

Polyphenol oxidases are involved in various substitution reactions of replacement of hydrogen atom with the hydroxy group at the ortho position which is not feasible through chemical synthesis (Fig. 2.27). Like laccases, these enzymes are also involved in polymerization of aromatic compounds; the biphenol products formed by the phenol oxidases reaction can be used in production of complex compounds with from substrates such as flavonols having industrial importance (Burton, 2003).

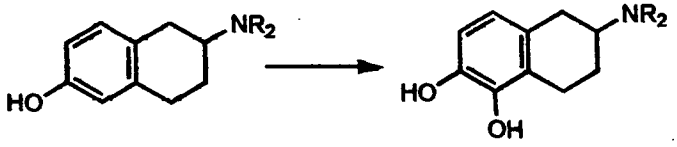
2.5.1.5. Peroxidases

Peroxidases are commonly found in plant, animal and microbial cells. These are haeme protein containing Fe(III) which undergoes redox reactions during the

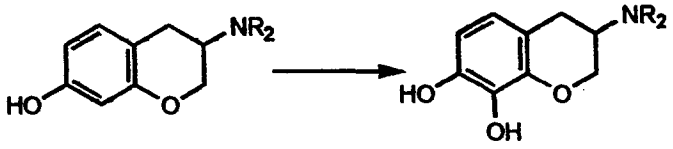
Fig. 2.27: Examples of typical PPO catalysed reactions



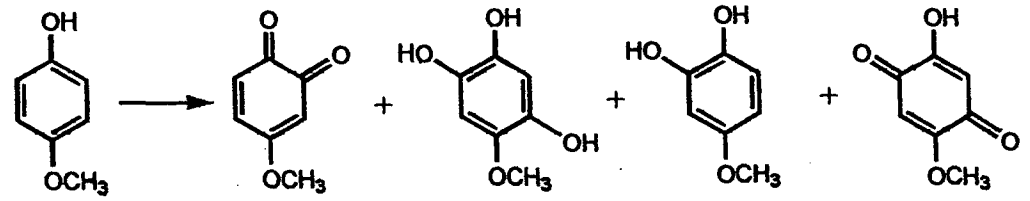
Tyrosine L-DOPA L-Dopaquinone



2-aminotetralins



Chromamaines

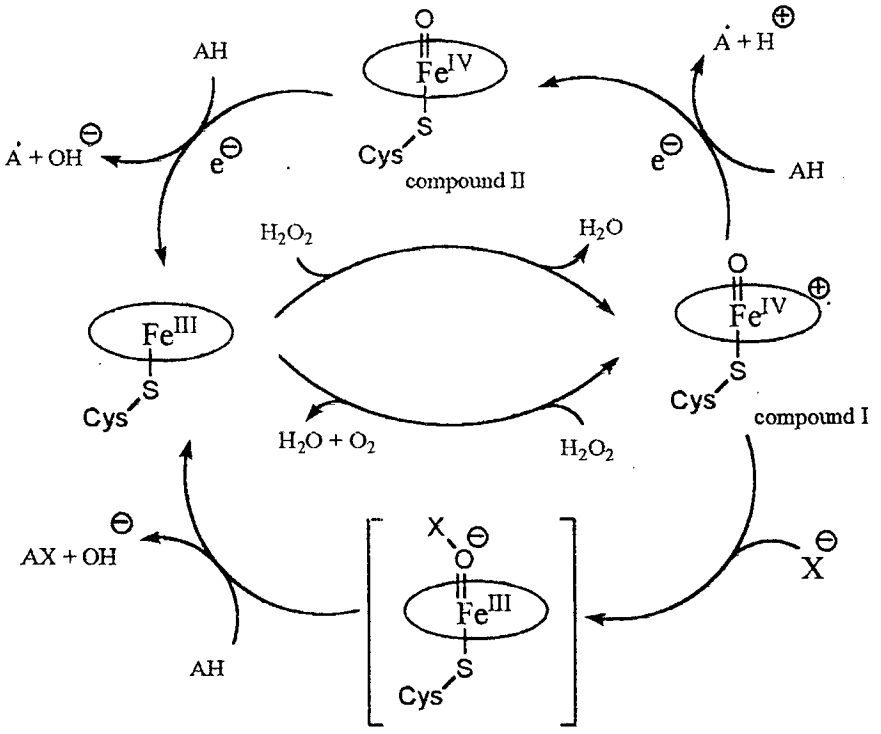


p-hydroxyanisole

catalytic reactions (van de Velde *et al.*, 2001). These proteins react non-selectively via free radical mechanism in the presence of hydrogen peroxide.

Colonna *et al.*, (1999), proposed the mechanism of its catalytic cycle has been shown if fig. 2.28. Compound-I is the oxidized state of enzyme peroxidase with ferryl oxygen (radical) with two electrons. The ferryl state gets back to the ferric state by two ways; 1) it gains two electrons, one each from two substrates or 2) oxidizes hydrogen peroxide thereby gaining two electrons, thus showing catalase activity.

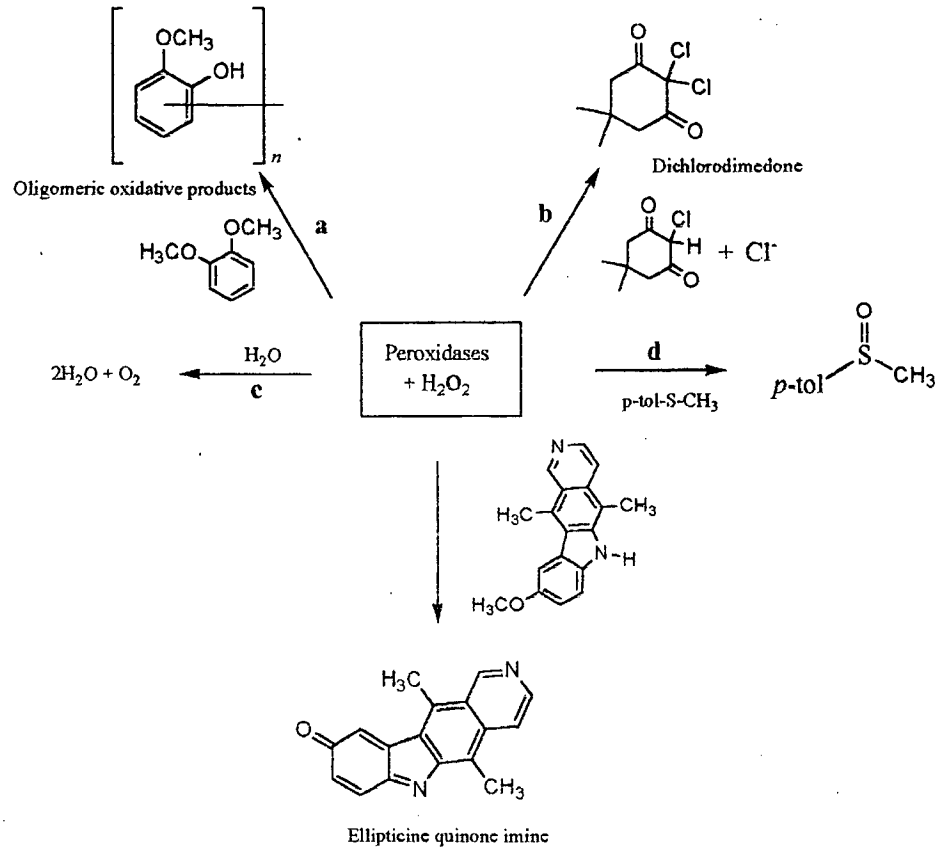
Fig.2.28: Catalytic mechanism of peroxidases.



Another form of peroxidase is isolated from the marine fungus *Caldariomyces fumago* which catalyses the halogenation of the substrate. This enzyme was found to be versatile as this could bring about catalysis of aromatic hydroxylation, epoxidation,

sulfoxidation and catalase (Colonna, *et al.*, 1999; van de Velde, *et al.*, 2001). Figure 2.29 shows participation of peroxidase in various reactions.

Fig.2.29: substrates and products of enzyme peroxidases.



Ellipticine quinone imine

2.6. ENVIRONMENTAL FACTORS

Various factors are responsible when biodegradation of the aromatic compounds is considered. Some of the important factors include - positive chemotactic behaviour of the bacterium towards the pollutant; bacterial ability to produce specific enzymes; the nature of the pollutant and environmental factors (such as oxygen (electron acceptor availability), pH, temperature, pressure and salinity.

2.6.1. *Oxygen*

2.6.1.1 Aerobic aromatic ring cleavage mechanism in bacteria

Bacterial response towards chemical substrates depends on various factors depending on its environment. Polluted waters such as aquifers and submerged sediment are normally anaerobic in nature. Bacterial interaction with contaminants will change depending on the availability of oxygen in the environment, accordingly, bacteria will follow aerobic or anaerobic type of degradation. Other factor equally significant is the terminal electron acceptor. Oxygen is the widely used electron acceptor under aerobic condition while Fe(III) also plays an equally important role.

Various aerobic reactions are catalyzed by enzymes such as oxygenases (monooxygenases, dioxygenases), laccases, phenol oxidases and peroxidases.

2.6.1.2. Anaerobic aromatic ring cleavage mechanism in bacteria

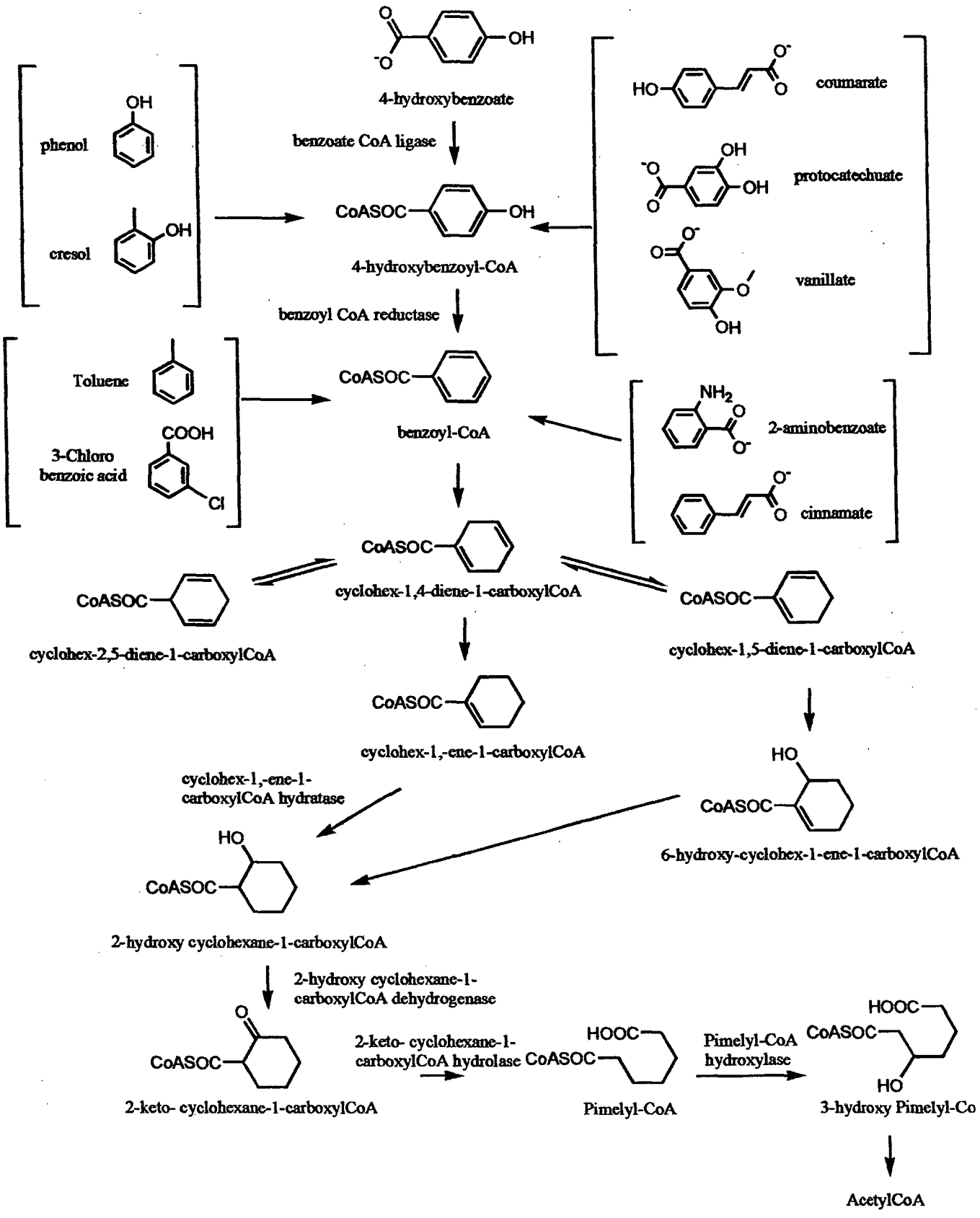
Anaerobic environment harbours a completely different community of microorganisms. These are found in aquifers, submerged sediments, polluted waters and in environments where the oxygen levels are depleted. In such conditions respiration of the organisms takes place with the terminal electron acceptors being nitrates, sulfates, Fe (III), carbondioxide or other acceptors (Chlorate, Mn, Cr, U, etc) (Diaz, 2004).

It was assumed that aromatic compounds would not be utilized under anaerobic conditions but it is surprising to find that these compounds are being utilized by ring cleavage pathway but follows a different set of reaction in the pathway. Harwood and Gibson (1997), reviewed the possible pathways followed for the degradation of aromatic compounds by various anaerobic bacteria. Fig.2.30 shows an overall pathway followed by microorganisms which involves significant steps; 1) CoA thioester formation, 2) ring reduction 3) introduction of a carbonyl group, 4) ring opening, 5) b-oxidation sequence leading to the conversion of the remainder of the molecule to acetyl-CoA.

Studies carried out by various researchers have shown that the aromatic compounds carrying different substituents are primarily carboxylated and thioesterified by anaerobic bacteria and which become available to be utilized by various anaerobic bacteria. Van Schiel and Young (2000), has shown the initial transformation of phenol to p-hydroxybenzoate by carboxylation in *Thauera aromatica* K172 (denitrifying bacteria), (via phenylphosphate and phenolate, to 4-hydroxybenzoate by enzyme phenolcarboxylase intracellularly) followed by thio-esterification, and ring cleavage. Hydroquinone was found to be anaerobically degraded via gentisate in a sulfate-reducing bacterium *Desulfococcus* sp. HY5. The carboxyl group of gentisate was thio-esterified and enzymatically transformed via ring cleavage (Gorny and Schink, 1994).

A recent review carried out by Widdel and Rabus (2000), has shown that there exists another mechanism by which the aromatic compounds are degraded. Amongst the denitrifiers, fumerate is used as the terminal electron acceptor. Aromatic compounds with alkyl chain such as toluenes, cresols, xylenes, cymenes, methyl naphthalenes

Fig. 2.30: Anaerobic degradation pathway of the benzene ring



(Beller *et al.*, 1992; Muller *et al.*, 2001; Achong *et al.*, 2001; Safinowski and Meckenstock, 2004) as couples with fumerate to give benzylsuccinate derivatives which are then converted to benzoate. On the other hand, aromatics such as ethyl benzene, propyl benzene showed their transformation to alcohols and naphthalene to 2-naphthanoic acid. The naphthalene ring cleavage pathway brought about by naphthalene degrading bacteria is proposed by Annweiler *et al.*, (2002), is shown in Fig.2.31.

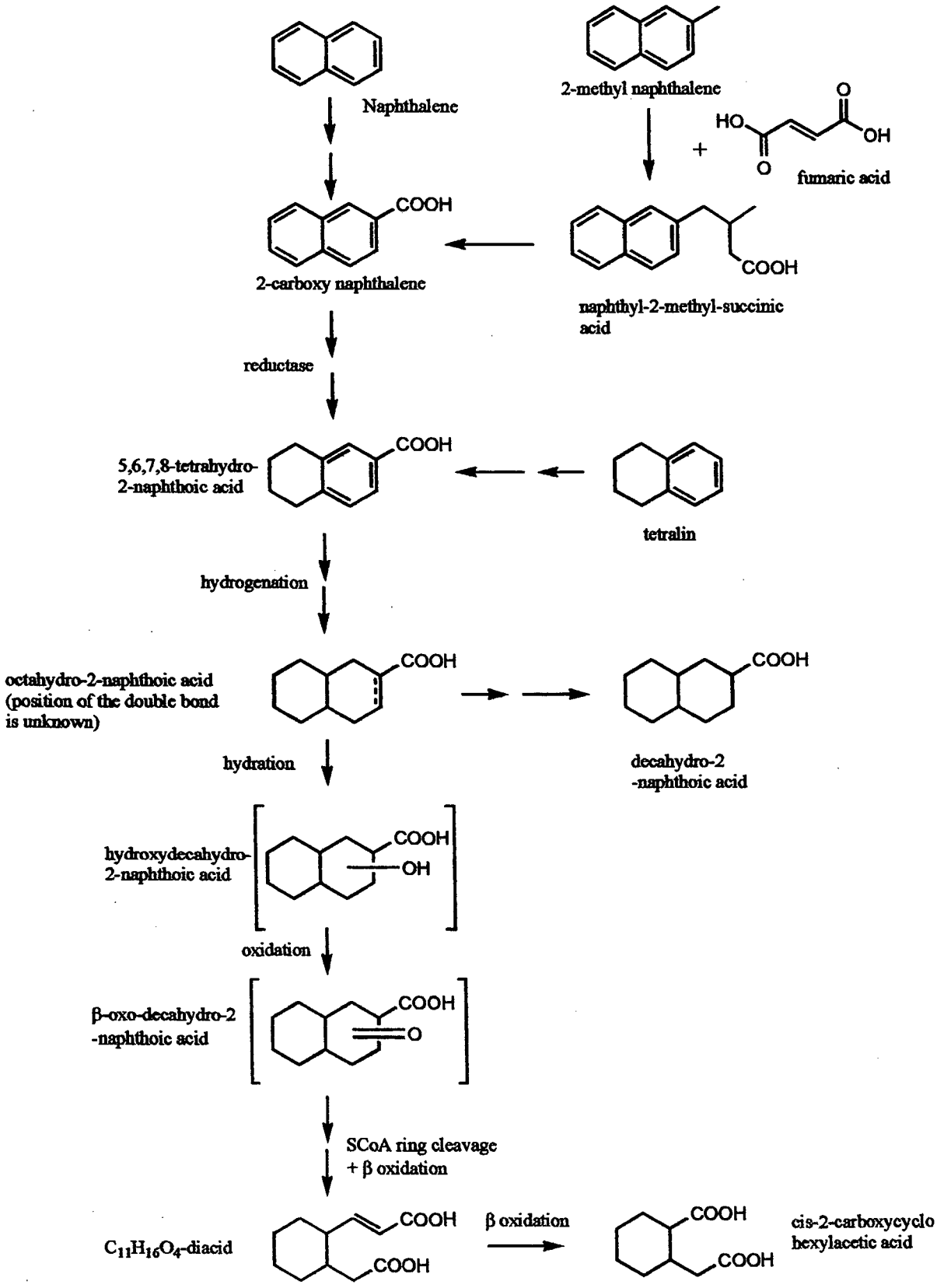
2.6.2. *Temperature*

2.6.2.1. High temperatures

Natural environments with high temperatures are observed at hot water springs, areas around active volcanoes, hydrothermal vents in deep oceans. Various other environments include composts, industrial storage areas containing utilizable effluents. The organisms growing optimally at high temperatures are called thermophiles while thermotolerant organisms consists of those that grow optimally at lower temperatures but can tolerate higher temperatures. Some of the thermophilic bacteria growing at moderate temperatures belong to genus *Bacillus, Geobacillus, Thermoactinomyces, Clostridium, Thermoanaerobacter, Thermoplasma;* extreme temperatures -*Thermus, Thermodesulfobacterium, Sulfolobus, Thermomicrobium, Dictyoglomus, Methanococcus, Sulfurococcus, Thermotoga;*, hyperthermophiles - *Methanoccus, Acidianus, Archaeoglobus, Methanopyru, Pyrobaculum, Pyrococcus* and *Thermococcus.*

Various PAH compounds such as fluorene, fluorathene, phenanthrene are considerably less soluble at mesophilic temperatures and Viamajala *et al.* (2007) have shown that there is an increase in the solubility with an increase in temperature and

Fig. 2.31: Anaerobic degradation pathway of naphthalene/methyl-naphthalene



has claimed to have isolated a thermophile *Geobacilli* from a compost which utilized phenanthrene. Phenol transformation to catechol followed by meta-cleavage was observed in *Bacillus stearothermophilus* (Kim and Oriel, 1995). Biodegradation of PCB by a thermophile, isolated and chracterised by Kimbara (2005), following the similar pathway with the addition of a dihydroxy group at 2,3 positions, followed by ring cleavage by dioxygenases.

2.6.2.2. Low temperatures

Psychrophilic bacteria include microorganisms that are able to grow optimally at low temperatures in the Artic, Antarctica and cold areas where there is formation of ice. Anthropogenic activities at the Arctic region is seen when petroleum products are used for heating and other mechanical activities leads to oil spills on ice. Transport of pollutants from other parts of the world which occur due to natural calamities and other anthropogenic activities to the Arctic regions by water currents is possible. Thus, it is of significance to understand the microbial population surviving in these conditions and their biodegradation activities. Mohn and Stewart (2000), studied the limiting factors for the biodegradation of hydrocarbons in these conditions. It has been found that the soil in the Arctic is barely seen when the ice melts during summer, the permafrost prevents movement of water which creates an active zone where most of the hydrocarbon exists and probably the site showing highest activity. Mohn also reported that the psychrotolerant bacteria, instead of psychrophilic, are the dominating population showing highest activity at higher temperatures i.e. at 15 – 20°C. This may suggest that the hydrocarbons become available to microbial attack at a higher temperature. Additional nutrients added such as nitrogen or heavy metals retard their ability to degrade the hydrocarbons. It is thus evident that bacteria bring

about degradation of aromatic compounds at such cold temperatures. Eriksson *et al.* (2001), showed a similar reaction where temperature plays an important role in hydrocarbon degradation. Studying the degradation at temperatures of 7, 0 and -5°C, hydrocarbon degradation was evident at a higher temperature. Eriksson *et al.* (2003), enriched a microbial population belonging to genus *Acidovorax*, *Bordetella*, *Pseudomonas*, *Sphingomonas*, and *Variovorax* from Arctic soil able to degrade PAH at 7°C anaerobically with nitrate as electron acceptor. The efficiency was 39% removal of 2-methylnaphthalene and fluorene at 7°C and up to 80% removal at 20°C under aerobic conditions after an incubation period of 90 days. PCBs have also been found at the Arctic and their degradation have been studied by Master and Mohn (1998), where isolates closely belonging to the genus *Pseudomonas* could breakdown PCB at 7°C, and their removal at higher temperatures were much higher (90% at 37°C).

2.6.3. *Salinity*

One of the natural extremities includes the environments with high salinity. Salinity varies with ecosystems, fresh water ecosystem shows negligible or no salinity whereas open seas show salinity of 30-35% salt concentration. Salt pans have salt concentration at saturation levels. Estuarine ecosystem shows a fluctuation in salt concentration due to constant mixing of sea water with fresh water. The general term given to the organisms that survive, tolerate and thrive in such environment is Halophiles which are included in a separate domain called Archaea (Woese *et al.*

1990), whereas organisms that can tolerate high salt concentrations but can even grow at environments with no or less salt concentrations are called halotolerant which belong to eubacterial domain. Some of the members of halophiles that were isolated from Dead Sea belonged to the family *Halobacteriaceae*, such as genus *Haloferax*, *Haloarcula*, *Halorubrum* (formerly *Halobacterium*) (Arahal *et al.* 1996). Pašić *et al.* (2005), have shown that bacteria belonging to some of the genera mentioned above thrive in salterns. These organisms belonged to obligate halophiles as they could not survive in medium at salt concentration below 15%. Halophilic archaea maintain an osmotic balance with the hypersaline environment by accumulating high salt concentrations, which requires salt adaptation of the intracellular enzymes (Ventosa *et al.*, 1998; Hough and Danson, 1999; Oren, 1999). Halophilic bacteria have adapted to the osmotic stress of high-salinity environments by actively accumulating K+, glycerol, betaine and ectoine within the cytoplasm (Peytona *et al.*, 2002; Woolard and Irvine, 1995; Galinski *et al.*, 1985; Jebbar *et al.*, 1992). Eubacteria are more promising degraders than archaea as they have a much greater metabolic diversity. Their intracellular salt concentration is low, and their enzymes involved in biodegradation may be conventional (i.e. not salt-requiring) enzymes similar to those of non-halophiles. The use of microorganisms able to degrade organic wastes in the presence of salt could prevent costly dilution to lower the salinity, or the removal of salt by reverse osmosis, ion exchange or electrodialysis before biological treatment. Thus these organisms play a vital role in the biodegradation and biomineralization of organic residues in saline environment. Nicholson and Fathepure (2004), has reported to have isolated a mixed population of halophiles from a brine soil which was dominated with the culture *Marinobacter* spp. The enriched cultures were tested to check their ability to degrade benzene, toluene, ethylbenzene or xylenes (BTEX

components). This culture had an ability to degrade all the components when introduced as pure compounds in the growth medium at a concentration of 20-30µmol at salt concentration of 2.5 M and at 30°C. It was observed that the enriched culture could degrade toluene the fastest (within a week) while benzene, ethylbenzene, or xylenes required roughly 2 to 3 weeks. Emerson *et al.* (1994), have isolated a halophile *Haloferax* D1227 that utilized aromatic compounds such as benzoate, cinnamate, and phenylpropanoate in the presence of 1.7–2.6 M NaCl at 45°C. Garcia, *et al.*, (2004), have reported to have isolated a halophile *Halomonas organivorans* a moderate halophile able to utilize a variety of aromatic compounds which includes benzoic acid, *p*-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenylacetic acid, phenylpropionic acid, phenol, *p*-coumaric acid, ferulic acid and *p*-aminosalicylic acid.

2.6.4. *pH*

2.6.4.1. Acidic pH

Naturally occurring acidic environments are present in various mining areas. These natural acidic conditions seem to have formed due to a combination of bacterial and aerobic oxidation process. The acidic condition arise in areas rich in pyrite ore. These are a kind of sulphide ore of iron, copper or zinc or a mixture of all three depending on the abundance of the ore. Chemical oxidation is instantaneous wherein the pyrites react readily with oxygen to give metal ions with a release of acid, mostly H_2SO_4. Further, the reactions are catalyzed by the acidophilic bacteria present most common being *Thiobacillus ferroxidans*, which oxidize the metal ions to serve as a source of energy within the cell. The oxidized metal inturn release the sulphur from the ore giving rise to more metal ions and more acid and thus the reactions become self sustaining and ongoing. In these conditions, the acidic products containing metals

of iron of respective ore is released to an extent that it pollutes the entire aquifer that comes in contact or could percolate down into the ground water thereby polluting it. Various iron ore mines, coal mines etc. can have such acidic conditions provided they have ore pyrites present. Mines not having such ore pyrite may not develop an acidic environment. The iron oxidizing strict acidophilic bacteria can also be detected in acid springs. *Sulfolobus* is another iron oxidizing bacteria detected in hot acid springs. Gonzales-Toril *et al.*, (2003), have reported that Tinto river in southwestern Spain is highly acidic with pH of 1.5-3.1 and has a high concentration of metal ions; iron contributes to a concentration up to 20g/lt, copper up to 0.7g/lt and zinc up to 0.56g/lt. which was due to naturally occurring metal pyrites upstream. Phylogenetic 16s rRNA analysis of the water body showed the presence of organisms related to *Leptospirillum* spp., *Acidithiobacillus ferrooxidans*, *Acidiphilium* spp., "*Ferrimicrobium acidiphilum*," *Ferroplasma acidiphilum*, and *Thermoplasma acidophilum*, microbes belonging to iron oxidizing group. More than 80% of the cells were affiliated with the domain *Eubacteria*, with only a minor fraction corresponding to *Archaea*. Other thermophilic bacteria belonging to genus *Acidisphaera spp.*, *Acidiphilium* and *Acidithiobacillus* spp. were isolated from acidic soil samples (pH ranging between 2.8-3.8) rich in sulfates in Yellostone national park, Montana, USA, which had a natural seep of hydrocarbons containing hexadecane (Namamura *et al.*, 2005). Stapleton *et al.* (1998), have reported the presence of indigenous acidophilic bacteria surviving at pH 2.0 at a runoff of a longterm storage in a coal-pile basin, that brings about 40% oxidation of aromatic compounds like naphthalene and toluene to carbon dioxide, thereby suggesting that the biodegradation can occur at acidic pH.

2.6.4.2. Alkaline pH

The pH plays an important role in the degradation of any compound. Optimum level of degradation is observed in neutral condition where the neutrophilic bacteria are abundant and gets acclimatized to the new introduced pollutant. pH is an important factor as it determines the ability of compound to dissociate. It has been observed that certain organic compounds such as chlorophenols and nitrophenols dissociate and their toxicity lowered with increase in pH (Holcombe *et al.*, 1980; Kishino and Kobayashi, 1995; Kulkarni and Chaudhari, 2006) and makes them vulnerable to microbial attack leading to its degradation. The most important criteria for degradation of such organic compounds is ability of microbes to survive and grow in such alkaline environments. The most stable naturally occurring alkaline environments are soda lakes with pH values up to 11.5. They are located in areas characterized by a unique combination of geological, geographical, and climatic conditions that diminish the significant buffering capacity of atmospheric CO_2 by the evaporative concentration of sodium carbonate (Kleinsteuber *et al.*, 2001). The soda lakes in the Rift Valley of Kenya and similar lakes found in other places on Earth are highly alkaline with pH values of 11 to 12. The Kenyan-Tanzanian Rift Valley contains a number of soda lakes whose development is a consequence of geological and topographical factors. The salinities of these lakes range from around 5% total salts (w/v) in the case of the more northerly lakes (Bogaria, Nakuru, Elmentiata and Sonachi) to saturation in the south (Magadi and Natron) with roughly equal proportions of Na_2CO_3 and NaCl as the major salts in some of the natural alkaline environments (Ulukanli and Diurak, 2002). Isolation of bacteria from environments otherwise hostile to neutrophilic bacteria has been reported. These organisms requiring alkaline condition as a prerequisite for their growth are called

alkalophiles/alkaliphiles and are able to tolerate pH values in a range of 8.0 to 12.0. Based on their optimal growth requirements, they are called as obligate alkalophiles (pH values 10.5-12.0) and alklotolerant (tolerates pH from 8.0 to 12.0 but has an optimal pH requirement of 8.0). Alkalophiles isolated from Magadi in Kenya belonged to the family *Halomonadaceae* grouped under γ-proteobacteria. These isolated bacteria were grouped under genus *Halomonas,* the various species that were isolated were *H. elongata, H. halodenitrificans, H. desiderata, H. cupida* and *H. magadii* (Duckworth *et al.,* 2000). Horikoshi (1991), had reported that the majority of bacteria belong to genus *Bacillus, Micrococcus, Corynebacterium, Pseudomonas, Flavobacterium,* Actinomycetes such as *Streptomyces, Nocardiopsis* and Yeasts. These belonged to the bacteria group that did not grow in the presence of high concentration of salt. Whereas, the species belonging to *Halomonas* grew well in the presence of salt. These were also called halotolerant alkalophiles. Alkalophiles were isolated from other parts of the world such as *Halomonas organivorans* were isolated from Spain (Garcia *et al.,* 2004), *Halomonas boliviensis* from Bolivia (Quillaguaman *et al.,* 2004), *H. koreensis* from Korea (Lim *et al.,* 2004). *Halomonas* species were also found active in cold conditions at Antartica and was named as *Halomonas glacie* by Reddy *et al.,* (2003). Another group of microorganisms growing in such an environment with high alkalinity and high salt concentration as a prerequisite was isolated and called Haloalkaliphiles. Well known archaea *Natronococcus* and *Natronobacterium,* were isolated from the salt lake from Magadi, Kenya (Tindall *et al.,* 1984; Mwatha and Grant, 1993). Bacteria belonging to genus *Halomonas* seem to share the salt tolerating characteristics with halophiles. Halotolerant alkaliphilic bacteria as mentioned by Garcia (2004), have the capability to degrade many aromatic

hydrocarbons. The versatile nature of these bacteria makes it unique and can be utilized to degrade various pollutants under extreme conditions.

2.7. ROLE OF BIOSURFACTANTS IN BIODEGRADATION OF AROMATIC COMPOUNDS

A large number of these compounds are insoluble and therefore not easily available for degradation/transformation by the microorganisms. The non-availability of the aromatic compounds could also be attributed to their adsorbed state to the soil particulate matter or other surfaces. Some of the hydrophobic compounds include aromatic and aliphatic hydrocarbons, resins, tars, etc., which naturally source from crude oil. These also include xenobiotic compounds released in nature through anthropogenic activities.

Bacteria have developed a strategy of utilizing such compounds to facilitate biodegradation by different mechanisms. Mechanisms so far known are production of biosurfactants, bioemulsifiers and direct cell-substrate attachment by the organisms. A few examples are elaborated here.

Microorganisms produce a variety of surface-active agents (or surfactants) which lower surface and interfacial tensions efficiently to allow emulsification with less energy and bind tightly to surfaces thereby stabilizing the emulsions. This facilitates the availability of the non-available compounds for degradation. The term 'biosurfactant' has been often used loosely to refer to other compounds like biopolymers which generally do not reduce interfacial tension but may prevent oil droplets from coalescing (Hommel, 1990). Biosurfactants also play an important role in regulating the attachment-detachment of microorganism to and from surfaces, which can increase aqueous dispersion of insoluble compounds by many orders of

magnitude, thereby increasing their chances of being degraded. In addition, emulsifiers are also involved in pathogenesis, quorum sensing and biofilm (Ron and Rosenberg, 2001).

Biodegradation requires uptake of the substrate by the cells, which in turn requires contact between the substrate and the cell. Contact is determined by two factors: (i) available substrate surface area and (ii) affinity of microbial cells for the substrate. Biosurfactants increase dispersion or surface area for microbial attachment, which increases biodegradation (Zhang and Miller, 1994). These can be divided into low-molecular-weight molecules that lower surface and interfacial tensions efficiently (Cooper and Zajic, 1980) and high-molecular-weight polymers that bind tightly to surfaces (Rosenberg and Ron, 1997). These are generally glycolipids which includes rhamnolipids (Al-Tahhan *et al*, 2000), trehalolipids (Li *et al.*, 1984) and sophorolipids (Cooper and Paddock, 1983). Some of the common high-molecular-weight compounds include emulsans, alasan, liposan mainly composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or a complex mixture of these biopolymers (Rosenberg and Ron, 1999).

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. While the bioemulsifiers allow easy mixing between the two immiscible phases, biosurfactants stabilizes the emulsion formed. They have a unique amphipathic property derived from their complex structures, which include a

hydrophobic/lipophilic portion (usually hydrocarbon (alkyl) tail of one or more fatty acids which may be saturated, unsaturated, hydroxylated or branched) attached to hydrophilic group by a glycosidic, ester or amide bond. Biosurfactants form spherical or lamellar micelles when surfactant concentration exceeds a compound-specific, critical micelle concentration (CMC). Hydrophobic compounds becomes solubilized in the hydrophobic cores of the micelles, which leads to the transfer of hydrophobic compounds from solid, liquid or sorbed state in to the water phase. There are furthermore reports of species-specific and energy-dependent uptake of biosurfactant-solubilized compounds, which points at a direct interaction of biosurfactant micelles with cell membranes (Beal and Betts, 2000 and Noordman and Janssen, 2002). Seeing that many biosurfactants represent constituents of cell envelopes (Neu, 1996), the possibility of a fusion between micelles and cells is indeed not far-fetched.

Utilization of biosurfactants has also been reported for mechanisms such as detachment form a substrate seen during conditions 1) depletion of utilizable carbon source from a mixture of compounds so that the cells could attach to another droplet of liquid containing the substrate or 2) depletion of oxygen level at the bottom of the biofilm (Neu, 1996).

A recent finding indicates the existence of horizontal transfer of high-molecular-weight emulsifiers from the producing bacteria to heterologous bacteria. Alasan, the exocellular polymeric emulsifier produced by *A. radioresistens* KA53, was shown to bind to the surface of *Sphingomonas paucimobilis* EPA505 and *A. calcoaceticus* RAG-I and change their surface properties. The transfer could be shown after incubation of the recipient cells with the purified emulsifier (Osterreicher-Ravid *et al.*, 2000). This horizontal transfer of bioemulsifiers from one bacterial species to

another has significant implications in natural microbial communities, co-aggregation and biofilms formation.

2.8. ROLE OF EPS IN BIODEGRADATION OF AROMATIC COMPOUNDS

Besides surfactants, bacteria producing film called biofilm overcome the problem of utilization of insoluble compounds by producing exopolymeric substances (EPS) also known as exopolysaccharides. Such an approach has been utilized to leach in the insoluble or immiscible compounds present in the environment as free or as surface sorbed compounds. Various authors have studied the role of EPS in utilizing insoluble aromatic compound in nature by bacteria (Moreno *et al.*, 1999; Obuekwe and Al-Muttawa, 2001; Janecka *et al.*, 2002; Rodrigues *et al.*, 2004).

Exopolysaccharides are high molecular weight organic macromolecules formed by polymerization of similar or identical building blocks, which may be arranged as repeating units within the polymer molecule. EPS may contain non-polymeric substituents of low molecular weight, which greatly alter the structure and physiochemical properties. The extracellular polysaccharide could compose of various sugars, amino sugars, sugar acids and carry organic substituents such as acetyl, succinyl or pyruvyl groups and other inorganic substituents such as phosphates and sulfates (Table 2.1). Proteins interact with polysaccharide compounds to form glycoproteins by glycosylic bonds or can be substituted with fatty acids to form lipoproteins (Wingender *et al.*, 1999).

These characteristics produce a wide heterogeneity among the polymer, thus representing a rich source of structurally diverse molecules with unique physical and chemical properties. Many of the polysaccharides are relatively soluble, and because of their large molecular mass, yield highly viscous aqueous solutions. As the presence

of uronic acid moieties confer a net negative charge, pyruvate residues contribute to the water binding property (Decho, 1990). However, polysaccharides such as hyaluronic acid can bind up to 1 kg water (gm polysaccharide) $^{-1}$. It is probable that many of the EPS in biofilms bind lesser quantities whilst some, like bacterial cellulose, mutan or curdlan, manage to exclude most water from their tertiary structure. Hydrophilicity is also dependent on their composition and their tertiary structure (Neu and Poralla, 1988). Authors have also reported in 1990 that the sugar monomers of polysaccharides can be hydrophobic or hydrophilic depending on the degree of hydroxylation. And some of the polymers contain both hydrophilic as well as hydrophobic regions.

Interaction of ions has been observed with the carboxylic groups on the EPS to yield networks of macromolecules which showed increased viscosity or gelation. Various cations may compete for the same binding site, as was shown by Loaec *et al.* (1997), Adsorption of heavy metal ions to the polymer has been observed by various authors (Farres *et al.*, 1997; Ferris *et al.*, 1989; Geddie and Sutherland, 1993; Mittleman and Geesey, 1985).

The EPS also contributes to the mechanical stability of the biofilms (Mayer *et al.*, 1999), enabling them to withstand considerable shear forces. Other structural properties of the polymer like rigidity or flexibility is based on the type of bonding in between the chains of the polymer; 1,4-β- or 1,3- β- linkages confers considerable rigidity, as seen in the cellulosic backbone of xanthan from *Xanthomonas campestris*. 1,2-α- or 1,6–α– yields more flexible structures. The presence of O-succinyl esters, O-acetyl or pyruvate ketals in the polysaccharide gives a varied conformation –

forming random coils or helicals. The long polysaccharide chain involves in a number of interactions between themselves and the substrate.

Due to the presence of the hydrophobic regions in the polymer made up of high glucosyl residue, it has an ability to desorb the insoluble compounds from the dormant surface in to itself thus making it available for the bacteria for mineralization and utilization.

Literature survey has shown that various studies have been carried out on biodegradation of aromatic compounds or substituted aromatic compounds containing nitro and amino groups. All these studies reported are under normal neutral conditions as such. Only a few reports are available on degradation of aromatic amines under alkaline condition. The present study was therefore been undertaken with a view to understand the interactions of aromatic amines under alkaline conditions with bacteria able to grow at pH 10.5.

CHAPTER III

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF ALKALOPHILIC BACTERIA

The isolation of alkalophiles needs a medium with pH above 9.5 and a source where the organism would be exposed to extreme conditions. Besides, it is also important to have the potent isolates which are exposed to a certain level of concentration of aromatic compounds. The present samples were therefore selected from the estuarine ecosystem, which are continuously facing the environmental changes with respect to salinity and discharge of effluents or oil from barges. Further, the samples available from the cruise of ONGC were also used for the present isolation, details of which are described herewith. The selection of the estuarine ecosystem as a sampling site was based on the severity of the condition where the organisms continuously face desiccation and heat due to tidal effect, mixing of fresh and saline water as well as due to increased exposure to xenobiotic contamination (Marinucci and Bartha, 1982).

3.1. MATERIALS AND METHODS

3.1.1. *Collection and processing of samples*

The source for the isolation of bacterial cultures for the present work has been the sediment and water samples collected from marine ecosystem around the Bombay High - oil platforms and the mangrove ecosystem.

Sampling was done during the periodical pollution monitoring programme carried by Oil and Natural Gas Commission (ONGC)-India, during the pre and post-monsoon periods of the year on board Sagar Paschimi. Various platforms were included as the sampling sites around which the sediment and water samples were collected. Sediment samples were collected using the Veen Van grab sampler (Heip *et al.*, 1977). Water samples were collected from the surface, middle and the bottom

layers using the Niskin sampler (Yokokwa and Nagata, 2005). Samples were collected at points around each platform with radii ranging from 250m to 2 km. The water samples collected from all these points were pooled to get cumulative individual samples of surface, middle and bottom layers, thus resulting in three water samples per station. Water samples were stored at refrigerated condition until the end of the journey (5-6 days). The sediment samples from different points around each station were pooled to give one sample per station and was stored in a deep freezer (-70°C).

Sediment and water samples were also collected from different mangrove ecosystems in Goa. The stations included the Panjim (banks of river Mandovi) and Palolem (beach). Water samples from these areas were collected in sterile polycarbonate bottles and sediment samples were collected from a depth of 4-5 inches around the roots and neumatophores of the mangrove vegetation using a plastic scoop.

Bacterial counts of water samples and sediment samples were determined by standard dilution plate count method.

3.1.2. *Physicochemical analysis of samples*

The physicochemical parameters of water samples such as pH, temperature, salinity, nitrites, nitrates and phosphates were determined as per standard methods (Grasshoff, 1983). Temperature and pH were checked on site at the time of collection.

3.1.2.1. Salinity

This method is based on titration of halide ions present in seawater against standard silver nitrate solution using potassium chromate as an indicator (Muller, 1983).

Ten ml of water sample was pipetted into a 250 ml conical flask, 20 ml of distilled water was added to it followed by few drops of potassium chromate indicator (Appendix II, B.1.). The contents were mixed and titrated against silver nitrate solution. Initially, a white precipitate appeared due to silver halide precipitation along with the solution turning yellow, the titration end point was noted when the colour of the solution changed to dirty orange and the colour persists for at least 30 sec. Silver nitrate was added drop wise till the colour in the flask started to show a dull red colour, the volume used was noted and salinity of the sample calculated using Harvey's table.

3.1.2.2. Nitrite content (NO_2)

Determination of nitrite in seawater is based on the reaction of NO_2 with an aromatic amine under acidic condition leading to the formation of diazonium salt which couples with a second aromatic amine to form an azo dye (Koroleff and Hansen, 1983).

Water sample (10ml) was taken into 50 ml graduated tubes and 10 ml of distilled water was added followed by 1ml of Sulphanilamine and 1ml of N-naphthyl ethylenediamine-di-hydrochloride (NEDA) (Appendix II, B.2.). The volume of the solution was made up to 50ml with distilled water and the absorbance was measured

at 540nm after 20min using a spectrophotometer (SHIMADZU, UV-1601). The concentration of nitrite was determined using standard curve of sodium nitrite.

3.1.2.3. Nitrate content (NO_3)

Nitrate estimation was carried out after its reduction to nitrite followed by the standard method of diazotization as explained earlier. The reduction of nitrate to nitrite is carried out in a heterogeneous system using cadmium granules (Koroleff and Hansen, 1983).

Ten ml of sample was taken in a 125ml polypropylene bottle and the volume was made up to 100ml with distilled water. Two ml of concentrated ammonium chloride solution (Appendix II, B.3.) was added to this solution, before passing it through the cadmium column. About 40ml of the solution was eluted through the column and concentration of nitrate was determined using the diazo coupling method.

3.1.2.4. Phosphate content (Koroleff and Hansen, 1983)

Inorganic phosphates present in water was made to react with acidic ammonium molybdate to give phosphomolybdic complex, which gives a blue coloured complex when reduced with ascorbic acid. The absorbance of the blue solution was measured at 660nm.

Undiluted water samples (50ml) was directly taken in graduated tubes, to which, 1ml of mixed reagent and 1ml of ascorbic acid was added and mixed well. After 15 min the absorbance of the solution was measured at 660nm using a spectrophotometer. Standard curve of phosphate was used to estimate the concentration of phosphate in water samples (Appendix II, B.4.).

3.1.3. *Total viable counts of bacterial isolates and alkalophiles*

Sediment samples (1 gm) were mixed with 10 ml of saline in a conical flask and were incubated on the shaker for 30 minutes at room temperature (30° ± 2°C). The flasks were then allowed to stand for 15min for the sediment to settle before serial dilution. The slightly turbid supernatant (1ml) and water samples (1ml) were serially diluted (10 fold serial dilution) with normal saline (Appendix I, A.1) and 0.1ml of appropriate dilutions were spread on plates containing – Nutrient agar (pH 7.0) for neutrophilic bacteria (Appendix I, A.3), Polypeptone Yeast extract Glucose agar (PPYG) (pH 10.5) (Gee *et al.*, 1995) for alkalophilic bacteria (Appendix I, A.5). The plates were incubated for 48 - 72hrs at room temperature 30°C ± 2°C and total viable counts were calculated for the samples processed. Predominant alkalophiles growing on the PPYG agar were picked up randomly using sterile toothpicks and transferred on to a fresh PPYG plate, considered as a master plate.

3.1.4. *Characterization of isolates*

The alkalophilic isolates were further screened for their tolerance and ability to grow at various levels of alkalinity by replica plating the isolates from master plate on PPYG agar (Gee *et al*, 1995) with pH values 6.0, 8.5, 10.5, and 12.0 (Appendix I, A.6.). The isolates that grew at pH 10.5 were considered and selected for further studies.

3.1.5. *Response of the isolates to aromatic amines*

The isolated alkalophilic cultures were spot inoculated on a defined medium – Mineral Salts Medium (MSM) agar medium (pH 10.5) (Sangodkar and Mavinkurve,

1991) (Appendix I, A.8.) incorporated with various aromatic compounds such as aniline, p-amino phenol, Diphenylamine, N,N-Diemthyl-1-Naphthylamine, p-Chloroaniline. The plates were incubated at room temperature and growth observed from one week up to a month. The alkalophilic cultures showing growth were further purified and used for further studies.

3.1.6. *Purification and maintenance of potent isolates*

Purification of the alkalophilic isolates was carried out by repeated streaking on a rich medium- PPYG, to obtain pure colony showing consistent cultural or morphological characteristics. The pure isolates were then reinoculated on the MSM agar with specific aromatic amine to check for their ability to grow. These isolates showing the desired characters were maintained on PPYG agar and MSM agar with the aromatic carbon sources were stored at 4°C and subcultured on fresh medium every month.

3.1.7. *Identification of selected isolates using polyphasic taxonomic approach*

The selected potent bacterial cultures were identified using physiological and biochemical tests, followed by molecular identification method based on 16s rRNA gene sequencing and microbial identification system (MIS) utilizing the Fatty acid methyl esters (FAME).

3.1.7.1. Morphological, biochemical and physiological characteristics

Colony and morphological characteristics of the isolates were noted. Physiological and biochemical characteristics were studied using the methodology for

the alkalophiles as described by Gee *et al* 1980. Media used for various biochemical tests were maintained at pH 9.5 (Appendix I, A.9).

3.1.7.2. Chemotaxonomic analysis

a) Whole cell lipid profile (Ross *et al.*, 1981)

Lipid analysis was carried out using the methods described by Ross *et al.*, (1981). Potent bacterial isolates under study were grown in PPYG broth (pH 10.5) for 24 hours. Cells were harvested by centrifuging at 10000rpm, washed with normal saline twice, centrifuged, and the pellet lyophilized. Cells (30-50 mg) were treated with methanol : toluene : conc. H_2SO_4 (3 : 3 : 0.1) and heated at 50°C for 24 hrs. The methylated fatty acids were extracted twice with 1.5ml of hexane. The organic layers were pooled, concentrated by nitrogen drying and the dried extracts were diluted with small amount of hexane. These hexane extracts were spotted on a TLC plate (Hamilton and Hamilton, 1987) (Appendix III, C.1) and run in a solvent system of petroleum ether (60-80) : diethyl ether (85 : 15). The methyl esters of fatty acids spots were detected by spraying 10% (w/v) dodecamolybdophosphoric acid in absolute ethanol as visualizing agent. The chromatograms were heated at 150°C-160°C for 10-15 minutes until blue spots appeared.

b) Fatty Acid Methyl Ester (FAME) analysis using Microbial Identification System (MIS)

The fatty acids of the bacterial cultures were extracted using the MIDI procedure of Schutter and Dick, (2000). This method uses four reagents and consists of four steps: saponification, methylation, extraction, and washing.

Pure bacterial culture was harvested from the PPYG agar plate (3/4th area) incubated for a period of 24 hrs, using a 4mm loop and smeared at the bottom of a test tube and mixed with 1 ml Reagent I (Appendix II, B.7.). Test tubes were vortexed for 5-10 seconds and placed in a 100°C water bath for 5 minutes and vortexed for 5-10 minutes followed by heating at 100°C for 25 minutes and cooled. During this cells were lysed and saponified (fatty acids cleaved from the cell lipids and converted to sodium salts). To convert fatty acids to methyl esters for increased volatility, 2 ml of reagent II (Appendix II, B.7.) was added to the tubes. The tubes were then incubated in a water bath for 10 min at 80°C and then cooled rapidly. To the cooled solution, 1.25 ml of reagent III (Appendix II, B.7.) was added, shaken gently for 10 minutes to extract the FAMEs from the acidic aqueous phase into the organic phase. The organic phase was transferred to a new test tube. To the clear organic phase 3 ml of reagent IV (Appendix II, B.7.) was added, mixed gently for 5 minutes and was allowed to stand for phase separation. Two thirds of the organic phase was transferred to a GC vial. The organic phase was analysed using gas chromatography (GC) with a Hewlett-Packard 5890 Series II (Palo Alto, CA) equipped with an HP Ultra 2 capillary column (5% diphenyl-95% di methylpolysiloxane, 25 mby 0.2) and a flame ionization detector. The temperature program ramped from 170° to 270°C at 58°C per min. Fatty acids were identified and their relative peak areas were determined using the MIS.

3.1.7.3. Isolation, amplification and sequencing of 16S rRNA gene (Pidiyar *et al.*, 2004)

a) Isolation and amplification

Chromosomal DNA was extracted from a single isolated bacterial colony picked from a PPYG plate incubated for 24 hrs. Isolation was carried out by suspending the bacterial isolate in 50µl of colony lysis buffer (Appendix II, B.8.). The reaction mixture was incubated at 55°C for 15 minutes followed by proteinase K inactivation at 80°C for 10 min. The reaction mixture was centrifuged at 15,000rpm at 4°C for 15 min. The supernatant containing genomic DNA was directly used as template in a polymerase chain reaction (PCR) reaction using a polymerase chain reaction (PCR) and universal eubacteria-specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'- TTC TGC AGT CTA GAA GGA GGT GWT CCA GGC -3'). in a 25µl final reaction volume, containing about 10ng of genomic DNA, 1X reaction buffer (Appendix II, B.8.), 0.4mM (each) deoxynucleoside triphosphates (Invitrogen), 0.5U of DNA Polymerase (New England Labs, UK) and the final volume was made to 25µl by using sterile nuclease free water. The PCR was performed in an automated Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, USA) under the following conditions: initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one and a half minute, and a final extension at 72°C for 10 minutes. The amplified PCR product was checked for the presence of the 1.5kb gene by carrying out electrophoresis of 5µl of the sample on 1% agarose gel in 1X TBE buffer (Appendix II, B.8.) and stained with ethidium bromide 0.5µg/ml. The amplified 16S rRNA gene PCR products were

directly sequenced after purification by precipitation with polyethylene glycol and NaCl (Appendix II, B.8.) at 37°C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet it was resuspended in 5µl of sterile nuclease free water. One microliter (50ng) of purified 16S rRNA PCR product was sequenced by 16S rRNA specific primer i.e. 16F27N, and 16R1525XP an internal primer 530F (5'-GTC CCA GCM GCC GCG G-3') was also used in addition to the other primers. The sample was sequenced by using BIG DYE Terminator cycle sequencing ready reaction kit (v3.1) in ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA).

b) Sequence analysis

The sequence data were compared with available 16S rRNA gene sequences at GenBank using the BLAST program National Center for Biotechnology Information; (http//:www.ncbi.nlm.nih.gov/BLAST) to determine the approximate phylogenetic affiliation. The 16S rRNA gene sequence of strain NRS-01 and NK2 was aligned with those of *Halomonas* species by using the Genedoc and Phyowin softwares. Evolutionary distance matrices were calculated using the algorithm of the Kimura two-parameter model (Kimura, 1980) within the PHYLOWIN software package, version 2.0. A dendogram was constructed using the neighbour-joining method (Saitou & Nei, 1987) available in the PHYLOWIN software package. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (500 replications) was performed.

3.2. RESULTS AND DISCUSSION

3.2.1. *Sampling sites*

Marine ecosystem at the Bombay High area provided a man-made polluted environment due to oil spillage at oil fields during routine work of crude oil extraction which was the basis of selection as sampling sites. The sampling sites included water and sediment samples from Bombay High region, an important offshore oil field of Maharashtra, west coast of India (Fig. 3.1a). The oil rigs are 300km away from Goa and about 160km away from the coast of Maharashtra. Dynamic activities during the extraction of crude oil from various platforms in the Bombay High Oil field area introduce a large amount of oil spills on the water body. Crude oil is a complex mixture of hydrocarbons ranging from straight chain simple alkanes to aromatic compounds ranging from benzene to more complex polyaromatic hydrocarbons (PAHs) (Atlas, 1975). The anthropogenic interference in this area also made it a very suitable environment for screening of heterotrophic microorganisms.

Two sites of mangrove ecosystem were also selected as sites of sample collection viz. Panjim which has a heavy influx of anthropogenic disturbance and Palolem which is secluded from or faces minimal intrusion (Fig. 3.1b). The uniqueness of this ecosystem is the absence of the fresh water contamination with the seawater which harbours the marine ecological community.

3.2.2. *Physicochemical characteristics of water samples*

The nutrient level in the water body determines the potential fertility of the water masses (Subramanyam and Sambamurthy, 2000). Addition of nutrients to the water body basically by nutrients runoff from land in to the sea is facilitated by the

Fig 3.1a: Sampling sites at the Bombay High region (Marine ecosystem)

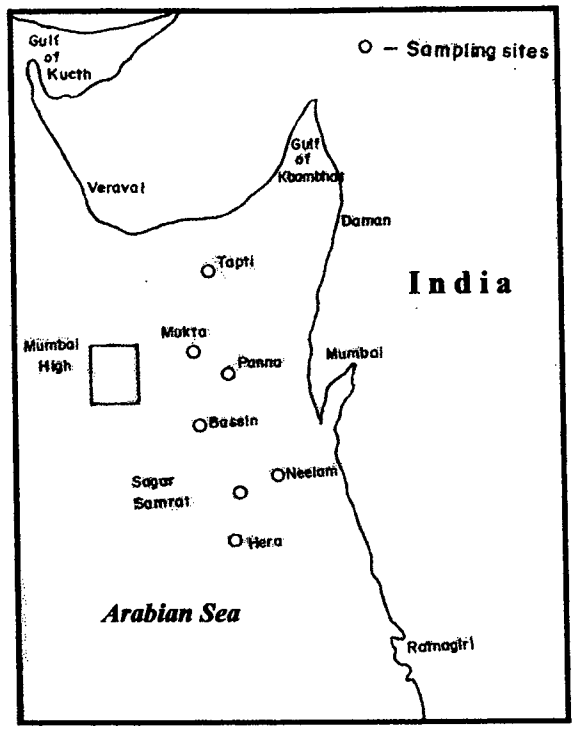
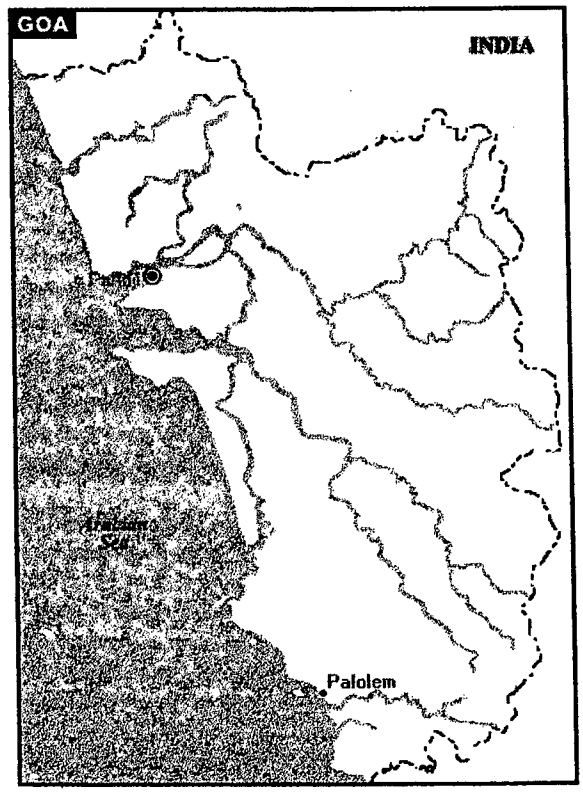


Fig 3.1b: Sampling sites at the Mangrove ecosystem in Goa



estuaries (McCarthy *et al.*, 2006). The distribution of nutrient elements in estuarine water is controlled, by nature of estuarine circulation, mixing and other physical process, together with biological, sedimentological and chemical processes (Aston, 1980). The biodiversity of microorganisms largely depends upon these characteristics and the nutrient availability in the ecosystem.

Seawater contains low concentrations of inorganic and organic nitrogenous compounds. The main inorganic forms are nitrate (1-500 µg/L), nitrite (< 0.1- 50 µg/L) and ammonia (< 1-50 µg/L). Sea water also contains low concentrations of dissolved and particulate organic nitrogenous compounds which associated with marine organisms as products of their metabolism and decay (Riley and Chester, 1980). The estuarine water samples collected from Panjim and Palolem for this study had nitrate content of 6 µg/L and 6.2 µg/L respectively. Nitrite content of the water samples was 4.3 µg/L and 5 µg/L and phosphate values were found to be 5.2 µg/L and 5.5 µg/L respectively. Nutrient levels of the Bombay High oil field area varied between 1.1 µg/L to 3 µg/L for nitrites, 1.6 µg/L to 8 µg/L for nitrates and 11.5 µg/L to 40.4 µg/L for phosphates. The temperature, pH and salinity of these samples ranged from 28-30°C, pH value of 7.3-7.8, and salinity of 33% at Panjim and 35% at Palolem respectively.

Salinity in the Arabian Sea has been reported to range between 34-37% (Subramanyam and Sambamurthy, 2000), while the nutrient content in the Indian ocean; the nitrate, nitrite and phosphate content was found to be 0-7 µg/L, 0-280 µg/L and 1 to 31 µg/L respectively (Lobban *et al.*, 1985) as seen in the present study. It has been reported that in surface water the concentration of nitrate was high at most of the places during monsoon and post monsoon, perhaps due to land run-off during

monsoon. Atmospheric precipitation also contains large amounts of ammonia and nitrate (Quasim and Sengupta, 1981).

3.2.3. *Microbiological analysis*

Total viable counts of the heterotrophic neutrophilic and alkalophilic bacterial population was enumerated from the water and sediment samples collected from marine and mangrove ecosystem by plating them on the specific media (Table 3.1a & b, 3.2). Colony counts were found to be much higher on the nutrient agar giving a colony count of 260 x 10^3 CFU/ml in SHP station as compared to the PPYG medium which showed 100 CFU/ml indicating the dominance of neutrophilic population over alkalophilic bacteria at the sampling sites. Horikoshi (1995), has reported that the ratio of alkalophiles to the neutrophiles found in soil is about 1:10 to 1:100. Sediment samples on other hand had a higher count averaging to 200 x 10^7 CFU/ml for neutrophiles and 100 x10^3 for alkalophiles, as compared to the water samples. Alkalophilic counts were found to be highest at stations Mukta giving a count of 4290 CFU for water samples, while station NQ gave a count of 452 x 10^3 for sediment samples.

3.2.4. *Characterization of alkalophiles*

Alkalophiles were grown in PPYG medium containing a final concentration of 1% sodium carbonate giving a final pH of 10.5 units to the medium. These isolates were rechecked for their ability to grow at neutral condition by plating them on the nutrient agar medium (pH 7.0) and incubated the plates for 24-48 hrs. Alkalophilic bacteria a group of microorganisms that thrive at alkaline conditions i.e. at pH above

Table 3.1a : Total viable counts for the water samples from ONGC

Water samples	Layer	pH	TVC $x10^3$	TVC $x 10^0$
LA	S	8.2	130	0
LB	S	8.3	102	100
TPP	S	7.8	6	390
	M	7.5	N.D	500
	B	7.5	N.D	980
BHN	S	8.2	0.7	40
	B	8.2	4	700
ICP	S	8.1	N.D	310
	M	8.2	N.D	10
	B	8.3	96	330
SHP	S	8	260	100
	M	8.1	N.D	710
	B	8.2	N.D	720
BHS	S	7.9	241	400
	M	7.8	N.D	410
	B	7.9	2.8	900
MAA	S	8.3	N.D	4290
	M	7.9	6.17	550
	B	7.7	41	290
PPA	S	7.8	46	N.D
	M	7.9	57	180
	B	8.2	52	200
BLQ	S	8.4	26.9	0
	M	8.2	28	0
	B	8.3	8.5	0
HRA	S	8.2	224	0
	M	8.2	93	0
	B	8.3	N.D	0
NLM	S	8.2	36	0
	M	8.3	63	0
	B	8.5	10.85	0
SR	S	7.6	90	0
	M	7.6	4	10
	B	7.8	12.115	10

Key	Abbreviations
S	Surface water
M	ddle water
B	ttom water
TVC	Total viable count (per ml)
N.D	Not determined

Table 3.1b : Total viable counts for the sediment samples from ONGC

Sediment samples	Neutrophiles TVC $x10^7$	Alkaliphiles TVC $x10^3$
LA	307.74	2.80
LB	76.84	0.20
ICP	440.00	4.80
NQ	103.61	452.90
BH1	69.40	4.00
BH2	35.20	28.35
BH3	110.17	2.10
BH4	153.32	Nil
IDA	139.81	Nil
BLQ	328.71	0.01
HRA	299.53	23.35
NLM	197.10	3.20
SR	N.D	5.30

Key Abbreviations
TVC Total viable count (per ml)

Table 3.2 : Total viable counts obtained from mangrove sediment and water samples

Sediment Samples	pH	Nutrient agar TVC	PPYG TVC x 10^0
Palolem	8.3	69 x 10^5	1020
Panjim	8	680 x 10^5	1240
H_2O samples Stations	pH	Nutrient agar TVC	PPYG TVC x 10^0
Palolem	7.9	1000 x 10^3	30
Panjim	7.6	2415 x 10^3	20

Keys	Abbreviations
PPYG	Alkaliphilic medium
TVC	Total viable count (per ml)

neutral conditions by 2 -3 units therefore the organisms growing optimally at this pH are called the alkalophiles, where as those that tolerate high pH values but grow optimally at near neutral conditions are called alkalitolerant. The salinity of the sea water also makes it favourable for another community of organisms to grow optimally at such a condition namely the halophilic bacteria (Satyanarayana *et al.*, 2005). The marine environment harbours bacterial community able to tolerate both high pH (alkalophilic) as well as high salt (halotolerant) called the haloalkalophiles (Arahal *et al.*, 1996, Fan *et al.*, 2004). This group of bacteria depends primarily on the presence of Na^+ ions for their respiration and growth (Kitada *et al.*, 1982) and are a diverse group of extremophiles (Kristjánsson and Hreggvidsson, 1995).

Predominant isolates were picked from the PPYG plates randomly and were replica plated on PPYG agar with pH values 6.0, 8.5, 10.5 and 12.0. The alkalophiles able to tolerate the pH range from 6.0 to 12.0 are grouped as alkalotolerant; those that were able to grow in the pH range 8.5 to 12.0 were alkalophiles, while those strictly growing between 10.5 and 12.0 were considered as obligate alkalophiles. The alkalophilic population from Bombay high was found to be highest comprising of 58.5%, alkalotolerant comprised 32% and only 9.5% of total population comprised of obligate alkalophiles (Table 3.3, Fig. 3.2a). The population of alkalotolerant bacteria showed dominance in the mangrove ecosystems with 47% followed by obligate alkalophiles with 28% and the alkalophilic population of 25% (Table 3.4, Fig 3.2b). Horikoshi (1999), has reported such an incidence where the extremophiles (alkalophilic bacteria) are present even in normal garden soil.

A total of 46 isolates growing in the pH range between 8.5 and 12.0 were selected for further screening for their response to aromatic amines.

Table 3.3 : pH tolerance of isolates obtained from ONGC sampling sites

water samples										sediment samples									
Is. No.	pH				Is. No.	pH				Is. No.	pH				Is. No.	pH			
	6	8.5	10.5	12		6	8.5	10.5	12		6	8.5	10.5	12		6	8.5	10.5	12
1	–	+	+	–	51	–	+	–	–	1	+	+	+	–	51	+	+	+	–
2	–	+	+	+	52	–	+	+	–	2	–	+	+	–	52	+	+	+	+
3	–	+	+	+	53	–	+	+	–	3	+	+	+	+	53	–	+	+	+
4	–	+	–	–	54	–	+	–	–	4	+	+	+	+	54	–	+	+	–
5	–	+	+	+	55	–	+	–	–	5	+	+	+	+	55	–	+	–	–
6	–	+	+	+	56	–	+	–	–	6	+	+	+	+	56	–	+	+	+
7	–	+	+	–	57	+	+	–	–	7	–	+	+	–	57	–	+	+	–
8	–	+	+	–	58	–	+	+	–	8	–	+	+	–	58	+	+	+	–
9	–	+	–	–	59	–	+	–	–	9	–	+	+	–	59	+	+	+	+
10	–	+	+	–	60	–	+	+	+	10	+	+	+	+	60	+	+	–	–
11	–	+	+	–	61	–	+	+	–	11	+	+	+	+	61	–	+	+	–
12	–	+	+	+	62	–	+	–	–	12	+	+	+	+	62	–	+	+	–
13	–	+	+	–	63	–	+	+	–	13	+	+	+	–	63	+	+	+	–
14	–	+	+	–	64	–	+	+	–	14	–	+	+	+	64	+	+	+	+
15	–	+	+	–	65	+	+	+	–	15	+	+	+	–	65	+	+	+	+
16	–	+	+	–	66	–	+	+	–	16	+	+	+	–	66	+	+	+	+
17	+	+	+	+	67	–	+	+	–	17	+	+	+	+	67	+	+	+	+
18	–	+	+	–	68	–	+	–	–	18	–	+	+	+	68	–	+	+	+
19	–	+	+	–	69	–	+	+	–	19	–	+	+	–	69	+	+	+	+
20	–	+	+	+	70	–	+	+	–	20	–	+	+	+	70	–	+	+	-
21	–	+	+	–	71	–	+	+	–	21	+	+	+	–	71	–	+	+	-
22	+	+	+	–	72	–	+	+	–	22	+	+	+	–	72	+	+	+	-
23	–	+	+	–	73	–	+	+	–	23	+	+	–	–	73	+	+	+	-
24	–	+	+	–	74	–	+	–	–	24	+	+	–	–	74	+	+	+	-
25	–	+	–	–	75	+	+	+	–	25	+	+	–	–	75	+	+	+	-
26	–	+	–	–	76	–	+	+	+	26	+	+	+	+	76	–	+	+	-
27	–	+	+	–	77	–	+	+	–	27	+	+	+	+	77	+	+	+	-
28	–	+	+	–	78	–	+	+	–	28	+	+	+	+	78	–	+	–	-
29	–	+	+	–	79	–	+	+	–	29	+	+	+	+	79	+	+	+	-
30	–	+	+	+	80	–	+	–	–	30	+	+	+	–	80	+	+	+	-
31	–	+	+	+	81	–	+	–	–	31	–	+	+	–	81	+	+	+	-
32	–	+	–	–	82	–	+	–	–	32	+	+	+	+	82	+	+	+	-
33	–	+	–	–	83	–	+	–	–	33	–	+	+	+	83	–	+	+	-
34	–	+	+	–	84	–	+	+	+	34	+	+	+	–	84	+	+	+	-
35	–	+	–	–	85	–	+	+	–	35	+	+	+	+	85	+	+	+	-
36	–	+	+	–	86	–	+	+	–	36	+	+	+	+	86	–	+	+	-
37	–	+	+	–	87	–	+	+	–	37	–	+	+	+	87	–	+	+	-
38	–	+	+	–	88	+	+	+	+	38	+	+	+	+	88	+	+	+	-
39	–	+	+	–	89	–	+	+	–	39	+	+	+	+	89	+	+	+	
40	–	+	+	–	90	–	+	+	–	40	+	+	+	–	90	–	+	+	
41	–	+	+	–	91	–	+	–	–	41	–	+	+	–	91	+	+	+	-
42	–	+	+	–	92	–	+	–	–	42	+	+	+	+	92	–	+	+	
43	+	+	+	+	93	–	+	+	–	43	–	+	+	–	93	–	+	–	
44	–	+	+	–	94	–	+	+	–	44	–	+	+	–	94	–	+	+	
45	–	+	+	–	95	–	+	+	–	45	–	+	+	–	95	–	+	–	
46	–	+	+	–	96	–	+	+	–	46	+	+	+	+	96	–	+	–	
47	–	+	+	+	97	–	+	+	–	47	–	+	+	–	97	–	+	+	
48	–	+	+	–	98	–	+	+	+	48	+	+	+	+	98	–	+	–	
49	–	+	+	–	99	–	+	–	–	49	+	+	+	+	99	–	+	+	
50	–	+	–	–	100	–	+	–	–	50	–	+	+	–	100	–	+	+	

Key	Abbreviations
+	Growth
-	No growth
Is. No.	Isolate Number

Table 3.4: pH tolerance of cultures isolated from the mangrove ecosystem

Is. No.	pH			
	6	8.5	10.5	12
1	-	+	+	+
2	+	+	+	+
3	-	+	+	+
4	-	-	+	-
5	-	+	+	+
6	-	-	+	+
7	+	+	+	+
8	+	+	+	-
9	-	-	+	-
10	-	+	+	+
11	-	+	+	-
12	+	+	+	-
13	-	-	+	-
14	-	+	+	-
15	-	-	+	-
16	+	+	+	+
17	+	+	+	+
18	-	-	+	-
19	-	+	+	+
20	-	+	+	+
21	+	+	+	+
22	-	-	+	-
23	-	-	+	-
24	-	+	+	+
25	-	+	+	+
26	+	+	+	+
27	-	-	+	+
28	+	+	+	+
29	-	-	+	-
30	-	-	+	+
31	-	+	+	-
32	+	+	+	+
33	-	-	+	-
34	-	+	+	-
35	+	+	+	-
36	+	+	+	+
37	-	-	+	+
38	+	+	+	-
39	+	+	+	-
40	+	+	+	+

Is. No.	pH			
	6	8.5	10.5	12
41	+	+	+	+
42	+	+	+	-
43	-	-	+	+
44	-	-	+	-
45	+	+	+	+
46	-	+	+	+
47	+	+	+	+
48	+	+	+	-
49	+	+	+	-
50	+	+	+	+
51	+	+	+	+
52	-	+	+	+
53	-	-	+	-
54	-	-	+	-
55	-	-	+	+
56	-	-	+	-
57	-	-	+	-
58	+	+	+	+
59	-	-	+	+
60	-	-	+	-
61	-	-	+	+
62	-	-	+	+
63	+	+	+	-

Key Abbrevations

\+ Growth

\- No growth

Is. No. Isolate Number

Fig. 3.2a: Percentage distribution of obligate alkalophiles (ONGC)

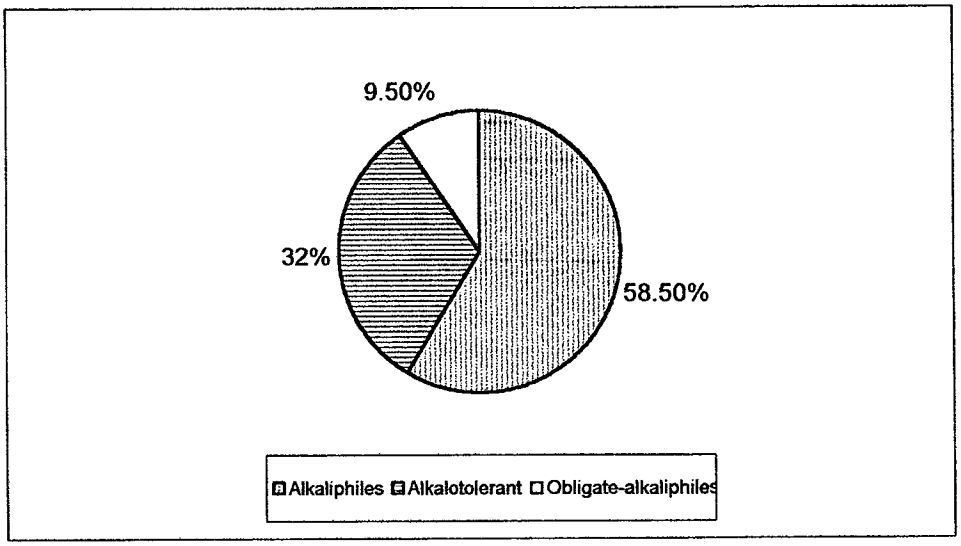
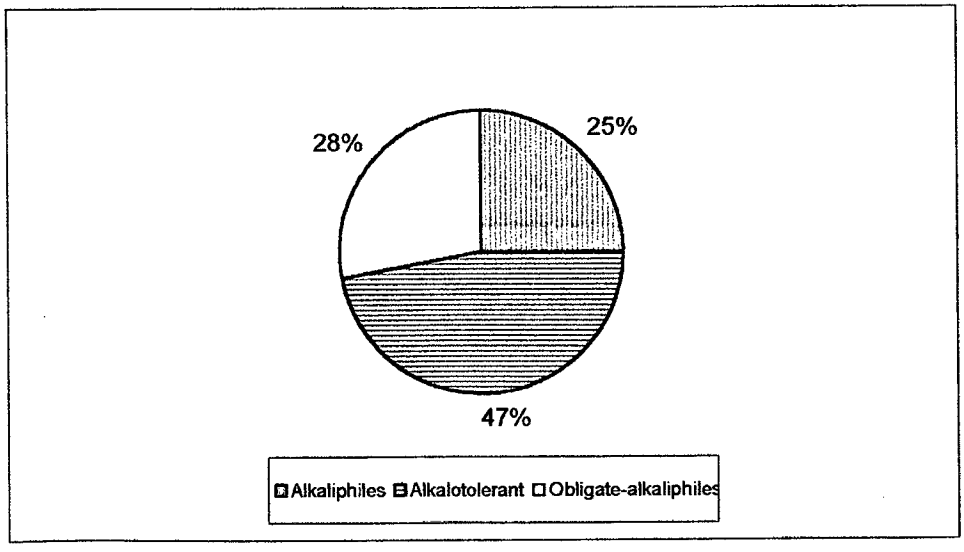


Fig. 3.2b: Percentage distribution of obligate alkalophiles (Mangrove ecosystem)



3.2.5. *Screening for potential isolates*

Alkalophilic bacteria are presently gaining importance in the degradation studies of aromatic pollutant especially for their use in waste water treatment plants (Margesin and Schinner, 2001). 46 alkalophilic isolates were incubated on MSM agar containing different aromatic amines (0.1%) (Aniline, Diphenylamine, p-aminophenol, N,N-Diemthyl-1-Naphthylamine and p-chloroaniline), (Table 3.5). It was interesting to note that maximum number of isolates (52%) were found to grow in the presence of aniline, followed by 26% each for N,N-Dimethyl-1-Naphthylamine and p-Chloroaniline, followed by p-Aminophenol (9%). None of the isolates were able to grow in the presence of Diphenylamine. These results show the toxicity level of the aromatic amines being dealt with increase in the following order- Aniline < N,N-Dimethyl-1-Naphthylamine < p-Chloroaniline < p-Aminophenol < Diphenylamine. It has been reported that toxicity of the aromatic compounds increases with increase in substitutions of groups on to the aromatic ring as well as with increase in length of side chain (Seirra-Alvarez and Lettinga, 1991).

Isolate No. 27 (Table 3.5) enriched from the mangrove sediment of the Panjim mangrove ecosystem was found to grow in the presence of most of the aromatic amines under consideration. On further subculturing, the isolate showed tolerance and ability to remain active on medium with N,N-Dimethyl-1-Naphthylamine alone, while it ceased to grow on other aromatic compounds. Isolate No. 24 (Table 3.5), isolated from the sediment of Lb station, was found to produce a colouration on the plate containing aniline (Fig. 3.3) during its growth. Both these isolates were therefore selected for further studies.

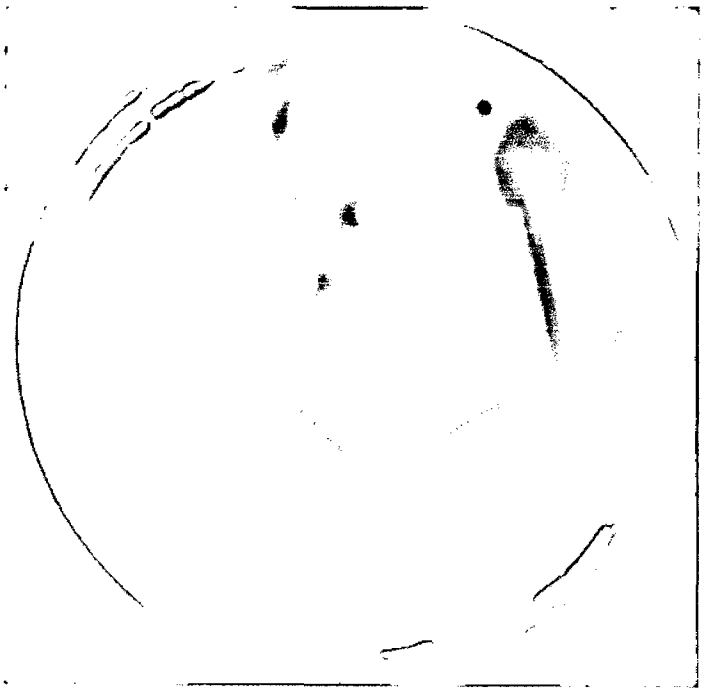
Table 3.5: Screening for isolates able to utilize aromatic amines

Isolate Name	Growth in MSM (pH 10.5) (with 0.1% substrate)				
	Aniline	DPA	PAP	NND	PCA
1	-	-	-	-	-
2	-	-	-	-	-
3	+	-	-	-	-
4	-	-	-	-	-
5	+	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	-	-	+	-	-
9	+	-	+	-	-
10	-	-	+	-	-
11	-	-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	+	-	-	-	+
16	+	-	-	-	-
17	+	-	-	-	+
18	+	-	-	-	-
19	-	-	-	+	-
20	-	-	+	-	-
21	+	-	-	-	-
22	+	-	+	-	+
23	+	-	+	-	-
24	+	-	-	-	-
25	+	-	-	-	-

Isolate Name	Growth in MSM (pH 10.5) (with 0.1% substrate)				
	Aniline	DPA	PAP	NND	PCA
26	+	-	-	+	-
27	+	-	+	+	+
28	-	-	-	-	-
29	-	-	-	+	-
30	-	-	+	+	-
31	+	-	-	+	+
32	+	-	+	-	-
33	-	-	-	-	-
34	-	-	-	-	-
35	-	-	-	+	+
36	+	-	-	-	+
37	-	-	-	+	-
38	+	-	-	-	+
39	+	-	-	-	-
40	-	-	-	+	-
41	+	-	-	+	+
42	-	-	-	-	-
43	+	-	-	-	+
44	+	-	-	+	+
45	-	-	-	+	-
46	-	-	-	-	+

Key	Abbrevation
DPA	Diphenylamine
PCA	P-Chloroaniline
NND	N,N-Dimethyl-1-Naphthylamine
PAP	p-Aminophenol
+	Growth
-	No growth

Fig. 3.3: Red colouration produced by NRS-01 during growth on MSM + Aniline (0.1%)



The aromatic amine p-aminophenol was oxidized under alkaline condition; hence, no further studies were undertaken with this compound.

3.2.6. *Purification and maintenance of the potential isolates*

Isolates 24 and 27 were streak purified on PPYG medium for a month. Colonies were observed for a period of time during purification for any changes in the cultural and morphological characteristics. The isolate giving a coloured reaction when grown on aniline was redesignated as NRS-01 and was selected for further studies.

Isolate 27 was incubated on MSM with N,N-Dimethyl-1-Naphthylamine (0.05% and 0.1%) for up to a month. Growth rate was found to be very slow on this medium as compared to a rich medium (PPY with 0.05% N,N-Dimethyl-1-Naphthylamine). Emphasis was given to the growth on MSM with the respective carbon source, where subsequent subculturing showed it's capability to tolerate the aromatic amine. This isolate was redesignated as NK2 and was selected for further studies.

Isolates NRS-01 and NK2 were maintained on PPYG medium and MSM with respective carbon source at an alkaline pH. Plates were stored in cold temperatures (4°C) and at room temperature (30±2°C). Periodically these cultures were subcultured to maintain viability and activity.

Aniline, the simplest aromatic amine, is used as a raw material in manufacturing a variety of products such as dyes, drugs, pesticides, rubber, with maximum utilization being carried out in the manufacture of azo dyes and pesticides (Boon *et al.*, 2001). In the environment, the anilines become susceptible to degradation and many neutrophilic bacteria have been reported to easily degrade aniline and their derivatives following a

common known pathway via catechol as an intermediate, with downstream pathways may proceed via ortho- or meta- cleavage pathway.

Pseudomonas sp. able to degrade anilines has been well studied by various authors (Anson and Mackinnon, 1984; Konopka *et al.*, 1989; Walker and Harris., 1969). Soil organisms *Comamonadaceae testosterone* I2 and *Delftia acidovorans* were able to transform chloroaniline to 4-chlorocatechol but only *Delftia* sp. could completely mineralize chloroaniline retaining no residue while the *C. testostreroni* was not able mineralize this compound (Boon, 2001), perhaps complete mineralization of this compound may be facilitated in the presence of aniline as a co-substrate in the growth medium as observed by You and Bartha (1982), for a *Pseudomonad* sp. Takenaka *et al.*, (2003), has reported *Bulkholderia* sp. AK-5 to degrade an aminophenol via ortho-cleavage.

As compared to aniline very little work has been reported with N,N,-dimethyl,1-Naphthylamine. This compound has been used as a laboratory reagent for estimating nitrites in ppb levels (Ohyama *et al.*, 2000). No reports have been found on the degradation of this compound either in neutral or under alkaline condition.

3.2.7. *Identification of the potent isolates*

The cultural and morphological characteristics of the two isolates were noted after growing on PPYG agar medium (pH 10.5) for 24 hrs (Table 3.6).

Isolates were identified following standard methods. One of the characteristic properties of the isolates was its tolerance to salt. Isolate NRS-01 was able to tolerate up to 25% of salt, while NK2 up to 15%, confirming them to be halotolerant alkalophiles (Kaye *et al.*, 2004). The culture was able to grow under temperatures 20°C-

Table 3.6: Colony characteristics of selected isolates

Colony characteristics	Isolates	
	NRS-01	NK2
Medium	PPYG	PPYG
Period of incubation	24 hrs	25 hrs
pH	10.5	10.5
Temperature	32°C	32°C
Size	15mm	4mm
Shape	Irregular (spreading)	Circular
Colour	Off-White	Slight pink
Opacity	Translucent	Translucent
Elevation	Flat	Low convex
Surface	Irregular	Smooth
Margin	Entire	Entire
Consistency	Butyrous after24hrs	Butyrous
	sticky after24hrs	
Motility	Motile	Motile
Gram Character	G-ve short rods	G-ve short rods

45°C and show a marked difference in their biochemical characteristics. The results were therefore compared with three other *Halomonas* species reported by Duckworth *et al.*, 2000 (Table 3.7).

The detection of eubacterial / archaeabacterial lipids is also used as a tool in identifying bacteria. The extracts got by following the extraction protocol specified earlier gave spots at Rf value 0.6 on thin layer chromatography, depicting the presence of lipid and confirming that the isolates belong to eubacterial group (Fig. 3.4).

The isolates were picked out and maintained on the respective media to be used for identification studies. These cultures were subjected to polyphasic taxonomical tools including the chemotaxonomy and 16S rRNA as basis for identification. As shown in table 3.6, the colony characterization of the organisms and the gram reactions indicated them to be gram-negative short rods, motile and growing at pH 10.5. The isolates were also able to tolerate high salt concentration with NRS-01 tolerating up to 25% salt while NK2 up to 15%. Both these isolates are therefore halotolerant alkalophiles. Further, the biochemical characteristics as shown in table 3.7, indicated both of them to be oxidase and catalase positive, facultative anaerobes capable of growing at pH 10.5, but also good growth between pH 8-12. The comparison of these isolates and their characterization indicated them to be belonging to genus *Halomonas*. This was confirmed by identification of lipids as shown in table 3.7 and fatty acid analysis using microbial identification systems.

Based on the morphological, biochemical and chemotaxonomical analysis, it is evident that the isolates NRS-01 and NK2 are identified as *Halomonas* sp. (Table. 3.7) however; the uniqueness of these isolates is the ability to tolerate aniline and NND respectively at a high concentration. To our knowledge so far, there have been no

Table 3.7: Identification of the isolated cultures (Duckworth A W *et al* 2000)

		Isolates				
	Biochemical Tests	**NRS-01**	**NK2**	***H. halmophila***	***H. Salina***	***H. meridiana***
	Oxidase	+	+	+	+	+
	Catalase	+	+	+	+	+
	Anaerobic Gr ($-NO_3$)	+	+	+	+	-
Growth	Salt conc(%) range	0-25	0-15	1-20	0-20	1-20
	opt salt (%)	< 1	< 1	ND	7.5	1-3
Growth	Temp (°C) range	20-45	20-45	20-45	20-45	20-55
	Opt Temp (°C)	30	30	37	37	37
pH	Range	7-12	8-12	ND	5-10	6-9
	optimum	10.5	10.5	7.5	7.2	7.6
	NO_3 reduction	+	+	-	-	-
	H & L	F.anaerobe	F.anaerobe	NA	NA	NA
	Growth on					
	Arabinose	-	+	-	-	+
	Dulcitol	-	+	-	-	+
	Erythritol	-	+	+	-	+
	D-Fructose	w+	w+	-	-	+
	D-Galactose	-	+	-	-	+
	D-Glucose	+	+	-	-	+
	Glycerol	w+	+	+	-	+
	Lactose	-	+	-	-	+
	D-Mannitol	-	+	NA	NA	NA
	D-Mannose	-	+	-	-	+
	D-Ribose	-	+	-	-	+
	D-Sorbitol	-	+	-	-	+
	D-Trehalose	-	w+	+	-	+
	Xylose	w+	-	-	-	+
	Rhamnose	-	w+	NA	NA	NA
	Sucrose	w+	+	NA	NA	NA
	Maltose	w+	+	NA	NA	NA

H. *halmophila* - ATCC19717; H. *salina* - ATCC 49509; H. *meridiana* - DSM 5425
+, growth/ test positive; w+, test showing a weak reaction;
NA, Not available; <1 less than 1

Table 3.7: Identification of the isolated cultures (Duckworth A W *et al* 2000) cont..

	Isolates				
Biochemical Tests	**NRS-01**	**NK2**	***H. halmophila***	***H. Salina***	***H. meridiana***
Growth on					
N-Acetylglucosamine	-	+	+	+	+
DL-α-Aminobutyrate	-	-	+	+	-
Benzoate	+	+	+	+	+
Citrate	+	+	-	-	+
Creatine	-	+	-	-	+
Ethanol	-	-	+	-	+
D-Glucosamine	+	w+	-	-	+
D-Glucuronate	+	+	+	+	+
D-Glucoronolactone	+	+	-	-	+
p-Hydroxybenzoate	-	+	-	+	+
DL-Malate	-	+	+	-	+
Oxalate	-	-	-	-	+
Pyruvate	+	+	-	+	+
Salicin	-	-	-	-	+
Sarcosine	-	+	-	-	+
Hydrolysis of					
Casein	+	+	NA	NA	NA
Esculin	-	-	-	-	+
Gelatin	-	+	NA	NA	NA
Starch	+	-	-	-	-
Tween 80	+	+	NA	NA	NA

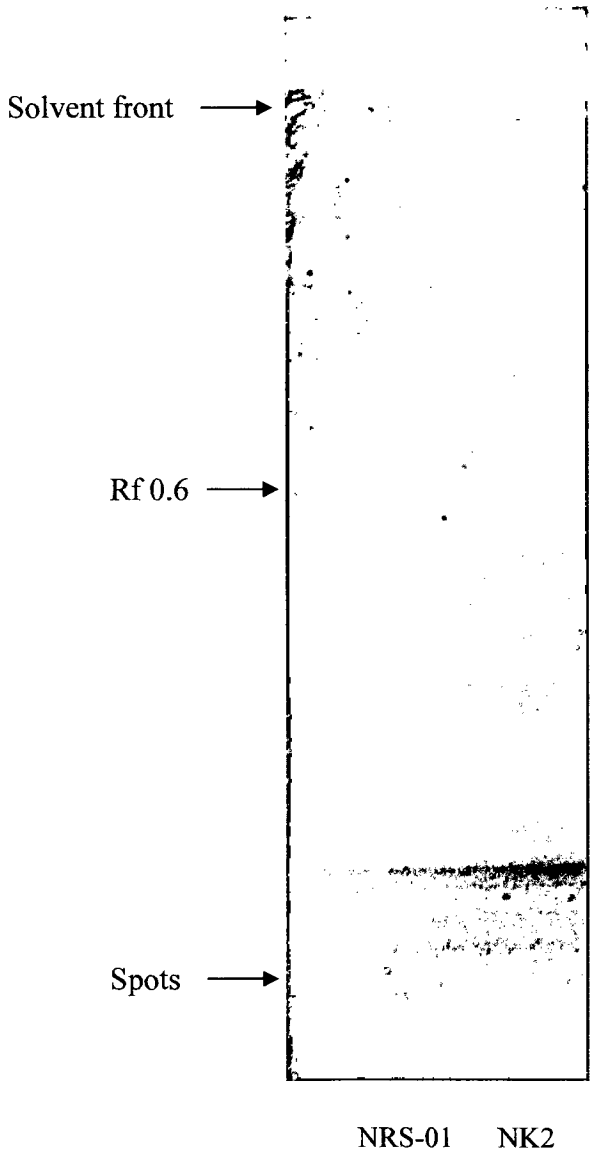
H. *halmophila* - ATCC19717; H. *salina* - ATCC 49509; H. *meridiana* - DSM 5425
+, growth/ test positive; w+, test showing a weak reaction; NA, Not available;

Table 3.7: Identification of the isolated cultures (Duckworth A W *et al* 2000) cont..

	Isolates				
Biochemical Tests	**NRS-01**	**NK2**	***H. halmophila***	***H. Salina***	***H. meridiana***
Growth on					
DL-Arginine	+	+	-	-	+
L-Asparagine	+	+	-	-	+
DL-Aspartic acid	+	+	-	-	+
L-Glutamine	+	+	-	+	+
L-Isoleucine	+	-	-	+	+
L-Leucine	+	+	-	+	+
DL-Lysine	+	-	-	+	+
L-Methionine	-	-	+	-	+
DL-Phenylalanine	+	+	-	-	+
L-Proline	+	+	-	+	+
L-Serine	+	+	-	+	+
L-Threonine	+	-	-	-	+
L-Tryptophan	-	-	-	-	-
L-Valine	+	-	-	+	+
Antibiotics					
Amikacin Ak10	R	R	NA	NA	NA
Amoxycilin Am30	S	S	NA	NA	NA
Ampicilin A10	R	R	NA	NA	NA
Bacitracin B10	R	R	R	R	R
Cephalothin Ch30	R	S	NA	NA	NA
Chloramphenicol C30	S	S	S	S	S
Ciprofloxacin Cf5	S	S	NA	NA	NA
Erythromycin E15	S	S	S	S	S
Gentamycin G10	S	S	R	R	R
Netilin Nt30	S	S	NA	NA	NA
Nitrofurantoin Nf300	S	R	NA	NA	NA
Novobiocin Nv30	R	R	R	R	R
Penicillin G P10	R	S	NA	NA	NA
Polymyxin B Pb300	S	S	S	S	S
Streptomycin S10	S	S	S	S	S
Tetracycline T30	R	R	NA	NA	NA
Trimethoprim Tr5	S	S	NA	NA	NA
Vancomycin Va30	R	R	R	R	R

H. *halmophila* - ATCC19717; H. *salina* - ATCC 49509; H. *meridiana* - DSM 5425
+, growth/ test positive; w+, test showing a weak reaction; NA, Not available;
R, resistant; S, sensitive

Fig. 3.4: TLC showing lipid profile of NRS-01 and NK2



reports on haloalkalophilic isolates tolerating these toxic pollutants. A few reports are however available on haloalkalophile *Halomonas organivorans* able to degrade phenol compounds through the catechol 1,2- dioxygenase pathway and protocatechuate 3,4-dioxygenase (Garcia *et al.*, 2005). Garcia *et al.*, (2004), had also previously reported the utilization of various other simple aromatic acids by *Halomonas organivorans*. Maltseva and Oriel (997), had reported the ability of a haloalkalophilic *Nocardioides* sp. strain M6 able to utilize 2,4,6-Trichlorophenol. Hinteregger *et al.* (1997), has reported the ability of a *Halomonas* sp. to degrade phenol via ortho-cleavage.

Although a large number of alkalophilic organisms have been studied, the focus has been basically on industrial importance of these cultures mainly with reference to enzymes, however there are no reports of alkalophilic degrading or tolerating high concentration of substituted aromatic compounds such as aniline.

Further, the identification was confirmed using FAME analysis and 16S rDNA sequencing. The Microbial Identification System (MIS) technique identifies the bacterial isolates based of the Fatty Acids Methyl Esters (FAMEs). The GC chromatogram and the fatty acid profiles are give in Table 3.8. Fatty acid profiles were matched with other *Halomonas* species, which complied with the presence of major fatty acids.

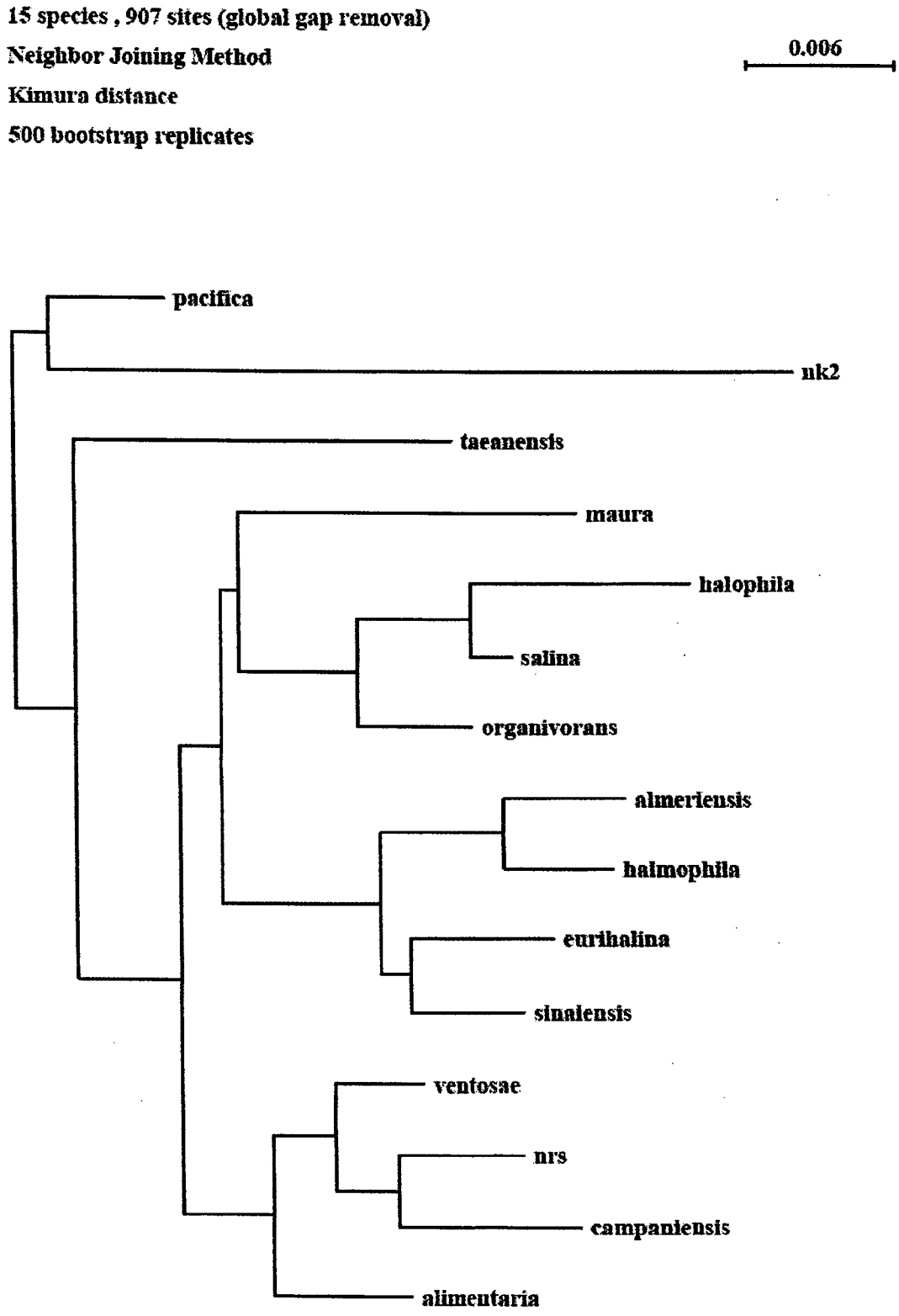
Further, the 16S rDNA gene sequence matched with that of genus *Halomonas*. The sequence was compared with other identified *Halomonas* isolates by drawing a dendogram (Fig 3.5). The results showed the isolate NRS-01 (Accession no. DQ202277) closely related to *H. campanieinsis* (similarity 97% through BLAST analysis) and NK2 (Bankit EF80950) closely related to *H. pacifica* (similarity 98%). The phylogenetic analysis of the 16s rDNA sequence was accomplished by PCR

Table 3.8: FAME profile of isolates NRS-01 and NK2

Lipid Fatty acid esters	NRS-01 % total fatty acids	NK2 % total fatty acids	*H. desiderata* % total fatty acids	*H. alimentaria* % total fatty acids
C10:0 and C10:0-3OH	3.59 / 0.59	2.47 / 0	2.13	2.4 - 0.2
C12:0 and C12:0-2OH	4.17 / 3.12(C12:0-3OH)	3.21 / 2.48(C12:0-3OH)	0.69 3-OH 4.26	0.1 - 5.2
C14:0	0.48	0.53	2.57	1.2
C15:0	0.29	–	N.A	0.6
C15:0 iso 2OH	4.97	10.88 / 0.40 (ISO-3OH)	N.A	N.A
C16:1	4.97 (16:1 w7c)	10.88 (16:1 w7c)	w7c 12.35	N.A
iC16:0	22.69	19.51	NC 16:0, 11.8	27 (16:0)
aiC16:0	–	–	N.A	N.A
nC16:0	0.89	0.2	N.A	N.A
nC17:0	2.39 (17:0 cyclo)	2.12 (17:0 cyclo)	N.A	0.2 (c17:0)
C17:1 cyclopropane	3.68 (17:1 ISO)	7.26 (17:1 ISO)	0.37	N.A
iC18:0	–	–	N.A	N.A
C18:0	0.46	1.28	0.3	12.9 (cyclo w7c)
C18:1	43.40 (18:1 w7c)	46.56 (18:1 w7c)	64	19.4 (C18:0 w7c)
C18:1 trans	–	–	N.A	N.A
C18:1 cis	–	–	N.A	N.A
C19:0	8.87 (19:0 cyclo w8c)	1.22 (19:0 cyclo w8c)	N.A	N.A
C19:1 trans	–	–	N.A	N.A
C19:1	–	–	N.A	N.A
C19:1 cyclopropane	–	–	1.23	N.A

Key Abbreviation
N.A Not available
– Absent

Fig. 3.5: Dendogram showing the isolates NRS-01 (nrs) & NK2 (nk2) closely related to *Halomonas* sp.



amplification of conserved region of the 1600bp gene using eubacterial universal forward and reverse primers.

The resulting PCR segment from each primer were then sequenced and optimally aligned. The phylogenies were constructed using PHYLOWIN phylogenetic analysis software. The dendogram was constructed using neighbour joining method (Saitou and Nei, 1987) (Fig. 3.5).

The sequence analysis accomplished by PCR amplification and phenotypic characteristics of the culture have been in good agreement for placement of the isolate in the genus of *Halomonas*. Sequences have been deposited in the gene bank and are identified with accession nos. DQ202277 for NRS-01 and EF080950 for NK2. These taxonomic tools confirmed that the isolates NRS-01 and NK2 belong to the *Halomonas* species.

The isolation of alkalophilic bacteria resulted in two potent haloalkalophiles which were identified as *Halomonas* and these isolates were capable of tolerating high concentration of aromatic amines, aniline and N,N-Diemthyl-1-Naphthylamine (NND) respectively, a characteristic which attributes to their novel metabolic system. The cultures are therefore unique in their response to these aromatic amines. Further studies were conducted on the metabolic implications, their interaction to aromatic amines and their possible use in detoxification of an untreated effluent. The results are compiled in the following chapters.

To our knowledge so far, there has been no reports on alkalophiles tolerating such high concentrations of aniline or N,N-Dimethyl,1-Naphthylamine.

CHAPTER IV

RESPONSE OF NRS-01/NK2 TO ANILINE/NND AND INDUSTRIAL EFFLUENT

Some of the effluents have a very high pH in the range of 10-13. Such effluents could be effectively treated with the organisms growing at such high pH values. In the present study, bacterial isolates were obtained which could grow under such alkaline conditions. These cultures were identified was *Halomonas* designated as NRS-01 and NK2 capable of growing in presence of aniline and N,N-Dimethyl,1-Naphthylamine (NND) respectively in an alkaline medium. Further studies were carried out on the response of the isolates to an industrial effluent as well as to the substrates aniline and NND. The results on these studies are described herewith.

4.1. MATERIALS AND METHODS

4.1.1. *Growth response of isolates to effluent*

4.1.1.1. Characteristics of effluent

Effluent samples were collected fresh from the source at the manufacturing site in a clean plastic container. Physico-chemical parameters such as pH, colour, odour and COD were noted along with its chemical composition.

4.1.1.2. Interaction of isolates NRS-01 and NK2 with effluent

The two isolates NRS-01 and NK2 were inoculated in PPYG as well as MSM containing sodium acetate as carbon source. The flasks were incubated on a shaker at room temperature for 24 hrs, 180rpm. 5% inoculum was subcultured in similar medium with 0.1% effluent incorporated in the medium serving as a sole source of carbon. Similar flasks were maintained and media were incorporated with sodium acetate as carbon source. Growth was observed as turbidity.

4.1.1.3. Enrichment of cultures growing in presence of effluent

Sediment samples collected from the mangrove ecosystems, Palolem and Panjim were used to enrich isolates able to tolerate or utilize the effluent. 2gms of sediment samples was inoculated in 100ml MSM (pH 10.5) medium with 1% effluent. The enrichment protocol followed is shown in fig. 4.1. At the end of the third subculture, 5% culture broth (Flask A_3) was inoculated in MSM with 0.1% effluent, in the presence or absence of sodium acetate, a source of carbon. Growth of the consortium was monitored as turbidity over a period of incubation to check their ability to tolerate, grow or utilize effluent as a source of carbon.

4.1.2. *Growth response of NRS-01 to aniline*

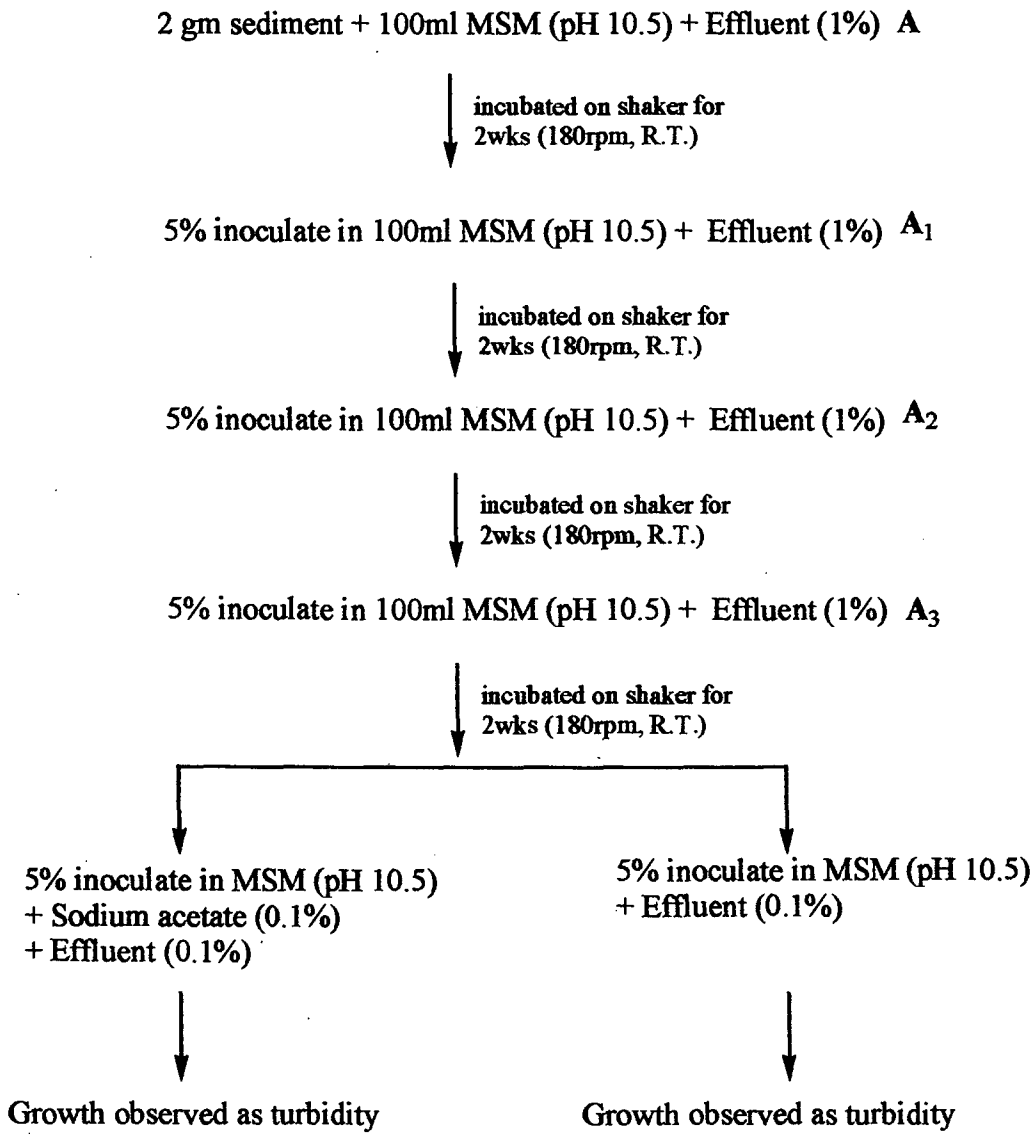
4.1.2.1. Growth medium

PPYG as well as mineral salts medium (MSM) was selected as the basal media during the growth optimization studies carried out with *Halomonas* NRS-01. pH of the medium was maintained at 10.5 using sodium carbonate prepared as shown in the Appendix-I (A.4, A.8). Freshly distilled aniline was incorporated in the media to a final concentration of 0.1% (approx. 11mM).

a) Solid medium

Alkalophilic isolate NRS-01 picked up from 24hr old PPYG plate was streaked on various media containing 0.1% aniline viz. 1) Mineral Salts Medium (MSM) - aniline used as a sole source of carbon, 2) MSM supplemented with (0.1%) glucose – glucose was used a carbon source, 3) MSM supplemented with sodium acetate – (0.1%) sodium acetate was used a carbon source and 4) PolypeptoneYeast

Fig. 4.1: Protocol showing enrichment of consortium/isolates towards effluent



agar (Appendix I, A.7)– 0.1% aniline was used as carbon source instead of glucose which has been usually used in this medium. The pH of the media used were maintained at 10.5. Petriplates were incubated at room temperature (30°C±2) and growth was observed periodically along with colour formation on the medium.

b) Liquid medium

Isolate NRS-01 was picked from a 24hr old PPYG agar medium and was inoculated in 1) MSM with aniline (0.1%) as sole source of carbon, 2) MSM containing aniline (0.1%) with sodium acetate (0.1%) as an additional carbon source and 3) MSM containing aniline (0.1%) with glucose (0.1%) as an additional carbon source. Bacterial response to aniline in all these conditions was studied observing growth based on the increase in turbidity along with the formation of colour in the medium observed visually.

i) Bacterial Adhesion To Hydrocarbons (BATH) Assay (Al-Tahhan *et al.*, 2000)

Culture pellet grown in MSM medium with 0.1% glucose aniline was used to determine the hydrophobicity of culture. Cells were harvested from various growth stages, viz. 0hrs, 18hrs (cells showing highest turbidity) and 48hrs (cells covered with a red product). Cells were washed twice with normal saline and a suspension was prepared in sodium phosphate buffer (pH 9.0) (Appendix II, B.18). To a cell suspension (2.5ml) of 0.6 O.D., 0.5ml of hexadecane was added to give a final volume of 3.0 ml in a suspension tube (16mm x 100mm test tube). This suspension was vortexed for 1min and was allowed to stand for 30min and 60min for complete

phase separation. O.D. of the aqueous phase was measured before vortexing and 30min and 60mins after vortexing, using Elico Colorimeter (450nm).

4.1.2.2. Effect of aniline Concentration

The ability of isolate NRS-01 to tolerate a high concentration of aniline was determined by inoculating 5% of the preculture grown in MSM with (0.1%) glucose and aniline (0.1%). Inoculation was carried out in MSM medium with similar concentrations of glucose but varying concentrations of aniline (1mM, 5mM, 10mM, 20mM, 50mM and 100mM). Growth was monitored at two-hourly intervals as turbidity. Colour formation in the medium was also monitored concomitantly with growth.

4.1.3. *Effect of physico-chemical parameters on the response of isolate NRS-01*

4.1.3.1. Effect of pH

The dependence of the alkalophile NRS-01 on pH was checked by growing the culture in medium at various pH values. A 5% inoculum grown in MSM with glucose (0.1%) was inoculated in various flasks containing MSM medium incorporated with glucose and aniline (0.1%) adjusted to pH values 7.4, 8.5, 9.0 and 10.5. Growth as turbidity and colour formation in the medium was monitored periodically.

4.1.3.2. Effect of salt

The halotolerant alkalophile NRS-01 grown in MSM medium with 0.1% glucose as a carbon source was used as an inoculum to study its growth response at

different salt concentrations. Inoculum (5%) was inoclutaed in MSM medium with various salt concentrations (0, 5, 10, 15, 20, 25%). Glucose was added as an additional carbon source in the presence of aniline at 0.1% concentration. Growth was observed as turbidity with the colour formation.

4.1.3.3. Effect of light

The red colour formation brought about by the isolate NRS-01 was checked in the presence and absence of light. 5% inoculum grown in MSM and glucose (0.1%) was inoculated to MSM with aniline (0.1%) and glucose (0.1%). For the reaction that needed to be carried out in dark, flasks were wrapped in black paper and were incubated on a rotary shaker (180rpm) at room temperature. Similar reactions were set up with MSMII (Appendix I, A.8.1). Colour formation was observed in the medium after an incubation period of 24-48hrs.

4.1.3.4. Effect of nitrate

A modified MSM medium with ammonium sulfate replacing ammonium nitrate was used to study the requirement of nitrates for colour formation. Modified MSM media containing glucose (0.1%) and aniline (0.1%) was inoculated with isolate NRS-01 picked up from the PPYG plate and was incubated for a period of 24 to 48hrs on a rotary shaker (180rpm) at room temperature. Colour formation brought about by the cells in the medium was observed.

4.1.4. *Growth response of NK2 to N,N-Dimethyl,1-Naphthylamine*

4.1.4.1. Growth medium

Growth optimization of isolate NK2 was carried out by growing the culture in PPY, as well as in mineral salts medium (MSM) in the presence of NND (0.05%). The utilization of NND as a source of nitrogen was studied using MSM II where ammonium nitrate was not added to the medium. The pH of the medium was maintained alkaline (10.5) using sodium carbonate. MSM medium was incorporated with 0.05% yeast extract, used as an additional carbon source to boost the growth of the isolate.

Starter inoculum was prepared by inoculating isolated colony of NK2 from a PPYG plate incorporated with 0.1% NND in MSM medium with 0.05% NND. The flasks were incubated for a period of 48 hrs and 5% of the culture broth (inoculum) was used to subculture in the similar medium. Growth was observed as turbidity and the disappearance of NND globules was observed visually. Culture grown in PPY was also used as a starter culture to inoculate PPY medium with 0.05% NND. Direct addition of the culture picked up from a PPYG plate also served as an inoculum.

The protocol that yielded best growth in the medium showing production of biosurfactant and a quick disappearance of NND was selected as an ideal condition and the appropriate medium was used to carry out further studies on NK2.

4.1.4.2. BATH assay

Isolate NK2 was grown in half strength PPY medium (Appendix I, A.7) for a period of 18hrs and 0.05% NND was added to the medium and was incubated for a period of 24hrs. Ten ml of the medium was centrifuged, washed with normal saline

and was used to carry out BATH assay. BATH assay protocol was followed as explained in section 4.1.2.1., (i).

4.1.4.3. Effect of incubation period on NND

Isolate NK2 was inoculated in 10 ml of half strength PPY medium in a 50 ml conical flask incubated on a rotary shaker at room temperature at 180rpm; 15 such flasks were maintained. After an initial incubation period of 18 hrs, a final concentration of 0.05% of NND was added to each flask and one flask was picked after every 48 hrs of incubation. Whole content of the flask was extracted in triplicates using diethyl ether. Extracts were pooled, dried over sodium sulfate, concentrated, dissolved in minimal amount of solvent and TLC was performed on silica gel-H (section 5.2.6.3). The solvent system used for TLC was Petroleum ether : acetone (96 : 4). The spots were visualized under UV-light.

4.1.5. *Effect of Physico-chemical parameters on the response of isolate NK2*

4.1.5.1. Effect of pH and salinity

Isolate NK2 was grown in half strength PPY medium, incubated at room temperature for 18hrs. In order to study the effect of pH, 5% of the culture broth was inoculated in half strength PPY media with pH values 7.0, 8.5, 9.5 and 10.5. Effect of salinity was studied by growing the culture in half strength PPY with NaCl concentrations of 0, 5, 10, 15%. After an incubation period of 18 hrs, NND (0.05% final concentration) was added to all the flasks. Flasks were incubated on shaker at room temperature, 180rpm. The formation of an emulsion or production of biosurfactant in the medium was considered as a positive response.

4.1.5.2. Biosurfactant production in the presence of other substrates

Isolate NK2 was grown in half strength PPY medium for a period of 18 hrs and was supplemented with other immiscible liquids such as hexadecane and paraffin oil, aromatic solids having naphthyl nucleus such as naphthalene and 1-naphthylamine as sources of carbon instead of NND. A final concentration of 0.05% of all these compounds were added. The flasks were incubated up to 20 days with visual monitoring on the production of the emulsion/biosurfactants by the culture.

4.1.6. *Isolation and characterization of biosurfactants*

4.1.6.1. Production of biosurfactant

Half strength PPY medium (1000ml) was inoculated with 18hr old NK2 culture grown on PPYG plate. PPY medium was incubated on a shaker at room temperature at 180rpm for a period of 18 to 20 hrs and NND was added to the culture broth and was further incubated for a period of 20 days on a shaker. The culture broth containing insoluble white biosurfactant produced by the organism was used for extraction.

4.1.6.2. Isolation of biosurfactant

a) Acidification method

The culture broth was acidified by dropwise addition of 10N HCl until the insoluble biosurfactant in the medium dissolved to give a clear supernatant. This culture broth was centrifuged to separate out the cell pellet and the cell free broth was alkalinized by dropwise addition of 10N NaOH to back precipitate the surfactant in

cooling centrifuge. The precipitate collected was purified and used for characterization studies.

b) Precipitation method

The broth containing the culture as well as insoluble biosurfactant was separated by initially dissolving the insoluble surfactant with a small amount of cold acetone. Cells were separated from the supernatant by centrifuging the culture broth at 10000rpm for 10min and the surfactant was precipitated using double volume of cold acetone (Cameotra *et al.*, 1990.). Acetone was added slowly and the contents in the flasks were mixed thoroughly during subsequent addition of acetone. On complete addition, the contents in the flasks were mixed thoroughly and stored overnight in cold (4°C) to bring about complete precipitation. Excess of culture broth from the flask was decanted followed by centrifugation in Remi cooling centrifuge at 4°C at 10000rpm for 15min to pellet out the surfactant. The precipitate collected was purified and used for characterization studies.

4.1.6.3. Purification of biosurfactant

The biosurfactant precipitate was collected and dissolved in minimum amount of sodium phosphate buffer pH 7.0 (Appendix II, B.18). The slurry was mixed thoroughly to facilitate complete dissolution and was centrifuged at 10000rpm at 4°C. The supernatant was collected and purified by dialysis or by diafiltration.

a) Dialysis

The dissolved biosurfactant was purified by carrying out dialysis in a 20 cm long and 2cm wide dialysis tubing having molecular weight cut-off of 12,000Da against double distilled water overnight at 4°C (Rodrigues *et al.*, 2006). Surrounding water was changed twice, after every 12 hrs. The dialysed biosurfactant was concentrated using amicon diafilter.

b) Diafiltration

Purification and concentration was also carried out using commercially available amicon diafilter having molecular weight cut-off of 3000Da (Bryant, 1990). The retanate was washed thrice with sodium phosphate buffer pH 7.0. The surfactant was concentrated to a volume of 1.5ml and was stored at -20°C until further use.

4.1.6.4. Assay of emulsification activity (Cooper and Goldengenberg, 1987)

Culture broth of NK2 grown in half strength PPY medium containing 0.05% NND and the purified biosurfactant was used to carry out emulsion activity. Four ml of culture broth was taken from a shake flask, centrifuged at 10000rpm at 4°C for 10 mins, filtered through a 0.22µm Millipore membrane filter and was used to check the emulsifying activity using hexadecane as the solvent. Emulsifier activity was measured by adding 6 ml of hexadecane to 4 ml of aqueous sample in a test tube (1.2cm x 12). The contents were vortexed at high speed for 2 min and the tubes were allowed to stand and measurements made after 24hr. The emulsion index (E_{24}) was calculated as the height of the emulsion layer (interphase), divided by the total height,

multiplied by 100. Uninoculated PPY medium served as a blank which was treated as explained above.

Emulsification activity of the purified biosurfactant was checked by dissolving 30µl of surfactant in 4ml of sodium phosphate buffer (pH 9.0) with 6ml of hexadecane. Activity was also checked with NND; to 3.0ml of phosphate buffer (pH 9.0) containing 5µl NND 20 µl of purified surfactant was added and vortexed. Sodium phosphate buffer (pH 9.0) was used as blank without adding purified biosurfactant.

4.1.6.5. Determination of the type of emulsification (Park and Kim, 2000)

The type of emulsification brought about by the surfactant produced i.e. whether it is a water-in-oil type or oil-in-water type was determined by adding either oil-O-red or crystal violet in two test tubes containing culture supernatant and hexadecane. Culture broth was taken from shake flask (3ml), centrifuged, filtered, and 1:1 diluted with sodium phosphate buffer pH 9.0. 2.5 ml of the diluted supernatant was taken in a suspension tube (15mm x 100mm), 0.5ml of hexadecane was added, and 2 mg of Oil-O-Red or crystal violet was added to two different tubes. Tubes were vortexed for 2-3mins and allowed stand for phase separation. The results were noted based on the emulsification and the dye transfer to either of the phases.

Simultaneously, purified biosurfactant was used to determine the type of emulsification by following the above mentioned protocol using NND instead of hexadecane. The tubes were vortexed for 1min and allowed to stand for phase separation. The emulsifying activity was determined by the turbidity and the colour dispersion in the aqueous medium.

4.1.6.6. Determination of the class of biosurfactant (Kuyukina *et al.*, 2001).

Class of biosurfactant was distinguished by carrying out qualitative analysis of protein and carbohydrates on the purified biosurfactant compound. Protein was determined by Bradford's method (Bradford, 1976) and carbohydrates were determined by total carbohydrate estimation method by Dubois *et al.*, (1956),

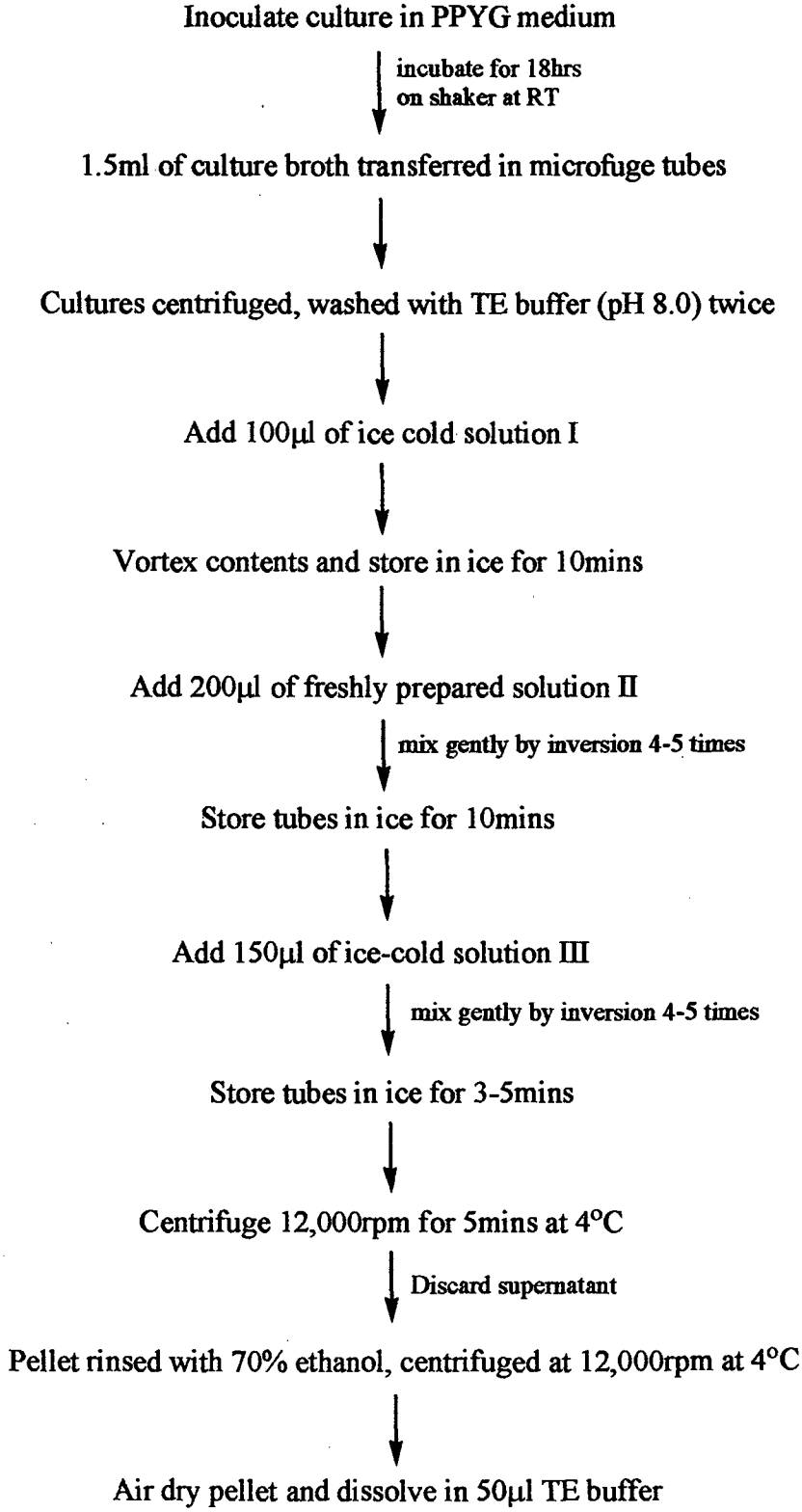
4.1.6.7. Determination of plasmid in NRS-01 & NK2

a) Alkaline Lysis Method (Birnboim and Doly, 1979): A single bacterial colony was transferred into 10 ml of PPYG broth (pH 10.5) and incubated overnight at 28°C at 180rpm. 1.5 ml of culture broth was taken in a microfuge tube and was centrifuged at 8,000rpm for 5 min at 4°C. The supernatant was discarded and the bacterial pellet was used to determine the presence of plasmid as shown in flowchart (Fig. 4.2). The composition of solutions I, II and III is given in Appendix II, B.14.

b) Gel Electrophoresis of plasmid DNA (Meyers *et al.*, 1976; Kado and Liue, 1981)

i) Preparation of agarose gel slabs: Agarose gel 0.8% (w/v)was prepared in 1X-TAE buffer (pH8.0) (Appendix-B.17) by heating in a microwave oven for 4min. The platform for electrophoresis was sealed on open sides with heat resistant adhesive tape. The comb was adjusted to 2mm above the gel slab and 1.5 cm from one end of the sealed side. To the molten agarose (50 ml), 5µl of ethidiumbromide (10mg/ml) (Appendix-B.15) was added to get final concentration of approximately 0.05µg/ml in molten agarose, poured into the platform to a thickness of 0.5 cm and allowed to set at room temperature. After the gel set, the comb and the adhesive tape were removed

Fig. 4.2: Plamid isolation by alkaline lysis method (Brimboim & Doly, 1979)



carefully. The gel slab was placed in the electrophoretic chamber and the 1X TAE buffer was poured in the chamber until the gel just submerged in the buffer.

ii) Loading of DNA sample: DNA sample (10µl) was mixed with 2µl of tracking dye (Appendix-II, B.16) on a parafilm and was loaded into the wells of agarose gel using a micropipette. A molecular weight marker (1µl) was mixed with tracking dye and was loaded in the adjacent well.

iii) Running of gel: The electrodes were connected to the power supply by means of connecting wires. The voltage was adjusted to 72V and the electrophoresis was carried out at constant voltage for 2 h.

iv) Visualisation of DNA: After electrophoresis the gel was observed on a UV photodyne transilluminator.

4.2. RESULTS AND DISCUSSION

4.2.1. *Studies with effluent*

Effluent sample collected from the pesticide industry had an alkaline pH of 13.0, a light yellow colour with aromatic odour. The important constituents used as a raw material reported in the effluent included trimethylamine, trimethylamine ethylbromide (TAEBr), bromochlorophenol and diethylaniline. The COD of the effluent was found to be 81,473mg/lt.

Isolated alkalophiles NRS-01 from marine ecosystem having an ability to tolerate high concentration of aniline and NK2 isolated from mangrove ecosystem, able to produce a biosurfactant in the presence of NND were used to study the interactions with the selected effluent. Isolates NRS-01 and NK2 showed growth in the form of turbidity in media (PPYG as well as MSM) containing 0.1% effluent, in the presence of additional carbon source whereas, growth was not observed in media devoid of either glucose or sodium acetate. This indicated the inability of the effluent to serve as a source of carbon for the isolates NRS-01 and NK2. However these isolates could grow in presence of the alkaline industrial effluent with an additional carbon source.

Enrichment techniques carried out from the sediment samples after 3rd enrichment yielded a consortium, which could grow in medium containing 0.1% effluent in the presence of a carbon source such as sodium acetate thereby able to tolerate the effluent. The consortium did not grow in the medium with effluent as a sole source of carbon thereby showing its inability to utilize it as a source of carbon.

It was interesting to find that the alkalophiles were able to tolerate an alkaline toxic effluent though other various pretreatments may be necessary for the effluent or a suitable cometabolic / gratuitic substrate be provided for the bacterial cultures or consortium in order to treat the effluent.

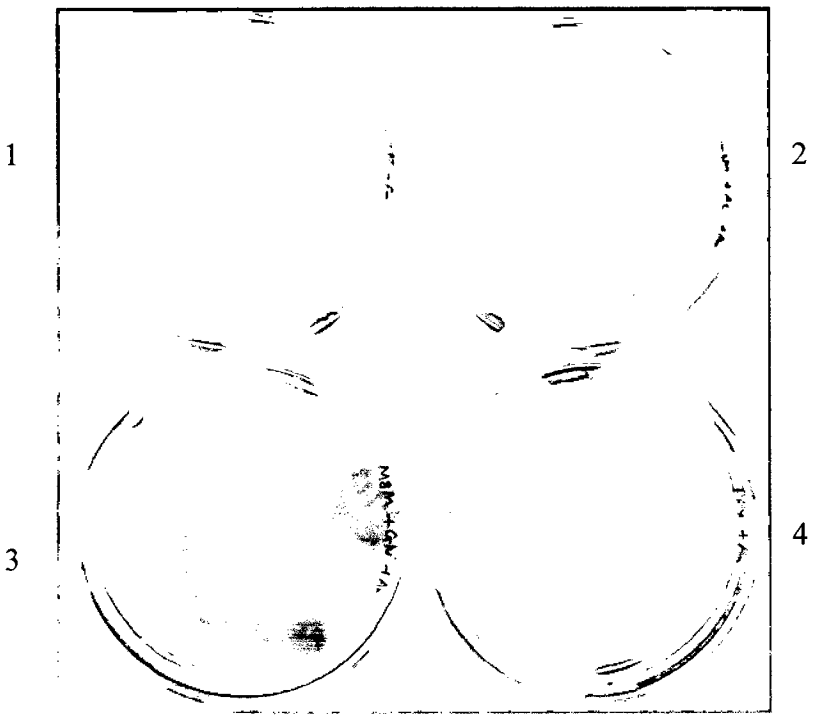
4.2.2. *Growth response studies of NRS-01 to Aniline*

4.2.2.1. Media

Initial studies during selection of the culture, NRS-01 was found to tolerate high concentrations of aniline and revealed the formation of a red colouration on the colony which diffuses in the surrounding medium. Such a response of the culture during its growth in the presence of aniline was interesting. Having known that aniline is the major component in the manufacture of dyes, it was of interest to understand the mechanism involved in colour formation at an alkaline pH. Various parameters include growth medium, pH, concentration of aniline, salinity and requirement of certain chemicals such as nitrates in the growth medium and the presence of light to study growth with colour formation.

Growth media included PPYG as well as MSM to observe growth and colour production by the isolate NRS-01 in presence of aniline. The pH of the media was maintained at 10.5. Although, the isolate showed a similar coloured reaction when grown on PPY agar as well as MSM agar medium, there was a difference in the incubation time for the development of colonies as well as colour. Growth was seen to decrease in the order of the media used; PPY + aniline (0.1%) > MSM + glucose (0.1%) + aniline (0.1%) > MSM + sodium acetate (0.1%) + aniline (0.1%) > MSM + aniline (0.1%). This indicates that aniline does not serve as a good source of carbon and energy, while it could be utilized by cometabolically using glucose or sodium acetate as a growth substrate which is evident from the fig. 4.3. Even though PPY showed good growth as well as colour formation, the colour intensity was found to be highest in the medium containing glucose, which may be evident for the requirement

Fig. 4.3: Growth of NRS-01 on PPYG and MSM containing aniline



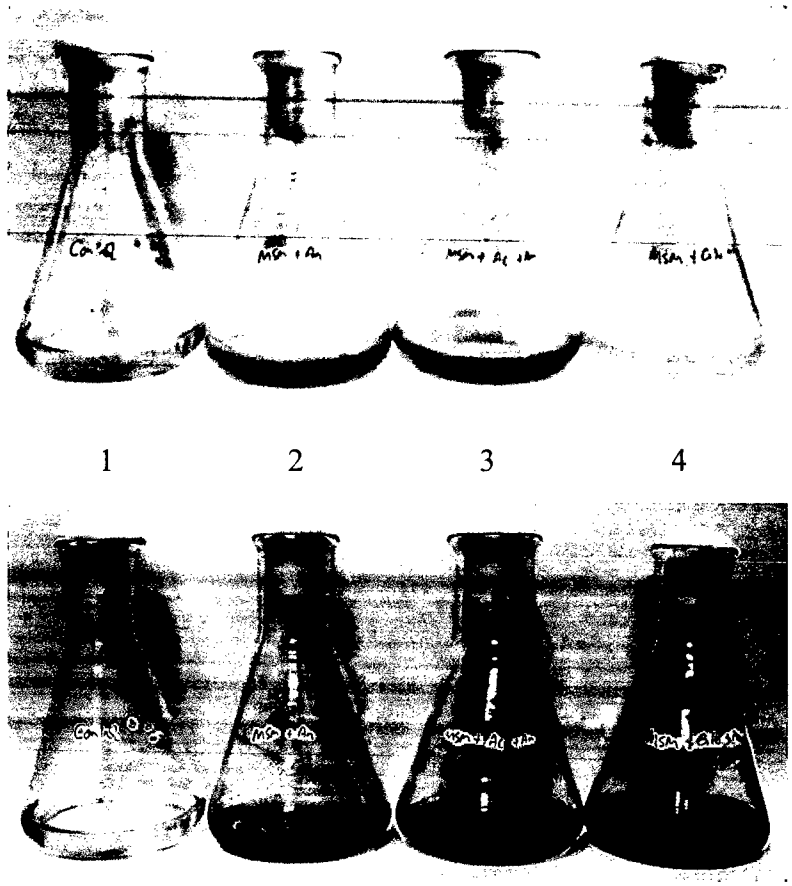
1. MSM + Aniline (0.1%)
2. MSM + Sodium acetate (0.1%) + Aniline (0.1%)
3. MSM + Glucose (0.1%) + Aniline (0.1%)
4. PPY + Aniline (0.1%)

of glucose or sodium acetate. The incubation period required to observe growth and colour formation was highest when aniline was supplied as a sole carbon source while shortest time was required when grown on PPY containing aniline.

While glucose or sodium acetate served as a source of easily utilizable carbon, microbes have used them as cometabolic carbon sources. The requirement of such compounds (glucose or acetate) to bring about anhancement in utilization/reaction of other aromatic compounds such as phenols by *Pseudomonas putida* has been reported by Tarighian *et al.*, (2003) and (Fakhruddin and Quilty 2005). Roberto *et al.*, (2005), reported the requirement of such carbon sources for the utilization of PAH under anaerobic condition by bacterial consortium.

MSM broth medium was utilized to study the bacterial growth response in liquid medium maintaining the concentrations of aniline at 0.1%. Growth in the form of turbidity and the colour formation was visually observed. On comparison with the control flask, it was evident that the colour formation was brought about by culture and not due to any chemical reactions among the media components and glucose or sodium acetate with aniline (Fig. 4.4). Within 24 hrs of incubation, flasks containing aniline as sole carbon source and the medium incorporated with sodium acetate showed a colour formation. While growth in both these flasks varied; growth was minimal in the flask containing aniline alone as compared to the flask containing sodium acetate. Although good growth was observed in the flask containing glucose there was no evidence of colour formation after 24hrs of incubation, but on further incubation, a red colouration was visible in medium while the intensity of the red colour increased in the other two flasks.

Fig. 4.4: Growth seen in MSM with aniline (in presence of acetate and glucose)



1. MSM + Aniline (0.1%) (Control)
2. MSM + Aniline (0.1%)
3. MSM + Sodium acetate + Aniline (0.1%)
4. MSM + Glucose (0.1%) + Aniline (0.1%)

BATH assay measures the partitioning of cells between aqueous and hydrophobic phases. BATH assay carried out did not show any significant adherence to hexadecane showing about 3 – 6% during the initial stages of growth, while adherence enhanced drastically with an increase in the incubation period. Adherence was found to increase to 63% after an incubation period of 48hrs where the red coloured compound was formed, which showed to enhance the hydrophobicity of the bacterial cell surface. An increase of hydrophobicity of the cell surface may be indicating a resistance towards certain hydrophilic compound which may be toxic to the cells (Deziel *et al.*, 1990; Carvalho *et al.*, 2005).

4.2.3. *Tolerance to aniline and effect of physico-chemical parameters on NRS-01*

Studying the various physico-chemical parameters required for growth and colour development, it was observed that the culture NRS-01 responded characteristically to their alkalophilic nature, showed excellent growth in medium with pH value 9.0 and 10.5 below which was grossly retarded thereby making it an important criteria to maintain the pH values above 9.0. According to fig. 4.5, optimum growth was observed at pH 9.0 as compared to 10.5, but pH 10.5 was selected for further studies for the intensity of red colour being high. With these results, it can be stated that it is necessary to maintain an alkaline environment to study the interaction of the isolate with aniline.

It has been reported that a typical alkalophile growing under alkaline conditions has its cytoplasmic pH 1-2 units lower than the external pH (ranging between 9-11) (Krulwich *et al.*, 1995; Krulwich 1997), a characteristic of obligate and

Fig. 4.5: Effect of pH on the growth of isolate NRS-01.

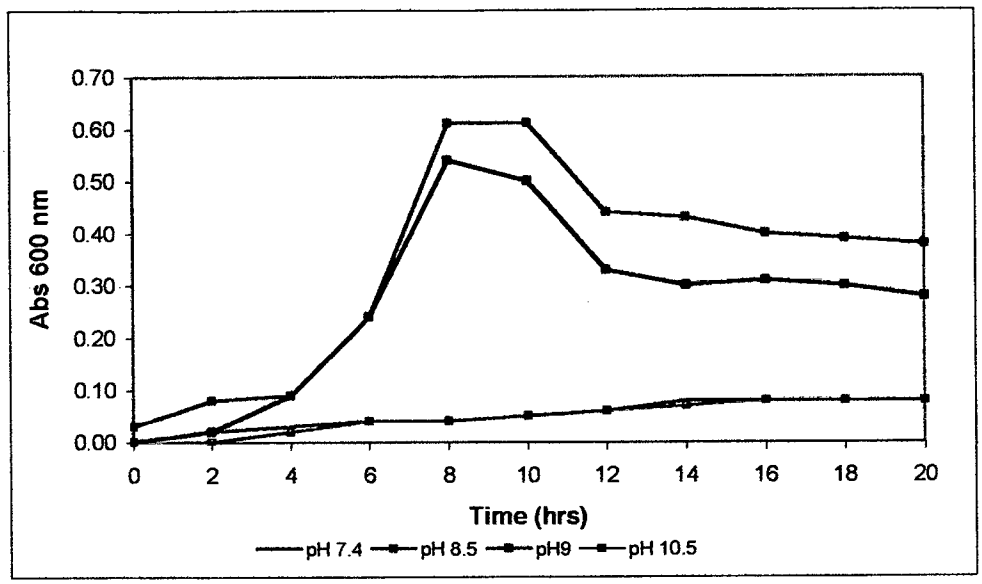
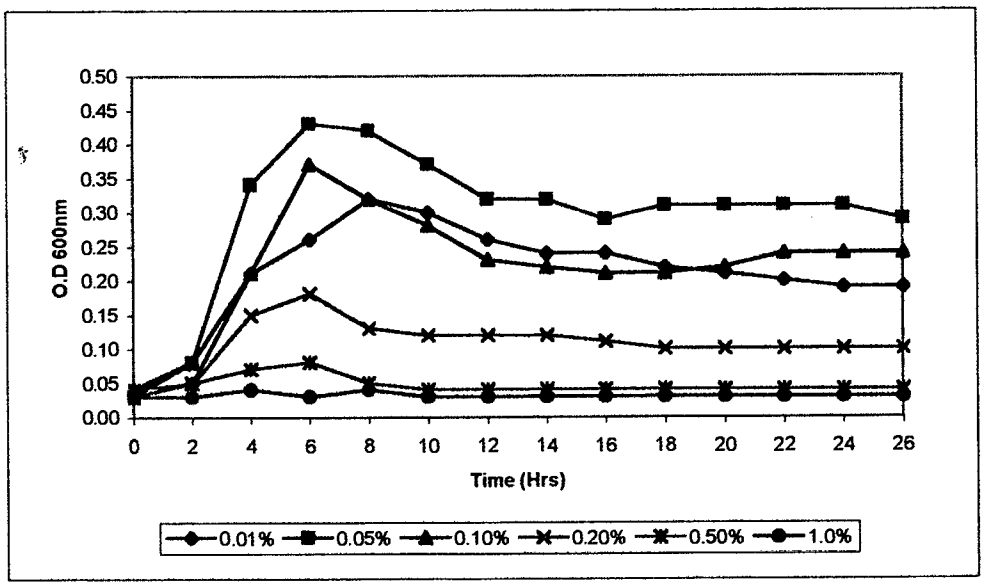


Fig. 4.6: Effect of aniline concentration on the growth of isolate NRS-01.



facultative alkalophiles but absent in alkali tolerant bacteria which cannot grow at pH value 9.0 (Guffanti *et al.*, 1980). Most alkalophilic bacteria require sodium ion for their growth, motility, sporulation (Guffanti *et al.*, 1978; Kitada *et al.*, 1982; Guffanti *et al.*, 1983; Kosono *et al.*, 1992; Kudo and Horikoshi., 1979) and for energy generation (Hicks and Krulwich, 1986). Thus presence of sodium ion and an alkaline condition are prerequisites for the normal physiological and metabolic working of an alkalophile.

Although the culture was found to be halotolerant, its interaction with aniline under alkaline and saline condition, was found to exert lot of stress on the isolate due to which the colour formation was observed to be delayed. Normal growth of the isolate was observed but the presence of the salt in the medium delayed the colour formation. The absence of added salt in the medium was found to be the most suitable condition for the colour product formation by the isolate NRS-01, while higher salinities showed colouration in the medium with no growth over extended periods of incubation.

Isolate NRS-01 was found to tolerate aniline at various concentrations in MSM. Growth curve studies (Fig. 4.6) showed good growth in the form of turbidity with aniline concentrations of 0.01% to 0.1%, while further increase in the aniline concentration retarded its growth. Although there was no growth, colour formation of lower intensity was observed along the range of concentrations above 0.1% aniline, but the intensities were found to be highest at 0.1% concentration. Best growth was observed with aniline concentration of 0.05%. Best growth with colour formation was observed in the MSM containing aniline and glucose at concentrations of 0.1%. This

medium combination was therefore used to carry out further studies. Various species of neutrophilic organisms such as *Pseudomonas* sp. (Ahmed *et al.*, 2001; Fukumori and Siant, 1997; Konopka *et al.*, 1989; You and Bartha, 1982; Zeyer and Kearney, 1982), *Nocardia* sp. (Bachofer *et al.*, 1975), *Commamonas* sp. (Boon *et al.*, 2001; Peres *et al.*, 1998), *Acinetobacter* sp. (Fujii *et al.*, 1997), *Phanerochaete chrysosporium* (Sandermann *et al.*, 1998), *Bacillus* sp. (Zissi *et al.*, 1997), *Burkholderia* sp. (Takenaka *et al.*, 2003) were found tolerant and degrade aniline or substituted anilines among which *Delftia* sp. AN3 was able to tolerate aniline at highest concentration of 53.8mM (Liu *et al.*, 2002).

It is a well-known fact that aniline gets photo-oxidized to quinones in the presence of light (Briviba *et al.*, 1993) with sequential transformation of aniline to para hydroxylated aniline to quinone. Nucleophilic reactions are known to occur between quionones and aniline to form anilinoquinones or 2,5-dianilinoquinones (Kutyrev, 1991). Thus it was important to know if light played any role in the initial photo-transformation of aniline to a compound, which in turn formed a precursor of the red compound. The light and dark reaction experiments performed did not show any significance of light in the formation of the red compound (Table 4.1). The results confirmed the role played by bacterial cells in the formation of the red compound.

One of the important nutrients required for the azo dye preparation is the nitrates/nitrites (Kiso *et al.*, 2006; Pasquali *et al.*, 2007). These nitrates normally couple with the primary aromatic amine to form a diazonium salt which further reacts with an aromatic compound such as aniline itself or other available aromatic

Table 4.1: Effect of incubation in light and dark condition on pigment

Medium	Condition	Colour formation
MSM + Glu + An	Dark	+
MSM + Glu + An	Light	+
MSMII + Glu + An	Dark	-
MSMII + Glu + An	Light	-

MSMII, MSM devoid of nitrogen; Glu, Glucose
An, Aniline

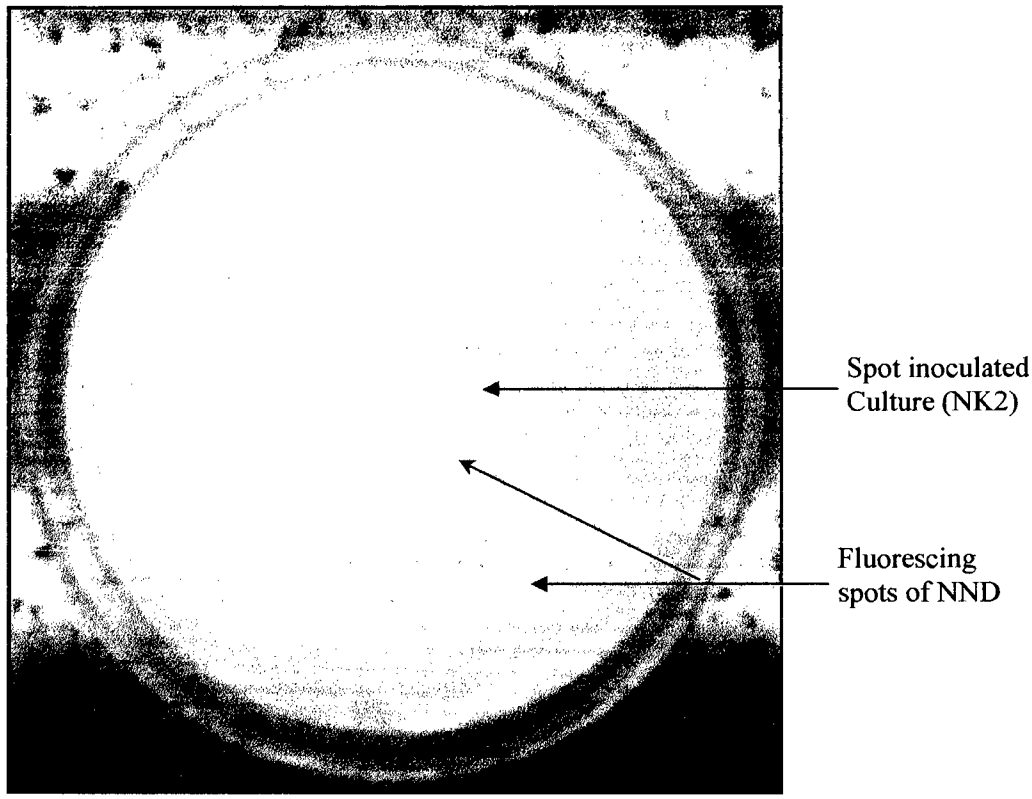
compounds forming an azo dye which is a coloured compound (Asad, 2007). Replacing ammonium nitrate with ammonium sulfate did not hinder the formation of the red coloured compound which indicates that the cells followed a different stratergy to produce this red coloured compound.

4.2.4. *Growth response studies of NK2 to N-Dimethyl,1-Naphthylamine (NND)*

Selection of medium for growth was based on the ability of the culture NK2 to grow and utilize NND as a sole source of carbon. Initial growth of the isolate on the PPYG plate containing NND (0.1%) (Fig. 4.7) showed better growth as compared to growth on MSM with 0.1% NND. Growth was found to be very slow taking up to almost a month on MSM broth with 0.05% NND as a carbon source. The culture was maintained on MSM with 0.1% NND as well as PPY with 0.1% NND. However, culture was picked up from a MSM plate having 0.1% NND and was subcultured on PPYG medium before using it for growth response studies.

Attempts to prepare a preinoculum by growing the culture in MSM with (0.1%) NND, did not yield a good growth. However, 5% of the culture broth taken from this flask was used as an inoculum to subculture in similar medium. It was observed that even on incubating the flask up to a period of 10 days did not yield growth, which was observed on addition of 0.05% of yeast extract to MSM broth containing 0.05% NND along with the production of a biosurfactant as an emulsion was formed. Emulsion formed in the medium dispersed the added amount of NND. Following this protocol as a subculturing technique did not yield consistent growth or emulsification. False positive results of emulsification were also observed in the medium maintained as control which did not contain bacterial culture. The test flasks

Fig. 4.7: Spot inoculation of isolate NK2 on PPY medium with 0.1% NND

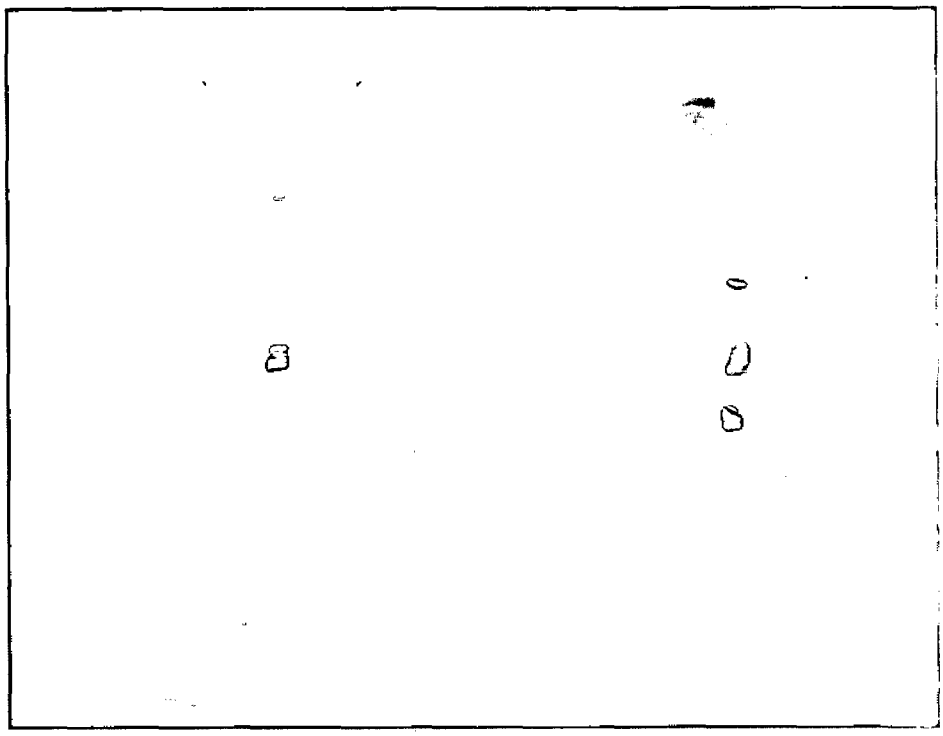


Petriplate visualized under UV

that showed the formation of the emulsion revealed the disappearance of NND after an incubation period of approximately one month. NND is an immiscible liquid with boiling point of 275°C, thus it was unlikely for the compound to volatilize from the medium. Utilization of NND as a source of nitrogen in the MSM medium was checked by utilizing MSM medium devoid of ammonium nitrate. The results showed inconsistency in growth as well as emulsification. Controls also showed the formation of emulsion as compared to the test flasks containing bacterial culture. On prolonged incubation in MSM medium containing yeast extract, the cells grown showed production of an insoluble white compound, but the protocol did not yield replicating results. Therefore, use of MSM was discontinued.

Inconsistency over growth and emulsification were solved by initially growing the isolate NK2 in half strength PPY medium (Appendix I, A.7) at pH 10.5. Addition of NND at a final concentration of 0.05% NND in the pregrown culture medium after an incubation of period of 18-24 hrs, showed the production of an insoluble compound which emulsified the added NND. Emulsification was observed to have initiated after an incubation period of 4-5 days. As the isolate produced the biosurfactant, the entire medium changed its colour from yellow (initial colour of medium) to off-white to near white (Fig. 4.8). Further studies were carried out by following the similar protocol of initially growing the cells in PPY medium. This indicates that a heavy load of bacterial cells was required to bring about a consistent production of biosurfactants.

Fig. 4.8: Flasks showing the production of white surfactant in PPY medium



Test Control

Test PPY + 0.05% NND + culture – incubation period 5 days

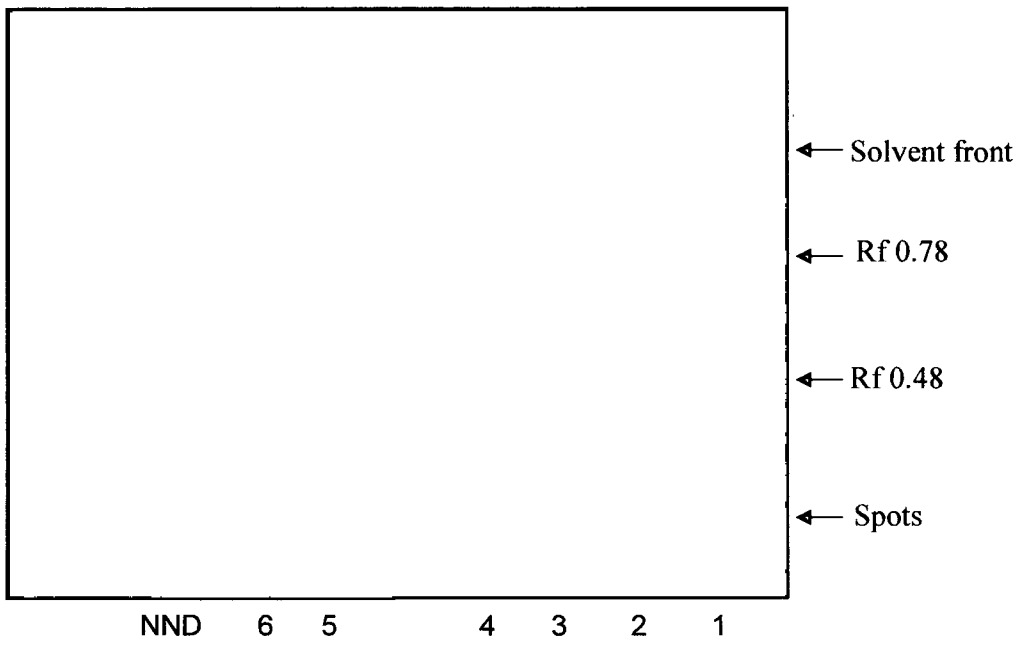
Control PPY + culture – incubation period 5 days

As NND was replaced with other immiscible substrates such as paraffin oil, hexadecane, or aromatic solids such as naphthalene or naphthaylamine in the growth medium, isolate NK2 did not produce the insoluble even after an incubation period of 20 days, which indicated that such a response was specific and brought about by the culture towards NND. Isolate *Halomonas* sp. NK2 was found to show a characteristic interaction with aromatic amine N,N-Dimethyl,1-Naphthylamine (NND) by the production of an insoluble compound in the PPY broth. This aromatic amine has not been studied either on degradation or on bacterial response point of view as it has been normally used as a laboratory reagent in detection and estimation of nitrites from samples in ppb quantities (Gusberti, F. A. *et al* 1984; Ohyama, T., *et al*., 2000).

As an insoluble biosurfactant was produced by isolate NK2 in the presence of NND, achieving cells free of biosurfactant to carry out BATH test was difficult. However, BATH assay of the isolate was determined of 24hrs old cells, after inoculation of NND in PPY medium as no surfactants were produced at this time of incubation. The percentage adherence showed by the isolate at these times was found to be 16%.

TLCs of the ether extracts (Fig. 4.9) showed the presence of the substrate (NND) in medium incubated up to a period of 8 days (Rf 0.78), after which, it was completely transformed to a product having a Rf 0.48. Further incubation completely removed the product by 25 days to 1 month. The degradation products of NND were not identified. Less or no information on the degradation pattern of NND is available either by neutrophiles or by alkalophiles.

Fig. 4.9: TLC of PPY broth with NK2 & NND at various periods of incubation



1 0hr/ 8 days incubated
2 12 days incubated
3 18 days incubated
4 21 days incubated
5 25 days incubated
6 1 month incubated
NND Standard

While studying the effect of other parameters such as pH and salinity on the growth and surfactant production by the isolate, it was found that growth was seen in all the medium having pH values neutral to alkaline, but emulsion was produced only in media maintained at pH 10.5 showing surfactant effect, whereas added NND were seen as globules at the base of the flasks with pH of the media less than 10.5. Results were observed initially after an incubation period of 72 hrs and were continued up to a period of 7 days.

Incubating the culture in half strength PPY medium with various salt concentrations, growth was observed in medium containing 0-10% salt concentrations showing an emulsification activity, while 15% salt did not support growth.

4.2.5. *Isolation and characterization of surfactant*

Of the two methods used to isolate the surfactant, acidification method involves a multiple step procedure of addition of acid and base which leads to loss of the surfactant as it could not be pelleted easily. On the other hand, precipitation procedure using cold acetone was simple, fast with a good recovery of the surfactant. Acidification procedure of biosurfactant recovery showed the ability of the surfactant to change its solubility along with a change in pH, whereas, in the precipitation method, the surfactant could be easily salted out using cold acetone. It has been observed that proteins show such reactions wherein they could be salted out or could alter their solubility by changing the pH (Scopes, 2000). This may indicate that the surfactant could be a protein based such as lipoprotein, glycoprotein or may be a pure protein compound such as polypeptide compound. Although less or no information on biosurfactant production is available in the presence of NND, biosurfactants with

peptide moieties produced by various organisms have been reported by many authors (Morikawa, 1993; Razafindralambo, 1996; Lin, 1997; Koch *et al*, 1991; Miller and Zhang, 1997; Turkovskaya *et al.*, 2001). The only information on polypeptide biosurfactant has been reported by Turkovskaya (2001).

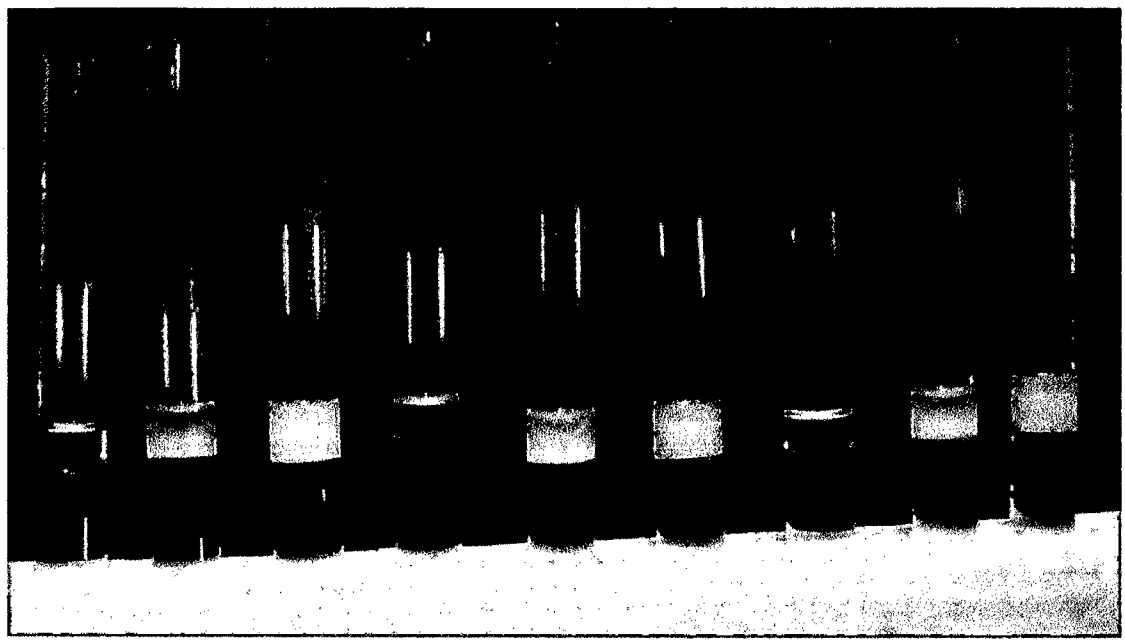
Initial attempts to dissolve the biosurfactant proved futile as the surfactant precipitate from the culture broth did not dissolve in water. Its insolubility in the culture broth may be attributed to high pH of the media along with the interaction between the surfactant and NND present in the medium to give a white emulsion. Water solubility was enhanced on separation and purification of the surfactant.

4.2.6. *Studies on biosurfactant*

Following the emulsification activity procedure by Cooper (1987), the emulsion index was found to be 0.013. Such a low value of E_{24} may indicate the inability of the pure surfactant or its presence in the culture broth to emulsify hexadecane. However, when supernatant/purified surfactant was vortexed with hexadecane (a total volume of 3ml with 0.5ml hexadecane), a thick interphase was observed which contradicts the emulsion activity assay results. The surfactant produced showed its emulsification activity under acidic, neutral and basic conditions (Fig. 4.10).

An interesting observation was noted while conducting experiments to determine the type of emulsion formed by the surfactant produced, on initial vortexing the contents in the test tubes formed a thick interphase, but, on allowing the tubes to stand for phase separation, no emulsion formed in the aqueous layer or in the organic solvent layer thus making it difficult to determine the type of emulsion

Fig. 4.10: Emulsification activity showed by the surfactant at pH conditions



A1 A2 A3 N1 N2 N3 B1 B2 B3

A1, B1, N1 Control tubes under acidic, neutral and basic pH.

A2, A3 Emulsification shown by under acidic condition

N2, N3 Emulsification shown by under neutral condition

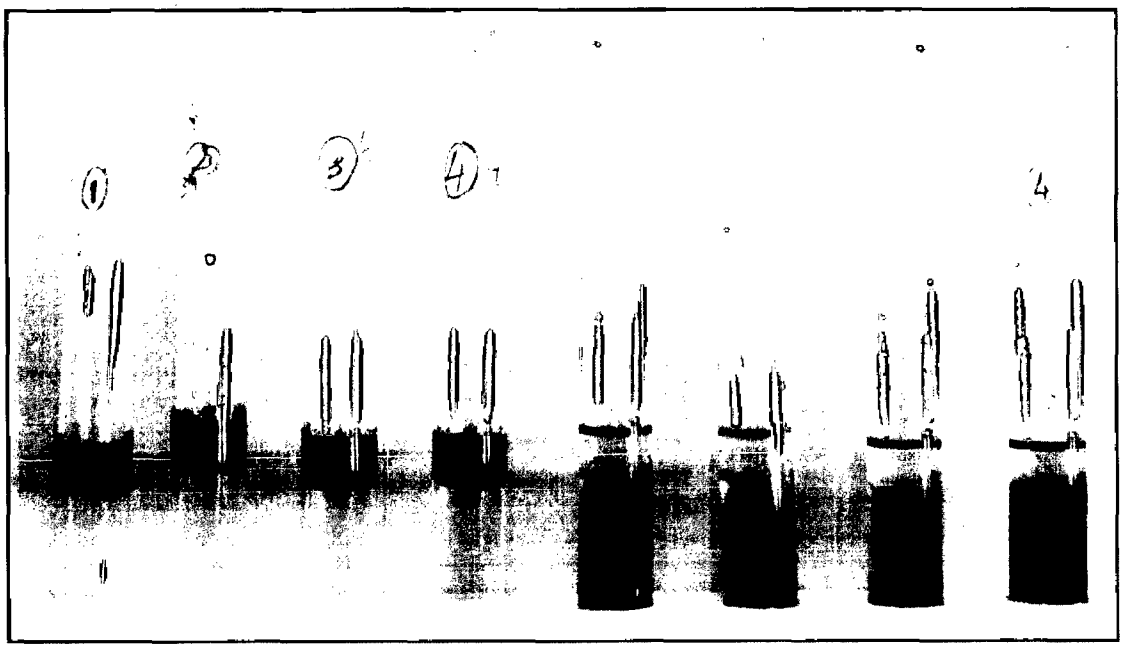
B2, B3 Emulsification shown by under basic condition

formed (water-in-oil or oil-in-water). Further, the interphase was comparatively thin to the interphase achieved during emulsification activity test (3ml volume). A significant observation was noted on further incubating the tubes used for determining the type of emulsion. The crystal violet present in the aqueous layer was decolourised, while, no decolourisation was observed in the organic layer containing oil-o-red (Fig. 4.11). On using NND in place of hexadecane as an organic substrate, emulsion activity showed the formation of an emulsion of oil-in-water type (Fig. 4.12) which was stable even after 24hrs. As the surfactant forms micelles with NND, a milky white emulsion is formed which may attribute to the insolubility of the surfactant.

The analytical procedures conducted on the biosurfactant revealed it to be a major protein constituent with no carbohydrate moieties. Further characterization of the surfactant was not carried out.

Most of the bacterial cells able to tolerate such high concentration of toxic compounds and its degradation are linked to an extra chromosomal plasmid. The absence of the plasmid in the isolate NRS-01 indicated that the property of tolerance to high concentration of aniline and production of the red compound was facilitated by chromosomal DNA. This characteristic also explained the reason for the culture not able to grow or produce intense colour on subsequent subculturing on solid media (MSM with aniline (0.1%)). Such a characteristic was not observed when the isolate was grown in the MSM in presence of glucose / acetate with aniline as they served as a source of carbon facilitating growth. Isolate NK2 showed the presence of plasmid of size 23kbp (Fig. 4.13), which may be contributing to the tolerance towards the aromatic amine, NND.

Fig. 4.11: Determination of the type of emulsion formed



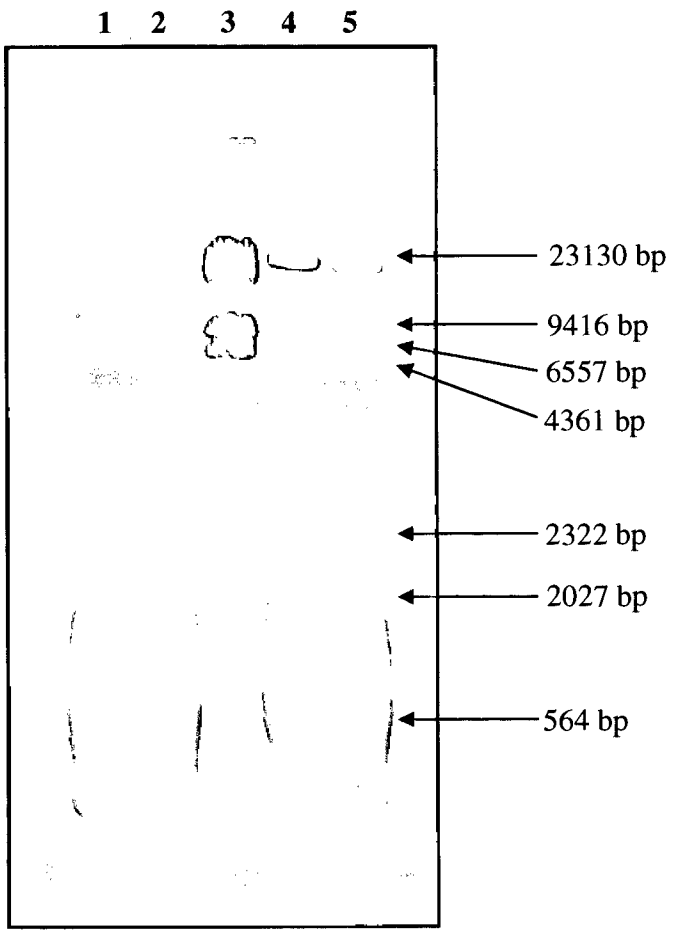
1 2 3 4 1 2 3 4

1 Culture broth (sample 1)
2 Culture broth (sample 2)
3 Centrifuged and filtered culture broth
4 Control (Sodium phosphate buffer pH 9.0)

Fig. 4.12: Emulsification shown by NK2 in the presence of NND

1 Control 1 - Phosphate buffer (pH 9.0)
2 Control 2 - Phosphate buffer (pH 9.0) + NND
3 Test 1 - Phosphate buffer (pH 9.0) + NND + pure surfactant
4 Control 2 - Phosphate buffer (pH 9.0) + NND + oil-O-red
5 Test 2 - Phosphate buffer (pH 9.0) + NND + pure surfactant + oil-O-red

Fig. 4.13: Determination of plasmid in NRS-01 and NK2



1, 2 NRS-01
4, 5 NK2
3 Molecular weight Ladder (λ DNA / *Hind* III Digest)

Both the cultures however, did not show any significant effect on the effluent. A consortium was therefore developed specifically which showed some effect on the effluent. However, further studies on this were not undertaken as the production unit of the herbicide industry had been discontinued.

NK2 however, showed a different response with the production of a biosurfactant which was responsible to the increase in cell substrate content. The biosurfactant was characterized as a protein compound.

The culture NRS-01 showed an unusual response in presence of aniline with the production of a red coloured product at 40hrs of incubation. This was preceded with aggregation of cells and homogenization with complete utilization of the carbon substrate – glucose.

The unusual response of NRS-01 on aniline with the production of red coloured product in the medium and simultaneous aggregation of the cells which is not reported hitherto, led to further studies. It was therefore of interest to study the effect of various concentration of aniline on NRS-01and studies of the enzymes which could be involved in such reactions. The results of which are compiled in the following chapter.

CHAPTER V

STUDIES ON EFFECT OF ANILINE ON ISOLATE NRS-01

Fate of aniline in the environment has been the subject of study and has resulted in the depiction of various ways for its detoxification by chemical, physical and biological methods (Lyons *et al.*, 1984, chapter II, Fig. 2.9). There have been many reports on degradation of aniline brought about by neutrophilic bacteria wherein aniline and its simple derivatives are transformed systematically by replacing the amino with hydroxyl group to form an intermediate – catechol; followed by cleavage of the benzene ring either at ortho- or meta-positions of the hydroxyl group, thereby mineralizing the aromatic hydrocarbon. Other responses observed in bacteria includes detoxification mechanism, wherein aniline is transformed to an anilide or condensed with other aromatic or aliphatic compounds to give a high molecular weight compound (Chapter VI, Figs. 6.8 & 6.9), whereas, the response of bacteria towards aniline in alkaline environment has not been well studied.

In the present study, it was observed that the isolated bacterium – *Halomonas* sp. NRS-01 produced a red coloured product in presence of aniline which gets diffused in the agar medium, (Chapter III, Fig 3.3). It was therefore of interest to study further the relation of this response to growth and concentration of aniline.

5.1. MATERIALS AND METHODS

5.1.1. *Preparation of preinoculum to study growth and utilization of aniline*

Preinoculum was prepared by making a suspension of the bacterial culture picked from a fresh PPYG plate (pH 10.5) incubated for a period of 24 hrs in normal saline (0.9% NaCl solution). The suspension was prepared so as to give an O.D. of 1.0 at 600nm (Shimadzu UV-1601 spectrophotometer).

5.1.2. *Growth response to aniline in the presence of an additional carbon source*

NRS-01 was inoculated in MSM medium (pH 10.5) containing sodium acetate and glucose at 0.1% as sole sources of carbon and incubated on a rotary shaker (180rpm) for a period of 18hrs at room temperature (30°C±2). 5% culture broth was inoculated in the liquid medium (MSM pH 10.5) with glucose or acetate as carbon source in the presence or absence of aniline. The flasks were incubated on a rotary shaker at room temperature, 180rpm. 5% inoculum was also inoculated in MSM containing glucose and sodium acetate as sole source of carbon at a final concentration of 0.1%. Growth was monitored as turbidity (600nm) and time at which the red colour appeared was noted.

5.1.3. *Growth response of the isolate using aniline as a sole source of carbon and nitrogen*

Cell suspension (0.1%) was inoculated in 20 ml basal medium (Mineral Salts Medium, pH 10.5) in a 100 ml side-arm flask with aniline at a final concentration of 0.1% (11mM). Glucose was added as carbon source to give a final concentration of 0.1%. Various growth conditions were maintained to study if aniline was used a sole source of carbon and/or nitrogen. A flask with medium devoid of nitrogen source (MSM-II) (Appendix-I, A.8.1) but containing aniline alone was used so that aniline could serve as a source of carbon and nitrogen, while, in the second flask, the medium (MSM-II) containing aniline as a sole of nitrogen and glucose as source of carbon. In the third flask, MSM medium containing 0.1% aniline as nitrogen source and glucose as carbon source was incorporated. The flasks were incubated on shaker at 180rpm,

30°C±2°C. Growth was noted as turbidity at various time intervals at 600nm using Elico Colorimeter.

5.1.4. *Utilization of aniline as a source of nitrogen*

Isolate NRS-01 was grown in MSM with glucose or sodium acetate (0.1%) as sole source of carbon. 5% of the culture broth was inoculated in MSM with sodium acetate or glucose containing aniline (0.1%) as a source of nitrogen. Growth was monitored as turbidity at 600nm and colour formation was noted.

5.1.5. *Growth response of the isolate to various concentrations of aniline.*

Utilization of aniline was studied concomitantly with growth of the culture. Cell suspension 0.1% (preinoculum O.D 1.0) was inoculated in a volume of 20 ml MSM, pH 10.5 in a 100 ml side-arm flask along with appropriate controls. Aniline was used at final concentrations of 0.05, 0.1, 0.5, 1, 5, and 11mM, with glucose as source of carbon at a concentration of 0.1%. The flasks were incubated at 30°C±2°C at 180rpm. One ml aliquots of culture broth were taken from the flasks and growth in terms of turbidity was noted every 12hrs at 600nm using Shimadzu UV-1601 spectrophotometer. The samples were centrifuged at 10,000rpm using Remi cooling centrifuge, supernatant scanned in the UV-VIS range and residual aniline was determined using the P-Dimethylaminobenzaldehyde (PDAB) method which is specific for primary aromatic amines.

5.1.6. *Determination of aniline*

5.1.6.1. p-Dimethylaminobenzaldehyde (PDAB) method

Aniline in the samples was determined using the procedure of Kupfer and Atkinson (1964). PDAB (1ml) (Appendix II, B.9.1) was added to the appropriately diluted samples made to a final volume of 5ml. Samples were mixed and incubated at room temperature for a period of 20min, and absorbance of the yellow colour developed was measured spectrophotometrically at 420nm. The concentrations of aniline present in the samples were determined using the standard graph plotted using aniline stock solution in alkaline MSM, ranging between 0 to 20µM. This method was also used for the detection and quantitative determination of indole; where the absorbance was read at 550nm.

5.1.6.2. Diazocoupling method (Thompson, 1970)

0.5ml of appropriately diluted sample was reacted with 1ml of 0.4% $NaNO_2$ for 3min at room temperature under acidic condition after adding 1ml of 1N HCl. Unreacted $NaNO_2$ was decomposed by adding 0.5ml (2%) ammonium sulfamate. The contents were allowed to react for 2min, followed by addition of 1ml N-Naphthylethylenediamine dihydrochloride (NEDA) (Appendix II, B.9.2). The contents were mixed and allowed to stand for 45mins at room temperature for completion of reaction and colour development, which was read at 560nm. Aniline concentrations were determined from a standard graph.

5.1.6.3 Hypochlorite method (Devi *et al.*, 2000)

To 5 ml of appropriately diluted sample, 0.2ml of phenol alcohol reagent was added and mixed, followed by addition of 0.2ml of sodium nitroprusside and 0.5ml oxidizing reagent (Appendix II, B.9.3). The contents were mixed and were allowed to form a blue colour for a period of 30min at room temperature. The blue colour developed was read at 640nm against a blank. Aniline concentrations were determined from a standard graph.

5.1.7. ***Characteristics of the culture broth (Response to aniline in MSM with glucose as source of carbon)***

Culture (0.1% of preinoculum) was inoculated in 300ml of basal medium containing final concentrations of 0.1mM and 11mM aniline in a 1000ml conical flask. Glucose was added as additional carbon source. The flasks were incubated at 30°C±2°C on a shaker at 180rpm. 5ml aliquots of the broth were collected from the flasks every two hours. Growth was determined as turbidity at 600nm using Shimadzu UV1601 spectrophotometer. The samples were then centrifuged at 10,000rpm and the supernatant was used to determine the residual glucose (Somogyi-Nelson method), production of extracellular proteins (Bradford's method), total carbohydrates (Phenol-sulphuric acid method) and to detect the formation of catechol (Arnow's method). Aniline concentration was determined using HPLC. Spectrofluorophotometric scan of the supernatants were taken to detect the presence of other products formed during growth using Shimadzu FC-5301 spectrofluorophotometer.

5.1.7.1. Reducing sugars estimation (Somogyi, 1952)

To 1ml of appropriately diluted samples, 1ml of alkaline reagent was added and kept in boiling water bath for 10mins. The samples were cooled under tap water and 1ml of arsenic-molybdate solution (Appendix II, B.10) was added. Total volume was made to 10ml with distilled water, mixed and absorbance noted at 510nm after 5 to 10mins using the Shimadzu UV-1601 spectrophotometer. Reducing sugars was estimated using a standard graph. Glucose was used to prepare a standard graph.

5.1.7.2. Protein estimation (Bradford, 1976)

Extracellular proteins were estimated following a modified Bradford's method. Bradford's reagent (100µl) (Appendix II, B.11) was added to 20µl of sample diluted to 400µl with distilled water. The reaction mixture was allowed to stand for 2 – 3 minutes before adding 100µl of 1N HCl and was diluted to a final volume of 1000µl. The absorbance was read at 590nm using Shimadzu UV-1601 spectrophotometer. Protein standard graph was prepared in MSM using Bovine Serum Albumin ranging between 0 – 5µg/ml.

5.1.7.3. Total carbohydrates (Dubois *et al.*, 1956)

One ml sample was mixed with 1ml of 5% aqueous phenol. The contents were mixed and the reaction mixture was kept for 5min. 5ml concentrated sulfuric acid was added to the reaction mixture, mixed and was allowed to stand for 5min for colour development (orange-yellow colour). The test tubes were cooled in an ice bath for 10 minutes before reading the absorbance spectrophotometrically at 490nm. The results were compared with standard prepared using glucose.

5.1.7.4. Detection of Catechol (Arnow, 1937)

One ml samples were acidified with 1ml of 0.5M HCl followed by addition of 1ml Nitrite-Molybdate solution (Appendix-II, B.12). 1ml of 1N NaOH was added after the tubes were incubated for 5mins. The contents were mixed and the final pink colour developed was read spectrophotometrically at 505nm after 15 – 30min.

5.1.8. *Aniline degradation studies*

Mineral salts medium (pH 10.5) with glucose (0.1%) (100ml) was taken in seven (250ml) flasks. 0.1% of the culture inoculum was inoculated and the flasks were incubated at room temperature (30°C±2°C) on the shaker at 180rpm. These flasks were removed from the shaker at different stages of growth and the time of incubation was noted. The culture broth from each flask was centrifuged at 10,000rpm to separate the cells. The supernatant was filtered through the Millipore membrane filter (0.22μ) before extraction. Cell pellet was washed with phosphate buffer (pH 9.0) twice and was used for extraction. Unreacted filtered broth was used to estimate residual aniline using HPLC.

5.1.8.1. Extraction of cell pellet

The washed cell pellets were suspended in phosphate buffer (pH 9.0) and extracted thrice using diethyl ether. The extracts were pooled, dried over sodium sulphate and vacuum evaporated. The residue was dried in a vacuum desiccator for a period of 15 minutes or until the residual moisture was completely removed and were redissolved or resuspended in a minimum volume of the diethyl ether which was used

for carrying out TLC studies. Rest of the residue was collected in acid cleaned dry screw capped glass vials and stored at a low temperature (4°C) until further use.

5.1.8.2. Extraction of supernatant

Filtered supernatants were extracted in series with pet ether (60° - 80°) and diethyl ether, followed by acidification to pH 2.0 using 10N HCl then extracted with pet ether and diethyl ether. All the solvents were distilled prior to use. All the extractions were carried out in triplicates using each solvent and the respective extracts were pooled, dried over sodium sulphate and were concentrated. The dried extracts were dissolved in a minimum quantity of respective solvent and were stored in acid cleaned screw capped vials.

5.1.8.3. Determination aniline in cell pellet extracts and culture broth (Liu, 2002)

Supernatants at various time intervals were taken from flasks containing 0.1mM and 11mM aniline filtered, appropriately diluted and the aniline was determined at 230nm by a UV-detector using HPLC equipped with C-18 reverse phase column (10cm long). The elution rate was maintained at 1.5ml/min using methanol : water (75 : 25) as an eluent. Under these conditions the retention time of aniline was about 1.5min.

The cell pellet extracts were filtered, diluted appropriately and analysed using HPLC as described above. The Retention time (Rt) of the eluted compounds were noted.

5.1.8.4. Spectrofluorophotometric analysis of supernatant

Non-extracted aliquots of supernatants were used to carry out spectrophotofluorimetric analysis. Samples were appropriately diluted with distilled water and were scanned to determine the optimum excitation and emission wavelengths. Using the optimum excitation wavelength, spectral analysis of the supernatant was carried out.

5.1.9. *Role of extracellular enzymes in the production of red coloured compound*

Isolate NRS-01 was inoculated in 100ml MSM medium with aniline (0.1%) and glucose (0.1%). The culture was incubated on the shaker at 180rpm at room temperature for a period of 30hrs (a stage where aggregation is initiated with no pink colour colour). 50ml of the culture broth was centrifuged under sterile condition, filtered through sterile 0.22μ Millipore membrane filter and filtrate was collected in a sterile flask. The original flask containing the whole medium with cells and the flask containing the filtrate were incubated further on the shaker for a period of 18-24hrs and the colour formation was noted.

5.1.10. *Dependence of the isolate NRS-01 on the carbon source for the production of fluorescent compound*

Isolate *Halomonas* sp. NRS-01 was inoculated in MSM medium containing aniline with glucose as carbon source (Flask A1) and in medium with glucose but without aniline (Flask B1). Similarly, the isolate was inoculated in MSM medium with sodium acetate as carbon source (Flasks C1 and D1). The flasks were incubated for a period of 18hrs and were subcultured in MSM and MSM-II medium with

glucose and aniline (Flasks Aa / Ba and Ab / Bb) and with sodium acetate and aniline (Flasks Ca/Da and Cb/Db). Fluorescence was observed visually under UV light (230 nm) and using a spectrofluorophotometer after an incubation period of 24, 30 and 48hrs.

5.1.11. *Rothera's Test*

Cells were grown in mineral salts medium with aniline (0.1mM as well as 11mM) and glucose as carbon source. Cells were simultaneously grown in benzoate as sole carbon source. Cells were harvested from aniline containing medium after an incubation period of 28-30hrs, after glucose in the medium was completely utilized. The cell pellet was washed, resuspended in phosphate buffer pH 8.0 and was used to carry out rothera's test. Benzoate grown cells were harvested after an incubation period of 16hrs, washed, resuspended in phosphate buffer pH 8.0 and used for rothera's test.

To 1ml cell suspension, 2ml of catechol solution (1mM aqueous) was added and vortexed for 5min. To this suspension, 0.5ml of toluene was added and shaken. The colour of the organic layer was noted 2-3min after shaking with toluene. Development of yellow colour in the organic layer would demonstrate the production of Hydroxy Methyl Semialdehyde (HMS) indicating a degradation product following a Meta-cleavage pathway. The test tubes were immediately wrapped with dark paper and incubated at 30±2°C for 3-4hrs. The mixture in the tubes was saturated with ammonium sulfate followed by addition of 5-6 drops of sodium nitroprusside (1% aqueous) along the walls of the test tube followed by addition of ammonia solution (0.5ml) along the walls of the tube. A purple ring developed at the interphase would

indicate the presence of β-ketoadipate- a degradation product of catechol following an Ortho-cleavage pathway. Phosphate buffer (pH 8.0) without cells was used as a control.

5.1.12. *Enzyme assays*

5.1.12.1. Aniline oxygenase (Fukumori *et al.*, 1997) / **Aniline 1,2 Dioxygenase assay** (Liu *et al.*, 2002)

Aniline oxygenase / dioxygenase activity was monitored based on oxygen uptake using a Clarke's electrode (oxygen electrode -Hansatech, United Kingdom). Isolate NRS-01 was harvested from MSM medium containing two different aniline concentrations (0.1mM and 11mM) in the presence of glucose as carbon source, incubated for 24hrs and between 30 and 32hrs (before initiation of cell aggregation and after complete utilization of glucose) respectively. Cells were washed and resuspended in 0.1M sodium phosphate buffer (pH 9.0), to a dry weight of 14mg ml^{-1}. Aniline-dependent oxygen uptake rate (aniline oxygenase and aniline-1,2-dioxygenase) was measured in the sodium phosphate buffer (1.5ml) containing 0.1 ml of the resting cells at 27°C after an endogenous rate was estimated. Aniline was routinely added at a final concentration of 3mM.

The rate of oxygen consumption by the culture grown in presence of glucose and benzoate as sole carbon sources was used to compare the oxygen consumption rates with that of aniline grown cells. The rate of oxygen consumption was measured as nmoles/min.

5.1.12.2. Aniline-Deaminase assay

a) Modified Weatherburn's method

The release of ammonia from aniline brought about by the enzyme aniline-deaminase was studied by a modified Weatherburn's method (Weatherburn, 1967 and Fukumori *et al.*, 1997). Culture NRS-1 grown in MSM with 0.1mM and 11mM aniline) (100ml) in presence of glucose, were washed and resuspended in 10ml of 0.1M sodium phosphate buffer (pH 9.0) and was used as resting cells to perform deaminase test. To 10m of resting cells, 0.6mM aniline was added and incubated on a shaker (180rpm).. After incubation for a period of 6hrs at room temperature, 0.5ml of the culture broth was centrifuged at 10,000 rpm, supernatant collected in a fresh tube and extracted with 0.3ml of chloroform. The ammonia released in the aqueous layer was estimated by the sensitive phenol-hypochlorite method to test for ammonia (Weatherburn's method) (AppendixII B.13).

To 20µl of the extracted sample, 5ml of reagent A and 5ml of reagent B (Appendix II, B.13) were added sequentially, mixed and were either incubated at room temperature for 40min or for 20min at 37°C. Absorbance of the blue colour developed was read at 625nm.

b) Conway method

Enzyme aniline-deaminase activity was studied by carrying out a modification of Conway method (Aoki *et al.*, 1983; Ballentine, 1957, Conway, 1933).

To the inner cavity of the Conway plate, 2-3 drops of methyl red indicator was added to 1ml of 0.001N H_2SO_4. The outer cavity contained 1ml of washed cell suspension (dry weight of 14 mg ml^{-1}-protocol as followed in section 5.1.12.1). Cells

used were obtained from a 24 hr old growth medium containing 0.1mM aniline, while cells grown in 11mM concentrations were obtained after an incubation period of 30hrs (no red colour not formed), and 45hrs (red colour formed). Aniline at final concentration of 3mM and saturated sodium carbonate solution were independently added to the outer cavity without mixing with the cell suspension or each other. The plate was covered immediately and the contents were mixed by shaking the plates in circular motion. The plates were incubated for 1hr at room temperature and the ammonia released was determined based on the amount of 0.0005N NaOH required to neutralize the residual unreacted sulphuric acid through an acid-base titration with an end-point colour change of the indicator turning from pink under acidic to yellow under alkaline condition. Appropriate controls were maintained.

5.1.12.3. Catechol 1,2 dioxygenase assay

Cells grown in 0.1mM and 11mM aniline in the presence of glucose were harvested after an incubation period of 24 and 32hrs respectively and washed twice with 0.1M sodium phosphate buffer (pH 8.0). Whole cells suspended in phosphate buffer as well as cell free lysate (CFL) (washed cells were sonicated under cold temperature at 196 decibels for 10 minutes with 1min break between pulses, centrifuged at 10,000rpm for 10min, supernatant was used as crude enzyme) were used to carry out the enzyme assay. Cells grown in benzoate were also checked for catechol-1,2-dioxygenase activity.

Catechol-1,2-dioxygenase enzyme assay was carried out spectrophotometrically as explained by Liu *et al.* (2002). The reaction mixture (total 3.0ml) contained 2.0ml phosphate buffer (pH 9.0), 0.6ml 1mM catechol, 0.2ml

deionized water and 0.2ml whole cells. Assay carried out using cell free lysate replaced the whole cells. The assay reactants without the cells or CFL were placed in reference and test cuvettes and the signals were neutralized. Whole cell or CFL were added in the test cuvette and the formation of 260nm peak was noted at fixed time intervals.

5.1.12.4. *Detection for the production of free radicals from aniline*

In order to detect the formation of free radicals of aniline, an indirect method was used wherein the free radicals were trapped using a second aromatic compound such as β-naphthol (Corke *et al.*, 1979). For the present study, this method was modified and β-naphthol- the radical trapping agent was added to resting cells. Cells were harvested from 100 ml of culture broth (30-32hrs of incubation), washed twice with 0.1M sodium phosphate buffer (pH 9.0) and resuspended in 20 ml of sodium phosphate buffer (pH 9.0) and was used as resting cells. Three flasks were maintained; first flask contained aniline as well as β-naphthol, flask2 contained only anline and flask 3 contained only β-naphthol in the basal phosphate buffer with cells. The pink colour formation was observed after various periods of incubation spectrophotometrically.

5.1.12.5. *Peroxidase enzyme detection*

One of the enzymes that bring about condensation reaction is peroxidase. The peroxidase activity in the cells was checked by following a method proposed by Elnemma, (2004). The protocol was modified in which, cell suspension played the role of hydrogen peroxide. 3.0 ml hydroquinone solution (0.25M) was treated with

0.5ml cell pellet in place of 0.1 ml of hydrogen peroxide. 0.1ml of (0.5%) ammonium molybdate was added. The reaction mixture was vortexed and allowed to stand. The results were noted as spectrophotometric scans in the range 350nm-700nm.

5.1.13. *Utilization studies of other related aromatic compound*

5.1.13.1. Oxygen uptake studies

Cells were harvested from the medium with 0.1mM aniline after an incubation period of 24hrs and after 30 and 36 hours of incubation from media containing 11mM aniline. Cells were prepared for the experiment as explained in section 5.1.12.1.The reaction mixture contained 1.25ml of 0.1M sodium phosphate buffer (pH 8.5), 0.2ml of washed cell suspension and 0.05ml of the respective substrates solution to make a final volume of 1.5ml. Endogenous respiration of the cells was noted and 0.05ml of a 5mg/ml stock solution of each substrate was added in to the reaction chamber. The oxygen utilized by the whole cells in the presence of the substrates was calculated as nmoles/min after correction with the endogenous respiration.

5.1.13.2. Growth tests on various aromatic amines

Isolate NRS-01 was tested for growth on agar medium containing 0.05% of various aromatic amines such as anisidine, p-aminobenzoic acid, p-toluidine, o,m,p-aminophenol, and non aromatic amines such as catechol, gentisate, homogentisate and sodium benzoate. The amines selected were the same used in the oxygen uptake studies. The amines were used as a sole source of carbon in MSM medium having pH 10.5. The plates were incubated for a period of 1wk and the results noted were in the form of growth and colour (pigment) formation.

5.1.14. *Whole cell protein profile (Native PAGE)*

Extraction of the whole cell proteins from the cells was carried out as reported by Nakamura *et al.,* 1991, with a slight modification. The cells were harvested from 50ml mineral salts medium containing aniline and glucose, washed and resuspended in 5ml phosphate buffer (pH 8.0). One ml of the suspension was used for the protein study. Cells harvested at various growth stages i.e. at 28-30hrs and at 40hrs were compared with cells grown in glucose and in PPYG medium.

To one ml of cell pellet, 100µl of 2% SDS was added, vortexed and was incubated for 1hr. The treated cells were further vortexed and to the reaction mixture, 100µl of sample treatment buffer (Appendix III, C.6) was added, vortexed and kept in a boiling water bath for 5 min, cooled and centrifuged at 10,000rpm. The aqueous phase was later separated and stored in a sterile vial at -20°C. Protein samples of 60 – 70µl was mixed with a loading dye in a 10% acrylamide gel (Appendix III, C.6). The gel was run with 25mV current for 4 hrs. The gel was then stained with coomassie blue stain, destained with destaining solutions (Appendix III, C.6), washed and dried using a gel dryer (Lyo Freezer, Heto, Germany). Gel drying was carried out at 45-50°C for 4hrs between the gel sealing plastic papers provided along with the instrument.

5.2. RESULTS AND DISCUSSION

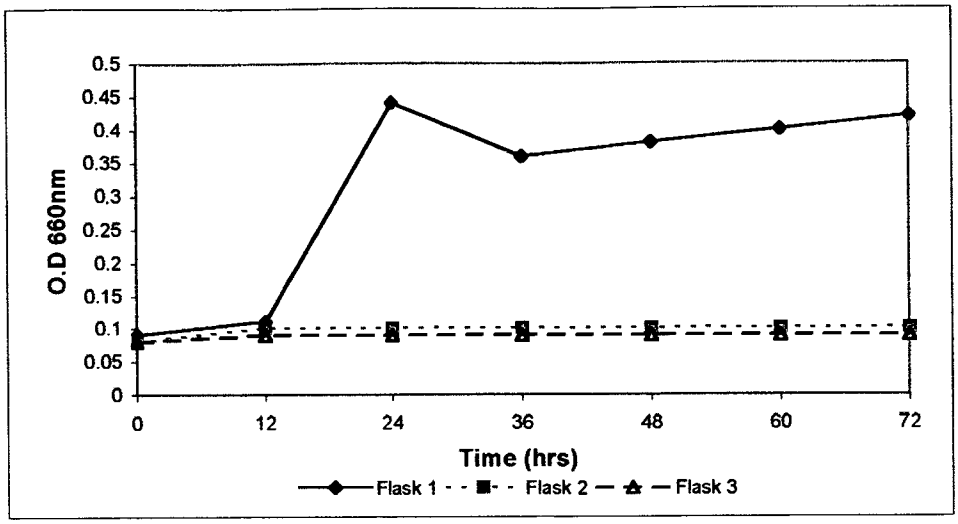
5.2.1. *Growth studies*

Isolate *Halomonas* sp. NRS-01 showed a difference in growth characteristics when incubated in medium where aniline was provided as a sole source of nitrogen and carbon plus nitrogen. Flask 1 containing glucose as an additional source of carbon

and ammonium nitrate as a source of nitrogen in response to aniline showed good growth (Fig. 5.1) having a long lag phase of 12hrs followed by exponential phase between 12hrs to 24hrs, with marked changes occurring in the culture broth after which the turbidity was found to decrease. A log phase extended to a period of 12hrs (incubation period of 24 hrs), during this period, the cells were found to aggregate but were released to normal after 36-48 hrs. This aggregation was accompanied with the formation of red colour on the cells at the onset of stationary phase at 24hrs. Flasks 2 & 3, where aniline served as a source of nitrogen and carbon plus nitrogen respectively did not support growth. Medium having ammonium nitrate as a sole source of nitrogen gave a tinge of yellow colour by the end of 72 hrs with no increase in growth as turbidity. While, flask 3 where aniline served as a sole source of carbon and nitrogen, the medium turned light pink (Fig. 5.2) after an incubation period of 12hrs which showed a similarity to the colour formation in flask 1 during its stage of release of aggregation. This colour did not intensify with an increase in period of incubation. These studies indicate the inability of the culture to utilize aniline as a sole source of carbon or nitrogen and require an additional carbon source to facilitate good growth in the presence of high concentration of aniline.

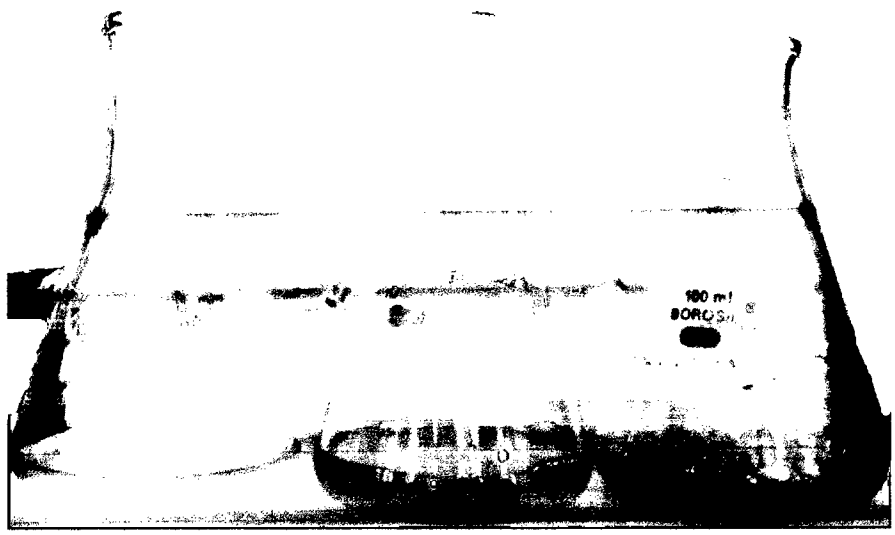
Two different carbon sources were used to compare their potency to facilitate growth of isolate NRS-01; glucose a six carbon sugar and easily utilizable 3-carbon compound sodium acetate. The significance of this study was to determine the requirement of a carbon source by the culture to produce good growth in presence of aniline. Flask 3 (Fig. 5.1) showed no growth in the form of turbidity, thereby rendering inability in studying various morphological, physiological and the

Fig. 5.1: Growth response of NRS-01 using aniline as a sole source of carbon and nitrogen



Flask 1 MSM + Glucose (0.1%) + Aniline (10mM) - Glucose as an additional source of carbon
Flask 2 MSM2 + Glucose (0.1%) + Aniline (10mM) - Aniline as sole source of nitrogen
Flask 3 MSM2 + Aniline (10mM) - Aniline as sole source of carbon and nitrogen

Fig. 5.2: Isolate NRS-01 grown in various growth conditions



Flask 3 **Flask 2** **Flaks 1**

interaction of culture with aniline. Addition of a carbon source was also aimed to bring about cometabolism to utilize aniline in the presence of glucose or sodium acetate and thus help in studying the transformation or degradation process. The utilization of glucose to facilitate the degradation of aromatic compounds has been demonstrated by Aranda *et al.*, (1999) where *Pseudomonas paucimobilis* utilized glucose to enhance the growth of 2,4,6-tricholorphenol. *Pseudomonas putida* utilized glucose to cometabolize other aromatic compounds was reported by Reber and Kaiser (1981); Horvath (1973).

Growth in the presence of glucose in the medium containing aniline (0.1%) showed a lag phase of 10hrs indicating the time required by the culture to adapt themselves to aniline, whereas in the absence of aniline, a short lag phase of 4 hrs was observed. Although growth differed in the period of lag phase, no change in the intensity of growth was observed showing its tolerance to high concentrations of aniline. Such an effect has been reported by Steinle *et al.*, 1998; Genthner and Bryant, 1987. Sodium acetate on the other hand, did not support good growth in the presence of aniline as compared to glucose, though it was utilized as a sole source of carbon in the absence of aniline. Figures 5.3a & b shows a comparative chart of growth in glucose and acetate in the presence or absence of aniline.

Bacterial growth depends on the availability of carbon and nitrogen source for its normal physiological and metabolic functioning. On incubating the culture in medium with aniline as a source of carbon and nitrogen along with an additional carbon source (glucose / acetate), showed a varied growth response. Flasks containing acetate as an additional carbon source (figure not shown) surprisingly showed a better

Fig 5.3a: Growth curve of NRS-01 in glucose in the presence and absence of aniline

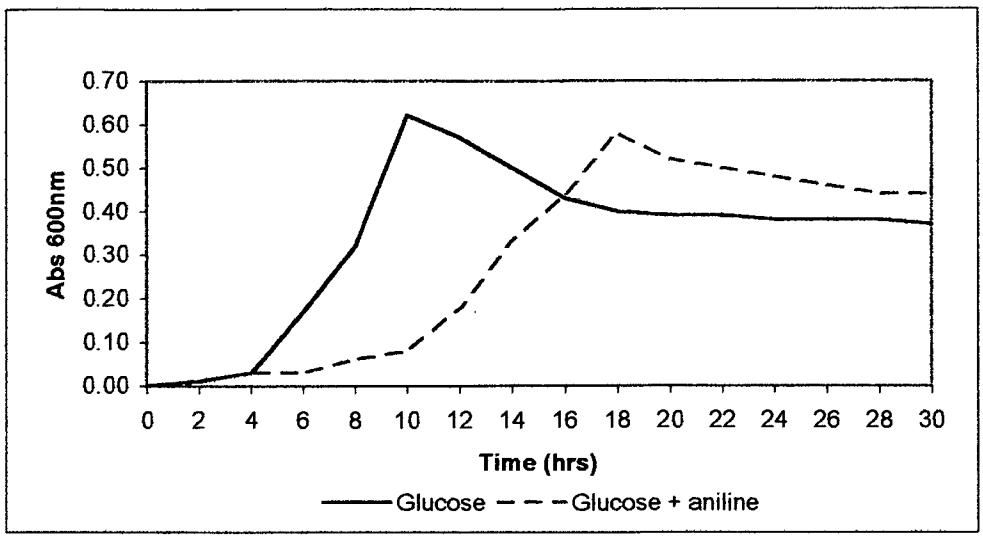
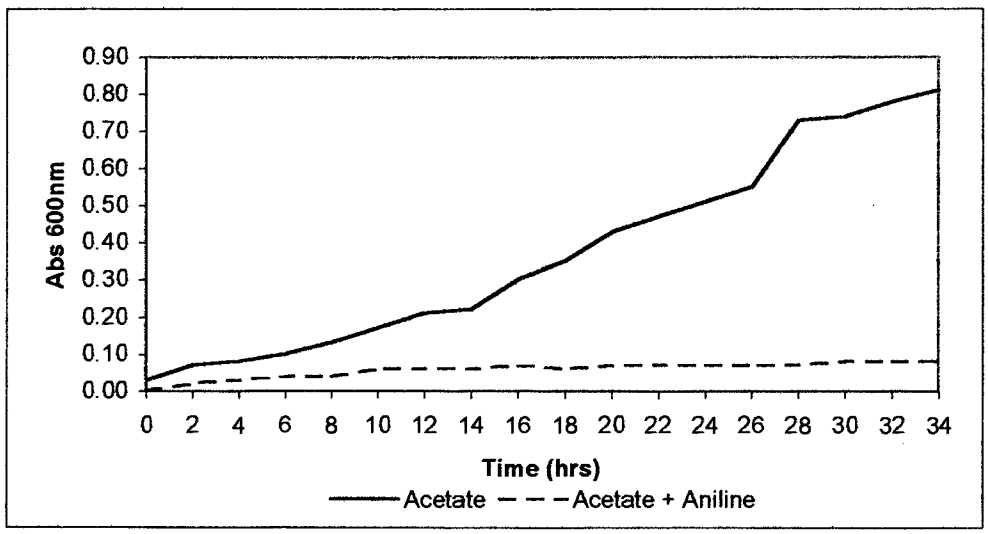


Fig 5.3b: Growth curve of NRS-01 in acetate in the presence and absence of aniline



growth in the absence of added nitrogen (MSMII) as compared to the growth in its presence (MSM). However, on further subculturing the isolate in a similar medium (MSMII) showed a retarded growth which indicated the carryover of added nitrogen from the original growth flask. Having glucose as a source of carbon in the medium devoid of nitrogen (MSMII) did not support growth while presence of added nitrogen (MSM), facilitated growth (Fig. 5.4). This inability of the bacterial culture to grow in MSM II in presence of aniline showed their inability to utilize aniline as a source of nitrogen. Bacteria such *Pseudomonas* utilizing aromatic amines as source of nitrogen has been reported by Mallavarapu *et al.*,1998; Oranusi and ogugbue, (2005); Cuskey *et al.*,1987.

Having shown good growth in the presence of glucose and aniline, as compared to sodium acetate, MSM medium was selected as basal medium with added nitrogen in the form of ammonium nitrate and glucose as carbon source to study its interaction with aniline.

Change in aniline concentration influenced the growth response of the isolate NRS-01. Several characteristic responses were visible during its growth in the presence of aniline, which included its general growth behaviour, production of a pigmented compound, production of metabolites.

Although growth was observed in all the concentrations of aniline added, the culture showed a difference in the lag phase; lowest being noted with 0.05mM concentration (not determined) and highest of 12hrs at 11mM concentration (Fig. 5.5a&b). The drop in the absorbance (turbidity) during the onset of stationary phase of growth at low aniline concentration (0.05mM) attributed to aggregation of cells.

Fig 5.4: Growth pattern of NRS-01 in MSM in the presence and absence of added nitrogen

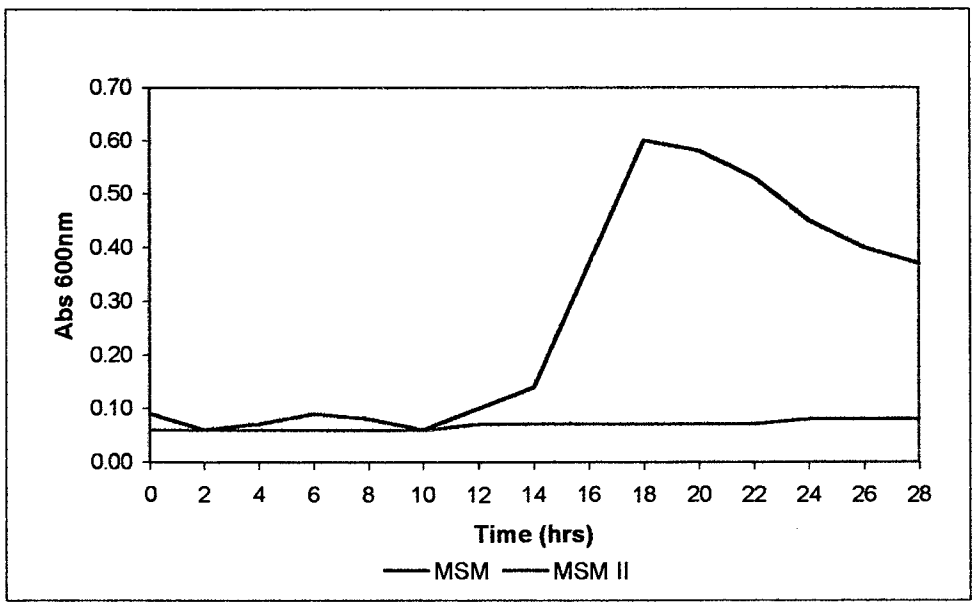
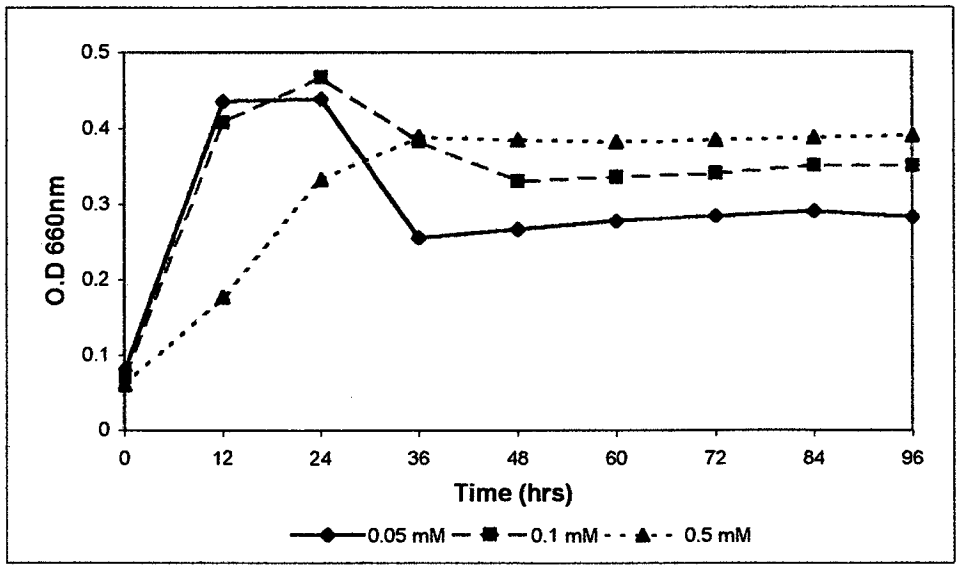
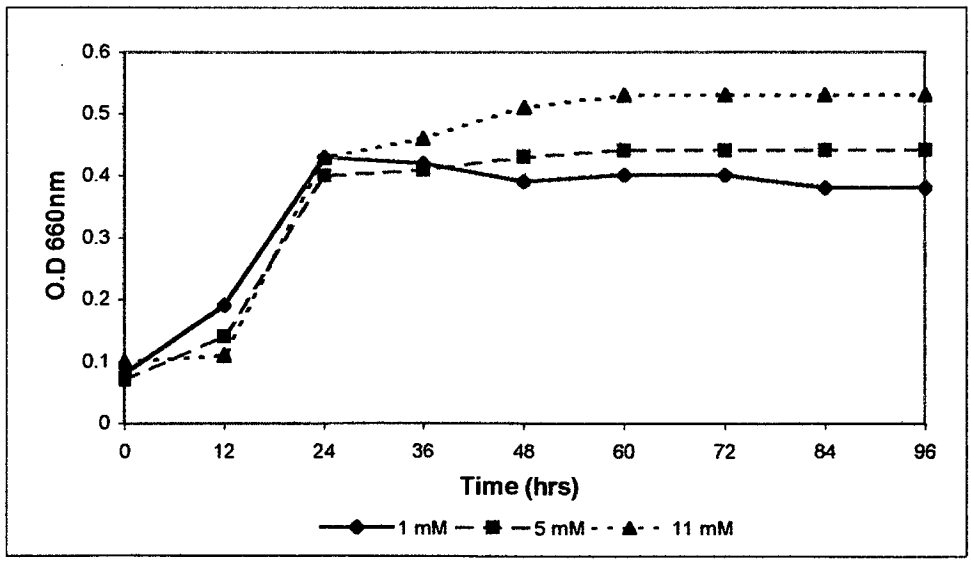


Fig 5.5: Graphs showing growth of NRS-01 at various aniline concentrations

a



b



These cellular aggregates formed at 0.05 and 0.1mM concentrations were found to be stable over a longer period of incubation (24hrs) as compared to those formed at a higher aniline concentration (above 0.5mM).

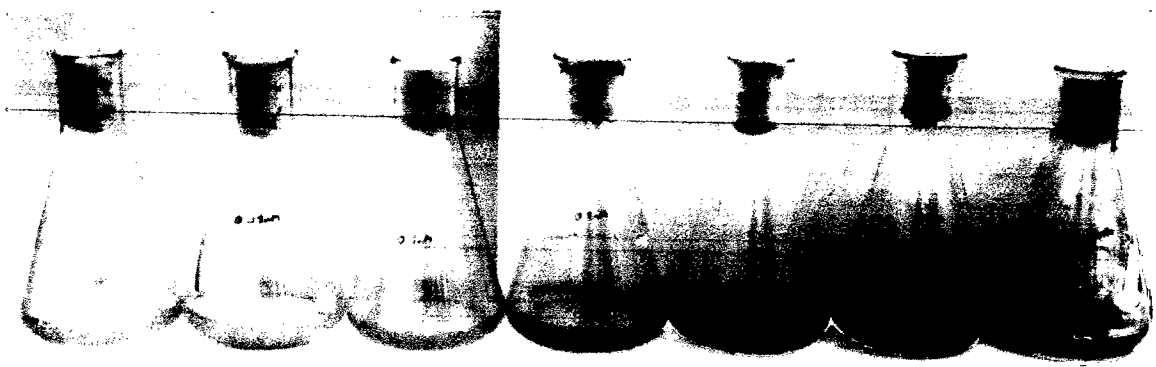
An interesting feature depicted by the isolate is the formation of a red pigmented compound in the medium of which the intensity increased with an increase in aniline concentration (Fig. 5.6). Red colour was negligible or not visible at concentration 0.05mM.

Spectrophotometric scans of the growth medium (Fig 5.7 a-f) revealed the production of a metabolite having absorption maxima of 260nm in the medium containing minimum quantity of aniline (Fig. 5.7a). The production of this metabolite was lowered with increasing aniline concentration with no production of this compound at aniline concentration of 11mM (Fig. 5.7f).

Studying the degradation of aromatic compounds, one of the intermediates that are produced during the ortho-cleavage of the aromatic ring is muconic acid which has a characteristic absorbance maximum at 260nm (Kojima *et al.*, 1961; Zeyer *et al.*, 1985). The production of muconic acid during the growth of NRS-01 in the presence of aniline and glucose was ruled out as the culture produced this metabolite (having an absorbance at 260nm) in the absence of aniline, indicating it to be a primary metabolite of the isolate and not a cleavage product of aniline.

Primary metabolites are the small molecules of living cells; they are intermediates or end products of the pathways of intermediary metabolism, building blocks for essential macromolecules or are converted into coenzymes (Demain, 2000). These include alcohols, amino acids, organic acids, sugars, polysaccharids, polyols, vitamins, etc. Such primary metabolites are reported to be produced by organisms

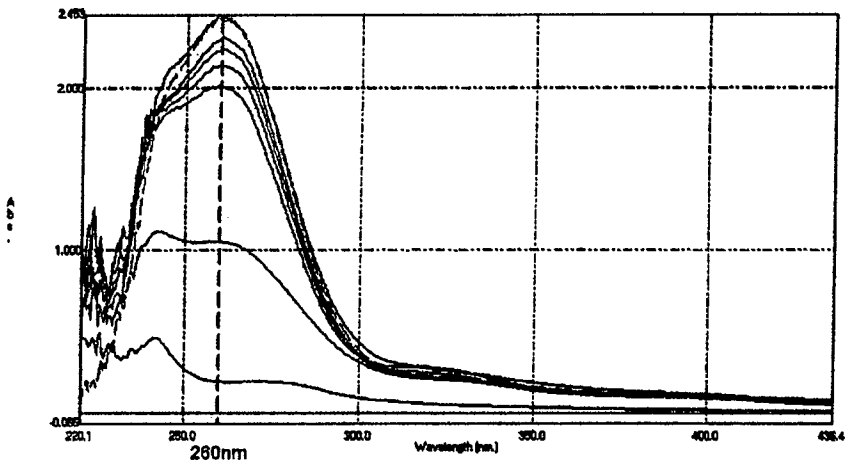
Fig. 5.6: Increasing concentrations of red compound in media with increasing aniline concentrations



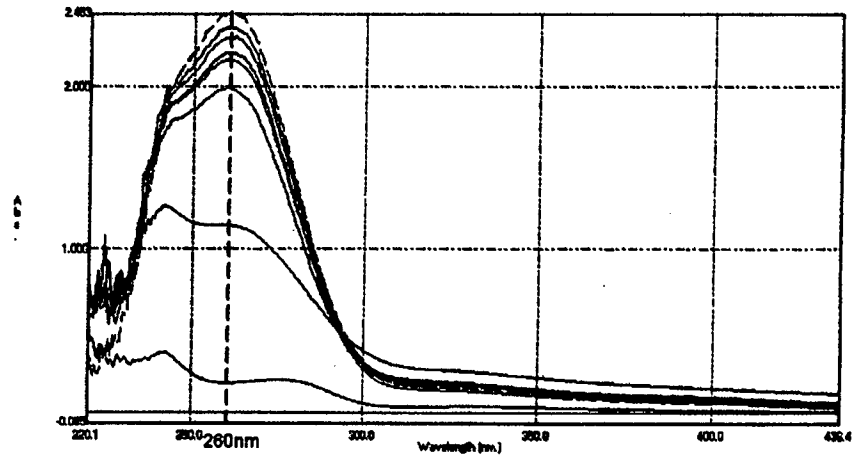
0mM 0.05mM 0.1mM 0.5mM 1mM 5mM 11mM

Fig. 5.7: Scans of supernatant showing 260nm peak at various incubation periods (with various aniline concentratio

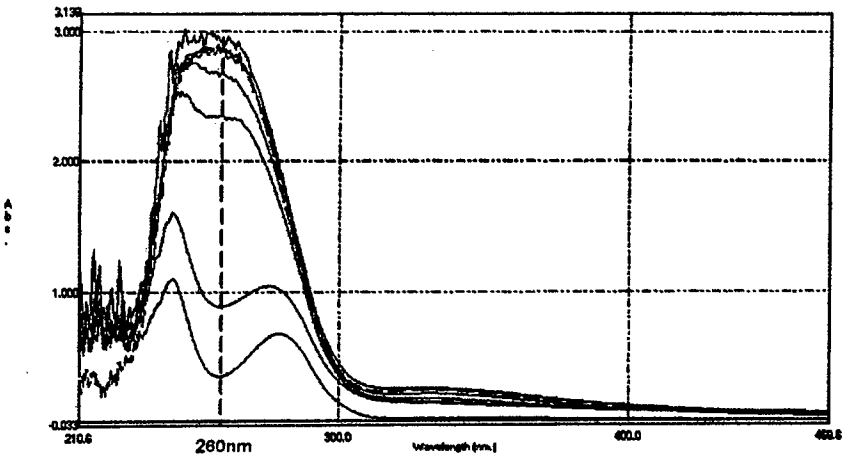
a-0.05mM



b-0.1mM



c-0.5mM



d-1mM

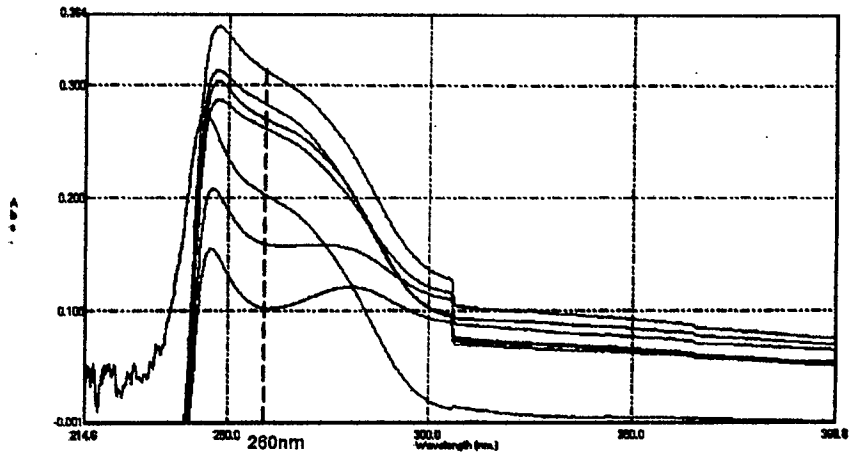
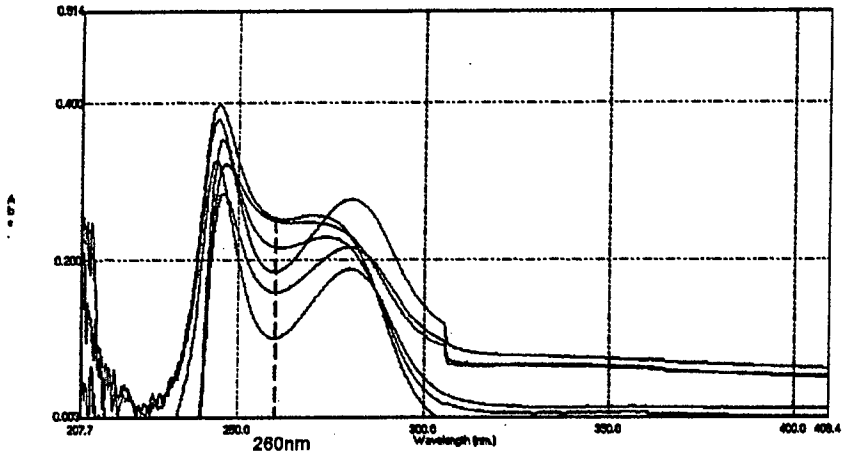
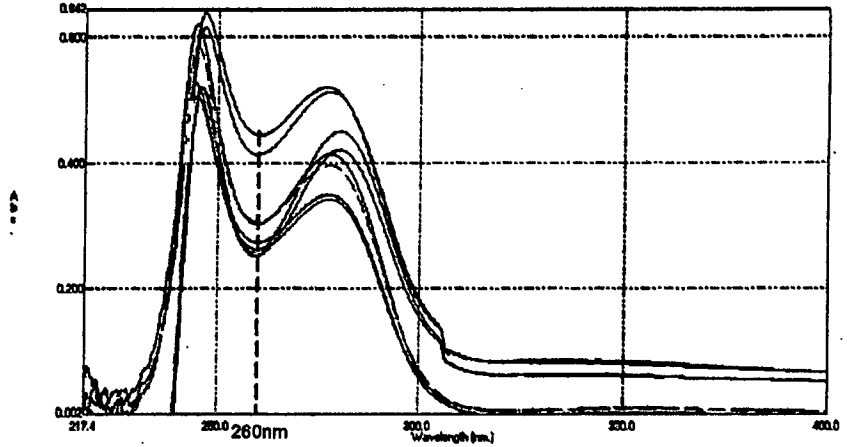


Fig. 5.7: Spectrophotometric scans of supernatant (various aniline concentrations) at various incubation periods

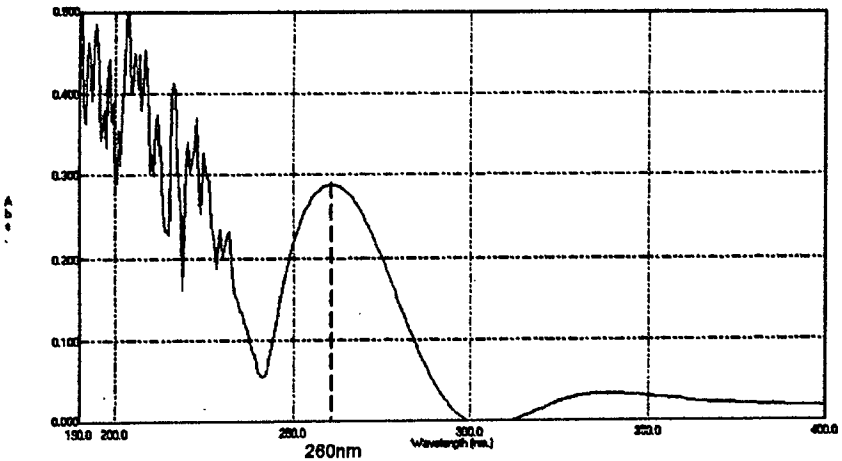
e-5mM



f-11mM



g-Cells grown in presence of glucose as sole source of carbon



such as *E. coli* (Carter *et al.*, 2003) which was genetically modified for commercial purposes. Guerra *et al.*, 2001, has reported the production of bacteriocins as primary metabolites by lactic acid bacteria in the presence of whey.

5.2.2. *Culture broth characteritics*

5.2.2.1. Isolate NRS-01 grown in 11mM aniline

The growth of the isolate in the medium containing 11mM aniline (fig. 5.8 a & b) showed a lag phase extending up to 14-16 hrs, followed by a sharp exponential phase between 18hrs to 28 hrs, followed by stationary phase observed from 28hrs wherein there was a sudden decrease in the turbidity due to the aggregation of cells in the broth which was found to be a characteristic of the isolate. As observed in growth response studies, the development of a red colouration on the cells was noted at 36^{th} hour of incubation which varied from 6 to 8 hrs from the initiation of aggregation. Further incubation (over 40hrs) dispersed the cell aggregates and the red colour intensified. Growth was monitored by standard plate count method after 28hrs as the spectrophotometric values yielded erratic results due to aggregation. It was observed that the stage where the turbidity was highest (27^{th} hour of incubation) had a bacterial population of $5x10^7$ CFU. Due to aggregation, the colony count was made after the aggregates dispersed i.e. at 72 hrs of incubation which showed a CFU value of $7 x10^7$. After 10 days, the colony count dropped to 16000 cells per ml.

Growth observed in fig. 5.8a was due the utilization of glucose which showed to decrease in concentration with an increase in bacterial population (monitored as turbidity). Depletion of total carbohydrates initiated at the 20^{th} hr of incubation correlating with the increase in growth as turbidity. At the 28^{th} hr of incubation,

Fig. 5.8a: Carbohydrate utilization and protein production compared with growth of NRS-01

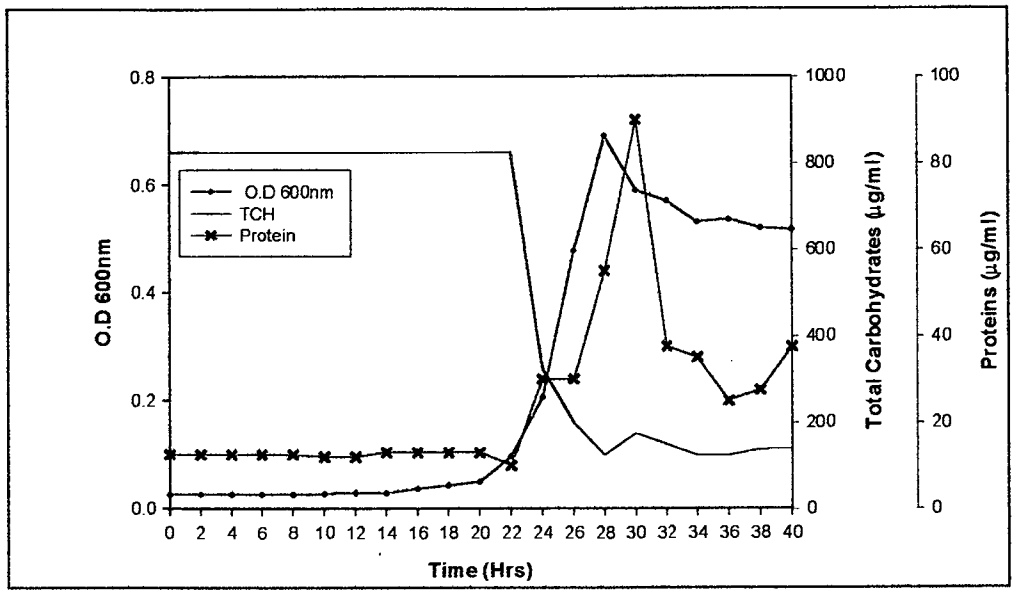
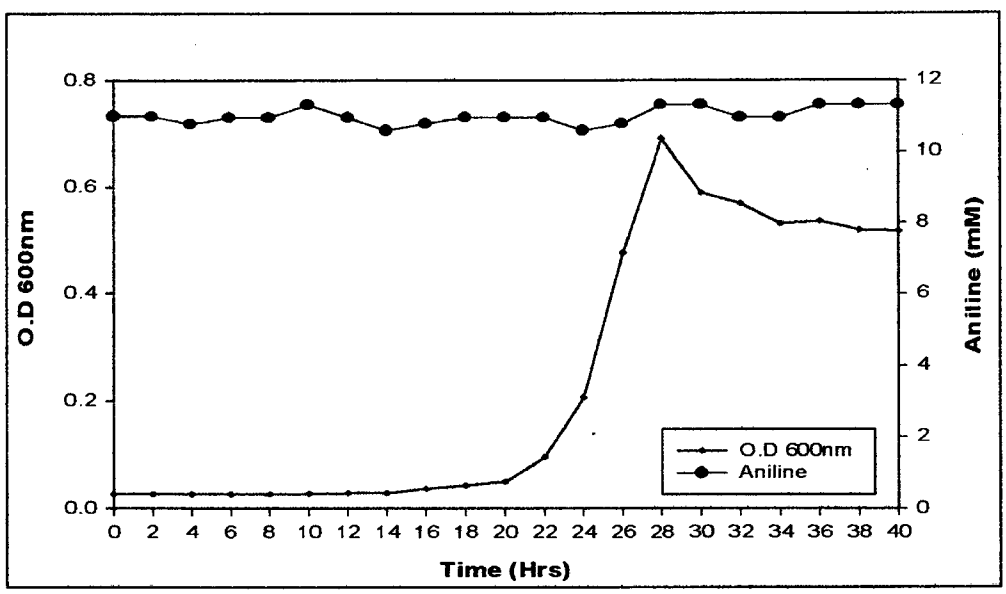


Fig. 5.8b: Aniline utilization compared with growth of NRS-01



complete depletion of the carbohydrate coincided with the onset of stationary phase of growth which led to cell aggregation. Comparing glucose or total carbohydrate concentration in the graph with that of aniline, suggested glucose was used as a primary source of carbon. The red colour formation on the cells did not reduce the aniline concentration, which suggested that the cells may be producing this compound as a mode of defense mechanism.

The extracellular protein content in the broth was found to be significantly low throughout lag phase, but, concomitantly increased with bacterial growth during the exponential phase with maximum concentration observed at 30hrs (Fig. 5.8a). There was a sharp decrease in protein content in the medium with its least concentration seen at 36^{th} – 40^{th}hr of incubation- a phase of growth during which the bacteria produced the red compound.

However, catechol remained undetected in the broth having aniline used as the carbon source. Arnow's method to determine the presence of catechol (an intermediate aromatic / aniline transformation) remained undetected. Rothera's test carried out on benzoate grown cells showed an O-cleavage of the benzene ring.

5.2.2.2. Response of NRS-01 grown in 0.1mM aniline

In comparison to growth characteristics of the isolate in 11mM aniline, the growth response of NRS-01 in presence of 0.1mM aniline showed slight difference (Fig. 5.9 a&b). The lag phase was found to be very short extending to 4 hrs, followed by an exponential phase between 4hrs and 16 hrs with a concomitant reduction of glucose up to the onset of stationary phase where a drop in the turbidity due to aggregation was observed. The aggregates formed were found to form to a larger

Fig. 5.9a: Carbohydrate utilization and protein production compared with growth of NRS-01

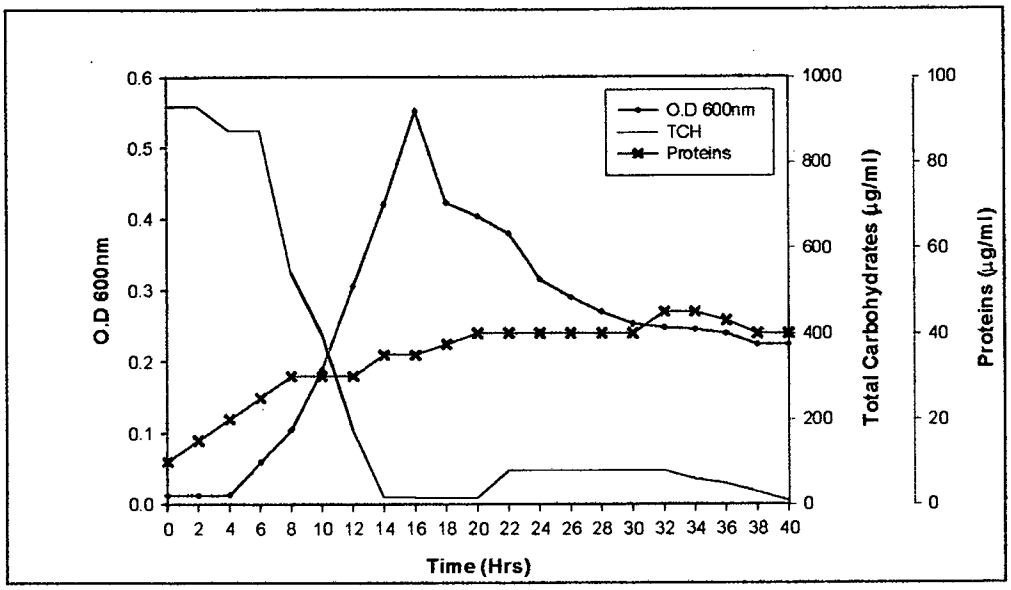
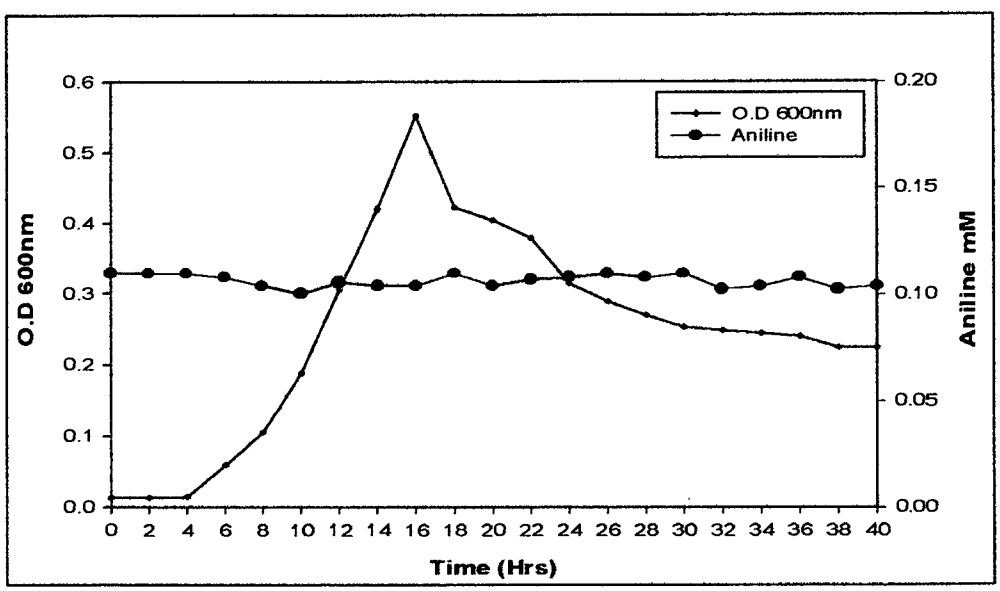


Fig. 5.9b: Aniline utilization compared with growth of NRS-01



extent with good stability as compared to the cell aggregates in 11mM aniline. The extracellular protein detected in the medium reached to a maximum of 40μg/ml as compared to 90 μg/ml when grown in the presence of 11mM aniline.

There was little or no red coloured product formed as compared to the intensity of the red compound produced in medium with 11mM aniline.

Aniline was not utilized as a carbon or nitrogen source even at low concentration (0.1mM) (Fig. 5.9b) or high concentrations (11mM) (Fig. 5.8b).

5.2.3. *Degradation / transformation studies of aniline*

Growth in aniline medium with 11mM concentrations could be divided into various stages; 0 hrs – experimental initiation stage; 18 hrs – growth (as turbidity) initiation stage; 28 hrs – a stage where the turbidity was found to be highest (onset of stationary phase); 30 hrs- beginning of aggregation; 36 hrs- pink colour formation observed in the broth; 40 hrs- prominent red colour was noted (Fig. 5.10) giving an overall picture of growth and the colour formation along with it. On centrifuging the culture broth, the cell pellet gave significant information of colour gradient formed on the cell surface. The colour appeared on the cells during 36th hour of incubation which intensified further on. After 40th hour of incubation, a deep red colour was found on the cell pellet with no pink colouration visible in the supernatant during these incubation periods, but acquired light yellow colour which fluoresced on exposure to UV light (260 nm). The fluorescence of the culture broth on comparison with the supernatant of the culture grown in MSM medium (MSM + glucose) and with uninoculated MSM (control) (Fig. 5.11) showed a bright blue fluorescence present only in medium with 11mM aniline.

Fig. 5.10: Transformation seen in MSM with (11mM) aniline and glucose

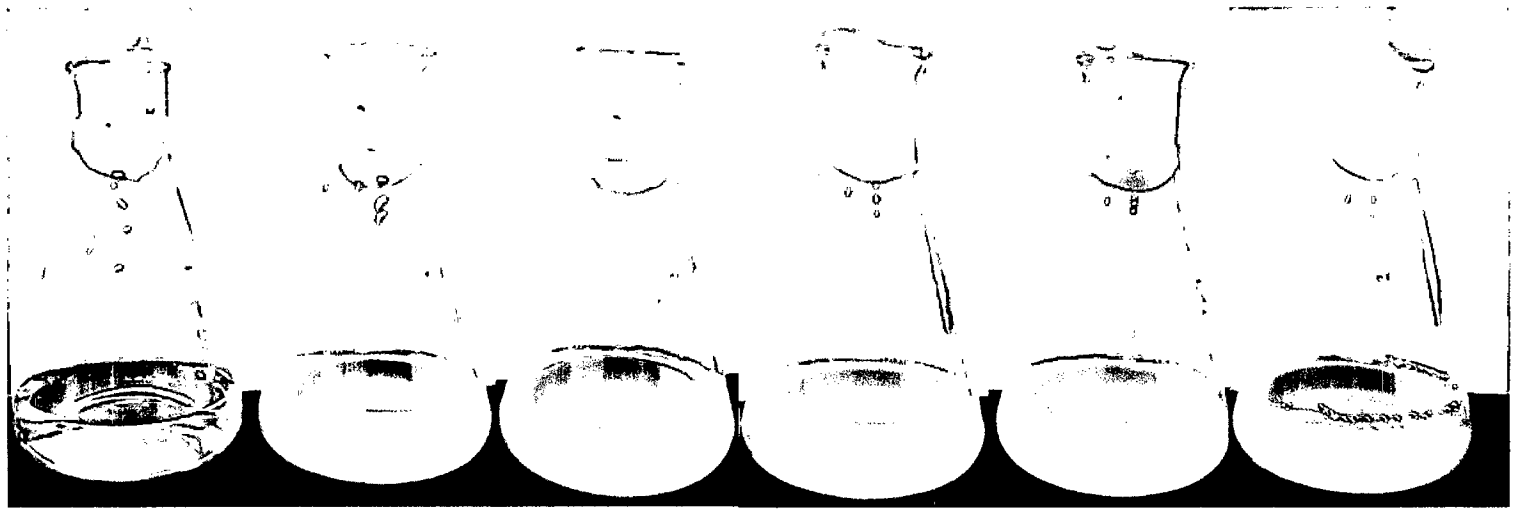
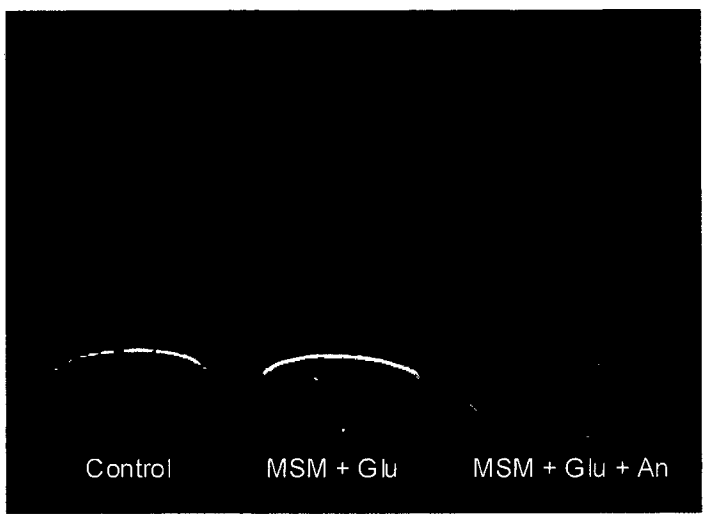


Fig. 5.11. Difference in fluorescence in MSM medium (NRS-01 grown in glucose and in aniline)

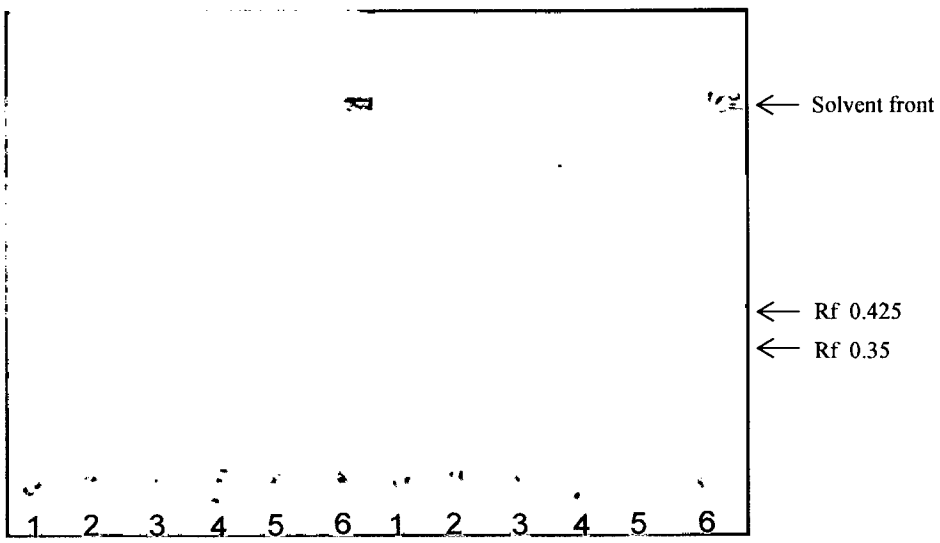


Thin layer chromatograms (Fig. 5.12), revealed the formation of red coloured compound formed on the bacterial cell surface (Rf 0.35) on incubation for 36hrs, which intensified on further incubation (40hrs). The production and accumulation of the red compound on the cell surface (and not present in the culture broth) confirms the hydrophobicity of the compound. A second compound (Rf 0.425) initially produced and detected on the bacterial cell surface (0-18hr, 1-4 on TLC, Fig. 5.12) was depleted during the formation of the red product and a new compound was produced at 36hr and 40hrs having very little difference in Rf values to 0.425.

Thin layer chromatography performed on the extracts from supernatants (figure not shown), did not show any significant production of compound during the growth of NRS-01 in presence of aniline. The TLCs were carried out with aniline and catechol as standards. The unacidified diethyl ether extracts showed the presence of aniline up to an incubation period of 40 hrs having Rf 0.6., whereas catechol (Rf 0.26) was not detected in extracts carried out using diethyl ether as well as pet ether and in unacidified and acidified conditions. The extracts did not show the presence of any compounds as spots on TLC that could be correlated with the production of the red compound extracted from the cell surface.

TLC carried out on the acidified pet ether extracts gave the presence of a fluorescent compound at Rf 0.186 on visualization under UV transilluminator (260nm). The production of this compound at its highest concentration was observed on 28hrs of incubation after which the concentration reduced over an incubation period of 36h and 40h (Fig. 5.13)

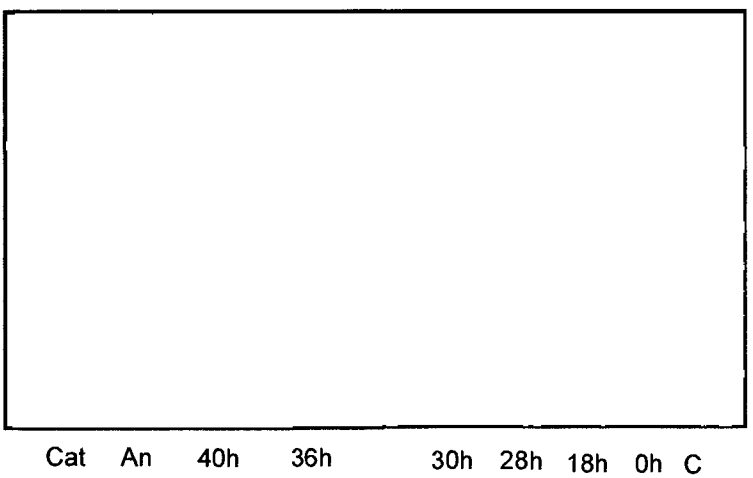
Fig. 5.12: TLC of ether extracts of cell pellet



Without visualizing agent visualizing agent- I_2 vapours

1 0hr; **2** 18hrs; **3** 28hrs; **4** 30hrs; **5** 36hrs; **6** 40hrs
Solvent (Hexane : acetone 80 : 20)

Fig. 5.13: TLC of petroleum ether extracts of acidified supernatant



Cat - Catechol; **An** - Aniline; **C** - Control

Solvent (Hexane : acetone 80 : 20)
Visualization using UV light

TLC carried out of the red product in comparison with other aromatic compounds such as azobenzene, biphenyl, o-aminophenol, p-aminophenol, m-aminophenol phenol, aniline and catechol, did not show any similarity on the basis of their Rf values (Table 5.1).

The HPLC pattern of the cell pellet extracts showed the presence of a single compound on 28hrs of incubation having retention time of 1.26min. On further incubation of the medium to 36 and 40hrs (pink colour observed), showed the presence of two peaks; one which was originally observed (Rt 1.26min) and a second compound was detected at a Rt of 4min. The compounds eluted did not correlate with standard aniline which had Rt of 1.5min.

On analyzing the supernatant samples by HPLC for the presence of aniline, a reduction of 53% was observed in medium containing 0.1mM aniline observed after an incubation period of 40hrs, where as supernatant with 11mM aniline did not show any reduction in its concentration after 40hrs. The medium with 0.1mM concentration aniline contradicts with the results obtained from chemical estimation where aniline was not found to have depleted in the medium over 40hrs of incubation. HPLC results correlated with the aniline concentration estimations in medium with 11mM aniline showing no depletion of aniline in the medium.

Results suggest the non-utilization of aniline when provided in the medium at a high concentration of 11mM, but inducing the cells to produce a red hydrophobic compound as a defense strategy towards aniline. Whereas, at lower concentration of 0.1mM, transformation of aniline by a process of rearrangement could be envisaged

Table 5.1: Rf of various aromatic compounds in comparison with TP2

Substrate	Rf	Colour (unstained)
Azobenzene	0.92	yellow
Biphenyl	0.92	colourless
o-aminophenol	0.36	colourless
p-aminophenol	0.13	light pink
m-aminophenol	0.13	light pink
aniline	0.54	colourless
catechol	0.23	colourless
TP2	0.36	red

Visualizing agent used: Iodine

due to which aniline seems to be detected by the diazotization method or by the PDAB method of estimation indicating the presence of a primary aromatic amine in the medium. Lendenmann and Spain (1996), has shown partial degradation of an aromatic amine, amino phenol followed by a rearrangement to form picolinic acid observed in *Pseudomonas pseudoalkaligenes*. The most common rearrangement of aromatic compound is the intermolecular shift of hydroxyl group of hydroxylamino compound to an aromatic amino compound (Nadeau *et al.*, 2003)

Growth medium of benzoate grown cells turned yellow after an incubation period of 18hrs. Rothera's test performed on the cells harvested from this flask showed an ortho-cleavage of the aromatic ring brought about by catechol 1,2 dioxygenase. This was confirmed by the purple ring formation at interphase, a characteristic reaction of β-ketoadipate (keto group present in the molecule) with sodium nitroprusside (Agarwal *et al.*, 1996; Kulkarni and Chaudhari, 2006) in the presence of ammonia. The culture grown in the presence of either 0.1mM or 11mM aniline concentration did not show any characteristic ring cleavage reaction.

5.2.4. *Studies on fluorescent compound*

The Spectrofluorophotometric analysis of the supernatant (Fig. 5.14) revealed the fluorescent compound to have excitation and emission wavelength of 310nm and between 380nm and 390nm respectively, while aniline showed a characteristic emission wavelength at 340nm. The concentration of the fluorescent compound increased from 0hr reaching to its highest concentration on 30 hours of incubation (a stage where aggregation was observed), which depleted on further incubation leading

Fig 5.14: Spectrofluorospectrometric scan of culture medium (MSM + Glu + 11mM Aniline)

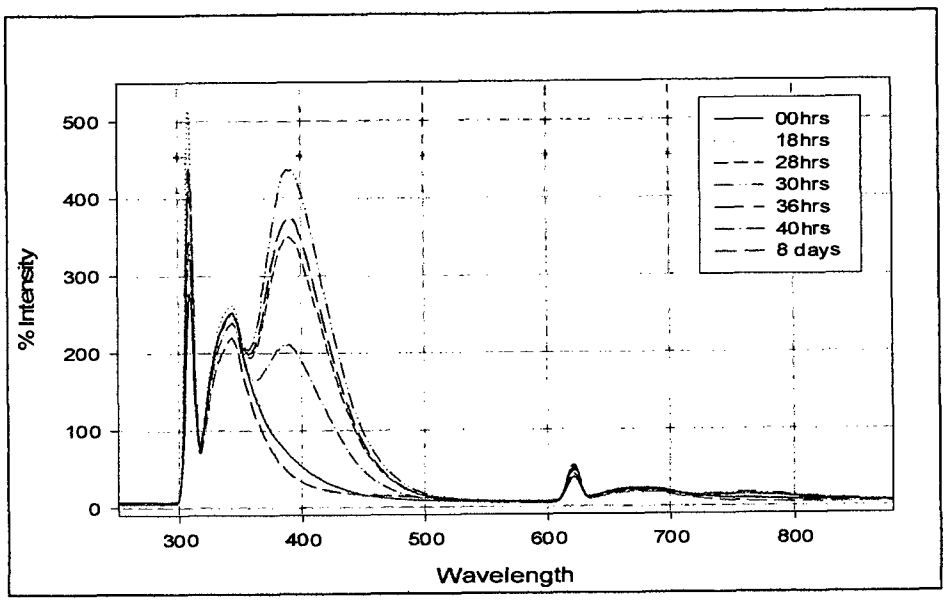
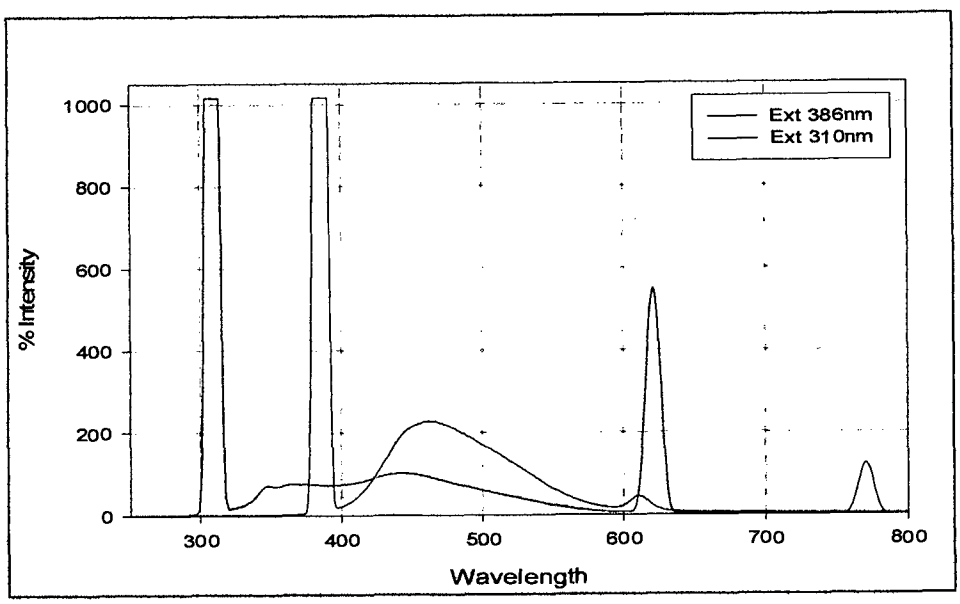


Fig 5.15: Spectrofluorospectrometric scan of culture medium (MSM + Glu)



to its complete removal from the broth after 8 days, whereas aniline was present in the supernatant even after 8 days of incubation. The results drawn were based on the changes in the intensities of the 380nm peaks depicted by the scans.

The culture medium, in which isolate NRS-01 was grown with 0.1mM aniline, did not show the presence of the fluorescent compound, indicating its production specifically at high concentrations of aniline.

Figure 5.15 shows a difference in the fluorescence of the broth when the culture was grown in MSM with glucose at different excitation wavelengths. Growth in glucose gave a greenish fluorescence as compared to bright blue fluorescence when grown in presence of high concentration of aniline (Fig. 5.11).

The culture grown in the MSM in the presence of glucose and aniline (Flask A1) and glucose (Flask B1) gave a good growth, but there was a production of blue fluorescence in the medium containing aniline. When subcultured in the MSM and MSMII with glucose and aniline, all the flasks (Aa, Ab, Ba and Bb) showed a production of a blue fluorescent compound, which was absent in flasks C1 or D1 or in subcultured flasks Ca, Cb, Da and Db (Table 5.2). It was thus observed that glucose played a role in the production of the blue fluorescent compound.

5.2.5. *Enzyme studies*

It was envisaged that the red coloured compound formed may be the result of aniline transformation by extracellular enzyme produced by the organism. This experiment was conducted to check for the effect of colour formation on removal of cells from the culture medium.

Table 5.2: Flasks showing production of blue fluorescent compound in the presence of glucose

24 hrs Incubation	Growth	Colour	Blue Fluorescence
A1	+++	Pink	+
Aa	+++	light pink	+
Ab	++	light yellow	+
B1	+++	off white	- (green fluor)
Ba	+++	light pink	+
Bb	++	light yellow	+
C1	-	no colour	-
Ca	-	no colour	-
Cb	-	light pink	-
D1	++	no colour	-
Da	+	light pink	-
Db	+	light pink	-

30 hrs Incubation	Growth	Colour	Blue Fluorescence
A1	+++	Pink	+
Aa	+++	light pink	+
Ab	++	yellow-pink	+
B1	+++	off white	- (green fluor)
Ba	+++	light pink	+
Bb	++	yellow-pink	+
C1	-	no colour	-
Ca	-	no colour	-
Cb	-	light pink	-
D1	++	no colour	-
Da	+	light pink	-
Db	+	pink	-

48 hrs Incubation	Growth	Colour	Blue Fluorescence
A1	+++	Pink	-
Aa	+++	light pink	-
Ab	++	light pink	-
B1	+++	off white	- (green fluor)
Ba	+++	light pink	-
Bb	++	light pink	-
C1	-	no colour	-
Ca	-	no colour	-
Cb	-	light pink	-
D1	++	no colour	-
Da	+	light pink	-
Db	+	pink	-

Formation of the red compound on the cells was observed after an incubation period of 36hrs. During incubation period between 28hrs (initiation of aggregation) and 36hrs (pink colour formation), it was assumed that the bacterial cells could be producing enzymes to biotransform aniline. As there was no depletion of aniline, either in 0.1mM or 11mM concentration maintained during study, the role of enzymes catalyzing aniline degradation was ruled out. However, its role in conversion of aniline to a chemically reactive compound was envisaged. Figure 5.16 depicts the colour formation in the flask containing the bacterial cells which was not observed in the filtrate, thereby projecting the role played by the cells in the production of the red compound.

Aniline was estimated from the broths of both the flasks and a blank using the diazo coupling method and the PDAB method at the time of separation and after a period of one month. Aniline concentrations in the medium over an incubation period one month showed a decrease from initial 11mM to 7.8mM & 8mM respectively in the medium devoid of cells, whereas, the flask containing cells showed a decrease from initial 11mM to 8.27mM & 8mM respectively. The control maintained showed a reduction in aniline concentration from 11mM to 7.25mM and 7mM respectively after one month of incubation which may probably be due to the role of environmental factors such as photo-oxidation (Lyons *et al.,* 1984)

5.2.5.1. Aniline Oxygenase/Aniline 1,2-dioxygenase assay (Oxygen uptake method-Clarke's Electrode)

The oxygen uptake electrogram (Fig. 5.17) represents a control and test reactions. Control (blue line) showed stabilization and saturation of molecular oxygen

Fig. 5.16: Dependence of the cell pellet for the formation of the red compound

Supernatant with cells
(pink colour due to cells)

Supernatant (filtrate)
(light yellow)

in the reaction chamber. The test reaction (pink line) showed an initial stabilization of oxygen level in the reaction chamber indicated by a straight line (parallel line) with respect to control (blue line). Addition of cell suspension demonstrated a downward inclination of the electrogram (5min-9min) indicating the utilization of molecular oxygen present in the reaction chamber, termed as endogenous respiration. On its stabilization, the cellular response towards a selected substrate such as glucose was studied by adding a fixed concentration into the reaction mixture (Fig. 5.17). As bacterial cells utilized glucose, molecular oxygen from the reaction chamber depleted in a much faster rate as compared to the endogenous respiration (10min-16min), the difference in oxygen uptake between the endogenous respiration and the substrate induced respiration gives the corrected rate of oxygen uptake solely induced by the substrate in terms of nmols/min.

When aniline was used as substrate, a contradictory effect of the oxygen uptake was observed, demonstrating a theoretical release of molecular oxygen into the reaction chamber instead of its depletion (fig 5.18) (12.5min-30min). Aniline oxygenase or dioxygenase involves an enzymatic reaction where the amino group is replaced by one or two hydroxyl groups respectively on the aromatic ring, which requires utilization of molecular oxygen. The practical observation points out to a defiance of such an activity thereby making it unable to determine the role of cells/enzymes in aniline degradation/transformation.

Cells incubated for 30 hrs where the pink colour just appears were found to be more active as compared to the cells incubated for 36 hours (a stage where a pink compound is more prominent and intense). The activity compared was based on the

molecular oxygen uptake during its endogenous respiration of the older cells. The addition of the substrate to the pink cells did not show any change in the rate of oxygen uptake. Substrates such as catechol, homogentisate, p-aminophenol and o-aminophenol showed oxidation when added in the reaction chamber in the absence of cells (control) as a result the cells did not show a correct rate of oxygen utilization. The oxygen uptake studies are shown in Table 5.3.

Cells grown in the presence of 0.1mM aniline also showed similar reactions when reacted with 3mM, 1.5mM, 0.5mM and 0.1mM as final concentrations of aniline. The pH value of the phosphate buffer used in the reaction cell was 7.5 and 9.0.

5.2.5.2. Aniline deaminase enzyme assay

Cells grown in medium in the presence of 11mM aniline, incubated for 30 hrs and 45 hrs gave different reactions. It was interesting to notice the release of ammonia when 30hrs cells were used, whereas plates maintained with 45hrs old cells showed utilization of a large volume of a 0.0005N NaOH as compared to the control. 30hrs old cells released ammonia from aniline there by requiring a volume of 1950 µl of NaOH. With 45hrs old cells, 4000 µl of NaOH was required to neutralize the acid, which was beyond control value, which gave contradictory results and the experiment could not be considered in the present study. The table 5.4a shows the amount of base utilized for neutralization of 0.001N H_2SO_4. Control required a volume of 2.5ml (2500µl) of 0.0005N NaOH to neutralize 1ml of 0.001N H_2SO_4.

Similar reaction was shown by cells grown in 0.1mM aniline. Cells incubated with 5mM aniline showed a requirement of a large volume of base to neutralize acid

Table 5.3: Oxygen uptake values (nmoles/min) of NRS-01 in the presence of various substrates

	30h cells			36h cells (pink cells)			controls
Substrate	**Endogenous**	**substrate induced**	**O_2 consumed**	**Endogenous**	**substrate induced**	**O_2 consumed**	
Aniline							
Benzoate	-2.161	-3.953	1.789	-0.47	-0.44	0	0
Gentisate	-3.037	-3.521	0.484	-0.167	-0.686	0.519	0
Catechol	-2.437	-35.005	-- *	-0.003	-17.491	-- *	129.7
Homogentisate	-2.26	-6.073	-- *	-0.037	-8.33	-- *	9.01
p-aminophenol	-5.552	-9.01	-- *	-0.187	-23.529	-- *	23.4057
o-aminophenol	-3.084	-31.864	28.78	-0.36	-15.93	15.57	15.57
m-aminophenol	-2.304	-0.366	0	-0.45	-0.002	0	0
Toluidine	-2.195	-0.567	0	-0.001	-0.001	0	0
p-aminobenzoic acid	-4.953	-0.361	0	-0.21	-0.016	0	0
Anisidine	-2.07	-0.8	0	-0.25	-0.138	0	0

--*, denotes the unavailability of correct reading as the controls show oxidation of substrate in the absence of cells

Oxygen uptake by cells in the presence of aniline

30 hrs cells		**36 hrs cells**	
endogenous respiration	-3.787	endogenous respira	-1.231
aniline (10 mins)	-1.227	aniline (10 mins)	-1.059
aniline (after 10 mins)	1.292	aniline (after 10 mi	-1.059

Table 5.4: Determination of enzyme aniline-deaminase (Conway method)

a) Cells grown in presence of 11mM aniline

	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
Cell Suspension	1 ml (30h)	1 ml (30h)	1 ml (45h)	1 ml (45h)	–
PO_4 buffer	–	–	–	–	1 ml
Na_2CO_3	1 ml	1 ml	1 ml	1 ml	1 ml
Aniline (5mM)	+	–	+	–	+
Amount of base	1950 µl	2500 µl	4000 µl	4000 µl	2500 µl

b) Cells grown in presence of 0.1mM aniline

	Plate 1	Plate 2	Plate 3	Plate 4
Cell Suspension	1 ml	1 ml	1 ml	–
PO_4 buffer	–	–	–	1 ml
Na_2CO_3	1 ml	1 ml	1 ml	1 ml
Aniline	5mM	0.5mM	–	5mM
Amount of base	9000 µl	2500 µl	2500 µl	2500 µl

2.5 ml (2500 µl) of 0.0005N NaOH was required to neutralize 0.001N H2SO4 in a simple acid base titration

as compared to control (Table 5.4b), whereas, no release of ammonia from aniline when incubated with 0.5mM aniline.

Weatherburn's method of detecting aniline deaminase is based on the estimation of released ammonia as a residue in the medium. Estimations carried out after a period of 12hrs and 24hrs revealed that there was no ammonia present in the medium, thereby suggesting the absence of enzyme aniline-deaminase.

5.2.5.3. Detecting Peroxidase enzyme in NRS-01

Peroxidase enzyme needs the addition of hydrogen peroxide to oxidize the substrate. Addition of hydrogen peroxide directly in a hydroquinone solution gives a pink colour in the presence of sodium molybdate which is p-benzoquinone an oxidized product. Where as, the cells showing peroxidase activity brings about the similar activity with the production of hydrogen peroxide. In the present study cells the colour formed in the tube containing hydrogen peroxide gave a deep pink colour showing an absorbance of 550nm, where as the tube containing the cells showed an absorbance at 437nm.

A second method to demonstrate the peroxidase activity for the formation of a coloured compound was by trapping the aniline radicals as described by Corke *et al.*, (1979), which utilized a coupling agent such as β-naphthol to give a coloured compound showing absorbance at 475nm, thereby preventing the formation of azo dye. In the present study, there was a reaction between the cells and β-naphthol as there was a colour formation when cells were incubated with β-naphthol in the

absence of aniline. For this reason, the activity of peroxidase by the cells could not be demonstrated. The formation of azo compounds due to the enzyme peroxidase has been demonstrated by Bartha, 1968; Bordealeau *et al.*, 1972 and Plimmer *et al.*, 1970.

5.2.5.4. Catechol 1,2 dioxygenase assay

Catechol 1,2 dioxygenase activity was demonstrated by benzoate grown cells on the formation of a 260nm peak demonstrated the conversion of catechol to cis,cis-muconic acid. The enzyme activity was found to be 95U/g. Whole cells as well as the cell free lysates from aniline grown cells (0.1 & 1mM concentrations) did not show catechol transformation during the catechol 1,2-dioxygenase assay.

5.2.6. *Utilization studies of other aromatic amines by isolate NRS-01*

The results pertaining to growth and colour formation in the presence of various other aromatic amines is shown in table 5.5. The aromatic amines viz. aminophenols, and anisidine did not favour growth but inducing the bacterial inoculum to produce a red colouration as observed in response to aniline. P-chloroaniline and diphenylamine did not support growth of the culture. Aromatic hydrocarbons (non-aromatic amines) such as tyrosine and benzoate however supported good growth, whereas, tryptophan was not utilized by the culture. Growth was observed on p-toluidine, p-Aminobenzoic acid when used as carbon source producing a yellow colouration. To summarize, isolate NRS-01 could assimilate p-toluidine and p-aminobenzoic acid as the sole source of carbon and nitrogen.

Table 5.5: Response of the isolate NRS-01 in presence of other aromatic amines

Substrate	Growth	Brown Colour
Aniline	+	+
p-amino phenol	-	+
m-amino phenol	-	+
o-amino phenol	-	+
PCA	-	-
DPA	-	-
Toluidine	+	+
p-aminobenzoic acid	+	-
p-Anisidine	-	+
Tyrosine	++	+
Benzoate	++	-
Tryptophan	-	-

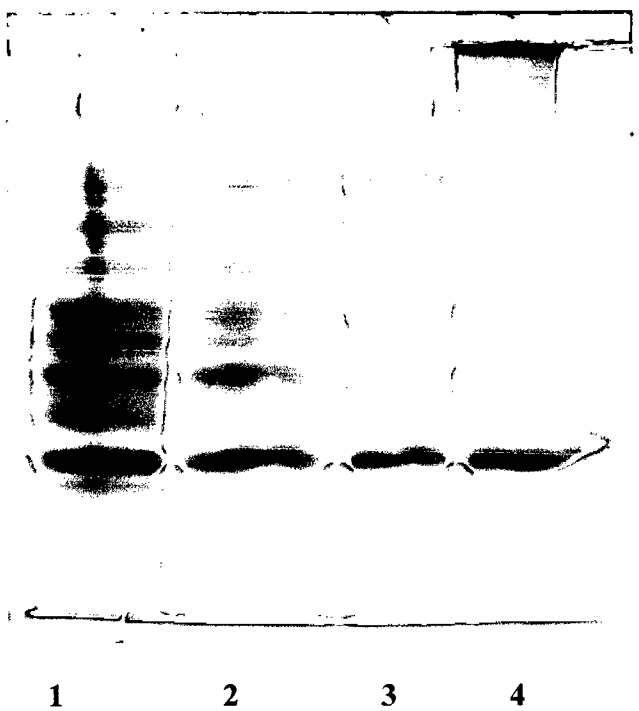
5.2.7. *Protein profile (Native PAGE) of NRS-01 grown in the presence of aniline*

The native PAGE gel (Fig. 5.19) shows a comparative description of the protein profile of the isolate NRS-01 grown in various growth media showing the effect of aniline.

The original protocol was slightly modified as the pink compound formed by the culture on the cell surface interfered with the extraction as well as resolution of protein bands during staining. The gel did not reveal the production of specific stress related proteins by isolate NRS-01 in presence of aniline all the media used. Protein bands on lane 3 (culture grown in MSM medium with glucose and aniline) showed the presence of similar protein bands as compared to the protein profile of the isolate grown in PPYG (lane 1) or MSM with glucose (lane 2). Protein profile in lane 4 showed absence of some bands, while there was a reduction in the intensity of bands present.

The growth of NRS-01 in presence of aniline and glucose, showed a lag phase sue to toxicity of aniline which was related to the concentration of aniline used. Further, the culture utilized glucose and during that period showed the production of a fluorescent compound with increase in protein concentration in the supernatant. The concentration of protein was found to reduce with increase in incubation period and formation of pink coloured compound on the cell surface. An interesting characteristic of the culture was its aggregation which was related to the production of protein and restoration and homogenization of the culture with the production of a red colouration on the cells. The effect of cells on the formation of red coloured product TP2 and the

Fig. 5.19: Native PAGE of isolate NRS-01 grown in different growth conditions



1. Culture grown in PPYG
2. Culture grown in MSM with glucose as sole carbon source
3. Culture grown in MSM with glucose and aniline (11mM) (Aggregation stage)
4. Culture grown in MSM with glucose and aniline (11mM) (pink cells)

fluorescent protein is observed only in the presence of cells indicating the role of NRS-01 in the formation of these products. It was therefore of interest to isolate and characterize the products which are formed by *Halomonas* in the presence of aniline.

CHAPTER VI

ISOLATION, PURIFICATION AND CHARACTERIZATION OF COMPOUNDS (TP2 & FP) PRODUCED IN RESPONSE TO ANILINE

The growth and the response studies of the isolate towards aniline showed that the halotolerant alkaliphile *Halomonas* sp. (NRS-01) was able to grow in the presence of aniline at concentrations from 0.05mM to 11mM, higher concentrations being toxic and inhibiting the growth. It was interesting to observe that in the presence of aniline a red coloured compound (TP2) was formed during the growth on glucose, the intensity of which increased with an increase in aniline concentration.

Further during growth of the culture in glucose and aniline production of an extracellular fluorescent compound (FP) was observed in the culture broth. This compound FP was detected in the culture medium until the production of TP2 by the cells. The fluorescent compound disappeared completely on further incubation but TP2 concentration was found to increase. It was therefore of interest to isolate and characterize these compounds.

6.1. MATERIALS AND METHODS

6.1.1. *Isolation of fluorescent compound (FP)*

6.1.1.1. Production of Fluorescent compound

Isolate NRS-01 was inoculated in MSM (1.0 litre) having aniline at 0.1% (11mM) concentration supplemented with 0.1% glucose. Flasks were incubated on a shaker at 30°C and at 180rpm and cells harvested before the formation of TP2 (30-32h). Culture broth was centrifuged at 10,000rpm and the supernatant was filtered through a 0.22μm Millipore membrane filter and then used for extraction of the fluorescent compound FP.

6.1.1.2. Isolation and detection of the fluorescent compound

The florescent compound was isolated using the method described below and the presence of fluorescent compound in all the extracts and residues was checked by exposing the liquid to UV-light to observe the blue fluorescence.

a) Precipitation methods

The above filtrate was saturated with ammonium sulfate (80% saturation) (Maier *et al*, 2004), or ice cold isopropanol (1:2 v/v) and kept overnight at 4°C. The precipitate formed in either case was collected by centrifugation at 10000rpm and dissolved in minimum volume of phosphate buffer and filtered. The supernatant & the dissolved precipitate were checked for the presence of fluorescent compound.

b) Solvent extraction methods

The filtrate was extracted with various solvents having different dielectric constants (polar and non-polar solvents) e.g. pentane, hexane, pet ether, chloroform and diethyl ether, and the organic phase was collected, dried over sodium sulfate. The aqueous and organic residues were checked for FP by observing under UV-light.

c) Evaporation

Filtrate of culture broth (150ml – 200ml) was taken in a 500 ml distillation flask and a procedure similar to simple distillation was carried out. The temperature maintained during the process was 60-70°C until roughly 15 ml of liquid remained in the flask. This concentrated residue was then extracted repeatedly with isopropanol and the isopropanol extracts were pooled, dried over sodium sulphate and vacuum

evaporated at 60°C to dryness. The resultant compound was dissolved in a minimum amount of acetone and stored at 4°C until further use.

6.1.1.3. Purification of the fluorescent compound

Purification of the fluorescent compound was carried out by column chromatography (Shriner *et al.*, 1980a). Silica gel (mesh size 160-200, Acme's synthetic chemicals) was used as the column matrix. The fluorescent compound was adsorbed on the silica gel and was introduced in the column for chromatographic separation. Solvent used for eluting the compound was a petroleum ether : acetone with sequential increase of acetone concentration from 2% to 30%. The elutes were collected as fractions and were checked for the presence of the fluorescent compound under ultraviolet light and on TLC (silica gel–H, Acme's synthetic chemicals) using petroleum ether as solvent system. Elutes with pure fluorescent compound were pooled and vacuum evaporated at 60°C and remaining moisture removed by placing the flask in a vacuum desiccator for 15-20 mins. The residual compound was resuspended in acetone and stored at low temperature (4°C) until further use.

6.1.2. *Isolation, purification and characterization of TP2 from cells*

6.1.2.1. Production of TP2

Isolate NRS-01 was inoculated in 1.0 litre MSM medium with aniline (0.1%) supplemented with 0.1% glucose and incubated on a shaker at room temperature (30°C) at 180rpm for 10 days. Cells were harvested by centrifuging the culture broth at a speed of 10,000 rpm, washed twice with 0.1M phosphate buffer (pH 8.0), resuspended in the same buffer and was used to extract TP2.

6.1.2.2. Extraction of TP2

Extraction of TP2 from the cell suspension was carried out using two different methods.

a. Extraction with Diethyl ether after growth

The cells were extracted with diethyl ether in a separating funnel. Extraction procedure was repeated until no colour was extracted into the organic phase. The organic extracts were pooled, dried over sodium sulphate and concentrated using vacuum evaporator at 60°C.

b. Biphasic extraction during growth with toluene

The washed cells suspended in phosphate buffer was layered with toluene in a conical flask and incubated on a shaker (60 rpm) for a period of 48 hrs. The entire mixture was then transferred in to a separating funnel for phase separation. The organic layer containing the red compound was separated, dried over sodium sulphate and evaporated using a combination of evaporation and drying under nitrogen.

The extracted dried compound was resuspended in a minimum amount of acetone and stored at -20°C until further use.

6.1.2.3. Purification of TP2

a) Preparative TLC (Dhar and Rosazza, 2000)

A slurry of silica gel (Silica gel-H) was prepared by mixing 10-15 g in 30ml distilled water and was poured on a clean and dry TLC plate (20 x 10 cm), swabbed with acetone and drawn into layer of 0.5 mm thickness. The plates were air-dried and

then activated at 110°C for 30min. After activation of plates, maximum amount of sample was spotted on the TLC plate and developed in pre-saturated solvent chamber containing the solvent system (Hexane : Ethyl acetate; 80 : 20). Solvent was allowed to run up to $3/4^{th}$ the length of the plate, plates were removed and were allowed to air dry. TP2, appearing as a red spot, was scraped from the plate and extracted by repeated washing of silica powder with diethyl ether, the extracts were pooled, concentrated using a vacuum evaporator and purified using flash Chromatography.

b) Purification using flash chromatography

(i) Column preparation

Flash chromatography utilized silica gel mesh size of 230-400 (grain size 0.023mm, Spectrochem, India). Column was plugged at the base with a small amount of cotton and silica gel powder was added to half the column length (2.5 cm dia x 40cm). Petroleum ether was used to homogenize the silica gel within the column using an inert gas such as nitrogen. Pet ether was filled in the space over the silica gel in the column and nitrogen gas was passed through the column. Due to the pressure created by the gas, there was a downward movement of the ether thereby forming slurry within the column. Ether was recycled back into the column and nitrogen gas passed through it. Repeated passage of ether in the column homogenized the silica and removed all the trapped air bubbles.

(ii) Running of the column and compound purification

The red product TP2 was weighed and was adsorbed on to silica gel three times its weight. This was used as the sample. Excess of ether was drained from the

column to retain a volume to give a height of 2-3 cms above the silica gel. Entire sample was then introduced in the column and layered uniformly. A cotton plug was placed on top of the sample and pressed gently with a glass rod to prevent its movement during addition of solvent. Solvent system used for the elution was 10% Ethyl acetate in petroleum ether. Fractions were collected at a fixed volume of 15 ml each in clean test tubes. Six fractions were collected and the ethyl acetate concentration was increased to 15%. A flow rate was maintained at 2 drops per second or 1ml per minute was maintained during the entire process. Fractions collected were checked on TLC for purity.

All the fractions giving a single spot of red compound were pooled, vacuum dried, weighed and used to carry out spectral analysis i.e. UV-VIS spectroscopy, NMR, IR and GC-MS.

6.1.2.4. Characterization of TP2

The purified compound was characterized for its elemental analysis and spectral characteristics (UV-VIS spectrophotometer).

a) Elemental analysis

Purified product (1mg) was analyzed for its elements Carbon, Hydrogen and Nitrogen using an automated elemental analyser Carlo-Erba 1100 analyzer. Further the presence of N was confirmed using sodium-fusion test.

b) Spectral analysis

Samples were dissolved in small volume of 95% ethanol and spectrophotometric scan was taken from 800 nm to 190 nm using UV1601 shimadzu spectrophotometer with 95% ethanol placed as a reference. The IR spectrum of compound was taken on Shimadzu FTIR – 8000S, Proton NMR spectra was recorded using tertramethylsilane (TMS) as internal reference on BRUKER AC-200 instrument operating at 200MHz and ^{13}C NMR spectra was recorded on BRUKER AC-200 instrument working at 50MHz. Samples contained 30mg of TP2 dissolved in $CDCl_3$.

c) Gas Chromatography-Mass Spectrometery (GC/MS)

Sample (TP2) was analyzed using gas chromatography-mass spectrometry (GC-MS) Shimadzu. Temperature of the column (X, TI, ID 0.32 mm) was varied from 60°C to 320°C. The spectra were used to understand and elucidate the structure of TP2.

6.2 RESULTS AND DISCUSSION

6.2.1 *Isolation and characterization of fluorescent compound (FP)*

NRS-01 when grown in the presence of aniline showed the formation of fluorescent compound which was observed at concentrations of 1mM to 11mM (0.1%) below which the presence was not detected. The formation of this compound showed its relation to the growth of NRS-01 in presence of aniline, depicting its relation to concentration of aniline. Such fluorescent compounds in the ecosystems during the growth of organism are known to be produced in response to various stress conditions, as signature molecules (Fujita *et al.*, 2004; Borchardt *et al.*, 2001; Camara

et al., 1998), quorum sensing molecules (Miller and Bassler, 2001; Atkinson *et al.*, 1999; Burgess *et al.*, 2002) especially in the microbial community structure, it was therefore of interest to study the characteristics of this compound.

The product being highly water soluble was not easily isolated with precipitation methods as well as Liquid-Liquid extraction as the fluorescence was detected in the filtrates, supernatant. The product FP was found to be highly water soluble as it was not easily isolated either by solvent extraction or by precipitation. However, it was interesting to note that the concentration of the filtrate at 60-70°C did not have any effect on the fluorescence of FP. Therefore, FP was obtained by evaporating the entire filtrate resulting in a concentrated residue which was easily extracted using isolpropanol. It was interesting to note that the fluorescence of this compound reduced after a period of 5 days and the compound turned pale brown in colour at room temperature and during spectral fluorimetric canalysis, the area exposed to the incident UV-rays changed the colour from colourless to light yellow to brown. Such phenomenon is seen with polyphenolic compounds. However, further characterization of the compound indicated it to be a protein.

The compound finally isolated as a liquid showed profound fluorescence. Characterization of the compound revealed to be a protein. The concentration of this compound was 85µg/ml and 90µg/ml respectively as per Bradford's (Bradford, 1976) and BCA (Smith, 1985). On isolation, the yield with reference to amount of culture broth taken was 15µl/1000ml where concentration of aniline was 0.1%. Further, on studying the fluorescence characteristics, the compound FP showed the excitation at 310 nm and emission at 380-390 nm and at 620nm. This protein-like compound when

acidified showed a considerable reduction in intensity of its emission at 380nm as shown in fig 6.1, with a simultaneous increase of the intensity at 620nm. Interestingly, the emission peak at 380nm was restored back on neutralization with a simultaneous restoration of the intensity at 620nm. However, such a significant change was not seen in the spectral data obtained on a UV-Vis Spectrophotometer (Fig. 6.2). Although its fluorescence was lost when acidified, neutralizing the compound restored its fluorescence; Fig. 6.1 shows the effect of acid and base on the fluorescent compound. Spectrophotometric scans did not reveal any significant change in the structural properties of the compound under acidic or alkaline conditions as it gave an absorption peak at 203nm (Fig. 6.2) in both the conditions.

Further, the molecule was found to be less than 3KDa indicating it to be a low molecular weight protein. Su (2005), has studied the presence of some low molecular weight fluorescent proteins in nature and its applications in biotechnology. One such protein called Green fluorescent proteins (GFP) isolated form a jelly fish *Aquorea victoria*, is being used as an important tool to study recombinant proteins. Such low Molecular weight proteins have been reported to be produced by bacteria in response to stress condition such as toxicity due to heavy metals (Reddy *et al*, 1990). Higham *et al*, 1986, have also reported on cadmium binding proteins produced by *P. putida* having a low molecular weight of 3.5 to 7 KDa. Fluorescent proteins have been isolated from certain marine bioluminescent bacteria namely *Photobacterium fischeri* producing a blue fluorescence-70KDa dimeric (Lee and Kopa, 1978; Lee *et al.*, 1979) and a yellow fluorescent protein (22KDa) from *Vibrio fischeri* strain Y-1 (Daubner C S *et al*, 1989). In both the organisms, the fluorescent proteins were found to be

Fig 6.1: Spectrofluorimetric scan of fluorescent product FP

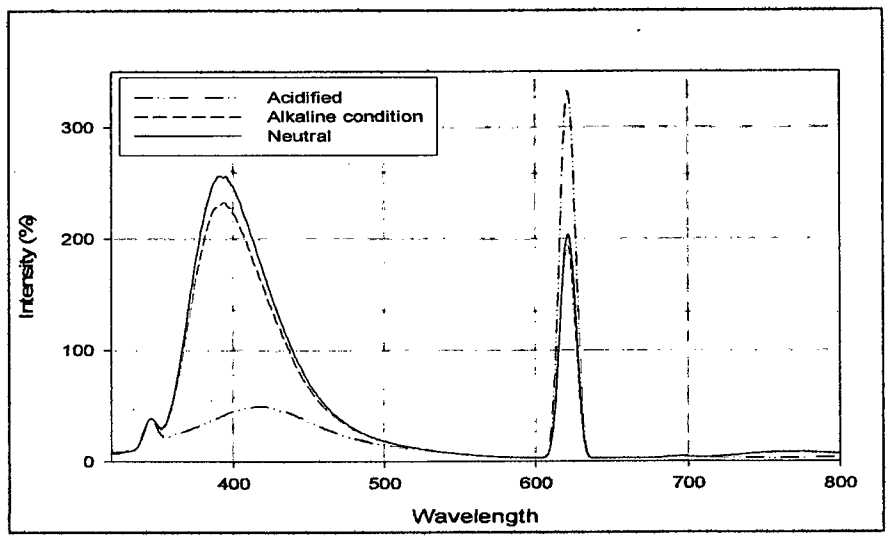
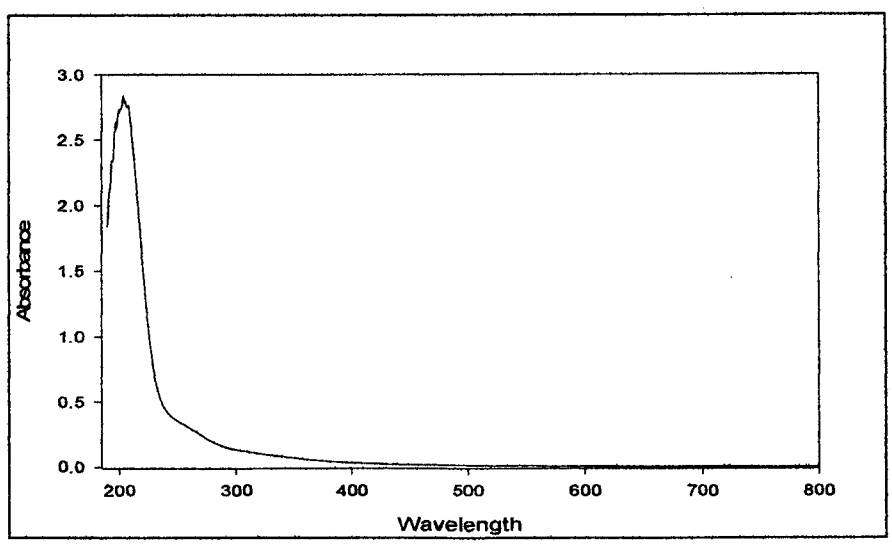


Fig 6.2: Spectrophotometric scan of fluorescent product FP



the bioluminescence activity. Hamdan *et al.*, 1991, has reported fluorescent proteins being produced such as fluorescent peptides which function as siderophores in environments deficient of iron (Reddy, 1990).

In the present study, the production of FP appears to be a mechanism of protection for the cells towards the exposure of toxic compound aniline. Reports on aniline toxicity in organisms have shown various methods or mechanisms of detoxification which involves either acetylation of the amino group converting aniline to acetanilide and formanilide analogues, or transformation of aniline to indole analogues, complexing of these analogues to give high molecular weight compounds (Tweedy *et al* 1970; Shankar *et al* 2006).

Significantly, the culture NRS-01 shows tolerance to aniline concentration up to 0.1%. However, at low concentration, the mechanism of tolerance appears to be different as compared to high concentration where in flask ranging from 0.1mM to 0.5mM and showing a very pale color, but above 1mM concentration the red colour product (Fig. 5.4, section: 5.3.3) was very prominent. This is collaborative with the formation of the fluorescent compound at high concentration. The role of stress induced proteins have been significantly established in response to various pollutants such as aromatic compounds and metals. for incidence occurring in organisms such as *Burkholderia xenovorans* LB400 (Denef. *et al.*, 2005) seen towards the presence of aromatic compounds such as benzoates and biphenyls, Phan-Thanh, (1996) reported the production of stress induced proteins in *Listeria monocytogenes* and cold shock proteins induced in *Bacillus subtilis* (Graumann, 1996).

In the present study however, the exact role of the fluorescent compound could not be established at the stage and further studies on its role need to be carried out establishing the significance of this protein. However, since the appearance of FP is seen prior to aggregation, it could be possible that it plays a role in aggregation. Bacterial aggregation is an important process which gives protection to the cultures from toxic chemicals and anti-microbials. This has been demonstrated in *Pseudomonas* in response to antibiosis and cold shock (Klebensberger *et al.*, 2006; Farrell and Quilty, 2002; Hoffman *et al.*, 2005).

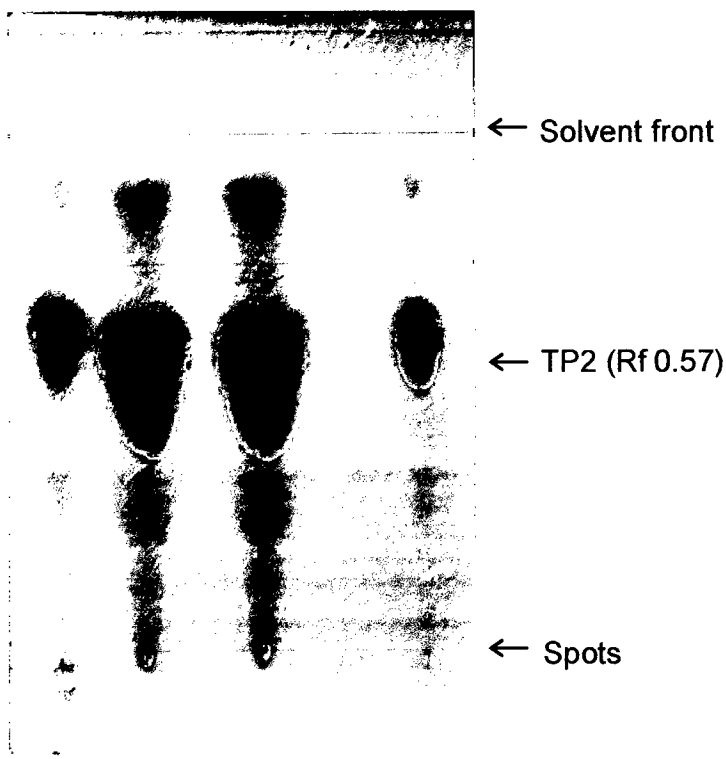
6.2.2. Isolation and characterization of TP2

Growth of NRS-01 in aniline depicted a typical response not only on the formation of an extracellular fluorescent compound but also in the formation of a coloured compound which was found to be associated with the cell surface.

The formation of this compound was also related to the aniline in the culture broth. It was interesting to note that with concentration as low as 0.05mM no pink colouration was detected. However, the cells acquired red colouration with concentration 0.1mM and above. The intensity of this compound increased with the increase in concentration and the time of incubation. TLC pattern of the extracted compound (Fig. 6.3) showed the compound having an Rf of 0.57.

Studies on microbial transformation of aniline at neutral condition have been reported with the formation of compounds which are coloured. Basically, pink or red compounds which have been identified as examples of azo compounds. Such azo compounds are formed with reaction between aromatic compounds and anilines. Some of the common azo dyes formed by such reactions are; 1) Reaction between the

Fig. 6.3: TLC showing TP2 a major compound accumulated



Solvent system – Hexane : Acetone (75 : 25)

β–naphthol and p-nitro aniline leads to the formation of para red and 2) Methyl red, a product formed from a reaction between N,N-dimethyl aniline and sulphanilamide (Morisson and Boyd, 1983). Prikryl *et al.*, 2007, have reported formation of a triazene on reaction between aromatic amines and 5-nitro-2,1-benzisothiazole-3-diazonium hydrogensulphate.

In the present study, it is evident that aniline induces a response of NRS-01 to overcome its toxicity by trapping the high concentration of aniline to form a red coloured product. Such a response is not seen at low concentration where the mechanism could be different. The aniline could either be transformed to a product which is less toxic or cells could form a barrier around them to avoid the cellular contact with aniline.

The response of this culture to aniline in presence of glucose has shown aggregation at the time where the concentration of glucose is completely utilized (Chapter V, Fig. 5.8a), these cellular floccules block the entry of toxic substances in to the cells. Such mechanisms have been noted in response to antibiotics where the cells develop resistance to antibiotics by forming aggregates (Voloshin *et al.*, 2004). Stewart and Costerton, (2001), have also reported the formation of such bacterial biofilms leading to aggregation in the presence of antibiotics making the culture resistant to it.

During this period, it appears that the cells are adapting themselves to survive in the medium containing in the presence of aniline, resulting in the formation of a red coloured product which intensifies with the increase in the incubation period.

Compound TP2 was found to be a water insoluble product that stuck on the walls of the glass vial forming needle shaped crystals with evaporation of solvent. The compound discolourized to yellowish green when acidified with 1N HCl and turned back to light red when neutralized with 1N NaOH. This reaction is normally used as a spot test for quinones (Finley, 1974). Allowing the treatment with 1N HCl for an increased period of time, the colour of the compound turned to dark violet-pink which did not revert back when neutralized.

The characterization of this compound using the spectral data and elemental analysis was undertaken to compare its structure in the known existing metabolites/azo compounds. The elemental analysis of the product showed 68.86% C, 4.94% H, and 13.48% N, and mass spectrum from which the empirical formula was derived to be $C_{23}H_{20}N_4O_3$ or $C_{21}H_{18}N_3O_3$.

Further, the spectrophotometric scan (Fig. 6.4) showed absorbance maxima of the red compound at 520 nm with other peaks at wavelengths 345nm, 260nm, 227nm and 203 nm. The IR spectrum of the compound (Fig. 6.5) showed the presence of carbonyl group at 1732 nm, the presence of N- or NH- group can be seen at peak formed at 3307nm, indicating the presence of a nitrogen group attached to the aromatic moiety. The NMR analysis (Fig. 6.6 a, b and c) showed the following results; chemical shifts in ^{1}HMR were found at 0.75ppm (3H), 1.25ppm (1H), 5ppm (1H), 5.5ppm (1H), 6ppm (1H), between 6.5-7ppm (12H) and 8.75 (1H). Chemical shifts in ^{13}CMR were found at 30ppm, 77, 91.4, 97,120.8, 122.9, 124.5, 125.2, 128.9, 129.47, 138, 149.37, 154.24, and 181ppm. The GC/MS (Fig. 6.7 a, b, c and d) showed the presence of two compounds based on their separation thereby giving their

Fig 6.4: UV-VIS scan of red compound -TP2

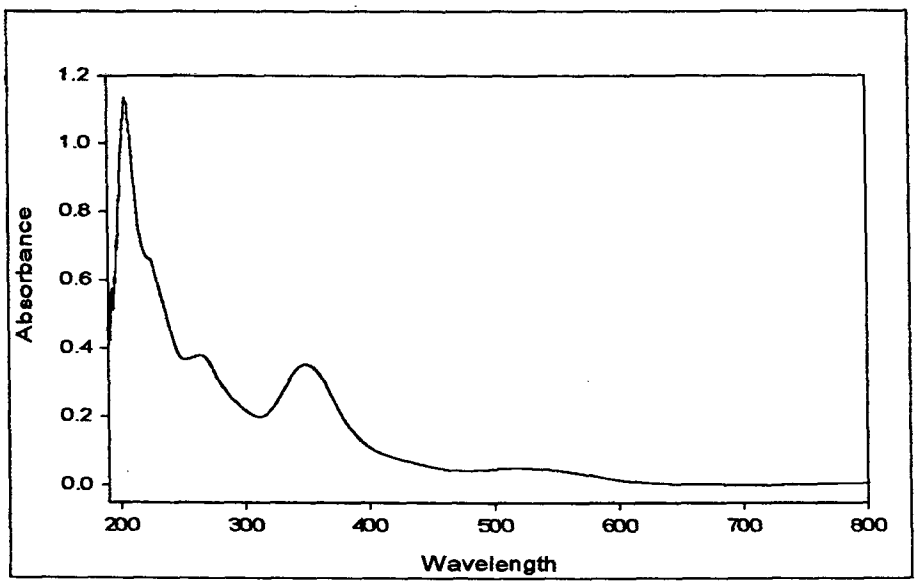
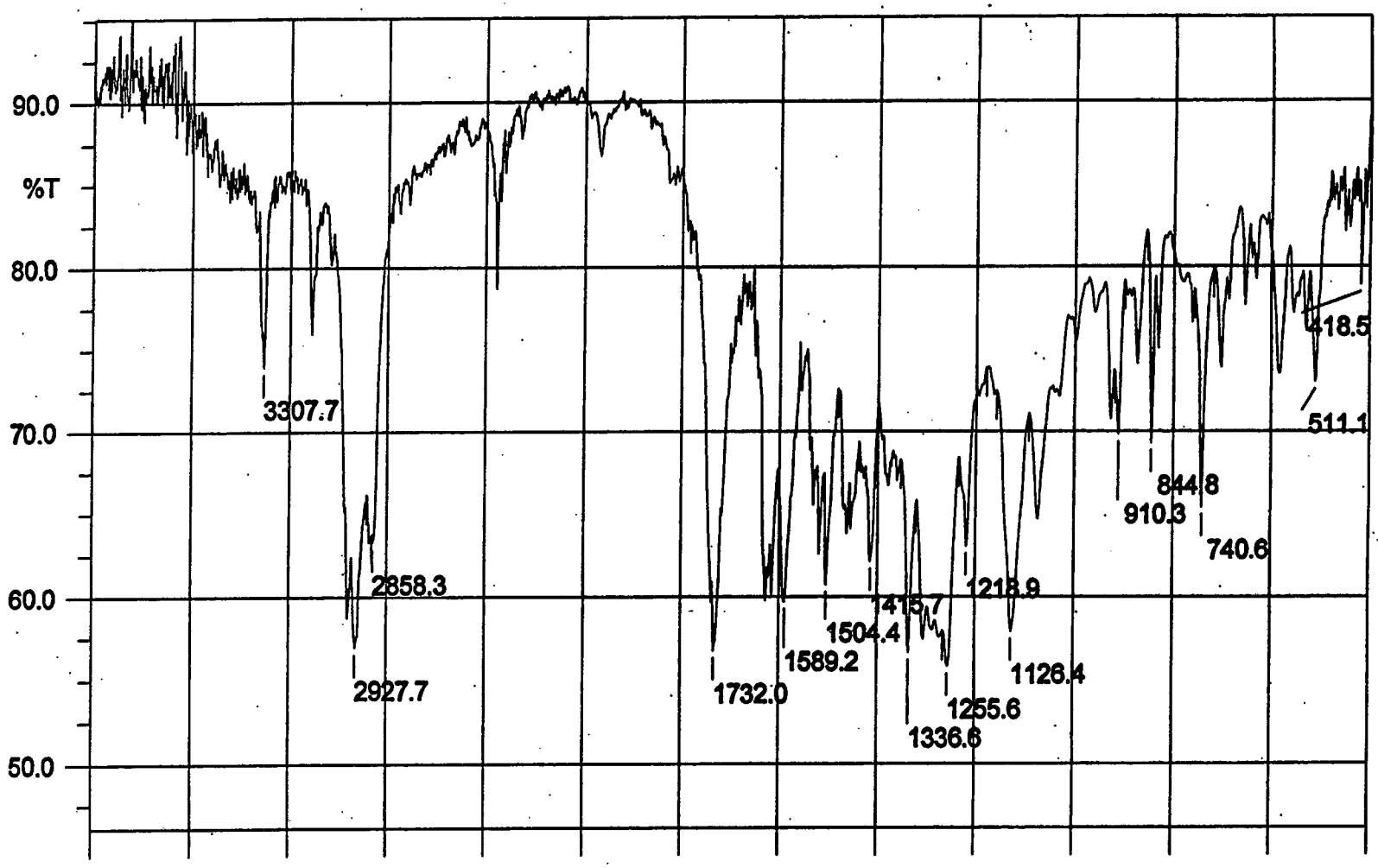


Fig. 6.5: IR Spectrum of TP2



SBHOR.IRS: TP2

Date:	03/17/2006	Time:	04:08:30	NScans:	25
Type:	HYPER IR	User:	Shimadzu Customer	Detector:	standard
Abscissa:	1/cm	Ordinate:	%T	Apodization:	Happ
Min:	401.17	Max:	3998.16	Range:	1/cm
Ndp:	1866	Data Interval:	1.92868	Resolution:	4.0
Gain:	auto	Aperture:	auto	Mirror Speed:	2.8

Fig. 6.6a: 1H-NMR spectrum of TP2

Acquisition Time (sec)	3.9584	*Comment*	Aarif Shaikh	*Date*	23 Jul 2006 10:42:08		
Date Stamp	23 Jul 2006 10:42:08			*File Name*	C:\WINDOWS\DESKTOP\NAVEEN\TP-2P_001001r		
Frequency (MHz)	200.13	*Nucleus*	1H	*Number of Transients*	32	*Origin*	av200
Original Points Count	16384	*Owner*	Administrator	*Points Count*	32768	*Pulse Sequence*	zg30
Receiver Gain	1290.20	*SW(cyclical) (Hz)*	4139.07	*Solvent*	CHLOROFORM-d		
Spectrum Offset (Hz)	1213.1884	*Sweep Width (Hz)*	4138.95	*Temperature (degree C)*	27.000		

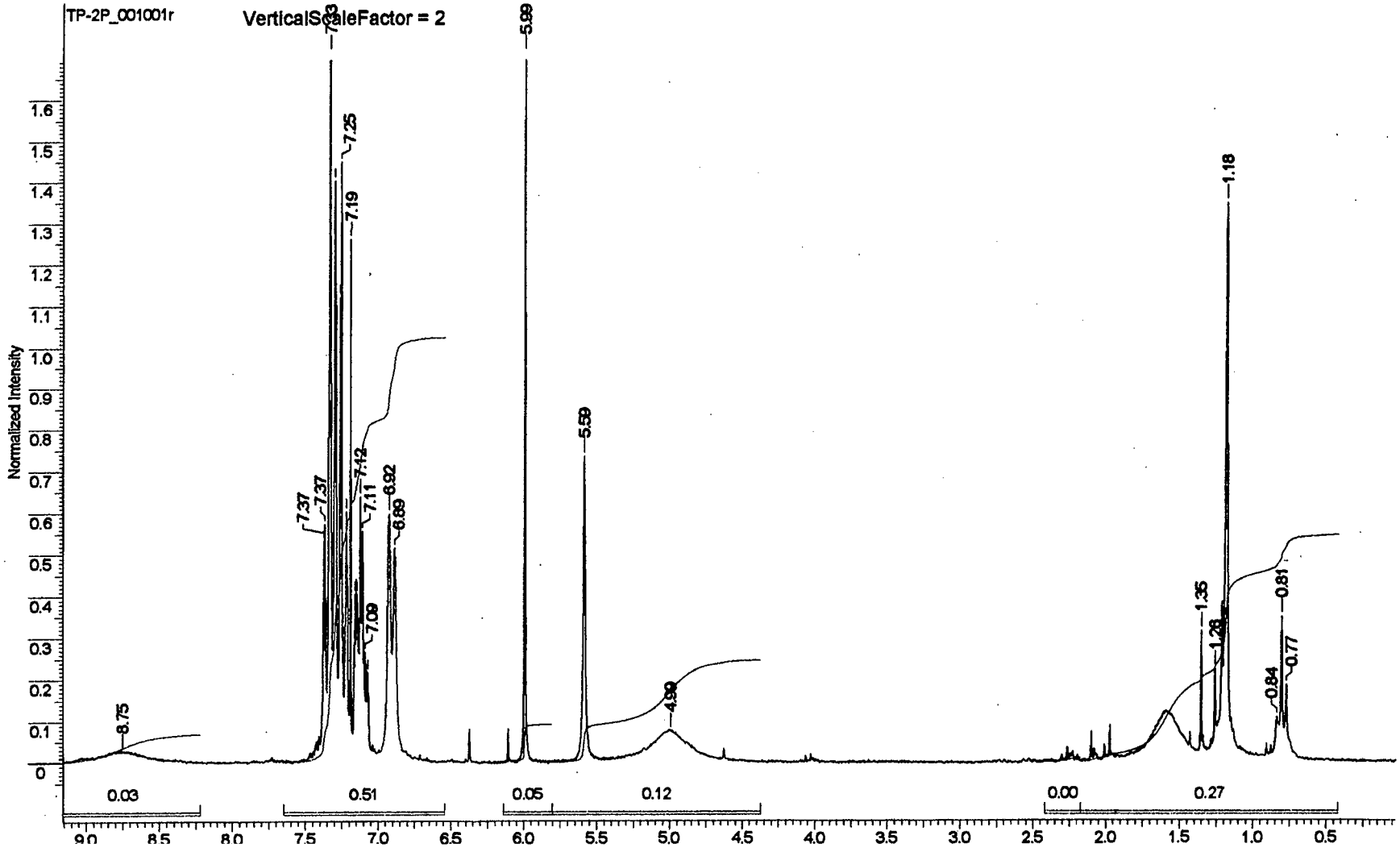


Fig. 6.6b: 13C-NMR spectrum of TP2

Acquisition Time (sec)	1.3664	*Comment*	Arif	*Date*	26 Jul 2006 22:49:36		
Date Stamp	26 Jul 2006 22:49:36			*File Name*	C:\WINDOWS\DESKTOP\NAVEEN\C-13-TP-2P_002001r		
Frequency (MHz)	50.32	*Nucleus*	13C	*Number of Transients*	640	*Origin*	av200
Original Points Count	16384	*Owner*	Administrator	*Points Count*	32768	*Pulse Sequence*	dept135
Receiver Gain	16384.00	*SW(cyclical) (Hz)*	11990.41	*Solvent*	CHLOROFORM-d		
Spectrum Offset (Hz)	5031.7993	*Sweep Width (Hz)*	11990.04	*Temperature (degree C)*	27.000		

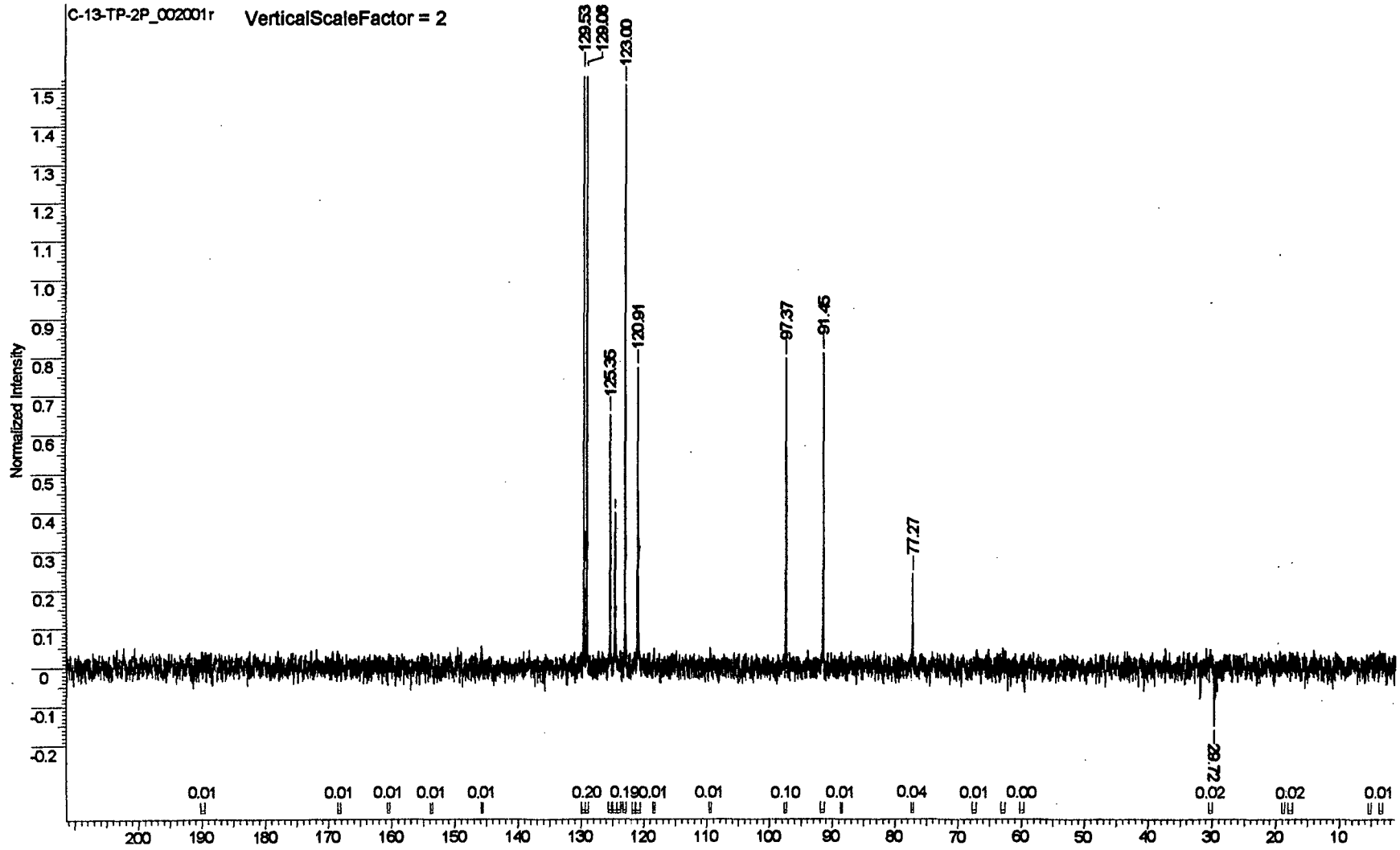


Fig. 6.6c: 13C-NMR spectrum of TP2

Acquisition Time (sec)	1.3664	*Comment*	Arif	*Date*	26 Jul 2006 22:13:20		
Date Stamp	26 Jul 2006 22:13:20			*File Name*	C:\WINDOWS\DESKTOP\NAVEEN\C-13-TP-2P_001001r		
Frequency (MHz)	50.32	*Nucleus*	13C	*Number of Transients*	1200	*Origin*	av200
Original Points Count	16384	*Owner*	Administrator	*Points Count*	32768	*Pulse Sequence*	zgpg30
Receiver Gain	161.30	*SW(cyclical) (Hz)*	11990.41	*Solvent*	CHLOROFORM-d		
Spectrum Offset (Hz)	5031.7993	*Sweep Width (Hz)*	11990.04	*Temperature (degree C)*	27.000		

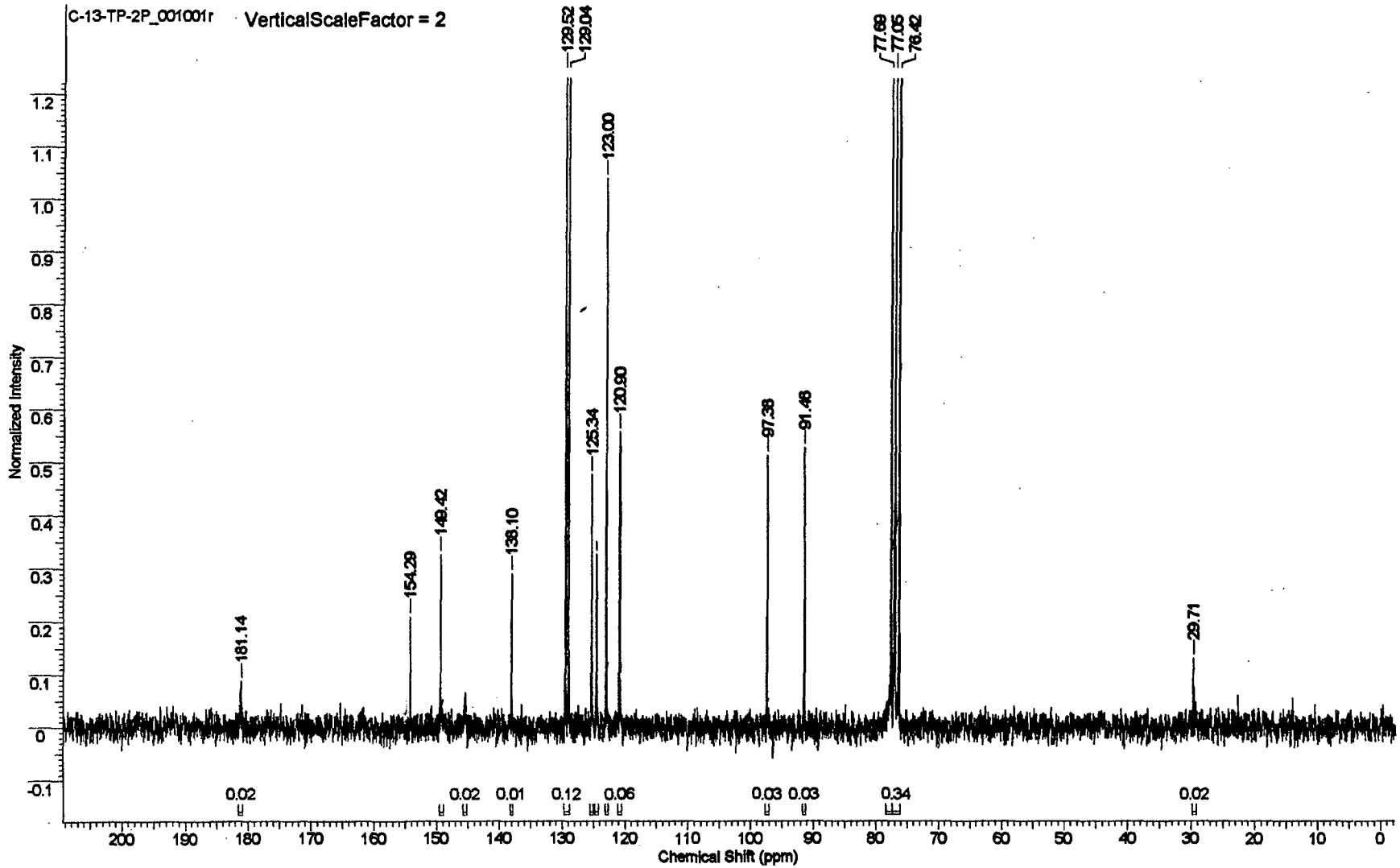
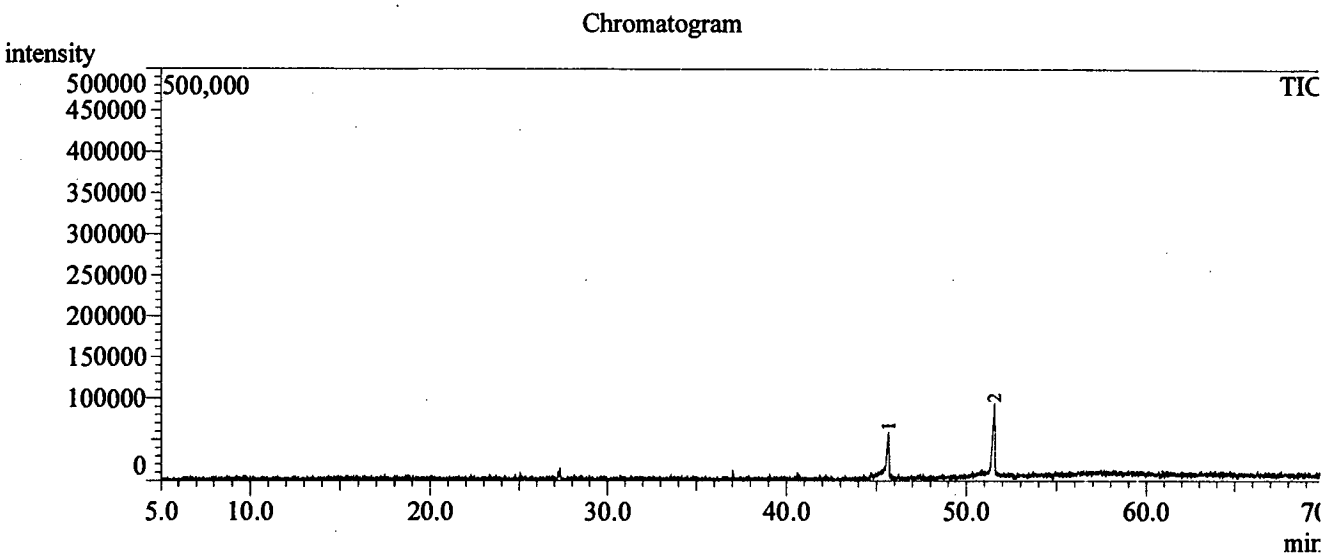


Fig. 6.7a: GC/MS profile of TP2

Sample Information

Analyzed :09-03-06
Sample Name :TP2

Analytical Data
Method : GC/MS Inj Volume (uL):1
Column: XTI-5,30m, ID 0.32mm Inj Temp:320 Column Temp:60-320



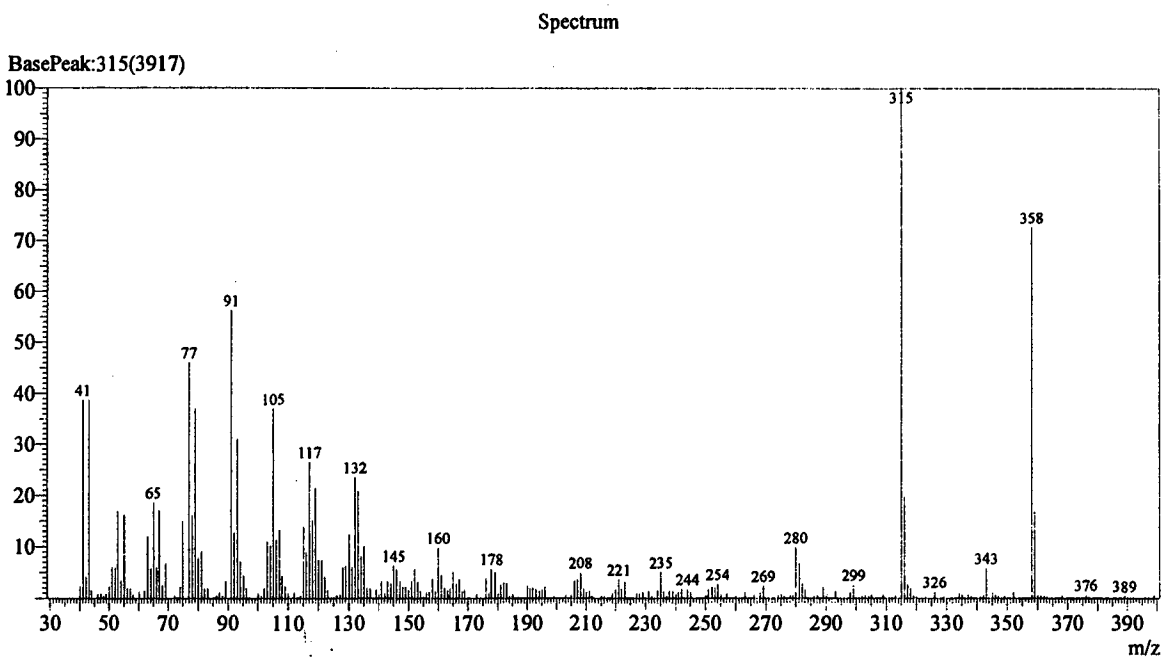


Fig. 6.7b: GC/MS profile of TP2

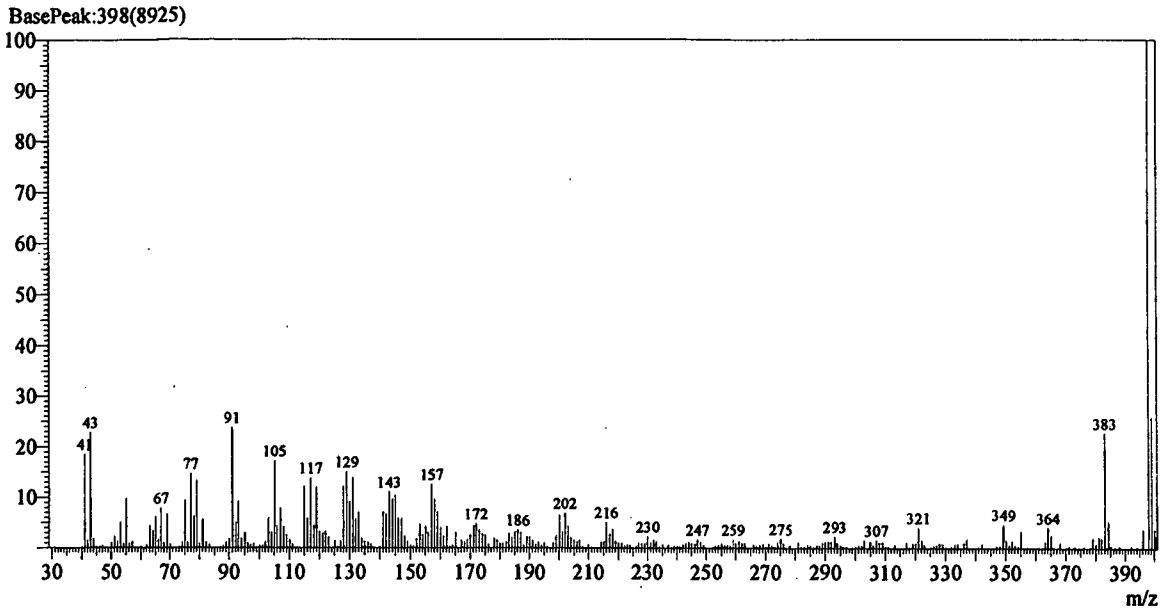


Fig. 6.7c: GC/MS profile of TP2

Library Search
<< Target >>
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RawMode:Single 45.683(5003) BasePeak:315.10(4342)
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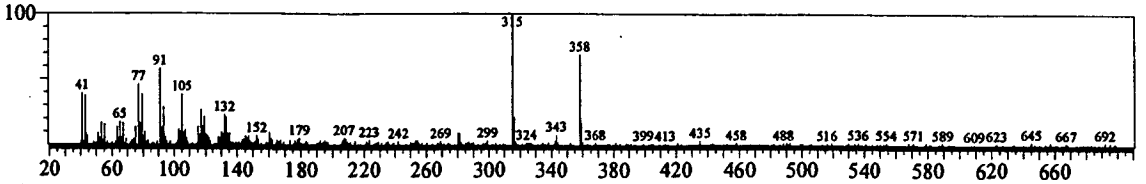

Hit#:1 Entry:259911 Library:WILEY7.LIB
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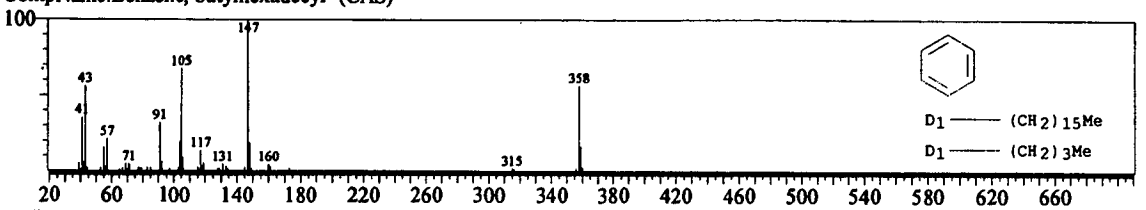
CompName:Benzene, butylhexadecyl- (CAS)



Hit#:2 Entry:137812 Library:NIST147.LIB
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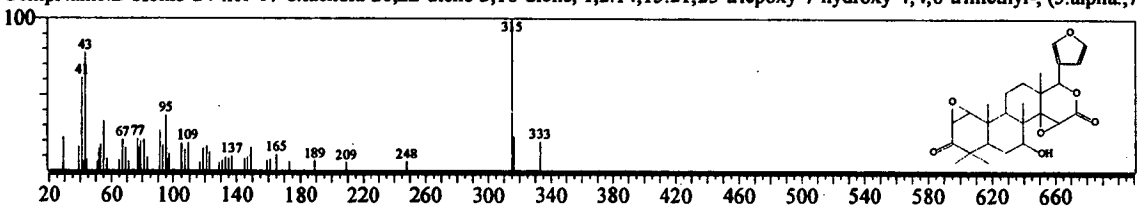
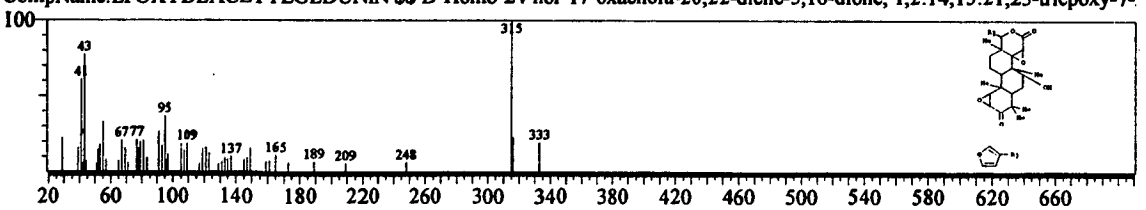
CompName:D-Homo-24-nor-17-oxachola-20,22-diene-3,16-dione, 1,2:14,15:21,23-triepoxy-7-hydroxy-4,4,8-trimethyl-, (5.alpha.,7



Hit#:3 Entry:306103 Library:WILEY7.LIB
SI:61 Formula:C26 H32 O7 CAS:35963-8-7 MolWeight:456 RetIndex:0
CompName:EPOXYDEACETYLGEDUNIN $$ D-Homo-24-nor-17-oxachola-20,22-diene-3,16-dione, 1,2:14,15:21,23-triepoxy-7-



Hit#:4 Entry:118598 Library:NIST147.LIB
SI:61 Formula:C26H46 CAS:55191-38-3 MolWeight:358 RetIndex:0
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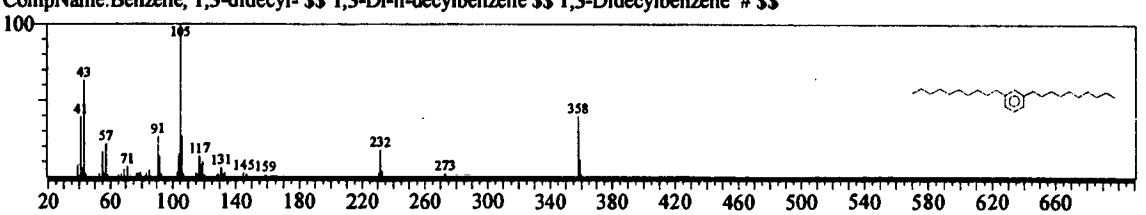

Hit#:5 Entry:114289 Library:NIST147.LIB
SI:61 Formula:C21H30O4 CAS:379-68-0 MolWeight:346 RetIndex:0
CompName:Pregn-4-ene-3,20-dione, 18,21-dihydroxy- $$ 11-Deoxy-18-hydroxycorticosterone $$ 18-Hydroxy-11-deoxycorticoster

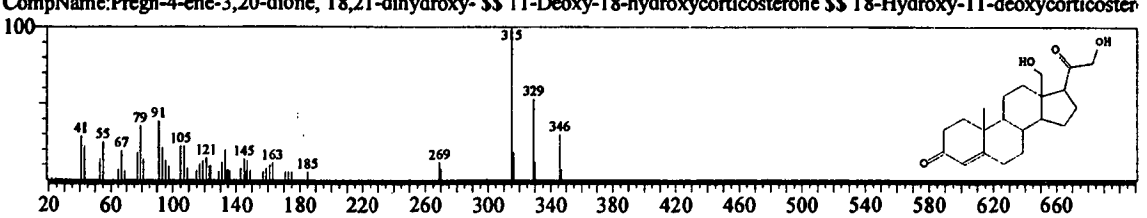

Fig. 6.7d: GC/MS profile of TP2

Library Search
<< Target >>
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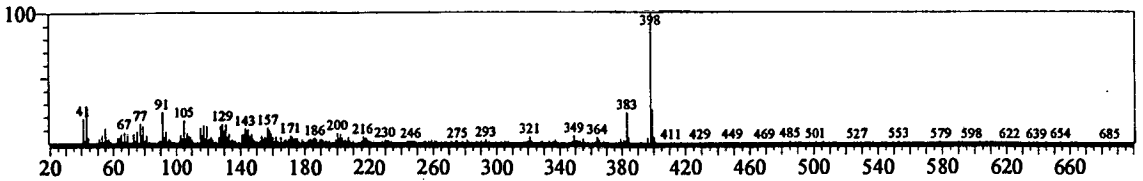

Hit#:1 Entry:142449 Library:NIST147.LIB
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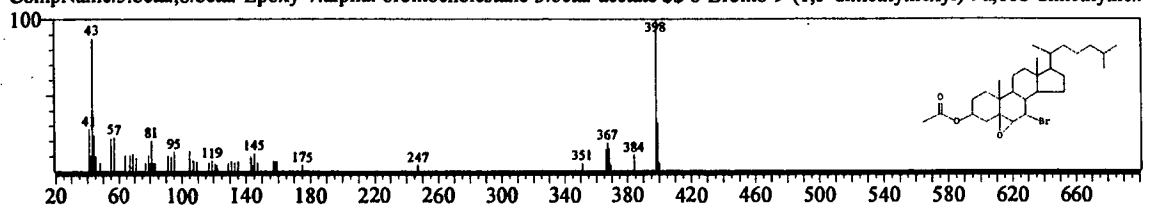

Hit#:2 Entry:129501 Library:NIST147.LIB
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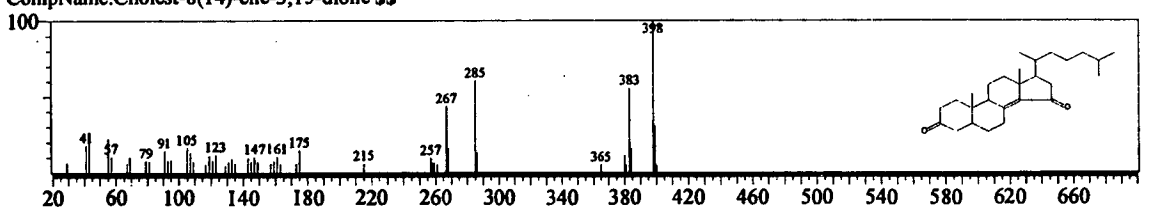
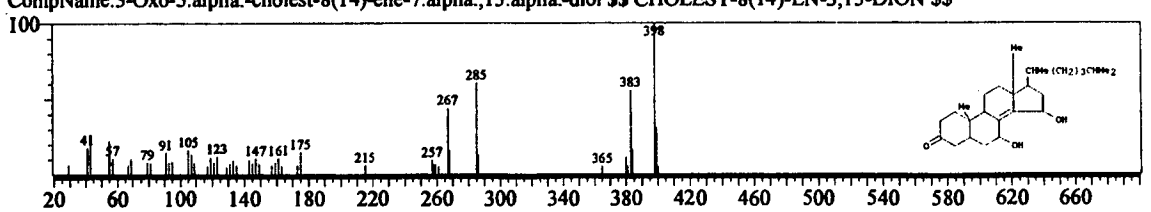


Hit#:3 Entry:283584 Library:WILEY7.LIB
SI:58 Formula:C27 H42 O2 CAS:69140-7-4 MolWeight:398 RetIndex:0
CompName:3-Oxo-5.alpha.-cholest-8(14)-ene-7.alpha.,15.alpha.-diol $$ CHOLEST-8(14)-EN-3,15-DION $$



Hit#:4 Entry:138024 Library:NIST147.LIB
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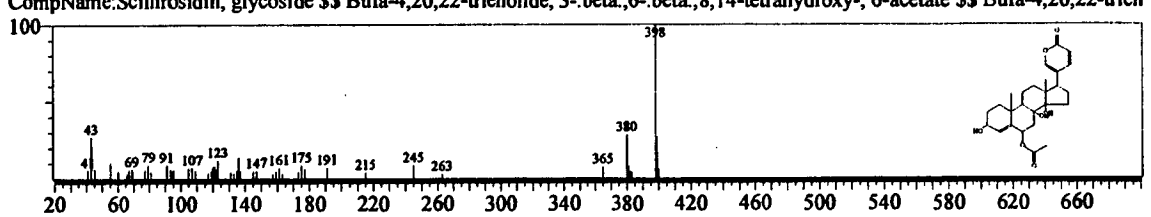
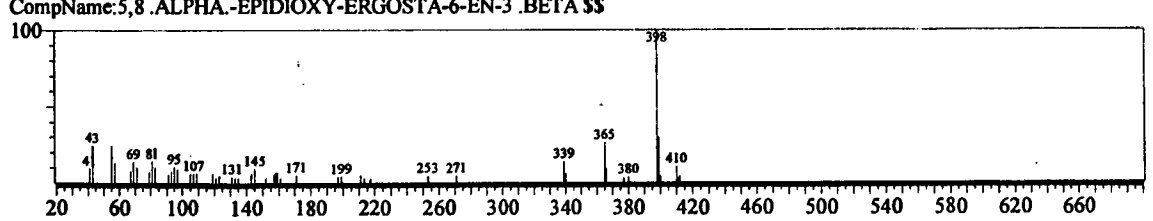
CompName:Scillirosidin, glycoside $$ Bufa-4,20,22-trienolide, 3-.beta.,6-.beta.,8,14-tetrahydroxy-, 6-acetate $$ Bufa-4,20,22-trien



Hit#:5 Entry:297796 Library:WILEY7.LIB
SI:56 Formula:C28 H46 O3 CAS:0-0-0 MolWeight:430 RetIndex:0
CompName:5,8 .ALPHA.-EPIDIOXY-ERGOSTA-6-EN-3 .BETA $$



giving their retention time at 45min and 51min. Further, the MS revealed the first eluted compound to have a base peak of 315 and the second compound had the base peak of 398. On comparing the mass distribution on the chromatogram with the mass spectra present in the library, the compound TP2 was found to have a 61%similarity to a basic the steroidal structure.

Reports on aniline transformation resulting in a high molecular weight compound (3E)-14-Hydroxy-3,7,11-trimethyl-3,5-tetradecadienyl-2-(hydroxymethyl)-1H indole-3-carboxylate have been reported by Shankar *et al*, (2006), having a molecular weight of 443 (Fig. 6.8). Thorn *et al.*, 1996 have reported that in the absence of catalysts, aniline undergoes nucleophilic addition reactions with the carbonyl functionality of the fulvic and humic acids and becomes incorporated in the form of anilinohydroquinone, anilinoquinone, anilide, heterocyclic, and imine nitrogens (Fig. 6.9a-k) In the presence of enzymes such as peroxidase, laccase and catalysts such as birnessite, aniline undergoes free radical coupling reactions together with nucleophilic addition reactions with the fulvic and humic acids (Fig. 6.9l-r). Among the condensation products unique to the catalyzed reactions are azobenzene nitrogens, iminodiphenoquinone nitrogens, and nitrogens tentatively assigned as imidazole, oxazole, pyrazole, or nitrile. Based on information available, the reactions of aniline transformation either in the environment or by different organism have been elucidated as given in (Fig. 6.9). The details have been used from a documented source available on the internet published by Pennington *et al.*, (2003), having the URL http://el.erdc.usace.army.mil/elpubs/pdf/trel03-2.pdf.

Fig. 6.8: Pathway showing complex formation with aniline (Shankar, 2006)

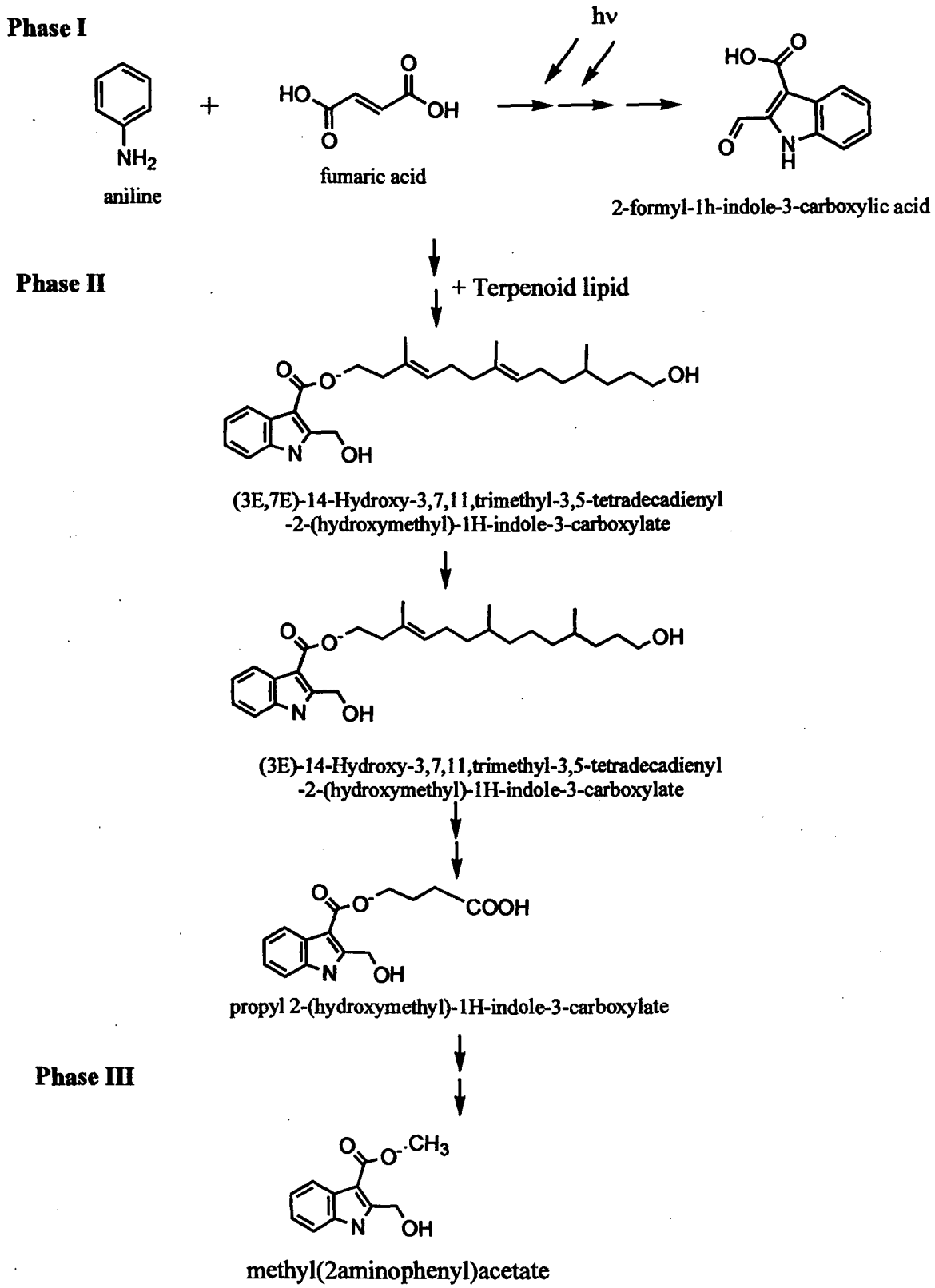
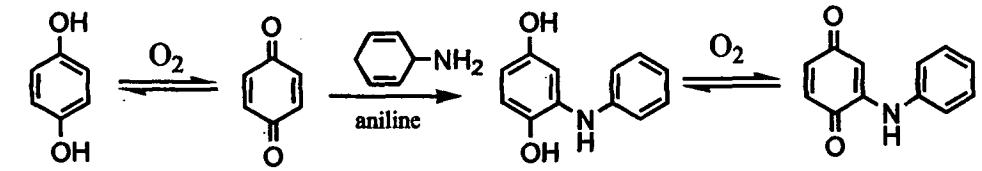


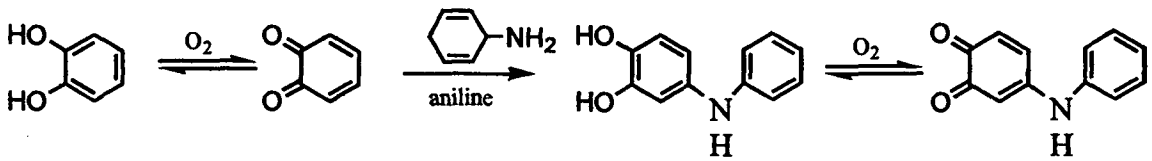
Fig. 6.9(a-h): Various addition reactions of aniline

a



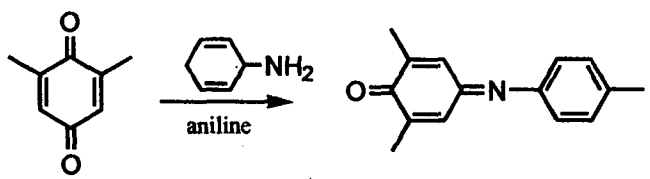
Benzene-1,4-diol Benzoquinone Anilinohydroquinone Anilinoquinone

b



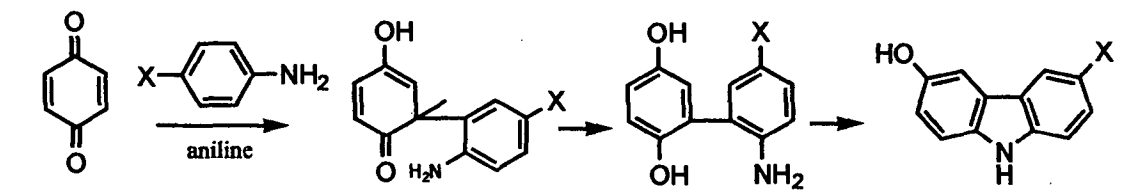
Benzene-1,2-diol Benzoquinone Anilinohydroquinone Anilinoquinone

c



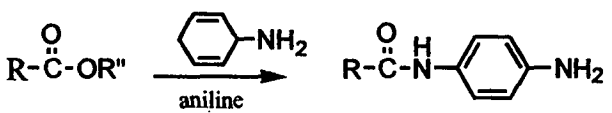
Benzoquinone Imine

d



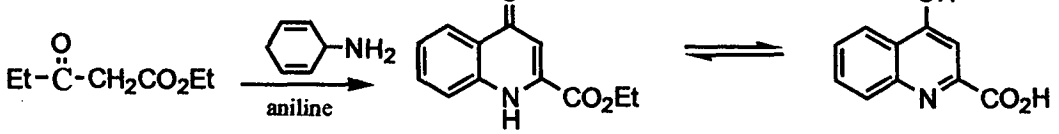
Benzoquinone Carbazol

e



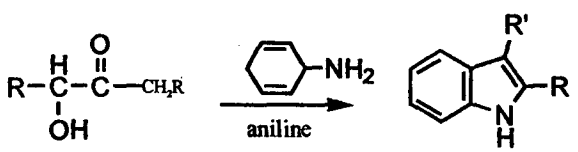
Anilide

f



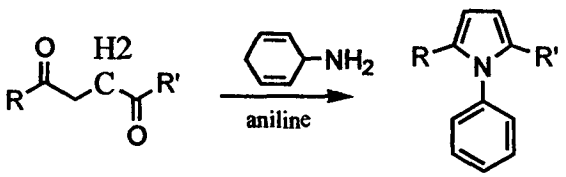
Quinolone

g



indole

h



N-phenylpyrrole

Fig. 6.9(i-o): Various addition reactions of aniline

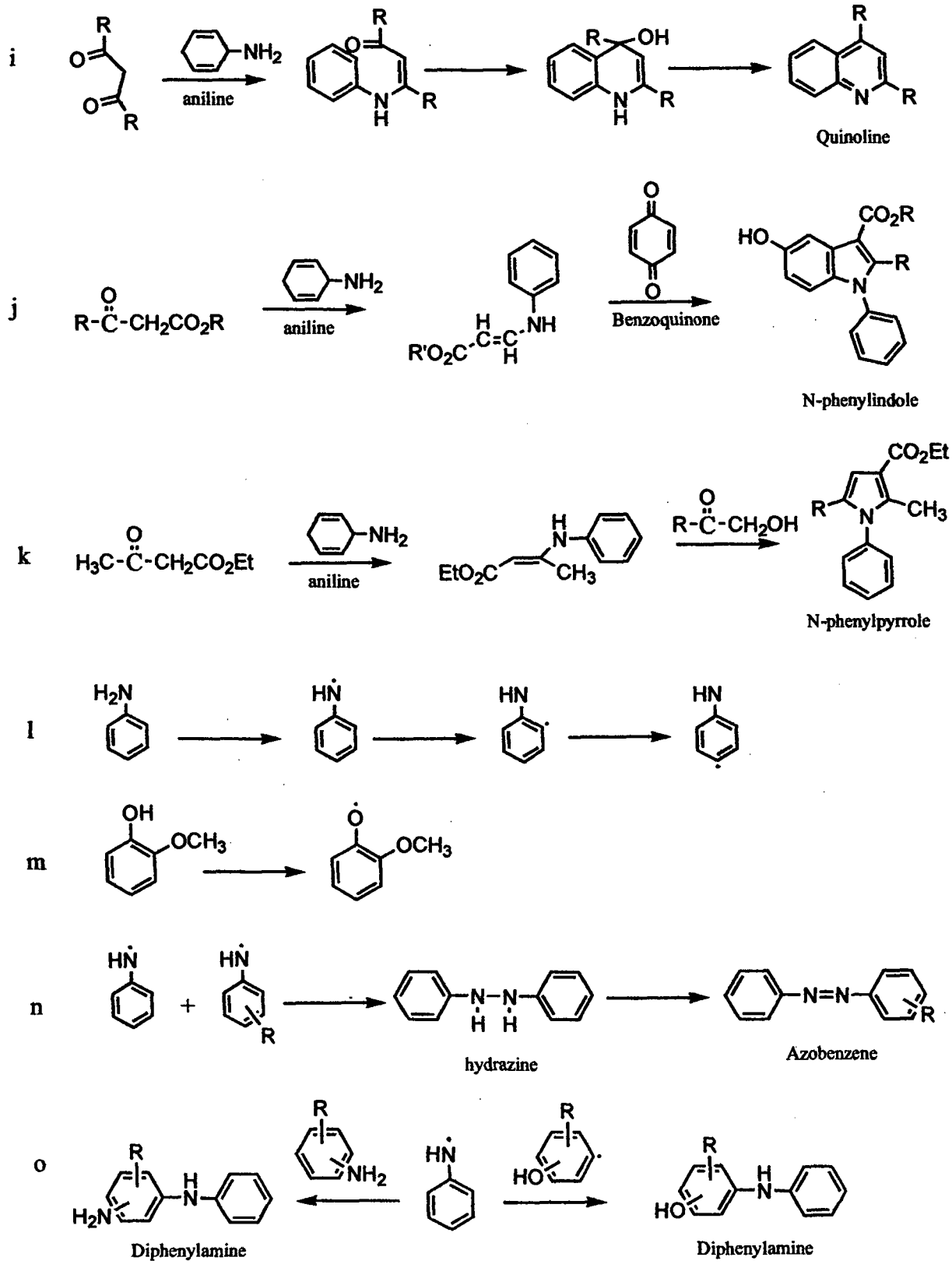
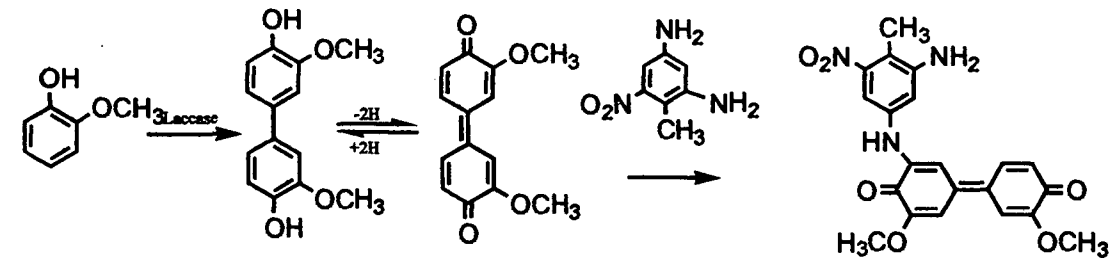


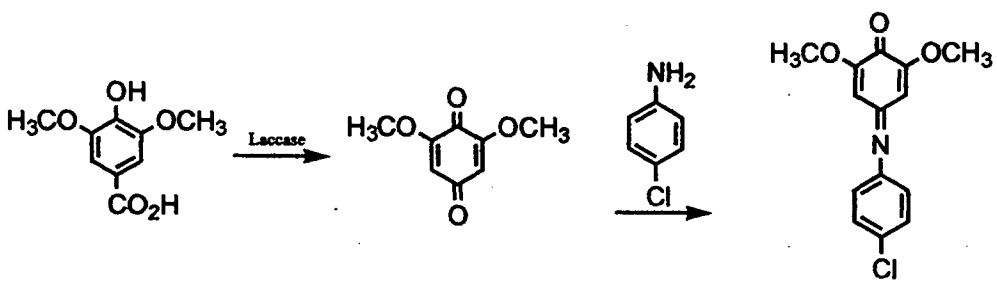
Fig. 6.9(p-r): Various addition reactions of aniline

p



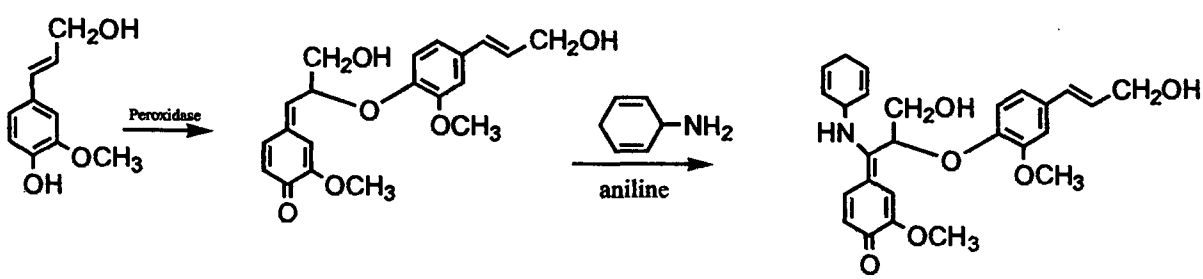
Aminoquinone

q



Imine

r



Characterization of the red compound (TP2) produced by NRS-01 in response to aniline during growth on glucose also appears to follow a similar path. It is interesting to note that the reactions at alkaline condition as well as neutral are not different. The general mechanisms of detoxification of developing resistance and tolerance to aniline appear to be similar. Based on the literature available and the characteristics of TP2, the following basic structures have been tentatively elucidated for TP2 formed by *Halomonas* sp. (NRS-01) in response to aniline at concentration above 1mM. However, based on the various analytical spectra data procured, the complete structure of the compound is yet to be elucidated.

The studies with biotransformation of aniline by bacteria under alkaline conditions have shown interesting results. The response of the isolates depicted at alkaline condition has confirmed the toxicity of this compound even in extremes of pH. Further, the bacterial detoxification mechanism appeared to be similar as reported under anaerobic system, converting the molecule to high molecular weight compound. The complexity of the substrate is also an added factor for production of high molecular weight compounds as in alkaline conditions, the diazonium salts are formed, which complexes with the hydroxylated aniline molecules.

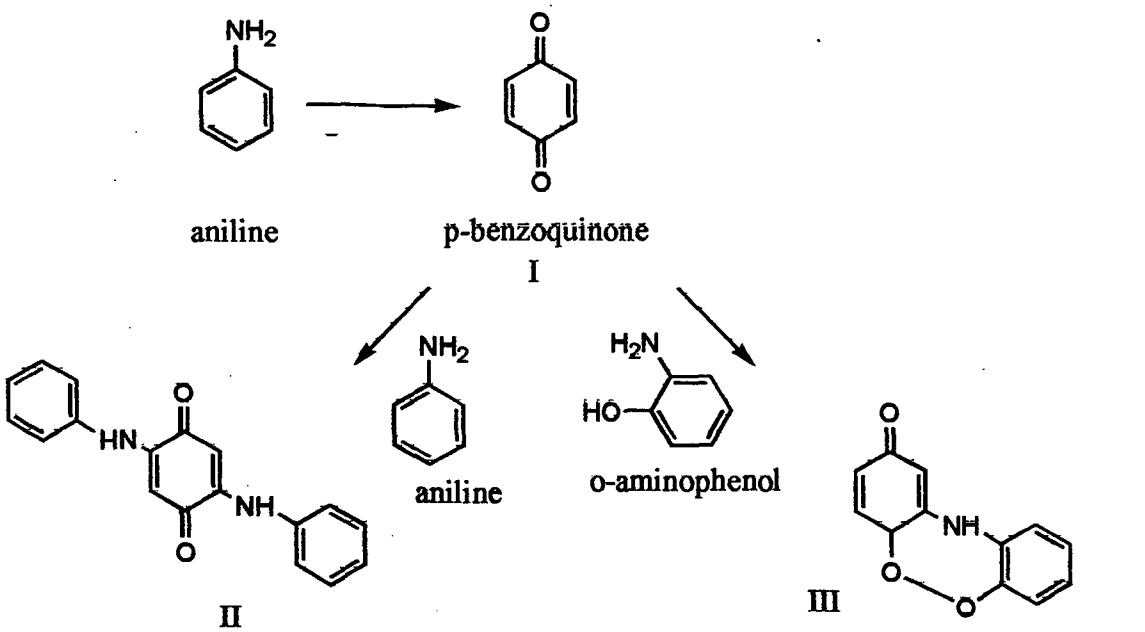
This study has shown that the microorganisms detoxify pollutants in the manner, which is common for the cellular systems irrespective of the environmental conditions.

It appears that the molecule is conveted to a keto compound, which fuses with unreacted aniline molecules, or with the molecule having hydroxyl group at the ortho-position to give a 3-ring compound.

Based on the IR data, there is a quinone to which 2 other aromatic rings are fused to give a high molecular weight compound. it is therefore envisaged that the molecule being biotransformed to give structure I (Fig. 6.10), which reacts with either unreacted/unmodified aniline molecules to give the structure as shown in structure II, or reacted with aniline which has hydroxyl group at ortho- position giving structure III.

The response of NRS-01 is unique and novel so no reports are available on such a response in presence of aniline under alkaline conditions.

Fig. 6.10: Proposed structures of TP2



2,5-Bis-phenylamino-[1,4]benzoquinone

5*H*,11a*H*-10,11-Dioxa-5-aza-dibenzo[*a*,*d*]cyclohepten-3-one

SUMMARY
&
FUTURE PROSPECTS

Summary

Aromatic amines are reported to be one of the most prominent environmental pollutants being released in the environment in the form of effluents or pesticides and the microbial interaction of various organisms on these aromatic has been well documented. The present work was undertaken to study the role played by alkalophilic bacteria on aniline and N,N-Diemthyl-1-Naphthylamine (NND) used as model aromatic amines, under alkaline condition.

The isolation of alkalophilic bacteria resulted in two potent halotolerant alkalophiles which were identified as *Halomonas* sp. NRS-01 and NK2 capable of tolerating high concentration of aromatic amines; aniline and N,N-Diemthyl-1-Naphthylamine (NND) respectively, a characteristic which attributes to their novel metabolic system. The cultures were therefore unique in their response to these aromatic amines.

Halomonas sp. NK2 showed a different response with the production of a biosurfactant which was responsible to the increase in cell substrate content. The biosurfactant was characterized as a protein compound. Culture NRS-01 showed an unusual response in presence of high concentrations of aniline with the production of a red coloured compound. This was preceded with aggregation of cells and production of a blue fluorescent compound, which was observed only in the presence of cells indicating the role of NRS-01 in the formation of these products.

The blue fluorescent compound was found to be proteinaceous with a low molecular weight of less than 3000Da. However, its exact role in the bacterial response towards aniline is yet to be understood. Spectral analysis of the red product indicated it to have a keto group belonging to a quinone molecule to which two other aromatic rings are fused giving a high molecular weight 3-ringed aromatic compound.

The studies with biotransformation of aniline by bacteria under alkaline conditions showed interesting results. The response of the isolates depicted at alkaline condition has confirmed the toxicity of this compound even in extremes of pH.

To our knowledge so far, there has been no reports on alkalophiles tolerating such high concentrations of aniline or N,N-Dimethyl,1-Naphthylamine. The response of NRS-01 is unique and novel as no reports are available on such a response in presence of aniline under alkaline conditions.

Future Prospects

Alkalophiles are a diverse group of bacteria having an enormous potential to survive and grow at pH conditions likely to kill other neutrophilic bacteria. Such characteristics are best suited for developing industrially potent bacterial strains for the production of commercially important molecules.

From the present work carried out, two such potent organisms (*Halomonas* sp.) able to tolerate alkaline condition (10.5) as well as high salt concentration (avg 10%) has been isolated showing the production of biosurfactant and a red compound on their interaction with NND and aniline respectively.

Future research on these cultures may be directed towards:

1) Use of biosurfactants produced by NK2 to enhance the bioavailability of toxic compounds for its degradation during environmental calamities such as oil slicks or other soil contamination.

2) Use of the blue fluorescent compound (FP) produced by NRS-01 as a bioindicator for early detection of pollution.

3) Identification of the red compound TP2 produced by NRS-01 as a medically important compound as quinones are used as anti-cancer drugs or used as a raw material for the production of other commercially important products.

APPENDICES

APPENDIX I

A GROWTH AND BIOCHEMICAL MEDIA

A.1. Normal Saline

Add 0.85gm NaCl to 100ml distilled water and autoclave.

A.2. Nutrient Broth

Peptone	10.0 gm
Beef extract	3.0 gm
Sodium-chloride	5.0 gm
Double Distilled Water	1000 ml

pH was adjusted to 7.0 with 0.1 N NaOH

A.3. Nutrient Agar

For nutrient agar, 1.5 gm of agar added to 100 ml nutrient broth. Digested in water-bath and sterilized accordingly.

A.4. Polypeptone Yeast Extract Glucose Broth (PPYG) for 1L

Peptone	5.0 gm
Yeast Extract	1.5 gm
Na_2HPO_4	1.5 gm
NaCl	1.5 gm
$MgCl_2$	0.1 gm
* Sodium carbonate (10%)	10 ml
* Glucose (10%)	10 ml
Double Distilled Water	1000 ml

* Glucose and sodium carbonate needs to be autoclaved separately and added to the autoclaved basal medium (contains all the ingredients except glucose and sodium carbonate) before dispensing.

A.5. Polypeptone Yeast Extract Glucose Agar (PPYG) for 1L

For PPYG agar, 1.5 gm of agar added to 100 ml PPYG basal medium. Digested in water-bath and sterilized accordingly. Glucose and sodium carbonate added before dispensing medium.

A.6. Polypeptone Yeast Extract Glucose Agar (PPYG) for 1L (used for characterization of isolates)

In order to achieve various pH values of the media, the basal medium is treated in the following way:

pH 6.5: pH adjusted to 6.5 using 1N HCl.

pH 8.5: 2.5ml of 10%Na_2CO_3 was added to the medium after autoclaving.

pH 10.5: 10 ml of 10% Na_2CO_3 was added

pH 12.0: 10 ml of 10% Na_2CO_3 was added and 0.5 ml of sterile 1N NaOH was added.

A.7. Polypeptone Yeast Extract Broth/Agar (PPY)

The composition of PPY medium (broth/agar) is similar to PPYG, except for the addition of glucose. Glucose is not added in basal PPY medium while preparing either agar or broth. To get half strength medium, PPY medium is diluted to a ratio of 1:1 with distilled water and autoclaved.

A.8. Mineral Salt medium (single strength) for 1L : (Sangodkar, U. M. X. *et al.,* 1991)

Dipotassium hydrogen ortho phosphate (12.6%)	100ml
Potassium dihydrogen ortho phosphate (18.2%)	20ml
Ammonium nitrate (10%)	20ml
Magnesium sulphate (1%)	20ml
Manganese sulphate (0.6%)	0.2 ml
Sodium molybdate (0.6%)	0.2 ml
Calcium chloride (dihydrate) (1%)	15 ml
Ferrous sulphate (green crystals)	0.06 gm
Double Distilled Water	1000 ml

For use: 10 ml of double-strength media made to 20 ml with distilled water and sterilized for 10 min at 120°C temperature and 15 lbs pressure. To prepare MSM agar, MSM broth is mixed with agar (1.5%) and autoclave accordingly.

A.8.1. Mineral Salt medium II (MSM II)

The composition of this medium is similar to A.8, with a difference that the medium is devoid of ammonium nitrate.

A.9. Biochemical medium used for identification (Gee *et al.*, 1980; Sneath *et al.*, 1986)

A.9.1. Hugh and Leifson's (HL) test

Peptone	2 gm
Yeast Extract	0.5 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Glucose	10 gm
O-cresol red	0.01 gm
Sodium Carbonate	10 gm
Agar	10 gm
Double distilled water	Make volume to 1000ml
pH	9.5

Medium was prepared, digested in a water bath, dispensed in test tubes and autoclaved for 20mins at 15psi. Sodium carbonate and glucose were autoclaved separately and added to the basal medium for HL medium. Test tubes were heated in a boiling water bath for 10mins and cooled immediately in an ice bath. 18hrs old cultures used as inoculum were stab inoculated. Growth and color change of the indicator dye was noted in the aerobic and anaerobic tubes. To check growth at anaerobic condition, test tubes were overlayered with sterile liquid paraffin.

Growth in medium maintained as aerobic and anaerobic condition demonstrate obligate aerobes and anaerobes respectively, while growth in both the tubes show a behaviour of facultative anaerobes. Cultures able to bring about a colour change in the medium indicate fermentative otherwise an oxidative reaction.

A.9.2. Catalase test

Three or four drops of 3% (v/v) hydrogen peroxide were mixed with a loopful of culture on a glass plate. Evolution of effervescence by liberation of free oxygen was indicative of catalase positive organisms.

A.9.3. Oxidase test

A filter paper strip was soaked in N,N,N,N-Tetramethylparaphenylenediamine (TMPD) dye. Fresh bacterial culture was smeared on the moist filter paper using a toothpick. Production of a deep purple colour in 5-10 seconds indicates a positive oxidase test

A.9.4. Nitrate reduction test

Medium composition

Peptone	5 gm
Beef extract	3 gm
KNO_3	1 gm
Double distilled water	Make volume to 1L
Na_2CO_3	10 mg
pH	9.5

Nitrate broth is inoculated with 18hr old culture and incubated at R.T. for 24-48h. After incubation, 5 drops of sulfanilic acid and 5 drops of α-naphthylamine were added (Appendix II, B.5). Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5mg of zinc dust. Uninoculated tubes served as the control.

A.9.5. Carbohydrate utilization test

Medium composition

Peptone	5.0 gm
Beef extract	3.0 gm
*Sugar	0.5 gm
D/w	To make 1L
pH	9.5 adjusted by using 10% Na_2CO_3 solution

* 10% sugar solutions were filter sterilized and incubated at room temperature for 24 hrs to check for contamination prior to use. A final concentration of 1% was added to the autoclaved medium (test tubes containing durhams tubes) before inoculating the cultures and incubated at R.T. for 24-48h. Growth in the medium was observed as turbidity and the fermentation reaction was noted by the formation of gas bubble in Durham's tubes. Uninoculated tubes serve as the control.

Growth on various other carbon sources were checked by using filter sterilized compounds and the medium did not contain Durham's tubes. Growth was checked by the formation of turbidity.

A.9.6. Gelatin liquefaction

Medium composition

Peptone	5 gm
Beef extract	3 gm
Gelatin	120 gm
Distilled water	To make 1L
Sodium Carbonate	10 gm
pH	9.5

Inoculate tubes and incubate at R.T. for 24-48 h, the tubes were refrigerated for 30 min and the solidification of medium was observed. Non-solidification of medium after refrigeration showed a positive test.

A.9.7. Casein Hydrolysis

Inoculate skimmed milk agar plates and incubate at R.T. for 24-48 h, then examine the plates for the presence or absence of a clear area around the colony. A clear area around the bacterial colony indicates a positive proteolytic activity.

A.9.8. Starch hydrolysis

Starch agar medium composition

Peptone	5 gm
Beef extract	3 gm
Soluble starch	2 gm
Agar	20 gm
Distilled water	Make volume to 1L
Sodium Carbonate	10 gm
pH	9.5

Inoculate starch agar plates by spot inoculation. Incubate the plates at R.T. for 24-48hr, then flood the plates with Gram's iodine for 1 min and pour off the excess stain. Clear zone surrounding the colony indicated a positive test.

A.9.9. Antibiotic tests

Commercially available antibiotic discs were used to check the sensitivity of organisms to various antibiotics.

APPENDIX II

B REAGENTS AND BUFFERS

B.1. Reagents for salinity estimation

1. $AgNO_3$ solution: Pure crystallized Silver nitrate (27.25 gm) was dissolved in 1L of Double Distilled Water. This solution was mixed and stored in amber coloured bottle.
2. Potassium chromate indicator solution: Pure Potassium chromate (10 gm) was dissolved in 100ml of double distilled water.

B.2. Reagents for nitrite estimation

1. Sulphanilamide: Sulphanilamide (1 gm) was dissolved in 10 ml concentrated HCl and the solution was made up to 100ml with Double Distilled Water.
2. N- (1- naphthyl) ethylene diamine dihydrochloride: In 100ml Double Distilled Water, 0.1 gm of reagent was dissolved.
3. Standard $NaNO_2$ solution: Pure analytical grade $NaNO_2$ (0.1725 gms) was dissolved in 250ml of Double Distilled Water (1ml contains 10 μg atom of NO_2-N).
4. Working solution - A: Above mentioned standard $NaNO_2$ (2.5ml) solution was diluted to 250ml with Double Distilled Water (1ml contains 0.1 μg atom of NO_2-N).
5. Working solution - B: Working solution A (50ml) was diluted to 500ml with distilled water. (1ml contains 0.01μg atom of NO_2 - N).

B.3. Reagents for nitrate estimation

1. Concentrated Ammonium chloride (NH_4Cl): NH_4Cl (62.5 gm) was dissolved in a 250ml volumetric flask with distilled water.
2. Diluted Ammonium chloride (NH_4Cl): The above concentrated NH_4Cl (5ml) was diluted to 200ml with distilled water.
3. Amalgamated Cadmium granules: This reagent (100gm) is treated with 500ml of 2% solution of $CuSO_4$. The amalgamated Cd granules were washed several times with distilled water and stored in diluted NH_4Cl.
4. Sulphanilamide: The reagent (1g) was dissolved in 10ml of concentrated HCl and the volume was made to 100ml with distilled water.
5. N-(1-naphthyl)-ethylene diaminedihydrochloride: The reagent (0.1gm) was dissolved in 100ml of distilled water to prepare the stock solution of the reagent.
6. Standard KNO_3 solution: KNO_3 (0.1g) was dissolved in 100ml distilled water.

7. Working solution of KNO_3 (standard): The above solution (2.5ml) was diluted to 250ml with distilled water.

B.4. Reagents for phosphate estimation

1. 9N H_2SO_4: Concentrated H_2SO_4 (25ml) was added to 75ml Double Distilled Water.
2. Ammonium molybdate solution: Ammonium molybdate (9.5gm) was dissolved in 100ml of Double Distilled Water.
3. Ascorbic acid: Ascorbic acid (7gm) was dissolved in 100ml of Double Distilled Water.
4. Potassium Antimonyl Tartarate: Potassium antimonyl tartarate (3.25gms) was dissolved in 100ml Double Distilled Water.
5. Mixed Reagent: Ammonium molybdate (22.5ml) solution, 100ml of H_2SO_4 and 2.5ml of Potassium Antimonyl tartarate solution was mixed together.
6. Standard phosphate solution: Potassium-di-hydrogen-ortho phosphate (KH_2PO_4) (0.1361gms) was dissolved in 100ml of double distilled Water.
7. Working Phosphate solution: The above solution (2.5ml) was diluted to 250ml of double distilled Water (1ml = 0.1 μg atom).

B.5. Bacterial staining

i) Gram staining
Reagents:

Crystal violet
Solution A-2g of crystal violet dissolved on 20ml ethanol
Solution B-0.8g ammonium oxalate dissolved in 80ml d/w
Mixed solution A and B and filtered through Whatman paper No. 1

Gram's iodine
Dissolved 1g iodine and 2g potassium iodide in 300 ml d/w. Filtered through Whatman filter paper No. 1 (diameter = 12.5 cm).

Safranine
2.5 g Safranine was dissolved in 10 ml ethanol made the volume to 100ml with d/w and filtered through Whatman filter paper No. 1.

Procedure
Prepared a heat fixed smear of the culture on a microscopic slide and flooded with crystal violet for a minute. Smear was washed with tap water and flooded with gram's iodine for a minute. The smear was decolorized with 60% ethanol prepared in distilled water after rinsing the smear under tap water, counter stained with safranine for 45 seconds. Washed with tap water, blot dried with tissue paper and examined under oil immersion.

(ii) Endospore staining (Schaeffer and Fulton's method):

Malachite green solution:
Dissolved 5g of malachite green in 100ml d/w. Filtered through Whatman filter paper no.1.

Safranine:
Dissolved 1g of Safranine in 100ml d/w. Filtered through Whatman filter paper no.1.

Procedure:
Prepared smear of the organism and heat fixed it. Flooded the smear with 5% malachite green solution. Hold the slide over a boiling water bath for 15 min to allow the penetration of the stain through calcium dipicolinate of spores. Washed with tap water and counterstained with safranine for 2min, washed with tap water and blot dried with tissue paper and examined under oil immersion objective.

Endospores appeared green with cells colored red.

(iii) Staining of metachromatic granules (Albert's method)

Staining Solution I: Albert's stain
Toluidene blue-0.15g
Malachite green – 0.2g
Glacial acetic acid – 1ml
95% ethyl alcohol – 2ml
D/w-100ml

Staining Solution II: Albert's iodine
Iodine- 2gm; KI-3gm; D/w-300 ml

Prepared a smear dry in air and fixed by heat. Stain with solution I for 5-7 mins. Do not wash out stain and stain with solution II for 2-3 mins. Wash, dry and examine vegetative cells stained green and metachromatic granules stained dark green.

B.6. Reagents for nitrate reduction

Solution A (Sulfanilic acid)

Sulfanilic acid	8 gm
Acetic acid (5N)	One part of glacial acetic acid added to 2.5 parts of d/w
D/w	to make 1L

Solution B (α-naphthylamine)

α-naphthylamine	5 gm
Acetic acid (5N)	1L

B.7. Fatty Acid Methyl Ester (FAME) analysis using Microbial Identification System (MIS)

Reagent I (Saponification reagent)

NaOH	75g
CH_3OH	25 ml
D/W	25 ml

Reagent II (Methylation reagent)

HCl (6N)	32.5 ml
Ch_3OH	27.5 ml

Reagent III (Extraction reagent)

Hexane	20 ml
Methyl-tertbutyl ether/ Diethyl ether	20 ml

Reagent IV (Base wash)

NaOH	0.6 g
D/W	50 ml

B.8. Isolation, amplification and sequencing of 16s rRNA gene

Lysis solution

Tris–HCl (pH 7.5)	10 mM
EDTA	10 mM
Proteinase K	50 µg /mL

1X Reaction Buffer

Tris–HCl (pH 8.8)	10 mM
$MgCl_2$	1.5 mM
KCl	50mM
Triton X-100	0.1%

10X TBE Buffer

Tris–HCl (pH 8.0)	108g
Boric acid	55g
EDTA (0.5M)	40 ml
D/W	make volume to 1000 ml

Dilute 1:10 with distilled water to get 1X TBE buffer.

Polyethyleneglycol - NaCl

PEG	20%
NaCl	2.5M

B.9. Reagents of Aniline estimation

B.9.1. p-Dimethylaminobenzaldehyde (PDAB) method (Kupfer *et al,* 1964)

PDAB reagent: Add 6mg p-Diamethylaminobenzaldehyde in 1 ml of 3N H_2SO_4.

B.9.2. Diazocoupling method (Thompson, J. F. 1970)

1. Sodium nitrite solution: 0.4% aqueous.
2. Ammonium sulfamate: 2% aqueous.
3. N-Naphthylethylenediamine dihydrochloride (NEDA): Final concentration of 0.6% in 95% ethanol.

B.9.3. Hypochlorite method (Devi, N P. *et al,* 2000)

1. Phenol reagent: 10% w/v phenol in ethanol.
2. Nitroprusside reagent: 0.5% w/v aqueous.
3. Oxidizing agent: 20 g of tri-sodium citrate 1 g of sodium hydroxide in 25 ml of 1.5 N sodium hypochlorite. Volume made to 100 ml with distilled water.

B.10. Reducing sugar estimation (Somogyi, M. 1952)

1. Alkaline reagent: $CuSO_4$ (4gm), Anh. Na_2CO_3 (24gm), Na K Tartarate (16gm), Anh. Na_2SO_4 (180gm). Dissolve in minimum amount of water and make the volume to 1000ml.
2. Arsenic-molybdate solution:
   Ammonium molybdate (Solution a)– 25gm in 450 ml distilled water, add 21ml concentrated sulfuric acid.
   Sodium Hydrogen Arsenate (Na_2H $AsO_4.7H_2O$) (Solution b) – 3gm in 25 ml distilled water.
   Add solution (a) and (b), mix and incubate at 37°C for 24-48 hrs.

B.11. Proteins estimation (Bradford, 1976)

Bradford reagent:: Coomassie blue G250 (100mg in 50 ml 95 % ethanol), 100 ml (85%) phosphoric acid. Make volume to 1000ml, store at 4°C.

B.12. Detection of Catechol (Arnow, L.E 1937)

Nitrite-Molybdate solution: 10gm $NaNO_2$, 10gm $NaMoO_4$, dissolve in minimum amount of distilled water and make up the volume to 100ml.

B.13. Weatherburn method to demonstrate deamination of aniline

1. Reagent A: Phenol (5gm), Na-nitroprusside (25mg), dissolve in minimum amount of distilled water and make up the volume to 500ml.
2. Reagent B: NaOH (2.5gm), Na-Hypochlorite (4.2ml), dissolve in minimum amount of distilled water and make up the volume to 500ml.

Store Reagents A and B in amber bottles in refrigeration.

B.14. Alkaline Lysis Method (Brinboim and Doly, 1979)

i) Solution I (pH 8.0): Glucose 0.9 gm
Tris-chloride 0.394 gm
EDTA 0.292 gm
Double Distilled Water 100 ml

ii) Solution II: SDS 1.0 gm
0.2N NaOH 100 ml

iii) Solution III (pH 5.0): 5 M Potassium acetate 60.0 ml
Glacial acetic acid 11.5 ml
Double Distilled Water 28.5 ml

B.15. Ethidium bromide solution: Ethidium bromide 10 mg; Distilled water 1 ml

Stock solution was prepared and kept in cool and dark place. The final concentration used for agarose gel was 5μg/ml.

B.16. Tracking dye: Bromophenol blue 0.25 gm
Sucrose 40 gm
0.1M EDTA 10ml
1% SDS 10ml
Distilled water 100 ml

The dye was stored at 4 °C.

B.17. Tris acetate EDTA (TAE) buffer (pH 8.0) (1X)

50x: Tris base 2.42 gm
0.5M EDTA 1ml

Trisbase and 0.5M EDTA was dissolved in 10ml of Double Distill water and pH was adjusted to 8.0 with glacial acetic acid (0.57ml) and the final volume was made up to 500ml.

B.18. Buffers

B.18.1. Phosphate buffer (0.1M)

Solution A: (0.2M monobasic hydrogen phosphate): 27.6g of NaH_2PO_4 dissolved in 1000ml distilled water.

Solution B: (0.2M dibasic hydrogen phosphate): 53.62g of Na_2HPO_4 dissolved in 1000ml distilled water.

X ml of A + Y ml of B mixed to obtain buffers of the desired pH

X	Y	pH
87.7	12.3	6.0
39.0	61.0	7.0
5.3	94.7	8.0
2	98	9.5

B.18.2. Tris-HCl buffer (0.05M)

Solution A: (0.05M Tris): 6.0g of Tris dissolved in 1000ml d/w.
Solution B: (0.05M HCl): 4.4ml of 11.35 N HCl added to 1000ml d/w.
70ml of A and 30ml of B mixed to obtain buffer of pH 9.0

B.18.3. Tris-HCl buffer (0.1M)

Add Xml of 0.2 mol/lit HCl to 50 ml of 0.2 mol/lit Tris-base and make volume upto 100ml.

X ml	pH
43	7.2
24	8.2
6	9.0

Add 6ml of 0.2 mol/lit HCl to 50ml of 0.2 mol/lit Tris and make volume upto 100 ml.

B.18.4. Carbonate-bicarbonate buffer (0.2 M)

Solution A (0.2 M anhydrous sodium carbonate): 21.2g of anhydrous sodium carbonate dissolved in 1000ml d/w

Solution B (0.2 M sodium bicarbonate): 16.8g of sodium bicarbonate dissolved in 1000ml d/w

27.5ml of A + 22.5ml of B, diluted to a total volume of 200ml with d/w to obtain buffer of pH 10.0

Carbonate-bicarbonate buffer (pH10) was sterilized by autoclaving for serial dilution technique.

APPENDIX III

C TECHNIQUES

C.1. Thin layer Chromatography (Hamilton and Hamilton, 1987)

TLC plates were washed, dried and made grease free by wiping the surface with acetone. The plates were placed firmly on a uniform surface with the help of an adhesive tape. A slurry of silica gel-H (Acme chemicals) was prepared by mixing 10g of the silica powder with 15 ml of distilled water to obtain a desired consistency. The slurry was poured on one end of the plate and using a clean glass rod, the slurry was spread along the length of the plate in one move. The plates were air dried and activated in the oven for a period of 45min at 100°C. The plates were allowed to cool in a desiccator before it could be applied and run.

C.2. Thin layer Chromatography (Shriner *et al* 1980)

TLC plates were carried out on microscopic slides prepared following a standard procedure (Shriner *et al* 1980). A pair of clean glass slides (placed back-to-back) was dipped in well stirred slurry of Silica gel G (Acmes synthetic chemicals) prepared in a mixture of 67vol%/33vol% chloroform/methanol and withdrawn slowly. The slides were separated, allowed to dry and were ready to spot in 5 -10 minutes. Cell pellet extract and the supernatant extracts were spotted along with aniline and catechol as standards for comparison along with the extracted samples and were developed in solvent system Hexane : Acetone (80 : 20). Visualization of the eluted spots was carried out in the presence and absence of iodine vapour.

Other aromatic amines (azobenzene, o-aminophenol, p-amino phenol, m-amino phenol, biphenyl and phenol) were also spotted along with the samples as standards to compare with the resolution factor (Rf) of the products formed.

C.3. Preparation of Oxygen electrode for oxygen uptake studies

Oxygen electrodes (Hansatech, UK) were prepared and calibrated as instructed in the manual.

The silver cathode and platinum anode were cleaned with moist aluminium oxide to get a shining metal appearance. Few drops of saturated KCl solution (17.5g in 100ml) was placed on the platinum dome and in the electrode cavity. Cigarette paper (1cm x 1cm) was placed over the dome followed by the placement of the membrane (1cm x 1cm) and both the films were held tightly to the electrode by fixing a o-ring using an applicator. Air saturated deionized water was used to calibrate the instrument which was achieved by determining the

air line and the zero-line. Membrane was maintained moist by adding deionized water into the reaction chamber.

Deionized water and phosphate buffer (50 ml, pH 8.5) was placed in a 250 ml conical flask and left on the shaker at 180rpm for aeration for one hour and was used as a basal medium for the oxygen uptake studies.

C.4. Column preparation for chromatography

The column used for chromatography was plugged with at the bottom with cotton and was filled with a small amount of petroleum ether (60-80 grade). Slurry of silica gel was prepared in petroleum ether and was poured in to the column with continuous tapping. Tapping was continued until the silica gel formed a uniform bed and the packing was homogenous with no air bubbles trapped in between. Silica gel packing was done up to half the length of the column. Petroleum ether was run through the column for a couple of times to ensure no bubbles were seen in the silica packing.

Samples were adsorbed on to the silica gel before loading in to the column. Samples were loaded with a small amount (1-2cm height) of solvent present above the silica gel. A cotton plug was placed directly over the sample to prevent disturbance while adding the solvent. Elution speed was maintained at 1-2 drop per second and aliquots were collected in clean test tubes.

Selection of solvent was based on the polarity and the compound to be separated.

C.5. Sodium fusion test (Shriner *et al.*, 1964)

A small piece of dried Na metal (0.05gm) was taken in sodium fusion tube. The tube was heated very gently over a low flame for about 20-30 seconds until the Na melts and turned black. TP2 dissolved in 95% ethanol was added carefully to the fusion tube containing fused Na metal taking care not to allow the sample to catch fire. The fusion tube was heated slowly until red-hot, and then plunged in an evaporating dish containing distilled water (1-2ml). The fusion tube was crushed completely using a glass rod and the resulting solution was boiled for few more minutes and filtered. The filtrate known as Sodium Fusion Extract (SFE) was used further for detecting the presence of Nitrogen.

To 2-3 ml of the SFE few drops of freshly prepared ferrous sulfate solution was added then acidified with 1n HCl. The results were noted based on the colour formation.

C.6. Polyacrylamide Gel elctrophoresis

C.6. Stock Solution for PAGE and SDS – PAGE:

Acrylamide-bis-acrylamide solution [Monomer solution]: 29% acrylamide and 1% (w/v) N,N methylene *bis* acrylamide was dissolved in warm d/w. Checked pH to be 7.0 and stored in dark bottles at 4°C and used within 30 days.

Resolving gel buffer [1.5 M Tris, pH 8.8]: Prepared by dissolving 18.615g Tris, in 70 ml d/w water and added 100 µls of 10% SDS in d/w. The pH of the solution was adjusted to 8.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Stacking gel buffer [1.0 M Tris pH 6.8]: Prepared by dissolving 12.11g Tris, in 70 ml d/w water and added 50 µls of 10% SDS in d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Ammonium per sulfate (APS, 10% w/v): Prepared by dissolving 0.1g of APS in 1.0 ml d/w. The solution was prepared afresh each time.

Electrophoresis buffer:

Composition of 1X buffer is as follows:

Tris	3.0g
Glycine	14.4g
SDS(10%)	10ml
D/w	to make 1000ml
pH	8.4

Sample buffer:

Composition of 4X buffer is as follows:

TriS- HCl (1 M pH 6.8)	0.04ml
Glycine	0.04g
SDS	0.004g
β-Mercaptoethanol	0.004ml
d/w	to make 10ml

Tracking dye:

50% sucrose	10 ml
Bromophenol blue	10mg

Staining Solution:
Coomassie Brilliant Blue G - 250 solution was prepared by dissolving 0.25g Coomassie Brilliant Blue G - 250 in 100ml of 25% methanol, 10% glacial acetic acid and 65% d/w.

Destaining Solution I

Methanol	40ml
Acetic acid	10ml
d/w	50ml

Destaining Solution II

Methanol	5ml
Acetic acid	7ml
d/w	88ml

Preparation of gel monomer

The composition of the resolving and stacking gels is as follows:

Solution	Resolving gel (10%) (ml)	Stacking gel (5%) (ml)
Monomer	2.5	0.33
1.5M Tris, pH 8.8	1.875	-
1.0M Tris, pH 6.8	-	0.625
10% SDS	0.075	0.025
10% APS	0.0375	0.025
D/w	0.003	1.525
TEMED	0.005	0.005

(a) **Preparation of sample:** 100 µls of cell pellet (containing 100 mg of protein) was mixed with 10µls of 25% SDS and boiled for 2 minutes at 100°C. 50µls of sample buffer was then added and boiled for 5 minutes at 100°C. After cooling, 20 µls of bromothymol blue was added and 50µls of the samples were loaded in the gel with SDS PAGE molecular weight markers (sigma- St.Louis, MO USA).

(b) **Procedure:** The PAGE and SDS - PAGE were carried out in a Bangalore Genei apparatus. After a pre-run for 10 minutes, 30µls of the samples containing 50µgms of proteins along with the standard molecular weight markers were loaded in the gel. The electrophoresis was carried out at a constant voltage of 80 V for stacking gel and 120 V for resolving gel till tracking dye (Bromothymol blue) reached the bottom of the gel. At the end of the run, the gel was stained by Coomassie blue.

(c) Staining and destaining procedure:

Coomassie blue staining: The gel was stained in Coomassie Brilliant Blue R-250 solution Staining was carried out overnight; followed by destaining under mild shaking using de staining solution I for 3-4 hours and destaining solution II for several hours till the protein bands became clearly visible with no background colour. The gels were dried and preserved between cellophane sheets.

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PUBLICATIONS

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1) Desai, R. S., Krishnamurthy, N. K., Mavinkurve, S. amd Bhosle, S. 2004. Alkaliphiles in estuarine mangrove regions of Goa, (Cemtral West Coast of India). IJSEM. 32(2): 177-180.

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Alkaliphiles in estuarine mangrove regions of Goa, (central west coast of India)

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Obligate alkaliphilic bacteria (28 strains) were isolated from various estuarine ecosystems of Goa. Most of these strains were found to be Gram positive, motile rods, capable of growth in aerobic condition upto *p*H 12, exhibiting optimum growth at *p*H 10.5. Isolates exhibited high buffering capacity confirming their alkaliphilic nature. Significantly high difference was noted in the buffering capacities of the alkaliphiles and the neutrophile indicating their ability to maintain their internal *p*H. The obligate alkaliphile A52 exhibited highest cytoplasmic buffering capacity of 8,500 nanomoles OH^- ions/*p*H unit/mg protein. The isolates also showed amylase (39%), protease (50%) and lipase (100%) activity under alkaline conditions. The present study depicts that such alkaliphilic bacteria also play an important role in the mineralization of organic matter under high *p*H conditions in natural ecosystems.

[**Key words:** Alkaliphiles, mangroves, buffering capacity, enzymes, biodiversity, Goa, bacteria, estuary]
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Introduction

Microorganisms and the archaea are found in the extreme conditions of temperatures, *p*H, pressures, salt and nutrient conditions. Alkaliphiles and haloalkaliphiles are one such diverse group of extremophilic microorganisms that thrive at very high *p*H and salt concentration. Organisms with *p*H optima for growth in excess of *p*H 8, usually between 9 and 10 *p*H are defined as alkaliphiles[1]. Obligate alkaliphiles are incapable of growth at neutral *p*H. Most of the organisms described to date as growing under high alkaline condition are prokaryotes, comprising heterogeneous collection of eubacteria with a few examples of archaebacteria. Alkaliphilic organisms (particularly prokaryotes) are widely distributed and can be found even in environments where overall *p*H may not be particularly alkaline[1]. Alkaliphiles grow well in *p*H range of 10-11 and are of ecological, industrial and basic bioenergetic interest[2]. A typical alkaliphile, which grows at an external *p*H of 10.5 or above does not allow its cytoplasmic *p*H to exceed a value of *p*H 9.5 even if the external *p*H is raised[3]. Alkaliphiles exist in a variety of unique environments that are hostile to other organisms and perhaps play a significant role in biotransformation occurring in such environments[1,4,5]. Several of these are entirely novel and have unique properties. Genes and genetic manipulations of industrially important enzymes of alkaliphiles have gained tremendous importance[6]. The marine and mangrove ecosystems offer a number of unique ecological niches for harboring such microorganisms and are reported to be rich in halophiles, alkaliphiles and haloalkaliphiles. The diverse organisms and their activities, specially their role in nutrient recycling has been documented[5]. We report here the isolation of obligate alkaliphiles from estuarine ecosystems of Goa, buffering capacity and the enzymic potentials of these alkaliphilic microorganisms.

Materials and Methods

Water and sediment samples were collected from various estuarine mangrove rich ecosystems of Goa in the area of Ribandar, Banastari, St. Cruz, Panjim, Merces during Oct 2000 and July 2002. The sampling sites were selected as they harboured rich mangrove forests, are affected by tidal fluxes and are water logged and also found to have econiches with alkaline *p*H. The media used for isolation, growth and maintenance of alkaliphilic bacteria was Polypeptone Yeast Extract Glucose (PPYG) Agar [7]. Solutions of 10% Glucose and 10% Na_2CO_3 were sterilized separately by autoclaving and the final *p*H of the medium was maintained at *p*H 10.5.

Water samples collected from each location were diluted ten fold in stabilized natural seawater (sterile) and plated on nutrient agar and PPYG (*p*H 10.5). Sediment samples collected from the subtidal regions of the estuarine ecosystem were suspended in stabilized natural seawater (sterile) at the ratio of 1:10

and incubated on the Orbitek shaker for 1 hr at 150 rpm. The suspension was allowed to settle and 0.1 ml of clear supernatant and the dilutions were spread plated on nutrient agar and PPYG (*p*H 10.5). The petri plates were incubated at room temperature and the colonies that appeared were counted. Predominant isolates obtained on PPYG (*p*H 10.5) were selected, purified and maintained on the same medium. Isolates growing on PPYG agar (*p*H 10.5), were replica-plated on PPYG agar with *p*H 7.0, 8.5, 10.5 and 12.0 to determine their *p*H tolerance and obtain obligate alkaliphiles. These obligate alkaliphiles (growing only at *p*H 10.5 and 12.0) were selected for further studies. The isolated obligate alkaliphiles were identified according to their morphological and biochemical characteristics based on Bergey's Manual[8]. The biochemical media used for identification was modified based on the alkaline conditions required for growth[7].

The ability of the isolates to hydrolyse polymers was determined using PPY agar *p*H 10.5, supplemented with starch, casein, tributyrin and cellulose instead of glucose. The cultures were streaked on the plates containing the substrates and incubated for 48 hrs. The hydrolysis of the substrates were detected by using standard methods[7].

Buffering capacities of selected isolates were determined by titration using 0.05 *M* KOH[9,10]. Cultures were grown in PPYG broth, incubated for 24 hrs on shaker and the cells harvested by centrifugation at 5000 rpm for 15 mins. The pellets were washed, resuspended in 10 ml of 0.2 *M* KCl solution and the protein content of this cell suspension was estimated using Folin Lowry's method[11]. Cell suspension corresponding to 5 mg of cell protein was taken in a beaker and the initial *p*H of the suspension was noted. The *p*H change at every addition of 10 µl of 0.05 *M* KOH was noted using calibrated *p*H analyser (Labindia). The whole cell buffering capacity (B_0) was measured as nanomoles of hydroxyl ions consumed to change 1 *p*H unit/mg protein. To determine the permeabilised cell buffering capacity (Bt), cell suspension in 0.2 *M* KCl corresponding to 5 mg of protein was treated with 10 ml of triton X-100, mixed, allowed to stand for 5 mins and centrifuged. The pellet obtained was washed with 0.2 *M* KCl to remove the permeabilizing agent, resuspended in 0.2 *M* KCl, titrated against 0.05 *M* KOH and change in *p*H noted. The permeabilised cell buffering capacity was noted as nanomoles of OH^- ions consumed to change 1 *p*H unit/mg protein. The cytoplasmic buffering capacity (Bi) was determined as a difference of Bo and Bt (Bi = Bo-Bt)[10]. Buffering capacity of a neutrophile was determined for comparison. Control titration was performed using the gram negative neutrophilic organism *Pseudomonas.*

Results and Discussion

Viable counts of general heterotrophic neutrophilic bacterial populations and alkaliphilic bacteria were enumerated from five different estuarine locations of Goa viz. Ribandar, Banastari, St. Cruz, Panjim and Merces (Table 1). The counts were much higher on nutrient agar as compared to PPYG medium, indicating the existence of neutrophilic and alkalotolerant bacteria. It has been reported that the ratio of alkaliphiles to neutrophiles[6] found in soil is about 1:10 to 1:100. Sediment samples had a higher count on nutrient agar as well as on PPYG (*p*H 10.5), as compared to the water samples. Highest counts of alkaliphilic bacteria were generally recorded from the Ribandar samples, which gave a viable count of 7×10^4 cfu/ml. It has been reported that the alkaliphiles depend on Na^+ ions for maintenance of internal *p*H[10]. It is noted that the concentration of Na^+ ions is very high in PPYG agar, the concentration best suited for organisms growing under alkaline conditions.

Predominant isolates (153) were picked up randomly from PPYG agar (*p*H 10.5) and replica plated on the same medium with *p*H 7.0, 8.5, 10.5 and 12.0. Isolates (28) growing only at *p*H 10.5 and 12.0 were considered to be obligate alkaliphiles and were selected for further studies.

Table 1—Total viable count of neutrophilic & alkaliphilic bacteria in samples taken from various mangrove ecosystems of Goa.

Sediment samples	Oct-2000 cfu/ml (× 102)		Jul-2001 cfu/ml (× 102)	
	N.A	PPYG	N.A	PPYG
Merces	7840	130	80	1
St. Cruz	2100	140	69	1
Panjim	6920	680	679	1
Banastari	5500	509	4	2
Ribandar	7500	700	210	4
Water samples	**cfu/ml (× 102)**		**cfu/ml**	
	N.A	PPYG	N.A	PPYG
Merces	130	3	100	2
St. Cruz	460	4	100	3
Panjim	410	2	241	2
Banastari	520	4	577	1
Ribandar	650	6	295	4

N.A - Nutrient agar; PPYG - Polypeptone yeast extract glucose agar; cfu - colony forming units

On the basis of morphological, biochemical and physiological characteristics, the cultures were identified as *Bacillus* (53%), *Corynebacterium* (21%), *Micrococcus* (7%), *Actinomyces* and *Flavobacterium* (Table 2). A majority of obligate alkaliphiles obtained during the study were found to be gram positive organisms confirming that they are better adapted to high *p*H as reported earlier [3,12-14].

Table 2—Identification status of alkaliphiles

Culture No.	Culture code	Tentative identification
A1a	RiMsX1a	*Corynebacterium*
A1b	RiMsX1b	*B. alcalophilus*
A3a	RiMsX3a	*B. laterosporus*
A3b	RiMsX3b	*Bacillus sp.*
A5	RiMsX5	*Actinomyces sp.*
A6	RiMsX6	*Actinomyces sp.*
A20	RiMsX20	*B. alcalophilus*
A27	RiMsX27	*B. alcalophilus*
A30	RiMsX30	*B. alcalophilus*
A37	PjMsX1	*B. alcalophilus*
A43	BnMsX7	*Micrococcus lylae*
A52	BnMsX12	*Micrococcus lylae*
A55	BnMsX15	*B. alcalophilus*
A59a	BnMsX19	*Bacillus sp.*
A59b	BnMsX19	*Bacillus sp.*
A61	BnMsX21	*B. schlegelii*
A62	BnMsX22	*B. schlegelii*
A64	BnMsX24	*B. stearothermophilus*
A65	BnMsX25	*Corynebacterium*
A66	BnMsX26	*Corynebacterium*
A67a	BnMsX27a	*Bacillus sp.*
A67b	BnMsX27b	*B. stearothermophilus*
A77	BnMsX28	*Corynebacterium*
A86	RiMsX86	*B. brevis*
A102	RiMsX102	*Corynebacterium*
A118	RiMsX118	*unidentified*
A129	RiMsX129	*unidentified*
A131	RiMsX131	*Flavobacterium*

Cytoplasmic buffering capacities and buffering by whole cells was examined in 12 alkaliphilic bacterial species. Acid base titrations were conducted on whole cells and cell permeabilised with triton X-100. Triton X-100 is the non-ionic detergent which specifically and selectively solubilises proteins of cytoplasmic membrane leaving the cell wall totally undisturbed[9].

The difference between buffering capacities of whole cells (B_0) and permeabilised cells (B_t) is interpreted as internal/cytoplasmic buffering capacity (B_i), which was found to be very high and stable for all the alkaliphiles selected during the study[10]. A consistent effect of permeabilisation treatment was noted to be a decrease in the buffering capacity for all tested cultures, indicating that certain components required for maintaining the buffering capacity are leached out on treatment with triton X-100[9].

Controlled titrations done with 0.2 *M* KCl indicate that KCl does not affect the changes in *p*H and hence is an ideal suspension medium for these studies. The amount of KOH required to bring about one *p*H unit change for whole cells and permeabilised cells of the 12 alkaliphilic isolates and a neutrophile was recorded. A significant difference was noted in the whole cell buffering capacity (B_0) of the neutrophile and the alkaliphiles. The amount of KOH required for the change of one *p*H unit was 150 nmoles/mg protein for the neutrophile while for the alkaliphiles it ranged from 2500 nmoles to 13,000 nmoles of OH ions (Fig. 1). Maximum whole cell buffering capacity was exhibited by the culture *Corynebacterium*.

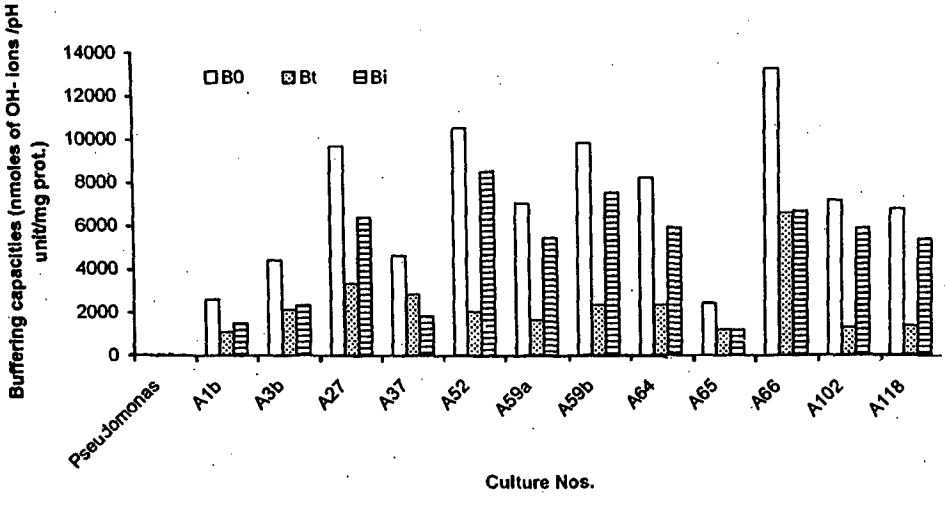

Fig. 1—Comparison of buffering capacities between the alkaliphilic cultures and the neutrophile. Bo-whole cell buffering capacity; Bt - permeabilised cell buffering capacity; Bi - cytoplasmic buffering capacity

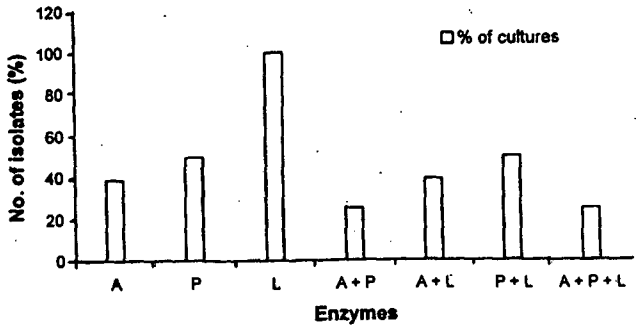

Fig. 2—Activity of enzymes exhibited by the isolates
A - Amylase; P - Protease; L - Lipase

Enzyme activities (Fig. 2) were checked using starch, skim milk, tributyrin and cellulose in the PPY medium as substrate and the production of amylase, protease, lipase and cellulase was noted respectively. It was noted that alkaliphiles producing amylase, protease and lipase were 39%, 50% and 100% respectively as compared to the cellulase production, which was however absent. Unique types of enzymes have been isolated from alkaliphiles, which have various industrial applications. It was observed that many of the isolates showed the production of more than one enzyme at alkaline *p*H as reported [15,16].

The present study has confirmed the presence of different genera of alkaliphilic organisms in estuarine mangrove ecosystems. The diversity of enzymes produced by these organisms such as amylase (39 %), protease (50%) and lipase (100%) activity under alkaline conditions indicates that such organisms also have a significant role to play in recycling organic matter in these ecosystems.

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