Chapter 5
Biodegradation of Aromatic Compounds by Alkaliphilic Bacteria

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5.1 Introduction

Mangroves, one of the specialized ecosystems of the tropical areas are nutritionally rich due to the continuous shedding of foliage which gets decomposed to form detritus matter. These are highly reproductive ecosystems which host a wide range of coastal and offshore marine organisms and provide a unique ecological niche for diverse bacterial communities (Ramanathan et al. 2008). These organisms, though have continuous nutrients, are affected by tidal variations, salinity and by anthropogenic substances added through run offs from the terrestrial ecosystem such as excess of fertilisers, pesticides and by the activities of various industries such as mining, shipbuilding, etc. The interaction of microbial flora with such substances has resulted in the proliferation of physiologically diverse microflora in marine ecosystems. Among these, are also the bacteria which have the ability to degrade hydrocarbons and xenobiotics. Mangrove ecosystem is also found to develop specialized niches having specific conditions such as high pH, increase in salinity and high nutrient content in the form of nitrogen and phosphorus (Robertson 1992).

5.2 Biodegradation of Aromatic Compounds

Biodegradation is the metabolic ability of microorganisms to transform or mineralise organic compounds into less harmful, nonhazardous substances which are then integrated into natural biogeochemical cycles. Organic compounds are often classified as biodegradable, persistent or recalcitrant, depending on their behaviour in the environment. Biodegradation means the biological transformation of an organic

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chemical to another (Blackburn and Hafker 1993; Liu and Suflita 1993). The monocyclic aromatic substances such as benzoate, toluene and phenol are more easily attacked than polycyclic aromatic hydrocarbons such as naphthalene, benzopyrene, anthracene, substituted aromatics, chlorinated biphenyls, etc. The intensity of biodegradation is influenced by several factors such as availability of nutrients, oxygen supply, pH value, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics and the pollution history of the contaminated environment (Margesin and Schinner 2001).

5.3 Incidence of Aromatic Compounds

The ever growing list of chemical contaminants released into the environment on a large scale includes numerous aromatic compounds such as petroleum hydrocarbons, halogenated and nitroaromatic compounds and phthalate esters. These compounds enter the environment through various paths such as (1) components of fertilisers, herbicides, pesticides, fungicides and insecticides that are used to increase food production, (2) pharmaceuticals used for health reasons (3) organic solvents used in industries and (4) synthetic organic xenobiotic chemicals. Combustion processes release others such as polycyclic aromatic hydrocarbons (PAHs), dibenzo-p-dioxins and dibenzofurans. The local concentration of a contaminant depends on:

a. The amount present and the rate at which the compound is released
b. Its stability in the environment under both aerobic and anaerobic conditions
c. The extent of its dilution in the environment
d. The mobility of the compound in a particular environment
e. Its rate of biological or non-biological degradation (Ellis 2000; Janssen et al. 2001)

5.4 Bacterial Transformation of Aromatic Hydrocarbons

Due to the hazardous effect of aromatic hydrocarbons, it is necessary to keep a check on the concentration of these substances in the environment. The control measure followed nowadays is the degradation of these substances by microorganisms. Degradation may occur either during the process of utilisation of the hydrocarbon as a carbon source or as a co-metabolic transformation by microorganisms. When the organism utilises the hydrocarbon as a carbon or energy source during its growth, the original compound is largely incorporated into the cell mass or is destroyed as carbon-dioxide. The parameters required for a degradation process to occur are:

a. A capable organism must be present
b. An opportunity must exist for requisite enzymes to be synthesized
c. Environmental conditions must be sufficiently suited for the enzymatically catalysed reaction to proceed at a significant rate

Microorganisms with the ability to degrade a wide variety of compounds like benzene, phenol, naphthalene, atrazine, nitroaromatics, biphenyls, polychlorinated biphenyls (PCBs) and chlorobenzoates have been isolated and characterised (Dickel et al. 1993; Sangodkar et al. 1989). Biotransformations of organic pollutants in the natural environment have been extensively studied to understand microbial ecology, physiology and evolution for their potential in bioremediation (Bouwer and Zchendir 1993; Chen et al. 1999; Johan et al. 2001; Kiran 2009; Mishra et al. 2001; Watanabe 2001). List of bacteria degrading the various aromatic compounds is as presented in Table 5.1.

5.5 Mechanism of Degradation of Aromatic Compounds by Bacteria

Aerobic degradation is dependent on the presence of molecular oxygen and is catabolized by enzymes that have evolved for the catabolism of natural substrates and exhibit low specificities (Dagley 1971). Oxygenases are a group of enzymes which catalyse the incorporation of molecular oxygen into various organic and inorganic substrates (Hayaishi 1966). Depending upon the type of enzyme catalysing the reaction, either one or two oxygen atoms are inserted into the molecule via an electrophilic attack on an unsubstituted carbon atom (Janssen et al. 2001). These oxygenases are classified into two groups:

a. ***Monooxygenases*** These enzymes catalyse the addition of a single atom of oxygen per molecule of substrate, e.g. Tyrosinase enzyme on substrate tyrosine.
b. ***Dioxygenases*** These enzymes catalyse the addition of two atoms of molecular oxygen per molecule of substrate, e.g. catechol 1,2 dioxygenase and catechol 2,3 dioxygenase on substrate catechol.

Aromatic compounds are ubiquitous growth substrates for microorganisms. Like some aliphatic compounds, aromatic compounds have chemical inertness which makes them difficult substrates. Benzoate is the most studied monocyclic aromatic compound. The aerobic degradation of benzoate follows various routes in microorganisms. Most of the bacteria aerobically degrade benzoate initially to catechol which then follows the *ortho*, *meta* or *gentisate* mode of ring cleavage (Murray and Williams 1974) (Fig. 5.1). Microorganisms utilise these compounds by dissimilating them in a step-wise manner as follows:

5.5.1 Initial Hydroxylation of Aromatic Ring Structure

Aerobic organisms introduce hydroxyl group in the aromatic ring using molecular oxygen as obligatory cosubstrate. The position whereby the group is incorporated decides the type of intermediate formed such as catechol, protocatechuate or gentisate (Fig. 5.1) (Bayley and Barbour 1984; Chapman 1972; Gibson and Subramanian

Table 5.1 Microorganisms involved in the degradation of various aromatic compounds

Aromatic compound	Application	Microorganisms involved in degradation	Reference
Various xenobiotics	Herbicides, pesticides	*Rhodococci*	Poelarends et al. (2000)
o-, m-, p-Cresols	–	*Pseudomonas putida*	Chapman (1972)
		Pseudomonas alcaligenes	Poh and Bayley (1980)
		Alcaligenes eutrophus	Hughes et al. (1984)
Xylenol	–	*Pseudomonas putida*	Chapman (1972)
		Pseudomonas alcaligenus	Poh and Bayley (1980)
Benzoate	Bacteriocide, fungicide	*Pseudomonas putida mt-2*	Williams and Murray (1974)
			Nakazawa and Yokoto (1973)
		Pseudomonas aeuroginosa	Kataeva and Golovlea (1990)
		Pseudomonas putida NCIB10015	Sala-Trepat et al. (1972)
		Azotobacter	Sala-Trepat and Evans (1971)
		Alkaliphilic *Bacillus*	Yumoto et al. (2003)
		krulwichiae	
Nitrophenols	Starting material in manufacture of pharmaceuticals, pesticides, explosives	*Arthrobacter sp.*	Zylstra et al. (2000)
Chlorinated ethylenes	Industrial solvents	*Pseudomonas stutzeri* OX	Ryoo et al. (2000, 2001)
Trichloroethylene			
Tetrachloroethylene			
	Polyaromatics hydrocarbons found in petroleum	*Geobacter*	Kazumi et al. 1995
	Kazumi et al. (1995)		
Monochlorinated & nonchlorinated aromatic compounds			Kiran (2009)
–	–	*Dietzia* sp	Wang et al. (2011)

Table 5.1 (continued)

Aromatic compound	Application	Microorganisms involved in degradation	Reference
Triclosan (5-chloro-2-2,4 dichlorophenoxy phenol)	Antimicrobial agent used in a wide range of consumer products like soaps, toothpastes, handcreams and plastics	Bacterial consortium	Hay et al. (2001)
Atrazine	Herbicide for control of broad leaf and grassy weeds	*Pseudomonas sp.* ADP	De Souza et al. (1998)
			Wackett et al. (2002)
Phenols and their derivatives	Used for manufacture in synthetic organic compounds	*Thauera aromatic*	Whiteley and Bailey (2000)
		Pseudomonas	Feist and Hegeman (1969)
		Pseudomonas putida MTCC	Bandhyopadhyay et al. (1999)
		Alcaligenus eutrophus	Hughes et al. (1984)
		Halomonas campisalis	Alva and Peyton (2003)
		Acinetobacter	Abd-El Haleem et al. (2003)
Nonyl phenol	used mostly in agricultural fertilisers	*Sphinogomonas sphinobium*	
	Tanghe et al. (1999)		
		Sphinogomonas amiense	Ushiba et al. (2003)
Mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid]	Herbicide	*Alcaligenes Ralstonia sp. CS2*	Smejkal et al. (2001)
2,4-dichlorophenoxyacetic acid	–	*Alcaligenes*	Clement et al. (2001)
3-chlorobenzoate	–	*Ralstonia eutrophus*	
Naphthalene	Used in fungicides, pesticides, detergents, dyes, moth balls, motor fuel and resins	*Pseudomonas sp*	Fuenmayor et al. (1998)
Aniline & p-chloroaniline	Production of polyurethanes, rubber, azo dyes drugs, photographic chemicals varnishes and pesticides	*Moraxella sp.* strain G	Zeyer et al. (1985)
		Pseudomonas putida	Fukumori and Saint (1997)
		Pseudomonas acidovorans	Loidl et al. (1990)
Anthracene	–	*Bacillus badius*	Ahmed et al. (2012)

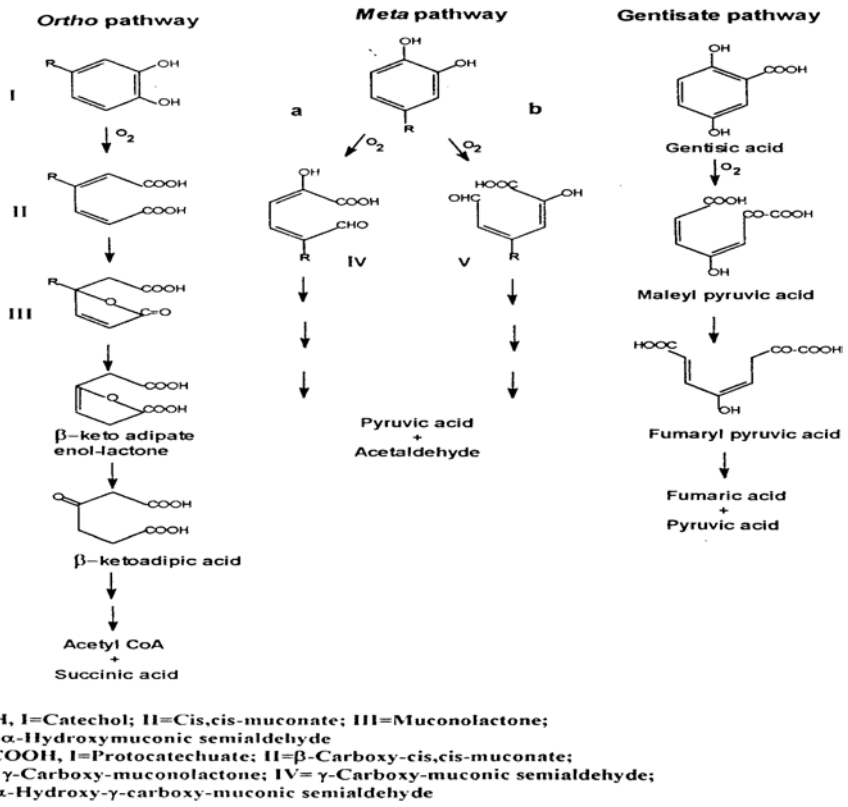

Fig. 5.1 Modes of ring cleavage in bacteria. (Adapted from Murray and Williams 1974)

1984; Stanier and Ornston 1973). The hydroxyl group is introduced either in *ortho* or *para* position. In the case of compounds such as resorcinol, gallic acid, etc. wherein two hydroxyl groups are already present, a third hydroxyl group must either be present or introduced before ring cleavage can occur (Bayley and Barbour 1984).

Cleavage of the aromatic ring is a key reaction in the oxidation of aromatic compounds. Dioxygenases capable of cleaving the aromatic ring occur widely in bacteria but differ in their mode of aromatic ring cleavage, specific inductions and substrate specificity (Kataeva and Golovlea 1990; Ornston 1971). Certain bacteria of the genus *Pseudomonas* synthesize several aromatic ring-cleaving enzymes that enable them to oxidize various aromatic compounds. For example, *Pseudomonas aeuroginosa* which grows on a wide set of aromatic substrates including p-xylene, synthesizes four dioxygenases namely pyrocatechase (Catechol 1,2 dioxygenase), metapyrocatechase 1 and 2 (Catechol 2,3 dioxygenases) and protocatechuate 3,4 dioxygenase (Kataeva and Golovlea 1990). The usual prerequisite for the oxidative cleavage of the aromatic ring by the action of dioxygenases where O_2 is used as a substrate is that, the ring contains two hydroxyl groups that are either *ortho* or *para* to one another.

5.5.2 Modes of Ring Cleavage

There are three distinct modes of ring cleavage

5.5.2.1 Ortho-Ring Cleavage

Cleavage of the bond between adjacent carbon atoms that carry hydroxyl groups is known as "*Ortho*" or "intradiol" cleavage and the pathway by which the product of such cleavage is metabolized is known as the *ortho* or β-ketoadipate pathway (Fig. 5.1). This kind of cleavage is brought about by catechol 1,2 dioxygenase which catalyses the cleavage of the aromatic ring of catechol to *cis*, *cis* muconic acid with the consumption of 1 ml of oxygen (Nakazawa et al. 1969; Nakazawa and Yokoto 1973).

5.5.2.2 Meta-Ring Cleavage

In the second mode of cleavage of the benzene nucleus, attack occurs between two carbon atoms, only one of which carries a hydroxyl group, the other carbon atom being either unsubstituted or substituted with other than a hydroxyl group (Fig. 5.1). This kind of cleavage is known as "*Meta*" or extradiol cleavage. In this case, the hydroxyl groups may be either *ortho* or *para* to one another and the enzymes catalysing such cleavages are designated as catechol 2,3 dioxygenases, which catalyse the cleavage of the aromatic ring of catechol to 2-hydroxymuconic semialdehyde (HMS) (Kojima et al. 1961). This enzyme catalyses the critical ring-cleaving step in the biodegradation pathways of many complex aromatic compounds. This enzyme has been reported in various microorganisms for catechol and cleaves the bond between carbon atoms 2 and 3 to yield the stoichiometric quantity of 2-hydroxymuconic semialdehyde (Evans 1971; Hayaishi 1966; Sala-Trepat et al. 1972).

5.5.2.3 Gentisate Pathway

This is the third mode of aromatic ring cleavage seen in compounds having the hydroxyl groups *para* to one another as in gentisic acid (2,5-dihydroxy benzonic acid). Oxidative cleavage is catalysed by gentisate 1,2 dioxygenase and the subsequent pathway is the gentisate pathway. Gentisate 1,2 dioxygenase is heat stable and inhibited by *o*-phenanthroline and 2,2′ bipyridyl (Crawford et al. 1975). Gentisate formed from a number of aromatic compounds is cleaved by this pathway (Fig. 5.1) (Harpel and Lipscomb 1990; Poh and Bayley 1980).

Benzoate ring cleavage is thus a central core for degradation of substituted and polycyclic aromatic compounds. Such ring cleavage could occur either by the *meta* or *ortho*-pathway. Oxygenative cleavage of catechol is then followed by a series of reactions leading to the formation of succinate and acetyl coenzyme A in case of

ortho-pathway, acetaldehyde and pyruvate in the *meta*-pathway and fumaric acid with pyruvic acid in the gentisate pathway (Nakazawa and Yokoto 1973; Murray and Williams 1974).

5.6 Degradation of Tyrosine

Tyrosine is a nonessential or sometimes called semiessential amino acid produced from phenylalanine. It is a constituent of aminosugars and aminolipids. It is known to increase the suppressant effects of phenyl propanolamine, ephedrene and amphetamine and helps to reduce body fat. It is the precursor of neurotransmitters like 3,4 dihydroxy phenylalanine (L-DOPA), dopamine, nerepinephrine and epinephrine. Tyrosine enters the mainstream pathways vital for the cells routine functioning. Besides this, it can also enter side pathways wherein the products produced may not be very harmful to the cell under normal conditions. Such a pathway is termed as the melanogenesis pathway. Here, tyrosine is first converted to DOPA and then dopaquinone, the intermediate metabolites in the production of melanin via the enzyme tyrosinase. Tyrosinase is fairly ubiquitous and was previously called monophenol monooxygenase with the terminology changed to phenolase, monophenol oxidase and as cresolase. Tyrosinase is a copper-containing monooxygenase, catalysing both *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Lerch 1987).

$$\text{Monophenol} + O_2 \rightarrow o\text{-quinone} + H_2O$$
$$2\ o\text{-diphenol} + O_2 \rightarrow 2\ o\text{-quinone} + 2H_2$$

It has been reported that the active site of the enzyme contains a pair of antiferromagnetically coupled Cu^{+2} ions. Its number in the standard enzyme nomenclature is (EC 1.14.18.1). Tyrosine is first *ortho*-hydroxylated to DOPA (Cresolase activity). L-DOPA is oxidized to dopaquinone (Catecholase activity), which undergoes further nonenzymatic oxidation and polymerization, yielding melanin. (Kelley et al. 1990).

$$\text{Tyrosine} + \tfrac{1}{2}\ O_2 \rightarrow \text{DOPA} \qquad (\text{Cresolase activity})$$
$$2\text{DOPA} + O_2 \rightarrow 2\ \text{Dopaquinone} + 2H_2O \quad (\text{Catecholase activity})$$
$$\text{Dopaquinone} \rightarrow \text{Leukodopachrome}$$
$$\text{Leukodopachrome} + \text{Dopaquinone} \rightarrow \text{Dopachrome} + \text{DOPA}$$

The overall reaction for tyrosinase activity is:

$$\text{DOPA} + \tfrac{1}{2}\ O_2 \rightarrow \text{Dopachrome}$$

The oxidation of tyrosine is preceded by a short lag which can be eliminated by the addition of a trace of DOPA as the oxidation of DOPA goes rapidly without a detectable lag.

Dopaquinone is converted to dopachrome through spontaneous autooxidation and finally to dihydroxyindole or dihydroxyindole-2-carboxylic acid (DHICA) to form eumelanin (brown–black pigment). The hydroxylation of tyrosine to DOPA can also be catalysed by the enzyme tyrosine hydroxylase which is found in the nerve cells. However, there are major differences between the two enzymes being:

a. They can be easily distinguished on the basis of their cofactor requirements. Tyrosinase requires DOPA, the product of the initial reaction to act as a cofactor for the reaction while tyrosine hydroxylase requires a tetrahydroprotein as cofactor. Neither enzyme can utilise the other cofactor as they are extremely specific (Hearing 1987; Pomerantz 1966).
b. Tyrosinase is a copper-containing enzyme while tyrosine hydroxylase contains iron and thus, they can be distinguished on the basis of inhibitors specific for either of these metals (Hearing 1987).

5.7 Products of Degradation of Aromatic Compounds

During the biodegradation of aromatic compounds, the number of products formed either as byproducts or intermediates include:

5.7.1 Quinones

Aromatic compounds which have two hydroxyl groups oriented either in *ortho* or *para* position can easily be oxidized to ketone like compounds called quinones (O'Leary 1976). Quinones are non-aromatic conjugated cyclo-hexadienones (Goodwin 1979). Varieties of quinones exist and are found in bacteria, fungi and higher plants. Four types of quinones described are: (1) Benzoquinones, (2) Naphthoquinones, (3) Anthraquinones and (4) Isoprenoid quinones.

5.7.2 Melanins

Melanin pigments occur widely in plants and animals and often in bacteria (Mason 1953). Apart from imparting intense pigmentation, which in certain cases is of taxanomic value, melanins play a role in increasing resistance of fungi towards lytic enzymes, detoxification of polyphenolics in root nodules, detoxification mechanism for pathogen *Cryptococcus neoformans* creating anaerobic conditions in nitrogen-fixing cultures and protecting cells due to antioxidant, antiradical and superoxide scavenging properties (Banulescu 2000; Barnett and Hageman 1983; Djordjeric et al. 1987; Margalith 1992; Mayer and Hazel 1979; Sadasivan and Neyra 1987; Shivprasad and Page 1989). Four basic kinds of melanins are:

5.7.2.1 Eumelanin

It is a black to brown insoluble material, consisting of highly polymeric cross-linked structures of several hundred monomeric units found in mushrooms and potatoes. The two types of eumelanin monomers known are dihydroxy indole (DHI) and dihydroxy indole carboxylic acid (DHICA) (Banulescu 2000).

5.7.2.2 Phaeomelanin

It is a yellow to reddish brown alkali soluble material and is found in the hair and feathers of fowls, encysted forms of certain strains of *Azospirillum brasilene*, black spot of potato, human hair, squid ink etc. (Sadasivan and Neyra 1987; Stevens et al. 1998).

5.7.2.3 Neuromelanin

DOPA is too hydrophilic to cross the blood–brain barrier and hence is converted to hydrophobic L-DOPA which can cross the blood–brain barrier, where it is reconverted to DOPA. This is then oxidatively converted to neuromelanin. Neuromelanins can act as chain-breaking antioxidants (Shivprasad and Page 1989).

5.7.2.4 Allomelanin

It is formed from nitrogen-free precursors such as acetate, catechol-1,8-dihydroxynaphthalene, etc. No tyrosine is involved in the synthesis of this pigment. The pathway for allomelanin formation is known as the polyketide biosynthetic pathway. Allomelanin is seen in watermelon seeds and in the filamentous fungus *Alternaria alternata* (Kimura and Tsuge 1993).

5.8 Degradation of Aromatic Compounds at Alkaline pH

Study of microbial degradation of aromatic compounds at alkaline pH is important in natural ecosystems where the fate and toxicity of these contaminants are unknown, but the existence of alkaliphilic microorganisms thriving at alkaline pH would support the functioning of the carbon cycle. Use of these microorganisms in the removal of aromatic compounds from alkaline and/or industrial wastewater will support the environmental concern of industries and environmentalists. The microbial degradation of aromatic compounds is important not only for the industrial applications of the respective enzymes involved, but also for studying struc-

tural, functional and genetic aspects of aromatic compound oxygenases. However, there is little information concerning alkaliphiles that degrade aromatic compounds, or concerning aromatic compound oxygenases isolated from them. Isolation of aromatic compound degrading alkaliphiles might enable the acquisition of new information not only on the taxonomy, physiology and ezymology of alkaliphiles, but also on aromatic compound oxygenases (Gibson and Subramanian 1984). Hydrocarbon biodegradation at alkaline pH is of interest for the bioremediation of industrial waste waters which are contaminated with aromatic or chlorinated hydrocarbons (Margesin and Schinner 2001).

Although much is known about the degradation of aromatic compounds at neutral pH, relatively very little information is available about such biotransformations occurring at an alkaline pH, more so with alkaliphilic bacteria. The term "Alkaliphilic" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and12, but cannot grow or grow only slowly at the near-neutral pH value (Horikoshi 1991). Alkaliphilic bacteria have been extensively studied with regard to their biotechnologically relevant extracellular enzymes and as model organisms to solve bioenergetic problems (Garcia et al. 1983; Krulwich and Guffanti 1989; Krulwich 1995). However, very little information is known about their capabilities of degrading aromatic compounds. One of the few available reports is on degradation of 4-chlorobenzoate by a facultatively alkaliphilic *Arthrobacter* sp. strain SB8 (Shimao et al. 1989). The bacterium dehalogenated 4-chlorobenzoate in the initial stages of degradation and metabolized it via 4-hydroxybenzoate and protocatechuate. The substrate concentration tolerated by this bacterium was as high as 150 mM. The degradation of phenol at pH 10 by bacteria isolated from a 2.6 g/l NaCl Lonar lake has been reported (Kanekar et al. 1999). To determine the feasibility of aromatic compound biodegradation in saline and alkaline conditions, the effect of pH and salinity on the biodegradation of phenol as a model aromatic waste compound by the haloalkalophilic bacterium *Halomonas campisalis* has been examined (Alva and Peyton 2003). Yumoto et al. (2003) have reported a halotolerant obligate alkaliphile *Bacillus krulwichiae* sp.nov.capable of utilising benzoate and m- hydroxybenzoate. Prior to this report, the degradation of 2,4 Dichlorophenoxyphenol by an alkaliphilic, moderately halophilic soda lake isolate *Halomonas* sp. EF 43 has been reported (Kleinsteuber et al. 2001). Three kinds of alkaliphilic bacteria able to utilise thiocyanate (CNS^-) at pH 10 were found in highly alkaline soda lake sediments and soda soils. Genetic analysis has demonstrated that both the heterotrophic and autotrophic alkaliphiles that utilised thiocyanate as a nitrogen source belonged to the gamma subdivision of proteobacteria (*Halomonas* group for heterotrophs and *Thioalkali vibrio* for autotrophs) (Sorokin et al. 2001). The heterotrophic and autotrophic alkaliphiles that grew with thiocyanate as a nitrogen source possessed a relatively high level of cyanase activity which converted cyanate (CNO^-) to ammonia and CO_2. Since it is relatively stable under alkaline conditions, cyanate is likely to play a role as a nitrogen buffer that keeps the alkaliphilic bacteria safe from inhibition by free ammonia which otherwise would reach toxic levels during dissimilatory degradation of thiocyanate.

Removal of aromatic compounds from alkaline or industrial waste water is an environmental concern for the industry. In addition, aromatics may be accumulating in soda lakes and unique natural systems where the fate and toxicity of these contaminants are unknown. It was, therefore, envisaged to review the degradative studies performed on aromatic compounds under alkaline conditions. Since benzoate is a central core for the degradation of substituted and polycyclic aromatic compounds, it was of interest to study the degradative pathway by alkaliphilic bacteria in the presence of sodium benzoate and tyrosine and note the various intermediates formed during their metabolism.

5.9 Degradation of Aromatic Compounds by Alkaliphilic Bacteria from Mangrove Ecosystems of Goa

5.9.1 Isolation of Alkaliphilic Bacteria and Screening for Aromatic Compound Degradation

Alkaliphiles from mangrove ecosystems of Goa namely Banastari, Merces, Panaji, Ribandar and St. Cruz, situated on the west coast of India, were studied with respect to biodiversity and their ability to biodegrade aromatic compounds under alkaline conditions. Samples collected from mangrove ecosystems were plated on polypeptone yeast extract glucose agar (PPYG) (pH 10.5) (Gee et al. 1980). Interestingly, all the samples were found to yield the presence of alkaliphilic bacteria (Desai et al. 2004). Predominant isolates (141) obtained on PPYG agar (pH 10.5) were purified, maintained and replica plated on four sets of PPYG agar plates of pH 7, 8.5, 10.5 and 12 for selection of obligate alkaliphiles. The plates were incubated for 48 h at room temperature and the isolates growing only at pH 10.5 and 12 were selected to be obligate alkaliphiles. Significantly, 20% isolates were found to be obligate alkaliphiles while 80% isolates were considered as facultative. Identification studies done by morphological, biochemical, chemotaxonomical and phylogenetic analysis revealed that 98% of the cultures were Gram-positive with 54% of the alkaliphilic cultures belonging to the genus *Bacillus*, 21% to *Corynebacterium*, 7% each to *Micrococcus* and *Actinomycetes*. Only one culture, designated as A-131 in the study was found to be Gram-negative short rods (Fig. 5.2) and was identified to belong to the genus *Flavobacterium* by 16S rRNA analysis. Further, 28 obligate alkaliphiles were screened for aromatic compound degradation by growing them in Mineral salts medium (MSM) with the aromatic compounds such as benzoate, tyrosine, phenylalanine, phenol, cresol, aniline, resorcinol, quinol and parachloroaniline. Interestingly, all the isolates grew luxuriantly when supplemented with benzoate, phenol, tyrosine and phenylalanine as sole source of carbon, whereas a few isolates exhibited the capability of utilising aniline, cresol and other phenolic compounds such as resorcinol, quinol and parachloroaniline.

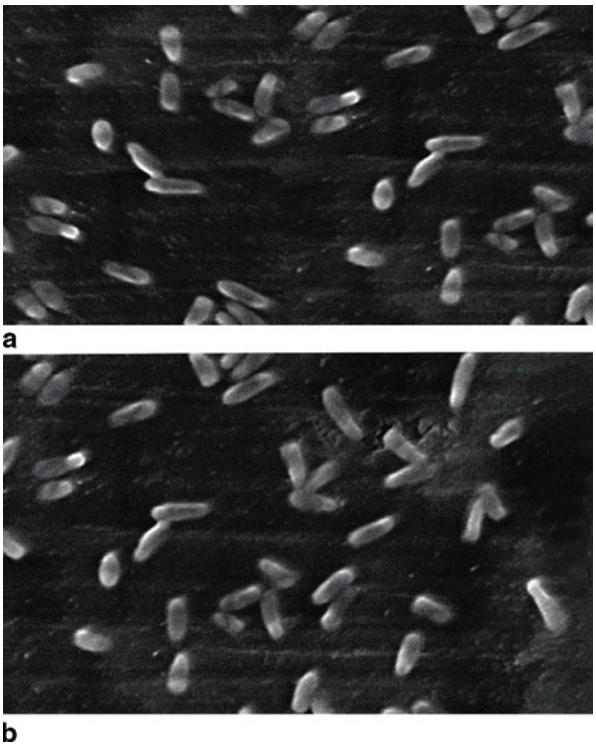

Fig. 5.2 Scanning electron micrographs of *Flavobacterium* A-131 in (**a**) MSM with 0.3 % benzoate and (**b**) MSM with 0.2 % tyrosine

5.9.2 Degradation of Sodium Benzoate by Flavobacterium Strain A131

Flavobacterium strain A-131 was inoculated in MSM at pH 10.5 containing sodium benzoate as the sole source of carbon at different concentrations (0.1–1 %). It was noted that the isolate could grow upto the concentration of 0.7 % sodium benzoate with the optimum concentration of 0.3 %. *Flavobacterium* A-131 grown in the presence of 0.3 % benzoate and extracted in ether showed a distinct spot of Rf of 0.9 of on thin layer chromatography (TLC) plate which turned black in colour and was corresponding to standard catechol, further confirmed by the addition of lead acetate to the supernatant which gave a white precipitate. Ring cleavage of aromatic substrate was studied by the modified Rothera's test (Offlow and Zolg 1974) which indicated that the *meta*-ring cleavage pathway was present in the *Flavobacterium* A-131 grown in benzoate as sole carbon sourceTherefore, the enzyme catechol 2,3 dioxygenase was assayed using whole cells as well as cell-free extract, and their specific activities were determined. Whole cells showed an activity of 0.38 U/mg as

compared to 1.08 U/mg shown by the cell-free extract. During the growth in MSM-containing benzoate, the culture showed very little growth and the initial colour of the medium was yellow, and later turned orange-red. The yellow and orange-red products formed during the metabolism of benzoate were analyzed further.

A distinct peak at 375 nm in the UV-visible scan was obtained with the appearance of a yellow colour in the medium, with the absorbance found to increase with deepening of the colour during growth. The orange-red supernatant formed during the metabolism of benzoate by *Flavobacterium* A-131 was tested for the presence of quinone (Finley 1974). The tests done showed that the orange-red product in the supernatant could probably be a quinone. Catechol is thus an important intermediate formed from benzoate metabolism by *Flavobacterium* A-131 which gets converted to a yellow-coloured intermediate and subsequently forms an orange-red product probably a quinone. From the above observations, we propose catechol to be the central metabolite in sodium benzoate biodegradation by *Flavobacterium* culture A-131 under alkaline conditions linking two pathways, namely the *meta*-cleavage pathway and the quinone formation, both of which operate simultaneously during benzoate biodegradation. The proposed pathway for the benzoate metabolism by *Flavobacterium* culture A-131 is as presented in Fig. 5.3.

Yumoto et al. (2003) have reported the utilisation of benzoate and *m*-hyroxybenzoate under alkaline conditions by the alkaliphilic bacterium *Bacillus krulwichiae*. The intermediates and the final product formed during the metabolism, however have still not been characterised and reported. *Rhodococcus* sp. K-37 and HA-99

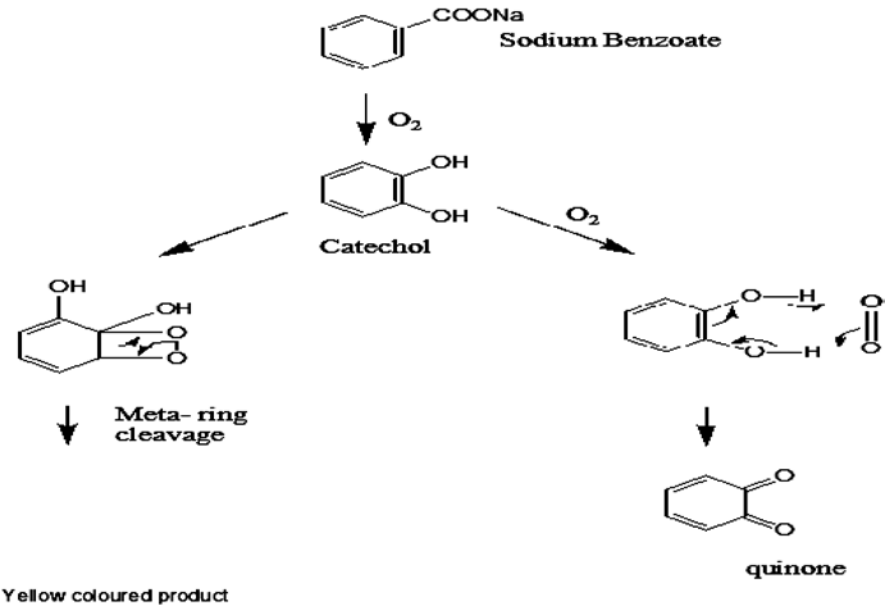

Fig. 5.3 Proposed pathway for metabolism of benzoate by *Flavobacterium* A-131

have also been reported to utilise benzoate and 3-hydroxybenzoate, phenol, biphenyl, 4-chlorobiphenyl and naphthalene under alkaline conditions (Maeda et al. 1998), but the intermediates or products formed during utilisation have not been discussed.

5.9.3 Degradation of Tyrosine by Flavobacterium Strain A-131

To check the degradation of tyrosine by *Flavobacterium* A-131, it was inoculated on MSM agar (pH 10.5) containing 0.1–1 % concentrations of tyrosine and incubated for 24 h at the room temperature. Interestingly, zones of clearance were observed depicting growth and tyrosinase activity (Fig. 5.4).

In liquid MSM medium with varied concentrations of tyrosine, growth and turbidity varied from pink colour within 16 h which deepened to a brown colour at 40 h (Fig. 5.5).

From the qualitative tests performed (Kelley et al. 1990) (Table 5.2), the pink-coloured compound could be a quinone, probably dopaquinone and the black-coloured product is similar to melanin which could be eumelanin as reported by Kelley et al. (1990). Degradation of tyrosine is known to occur by the production of tyrosinase, a copper-containing monooxygenase. From the qualitative tests performed, it can be concluded that there exists an enzyme which produces DOPA from tyrosine and further oxidizes it to dopaquinone. DOPA autooxidation and polymerization then converts dopaquinone to melanin which is the brown-coloured product turning black in colour. The tyrosinase (oxidase) activity was found to be present in whole cells and cell-free extract with an activity of 0.23 U/mg with 0.55 U/mg, respectively. Many reports are available which support the above data (Lerch

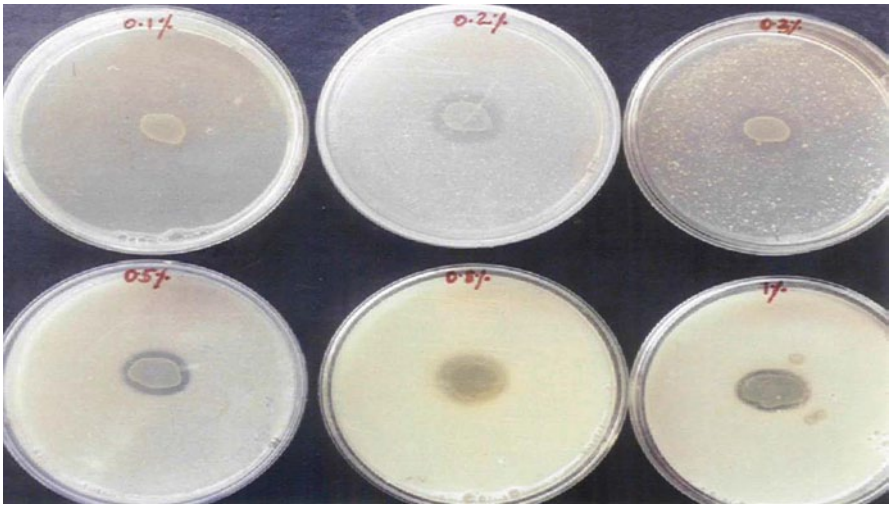

Fig. 5.4 Demonstration of tyrosinase activity by *Flavobacterium* A-131

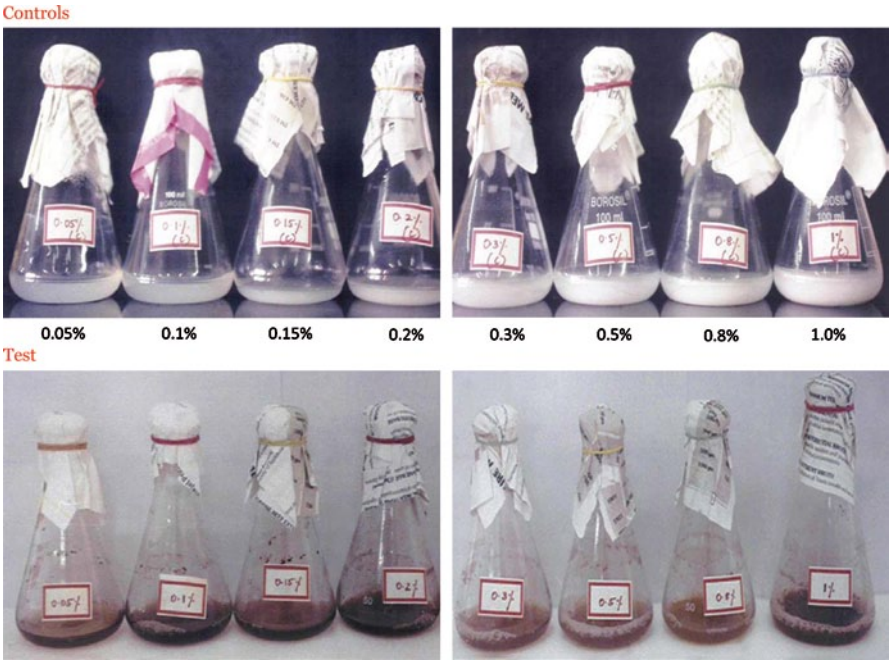

Fig. 5.5 Growth of *Flavobacterium* A-131 in MSM with tyrosine as a sole source of carbon (40 h incubation)

Table 5.2 Qualitative tests for the detection of metabolic products of tyrosine

S. no	Tests	Control*	Culture supernatant	Black
		(0.1 % DOPA)	Pink	
1	Supernatant + 6N HCl (pH 2)	Pink to colourless	Pink to colourless	Black to colourless
2	(1) + 10N NaOH (pH 12)	Colourless to pink	Colourless to pink	Colourless to black
3	Supernatant + 2 % sodium dithionite	Pink to colourless	Pink to colourless	Black to colourless
4	(3) + when aerated for 2 h	Colourless to pink	Colourless to pink	Colourless to black
5	Supernatant + pinch of sodium borohydride	Pink to colourless	Pink to colourless	Black to colourless
6	Supernatant + H_2O_2	Pink to colourless	Pink to colourless	Black to colourless

* Control (0.1 % DOPA) was incubated at the room temperature till the pink colour appeared

1987; Kelley et al. 1990). Thus, the pathway of tyrosine → DOPA → dopaquinone → dopachrome → melanin is followed by *Flavobacterium* A-131 for degradation of tyrosine and is carried out by the enzyme tyrosinase. Reports have shown ex-

tensive studies of aromatic compound degradation with Gram-positive bacteria at neutral pH. However, limited work has been done with Gram-negative bacteria (Kelley et al. 1990). In view of this, the culture *Flavobacterium* A-131 is a novel isolate from the mangrove ecosystem showing growth and metabolism of aromatic compounds only at alkaline pH. It is a versatile alkaliphilic bacterium having the ability to degrade a wide range of aromatic compounds, including monoaromatic, polyaromatic, chlorinated and nitrocompounds exhibiting the formation of coloured intermediates when grown in sodium benzoate and tyrosine as a sole source of carbon. The culture has been deposited at the National Chemical Laboratory, Pune, with the accession number NCIM 5243.

5.10 Conclusions and Future Prospects

The mangrove ecosystems harbour obligate alkaliphilic bacteria which are capable of producing a wide range of enzymes with the potential of degradation of aromatic compounds. The presence of these bacteria indicates that they play an important role in the biogeochemical cycles as well as pollution abatement. Search for these isolates from such ecosystems using various techniques and media could result in isolating highly potential obligate alkaliphiles including *Flavobacterium* A-131. Further research in this area can, therefore, be directed towards:

a. Derivitisation, purification and characterisation of the yellow- and orange-coloured products obtained from benzoate degradation and the dopaquinone obtained from tyrosine degradation.
b. Purification and characterisation of the enzymes catechol 1,2 dioxygenase, 2,3 dioxygenase and tyrosinase obtained from *Flavobacterium* A-131 with respect to enzyme assays and substrate specificities.
c. Utilisation of *Flavobacterium* A-131 in the treatment of industrial effluents containing aromatic compounds for bioremediation.

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