

Full Length Research Paper

Utilisation of Toxic Aromatic Compounds by Alkaliphilic Bacteria Isolated from Mangrove Ecosystems of Goa

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Alkaliphilic bacteria are bacteria which are capable of growth at high pH. Industrial waste effluents possessing a very high pH and containing various kinds of toxic and hazardous aromatic compounds can be treated using alkaliphiles, which exhibit an enormous potential of degrading these aromatic compounds thus helps in clearing the pollutants, which are mainly present in waste effluents which have a high pH. Therefore employing alkaliphiles for biodegradation of toxic compounds present in the industrial waste effluents would be of great help. In this study alkaliphiles were isolated from various mangrove ecosystems of Goa, West Coast of India. The alkaliphiles were isolated on PPYG medium (Polypeptone Yeast Extract Glucose) medium of pH 10.5. These alkaliphiles were also screened for their ability to degrade a wide range of aromatic compounds and it was observed that most of the alkaliphiles utilized these aromatic compounds especially benzoate phenol and tyrosine as sole source of carbon. Further one of these obligate alkaliphiles also showed an excellent degradative pathway for degradation of benzoate producing HMS (Hydroxy Muconic Semialdehyde) as a metabolite of degradation.

Keywords: Alkaliphiles, high pH, Bioremediation, Aromatic compounds, HMS.

INTRODUCTION

Mangroves 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. They are nutritionally rich due to the continuous shedding of foliage which gets decomposed to form detritus matter (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 an excess of fertilizers, 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.

Due to the hazardous effect of aromatic hydrocarbons, it is necessary to monitor 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 utilization of the hydrocarbon as a carbon source or as a co-metabolic transformation by microorganisms.

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 environment 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

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for studying structural, functional and genetic aspects of aromatic compound oxygenases. However, there is little information concerning alkaliphilic bacteria 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 enzymology 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). Degradation of aromatic compounds at alkaline pH is significant as alkaliphilic bacteria are more tolerant to toxic compounds and have better bioavailability that can improve the efficiency of biodegradation in control and removal of toxic pollutants from the environment.

MATERIALS AND METHODS

Collection and analysis of samples

Sediment and water samples were collected from various mangrove ecosystems of Goa. pH of the various samples was tested using the pH paper on the site of collection and using LABINDIA pH analyzer (PHAM) in the laboratory.

Enumeration of Neutrophiles and Alkaliphiles

Neutrophiles were isolated on nutrient agar (pH 7.0). For isolation of alkaliphiles three different media were used.

- (i) Polypeptone Yeast extract glucose agar –PPYG (pH 10.5) (Horikoshi 1991)
- (ii) Horikoshi-I
- (iii) Horikoshi-II

Soil sediments were suspended in sea-water at the ratio of 1:10 and incubated on the Orbitek shaker for 1h at 150 rpm, the suspension was allowed to settle and 0.1ml of supernatant used for plating or preparation of dilution. For viable count on Nutrient Agar, water samples and sediment supernatants were diluted serially tenfold in sea water. Viable count of alkaliphiles was determined by plating out water samples and sediment supernatant directly without any dilution on the respective media plates. The plates were incubated for 24-48 hrs at room temperature for neutrophiles and alkaliphiles. Colonies formed were counted and the total viable count was determined.

Screening of alkaliphiles for pH preference

Predominant isolates from PPYG agar pH 10.5 were

replica plated on four sets of PPYG agar plates of pH 7, 8.5, 10.5 and 12 to obtain 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.

Growth of alkaliphiles on various aromatic compounds

The selected alkaliphilic isolates were screened for their ability to degrade aromatic compounds. The isolates were grown on Mineral Salt Medium (MSM) supplemented with 0.1% of aromatic compounds such as benzoate, phenol, tyrosine, aniline, cresol, phenylalanine, resorcinol, and quinol as the sole source of carbon. The plates were incubated and the results were checked after 48hrs of incubation.

Determination of optimum concentrations of phenol, benzoate and tyrosine

Benzoate, phenol and tyrosine were added in concentrations of 0.2, 0.3, 0.5, 0.7, 0.8 and 1% in Mineral Salt Medium of pH 10.5 using 10% Na₂CO₃. The obligate alkaliphilic cultures were inoculated in this medium and incubated at 37°C. Growth of the cultures was monitored visually after 24 to 48hrs of incubation at 37°C. The substrate concentration at which the cultures exhibited good growth were selected as the optimum concentration of substrate for culture.

Qualitative and quantitative tests for detection of transformation products

Qualitative and quantitative tests were carried out to determine the transformation products formed by the alkaliphilic isolate in MSM medium containing benzoate and phenol respectively.

1) Precipitation of Catechol: (Clarke et al., 1975)

0.3 ml of (20% w/v), lead acetate solution was added to 1 ml of culture supernatant. Presence of catechol gave a white precipitate.

2) Colorimetric estimation of Catechol: (Evans, 1971)

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

3) Rothera's test: (Offlow and Zolg 1974)

Ring cleavage of aromatic degradation was studied by modified Rothera's method. Culture cells grown in MSM with phenol, were centrifuged at 6000 rpm for 20 minutes at 4°C. Pellets were washed twice and

resuspended in 0.05M phosphate buffer pH 7 to an absorbance of 4.0 at 540 nm. To 2 ml cell suspension, 0.5 ml of toluene and 3 ml of 13.3mM catechol (final concentration = 5mM) were added. After 3 minutes the colour change was noted. The appearance of yellow colour indicates the presence of meta ring cleavage enzymes. The mixture was further incubated on a shaker for 12 hours following which, 1 g of ammonium-sulphate was added and the solution was mixed well. 5 drops of freshly prepared 1% sodium-nitroprusside solution and 0.5ml of liquor ammonia were then added slowly along the side of the tube and the colour change was recorded. Presence of ortho ring cleavage enzymes results in a purple ring formation.

4) Spectrophotometric analysis of the coloured intermediate metabolites formed during benzoate and phenol degradation:

The coloured intermediates (black and yellow products) formed during benzoate degradation by the alkaliphile at pH 10.5 were then analysed spectrophotometrically using the Shimadzu UV-Visible Spectrophotometer. The UV-visible scans of the black coloured and yellow coloured intermediate were taken.

RESULTS AND DISCUSSION

Distribution and total viable count of alkaliphiles

Samples collected from mangrove ecosystems were plated on Nutrient agar and Polypeptone Yeast extracts Glucose agar for the isolation of alkaliphiles. Polypeptone. Yeast extract Glucose agar pH 10.5 (pH was maintained using Na_2CO_3) and showed good growth of alkaliphiles. It is well documented that Na^+ is crucial for the growth of most alkaliphiles (Krulwich and Guffanti 1989). The high Na^+ ion content of PPYG was perhaps contributing to the growth of obligate alkaliphiles, Similar results have been reported earlier with reference to the alkaliphilic count from mangrove ecosystems (Desai *et al.*, 2004). PPYG medium pH 10.5 was hence selected as the specific medium for the further studies. It has been reported that Na^+ ions are essential for maintenance of the cytoplasmic pH which remains 1-2 units below external pH. Unlike pH homeostasis in non alkaliphiles, which can be coupled to Na^+ (perhaps to avoid detrimental reductions in the cytoplasmic K^+). Thus alkaliphiles capacity for Na^+ extrusion may have to be protected for this crucial function under conditions other than Na^+ stress. In fact, routes that make cytoplasmic Na^+ available i.e. complete the Na^+ cycle are necessary for pH homeostasis independently of other functions to which they relate (Krulwich and Guffanti 1989). The samples showed the presence of alkaliphilic bacteria and gave high counts on PPYG agar.

Screening for obligate alkaliphiles

The predominant isolates obtained on the PPYG agar were further screened for selection of obligate alkaliphiles. These isolates were plated on PPYG agar of pH 12. The isolates growing at pH 10.5 and 12 were selected to be obligate alkaliphiles. Twenty-five predominant isolates were obligate, the remaining isolates were considered to be facultative.

Screening and selection of benzoate, phenol and tyrosine degrading alkaliphiles

The medium used was Mineral Salt Medium (MSM) which was sterilised separately. The pH of the medium was adjusted to 10.5 using Na_2CO_3 solution. To this medium, various concentrations of phenol, benzoate and tyrosine ranging from (0.05% to 1%) was added. In this present investigation, attempts were made to isolate benzoate and phenol degrading microorganisms from mangrove soil. Obligate alkaliphiles obtained were tested for their ability to grow on benzoate and phenol by inoculating in MSM agar as well as broth containing various concentrations of benzoate and phenol. All the obligate alkaliphiles obtained showed growth on MSM medium containing phenol and benzoate as the sole source of carbon, but only one of the isolates showed excellent growth at higher concentrations of phenol and benzoate and this isolate were selected for further studies. The concentration of phenol and benzoate of 1% and above inhibits the growth of the microorganisms indicating that the increased concentration of substrate is toxic for the microorganisms and the microorganism is not able to utilise it for its metabolism.

Detection of the transformation products formed due to benzoate and phenol degradation

Qualitative and quantitative tests were carried out to determine the transformation products formed by isolate MSC in MSM medium containing phenol and benzoate. The alkaliphilic isolate was inoculated in Mineral Salt Medium of pH 10.5 containing phenol and benzoate respectively as the sole source of carbon. It was noted that the isolate exhibited excellent growth at an optimum concentration of 0.2% benzoate and 0.2% phenol respectively. Therefore, it was grown at this concentration that it was observed that during growth of the culture the medium turned light black in colour. So it was of interest to investigate this particular product formed during benzoate metabolism. It has been reported in literature that during degradation of most aromatic compounds, the central intermediate formed is catechol which is black in colour.

A) Test performed to identify Transformation Product I (TPI)

The black coloured supernatant on the addition of lead acetate gave a white precipitate. This was compared with the control i.e. MSM medium with phenol and benzoate which did not give a white precipitate. This confirmed that the Transformation Product I (TPI) was catechol. From the above observations we propose catechol to be the central metabolite in benzoate biodegradation by the alkaliphilic isolate under alkaline conditions. Catechol is thus an important intermediate formed from benzoate metabolism. Catechol formed in the culture broth from phenol is detected by a white precipitate with lead acetate.

B) Rothera's test

Ring cleavage of aromatic substrate catechol was studied using the modified Rothera's test. Culture cells grown in MSM with benzoate and phenol, were centrifuged at 6000 rpm for 20 minutes at 4°C. Pellets were washed twice and resuspended in 0.05M phosphate buffer pH 7 to an absorbance of 4.0 at 540nm. To 2 ml cell suspension, 0.5 ml of toluene and 3 ml of 13.3mM catechol (final concentration = 5mM) were added. After 3 minutes the colour change was noted. The appearance of yellow colour indicates the presence of meta ring cleavage enzymes. This indicated the presence of meta-ring cleavage pathway in the alkaliphilic isolate grown in benzoate and phenol as the sole source of carbon.

C) Transformation Product II (TPII)

On further incubation of the alkaliphilic isolate in MSM broth under shaker conditions i.e 96h, a yellow colour formation was observed in the medium. The yellow coloured product formed after further incubation of catechol was tentatively identified as HMS (Hydroxy Muconic Semialdehyde) (Transformation Product II).

D) Spectroscopic analysis of the intermediate metabolites formed during phenol and benzoate degradation by the alkaliphilic isolate

Benzoate and phenol were found to be transformed into catechol by the alkaliphilic bacterial strain at the end of approximately 12 hours of incubation. Spectrophotometric and colorimetric methods were unsuccessful for catechol detection possibly due to the

interference with other degradation products. After 72 hours the black coloured compound became lighter and was converted to a yellow colour within 96 hours of incubation. Therefore it was of interest to study this yellow coloured product. The UV-visible scan of the yellow supernatant showed a distinct peak at 375nm (Figure 1). The yellow product formed from catechol could be 2-hydroxymuconic semialdehyde (HMS); a meta ring cleavage product. A distinct peak at 375nm 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, indicating the production of HMS as also obtained from *Pseudomonas cepacia* AC 1100 (Ghadi and Sangodkar 1994) and *Pseudomonas mendocina* P₂d (Parulekar 2001).

E) Tentative benzoate/phenol biodegradation pathway by the alkaliphilic isolate

Figure 2 indicates the proposed degradation of benzoate and phenol by the alkaliphilic isolate. Benzoate and phenol in MSM medium inoculated with this bacterium resulted in the formation of different metabolites. The initial reaction which is reported to be catalysed by monooxygenase resulted in the formation of catechol. In the next step ring fission occurred due to dioxygenase enzyme resulting in the formation of Hydroxyl Muconic Semialdehyde (HMS). The formation of these intermediates could be attributed to enzymes like monooxygenase and catechol 2, 3 dioxygenase and finally the aliphatic moieties may completely be oxidised via TCA cycle.

CONCLUSIONS

Obligate alkaliphilic bacteria which are capable of producing a wide range of enzymes with the potential of degradation of aromatic compounds are present in the mangrove ecosystems. The presence of these bacteria indicates that they play a major role in the biogeochemical cycles as well as pollution abatement. These obligate alkaliphiles have a remarkable potential for application in bioremediation of toxic aromatics and in waste water treatment, especially in detoxification of aromatics as wastes.

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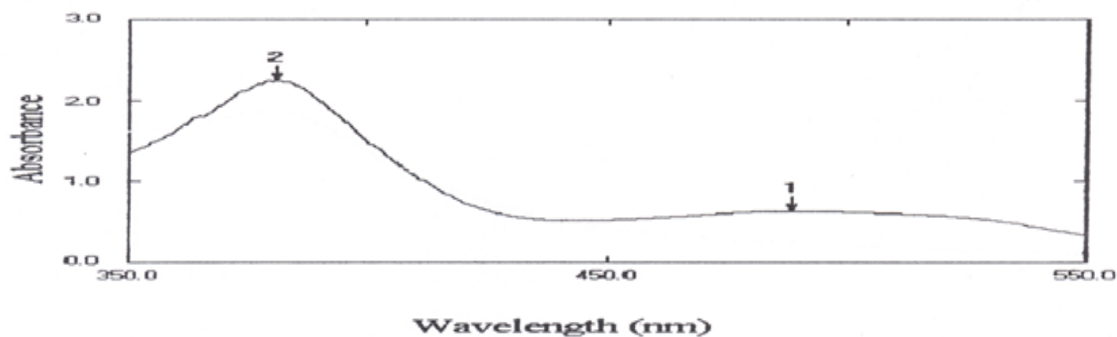


Figure 1. UV-visible scan of the yellow coloured supernatant.

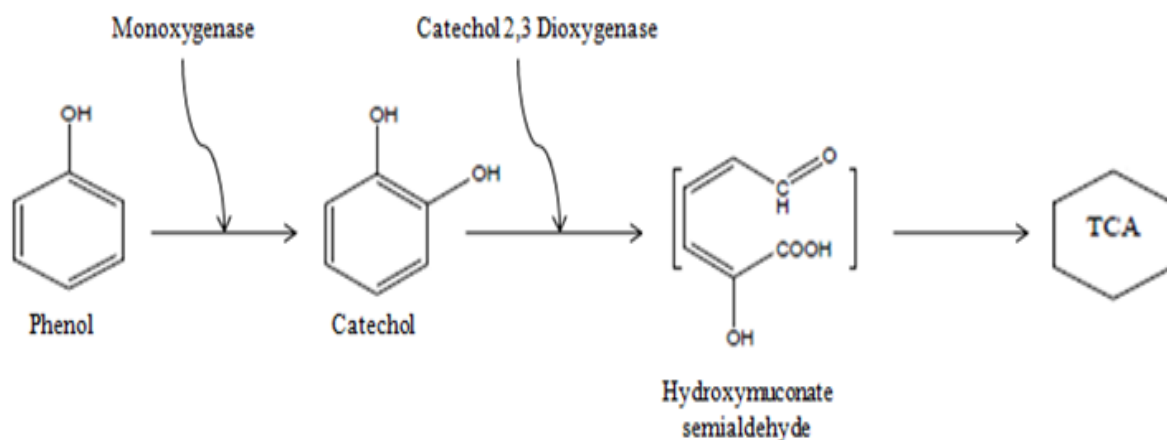


Figure 2. Proposed phenol degradation pathway by the alkaliphilic isolate.

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