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Ortho and meta cleavage dioxygenases detected during the degradation of phenolic compounds by a moderately halophilic bacterial consortium

Krishnaswamy Veenagayathri and Namasivayam Vasudevan

Centre for Environmental Studies, Anna University, Chennai-25

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Phenolic compounds are carcinogenic and toxic environmental pollutants which are massively discharged to the terrestrial and marine environment from uncontrolled industrial activities. A moderately halophilic bacterial consortium was isolated from different phenol contaminated sites and marine environment, the degradation pathway followed during the degradation of phenol and 4-chlorophenol by the bacterial consortium was investigated. During the degradation of phenol it was found that only catechol 1, 2 dioxygenase activity was pronounced with a maximum specific activity of 0.425 $\mu\text{m}/\text{min}/\text{mg}$ of the protein showing the *ortho*-cleavage pathway at pH 7. In the degradation of 4-chlorophenol it was found that only catechol 2,3 dioxygenase activity was pronounced with a maximum specific activity of 0.412 $\mu\text{m}/\text{min}/\text{mg}$ of the protein at pH 7 showing the consortium followed *meta*-cleavage pathway during the degradation of the substrate. The cell free extracts of bacterial consortium showed *cis-cis* muconic acid as the intermediate metabolite during the degradation of phenol and 4-chlorocatechol and 5-chloro-2-hydroxymuconate as intermediates during the degradation of 4-chlorophenol, which proved that the moderately halophilic bacterial consortium followed *ortho*-cleavage on phenol degradation and *meta*-cleavage during the degradation of 4-chlorophenol.

Keywords: Halophiles, Biodegradation, Phenol, 4-Chlorophenol, Dioxygenase enzyme, Saline environment, Bacterial consortium

INTRODUCTION

Phenol and its derivatives are found in wide variety of wastewaters including those from the oil refining, petrochemical, coke and coal gasification industries. As a result, these compounds are commonly encountered in industrial effluents and surface water. Biodegradation has been chosen as a method to remediate environments contaminated by phenolic compounds, which is massively discharged from uncontrolled industrial waste disposal. A number of aerobic phenol degrading bacteria have been described previously, (Dua et al 2002, Lovely 2003, Wackett 2000, Watanabe 2001, Prpich and Dauglis 2005, Karigar et al 2006) however, little information is only available on the degradation of phenol by moderately halophilic bacterial strains (Hinteregger et al 1997, Garcia et al 2005, Munoz et al 2001, Alva and

Peyton 2003).

The aerobic degradation pathways in bacteria and yeast involve the occurrence of vicinal diols as substrates of ring-cleaving enzymes. Thus, the first step of phenol degradation is a hydroxylation of phenol to catechol. Catechol can undergo fission either by an intradiol or an extra-diol type of cleavage (*ortho*- or *meta*-fission). *Meta*-fission leads to 2-hydroxymuconic semialdehyde and further to formate, acetaldehyde, and pyruvate. The final products of both the pathways are molecules that can enter the tricarboxylic acid cycle (Powlowski and Shingler 1994; Harayama et al. 1992). For complete degradation of chlorinated aromatic compounds to occur, two steps are necessary, cleavage of the aromatic ring and the removal of the chloride atom (Hagglblom, 1990). The initial step in the aerobic degradation of monochlorophenols is their transformation to chlorocatechols. Chlorocatechols are the central metabolites in the aerobic degradation of a wide range of chlorinated aromatic

*Corresponding author E-mail: veenagayathri@yahoo.com

compounds. The identification of by-products formed during the biodegradation process of phenol and chlorophenol is essential for a better understanding of the degradation mechanism. We were interested particularly in isolating a bacterial consortium which could utilize phenolic compounds under saline conditions. The present study was centered mostly on ring cleaving dioxygenases. In this study the moderately halophilic bacterial consortium was analysed for the type of ring cleavage dioxygenase physiologically induced during growth in the presence of phenolic compounds as a sole carbon and energy source. The specific activities of dioxygenase enzyme were performed during the degradation of phenol and 4-chlorophenol as model compounds by the bacterial consortium.

MATERIALS AND METHODS

Composition of the culture medium

The bacterial consortium was grown in mineral salts medium of (g/L) NaCl 50.0, KH₂PO₄ 0.25, NH₄Cl 1.0, Na₂BO₇ 2.0, FeCl₃ 0.0125, CaCl₂ 0.06 and MgCl₂ 0.05 with 10 mg/L of yeast extract, adjusted to pH -7 and distilled water – 1L (Alva and Peyton 2003). The medium was autoclaved, cooled to room temperature and was amended with respective phenolic compound through a sterile filter (0.45 µm) in 250 ml Erlenmeyer flasks. The chemicals and reagents (Analar grade) used in the study were purchased from Merck, India.

Bacterial consortium

Soil samples were collected from different ecosystems in chennai such as salt pan, Puliket marine back water lake; Sea harbour (chennai), tannery affected soils and soil from sea food industries. The bacterial consortium was isolated by enrichment culture technique, where the soil sample (300g wet weight) was mixed in sterile distilled water (1:1 w/v) for 1 h at room temperature.

During the initial adaptation stage the consortium was enriched with phenol 50 mg/L (Concentration of Phenol) and they were biochemically characterized, having six strains, of which four strains were gram positive and two strains gram negative. Further analyses by cloning and 16S rRNA gene sequence analysis, identified the isolates as *Bacillus cereus*, *Arthrobacter sp.*, *Bacillus licheniformis*, *Halomonas salina*, *Bacillus pumilus* and *Pseudomonas aeruginosa* (VeenaGayathri and Vasudevan 2010).

Total Protein

For analysis of total cell protein, samples were

centrifuged at 12,000 rpm for 10 mins and washed with fresh (substrate-free) mineral medium, then centrifuged and washed few times to remove the substrate. The pellet from each sample was then disrupted by sonication at 30 % amplitude for a total of 3 minutes (1.5 min x 2) on an ice-water bath. Sample (0.5 ml) was added to 0.5 mL Coomassie Blue protein dye and the absorbance was measured at 595 nm. The total protein concentration was determined by calibration with bovine serum albumin standard according to Bradford (1976).

Preparation of Enzyme Extract and Dioxygenase Activity

The bacterial consortium grown individually on phenol (100 mg/L), and 4-chlorophenol (25 mg/L) at an optimum salinity of 5 % was harvested at the exponential phase (48 h) by centrifugation and washed 0.033 M Tris –HCl buffer (pH 7.6) to remove the salts and resuspended in the same buffer. Cells were disrupted by using the tip sonicator Bandelin Sonopuls GM 200. Polyvinylpyrrolidone (PVP) was added to the suspension to remove the phenolics. The samples were centrifuged at 15,000 rpm for 30 min at 4 °C and the pellet was discarded. The clear supernatant solution was used as crude enzyme assay. The cell free extract was kept in ice and assayed for dioxygenase activity.

Catechol 1,2 dioxygenase (Type I) activity on the degradation of phenol was measured by following the formation of cis,cis- muconic acid, the ortho-cleavage product of catechol. The following reagents were added in the quartz cuvette 2mL of 50mM Tris HCl buffer (pH 8.0); 0.7 mL distilled water 0.1 mL ,100 mM 2-mercaptoethanol; 0.1 mL cell-free extract. The contents of the cuvette were mixed by inversion and 0.1 mL catechol (5µM) was then added and the contents were mixed again. Catechol 1,2 dioxygenase was assayed following the formation of cis,cis muconic acid. The increase in the absorbance at 260 nm and decrease in the absorbance at 278 nm over a period of 5 mins were followed in a Spekol spectrophotometer (Ngai et al 1990).

Catechol 1,2-dioxygenase (Type II) activity on the degradation of 4-chlorophenol was measured by following the formation of 2-chloromuconic acid, the ortho- cleavage product of 3-chlorocatechol. The procedure used was as same as Type I activity, with 3-chlorocatechol (5µM) being used in the place of catechol (5µM) (Farrell and Quilty 1999).

Catechol 2,3-dioxygenase activity on the degradation of phenol and 4-chlorophenol, was measured by following the formation of 2-hydroxymuconic semialdehyde, the meta- cleavage product of catechol, or the formation of 5-chloro-2-hydroxymuconic semialdehyde, the meta-cleavage product of 4- chlorocatechol. The following reagents were added to a cuvette: 2 ml 50 mM Tris-HCl

buffer (pH 7.5); 0.6 ml distilled water; 0.2 ml cell free extract. The contents were mixed by inversion and 0.2 ml catechol or 4-chlorocatechol (50 mM) was added and mixed with the contents. The production of 2-hydroxymuconic semialdehyde was followed by increase in absorbance at 375 nm over a period of 5 mins, while the production of 5-chloro-2-hydroxymuconic semialdehyde was followed by the increase in absorbance at 380 nm over the same period (Farrell and Quilty 1999). Protein estimation was measured by Bradford's method by using bovine serum albumin as a standard (Bradford 1976). Specific enzyme activities are reported as $\mu\text{mol}/\text{min}/\text{mg}$ protein. All assays were performed in duplicates.

Enrichment of bacterial consortium

Phenolic compounds were dissolved in dichloromethane and added to 250 mL conical flask and after the evaporation of dichloromethane, the mineral medium (100 mL) was added. The bacterial consortium containing 5 mL of 10^4 to 10^5 cfu/mL was added to the mineral medium containing phenol as sole carbon source. The conical flask was kept in shaker at 150 rpm with 37°C as incubation temperature. After growth was visualized under microscope, 5 mL of enrichment culture was transferred to a fresh medium and incubated under the same conditions. Subsequent identical transfer of culture was performed in the respective Phenolic compound containing medium to enrich the bacterial consortium. The cultures were maintained as glycerol stock and as well as in phenol containing medium.

Phenolic compound degradation by the bacterial consortium

For the degradation study, the bacterial consortium was inoculated in mineral medium containing phenolic compound. Different compositions used in the degradation of Phenolic compounds were 1) Medium + Phenol + bacterial consortium; 2) medium + Phenol and 3) medium + bacterial consortium where 2) and 3) served as controls. Bacterial consortium was added at concentrations of 10^4 to 10^5 cfu/mL in the medium. The culture prepared in duplicates were incubated at 37 °C in shaker at 150 rpm and extracted at every 24 h time interval for 5 days. The culture samples were extracted twice with dichloromethane (v/v) after acidification to pH 2.5 with 1 N HCl. The extracts were filtered through anhydrous sodium sulphate and condensed to 1mL using rotavapour unit (Buchi, Germany) and analysed in gas chromatography (GC). These condensed samples were used in TLC (thin layer chromatography) and gas chromatography-mass spectrometry (GC-MS) to analyse

the metabolites formed during Phenolic compound degradation.

Analysis of metabolites by GC-MS

During the degradation study, the different kinds of metabolites formed were identified using thin layer chromatography. The condensed samples were loaded on TLC plates using a 10 μL capillary tube. The chromatograms were run in different solvents for migration of the Phenolic compounds. Different solvent mixtures such as: benzene/hexane (50:50), benzene/acetone (50:50) and benzene/acetone/acetic acid (80:10:10) were used to identify the metabolites. After removing the plates from the substrates were identified using UV detection at 254 nm. For visualization, plates were sprayed with FeCl_3 (2% in ethanol). GC-MS analysis was performed with GC-MS-QP2010 [SHIMADZU] with an inert mass selective detector and a computer workstation was used for the phenolic compounds analysis. The samples were silylated before analysis. The GC-MS was equipped with: an Agilent DB-5 capillary column (30m x 0.25mm id x 0.25 μm); with an injection volume of 1 μL , split ratio of 20 injection at 280°C and an ion source temperature at 200°C. Oven operating temperature was 80°C with the holding time of 1 min, 300 °C for 2 mins with the total time of 41.67 mins. The masses of primary and secondary phenolic compound ions were determined by using the scan mode with an impact ionization (70 eV, 200°C) for pure phenolic compound standards (Merck). Qualitative analysis of phenols was performed by using the selected ion monitoring (SIM) mode. Fragmented products were identified using computer station library search. Retention time of the fragmented products are further compared and confirmed by analyzing authentic standards. Helium was used as the carrier gas. Standards from Sigma Aldrich were used for the phenolic compounds and their metabolites. A GC-MS library search was used to confirm the metabolites without standards.

RESULTS AND DISCUSSION

Dioxygenase Activity on phenol

A batch study was conducted with an optimum phenol concentration of 100 mg/L to detect the presence of catechol 1,2 dioxygenase and catechol 2,3 dioxygenase produced by the bacterial consortium at 50 g/L of NaCl. The degradation of phenol (100 mg/L) by the bacterial consortium at optimum salinity of 50 g/L data is not shown. To study the effect of substrate on catechol 1,2 dioxygenase activity, experiments were conducted on degradation of phenol (100 mg/L) by the bacterial consortium on the 2nd day in their log phase at 50 g/L

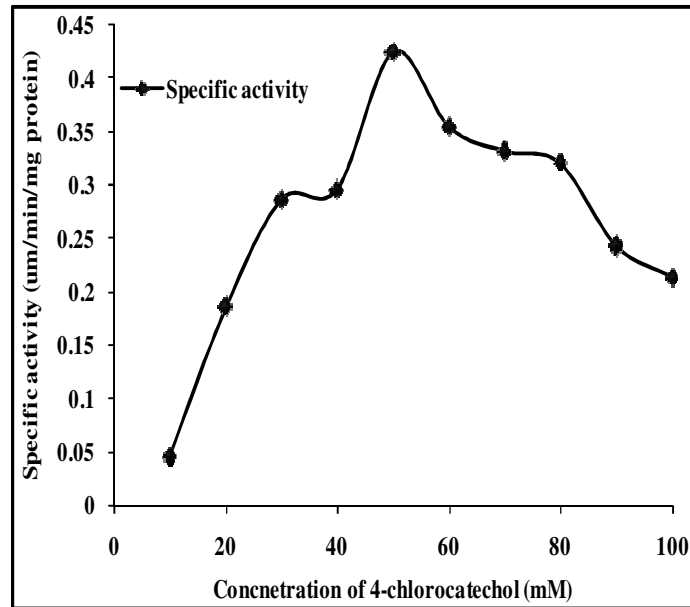


Figure 1. Effect of substrate (Catechol) concentration on catechol 1,2 dioxygenase activity

NaCl. The effect of substrate (catechol) concentration on the activity of catechol 1,2 dioxygenase is shown in the Figure 1. Substrate concentrations were used from 1 μm – 10 μm . It was observed from the figure that the highest catechol 1,2 dioxygenase activity of 0.379 $\mu\text{m}/\text{min}/\text{mg}$ of the protein was achieved at 5 μm of catechol, from 6 μm of the substrate concentration there was no increase in the activity of catechol 1,2 dioxygenase. Hence, for the further studies 5 μm of catechol was used as the substrate. Enzyme assays conducted with phenol (100 mg/L) as the substrate at 50 g/L NaCl, suggested that the consortium produced only catechol 1,2 dioxygenase showing the *ortho*-cleavage pathway. There was no catechol 2,3 dioxygenase activity detected when phenol was used as the substrate by the bacterial consortium.

Hinteregger and Streichsbier (1997) reported the degradation of phenol (100 mg/L) by *Halomonas* sp. at 50 g/L NaCl, where the catechol 1,2 dioxygenase activity was 760 nm/min/mg of protein, there was no catechol 2,3 dioxygenase activity and they showed that there was only *ortho*- pathway during the degradation of phenol. Use of the *ortho*- pathway is reported to be more efficient in carbon conversion to cell mass (growth yield) than the *meta*-pathway. The meta pathway utilizes phenol at a higher rate but results in lower cell yields (Kiesel and Muller 2002).

Effect of pH on degradation of phenol with catechol 1,2 dioxygenase activity

To determine the effect of pH on catechol 1,2

dioxygenase produced by the bacterial consortium, its activity was measured with 5 μm of catechol at different pH from 5 to 8. The specific activity was 0.085 $\mu\text{m}/\text{min}/\text{mg}$ of protein at pH 5 during the log phase (on 2nd day) with total protein yield of 22.4 mg/L. However, activity increased to 0.268 $\mu\text{m}/\text{min}/\text{mg}$ of protein, at pH 6 with the maximum protein yield at the end of 3rd day to 25.4 mg/L. The specific activity of catechol 1,2 dioxygenase was maximum at pH 7 (0.425 μm), with total protein of 30.3 mg/L. (Figure 2). Further increase in pH resulted in decrease in specific activity and finally it gave only 0.322 $\mu\text{m}/\text{min}/\text{mg}$ of protein at pH 8 an total protein of 22.5 mg/L on 3rd day. The optimum pH for the enzymatic action of catechol 1,2 dioxygenase is pH 7. This showed that the bacterial consortium was able to degrade the substrate at neutral pH with the production of catechol 1,2 dioxygenase.

Briganti et al (1997) reported phenol degradation by *Acinetobacter radioresistens*, where the purified catechol 1,2 dioxygenase showed that the optimum activity was in the range of pH 6.0 to 8.5 under non-saline conditions. Alva and Peyton (2003) reported degradation of phenol and catechol by the haloalkaliphilic *Halomonas campisalis*, where they showed the presence catechol 1,2 dioxygenase at pH 8. Two *Halomonas* sp. have been reported to use the *ortho*-pathway for the biodegradation of aromatics under saline conditions and at neutral pH (Hinteregger and Streichsbier 1997, Rosenberg 1983).

Garcia et al (2005) reported the degradation of phenol by *Halomonas organivorans*, the specific activity achieved during the degradation was 0.061 $\mu\text{m}/\text{min}/\text{mg}$ of

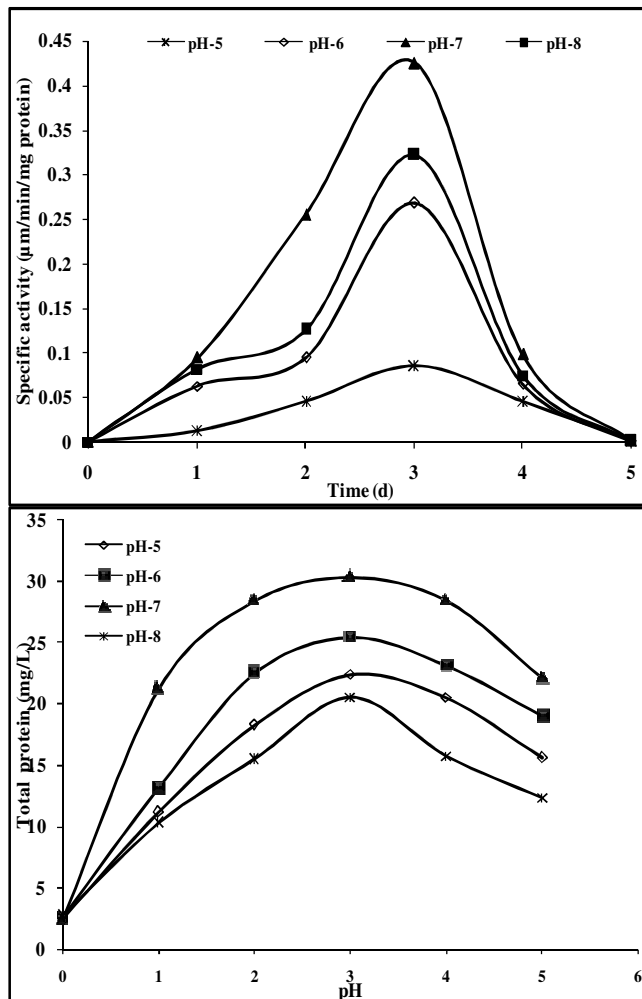


Figure 2. Effect of pH on catechol 1,2 dioxygenase activity by the bacterial consortium during the degradation with phenol

the protein at 10 % NaCl. In present study on phenol degradation by the bacterial consortium showed 0.425 $\mu\text{m}/\text{min}/\text{mg}$ of the protein at the end of 2nd day, which proved that the consortium could produce a higher specific activity, this might be due to the coexistence of different bacterial strains in the consortium.

Dioxygenase activity on 4-Chlorophenol (4-CP)

To detect the presence of dioxygenase activity from the bacterial consortium during the utilization of 4-chlorophenol, experiment was performed at an optimum concentration of 25 mg/L of 4-CP under saline conditions at 50 g/L of NaCl (data not shown). Experiments conducted during the degradation of 4-CP, showed that there was only pronounced catechol 2,3 dioxygenase activity, which showed that the bacterial consortium utilized 4-chlorophenol by meta-cleavage pathway. The

effect of substrate concentration on the activity of catechol 2,3 dioxygenase is demonstrated in the Figure 3. Substrate concentrations were used from 10 mM – 100 mM of 4-chlorocatechol. It was observed from the figure that the highest catechol 2,3 dioxygenase activity of 0.425 $\mu\text{m}/\text{min}/\text{mg}$ of the protein was achieved at 50 mM of 4-chlorocatechol, from 60 mM of the 4-chlorocatechol concentration there was no further increase in the activity of catechol 2,3 dioxygenase. Hence, 50 mM of 4-chlorocatechol was used as the substrate for dioxygenase assay.

Effect of pH on the degradation of 4-CP with catechol 2,3 dioxygenase activity

To study the effect of pH on catechol 2,3 dioxygenase the activity of the enzyme was measured with 50 mM of 4-chlorocatechol as optimum at different pH from 5 to 8.

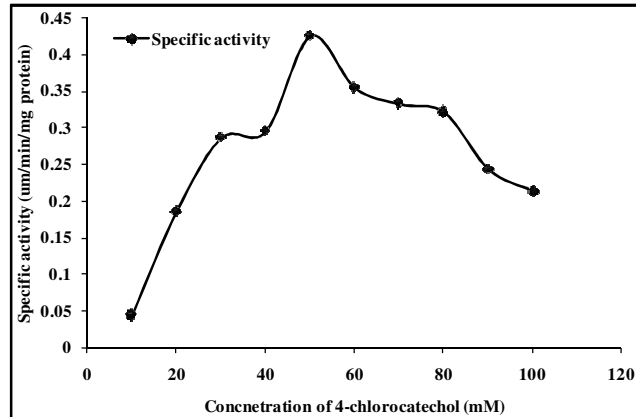


Figure 3. Effect of substrate (4-chlorocatechol) concentration on catechol 2,3 dioxygenase activity

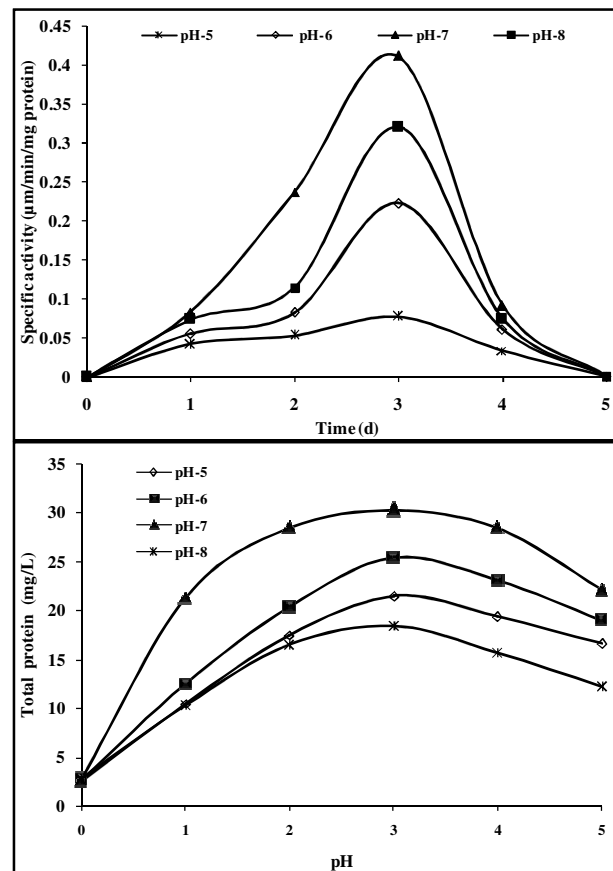


Figure 4. Effect of pH on catechol 2,3 dioxygenase activity by the bacterial consortium during the degradation with 4-CP

The enzyme activity was less ($0.078 \mu\text{m}/\text{min}/\text{mg}$ of protein) at pH 5 at the on 2nd day with total protein of $21.5 \text{ mg}/\text{L}$. However it increased at pH 6 the activity was 0.223 with an increase in protein content to $25.4 \text{ mg}/\text{L}$. The specific activity of catechol 2,3 dioxygenase was maximum at pH 7 which showed highest activity of 0.412

$\mu\text{m}/\text{min}/\text{mg}$ of protein with $30.3 \text{ mg}/\text{L}$ of total protein (Figure 4). Further increase in the pH resulted in a decrease in enzyme activity and at pH 8.0 the enzyme activity was $0.321 \mu\text{m}/\text{min}/\text{mg}$ of protein with $22 \text{ mg}/\text{L}$ of total protein on 3rd day. The optimum pH for the enzymatic action of catechol 2,3 dioxygenase was similar

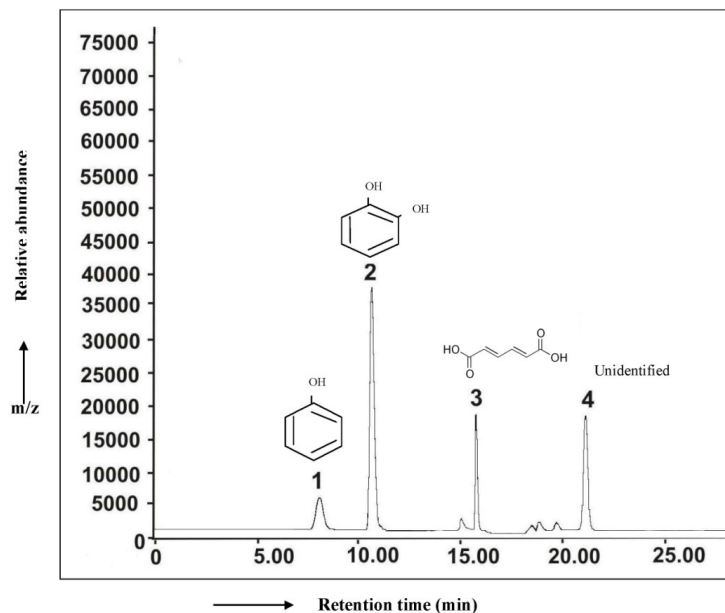


Figure 5. GC-MS Spectrum of the metabolites formed during the degradation of phenol

to that of catechol 1,2 dioxygenase at neutral pH 7 and the activity was retarded at pH 8. This experiment further confirmed that the bacterial consortium was able to degrade the phenols with the intradiol enzymes at neutral pH. The levels of catechol 2,3-dioxygenase activity detected during the degradation of 4-Chlorophenol disappeared only after 4-chlorophenol was almost degraded by the mixed culture. Hollender et al (1997) reported degradation of 1.8 mM 4-CP via the *meta*-cleavage pathway by *Comanomonas testosteroni* JH5, where catechol 2,3 dioxygenase activity was 173 mU/ mg of the protein, while catechol 1,2 dioxygenase activity was less than 1. O' Sullivan (1998) reported the degradation of 4-CP (1.56 mM) by mixed culture containing two *Pseudomonas* species where the degradation took place via the *meta*-cleavage pathway. Farrell and Quilty (1999) reported the degradation of 4-CP via *meta* cleavage pathway, with catechol 2,3 dioxygenase activity of 0.096 $\mu\text{m}/\text{min}/\text{mg}$ of the protein, by a mixed microbial community under non-saline conditions. As shown in the figure, the present study showed that the catechol 2,3 dioxygenase activity was 0.412 $\mu\text{m}/\text{min}/\text{mg}$ of the protein in the log phase of 48 h by the bacterial consortium, which is comparatively higher than reported by earlier workers (Farrell and Quilty 1999, Sahinkaya and Dilek 2005).

Detection of intermediates during the degradation of phenol and 4-chlorophenol

Intermediates formed during the degradation of phenol

(100 mg/L) was analysed by Gas Chromatograph-Mass Spectral (GC-MS) analysis. Analyses of the 72 h supernatant, from the growth of bacterial consortium on phenol, showed the presence of the metabolites. A comparison of the mass spectra of extracted compounds with the standards (phenol and catechol) showed that the peaks in Figure 5 are phenol (peak 1), catechol (peak 2) and *cis, cis*-muconic acid (peak 3), indicating that the phenol degradation by the bacterial consortium followed via *ortho*-cleavage.

The GC-MS chromatogram showed four peaks, first being the parent compound phenol at retention time of 8.35 min; corresponding mass analyses yielded m/z (25,39,55,66,74,94), followed by peak 2 with a retention time of 9.863 min represented catechol, intermediate compound of phenol, with the masses m/z (40,53,64,81,92,110,112,136,151,166,207). At the retention time of 15.925 min peak 3 was observed which represented the *ortho*-cleavage product *cis-cis* muconic acid with m/z (97,137,163), the peak 4 was an unidentified product (Figure 5).

Hinteregger and Streichsbier (1997) studied the degradation of phenol at optimum salinity of 50 g/L where they reported that the disappearance of phenol was accompanied by accumulation of *cis-cis* muconic acid, which is a dead end product of *ortho*-cleavage pathway. Oren et al (1992) reported the same *ortho*-pathway in benzoate degradation by a marine bacterial isolate *Pseudomonas halodurans*, which exhibited a similar behaviour of tolerating increased salt concentrations from 17 g/L NaCl to 150 g/L in synthetic sea water, but there was no further intermediate products in the *ortho*-

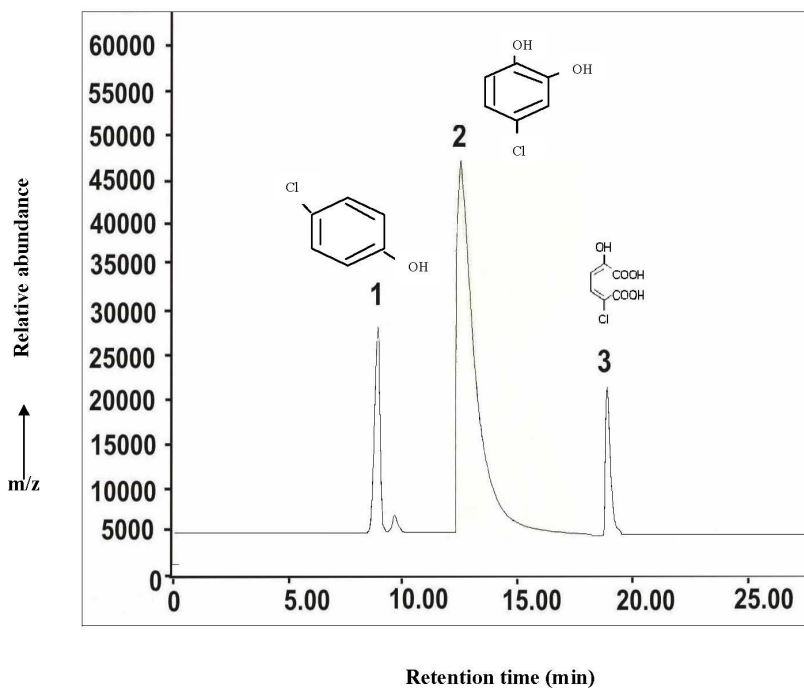


Figure 6. GC-MS Spectrum of the metabolites formed during degradation of 4-CP

cleavage pathway. Many researchers have reported the production of catechol and *cis-cis* muconic acid as intermediates of *ortho* pathway during the degradation of phenol under non-saline conditions (Müller and Babel 1996, Bastos et al 2000b, Tsai et al 2005, Alva and Peyton 2003). In the present study formation of *cis-cis* muconic acid as the metabolite during the degradation suggested that the degradation of phenol was by *ortho*-cleavage pathway by the bacterial consortium under saline conditions.

To identify the metabolites from the degradation of 4-CP, 72 h culture supernatant were analysed in GC-MS. The mass spectra were compared with the standards (4-CP, 4-Chlorocatechol), which showed that the peaks as shown in Figure 6.

The chromatogram showed three peaks, first was the parent compound 4-CP at retention time of 8.92 min; corresponding mass analyses yielded m/z (26,39,50,65,73,92 99.8,100,128). The second peak formed at the retention time of 14.131 represented the intermediate compound 4-Chlorocatechol (40,51,63,83,87,98,115,126,144,170,185,200,259,274) and peak three was the final degraded product of 4-CP, 5-chloro-2-hydroxymuconate at the retention time of 17.25 with the following masses (48,63,75,83,97,114,131,158,174,184,191,199). The structures of the compounds are represented on top of the peak in Figure 6. During the degradation of 4-CP, the degradation pathway is initiated by the hydroxylation of 4-CP to the corresponding 4-

chlorocatechol. The 4-chlorocatechol then undergoes a *meta*-ring cleavage by catechol 2,3 dioxygenase to produce 5-chloro-2-hydroxymuconic semialdehyde, which in turn is converted to 5-chloro-2-hydroxymuconate (El-Sayed et al 2009). In the present study, the GC-MS analysis of the degradation of 4-CP, showed that the key intermediate 5-chloro-2-hydroxymuconic semialdehyde was formed this suggest that the consortium followed *meta*-cleavage pathway during the degradation. Activated aromatic compounds undergo ring cleavage reactions via lower pathway and are further processed to give molecules that can eventually enter the tricarboxylic acid cycle (Cafaro et al 2004). Degradation of 4-CP by pure and mixed culture and the metabolites formed during the degradation was reported by many other researchers (Hollender et al 1997, Farrell and Quilty 1999, Yang and Lee 2008). Nordin et al (2005) reported *A. chlorophenolicus* A6 degraded 4-CP via hydroxyquinol. Moreover, hydroxyquinol was removed from cell extracts derived from 4-CP-grown cells but not from extracts of cells grown on succinate. El Sayed et al (2009) reported that degradation of 4-CP by production of the 4-chlorocatechol and 5-chlorohydroxymuconic acid as intermediates of *meta*-cleavage pathway by *Bacillus subtilis* OS1, while the cell free extracts of *Alcaligenes* OS2 showed modified *meta*-cleavage pathway. According to the results on the dioxygenase enzyme activity, it can be concluded that the bacterial consortium followed *ortho*-cleavage pathway during the degradation of phenol and *meta*-cleavage pathway during the

degradation of 4-CP.

REFERENCES

- Alva V, Peyton BM (2003). Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity, Environ. Sci. Technol. 37: 4397-4402.
- Bastos AER, Moon DH, Rossi A, Trevors JT, Tsai SM (2000b). Salt tolerant phenol-degrading microorganisms isolated from Amazon soil samples. Arch. Microbiol. 174: 346-352.
- Bradford MM (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
- Briganti F, Pessione E, Giunta C, Scozzafava A (1997). Purification, biochemical properties and substrate specificity of a catechol 1,2-dioxygenase from a phenol degrading *Acinetobacter radioresistens*. FEBS Letters 416: 61-64.
- Cafaro VV, Izzo R, Scognamiglio E, Notomista P, Capasso A, Casbarra P, Pucci, Donato AD, (2004) Phenol hydroxylase and toluene/o-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. Appl. Environ. Microbiol. 70: 2211-2219.
- Dua M, Singh A, Sethunathan N, Johri AK (2002). Biotechnology and bioremediation: Successes and limitations. Appl. Microbiol. Biotechnol. 59: 143-152.
- El-sayed WS, Ismaeil M, El-Beih F (2009) Isolation of 4-Chlorophenol-Degrading Bacteria, *Bacillus subtilis* OS1 and *Alcaligenes* sp. OS2 from Petroleum Oil-Contaminated Soil and Characterization of its Catabolic Pathway. *Aus. J. Basic. Appl. Sci.* 3 (2): 776-783.
- Farrell A, Quilty B (1999) Degradation of mono-chlorophenols by a mixed microbial community via a meta-cleavage pathway. *Biodegradation* 10: 353-362.
- García MT, Ventosa A, Mellado E (2005). Catabolic versatility of aromatic compound-degrading halophilic bacteria. *FEMS Microbiol. Ecol.* 54: 97-109.
- Haggblom MM, Nohynek LJ, Salkinojalonen MS (1998b). Degradation and o-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. *Appl. Environ. Microbiol.* 54: 3043-3052.
- Harayama S, Timmis KN (1992). Aerobic biodegradation of aromatic hydrocarbons by bacteria, In: Sigel H. and Sigel A. (eds.), *Metal ions in biological systems, Degradation of environmental pollutants by microorganisms and their metalloenzymes*, Marcel Dekker Inc., New York, 28: 99-165.
- Hinteregger C, Streichsber F (1997) *Halomonas* sp., a moderately halophilic strain, for biotreatment of saline phenolic wastewater. *Biotechnol. Lett.* 19 (11): 1099-1102.
- Hollender J, Hopp J, Dott W (1997) Degradation of 4-chlorophenol via the meta-cleavage pathway by *Comomonas testosteroni* JH5. *Appl. Environ. Microbiol.* 63: 4567-4572.
- Karigar C, Mahesh A, Nagenahalli M, Yun DJ (2006). Phenol degradation by immobilized cells of *Arthrobacter citreus*, *Biodegradation* 17: 47-55.
- Kiesel B, Muller RH (2002). The meta Pathway as a Potential Energy-Generating Sequence and its Effects on the Growth Rate during the Utilisation of Aromatics. *Acta.Biotechnol.* 3-4: 221-234.
- Lovely DR (2003) Cleaning up with genomics: applying molecular biology of self-bioremediation, *Nat. Rev. Microbiol.* 1: 35-44.
- Müller RH, Babel W (1996). Growth rate-dependent expression of phenol assimilation pathways in *Alcaligenes eutrophus* JMP 134-the influence of formate as an auxiliary energy source on phenol conversion characteristics. *Appl. Microbiol. Biotechnol.* 46 (2):156-162.
- Munoz JA, Perez-Esteban B, Esteban M, de la Escalera S, Gomez MA, Marti'nez-Toledo, MV, Gonzalez-Lopez J (2001). Growth of moderately halophilic bacteria isolated from sea water using phenol as the sole carbon source. *Folia Microbiol (Praha)*, 46: 297-302.
- Ngai KL, Neidle EL, Ornston LN, (1990) Catechol and chlorocatechol 1,2-dioxygenase, In: Lidstrom M.E. (eds.), *Hydrocarbon and methylotrophy*, Meth. Enzymol. 188: 122-126.
- Nordin K, Unell M, Jansson KJ (2005). Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6, *Appl. Environ. Microbiol.*, 71: 6538-6544.
- O' Sullivan M (1998). The degradation of phenol and monochlorophenols by a mixed microbial population, Ph.D. Thesis, Dublin City University.
- Oren A, Gurevich P, Azachi M, Henis Y (1992). Microbial degradation of pollutants at high salt concentrations. *Biodegradation*, 3, :387-398.
- Powlowski J, Shingler V (1994), Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF600. *Biodegradation* 5: 219-236.
- Prpich GP, Daugulis AJ (2005). Enhanced biodegradation of phenol by a microbial consortium in a solid-liquid two-phase partitioning bioreactor. *Biodegradation*, 16: 329-339.
- Sahinkaya E, Dilek FB (2005) Biodegradation of 4-Chlorophenol by acclimated and unacclimated activated sludge—evaluation of biokinetic coefficients. *Environ. Res.* 99: 243-252.
- Tsai S C, Tsai LD, Li YK (2005). An isolated *Candida albicans* TL3 capable of degrading phenol at large concentration. *Biosci. Biotechnol. Biochem.* 69 (12): 2358-2367.
- Veena GK, Vasudevan N (2010). Enrichment and Isolation of Phenol degrading Moderately Halophilic Bacterial Consortium from Saline Environment. *J. Biorem. Biodeg.* Vol 1 (1) 1-6.
- Wackett LP (2000). Environmental biotechnology Trends *Biotechnol.* 18:19-21.
- Watanabe K (2001) Microorganisms relevant to bioremediation. *Curr. Opin. Biotechnol.* 12: 237-241.
- Yang CF, Lee CM (2008). Enrichment, isolation, and characterisation of 4-chlorophenol- degrading bacterium *Rhizobium* sp. 4-CP-20. *Biodegradation*, 19: 329-336.