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Biodegradation of Phenol in Batch Culture by Pure and Mixed Strains of *Paenibacillus* sp. and *Bacillus cereus*

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Abstract

The bacterial strains, *Paenibacillus* sp. (AY952466) and *Bacillus cereus* (DQ002384), have proven capacity to degrade lignin and pentachlorophenol. In the present study, both strains were screened at different concentrations of phenol on mineral salt agar medium in the presence of glucose. At optimized condition (pH 7.5 ± 0.2, 37 ± 1°C, 120 rpm, 1% glucose w/v), it was observed that both *Paenibacillus* sp., *B. cereus* and its mixed culture degraded phenol (500 mg/l) up to 53.86%, 91.63% and 67.76% within 168 h of incubation, respectively. Phenol degradation was routinely monitored spectrophotometrically and further confirmed by HPLC. Catechol and 2-hydroxy muconic semialdehyde were identified as intermediate products from degraded samples using GC-MS. It was also observed that, in the absence of glucose, bacterial strains were unable to utilize phenol indicating the phenomenon of co-metabolism.

Key words: degradation, environmental condition, mixed culture, phenol

Introduction

Phenol, a compound regarded as a priority contaminant by the U.S. Environmental Protection Agency (Keith and Telliand, 1979; Sung *et al.*, 2000), is a characteristic pollutant in effluents (containing 5–500 mg/l or higher) from crude oil, coal conversion processes and pulp paper mill has also been detected recently in river water (Paxéus, 1996; Chandra *et al.*, 2006; Singh *et al.*, 2008). The extensive use of phenol has led to a widespread contamination of soil, groundwater and this affects living organisms owing to the toxicity of the compound. Good solubility of phenol in water and its high content in sewage water testing shows the high probability of phenol acting as a water pollutant, deteriorating the organoleptic qualities of water. A 1.0 µg/ml and higher concentration of phenol inhibited the photosynthesis of diatoms and blue green algae. Phenol concentrations in the range of 100–400 µg/ml caused complete inhibition of photosynthesis (Kostyeav, 1973).

In treating phenolic compounds, the biological method has attracted more attention than physical and chemical methods because many different types of microorganisms are known to utilize phenol as their sole carbon and energy source (Monteiro *et al.*, 2000; Begoňa Prieto *et al.*, 2002; El-Sayed *et al.*, 2003). Moreover, biodegradation may be preferred due to lower costs and the possibility of complete mineralization.

The literature on phenol biodegradation by mixed or pure cultures reports that many bacteria but likely not all of them metabolize phenol (Teixeira and Mata, 1992; Monteiro, 1998). Pseudomonads are the most widely distributed bacteria known for the biodegradation of phenolic compounds (Allsop *et al.*, 1993; Bandhyopadhyay *et al.*, 2001; Sa and Boaventura, 2001; Annadurai *et al.*, 2002). In addition to this, different researchers have reported phenol-degrading microorganisms (bacteria) from the natural environment *i.e. Thermophyllite bacilli* (Alexeyev, 1973), *Bacillus stearothermoleovorans* (Mutzel *et al.*, 1996).

Lignin, pentachlorophenol (PCP) and phenol are major environmental pollutant discharged from pulp and paper mills. Phenol as monophenols from lignin are the major part of cell wall of plant converted into chlorophenolic compounds during the bleaching process in the paper pulp industry. Among these various chlorophenols, PCP is the most abundant and toxic (Singh *et al.*, 2008). So, there should be priority in

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degrading phenol before it converts to its higher toxic form PCP. In our previous studies, we successfully degraded kraft lignin and PCP using *Paenibacillus* sp. and *Bacillus cereus* (Chandra *et al.*, 2006; Chandra *et al.*, 2007). In the present study, we are investigating the degradation of phenol by pure and mixed bacterial strains *Paenibacillus* sp. and *B. cereus*.

Experimental

Materials and Methods

Chemicals. The chemicals used for preparation of media were procured from HiMedia, India. All reagents used were of analytical grade. Phenol, Catechol and 2-hydroxy muconic semialdehyde (2-HMS) were purchased from Sigma, Germany. All solutions were prepared in Milli-Q water (Milli-Q Ultrapure Water Purification System).

Origin of strains. *Paenibacillus* sp. (AY952466) and *B. cereus* (DQ002384) were isolated from pulp paper mill effluent sludge and were identified based on biochemical and 16S rRNA gene sequencing (Chandra *et al.*, 2006; Chandra *et al.*, 2007).

Screening for phenol tolerance. Screening of phenol tolerance was done using nutrient enrichment technique in mineral salt medium (MSM) of following composition in g/l: Na_2PO_4, 1.6; KH_2PO_4, 0.4; NH_4NO_3, 0.5; $MgSO_4 \times 7H_2O$, 0.2 (autoclaved separately); $CaCl_2$, 0.025; $FeCl_3$, 0.0025 and purified agar, 18 at pH 7.5±0.2 amended with different concentrations of phenol (100, 200, 300, 400, 500, 600 mg/l) by dissolving in 0.1% ethanol along with and without 1% glucose (w/v) as additional carbon source (autoclaved separately), *Paenibacillus* sp. and *B. cereus* was plated onto MSM agar plate. The plates were incubated at 37±1°C for 5 days. On the basis of their phenol tolerance, 500 mg/l were selected for further degradation studies.

Biodegradation studies. The degradation studies were performed by inoculating 1% inoculum of *Paenibacillus* sp. (2.5×10^3 CFU/ml), *B. cereus* (2.7×10^3 CFU/ml) and its mixed culture (2.3×10^3 CFU/ml) into 250 ml Erlenmeyer shake flask containing 99 ml MSM supplemented with 500 mg/l phenol at pH 7.5 ± 0.2 in presence of 1% (w/v) glucose as carbon source, incubated at 37 ± 1°C in a refrigerated incubator shaker at 120 rpm up to 168 h. The stability of pure and mixed culture in experimental and control (without inoculum) was optimized at different environmental conditions (*i.e.* temperature, pH and aeration) to ensure that the maximum disappearance of phenol was occurred. The culture samples (5 ml) were taken under aseptic conditions.

Growth of bacterial cells was determined by measuring absorbance (620 nm) at 24 h interval. In order to estimate the phenol concentration, cells were centrifuged at 5000×*g* for 20 min (Remi C-24, India) at 4°C to separate the biomass and the supernatant was collected. Simultaneously, dried biomass was determined during phenol removal at every 24 h interval up to 168 h incubation and was compared to that of the control.

The quantification of phenol was done using the colorimetric method of Martin (1949). Phenol reacts with 4-aminoantipyrin (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-on) and forms a red indophenol dye under alkaline conditions. This dye was measured spectrophotometrically at a wavelength of 460 nm by UV-Vis spectrophotometer (GBC Cintra-40, Australia) at every 24 h interval up to 168 h incubation. The degraded phenol was quantified by comparing respective standard curve of phenol (0–500 mg/l). Simultaneously, degraded samples were scanned between 300–700 nm as compared to control sample.

The pH of the medium was measured by ion analyzer (model 960, Thermo Orion) using calibrated selective electrode (9172 BN). Dissolved oxygen (D.O.) was measured as partial oxygen pressure using a Clark-type polarographic DO probe model-835A of Thermo Orion according to the manufacture's instructions (detection level, 0.1 mg/l). All experiments were carried out in triplicate; simultaneously a control was also performed in triplicates. The values were presented as mean ± SD (n = 3).

HPLC analysis. For phenol estimation, 1.5 ml of pure and mixed culture medium (from degraded and control flask after 168 h incubation) was transferred in Eppendorf tubes and centrifuged at 12,000×g at 4°C for 10 min, discard the pellet and supernatant was collected for phenol estimation. Phenol was determined using HPLC system (Model 515 pump, Waters, USA) equipped with UV-Vis (Model 2487, Waters, Australia) detector operating at 270 nm. Samples were injected with loop (20 µl) equipped with pump controller module using millennium power software ($M\text{-}32^R$). For phenol, the sample was analyzed using a symmetry C-18 column (4.6×150 mm, 5 µm particle size) obtained from Waters. The mobile phase was composed of 55% (v/v) distilled water and 45% (v/v) acetonitrile and flow rate was set at 1 ml/min. Percentage of degradation was calculated by comparing peak area of the degraded and control sample (Geng *et al.*, 2006).

GC-MS analysis. The same extracted sample given in above section were used for GC-MS analysis for qualitative estimation of phenol and its metabolites degraded by pure and mixed culture at 168 h incubation period along with experimental control. For GC-MS analysis, 2 µl of control and degraded sample by pure and mixed culture were injected in GC-MS equipped with a splitless injector and a PE Auto sys-

tem XL gas chromatograph interfaced with a Turbomass spectrometric mass selective detector system were used. The MS was operated in the EI mode (70 eV). Helium was employed as the carrier gas and its flow rate was adjusted to 1 ml/min. The analytical column connected to the system was a HP-5 capillary column (length-30m × 0.20mm, 0.11 mm film thickness). The column head pressure was adjusted to 12 psi. The GC column temperature was programmed at 250°C. A solvent delay of 3 min was selected. The injector temperature was set at 250°C. The GC-MS interface was maintained at 250°C. The oven program was 70°C–1°C/min-100°C. The MS was operated in the total ion current (TIC) mode, scanning from m/z 30 to 400. In the full scan mode, electron ionization (E1) mass spectra in the range of 30–400 (m/z) were recorded at electron energy of 70 eV. The metabolic intermediates were derived from phenol degradation identified by comparing their retention time (RT in min) and mass spectra with library of the National Institute of Standard and Technology (NIST), USA or by comparing the RT with those of authentic standards available.

Results and Discussion

Screening for phenol tolerance. *Paenibacillus* sp. and *B. cereus* showed luxuriant growth on phenol amended MSM agar in presence of glucose 1% (w/v), whereas no growth was observed in absence of glucose. The strains showed fast and luxuriant growth at various concentrations of phenol (100–500 mg/l) however, at 600 mg/l phenol, no growth was observed. Phenol has a potentially inhibitory effect on cell growth, *i.e.*, if the concentration of phenol in the medium is high enough it will cause substrate inhibition (Yang and Lee, 2007; Molin and Nilsson, 1985).

Degradation of phenol by pure and mixed culture. Both strains used for biodegradation study were pre-identified as *Paenibacillus* sp. and *B. cereus* based on biochemical and 16S rRNA gene sequencing in our previous works (Chandra *et al.*, 2006; Chandra *et al.*, 2007). When the degradation assay was carried out in MSM by pure and mixed culture at pH 7.5±0.2 in batch shake flasks at 37±1°C, 120 rpm a marked increase in optical density at 620 nm revealed that growth initially showed log phase then reached maximum at 72 h for *B. cereus*, and then decline phase as shown in (Fig. 1a), simultaneously there is a concomitant increase in biomass. Simultaneously, small lag phase up to 24 h were observed, growth reached maximum at 48–72 h for *Paenibacillus* sp. and mixed culture and then decline phase was reached (Fig. 1a). In addition, no growth and degradation was observed in control sample during experiment.

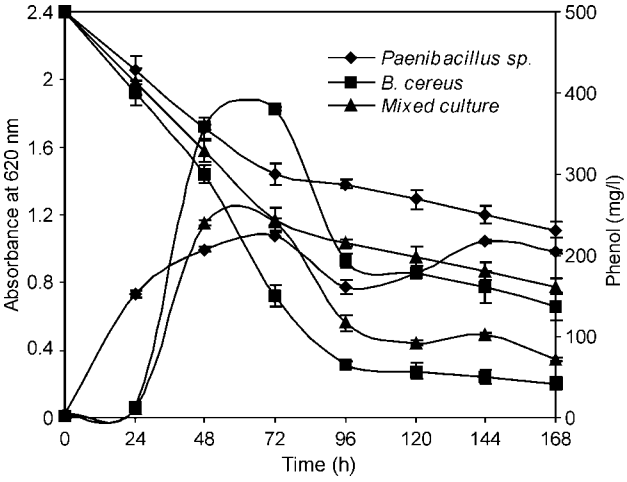

Fig. 1a. Growth curve and degradation of phenol in 1% glucose containing MSM in the presence 500 mg/l of phenol at optimized condition (pH 7.5±0.2, 37±1°C, 120 rpm).

(◆–◆) indicates absorbance at 620 nm and phenol concentration of *Paenibacillus* sp., (■–■) indicates absorbance at 620 nm and phenol concentration of *B. cereus* and (▲–▲) indicates absorbance at 620 nm and phenol concentration of their mixed culture. All the experiments in this study were performed in triplicate and values are the mean of three replicates.

The rate of phenol degradation was not favored by the variation at different environmental conditions when compared to the optimized condition as shown in Figure 1c. Alteration of optimized condition might be inhibitory to the activity of the enzymes responsible for phenol degradation in bacteria. The degradation of phenol (500 mg/l) was observed by *Paenibacillus* sp. (53.86%), *B. cereus* (91.63%) and mixed culture (67.76%) as compared to control respectively,

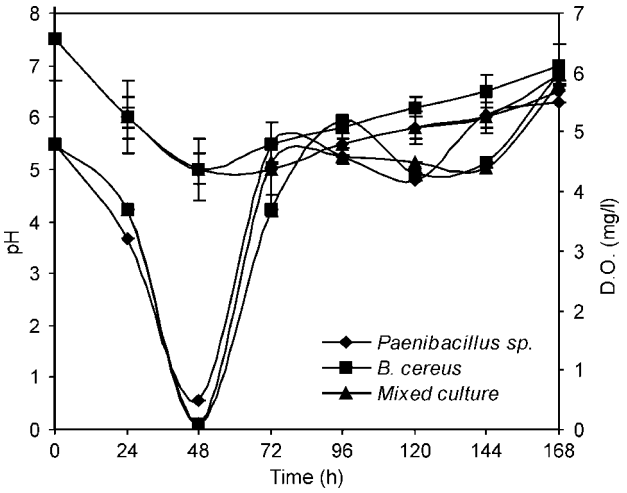

Fig. 1b. Determination of pH and D.O. during phenol degradation in 1% glucose containing MSM in the presence 500 mg/l of phenol at optimized condition (pH 7.5±0.2, 37±1°C, 120 rpm).

(◆–◆) indicates pH and D.O. during phenol degradation of *Paenibacillus* sp., (■–■) indicates pH and D.O. during phenol degradation of *B. cereus* and (▲–▲) indicates pH and D.O. during phenol degradation of their mixed culture.

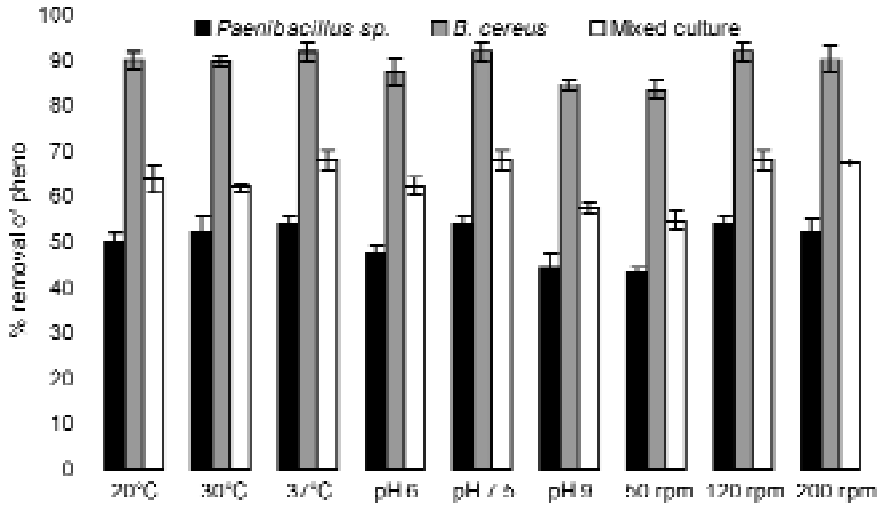

Fig. 1c. Effect of temperature (20, 30 and 37°C) , pH (6, 7.5 and 9) and aeration rate (50, 120 and 200 rpm) on % removal of phenol in MSM in the presence of (phenol-500 mg/l) degraded by *Paenibacillus* sp., *B. cereus* and their mixed culture at 168 h.

within 168 h incubation at optimized condition (pH 7.5±0.2, 37±1°C and 120 rpm) in presence of glucose as showed in Figure 1a. Thus, this phenomenon is glucose dependent and a result of co-metabolism process. In a similar study, Monteiro *et al.* (2000) observed that glucose was utilized initially followed by phenol degradation.

The above data were supported by scanning of degraded phenol sample at 168 h incubation (data not shown). *B. cereus* was found most effective for phenol degradation as compared to *Paenibacillus* sp. and mixed culture. Whereas, in mixed culture, *Paenibacillus* sp. and *B. cereus* antagonized each other, thus a suppress degradation of phenol was observed (Fig.1a). In another study, Gallego *et al.* (2003) observed competition between bacterial strains for the removal of substrate in a batch reactor.

The phenol degradation by pure and mixed culture showed that there is decrease of pH from 7.5 to 5 at 48–72 h and further, the pH value increased up to neutral (Fig. 1b) concomitantly D.O. of the media were changes from 4.8 to 0.5 mg/l for *Paenibacillus* sp., 0.1 mg/l for *B. cereus* and 0.1 mg/l for mixed culture (Fig. 1b) respectively at 48 h as compared to the control. It was observed that the cell density increased with the decrease in substrate concentration.

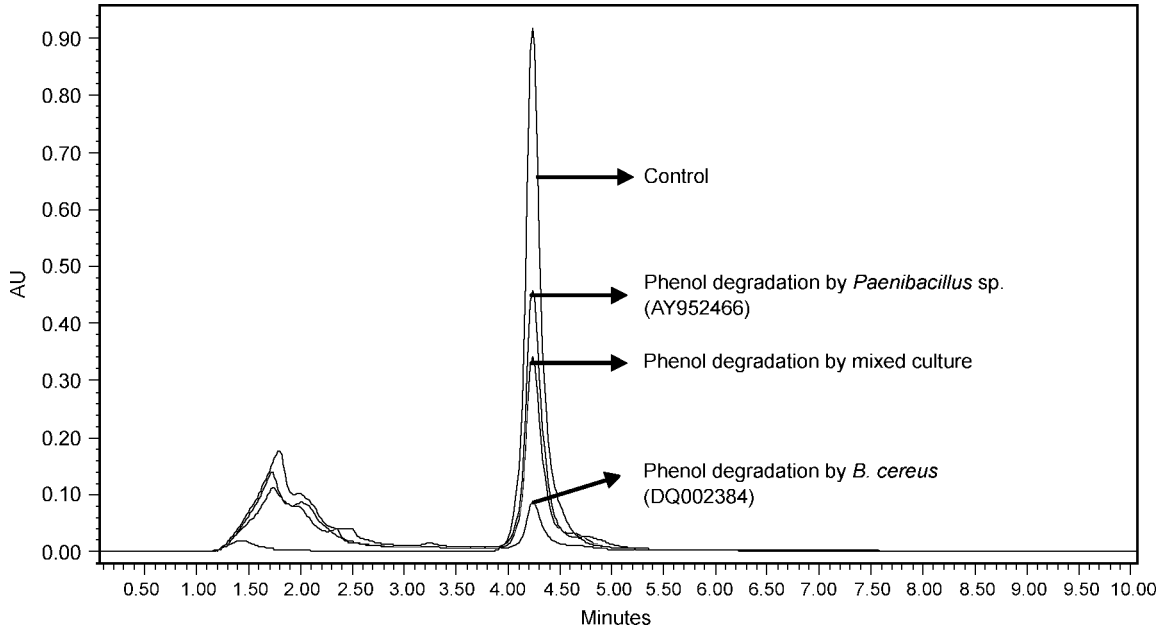

Fig. 2. Comparative HPLC chromatogram of phenol degrading pure [*Paenibacillus* sp. and *B. cereus*] and their mixed culture compared with control at 168 h at optimized condition.

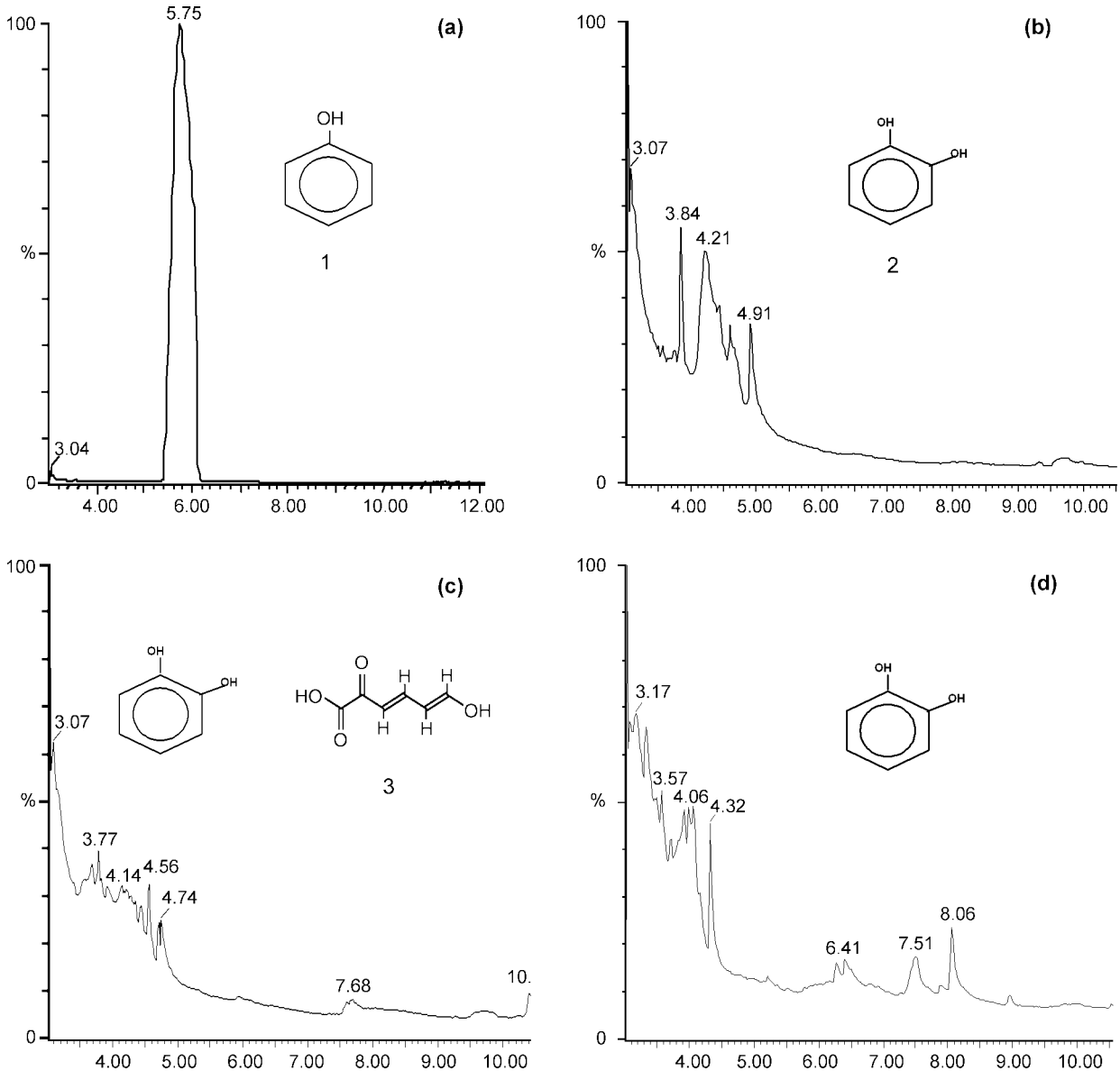

Fig. 3 (a-d). GC-MS chromatograms of phenol and its intermediate products after extraction at 168 h (a) control, (b) *Paenibacillus* sp., (c) *B. cereus* and (d) mixed culture at 168 h incubation period. Abbreviations 1. Phenol, 2. Catechol and 3. 2-HMS.

The biomass growth directly related to the utilizing of the carbon source was one of the important factors to be studied during biodegradation. After the exponential growth phase, the pH increases up to neutral. Similar results have been reported by Lallai *et al.* (1988) and Monteiro *et al.* (2000).

In all experiments, the incubation mixtures were kept in cotton-plugged shake flasks. Under these conditions, atmospheric oxygen is admitted to the system. As degradation was not apparently affected by low O_2 concentration, inhibition is more likely to be a metabolism dependent event. The decrease of D.O. may be attributed to glucose fermentation, contribute to decrease its concentration in the medium (Ramalho *et al.*, 2004).

The degradation of phenol was further monitored by HPLC. A peak of phenol was observed in control sample (retention time of 4.235 min). Degraded samples showed a small peak of phenol after 168 h as compared to control at 4.237 min by *Paenibacillus* sp., 4.241 min by *B. cereus* and 4.236 min by mixed culture (Fig. 2) with additional peaks at 3.243 min by *Paenibacillus* sp., 3.201 min by *B. cereus* and 3.206 min by mixed culture of catechol. 2-HMS peaks were observed at 3.702 min by *B. cereus*. This observation correlated well with the biotransformation of phenol. A comparative HPLC chromatogram of phenol degradation by pure and mixed culture is shown in Figure 2.

At 168 hr incubation, 230.70, 42.0 and 161.20 mg/l of phenol concentrations were remained in samples degraded by *Paenibacillus* sp., *B. cereus* and mixed culture, respectively. Moreover, 2.53, 1.43 and 1.03 mg/l of catechol concentration were present in samples

of *Paenibacillus* sp., *B. cereus* and mixed culture, respectively. Whereas, 1.28 mg/l of 2-HMS was present in the sample degraded by *B. cereus*.

Furthermore, there is formation of yellow colour intermediate of 2-HMS in the phenol catabolism, accumulated in flask media by bacterial action (figure not reported) might be largely attributed to intermediate product formation by enzyme action. The additional peaks found during HPLC analysis confirmed it. A similar finding was reported by Molin and Nilsson (1985). *P. putida* ATCC 11172 degraded phenol by the meta cleavage pathway (Clarke and Ornston, 1975), which was indicated by the accumulation of 2-HMA.

GC-MS analysis. GC-MS analysis of degraded sample by pure and mixed culture in extractable products showed that in control sample, only phenol (RT = 5.75 min) is identified (Fig. 3a) whereas, in degraded sample different intermediates were identified using the NIST mass spectral database. Catechol (RT = 3.07 min) along with some unknown peaks at RT 3.84, 4.21 and 4.91 min were observed in degraded sample by *Paenibacillus* sp. at 168 h (Fig. 3b). Similarly catechol (RT = 3.07 min), 2-HMS (RT = 7.68 min) and some unknown peaks at RT 3.77, 4.14, 4.56, 4.74, 10.41 min were observed in degraded sample by *B. cereus* as showed in Figure 3c. Catechol (RT = 3.07 min) and unknown peaks at RT 3.17, 3.57, 4.06, 4.32, 6.41, 7.51 and 8.06 min were observed in degraded sample by mixed culture (Fig. 3d). The results show that the phenol was degraded into catechol, 2-HMS and some unknown intermediates. Catechol and 2-HMS, the two known key catabolic intermediate of phenol dissimilation are less toxic then its parental compound (Gibson and Subramanian, 1984) and can be widely used in manufacturing novel pharmaceuticals, agricultural chemicals, food additives and even synthetic plastics (Molin and Nilsson, 1985; Shirai, 1986). These factors indicate that *Paenibacillus* sp. and *B. cereus* metabolized phenol using the meta-pathway, which is controlled by two key enzymes. Phenol hydroxylase converts phenol into catechol, which is simultaneously transformed into 2-HMS by the meta-ring cleavage activity of catechol 2,3-dioxygenase enzyme (Hughes *et al.*, 1984). *B. cereus* was most potential aerobic phenol degrading bacterial strain then *Paenibacillus* sp. Therefore, *B. cereus*, in particular, *Paenibacillus* sp., can be used, in general, for phenol degradation. Moreover, these bacterial strains can be used for degradation of phenol, PCP and lignin containing industrial effluent.

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