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Degradation of 4-Chlorophenol by a Moderately Halophilic Bacterial Consortium under Saline Conditions

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Authors' contributions

This work was carried out in collaboration between both authors. This work was carried out in Environmental Microbiology Laboratory author KV performed the literature search, designed and executed the experiments and wrote the manuscript. Author NV supervised the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To investigate the degradation of 4-chlorophenol by a moderately halophilic bacterial consortium and to study the effect of nitrogen sources on the degradation of 4-chlorophenol.
Study Design: 4-chlorophenol degradation by moderate halophiles.
Place and Duration of Study: Centre for Environmental studies, Microbiology Lab, Anna University, Chennai, Tamil Nadu, between Jan 2009 and May 2009.
Methodology: 4-chlorophenol was degraded with the moderately halophilic bacterial consortium in the minerals salts medium and degradation was analysed by gas chromatography. Effect of NaCl concentrations and alternate nitrogen sources on the degradation of 4-chlorophenol was determined.
Results: The bacterial consortium was able to degrade 4-Chlorophenol at a range of NaCl concentrations, where the optimum degradation was obtained at 50 g/L of NaCl. Addition of alternate nitrogen sources like tryptone and urea did not enhance the degradation of 4-Chlorophenol.
Conclusion: Bacterial consortium degraded 4-chlorophenol at optimum NaCl concentration of 50 g/L of NaCl. Addition of yeast extract as nitrogen source showed higher degradation

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than the nitrogen sources.

Keywords: Biodegradation; 4-Chlorophenol; halophilic bacterial consortium; saline environment.

1. INTRODUCTION

Chlorophenols are the most commonly encountered chloroaromatics and widely used as biocides, flame retardants, wood treatment agents, solvents and in synthetic chemistry. Significant amounts of chlorophenols can be formed and subsequently released into the environment as by-products of chlorine bleaching process in the pulp and paper industry [1], the chlorination of waste-water and drinking-water, and the incineration of municipal waste [2]. Most of chlorinated phenolic compounds are resistant to microbial degradation in sewage plants and accumulate in soil and water after they have fulfilled their useful function. In fact, chlorophenols are also found in drinking waters as a result of chlorination in water treatment facilities. Chlorophenols are toxic for a wide range of organisms [3], a property that accounts for many of their uses as antiseptics and disinfectants [4]. Due to their high toxicity, mutagenic and carcinogenic properties, chlorophenols are considered to be priority pollutants, both by the Unites States Environmental Protection Agency (USEPA) and the European Union (EU).

The misuse, accidental spillage and improper disposal of these toxic compounds have resulted in intensive pollution [5]. In water, chlorophenols sorbs onto particulate material and, if not degraded, eventually end up in sediments. The annual global demand for chlorophenols is ~100 kilo tonnes [6], and the global production of higher and lower chlorinated phenols is ~25-30 kilo tonnes and ~60 kilo tonnes, respectively [7]. In treating chlorophenols, the biological method has attracted more attention than physical and chemical methods because of relative inexpensive cost and less secondary pollution [8]. Conventional microorganisms cannot survive under the saline conditions. There is now significant interest in the physiological capabilities and uses of extreme conditions of pH, temperature, salinity and pressure [9].

Halophiles are group of extremophiles that not only thrive in but requires salinity for their growth. Halophiles, thrive at high salt concentrations of at least 0.2 M [9, 10, 11,12]. Moderate halophiles grow optimally between 30 and 150 g/L NaCl, while extreme halophiles thrive above 150 g/L NaCl [9]. These halophiles grow optimally, in media containing between 3 % and 15 % NaCl [13] and constitute a very interesting group of microorganisms with great potential for use in biotechnology.

Biodegradation of chlorophenols have been extensively studied, under non–halophilic conditions, and there are reports that several micro-organisms (fungi and mainly bacteria) are able to degrade these compounds, especially strains of the genera *Alcaligenes* [14], *Ralstoni* [15] and *Burkholderia* [16], *Arthrobacter ureafaciens* CPR706 [17], *Arthrobacter* ,[18] *Arthrobacter chlorophenolicus* A6 [19]. But there is no report on the degradation of chlorophenols by bacterial consortium under saline conditions.

The present study investigates the degradation of 4-chlorophenol (4-CP) by the moderately halophilic bacterial consortium under saline conditions. The bacterial consortium was studied for its ability to degrade 4-chlorophenol (4-CP) at different NaCl concentrations and was also

studied by using alternate nitrogen sources and additional salts. Further, the intermediates formed during the degradation of 4-chlorophenol (4-CP) was studied to detect the metabolic pathway followed by the bacterial consortium.

2. MATERIALS AND METHODS

2.1 Bacterial consortium and culture preparation

Bacterial consortium isolated from saline environment was initially adapted and enriched with phenol as the sole carbon source. There were about six strains of which four strains was gram positive and two strains were gram negative. Bacterial consortium was grown with 4-chlorophenol (4-CP) from 25 mg/L to 100 mg/L, with the mineral salts medium of (g/L) NaCl 50.0, KH_2PO_4 0.25, NH_4Cl 1.0, Na_2BO_7 2.0, $FeCl_3$ 0.0125, $CaCl_2$ 0.06 and $MgCl_2$ 0.05 with 10 mg/L of yeast extract, adjusted to pH -7 and distilled water – 1L [20]. The medium was autoclaved, cooled to room temperature and was amended with 4-chlorophenol through a sterile filter (0.45 µm) in 250 ml Erlenmeyer flasks. NaCl concentrations were varied as 30 g/L, 50 g/L and 70 g/L in the mineral salts medium. The chemicals and reagents (Analar grade) used in the study were purchased from Merck, India.

2.2 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 were measured at 595 nm. The total protein concentration was determined by calibration with bovine serum albumin standard [21].

2.3 Degradation of 4-chlorophenol (4-CP)

For the degradation study, mineral salts medium containing 4-CP was inoculated with the bacterial consortium. Different conditions used for the degradation of 4-CP were (i) medium + 4-CP + bacterial consortium; (ii) medium + 4-CP and (iii) medium + bacterial consortium, with (ii) and (iii) serving as controls. The bacterial consortium was added to the medium at concentrations of 10^4–10^5 cfu/mL. The culture was incubated at 37 ºC with shaking at 150 rpm and extracted every 24 h interval for 5 days.

Increase in protein yield was taken as a confirmation of the ability of the consortium for utilization of 4-CP. 4-CP was added to the medium at different concentrations (25 mg/L to 100 mg/L). Cell morphology and the motility of cells were examined by light microscopy. Alternatively the cells were also plated on nutrient agar and viable cell count (cfu/mL) was counted. Each culture flasks was acidified with pH 2.5 with 1N HCl and extracted twice with dichloromethane (v/v). The extracts were filtered through anhydrous sodium sulphate and condensed to 1 mL in a rotavapour (Buchi, Germany) for further gas chromatographic analysis.

2.4 Gas Chromatographic Analysis of the Degradation Products

The ability of the consortium to utilize 4-Chlorophenol as sole carbon source was determined by growing it in the mineral medium containing different concentrations of 4-Chlorophenol (25, 50, 75, 100 mg/L). The cell suspensions were clarified by centrifugation at (10,000 rpm for 15 min, 6ºC). The culture supernatant was extracted with dichloromethane, condensed and filtered through a 0.2 mm Gelman filter acro disc, prior to analysis in gas chromatograph (Chemito GC Model No 1000) equipped with FID detector and capillary column (Varian Chromopak capillary column CP SIL 8 CB, 30m X 0.32 mm . Nitrogen was used as a carrier gas, injector temperature 220ºC, detector temperature 250ºC and the oven temperature of the column was maintained at 150ºC. Standard solutions of different 4-Chlorophenol was used for reference. The samples were injected and the rate of utilization of 4-Chlorophenol was calculated based on the peak area percent and retention time.

2.5 Effect of Alternate Nitrogen Sources

The growth medium containing (100 mg/L) of yeast extract was replaced with different nitrogen sources such as tryptone and urea. Degradation of 4-CP was monitored at regular intervals.

2.6 Bacterial Strains Used in the Degradation Study

The bacterial strains used in the present study were *Bacillus cereus, Arthrobacter sp., Bacillus licheniformis, Halomonas salina, Bacillus pumilus* and *Pseudomonas aeruginosa* [22].

3. RESULTS AND DISCUSSION

3.1 Effect of NaCl Concentrations on the Degradation of 4-CP

Degradation experiments were conducted with 4-CP (25 mg/L) as the sole source of carbon and energy at different NaCl concentrations (Fig. 1). It was observed at 5% NaCl the degradation of 4-CP was 93% which was achieved at the end of 4 days, were the maximum protein yield was 37.4 mg/L. At 3% NaCl the degradation was 87%, which reduced to 72% with 7% NaCl, with increase in the protein yield to 35.2 mg/L and 28 mg/L respectively. Increase in NaCl concentration to 10% showed less growth and degradation (data not shown). The reduction in the degradation of 4-CP above 7% NaCl may be due to higher salt concentrations, which would have affected the metabolic rate of bacterial cells; this was also supported by few studies [9,23,24]. The optimum growth of the bacterial consortium on utilizing 4-CP was at 5% NaCl. Degradation of phenol studied by the researchers [25] in synthetic wastewater, showed that the degradation of the substrate was 99 % at 15% NaCl. According to the work carried out by the author [26] the degradation of phenol in a saline wastewater was carried out by the bacterial strain *Halomonas sp.* which was able to degrade at an optimum salinity of 5%. From the results of the present study it was proved that the bacterial consortium was able to degrade 4-CP (25 mg/L) up to 93% at optimum salinity of 5% in 4 days.

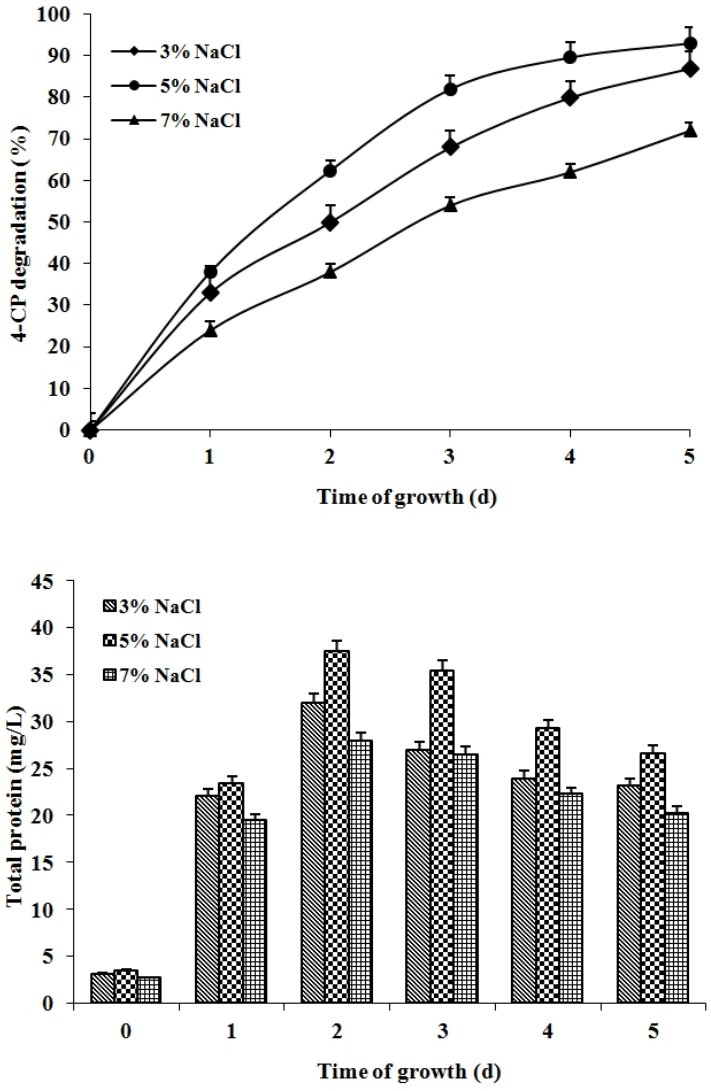

Fig. 1. Degradation of 4-CP at different NaCl concentrations by the bacterial consortium

3.2 Degradation of Different Concentrations of 4-CP

To study the ability of the bacterial consortium to degrade different concentrations of 4-CP at optimum salinity of 5% NaCl, the growth of the bacterial consortium and degradation was studied with different concentrations of 4-CP ranging from 50 to 100 mg/L. Initially the degradation increased with 50 mg/L and 75 mg/L of 4-CP, with a steady increase in the protein yield. When the concentration of the substrate increased to 100 mg/L the growth of the consortium reduced, with decrease in degradation of 4-CP as shown in Fig. 2.

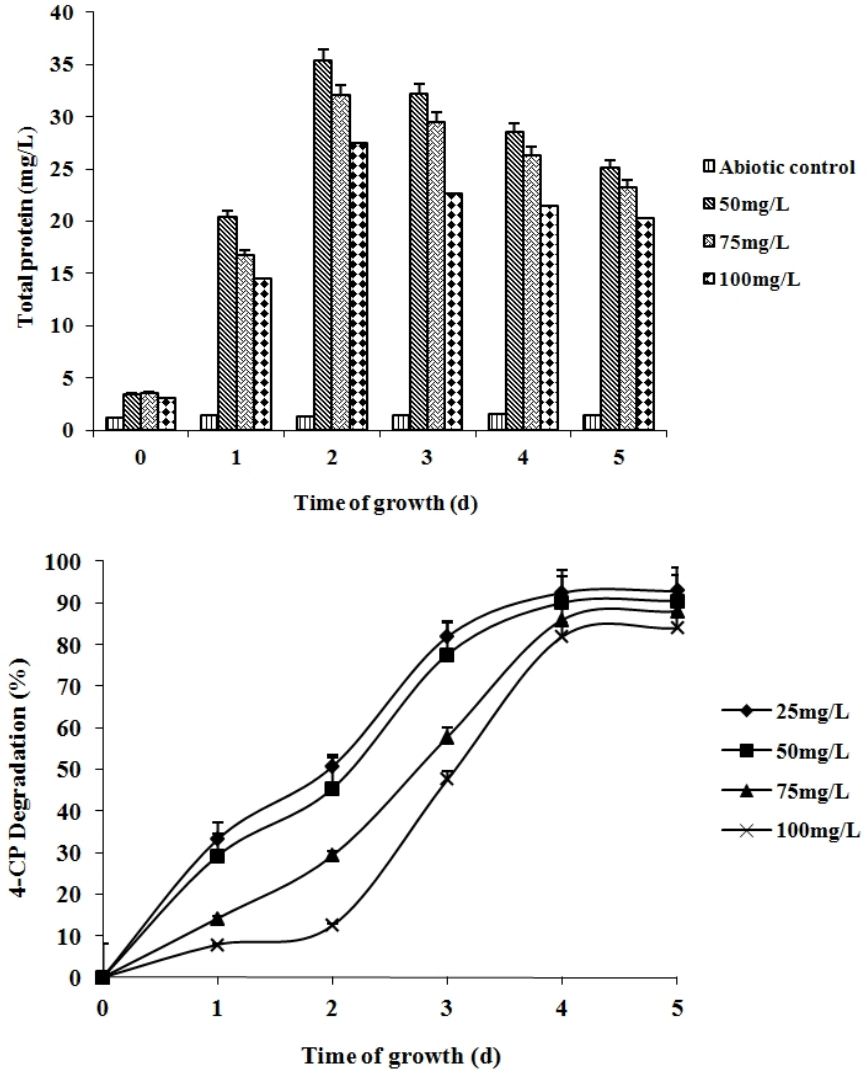

Fig. 2. Degradation and protein yield by the bacterial consortium on different concentrations of 4-CP at 50 g/L NaCl

When the concentration of 4-CP was increased to 50 and 75 mg/L the degradation was 90% and 88% by gradual decrease in the protein content at the end of 2 days to 35.4 mg/L and 32.1 mg/L respectively. At higher concentration of 100 mg/L the degradation still reduced to 84% with respective decrease in the protein content on the second day to 27.5 mg/L and there was no further degradation observed by gas chromatographic analysis. At concentration above 75 mg/L of 4-CP there was reduction in the rate of biodegradation, this could be due to the toxicity of the compound. Work reported by the researcher [3] on the degradation of chlorophenol showed that the cytotoxicity of chlorophenols was attributed at elevated concentrations of chlorophenol. *Nocardioides* sp. strain M6 could degrade 2,4-DCP (50 mg/liter) and 2,4,5-TCP (50 mg/liter) under saline conditions [27]. *Comanomonas testosteroni* JH5 could degrade 4-CP under non-saline conditions, where above 1.24 mM (158 mg/L) concentration the degradation was inhibited [14].

Biodegradation of chlorophenols have been studied under non-saline conditions by pure cultures and by mixed cultures, which were initially grown on various growth substrates, including phenol, toluene and other more common carbon sources [28,29,30].

4-CP degradation was reported with mixed cultures which proved to be more efficient than pure species [31]. *Pseudomonas putida* CP1 completely degraded monochlorophenols (2- and 3- Chlorophenols) via an *ortho*-cleavage pathway up to 1.56 mM and 4-CP at 2.34 mM [32]. The strain *Comanomonas testosteroni* CPW301 was able to degrade 4-CP at 0.75 mM (96 mg/L) in 340 h and 0.9 mM (115 mg/L) was inhibitory [17]. *Arthrobacter chlorophenolicus* could degrade 100 mg/L 4-CP within 24 h [18].

Rhizobium sp. was able to utilize 100 mg/L of 4-CP in 4 days under non-saline conditions, concentrations above 240 mg/L was inhibitory [33]. They also proved that mixed culture had better capability to degrade 200 mg/L of 4-CP in 7 days. In present study the bacterial consortium could degrade 4-CP up to 100 mg/L under saline conditions at 50 g/L NaCl, this shows the ability of the consortium to grow and tolerate 100 mg/L of 4-CP as the sole carbon source equivalent to non-halophilic strains.

Results from the previous literature are on degradation of 4-CP by pure and mixed cultures under non-saline conditions. These non-halophilic strains have degraded 4-CP either in the presence of additional carbon sources or the time taken for degradation of the substrate was more than 5 days. In the present study the bacterial consortium was able to degrade up to 100 mg/L of 4-CP under saline conditions within 4 days. The growth of the bacterial consortium did not show any lag phase and was able to degrade 4-CP up to 75 mg/L concentration.

3.3 Effect of Alternate Nitrogen Sources on the Degradation of 4-CP

Increase in salinity and depletion of nutrients inhibit the growth of the bacterial cells. To support the growth of bacterial cells, nutrients such as glucose, yeast extract and acetate were added as additional carbon source [34]. In the present study (0.01%) of yeast extract was added as the nitrogen source in the mineral salts medium to enhance the degradation efficiency, to evaluate alternate nitrogen sources tryptone and urea were used.

At 25 mg/L of 4-CP the bacterial consortium was able to show maximum degradation of the substrate of about 93%, hence this optimum concentration was taken to further evaluate the degradation efficiency of alternate nitrogen sources in the mineral salts medium. The nitrogen sources like tryptone and urea (10 mg/L) were added individually to the mineral salts medium by replacing yeast extract. The effect of the alternate nitrogen sources on the degradation of 4-CP is given the Fig. 3. The degradation of 4-CP was influenced by altering the nitrogen sources, where the degradation was reduced markedly. In the presence of tryptone as the nitrogen source the degradation reduced to 89% with a maximum protein content of 27.5 mg/L, in the presence of urea the degradation still reduced to 83% with corresponding protein yield of 24.3 mg/L. The results showed that altering the yeast extract with other nitrogen sources like tryptone and urea reduced the degradation of 4-CP.

Pseudomonas putida CP1 could degrade 4-CP in the presence of yeast extract where the rate of removal of chlorophenol (200 mg/L) was increased up to a value of 0.5% (w/v) under non-saline conditions [35]. In the present study, the bacterial consortium had the capability to degrade 75 mg/L of 4-CP with more than 90% degradation in the presence of very less

amount of yeast extract under saline conditions. This shows that the bacterial consortium had the ability to degrade the substrate even under salt stress.

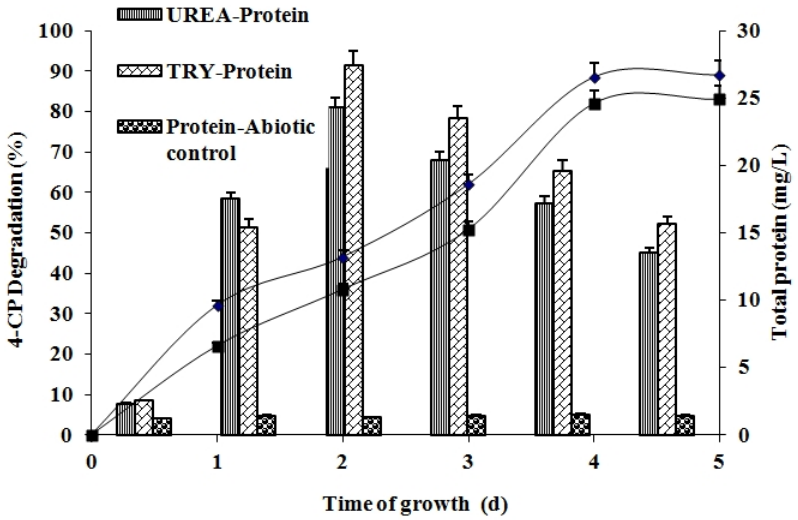

Fig. 3. Effect of nitrogen sources on the degradation of 4-CP (25mg/L) at 50 g/L NaCl

3.4 Identification of Metabolites during the Degradation of 4-Chlorophenol

To identify the metabolites from the degradation of 4–Chlorophenol, 72 h culture supernatant was analysed in GC-MS. The mass spectra were compared with the standards (4-Chlorophenol, 4-Chlorocatechol), which showed that the peaks in (Fig. 4) are 4-CP (peak 1), 4-Chlorocatechol 268 (peak 2) and 5-chloro-2-hydroxymuconate (peak 3), indicating that the 4-CP aromatic ring was broken down to form its intermediates by *meta* cleavage pathway. 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 in the top of the peak in (Fig. 4). It was observed from the GC-MS analysis during degradation of 4-CP by the bacterial consortium initiates the degradation pathway by hydroxylation of 4-CP to the corresponding 4-Cholorocatechol. The produced 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 [36]

The degradation of 4-CP was studied by acclimated and unacclimated activated sludge's using aerobic batch reactors under non-saline conditions [37]. Complete removal of 4- CP (300 mg/L) with acclimated culture led to formation of 5-chloro-2-hydroxymuconic semialdehyde, which was further metabolized, indicating complete degradation of 4-CP via a *meta*-cleavage pathway.

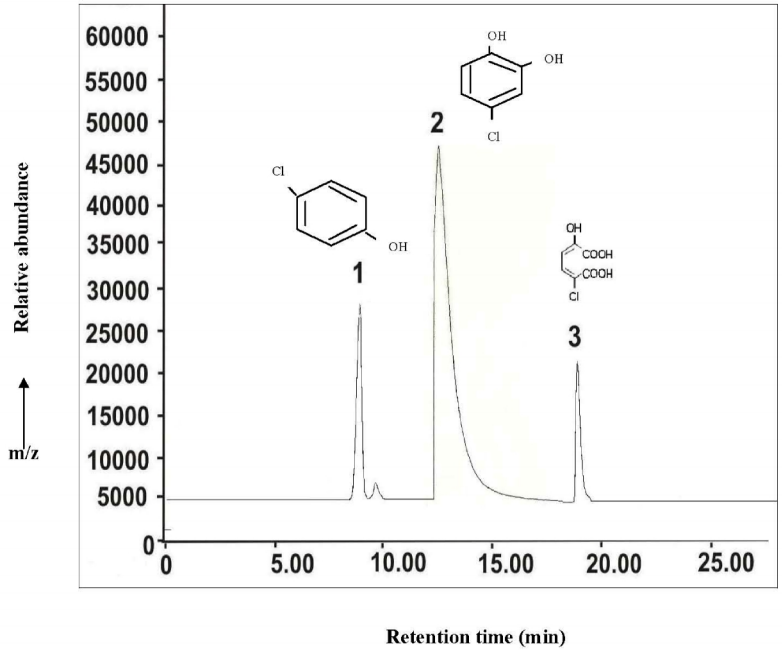

Fig. 4. GC-MS Chromatogram of the metabolites formed during the degradation of 4-CP

Degradation of 4-CP by pure and mixed culture and the metabolites formed during the degradation was reported by many other researchers [14, 31, 33]. *Alcaligenes* sp. OS2 catabolised 4-CP through extradiol *meta*-ring cleavage pathway, were 4-Chlorocatechol and 5-chlorohydroxymuconate were identified as metabolites [36]. *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 [38].

4. CONCLUSIONS

The bacterial consortium was able to degrade 4-Chlorophenol at a range of NaCl concentrations from 30 g/L to 70 g/L, where the optimum degradation was obtained at 50 g/L of NaCl. The consortium was able to utilize up to 100 mg/L of 4-Chlorophenol at optimum concentration of 50 g/L NaCl. Addition of alternate nitrogen sources on the mineral salts medium like tryptone and urea did not show much impact on the degradation of 4-Chlorophenol.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

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