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# ISOLATION AND CHARACTERISATION OF PHENOL AND CRESOL DEGRADING PSEUDOMONADS

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A number of bacterial strains capable of degrading phenol were isolated by enrichment technique out of which five were selected for further studies. Their taxonomical positions were determined by studying their morphological growth and biochemical characteristics. Among them, *Pseudomonas* sp. CPCI, *P. aeruginosa* CoPC3, *P. aeruginosa* CoPC4 and *Pseudomonas* sp. SoPC5 degraded 1000 ppm of phenol whereas *P. stutzeri* SPC2 could degrade only 500 ppm of phenol. The former four strains also degraded all the three isomers of cresol while strain SPC2 did not utilize any of the cresols. Strain SoPC5 degraded 1000 ppm of p-cresol and 500 ppm each of o- and m-cresol. Strains CPCI, CoPC3 and CoPC4 degraded 500 ppm of the different cresols. Strains CPCI, CoPC3, CoPC4 and SoPC5 degraded phenol through metapathway and the cell-free extracts showed fairly high levels of C2, 3-D activities and very low levels of C1, 2-D activities whereas strain SPC2 degraded phenol through ortho-pathway and showed fairly good C1, 2-D activity and showed no C2, 3-D activity.

Most of the phenolic compounds are toxic and cause serious environmental pollution if discharged without proper treatment. Phenols find their way into the environment through effluents from coal and coke gasification plants, petroleum refineries, plastic and pharmaceutical industries etc (1). Biodegradation by microorganisms is thought to be potentially the most efficient method to eliminate these compounds from industrial effluents. A number of microorganisms capable of degrading phenol and cresols have been reported by several workers. It includes the bacteria *Bacillus stearothermophilus* (2), *Brevibacterium fuscum* (3), a number of *Pseudomonas* sp (4-7), number of yeasts including *Candida tropicalis* (8) *Trichosporon cautaneum* (9) etc. The present study deals with the isolation and characterization of different *Pseudomonas* strains which can degrade phenol and the isomers of cresol.

## MATERIALS AND METHODS

The samples were collected from municipal sewage, pesticide contaminated soil, and from a column which was maintaining for a long period with different substrates such as acetone, benezene, phenol, etc. A few contaminants which were found to grow on hexachlorocyclohexane sprayed plates were also picked up and enriched. Shake flask enrichment technique was followed for isolating the strains capable of degrading phenol and cresols. The composition of the Mineral Medium (MM) used was as follows (per litre): $KH_2PO_4$, 2.72g; $Na_2HPO_4$, 3.56g; $(NH_4)_2SO_4$, 1.5g; $MgSO_4.7H_2O$, 0.2g; $Ca(NO_3)_2$, 0.1g; Trace minerals, 1ml and the carbon source at 200 ppm level unless otherwise mentioned.

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5ml of the samples along with 10mg phenol were added in four different 250 ml flasks containing 50 ml of the sterilized MM and incubated at 30°C on a rotary shaker (150 rpm) for 5 days. 1 ml of the aliquote was subcultured into fresh medium every 5 days for three weeks. An appropriately diluted sample of the culture broth was then plated on MM agar (2%) containing 200 ppm of phenol. Different colonies were picked up and maintained. Identification of the selected five cultures was carried out according to Bergey's Manual of Systematic Bacteriology (10).

Degradation of phenol, the three isomers of cresol and other aromatic compounds by the different strains of bacteria was tested by growing them on MM with different concentration of the respective substrates. The growth of the organism was determined turbidimetrically taking absorbance at 550 nm at different intervals of growth. Phenol, o-cresol and m-cresol present in the culture medium was estimated by 4-amino antipyrene colorimetric method (12). p-cresol was estimated by using HPLC.

Various enzyme activities were determined using cell extracts which were prepared as follows. Cells grown with 200 ppm of phenol were harvested at exponential phase, washed twice with phosphate buffer (50 mM, pH 7.5) and resuspended in 3 ml of the suitable buffer. This was subjected to sonication for 5 min. with intermittent bursts of 30 seconds with an interval of 1 min. each by Labsonic 2000 sonicator (B.Braun, Germany). The cell debris was removed by centrifugation for 20 min. at 15000 rpm. All the operations were carried out at 4°C. The supernatant was used for various enzyme assays. The protein content of the cell free extracts was estimated by the method of Lowry et al (13).

Catechol 1,2-oxygenase (EC.1.13.1.1) assay was carried out following the method of Nakazawa and Nakazawa (14) using the cell extracts prepared in 50 mM Tris-HCl (pH 9.0). Catechol 2,3 oxygenase (EC.1.13.1.2) assay was carried out following the method of Nozaki (15) using cell extracts prepared in 50 mM phosphate buffer to which acetone was added to a final concentration of 10% (v/v) to stabilise the enzyme.

## RESULTS AND DISCUSSION

After 4 transfers in the enrichment flasks microorganisms capable of degrading phenol got established. These mixed cultures were then plated out and morphologically distinct individual colonies were picked up. Thus, from sewage enriched flask five different types, from the column two types, from the contaminated plates two types and from soil a single type colonies were isolated and they were individually tested for their phenol degrading ability. Out of these 10 isolates, 5 were selected for further studies. These were found to utilise phenol at higher concentrations. The different organisms were designated as CPC1 (contaminant), SPC2 (sewage) CoPC3 and CoPC4 (column) and SoPC5 (soil). The morphological, cultural and biochemical characteristics of the five strains were studied to establish their taxonomical positions (Table 1). Two isolates CoPC3 and CoPC4 were identified as *Pseudomonas aeruginosa* and SPC 2 as *P.stutzeri*. The two other strains CPCI and SoPC5 were found to belong to the genus *Pseudomonas* but their species positions could not be fixed.

Table 1 Morphological and biochemical characteristics of the isolates

| Character | Strain | | | | |
|---|---|---|---|---|---|
| | CPC1 | SPC2 | CoPC3 | CoPC4 | SoPC5 |
| Cell Shape | Cocco-bacilli | Cocco-bacilli | Cocco-bacilli | Cocco-bacilli | Cocco-bacilli |
| Gram reaction | - | - | - | - | - |
| Oxidase test | - | + | - | + | - |
| Catalase | + | + | + | + | + |
| Oxidative | + | + | + | + | + |
| Urease | + | + | + | + | + |
| Citrate utilization | + | + | + | + | + |
| Gelatin hydrolysis | + | - | - | - | + |
| Lipolytic reaction | + | + | + | + | - |
| Starch hydrolysis | - | - | - | - | - |
| Nitrate reduction | - | + | - | - | - |
| Sucrose fermentation | - | - | - | - | - |
| $H_2S$ production | + | - | - | - | - |
| Casein hydrolysis | + | - | - | - | + |
| Growth 5°C | - | + | + | + | + |
| 41°C | - | + | + | + | + |
| Arginine utilization | + | + | + | + | + |

Table 2. Growth of different *Pseudomonas* strains on phenol as the sole source of carbon and energy

| Strain | 0hr | 6hr | 24hr | 138hr | 144hr |
|---|---|---|---|---|---|
| CPC 1 | 0.051 | 0.014 | 0.069 | 0.865 | 0.805 |
| | (973) | (875) | (790) | (0) | (0) |
| CoPC 3 | 0.043 | 0.042 | 0.077 | 0.975 | 0.913 |
| | (965) | (860) | (730) | (0) | (0) |
| CoPC 4 | 0.048 | 0.032 | 0.050 | 0.134 | 0.830 |
| | (930) | (850) | (790) | (710) | (0)] |
| SoPC 5 | 0.045 | 0.045 | 0.075 | 0.656 | 0.603 |
| | (955) | (910) | (540) | (0) | (0) |

Data in parenthesis is the residual substrate (phenol) present

* Absorbance at 550 nm (Biomass)

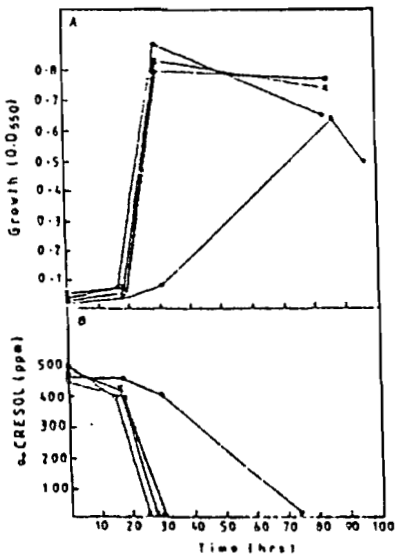

Fig.1 Degradation of o-cresol by different strains of *Pseudomonas*
A. Growth ($OD_{550}$) of the strains
B. Residual o-cresol
-x-x- CPCI; -□- □- CoPC3
-o-o- CoPC4;-●-●- SoPC5

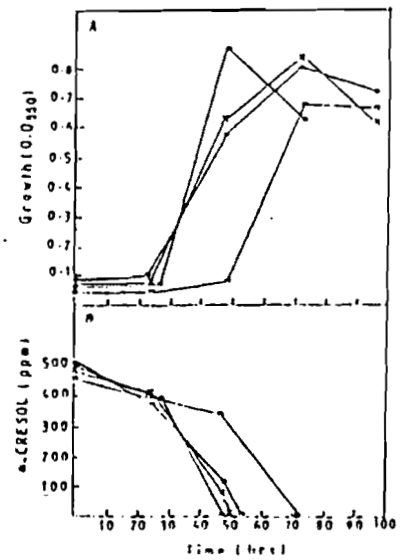

Fig.2. Degradation of m-cresol by different strains of *Pseudomonas*
A. Growth ($OD_{550}$) of the strains
B. Residual m-cresol
-x-x- CPCI; -□- □ CoPC3
-o-o- CoPC4; -●-●- SoPC5

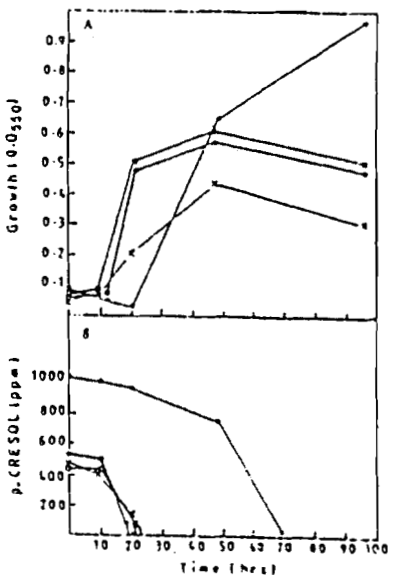

Fig.3. Degradation of p-cresol by different strains of *Pseudomonas*
A. Growth ($OD_{550}$) of the strains
B. Residual p-cresol
-x-x- CPCI; -□- □- CoPC3
-o-o- CoPC4; -●-●- SoPC5

Table 3 Ring-cleaving enzyme activities of the cell extracts of different *Pseudomonas* strains. Details of the experiment were as given in the text

| | Enzyme activity (Units. $mg^{-1}$ protein) | |
|---|---|---|
| | C1,2-D | C2,3-D |
| CPC1 | 0.0001 | 0.4810 |
| SPC2 | 0.3857 | 0.0000 |
| CoPC3 | 0.0625 | 0.2550 |
| CoPC4 | 0.0001 | 0.3349 |
| SoPC5 | 0.0334 | 0.2517 |

Table 4 Utilization of various aromatic compounds by different strains of *Pseudomonas*.

| Compounds (10 ppm) | Strain | | | | |
|---|---|---|---|---|---|
| | CPC1 | SPC2 | CoPC3 | CoPC4 | SoPc5 |
| Phenol | + | + | + | + | + |
| oCP | - | - | - | - | - |
| mCP | - | - | - | - | - |
| pCP | -a | -a | -a | -a | -a |
| 2,4-DCP | - | - | - | - | - |
| 2,6-DCP | - | - | - | - | - |
| PCP | - | - | - | - | - |
| 0-cresol | + | - | + | + | + |
| m-cresol | + | - | + | + | + |
| p-cresol | + | - | + | + | + |
| 2,3-dimethylphenol | + | - | + | + | + |
| 3. CBA | - | - | - | - | - |
| 4. CBA | - | - | - | - | - |
| 2,4-D | - | - | - | - | - |
| 2,4,5-T | - | - | - | - | - |

a - pCP was partially metabolized to a dead-end metabolite, chloro-hydroxy-muconic semi aldehyde

The comparative ability of these five strains to degrade different levels of phenol and isomers of cresol was tested. Among these, four strains viz., CPC1, CoPC3, CoPC4 and SoPC5 utilized phenol upto a maximum concentration of 1000 ppm whereas the strain SPC 2 could utilize only 500 ppm. The former four strains also utilized all the three isomers of cresol and the latter strain (SPC2) did not degrade any of them. All the cresol utilizing strains could grow on all the isomers of cresol upto a concentration of 500 ppm and the strain SoPC5 utilized upto 1000 ppm of p-cresol. Table 2 and Figs. 1-3 show the pattern of growth and residual substrate after different periods of growth of the different bacterial strains.

A number of reports are available on organisms which utilize both phenol and cresols. These include the bacteria *Bacillus strearothermophilus, Brevibacterium fuscum, Streptomyces setonii*, strains of *Pseudomonas, Alcaligenes, Arthrobacter*, yeasts *Candida tropicalis, Trichosporon cutaneum* etc., among which the majority of the reports are on the genus *Pseudomonas*. *P. putida* strain U of Dagley and Gibson (16) is able to degrade phenol and the isomers of cresol. There are also reports that cresols are metabolized by certain fluorescent species of *Pseudomonas* (17). Hinteregger et al reported the degradation of phenol and cresols by *Pseudomonas putida* EKII. It utilized phenol upto a concentration of 1000 ppm as the sole source of carbon and energy. A *Pseudomonas* sp. strain CP4 isolated in our laboratory could utilize phenol and cresols at very high concentrations in the order: p-cresol (2000 ppm) >phenol (1500 ppm) > o-cresol (1400 ppm) > and m-cresol (1000 ppm). Generally bacterial strains have been shown to utilize phenol through meta-pathway. However, there have been sporadic reports on phenol degradation by bacterial species through ortho-and modified ortho-pathways (18). In the present case, 4 of the *Pseudomonas* strains viz., CPC1, CoPC3, CoPC4 and SoPC5 degraded phenol through meta-pathway whereas the *P.stutzeri* strain SPC2 followed ortho-pathway.

The activities of the ring-cleaving enzymes C1, 2-D and C2, 3-D were estimated in the extracts of cells grown on 500 ppm phenol (Table 3) strains CPC1, CoPC3, CoPC4 and SoPC5 showed fairly good levels of C2,3-D activity the highest being that of strain CPC1. Very low levels of C1,2-D activity was also observed. On the contrary strain SPC2 did not have any C2, 3-D activity, but it showed good activity of the ortho-cleaving enzyme C1, 2-D.

Among the different aromatic compounds tested only phenol and cresols were utilized as the carbon source by the bacterial strains (Table 4). None of the chloroaromatic compounds were utilized by any of the strain. However, all the strains except *P. stutzeri* SPC2 partially converted p-chlorophenol to a yellow coloured dead end metabolite which was tentatively identified as chloro hydroxy muconic semialdehyde.

It would be possible to improve the phenol-degrading ability by acclimatizing the cells gradually to higher levels of phenol. Such a phenomenon has been reported by Masque et al and also in our laboratory on *Pseudomonas* sp. CP4 (personal communication). It was interesting to see one of the strains *P.stutzeri* SPC2 catabolized phenol through ortho-pathway which is rarity among bacteria.

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