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Isolation of Bacterial Strains Degrading High Concentration of Phenol from Wastewater Contaminated Sites

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Bacterial strains were isolated from wastewater contaminated sites, which had the ability to utilize higher concentration of phenol of 1000 mg/L as carbon and energy source at neutral pH under aerobic conditions. Phenol degrading strains were isolated by employing enrichment culture technique a basal salt (BS) medium supplemented with different phenol concentrations as a sole carbon source. The results showed that some of the strains could use phenol as sole carbon source at very higher concentration. Several strains were isolated of which only six strains were able to tolerate very high concentration of phenol. The isolates were *Pseudomonas cepacia*, *P. fluorescens*, *Pseudomonas* sp., *P. stutzeri* and *P. putida*. The strains had additional abilities to degrade catechol and resorcinol. Of the six strains *Pseudomonas putida* was able to degrade phenol (1000mg/L) individually up to 89%.

Key words: Biodegradation, Phenol, Wastewater.

Phenolic compounds are hazardous pollutants and they enter the environment through the decomposition of attached algae, phytoplankton (Babich and Davis) and through wastewater discharges from a variety of industries like leather, phenol–formaldehyde resin, oil refinery, coking plant, pharmaceutical, coal conversion, etc**.** (Arutchelvan *et al.,* Buikema *et al.,* Dailey *et al.,*)

Phenol has been also detected in groundwater as a result of leaching through soil after a spill of phenol (Delfino and Dube, 1976), from landfill sites (Clark and Piskin, 1977), and from hazardous waste sites (Plumb, 1987). Phenol is lethal to fish even at relatively low levels, e.g. 5-25mg/L, depending on the temperature and state of maturity of rainbow trout (Brown *et al.,*1967).

Phenol is a listed priority pollutant by the U.S. Environmental Protection Agency (EPA, 1979) and is considered to be a toxic compound by the Agency for Toxic substances and Disease Registry (ATSDR, 2003).The adverse effects of phenol on health are well documented (Calabrese and Kenyon, 1991) and death among adults has been reported with ingestion of phenol ranging from 1 to 32 g (Prpich and Daugulis, 2005).

Several treatment methods are available for removal of phenol content in wastewater. The technologies for the treatment of wastewater containing phenol include chlorination, ozonation, adsorption, solvent extraction, membrane process, coagulation, flocculation and biological treatment (Klein and Lee 1978). But

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physicochemical methods have proven to be costly and have the inherent drawbacks due to the tendency of the formation of secondary toxic materials such as chlorinated phenols, hydrocarbons, etc. Hence the biological method performs significantly on the degradation of phenol.

In spite of the toxic nature of phenol, microbial degradation of phenol was well documented by wide range of bacteria and fungal cultures such as *Acinetobacter calcoaceticus* (Paller *et al.,* 1995); *Alcaligenes eutrophus* (Hughes *et al.,* 1984; Leonard and Lindley, 1998); *Pseudomonas putida* (Hill and Robinson, 1975; Kotturi *et al.,* 1991; Nikakhtari and Hill, 2006); and *Burkholderia cepacia*G4 (Folsom *et al.,*1990 , Solomon *et al.,*1994).*Cryptococcus elinovic* (Morsen and Rehm, 1990), *Fusarium flocciferum* (Anselmo *et al.,* 1985), *Candida tropicalis* (Ruiz-ordaz *et al.,* 2001, Chang *et al.,* 1998)

Objectives of the present studies were to isolate and characterize phenol degrading bacterial strains from different phenol contaminated sites, to test biodegradation potential at very high phenol concentrations.

MATERIAL AND METHODS

Enrichment and isolation of the bacterial strains

Soil samples were collected from different wastewater contaminated sites in Chennai such as refinery wastewater, refinery dump site, mine wastewater, tannery affected soils.. The bacterial strains were 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. Five ml of this suspension was inoculated in 100 ml of the mineral medium containing (in g/L) NaH_2PO_4 -6 KH_2PO_4 -3, Yeast Extract- 200 mg, NaCl-0.5, and $CaCl_2$ 0.01 and $MgSO_4$ 0.5, the medium was adjusted to pH -7 (Bettmann *et al.,*). The medium was autoclaved, cooled to room temperature and was amended with phenol through a sterile filter (0.45 µm) in 250ml Erlenmeyer flasks. The concentrations of phenol used in the study were 100, 500 and 1000mg/L. During all these experiments, after observing the turbidity at an interval of 24h, 5ml aliquots was aseptically inoculated into 100ml of liquid medium supplemented with increasing phenol concentrations. The chemicals and reagents used in the study were analytical grade.

Growth on phenol

Growth of the bacterial strains were studied by measuring the optical density at 600nm.After growth at 100mg/L of phenol, selection for efficient strains continued by successive inoculation into 100ml of saline medium supplemented with increasing concentration of phenol. Phenol was measured colorimetric method based on its rapid condensation with 4 amino antipyrine (4 amino 2-3 dimethyl-1- phenyl-3-pyrozolin-5-one).The culture sample was filtered through a 1.2 µm filter (Whatman) and filtrate was used determine phenol concentration (Yang and Humphrey 1975).

RESULTS AND DISCUSSION

Isolation of bacterial strains

Soil samples were collected from different wastewater contaminated sites to isolate phenol degrading bacterial strains. Several bacterial strains were isolated which could degrade phenolic compounds. Initially, during the isolation period the isolated several bacterial strains were viable after repeated subculture on the mineral medium, only few gram negative rods prevailed. However, when each of them was cultured in a pure culture only 6 strains survived and degraded phenol (IS1, IS2, IS3, IS4, IS5, and IS6). The biochemical and physiological characterization of the organisms are given in Table 1.

The biochemical and physiological characterization of the organisms are given in table 1. The bacterial strains which were able to grow on phenol were 6 gram negative rods and all the six strains were motile. The identification of bacteria was carried out based on phenotypic characteristics, such as gram staining, morphology, motility and nutritional requirements which showed that all the isolate which were bale to tolerate high concentration of phenol belong to the genus *Pseudomonas*. The other characteristics of the microorganisms used in the study were compared with Bergey's manual. By comparison the isolates were found out to be IS1-

Pseudomonas cepacia, IS2- *Pseudomonas fluorescens*, IS3, IS5 -*Pseudomonas* Sp., IS4 – *Pseudomonas stutzeri*, IS6- *Pseudomonas putida*.

Biodegradation of phenol

Phenol is marked as one of the toxic organic pollutants and bacterial genera capable of degrading phenolic compounds in the environment are important soil bacteria that can play an important role in degrading phenoilc compounds. Currently, a number of bacteria were discovered to have excellent capability of phenol degradation. Identification of these bacteria showed the dominance of genus *Psesudomonas*, especially *Pseudomonas putida* mainly because of its spread distribution in soils. Besides, many other genera of bacteria were described as degrading strains of phenolic compounds, including *Agrobacterium*, *Burkholderia*, *Acinetobacter*, *Ralstonia*, *Klebsiella*, *Bacillus*, *Rhodococcus* Koutny *et al.,*. From this study the results of isolation of organisms suggested that the main group of bacterial strain isolated from different sites only Genus *Pseudomonas* dominated and could degrade very high concentration of phenol which is in accordance to other literature.

Batch studies were conducted with different initial concentrations of phenol 100 mg/L, 500 mg/L and 1000 mg/L. Fig.1 showed the results of cell growth and phenol biodegradation with individual bacterial strains with 100 mg/L concentration. No lag phase was observed for initial phenol concentration of 100 mg/L as shown in Fig. 1.When the concentrations were increased to 500 mg/L and 1000 mg/L the lag phase also increased given in Fig 2 and 3.

Removal efficiency of phenol varied markedly with individual strains of the genus *Pseudomonas,* after a period of 96 hours at 100mg/L phenol the removal efficiency by IS6 was 96 %, followed by IS3 90 %, while IS2 could degrade 87 % and IS5, IS1 showed 85 % and 84 % respectively and the least removal efficiency was showed by IS4 of 83 %.With the increase of growth O.D, biodegradation potential of the strain promoted prominently. With the augmentation of the inoculum concentration, the phenol removal efficiency of the strains increased gradually, but cell growth was not proportion with phenol degradation, which demonstrated there was no essential association between growth and phenol degradation as previous reports, especially at high phenol concentration Clauben *et al.,*.

Fig.2 depicts the removal efficiency and growth of the bacterial strains at 500 mg/L of phenol. The degradation percentage of IS6 which

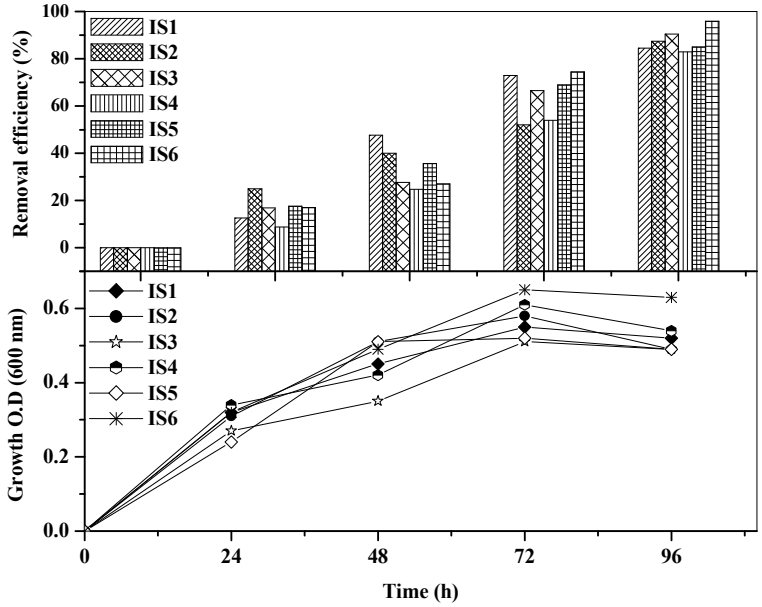

Fig. 1. Growth response and removal efficiency of phenol (100 mg/L) by the isolated bacterial strains

efficiently removed 89 %, IS3 showed 86 % and the removal markedly reduced for the other isolates to 81% for IS5, 73 %, (IS4, IS1) respectively, it was found IS2 was the least to remove phenol of about 64 % in 96 hours. The bacterial strains could grow on very high concentration of phenol of 1000 mg/L, which is shown in Fig 3. Highest removal efficiency was shown by IS6 of 87 %, followed by IS3 84 %, IS5, IS4 (78 % and 69 %), and IS1 showed 64 % and least was IS2 (58%).

The bacterial strains showed a longer lag phase, when manifested with high phenol concentration of 1000 mg/L. Reason may be due to the higher phenol concentration, the stronger the substrate inhibition resulted. It exhibited a remarkable augment of substrate inhibition, which could be also demonstrated by the longer lag phase of cell growth. Hence, cell growth was out of proportion to phenol degradation as previous reports, particularly at high phenol concentration Clauben *et al.,* although phenol was consumed

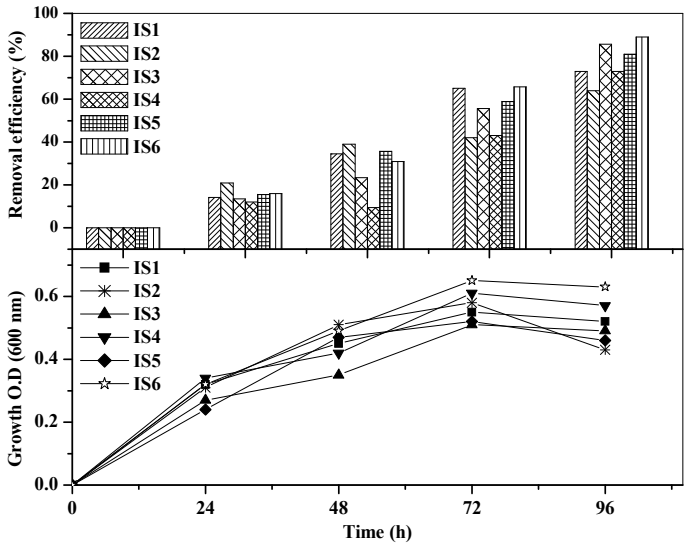

Fig. 2. Growth response and removal efficiency of phenol (500 mg/L) by the isolated bacterial strains

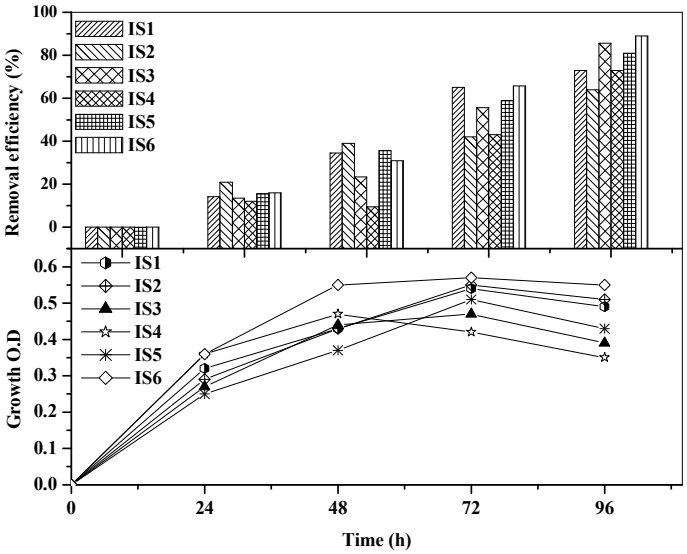

Fig. 3. Growth response and removal efficiency of phenol (1000 mg/L) by the isolated bacterial strains

Table 1. Biochemical characteristics of the isolated Bacterial strains

Characteristics	IS 1	IS 2	IS 3	IS 4	IS 5	IS 6
Gram reaction	–	–	–	–	–	–
Motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Indole production	+	+	–	–	–	+
Methyl red	+	+	–	+	–	+
Voges-proskauer	–	–	–	–	–	–
Citrate	+	+	+	+	–	+
Nitrate reduction	+	+	+	+	–	+
Gelatin	–	+	+	–	–	–
Lysine	+	–	–	–	–	–
Arginine	–	+	+	+	+	+
Ornithine	+	–	–	–	–	–
Pyocyanine	–	–	–	–	–	–
Pyoverdin	–	+	–	–	–	+
Polyhydroybutyrate	+	–	–	–	–	–
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Sucrose	–	–	–	–	–	–
Malonate	+	+	–	–	–	–
Mannitol	+	–	–	–	–	–
Arabinose	–	–	–	–	–	–
Xylose	+	+	–	+	–	–
Rhamnose	–	–	–	–	–	–
Tween 80	–	–	–	+	–	–
Starch	–	–	–	+	–	–

mainly for assimilation into biomass and energy for cell growth and maintenance (Venkataramani *et al.,*). Besides, the production and accumulation of various intermediates may also be responsible for the decreased cell mass yield Allsop *et al.,*and Hao *et al.,*

CONCLUSION

Phenol degrading bacterial strains were isolated from different waste water contaminated sites. Only six organisms belonging to the genus *Pseudomonas* could utilize higher concentration of phenol as the carbon and energy source. The isolates were *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas* Sp., *Pseudomonas stutzeri*, and *Pseudomonas putida*. The strains had additional abilities to degrade catechol and resorcinol. Further, these cells were exceptional in being used continuously for a week with out the loss of their degradative ability. Since the bacterial strains were capable of degrading phenol, catechol and resorcinol there exist a possibility for its use in development of microbial technology for decontamination of phenolic wastes.

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