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Full Length Research Paper

Isolation and characterization of phenol degrading *Xanthobacter flavus*

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A soil bacterium isolated from a contaminated site degraded phenol as the sole carbon and energy source was identified as *Xanthobacter flavus* MTCC 9130. This microbial strain was able to tolerate phenol up to 1100 mg/l concentration. The lag phase increased with the increase in phenol concentration. The optimum growth temperature was 37°C. The organism could degrade completely within 120 h when initial concentration was less than 600 mg/l. Enzyme assay through cell free extract showed the presence of catechol-1,2-dioxygenase. The specific activity was 0.146 µmol/min/mg protein. However higher concentrations of phenol in the medium showed a negative effect on the growth of the bacterium. Hence *X. flavus* can be effectively used for bioremediation of phenol-contaminated sites.

Key words: Bioremediation, catechol-1, 2-dioxygenase, ortho-pathway, phenol, *Xanthobacter flavus.*

INTRODUCTION

Phenol is a natural as well as man-made aromatic compound and one of the widely used organic compounds in existence. Annual production of phenol is approximately 1.25 billion kg (Stephen et al., 1983). Phenol and its derivatives are used in industry for the production of compounds like resins plastics, disinfectants, paints, dyes antioxidants, perfumes, etc. These have been reported as highly toxic and hazardous to living organisms (ATSDR, 1998). Due to their potential toxicity and persistence in the environment, rapid removal and detoxification is urgently needed. Microbial uptake and mineralisation of phenol has been studied extensively (Dua et al., 2002; Lovely, 2003; Wackett, 2000; Watanabe, 2001). A large number of bacterial fungal genera posses the capability to degrade most of the organic pollutants. Phenol degrading aerobic bacteria are capable of converting phenol into non-toxic intermediates of tricarboxylic acid via ortho or meta pathway (Powlowski and Shingler, 1994). Catechol, a dihydroxy that substituted aromatic compound and key intermediate in the phenol biodegradation, is cleaved by ring cleavage dioxygenases. Cleavage can occur either between two hydroxyl groups (ortho) or proximal to one of the two hydroxyl groups (meta) (Harayama and Timmis, 1992). Catechol-1, 2-dioxygenase catalyses the ortho cleavage of catechol and generates cis-cis muconic acid (Babu et al., 1995); it is transformed into tricarboxylic acid, and fed into tricarboxylic cycle for further metabolism.

To date several bacterial strains such as *Acaligenes eutrophus* (Hinteregger et al., 1992), *Pseudomonas* sp. (Neumann et al., 2004), *Streptomyces* sp. (Antai and Cranford, 1983), *Brevibacillus* (Yang and Lee, 2006), and *Serratia plymuthica* (Pradhan and Ingle, 2007) have been reported to degrade phenol but there are no reports on degradation of phenol by *Xanthobacter* belonging to Xanthobacteriaceae family. We have not come across any publication reporting degradation by *Xanthobacter*.

In this study, a bacterium identified as *Xanthobacter flavus* was isolated from contaminated site near the dye industries soil samples. It was capable of degrading

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Abbreviations: MSM, mineral salts medium; **IMTECH**, Institute of Microbial Technology; **MTCC**, Microbial Type Culture collection; **PAH's**, Polyaromatic hydrocarbons, **HAC's** haloaromatic compounds.

phenol and some other phenolic compounds as sole source of carbon and energy. Further growth behavior of the organism was studied using phenol as substrate.

MATERIALS AND METHODS

Growth medium

Bacteria were cultivated in minimal salt medium. This MSM medium contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), $CaCl_2.2H_2O$ (1 M) and $MgSO_4.7H_2O$ (1 M) in 1000 ml double distilled water. Phenol (analytical grade) was used as a sole source of carbon and sterilized phenol solution was added directly to MSM at a concentration of 600 mg/l.

Isolation of bacterial culture by enrichment method

X. flavus was isolated by enrichment culture technique using soil samples from Jaipur disrict, Rajasthan in India. A 5 g soil sample was suspended in 100 ml of minimal medium (Sambrook et al., 2001) containing 600 mg/l of phenol as sole source of carbon and incubated in 250 ml flasks at 37°C on an orbital shaking incubator at 200 rpm for a period of 10 days. After incubation period, the soil particles were allowed to settle and 5 ml of particulate free suspension was then used to inoculate a 100 ml minimal medium containing 60 mg phenol. Four such transfers were made and every time the enriched population was plated on minimal medium plates containing phenol as sole carbon source. After fourth transfer, a pure isolate capable of growth on phenol was obtained.

Identification of bacteria

Colony morphology of the bacterium was determined by cultivating the isolate on minimal salt agar medium supplemented with 600 mg/l phenol. Physiological and biochemical tests were done following the methods of Cappuccino and Sherman (2002). The organism was further conformed from MTTC, IMTECH, Chandigarh, India.

Phenol degradation studies

Phenol degradation ability of *X. flavus* was studied by culturing in MSM medium with different phenol concentrations ranging from 100 - 1100 mg/l, for a period of 10 days at 37°C. For each experiment freshly prepared inoculum of O.D 0.1 was used. The samples were analyzed regularly for phenol degradation and the residual phenol concentrations were determined by 4-amino antipyrine method (Yang and Humphrey, 1975). Growth behavior of the isolate with initial phenol concentration of 600 mg/l was monitored spectrophotometrically (UV-Vis 117,Systronics, Romania) at 600 nm by collecting the culture at every 4 h interval for a period of 120 h.

Enzyme assay

To attain required cell biomass for enzymatic assay *X. flavus* was grown aerobically to mid-log phase and cells were harvested by centrifuging at 10,000 g for 10 min at 4°C. Cell pellet was washed twice with saline and resuspended in 10 mM Tris-Hcl buffer (pH-8). This cell suspension was homogenized using silica beads (0.5 mm) in a mini bead beater (Biospec products, US) for 8 min. Cell debris was removed by centrifugation at 20,000 g at 4°C for 20 min. The supernatant was used for enzyme assay. The assay of catechol-1,2-dioxygenase was carried out by measuring the increased concentration of cis-cis muconic acid. The reaction mixture contained 2.8 ml of 0.1 M sodium phosphate buffer, 120 µl 0.1 mM catechol, 80 µl cell free extract and incubated at 37ºC for 30 min. Increase in the absorbance at 260 nm was monitored spectrophotometrically for every 30 s for 5 min. The specific activity was measured as µ moles of cis-cis muconic acid formed /min/mg of protein (Pradhan and Ingle, 2003). Protein was determined using Bradford method (Bradford, 1976).

Table 1. Morphological and biochemical characteristics of isolate Xanthobacter Flavus.

Characteristics	Isolate (Xanthobacter flavus)
Gram Staining	Negative
Cell Shape	Coccobacilli or Rod
Motility	Negative
Oxygen Requirement	Aerobic
Colony	Yellow color slime formation
Catalase	Positive
Nitrate Reduction	Negative
H_2SProduction	Negative
Indole Production	Negative
Fermentation Test:	
Dextrose	Positive
Lactose	Positive
Sucrose	Positive
Gelatin Liquefaction	Negative
Starch Hydrolysis	Negative
Oxidase	Positive
Citrate Utilization	Positive
Growth Temperature	37° C

RESULTS AND DISCUSSION

Isolation and characterization of phenol degrading isolate

A bacterium capable of degrading phenol was isolated from the soil sample collected near dye industries. Four isolates that were able to utilize phenol as sole source of carbon were obtained from the enriched population grown in MSM medium, supplemented with phenol. However, only one strain capable of utilizing phenol completely within 120 h was characterized using morphological and biochemical properties listed in Table 1. By comparing these characteristics with those mentioned in Bergey's manual of systematic Bacteriology, the bacterium was identified as *Xanthobacter*. Further it was conformed by MTCC, IMTECH, Chandigarh, India as *X. flavus* 9130. It is a Gram negative, non-motile, cocobacilli showing growth under aerobic conditions with the optimum temperature of 37°C. Colonies of *Xanthobacter* on minimal salt agar with phenol appeared yellow colored,

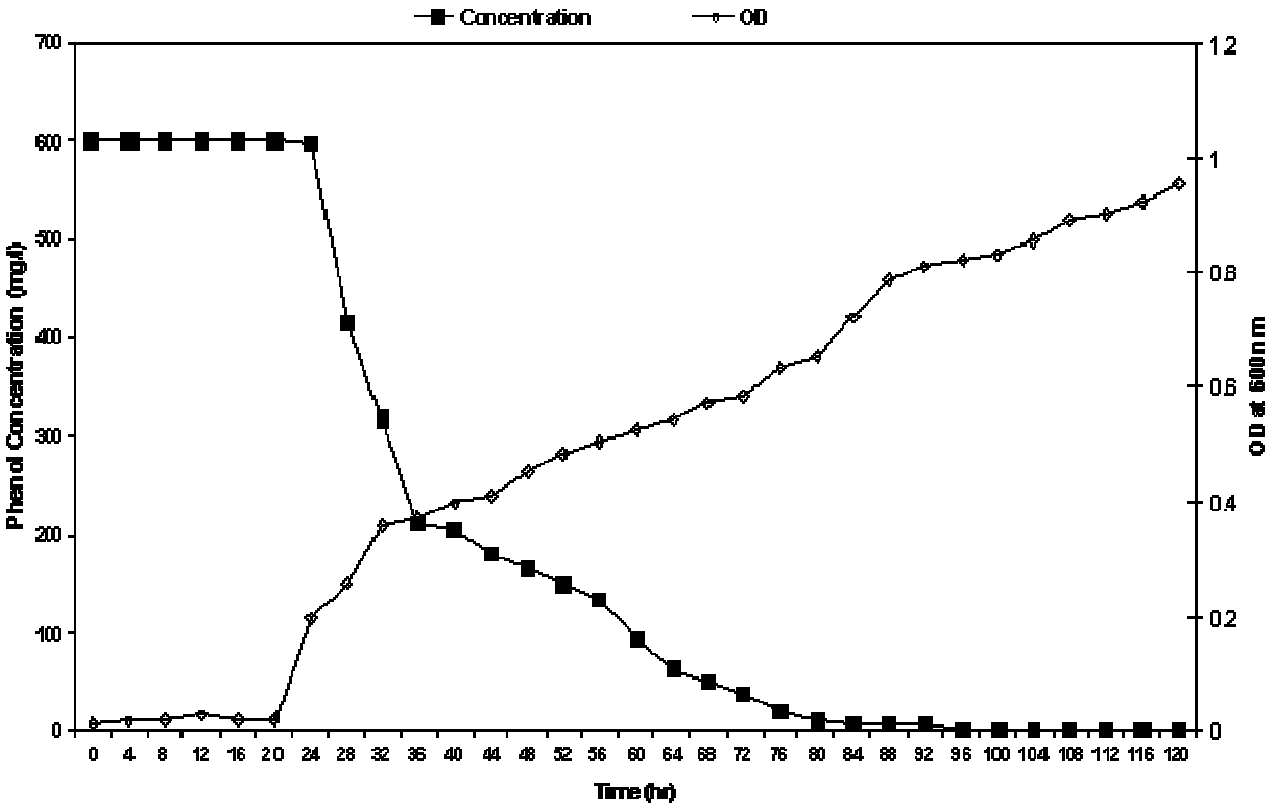

Figure 1. Growth behaviour of Xanthobacter flavus on phenol supplemented mineral salt media.

slime formation and on nutrient agar colonies were white, round and transparent.

Biodegradation of phenol

X. flavus showed a short period of lag phase for 20 h (Figure 1). When initial concentration of phenol was 600 mg/l, complete utilization of phenol was within 120 h with a corresponding increase in cell biomass. The approximate doubling time (generation time) of the cell was 28 h, elucidated from the growth behavior of the isolate. This bacterium was able to tolerate the phenol concentration up to 1100mg/l. However, the strain was not able to adapt to higher concentrations (>650 mg/l) of phenol (Figure 2). There was a rapid removal of phenol by the organism in MSM, when added at lower concentrations (<600 mg/l).

Xanthobacter 127W has ability to degrade PAHs and HACs (Hirano et al., 2004) and xanthobacter SP Py2 is capable of degrading propene, benzene and phenol and its unique alkene monooxygenase has been shown to be responsible for aromatic hydrocarbon degradation (Zhou et al., 1996). another strain, *Xanthobacter* 14p1 (Speiss et al., 1995) was able to degrade cholorobenzene.

Enzyme activity

Aerobic and anaerobic catabolism of phenol in micro-organisms is mediated by catechol dioxygenases. These are critical enzymes involved in the ring cleavage of catechol, during the biodegradation of phenol. The cell free extract of *Xanthobacter* has shown catechol-1,2-dioxygenase activity, indicating that the oxidation of catechol takes place through intradiol (ortho) cleavage. Specific activity of catechol-1,2-dioxygenase was 0.142 µ mole/min/mg protein. Total protein in the cell free extract was 84 mg/ml. *Xanthobacter* showed a high catechol-1,2-dioxygenase activity but moderate specific activity (Table 2). In this study, cell free extract showed high protein content, with an increase in enzyme activity and a moderate specific activity. Catechol 2,3 dioxygenase activity was not detected in phenol grown cells of *Xanthobacter*.

Conclusion

Several bacterial strains belonging to the species of *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Rhodococcus*, etc (Chitra et al., 1995; Balasankar and Nagarajan, 2000;

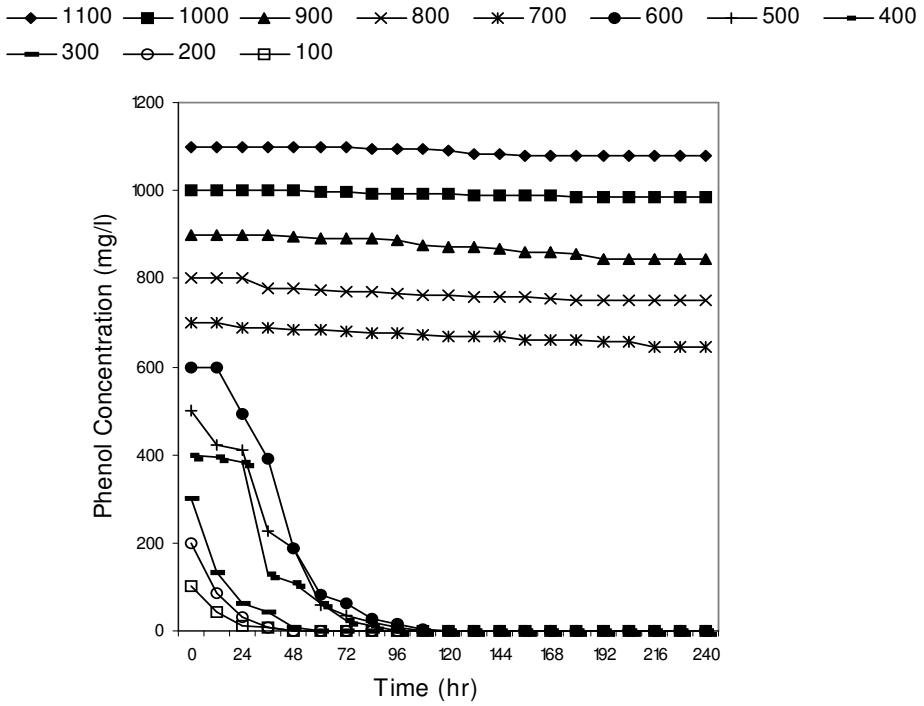

Figure 2. Phenol tolerance ability of Xanthobacter flavus grown under varied concentrations.

Table 2. Enzyme activity in cell free extract, during phenol degradation by Xanthobacter flavus.

Enzyme	Specific activity (µ moles/min/mg protein)
Catechol-1,2-dioxygenase	0.142
Catechol-2,3-dioxygenase	0.00

Grit et al., 2004) were reported for degrading phenol and its derivatives. Till date several works are in progress to isolate new and efficient microbial strains that have ability to degrade phenol. We report here the isolation and characterization of phenol degrading bacteria isolated from soil sample near the contaminated sites of dye industries in Jaipur District, Rajasthan, India. The present results and findings indicate that *X. flavus* has the potential to utilize phenol as sole source of carbon and energy. The phenol degradation capability by *X. flavus* makes it an interesting organism for bioremediation of phenol-contaminated studies.

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