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Phenol degradation by immobilized cells of *Arthrobacter citreus*

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Abstract

An aerobic microorganism with an ability to utilize phenol as carbon and energy source was isolated from a hydrocarbon contamination site by employing selective enrichment culture technique. The isolate was identified as *Arthrobacter citreus* based on morphological, physiological and biochemical tests. This mesophilic organism showed optimal growth at 25 °C and at pH of 7.0. The phenol utilization studies with *Arthrobacter citreus* showed that the complete assimilation occurred in 24 hours. The organism metabolized phenol up to 22 mM concentrations whereas higher levels were inhibitory. Thin layer chromatography, UV spectral and enzyme analysis were suggestive of catechol, as a key intermediate of phenol metabolism. The enzyme activities of phenol hydroxylase and catechol 2,3-dioxygenase in cell free extracts of *Arthrobacter citreus* were indicative of operation of a *meta*-cleavage pathway for phenol degradation. The organism had additional ability to degrade catechol, cresols and naphthol. The degradation rates of phenol by alginate and agar immobilized cells in batch fermentations showed continuous phenol metabolism for a period of eight days.

Introduction

A wide variety of synthetic chemicals have found their way into the ecosystem as a consequence of industrial activities, agricultural application and use in domestic purposes. Several compounds belonging to different classes of chemicals such as aromatics, halo-aromatics, nitro aromatics, sulpho-aromatics, polychlorinated biphenyl, polycyclic aromatic hydrocarbons are found to be in environmental contaminants and have been labeled as priority pollutants (OECD, 2001). Among the aromatics, phenols and their derivatives such as nitrophenols and chlorophenols have been placed in the list of priority contaminants by the U.S Environmental Protection Agency (Keith & Telliand 1979). The phenolics comprise characteristic pollutants in wastewaters and effluents discharged from petrochemical, textile, tannery and coal gasification units (Shashirekha et al. 1997). Since these compounds are toxic even at low levels, they pose a threat to the biosphere and especially to aquatic life. Biodegradations of phenolics by certain anaerobic, aerobic bacteria and fungi have been reported (Buswell, 1975; Furukawa et al. 1978; Knoll & Winter 1987; Abramowicz 1990; Baker & Herson 1994; Gil et al. 2000; Soroka 2001; Shimoni et al. 2002; Tixier et al. 2002; Pandey et al. 2003; Shimoni et al. 2003; Margesin et al. 2004).

Diverse microorganisms, including many species of bacteria and fungi have evolved the metabolic capacities to degrade hydrocarbons. Bacteria are often the dominant hydrocarbon degraders in aquatic systems. The most prevalent bacterial hydrocarbon degraders belong to the genera *Pseudomonas, Achromobacter, Flavobacterium, Nocardia, Arthobacter*. Other genera of bacteria

capable of degrading hydrocarbons include *Actinomyces, Aeromonas, Alcaligenes, Coryne bacterium, Lactobacillus, Mycobacterium, Sarcina, Streptomyces and Xanthamonas* (Atlas & Cerniglia 1995). Among the prokaryotes, the photosynthetic oxygen-evolving cyanobacteria would be ideal for the treatment of effluents containing aromatic compounds since they would hasten the process of biodegradation through oxygenation and reduction of Biological Oxygen Demand (BOD). In addition, they possess advantages over other bacteria and green algae by their trophic independence for nitrogen as well as carbon (Carr & Whitton 1982). Marine cyanobacteria have already been reported to be useful in treating effluents from industries producing antibiotics (Subramanian et al. 1994), Ossein (Uma & Subramanian 1990) and chlor-alkali (Uma & Subramanian 1994). Identification of phenol-degrading cyanobacteria is important in view of their pivotal role in effluent treatment and recycling processes. There are several reports on the cyanobacterial degradation of aromatic hydrocarbons (Cerniglia & Gibson 1977; Ellis 1977; Gibson 1982; Narro et al. 1992) and xenobiotics (Kurtitz & Wolk 1995). Phenol was found to be effectively removed and degraded by the marine cyanobacterium *Phormidium Valderianum* BDU 30501 (Shashirekha et al. 1997) showing the production of inducible intracellular phenol degrading enzymes.

The bacterial catabolism of phenol and cresols have been studied in detail and catabolic sequences have been identified. Phenol is degraded by bacteria via catechol (Dagley 1971) whilst, p-cresol is catabolized either via 4-methyl catechol (Bayly et al. 1966) via protocatechuate (Hopper & Taylor 1975). It has been shown that the mold, *Scedosporium apiospermum* is capable of productively utilizing phenol and p-cresol as carbon sources and the catabolic sequences employed for the degradation of these compounds has also been proposed (Clauben & Schmidt 1998).

Degradation of monochloro-phenols as sole source of carbon in aerobic batch cultures has been examined by a mixed microbial community (Farell & Quilty 1999). The influence of supplementary conventional carbon sources on enhancing the biotransformation rates of phenol as the primary substrate and 4-chlorophenol as a non-growth substrate has been studied by medium augmentation with conventional carbon sources (Loh & Wang 1998). These fundamental studies about pollutant degradations by microorganisms have opened up a potential treatment technology called bioremediation (Atlas & Unterman 1999). However, the development of biotechnology for the removal of phenols from the industrial effluents remains to be adequately addressed even today.

Various approaches are being developed to treat the phenolic effluents (Kratanov 2000). The constraints are the availability of the suitable microorganisms that can overcome their culturing limitations from their natural habitats to the effluent conditions. The method of cell immobilization seems to be promising in the development of the biotechnology for the removal of not only phenolics but also other xenobiotic bearing effluents (Murugesan 2003). The immobilized cells also have valuable application in industries for production of biochemicals (Cheetam 1980; Bisping & Rehm 1988). Since the entrapped cells remain viable for a considerable duration they would be a better alternative against free cells for the bioremediation of variety of toxic organics from effluents (Bettmann & Rehm 1984, 1985; O'Reilly & Crawford 1989; Westmeier & Rehm 1985, 1987; Belfanz & Rehm 1991; Menke & Rehm 1992; Kochar & Kahlon 1995; Lee et al. 1994; Ferschl et al. 1991 and Sahasrabudhe et al. 1988).

In this communication we report the biodegradation of phenol by *Arthrobacter citreus*. This is a new agent for biodegradation of pollutants and is capable of mineralizing phenol and its derivatives as the sole carbon and energy sources. We have also carried out immobilization of *Arthrobacter citreus* in two matrices to show its applicability in bioremediation of phenol containing effluents.

Materials and Methods

Organism Source, Culture Conditions and Identification

The soil collected from hydrocarbon contamination site was used to isolate microorganism adopting selective enrichment culture technique. The bacterial strain was grown on mineral salt medium (MSM) containing (g l^{-1}) K_2HPO_4, 1.6;

KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 1.0; Mg $SO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4$. H_2O, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.05; $MnSO_4 \cdot H_2O$, 0.05; $Na_2WO_4 \cdot 2\ H_2O$, 0.05; supplemented with phenol up to 5 mM as the sole source of carbon and energy (Ganji & Pujar 1992). In the metabolic studies, growth substrate (2–5 mM) was incorporated to the sterilized MSM asceptically. The flasks were inoculated with 5% (v/v) of inoculums and incubated on a rotary shaker (110 rpm) at 25 ± 2 °C. Un-inoculated flasks were incubated in parallel as controls. Purified bacterium was identified and characterized based on the morphological, physiological and biochemical features as described in Bergey's Manual of Systematic Bacteriology (Keddie et al. 1986).

Measurement of Cell Growth

The growth curve of the organism was followed by measurement of the optical density at 600 nm. The viable counts were established by pour plate technique. The growth response of the organism on other substrates was assessed by harvesting phenol-metabolizing cells by centrifugation 5000 rpm at 5 °C for 10 min. The cell pellet obtained was resuspended in the sterile MSM containing different substrates (5 mM each). All operations were performed under aseptic conditions. All the results represent data from at least three independent and repetitive experiments.

Degradation of phenol

The degradation of phenol was followed by solvent extraction of the residual phenol from the spent medium at specific time intervals followed by its estimation by a colorimetric method using 4-aminoantipyrine and potassium ferricyanide according to the American Public Health Association Manual (APHA 1992).

Isolation and Identification of Metabolites

Metabolic intermediates were isolated using solvent extraction of the spent culture medium. After acidification to pH 2.0, the spent broth was extracted with ethyl acetate twice. The extract obtained was subjected to Thin Layer Chromatography (TLC) on silica gel precoated sheets (Alugram Sil G 0.20 mm thick layer, Macherey-Nagel GmbH & Co, Germany) using solvent systems i) 2-propanol / ammonia / water (20: 1: 2 v/v) and ii) Benzene / methanol / acetic acid (90: 25: 5 v/v). Preparative TLC was performed to purify the metabolites. The spots on the chromatogram were visualised under UV lamp or by spraying with a mixture of potassium ferricyanide and 2% $FeCl_3$ solution to detect hydroxylated metabolites. The separated metabolites were scraped off from chromatograms, eluted with methanol and subjected to Ultra-violet (UV) spectral analysis (UV-VIS Spectrophotometer, Model SL 159, ELICO, India).

Preparation of cell free extracts

Cells were harvested after 12 h growth period (logarithmic growth phase) and lysed by sonic waves produced by Vibra-Cell ultrasonic cell processor model 130, (Sonics & Materials Inc, USA) for a total time of 3 min. The lysate was centrifuged at 10000 rpm for 20 min at 4 °C, and the supernatant obtained was used as the cell free extract for enzyme assays. The protein content in the crude enzyme was determined by the method of Bradford using bovine serum albumin as the standard (Bradford 1976).

Immobilization of microorganism

The phenol degrading bacteria was harvested after 12 h of growth from 1 litre of culture medium. The cell pallet obtained by centrifugation at 5000 rpm for 10 min at 4 °C, obtained was used for immobilization in alginate and agar matrices.

The alginate entrapment of cells was performed according to Bettemann and Rehm (Bettemann & Rehm 1984). Alginate (4% w/v) was solubilised in boiling water and autoclaved at 121 °C for 15 min. A 10 ml of bacterial cell suspension was added to 100 ml of sterilized alginate solution and mixed by stirring on a magnetic stirrer. This alginate-cell mixture was extruded drop by drop into a cold, sterile 0.1 M calcium chloride solution through a burette. The gel beads formed were left in the solution for one hour before being filtered off. The beads were washed with distilled water and used for experiments.

Agar entrapment of cells was carried out in sterile 4% (w/v) agar saline solution (Manohar &

Karegoudar 1998). Briefly, 12 hour bacterial cell suspension (10 g wet weight) was mixed with 10 ml of saline and extruded into sterile agar-saline solution to obtain cell-entrapped beads. These agar beads were washed successively with distilled water and saline for further applications.

Fermentation process

Free cells
Batch fermentations of free cells (1.2×10^{12} cells) were performed in a 100 ml conical flask containing 25 ml of MSM with 22 mM of phenol. The process was carried out at 25 °C on a rotary shaker at 150 rpm for the desired incubation period.

Immobilized cells
A 25 g each, of the alginate and agar immobilized beads containing 2.1×10^{11} cfu g^{-1} and 2.3×10^{11} cfu g^{-1} beads, respectively, were added to 500 ml conical flask containing 125 ml of mineral salt medium to which 22 mM phenol was supplemented. The fermentation was carried out at 25 °C on a rotary shaker at 150 rpm for the desired incubation period. Samples of the culture broths were taken each day for residual phenol analysis. Control flasks were incubated in parallel under the same conditions to ascertain the evaporation losses of phenol.

Enzyme Assays
Phenol hydroxylase enzyme activity was measured with UV-VIS Spectrophotometer (ELICO, India) in quartz cuvettes at 25 °C by following the substrate dependent NADH consumption at 340 nm (Clauben & Schmidt 1998). The reaction mixture of 1.0 ml contained 50 mM sodium phosphate buffer, pH 7.0, 50 μg enzyme and 75 μmol of substrate (phenol or catechol). Catechol 1,2-dioxygenase activity was measured as an increase in absorbance at 260 nm by following the formation of *cis-cis* muconic acid, the *ortho* cleavage product of catechol. The catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*–cleavage product of catechol. spectrophotometrically at 375 nm (Sala-Trepat & Evans 1971). The enzyme activities were expressed as μmoles of product formed per min per mg of protein.

Determination of ring cleavage
Mode of ring cleavage of catechol in cells and cell free extracts was determined by measuring the formation of red nitroprusside complex at 546 nm owing to the formation of 3-oxoadipate from catechol using modified Rothera's method (Kaschabek & Reineke 1992).

Results

Identification of the Organism

Microorganisms have the ability to adapt to a variety of environmental conditions. In the present work, the preliminary objective was to isolate a microorganism that could grow on phenol. A microorganism was obtained that could metabolize phenol up to 22 mM, by adopting the enrichment culture technique. The classification of bacteria was carried out entirely based on phenotypic characteristics, such as cell wall type, morphology, motility, and nutritional requirements. The isolated phenol metabolizing strain was thus subjected to various morphological, physiological and biochemical tests. The results of the tests showed that the strain growing on phenol is Gram positive, exhibits typical rod–coccus transition, aerobic, motile and assimilates glucose, galactose, lysine, threonine, alanine and does not require vitamin. These results show that the strain degrading phenol is *Arthrobacter citreus* according to Bergey's Manual of Determinative Bacteriology (Keddie et al. 1986).

Phenol Degradation and Identification of Metabolites

Studies on the degradation of phenol by *Arthrobacter citreus* indicated that 5 mM phenol utilisation occurred in 24 h (Figure 1.). The organism tolerated phenol concentration up to 22 mM, but at higher concentrations there was a decline in the growth due to substrate inhibition. The ethyl acetate fractions on TLC analysis indicated the increased accumulation of catechol with time in the initial aliquots, but later aliquots contained less catechol owing to its further metabolism. The R_f value from TLC analysis (0.85), and the UV spectrum (λ_{max} 270) of the isolate agreed well with the authentic sample of catechol. The bacterium

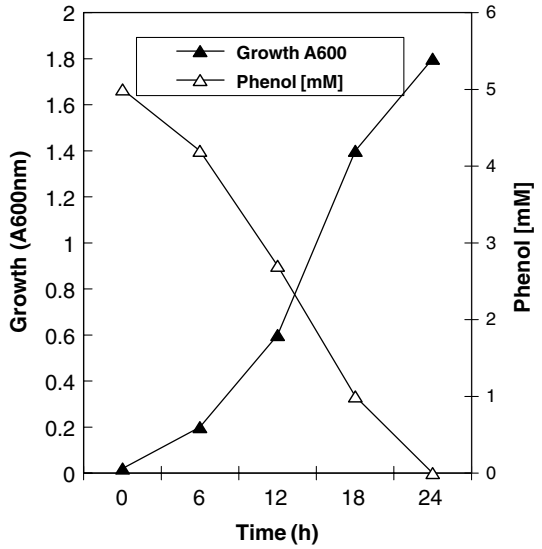

Figure 1. Growth response of *Arthrobacter citreus* and degradation of phenol.

was able to utilize other compounds including cresols and naphthol upto 5 mM concentrations from medium (Figure 2).

The degradation of phenol by free, alginate, and agar immobilised *Arthrobacter citreus* was compared (Figure 3.). The data obtained from the degradation of phenol with immobilized cells, suggest that the rate of degradation of phenol was unaffected on immobilization and these cells could be used continuously for one-week period with out loss of their bidegradative property.

Enzyme studies

The specific activities of phenol hydroxylase, catechol 1,2–dioxygenase and catechol 2,3–dioxygenase in cell free extracts were found to be 46, 2 and 54 IU respectively (Table 1). However, glucose grown cells did not possess these activities in appreciable quantity. Ring cleavage test revealed that catechol is further metabolized by *meta*-pathway.

Discussion

This study demonstrates that the bacterial strain, *Arthrobacter citreus* isolated from a petroleum contamination site is able to metabolize phenol in free, as well as aliginate and agar immobilized fermentation cultures. This follows that; the indigenous microflora already present at a polluted site can often adapt to degrade the contaminants if given sufficient time. Acquisition of degradative abilities by selective enrichment has been seen in laboratory ecosystems for many organic compounds and heavy metals (Alexander

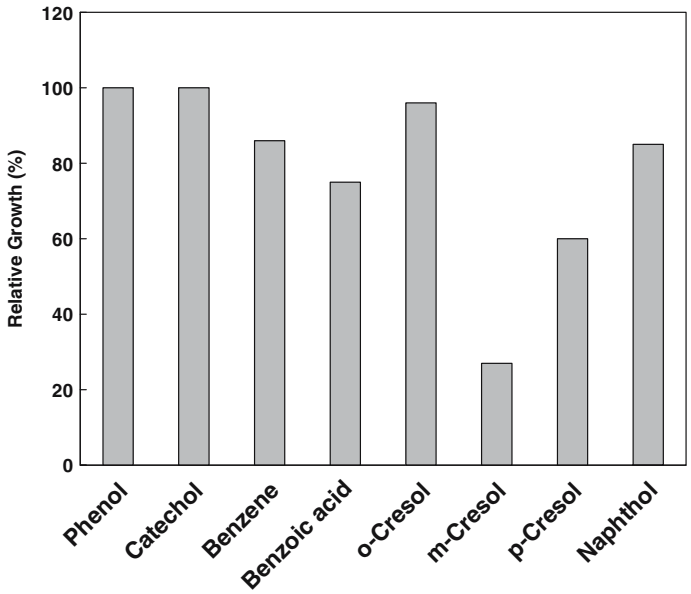

Figure 2. Utilization of various compounds as substrates by *Arthrobacter citreus.*

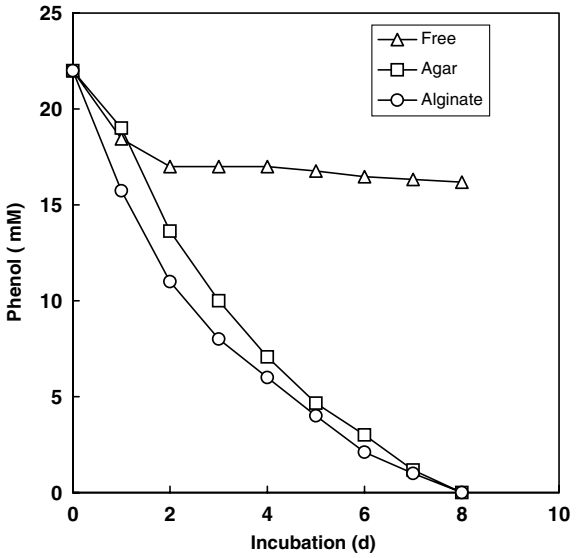

Figure 3. Rate of phenol degradation by immobilized *Artrobacter citreus*.

Table 1. Specific activities of enzymes involved in degradation of phenol by *Arthrobacter citreus*

Enzyme	Specific Activity[a]
Phenol hydroxylase	46
Catechol 1,2–dioxygenase	02
Catechol 2,3–dioxygenase	54

[a]Specific activity are derived from three independent sets of experiments and are expressed as n mol. min^{-1}. mg^{-1} of protein.

1999; van der Meer et al. 1992). In consistent with this observation a bacterial strain degrading phenol has been isolated from the contamination site adopting the selective enrichment technique. The other advantage of applying the bacterial systems for effluent remediation is that they possess a higher rate of biodegradation than the fungi (Prenafeta Boldu et al. 2001). In this study the bacteria degraded phenol without the lag phase. This is not unexpected, as the culture used is the one that has already been adapted to grow on phenol. A mixed culture which is known to degrade 4-chlorophenol showed a typical growth curve without the lag phase (Farrell & Quilty 1999). The slow growers often follow an initial lag phase due to the delay in the expression of the desired genes required for the succeeding phases of growth. The lag phase has been observed for the soil fungi *Cladophialophora* sp. degrading toluene, ethylbenzene, and xylene (Prenafeta Boldu et al. 2004).

The metabolism of phenol by the isolated strain occurred through the involvement of catechol as the key intermediate. It has been shown in general that, the compounds containing a hydroxyl group, such as phenols, salicylate and hydroxybenzoate, are monohydroxylated as the initial transformation (Häggblom, 1992, Mishra et al. 2001). The addition of a single hydroxyl group to an aromatic ring is typically performed by a monooxygenase enzyme, which reduces two atoms of dioxygen to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NAD(P)H (Häggblom 1992; Harayama et al. 1992). Monooxygenases, as well as dioxygenases, require cofactors capable of reacting with dioxygen as reactions occur directly between dioxygen and carbon in organic compounds (Harayama et al. 1992). This cofactor is commonly iron (Harayama et al. 1992). In this study, the isolation of metabolites by preparative TLC, and the cell free enzyme extract analysis demonstrated that phenol is initially hydroxylated by phenol hydroxylase to catechol, which is the substrate for the next catabolic enzyme catechol 2,3-dioxygenase. There are two major mechanisms that can be distinguished for ring cleavage of aromatics, and they are performed by enzymes that have evolved independently of one another (Eltis & Bolin 1996; Que & Ho 1996). The ring cleavage studies showed the operation of the *meta* cleavage pathway for the phenol degradation by *Arthrobacter citreous*. In extradiol or *meta*-cleavage reaction, the ring is opened just outside the hydroxyl groups. The product of *meta*-cleavage of catechol is hydroxymuconic semialdehyde. This ring-cleavage mechanism is commonly found in degradation of methylated monoaromatics, in degradation of PAHs and for cleavage of the first ring in PCB degradation (Mishra et al. 2001). The second major ring cleavage mechanism is called intradiol or *ortho* cleavage and takes place between two hydroxyl groups on an aromatic ring. The product of intradiol cleavage of catechol is *cis,cis*-muconate. *Ortho* cleavage is the dominant ring cleavage mechanism in degradation of chlorinated compounds, as extradiol cleavage of halocatechols may produce dead-end or suicide metabolites (Bartels et al. 1984; Häggblom, 1992; Ganji et al. 1993). After ring cleavage, the products are

transformed and funneled into the citric acid cycle, the central metabolic pathway for energy of the cell. *Meta* ring cleavage yields compounds such as pyruvate, acetaldehyde, oxaloacetate, malate or succinate semialdehyde, whereas *Ortho* cleavage of catechol yields products that are transformed into 3-oxoadipate, eventually yielding acetyl-CoA and succinyl-CoA (Harayama & Timmis, 1989). Thus phenol is expected to undergo complete mineralization through these sequences.

The use of biological systems for bioremediation is more cost-effective than traditional cleaning techniques such as waste incineration; possible savings have been estimated to 65–85% (Zechendorf, 1999). Among fungi, the white-rot fungi such as *Phanerochaete* and related genera have the ability to produce extracellular peroxidase enzymes that act on a variety of organic compounds (Alexander 1999; Cameron et al. 2000; Reddy & Gold 2000). Phytoremediation is a technique that employs plants to accumulate and eventually remove environmental contaminants. However, a disadvantage associated with fungal and plant bioremediation is that it tends to be slow (Alexander 1999). It is often possible to overcome the pollution problems using a variety of laboratory methods to obtain optimized microbial strains. Most of the studies so far carried out depend on the use of the cell free systems. The drawback with these systems is, that they fail to be viable for longer periods for the purpose of effluent treatment or industrial applications. In this context, the procedures involving cell immobilization can be a better alternative. Varieties of microorganisms have been immobilized by entrapment methods. The potential of immobilizing cells for industrial and biotreatment applications is of great value (Cheetam 1980: Bisping & Rehm 1988). Since the immobilized cells entrapped at the optimal conditions remain viable for long time they are better choice to the cell free systems. When bacteria are immobilized they also excel the disadvantages of fungal and plant bioremediation methods. In the present investigation, the bacterial strain *Arthrobacter citreus* on immobilzation in alginate and agar as matrices proved useful for developing the biotechnology for treating phenol-containing effluents. The immobilized cells could remove phenol for eight days in comparison to free cells which could perform this only for a day.

Conclusions

The microorganism isolated from contaminated site used phenol as sole source of carbon and energy. This was identified and characterized as *Arthrobacter citreus*. This mesophilic organism showed optimal growth at 25 °C and at pH of 7.0. The substrate concentrations up to 22 mM were well tolerated by *Arthrobacter citreus*. The organism had additional abilities to degrade catechol, cresols and naphthol. The isolation of catechol as a metabolic intermediate suggests that phenol is hydoxylated initially by a monooxygenase. This is further cleaved in a 2,3-dioxygenase mediated reaction, where it is metabolised by *meta*-cleavage pathway. Alginate and agar immobilised *Arthrobacter citreus* exhibited all the properties of free cells. Further, these cells were exceptional in being used continuously for a week with out the loss of their degradative ability. The study also reveals that *Arthrobacter citreus* can efficiently degrade phenol even at higher concentrations following immobilisation. Since the bacterium is capable of degrading phenol, cresols and naphthol there exist a possibility for its use in development of microbial technology for decontamination of phenolic and hydrocarbon wastes.

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