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Biodegradation of Phenolic Pollutants

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Abstract

Phenolic compounds are important constituents of petrochemical waste waters arising from transformation processes. Phenol together with substituted halophenols, nitrophenols and alkyl phenols are the main constituents. An *Arthrobacter citreus* spp. strain MSA - 2005 initially selected for growth at the expense of phenol as the sole source of carbon and energy, was enriched with phenolic compounds such as 4-nitrophenol, 4-chlorophenol and 4-methyl phenol. Metabolization of these compounds upto their maximum levels in the mineral salt medium was studied. The complete metabolization of 4-nitrophenol upto 15mM, 4-chlorophenol up to 12mM and 4-methyl phenol upto 6mM against phenol upto 22mM was achieved in 24 hours. The metabolism of these compounds by the isolated strain occurred through the involvement of catechol derivatives as key intermediates which are further hydrolysed to hydroxy quinol which enters the β-keto adipate pathway. Results indicate that the cells contain mono and di-oxygenase enzymes necessary for these conversions. The degradation rates of these phenolic compounds, by agar immobilized cells has also been reported.

Keywords: Phenols degradation, *Arthrobacter citreus*, immobilization

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Introduction

Aromatics, halo-aromatics, nitro-aromatics, polyacrylic aromatics are found to be environmental contaminants. Among the aromatics, phenols and their derivatives such as nitrophenols and chlorophenols have been labelled as priority pollutants. The phenolics comprise characteristic pollutants in waste waters and effluents discharged from petrochemical, textile, tannery and coal gasification units [1]. These are toxic and recalicitrant even at low levels and hence pose a threat to the biosphere.

Bioremediation exploits the catabolic contaminants into eco-friendly compounds. Diverse microorganisms, including many species of bacteria and fungi have evolved the metabolic capacities to degrade aromatics. The most prevalent aromatic degraders belong to the genera *Pseudomonas, Moraxella, Nocardia, Arthrobacter*. Various approaches are being developed to treat the phenolic effluents [2, 3]. The constrain is the availability of a suitable microorganism that can overcome their culturing limitations from their natural habitats to the effluent conditions.

An aerobic microorganism with an ability to utilize phenol as carbon and energy source was isolated from a contamination site by employing selective enrichment culture technique. The isolate was identified as *Arthrobacter citreus* species based on morphological, physiological and biochemical tests. The organism metabolized phenol upto 22mM concentrations. TLC, UV spectra and enzyme analysis were suggestive of catechol, as a key intermediate of phenol metabolism. The enzyme activities of the key enzymes in cell free extracts were indicative of operation of *meta*-clevage pathway for phenol degradation [4]. The organism had additional ability to degrade cresols, naphthol, benzene and benzoic acid.

The present study reports the use of *Arthrobacter citreus* strain in the degradation of 4-nitrophenol, 4-chlorophenol and 4-methyl phenol. This serves as an agent for biodegradation of pollutants and is capable of mineralizing phenolic compounds as the sole source of carbon and energy. The *Arthrobacter citreus* cells induced with phenol is capable of degrading 4-nitrophenol, 4-chlorophenol and 4-methyl phenol to 4-nitro catechol, 4-chlorocatechol and 4-

methyl catechol respectively which are further hydrolyzed to hydroxyquinol and then to β-ketoadipate. In this report we have an alternate pathway for the degradation of these phenolic derivatives by the strain *Arthrobacter citreus* through *meta* clevage.

The method of cell immobilization seems to be promising in the development of biotechnology for the removal of various xenobiotic bearing effluents [5]. Since the entrapped cells remain viable for a considerable duration, they would be

Better alternative against free cells for the bioremediation of variety of toxic aromatics from effluents. Immobilization of *Arthrobacter citreus* has also been carried out to show its applicability in the bioremediation of phenolics.

Materials and Methods

The bacteria were isolated from the soil collected from contaminated site. The organism was maintained and propagated on nutrient-agar and substrate-mineral salt media. For purification of the bacterial strain, the microorganism was grown on nutrient agar medium. For metabolic studies the bacterial strains were grown on mineral-salt medium (MSM) containing (g l^{-1}): K_2HPO_4, 1.6; KH_2PO_4, 0.2 ; $(NH_4)2\ SO_4$, 1.0 ; $MgSO_4$. $7H_2O$, 0.2 ; NaCl, 0.1 ; $CaCl_2$. $2H_2O$, 0.02 ; $FeSO_4$. H_2O, 0.01; Na_2MoO_4. $2H_2O$, 0.5; $MnSO_4.H_2O$, 0.5; $Na_2WO_4.2H_2O$, 0.5. The growth substrate (5mM phenol) was supplemented to the sterilised mineral salt medium aseptically. The flasks were then inoculated with 5% of inoculum aseptically and were incubated at 25°C (± 2°C) on a rotary shaker for 24 hours. Uninoculated flasks were incubated in parallel as controls.

Growth study of the organism

The bacterial cells isolated by substrate enrichment culture were used for the growth study. Various phenolic compounds served as source of carbon and energy. The cells were freed from adhering substrate by centrifugation (5000 rpm) at 5° for 20 min. The cells were repeatedly washed with 0.05M phosphate buffer (pH 7.0) and centrifuged. The cell pellet obtained was finally resuspended in the sterile mineral-salt medium. Suitable aliquots (2.0ml) of this cell

suspension were inoculated to the flasks containing different phenolic compounds (5mM each of 4-nitrophenol, 4-chlorophenol and 4-methyl phenol). The growth of the organism was measured turbidometrically by monitering the OD at 600nm at different incubation periods. 4-nitrophenol, 4- chlorophenol and 4-methyl phenol utilization was followed by estimating residual substrate colorimetrically at 600nm.

Characterization of metabolites

In order to know the microbial degradative patterns, the bacteria were grown on MSM containing the substrate. Substrate grown cells were transferred to 500ml Erlenmeyer flask containing 100 ml of 0.05M phosphate buffer (pH 7.0). The spent media was examined for the accumulation of metabolic intermediates at regular intervals. The metabolic intermediates were isolated from the spent broth using organic solvent. It was performed on silica gel plates (0.25mm thickness using 2-propanol; Ammonia; water (20:1:2 V/V) solvent system. The spots on the chromatogram were visualized by exposing the TLC plates to iodine vapours.

The metabolite extract of the replacement culture was characterized by spectral methods using methanol as the solvent.

Degradation of phenolic compounds

The ability of the organism to degrade of 4-nitrophenol, 4-chlorophenol and 4- methyl phenol was determined by solvent extraction of the residual 4-nitrophenol from the spent medium at specific time intervals. Growth was measured by determining OD at 600nm with SL 159, ELICO Spectrophotometer.

The extract was dried and the metabolites were purified by TLC and characterized by UV-Spectroscopy.

Immobilization

Immobilization is the technique of binding biocatalysts to a carrier as a means of increasing their activity and stability, improving the technological application of the reaction. Enclosure in organic gel forming matrices such as calcium alginate, agar are the possible means of immobilization [6].

Agar entrapment of cells was carried out in sterile 4% (w/v) agar saline solution. 12 hour bacterial cell suspension was mixed with 10ml of saline and extruded into sterile agar-saline solution to obtain cell-entrapped beads. These agar beads were washed repeatedly with distilled water and used for further applications.

Batch fermentation was performed for both freely suspended cells and immobilized cells. About 50g of beads with cells and without cells were taken separately along with 50ml of 0.1M phosphate buffer (pH 7.0) to which the phenolic compounds were added. The fermentation process was carried out along with a control with 50ml of buffer and the substrate used at room temperature on a rotary shaker at 150rpm for four hours. Samples of the culture broth were taken every hour for the analysis of the substrate. The experiment was repeated using 4-nitrocatechol, 4-chlorocatechol and 4-methyl catechol as substrates. The formation of catechol derivatives due to the degradation of phenolic derivatives and subsequent degradation of the same was checked by TLC.

Results and Discussion

Description of the organism

Based on phenotypic characteristics such as cell wall type, morphology, motility and nutritional requirements the bacteria capable of degrading phenolics is classified as *Arthrobacter citreus* strain. The strain growing on phenolics is Gram positive, exhibited typical rodcoccus transition, aerobic, motile and assimilated glucose, galactose, lysine, threonine, alanine and did not require vitamin. The organism is a mesophile and has optimal growth at pH 7.0 and at 25°C.

Degradation of phenolic compounds and identification of metabolites

Studies on the degradation of phenolic compounds by Arthrobacter citreus strain indicated that 4.2mM 4-nitrophenol, 3mM 4-chlorophenol and 1.8mM 4-methyl phenol utilization occurred in 24 hours. The organism tolerated 4-nitrophenol concentration upto 15mM, 4-chlorophenol concentration upto 12mM and 4-methyl phenol concentration upto 6mM. TLC analysis and

spectrophotometric analysis of the metabolic intermediates suggest that 4-nitrophenol is degraded to 4-nitrocatechol, 4- chlorophenol to 4-chloro- catechol and 4-methyl phenol to 4-methyl catechol and then to hydroxyquinol owing to further metabolism. The Rf value and the UV spectrum of the metabolite agreed well with the authentic sample of 4-nitrocatehol, 4-chlorocatechol and 4-methyl catechol as intermediary metabolites in the metabolism of the phenolic compounds. Replacement culture methods also support the role of 4-nitrocatechol, 4- chlorocatechol and 4-methyl catechol as intermediary metabolites in the metabolism of phenolic compounds.

Results presented here indicate that at least one monooxygenase enzyme is able to hydroxylate substituted phenols. The product of the initial reaction undergoes an oxygenase catalyzed removal of the substituent group forming hydroxyquinol. Hydroxyquinol undergoes ring fission to maleylacetic acid, which can be readily degraded via β-Ketoadipic acid. Studies with cell extracts clearly show that a dioxygenase enzyme catalyses the subsequent conversion of hydroxyquinol to maleylacetic acid. The *Arthrobacter citreus* studied here appears to use the monooxygenase catalyzed reaction since it is clear that the system converts 4-nitrocatechol, 4-chlorocatechol and 4-methyl catechol to hydroxyquinol.

This study demonstrates that the bacterial strain, *Arthrobacter citreus* isolated from the contamination site is able to metabolize phenolics in free as well as agar immobilized fermentation cultures. In mixtures the phenol induced organism preferred 4-nitro phenol over 4-chlorophenol and 4-methyl phenol.

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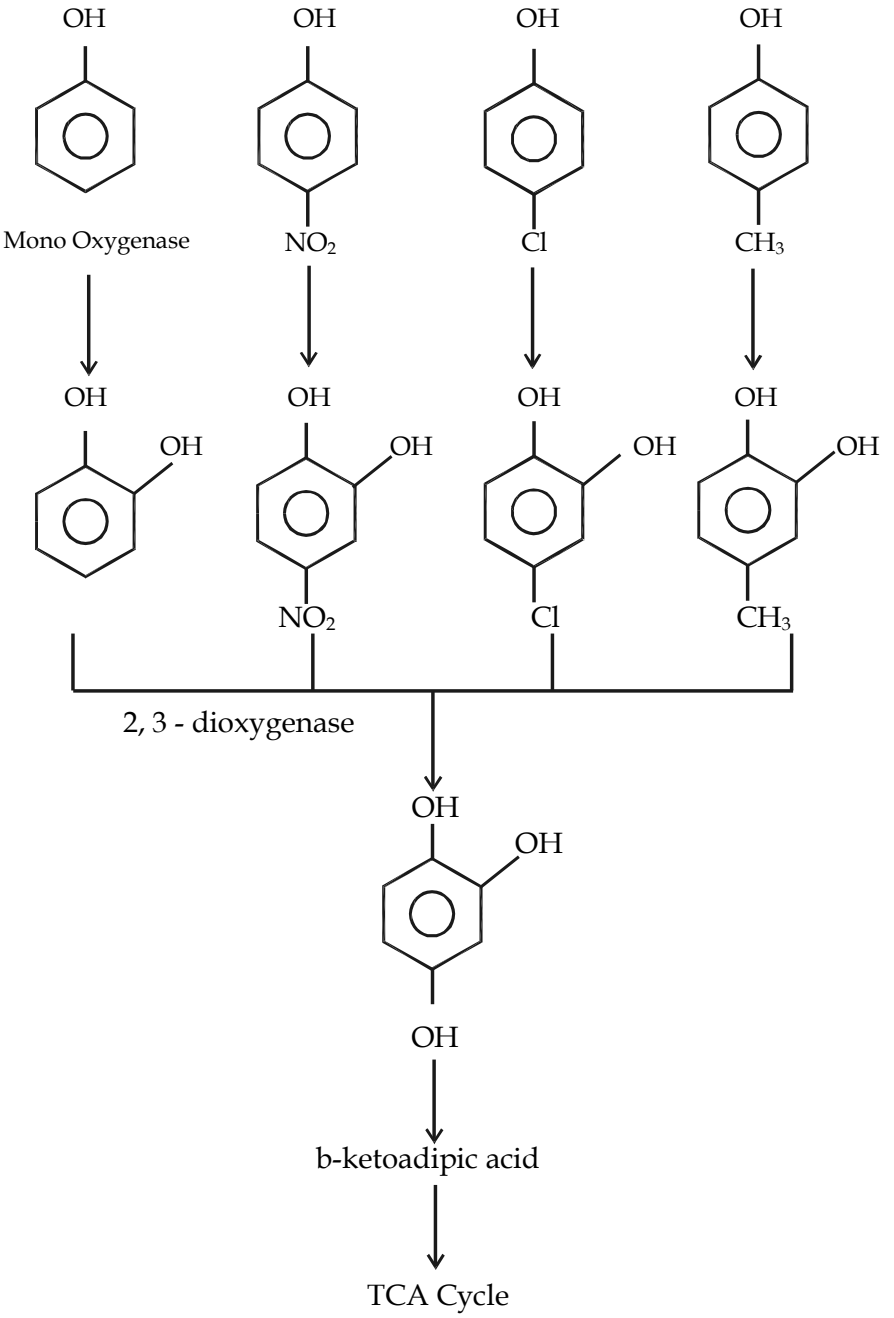

Proposed pathway for the degradation of phenolics by *Arthrobacter citreus*

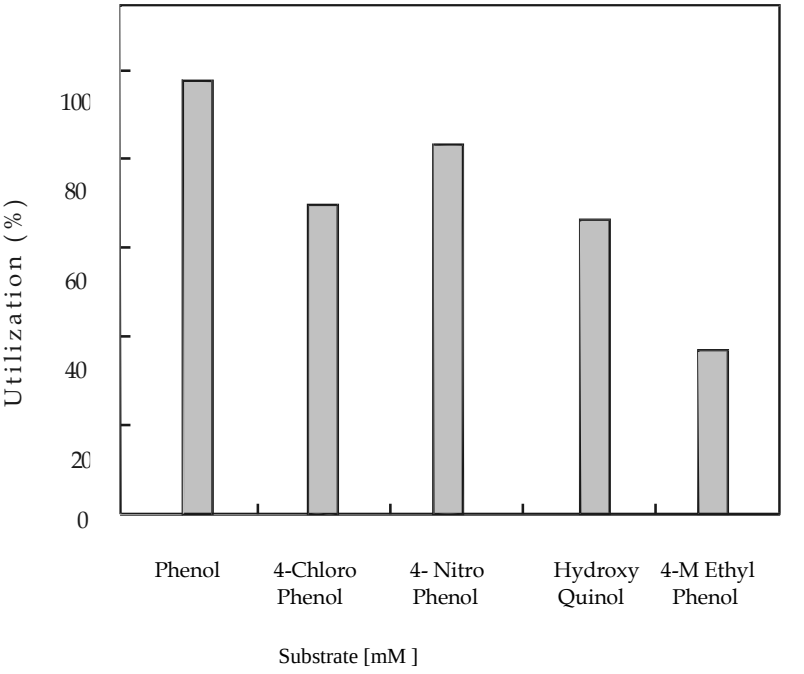

Fig.-1: Utilization of phenolics as substrates by *Arthrobacter citreus*

Conclusion

The present study reports that the isolated, *Arthrobacter citreus* strain which has already been adapted to grow on phenol has an additional ability to degrade 4- nitrophenol, 4-chlorophenol and 4-methyl phenol. The organism also has additional capacity to utilize benzene, benzoic acid and naphthol as substrates.

The metabolism of the phenolic compounds by this strain occurred through the involvement of catechols as key intermediates. The isolation of hydroxyquinol as a metabolic intermediate suggests that the phenolic compounds are hydroxylated by a monoxygenase which are further cleaved in a dioxygenase mediated reaction where they are metabolized by β-keto adipate pathway.

The study reveals that *Arthrobacter citreus* strain can efficiently degrade the phenolic compounds even at higher concentrations. The bacteria can often adapt to degrade the contaminants if given

sufficient time. Since the organism is capable of degrading 4-nitrophenol, 4-chlorophenol, 4-methylphenol and other toxic aromatics, there is a high potential for its use in the development of microbial technology for bioremediation. Understanding the physiology and genetics of degradation of phenolics will be helpful in designing strategies for bioremediation of contaminated environments.

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References

[1] S. Shashirekha, L. Uma, and G. Subramanian, "Phenol degradation by the marine *Cyanobacterium Phormidium valderianum BDU 30501*," *Journal of Industrial Microbiology and Biotechnology,* vol. 19, pp. 130-133, 1997

[2] A. Krastanov, "Removal of phenols from mixtures by co-immobilized laccase/tyrosinase and Polyclar adsorption," *Journal of Industrial Microbiology and Biotechnology,* vol. 24, pp. 383-388, 2000.

[3] V. L. Santos and V. R. Linardi, "Phenol degradation by yeasts isolated from industrial effluents," *J Gen Appl Microbiol,* vol. 47, pp. 213-221, Aug 2001.

[4] C. Karigar, A. Mahesh, M. Nagenahalli, and D. J. Yun, "Phenol degradation by immobilized cells of *Arthrobacter citreus*," *Biodegradation,* vol. 17, pp. 47-55, Feb 2006.

[5] K. Murugesan, "Bioremediation of paper and pulp mill effluents," *Indian J Exp Biol,* vol. 41, pp. 1239-48, Nov 2003.

[6] S. S. Manohar and T. B. Karegoudar, "Degradation of naphthalene by cells of *Pseudomonas spp*. strain NGK 1

immobilized in alginate, agar and polyacrylamide," *Applied Microbiology and Biotechnology,* vol. 49, pp. 785-792, 1998.