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Biodegradation of high amounts of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol by *Aspergillus awamori* cells

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Abstract

The mycelium (or conidia) of *Aspergillus awamori* NRRL 3112 was investigated for its ability to degrade phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol in high concentrations. The biodegradation studies were performed in a liquid medium with the phenolic compounds as a sole carbon and energy source. The organism had mineralized phenol concentration of 0.3 g/l in 60 h, 0.6 g/l in 72 h and 1.0 g/l in 7–8 days. *A. awamori* had fully degraded catechol concentration of 1.0 g/l in 82 h, 2.0 g/l in 108 h and 3.0 g/l in 124 h. Five days are sufficient for complete biodegradation of 1.0 and 2.0 g/l 2,4-dichlorophenol. The higher concentration of 3.0 g/l was degraded at a rate of 85% for 6 days. The degradation of 2,6-dimethoxyphenol goes slow and only 1.0 g/l concentration was fully degraded for 7 days. In case of 2.0 and 3.0 g/l no complete degradation can be observed even after 8 days of the process. Successfully simulated phenols degradation profiles in all studies were obtained by Haldane-type kinetics. The values of endogenous or decay and yield coefficients for all phenols at different concentrations were also determined.
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Keywords: *Aspergillus awamori*; Biodegradation; Phenol; Catechol; 2,4-Dichlorophenol; 2,6-Dimethoxyphenol

1. Introduction

Phenol and phenolic compounds are well known components in a wide variety of waste waters including these from coal conversion processes, coking plants, petroleum refineries and several chemical industries, as pharmaceuticals, resin and dye manufactures [1,2]. Chlorophenols constitute a significant category of pollutants and are also major components of paper pulp bleach plant effluents. In addition 2,4-dichlorophenols have been extensively used as wood preservatives and pesticides and as precursors for the synthesis of herbicides [3]. Because of the improper treatment of these materials, they have widely contaminated soil and groundwater and their toxicity seriously affects living organisms.

The metabolism of aromatic compounds, particularly phenol and its derivatives, has been intensively studied in prokaryotic microorganisms. Chitra et al. [4] have studied the removal of phenol using a mutant strain of *Pseudomonas*. Pal et al. [5] have worked with the biodegradation of 2,4,6-trichlorophenol and 2,4,5-trichlorophenol by *Phanerochaete chrysosporium* in batch and as well as in continuous reactor systems. Bandhyopadhyay et al. [2] have used *Pseudomonas putida* to degrade phenol in water in the concentration range 100–1000 ppm. They observed that the inhibition effects became predominant above the concentration of 500 ppm. Annadurai et al. [6] have used the Box-Behnken design for the development of an optimized complex medium for phenol degradation using *Pseudomonas putida*. They have used four variables viz., maltose, phosphate, pH and temperature to identify the significant effects and interactions in the batch studies. Sa and Boaventura [7] have studied the biodegradation of phenol in a batch reactor using a pure culture of *Pseudomonas putida* DSM 548. They have conducted experiments to determine the kinetics of biodegradation. They have confirmed that the Haldane equation adequately describes the cell growth kinetics. Kavitha and Beebi [8] have studied the biodegradation of phenol by unadapted mixed microbial culture,

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isolated from soil, in a packed bed reactor using peat media. Reardon et al. [9] worked on the mathematical modeling of the mixed substrate kinetics of *Pseudomonas putida* F1 growing on benzene, toluene, phenol and their mixtures. They observed that benzene and toluene were better growth substrates than phenol, resulting in faster growth and higher yield coefficients. They reported that benzene and toluene biodegradation kinetics were well described by Monod model. Monod model was also used to characterize phenol degradation with a small degree of substrate inhibition. They concluded through their experiments that phenol had little effect on the biodegradation of either toluene or benzene.

After reviewing the literature on the degradation of phenol, it was found that most of the work was done using bacterial strains and some yeast [10,11]. The use of fungal strains for the degradation is relatively untouched area. Mycelial fungi such as *Fusarium floccieferum* [12], *Aspergillus fumigatus* [13] and *Graphium* sp. [14] have been cited for their potential for phenol degradation. Little is known about phenol metabolism in mycelial fungi. In all of these studies, phenol was metabolized by the β-ketoadipate pathway, through *ortho*-fision of catechol.

Very little is known for the degradation of phenol derivatives, especially chlorophenols by fungi. The fungus *Phanerochaete chrysosporium* was found to be able to degrade ρ-cresol, phenol [15], 2,4-dichlorophenol [16] and 2,4,5-trichlorophenol [5]. Degradation of catechol and 2,6-dimethoxyphenol was not studied. The results for the capabilities of microorganisms, especially fungi to degrade high amount of phenol and its derivative are not sufficient.

Fungal whole cells are gaining importance for their use in the waste water treatment systems and the potentiality of a fungal strain *Aspergillus awamori* to degrade high amount of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol is taken up for this study. Also the microorganism growth kinetics using Haldane's growth model was investigated.

2. Materials and methods

2.1. Microorganism and growth medium

A strain of *A. awamori* NRRL 3112, obtained from US Department of Agriculture, Illinois, USA was used through out this study. The organism was grown on slants on a medium of the following composition—malt extract 3.0 g/l, yeast extract 3.0 g/l, peptone 5.0 g/l, glucose 10 g/l and agar 20 g/l. The organism on the slants was allowed to grow for 72 h at 30 °C and then stored at 4 ± 1 °C for further use.

2.2. Medium for degradation studies

The studies on the biodegradation of phenol were carried out in the Czapekdox medium, which had the following composition (g/l) sodium nitrate 2.0, potassium phosphate (dibasic) 1.0, potassium chloride 0.5, magnesium sulfate heptahydrate 0.5, and ferrous sulfate heptahydrate 0.01. The initial pH of the medium was adjusted to 5.5 using 1 N NaOH or 1 N HCl. The minimal medium consist as a sole carbon source 2,4-dichlorophenol, 2,6-dimethoxyphenol and catechol in concentrations of (g/l) 1.0, 2.0 and 3.0 and phenol in concentrations of (g/l) 0.3, 0.6 and 1.0.

2.3. Growth of the organism

The 14-day culture in spore form, from the slants was used as inoculum for the liquid medium (1×10^5 conidia/ml medium). To find out the growth phases of the organism, the flasks containing 50 ml liquid medium were inoculated with equal volume of inoculum (1×10^5 conidia/ml medium) and agitated on a shaker (240 rpm) at 30 °C. Samples were taken at every 12 h interval and centrifuged at 5000 rpm for 20 min to separate the cells. The wet weight of the cells was determined. The number of conidia in the samples was counted by a standard microscope method.

2.4. Analytical methods

The dry weight of the cells was determined by ULTRA X apparatus for drying.

The content of phenols was determined by using Folin-Ciocalteu reagents and confirmed by HPLC. A 1 ml of the sample or the standard solutions was added to 10 ml distilled water and 1.0 ml of Folin-Ciocalteu reagents. The mixture was then allowed to stand for 5 min and 2.0 ml sodium carbonate was added to the mixture. After 1 h in a dark place the absorbance at 750 nm was measured. The HPLC analyses were performed in C_{18} 10 μm Bondapac Column (3.9 mm × 300 mm) and Waters 484 UV detector (260 nm). The mobile phase was methanol–water (70:30), flow rate 0.2 ml/min and 22 °C.

Protein concentration in supernatant was determined by the method of Lowry with bovine serum albumin as protein standard.

2.5. Enzyme assay

Enzyme activities were determined in cell-free extracts and in the liquid phase of the culture media after 24 and 72 h of degradation process. Cells were harvested, washed twice in 50 mM Tris–SO_4 buffer, pH 7.5 and broken by grind and then cell debris were removed by centrifugation at 5000 g for 20 min. The cleared supernatant solution was used for both enzyme and total protein assays.

Phenol hydroxylase (EC 1.14.13.7.) activity was assayed spectrophotometrically (LKB UV–vis Ultraspec 1000), following NADPH absorbance at 340 nm [17]. The activity of catechol 1,2-dioxygenase (EC 1.13.11.1.) was determined by measuring the rate of *cis,cis*-muconic acid accumulation at 260 nm [18]. One unit of activity is defined as the amount of enzyme transforming 1 μmol of substrate in 1 min under assay conditions. Specific activities were expressed as units (U) per mg total protein.

3. Result and discussion

3.1. Degradation studies

Microorganisms capable of degrading one aromatic compound are often able to degrade other similar compounds. In a previous work [19] *A. awamori* NRRL 3112 utilized phenol at concentration up to 1000 ppm for its development. Recent investigations also showed that *A. awamori* could grow in a minimal medium comprising except phenol also some phenol derivatives (2,4-dichlorophenol, 2,6-dimethoxyphenol and catechol) as a sole carbon source. The experiments were carried out in order to determine the capability of this microorganism to grow and degrade high amounts of these phenolics and to study the microorganism growth kinetics using Haldane's growth model. The synthetic media supplemented with phenolic derivatives (up to 3.0 g/l) were used for the cultivation and degradation analyses.

The phenol degradation data at initial concentrations of 0.3, 0.6 and 1.0 g/l are given in Fig. 1. Experiments conducted with phenol as a sole carbon source indicated that the organism was capable of utilizing phenol as a source of carbon for its growth up

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