

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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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 flocciferum* [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*-fission 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 *p*-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 C₁₈ 10 μ m Bondapac Column (3.9 mm \times 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–SO₄ 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 μ mol 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

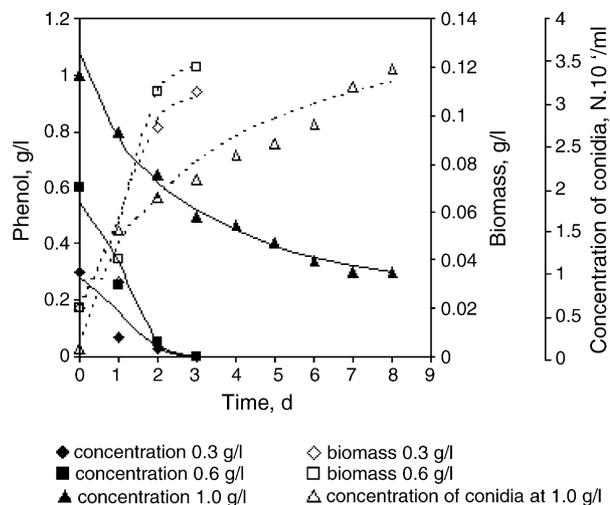


Fig. 1. Experimental data and Haldane model for growth and phenol degradation process of *Aspergillus awamori* NRRL 3112.

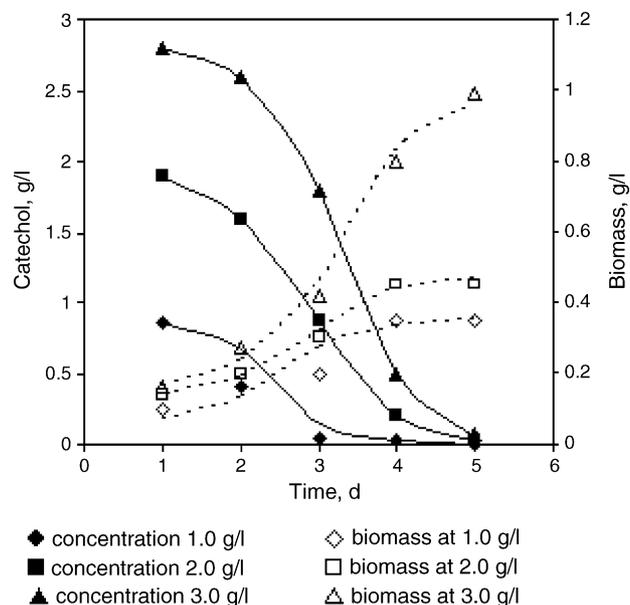


Fig. 2. Experimental data and Haldane model for growth and catechol degradation process of *Aspergillus awamori* NRRL 3112.

to concentration of 0.6 g/l. Above this concentration no mycelial biomass was formed. In case of 1.0 g/l concentration of phenol only conidia can occur in the culture media. The fact that the strain grows in a mycelial form at 0.6 g/l phenol and only in a spore form at 1.0 g/l clearly shows that the spore form of growth is a way of culture survival at toxic substrate concentrations. Data about the physiological activity of the spores of other fungi, capable of organic compounds transformation, were published [20]. The cells utilized phenol with varying rates depending on phenol concentration. 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. Beyond a phenol concentration of 1.0 g/l, the organism grows very slowly only in a spore form and beyond the concentration of 2.0 g/l the organism did no grow. This may probably be due to the inhibition caused by excessive phenol concentration. The rate of degradation decreased progressively with increase in phenol concentration above 0.6 g/l. Similar kind of observation was made by Santos et al. [14] and Kennes and Lema [15] using *Graphium* sp. and *Phanerochaete chrysosporium* respectively. They reported that the fungi were capable of degrading phenol up to a concentration of 0.3 and 0.05 g/l in batch system. However, in the present study, the cells could degrade phenol only up to a concentration of 1.0 g/l. Thus *A. awamori* NRRL 3112 can be compared with *Trichosporon cutaneum*, the most investigated microorganism among the eucaryotes for phenol degradation [11]. At concentrations of phenol under 1.0 g/l the lag phase of the growth was 24 h independently of the concentration, but the biomass at concentration of 0.6 g/l is higher than at concentration of 0.3 g/l (Fig. 1).

Similar kind of observation was made in case of catechol degradation. The results are presented in Fig. 2. The lag-phase of as long as 24–30 h had been reported to occur during degradation of catechol in case of all used concentrations (1.0, 2.0 and 3.0 g/l). After this period the specific growth rate was found to increase with the increase of substrate concentration. Thus at the end of the process the mycelial biomass formed at 3.0 g/l catechol is three times more than in case of 1.0 g/l catechol used.

At each of the initial concentrations there was a period of exponential growth when the substrate was being consumed at faster rate. *A. awamori* had fully degraded catechol concentration of 1.0 g/l in 82 h, 0.2 g/l in 108 h and 3.0 g/l in 124 h (Fig. 2). The versatility of *Rhizobium* sp. to degrade catechol is cited [21] but at initial concentration much lower than applied in this study. Kumar et al. [22] also reported degradation of catechol by *Pseudomonas putida* at concentration of 0.5 g/l for 95 h. It should be emphasized that *A. awamori* NRRL 3112 grow very well with formation of mycelial biomass at very high catechol concentration (3.0 g/l) as a sole carbon source and it is able to fully degrade this amount of catechol.

The biodegradation of 2,4-dichlorophenol and 2,6-dimethoxyphenol were also conducted. The results are shown in Figs. 3 and 4, respectively. Both substrates are used at initial concentration of 1.0–3.0 g/l. 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 (Fig. 3). 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 (Fig. 4). It was found that 24 h are needed to start the process of degradation at the highest concentrations of phenolic compounds. This time is required for adaptation of the microorganism to this very high phenolic concentration and no mycelial biomass was formed. Only increase in the number of conidia occurred in case of all concentrations tested for phenolic compounds.

In case of all investigated compounds it was also observed that towards the end of the substrate consumption curve, there is a region of relatively less rate of substrate degradation. The fall in pH of the solution may be the reason for that. Decrease in pH of the solution had previously been reported when phenol was metabolized by mixed culture [23]. Low values of both

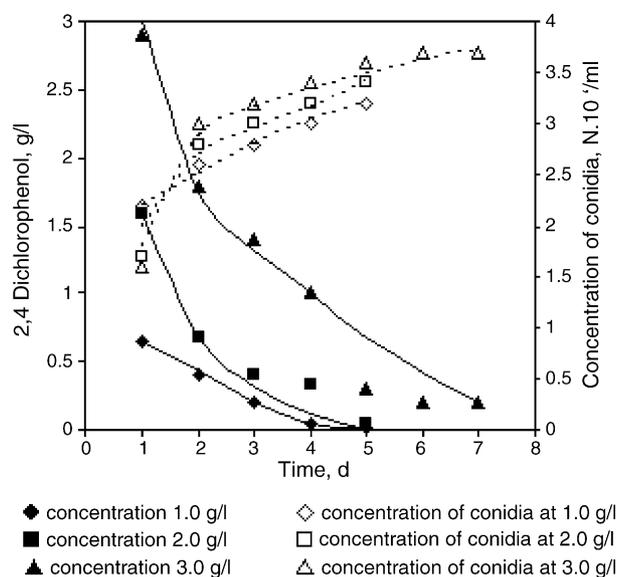


Fig. 3. Experimental data and Haldane model for growth and 2,4-dichlorophenol degradation process of *Aspergillus awamori* NRRL 3112.

oxygen and pH may affect the kinetics of substrate consumption adversely [24].

3.2. Growth kinetics

Models of the cultivation process of mycelial fungi in media, comprising phenols as a sole carbon source, however, have not been found in the available literature. For these reasons, several single-substrate inhibition models (Edwards, Andrews, Haldane, and Aiba) were investigated in order to determine the mathematical expression of *A. awamori* growth rate.

According to the research reports on similar batch cultivation processes [25] and nonlinear analysis of the experimental data, Haldane-type inhibitory growth kinetics was considered to be

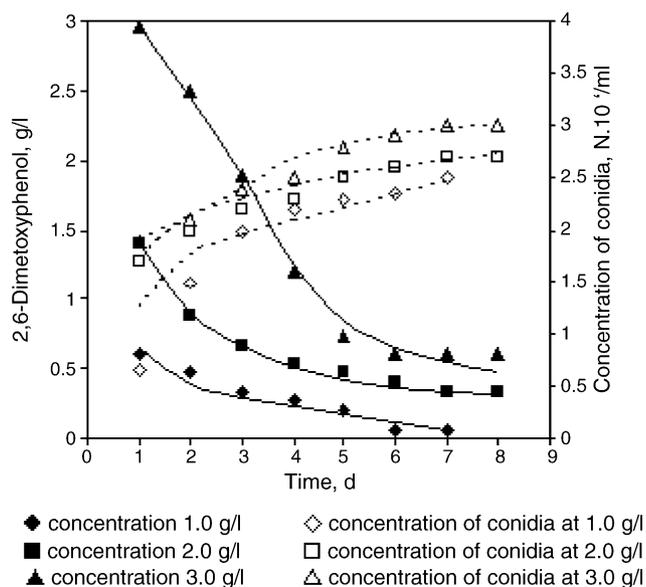


Fig. 4. Experimental data and Haldane model for growth and 2,6-dimethoxyphenol degradation process of *Aspergillus awamori* NRRL 3112.

the best fit mathematical model for the present study:

$$\frac{dX}{dt} = \mu X, \quad \frac{dS}{dt} = -k \frac{dX}{dt} \quad (1)$$

$$\mu = \frac{\mu_{\max} S}{K_s + S + (S^2/K_i)} \quad (2)$$

where μ_{\max} is the maximum specific growth rate; K_s the saturation coefficient; K_i the inhibition coefficient; k denotes the proportional coefficient of phenol utilization to cell growth. The unknown kinetic coefficients (μ_{\max} , K_s , K_i and k) were evaluated for each investigated phenolic compound and concentration using a nonlinear optimization approach. The specific degradation rate of the substrate ($Q \text{ h}^{-1}$), was determined as follows:

$$\frac{dS}{dt} = QX \quad (3)$$

where S and Q are the substrate concentration and the specific degradation rate, respectively.

In this study, the unknown parameters were estimated using the optimization procedure for direct search by the method of Hooke and Jeeves [26] where the parameter identification problem was reduced to minimization of a given performance index:

$$J = \int_{t_0}^{t_f} [(X_{\text{mod}} - X_{\text{exp}})^2 + (S_{\text{mod}} - S_{\text{exp}})^2] dt \quad (4)$$

where X_{mod} , S_{mod} and X_{exp} , S_{exp} are the model and experimental data for biomass and substrate concentrations respectively; t_0 the initial process moment and t_f is the final process moment. For that purpose the experimental data were approximated with spline functions from third order. The nonlinear optimization procedure is highly sensitive to the initial values and the variation intervals of the model parameters. For this reason, the search for the values of the kinetic constant was constrained within boundaries predetermined on the basis of the process knowledge and experimental data. Kinetic model on growth and degradation of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol at all tested concentrations by *A. awamori* NRRL 3112 were similar to those obtained from the experiments (Figs. 1–4).

The model kinetic coefficients of the growth and phenols degradation processes of *A. awamori* NRRL 3112 strain are given in Table 1. Q and K were calculated only in case of mycelial biomass formation when the microorganism grows on phenol and catechol containing media. The highest specific rate of degradation (Q) was observed when the *A. awamori* grows in a media containing 0.6 g/l phenol and 2.0 g/l catechol. The data presented in Table 1 showed no direct correlation between efficiency of phenol and catechol degradation and the biomass productivity. Similar observation was made by other authors [25] in case of phenol degradation by *T. cutaneum* strains. This was considered to be due to the re-directing of phenol and catechol degradation derivatives to metabolic pathways other than those directly involved in biomass formation. Analogous suggestions have already been reported for *Aspergillus fumigatus* [13] and have been supported by HPLC analysis in our study (data not shown because of unknown intermediate products formation during degradation process).

Table 1
Comparison of the model kinetic parameters of growth and different phenols degradation of *Aspergillus awamori* NRRL 3112

Substrate	Concentration (g/l)	μ_{\max} (h ⁻¹)	K_s (g/l)	K_i (g/l)	K (g/g)	Q (h ⁻¹)
Phenol	0.3	0.2328	1.013	1.023	0.131	0.03381
	0.6	0.1116	0.916	1.088	0.229	0.0589
	1	0.0169	1.006	1.069	–	–
Catechol	1	0.0632	0.988	0.994	0.133	0.0239
	2	0.0472	0.906	0.986	0.238	0.0402
	3	0.0406	1.056	1.093	0.139	0.0278
2,4-Dichlorophenol	1	0.0261	0.986	1.015	–	–
	2	0.0273	0.985	0.942	–	–
	3	0.0115	0.986	1.015	–	–
2,6-Dimethoxyphenol	1	0.0251	0.944	1.041	–	–
	2	0.0058	1.030	0.917	–	–
	3	0.0056	0.986	1.015	–	–

The increase in the concentration of all substrates leads to a decrease in μ_{\max} (Table 1). This may be considered an indirect indication for growth inhibition at some value of S for each of the phenolic compounds.

The analysis of $\mu(t)$ and $\mu(S)$ reveals the presence of a peak value in the growth rate at 2.0 and 3.0 g/l catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol. However, at substrate concentration of 1.0 g/l no peak in the growth rate occurs.

Apparently the critical concentration of the tested phenolic compounds, at which inhibition is observed, is within 1.0 and 2.0 g/l.

Since $S(0)$ and K_i have approximately the same value at low substrate concentrations (1.0 g/l), the squared term in (2) weakly effects μ values. Though K_i is not significantly changed at higher initial concentrations, the effect of S^2/K_i increases four-folds at 2.0 g/l and nine folds at 3.0 g/l. The higher effect of the squared term explains the presence of an extremum of the function $\mu(S)$.

The close values of K_s (Table 1) evaluated for all tested phenolic compounds, indicate that the cell growth is limited at initial concentrations of about 1.0 g/l in spite of the differences in the chemical composition and structure of the substrates.

The values of K and Q increase up to 2.0 g/l substrate concentrations. When 3.0 g/l catechol was used a decrease in their values is noticed.

No growth inhibition was detected when phenol was applied as a substrate.

3.3. Enzyme activities

The present study demonstrates that *A. awamori* NRRL 3112 has all the properties of an efficient phenol-degrading microor-

ganism. The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s).

Little is known about phenol metabolism in mycelial fungi [13,14]. In most of these studies phenol was metabolized by the β -ketoadipate pathway, through *ortho*-fission of catechol. The activities of the first two enzymes degrading phenol were investigated in this study. Mycelia grown by 24 h on 0.6 g/l phenol exhibited extra cellular phenol hydroxylase (0.35 U/mg protein) activity. No phenol hydroxylase activity was found in case of the other phenolic substances. The specific catechol 1,2-dioxygenase activities in cell free extract and culture media are shown in Table 2. The intracellular activity was observed after 24 h of the process when the phenol hydroxylase was secreted in the media. After this period the catechol 1,2-dioxygenase is also secreted in the culture media and it is obviously that in case of catechol degradation this activity is twice more than in case of phenol degradation (Table 2). The 2-hydroxymuconic semi-aldehyde, the product of catechol 2,3-dioxygenase action on catechol oxidation, was not detected by HPLC analysis used in this study. Catechol was oxidized by means of the *ortho*-pathway and then cleaved by intradiol mechanism leading to 3-oxoadipate. It seems that this mechanism of phenol and catechol degradation is preferable for the mass of the fungi investigated [13,14,25]. This mechanism may contribute to metabolic adaptation of ubiquitous fungus found in nature, such as *Graphium*, *Aspergillus* and *Penicillium*, exposed to xenobiotic and aromatic compounds.

Presence of catechol 1,2-dioxygenase activity in the culture media containing 2,4-dichlorophenol (Table 2) indicates that the degradation pathway of this compound is different from that, proposed for *Phanerochaete chrysosporium* [16]. Neither of the both enzyme activities tested in this study was found in

Table 2
Catechol 1,2-dioxygenase activity in the cell-free extract and culture media of *Aspergillus awamori* NRRL 3112 grown on different phenolic compounds

Phenols	Specific intracellular activity (U mg ⁻¹ protein)	Extra cellular activity (U ml ⁻¹)	Specific extra cellular activity (U mg ⁻¹ protein)
Phenol	0.065	0.004	0.067
Catechol	0.120	0.008	0.122
2,4-Dichlorophenol	–	0.013	0.043
2,6-Dimethoxyphenol	–	–	–

culture media during the degradation of 2,6-dimethoxyphenol. The above proposed mechanism is also inapplicable in case of degradation of this compound by *A. awamori* NRRL 3112. The data obtained in this study are not sufficient to explain the exact mechanism of degradation of 2,4-dichlorophenol and 2,6-dimethoxyphenol by the investigated strain of *A. awamori*. This will be the objective of the future work with this strain.

4. Conclusions

Our work shows that *A. awamori* NRRL 3112 has a great potential for biodegradation of phenol, catechol, 2,4-dichlorophenol, 2,6-dimethoxyphenol and possibly other related aromatic compounds, at very high concentrations. From the data presented in Figs. 1–4 it can be concluded that the applied model is appropriate to give a good description of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol degradation profiles for *A. awamori*, even in case of spore form growth. Besides, such mathematical models could successfully be used to predict the rate of phenols biodegradation under industrial conditions. The values of endogenous or decay and yield coefficients for all phenols at different concentrations were also determined. The above parameters are invariably required for the design and simulation of batch and continuous bioreactors treating phenolic wastewaters. This potential strain of *A. awamori* NRRL 3112 will be further studied for possible utilization on industrial effluent treatment and decontamination of natural areas.

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