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BIODEGRADATION OF MIXED PHENOLIC COMPOUNDS BY A MICROBIAL ASSOCIATION OF *ASPERGILLUS AWAMORI* AND *THERMOASCUS AURANTIACUS*

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ABSTRACT

Biodegradation of four mixtures of phenolic compounds as carbon sources achieved by a microbial association between *Aspergillus awamori* and *Thermoascus aurantiacus* had been studied: phenol + 2,4-dichlorophenol, phenol + 2,6-dimethoxyphenol, phenol + diphenylamine, phenol + catechol. The total concentration of phenolic compounds in each mixture was 0.4%, of which 0.2% was phenol and 0.2% - the second phenolic derivative. On a medium comprised of phenol + 2,6-dimethoxyphenol a positive interaction between the two cultures was observed, as demonstrated by the increased number of conidia in the mixed medium, outnumbering conidia count in the respective monocultures, the synergistic laccase production in the mixed medium, and the greater degree of biodegradation of the phenolic mixture as opposed to that achieved by the monocultures alone – 1.7 times bigger than *T. aurantiacus* and 2.5 bigger than *A. awamori.* On a medium containing phenol + 2,4-dichlorophenol the two species established a neutral type of interaction, while for the remaining experimental media the two microbial populations were involved in competitive interactions.
A 64 % biodegradation of 4.0 g/l mixture of phenol and 2,6-dimethoxyphenol was achieved by the microbial association between *T. aurantiacus* and *A. awamori.*

KEYWORDS
Aspergillus awamori, Thermoascus aurantiacus, microbial association

INTRODUCTION

Bioremediation technologies most often take advantage of the ability of various bacteria to clean the environment, this being the reason why scientific information about bacterial bioremediation is constantly expanding, while insufficient attention is still paid to bioremediation conducted through yeasts and fungi. Fungi are famous for their wide incidence and the outstanding capacity of degrading complex and inert natural products like lignin, chitin and cellulose. They adapt more easily than bacteria and are capable to grow in extreme conditions, like nutrient deficiency, low pH, limited water supply, etc. [1]. And not on the least, there comes the ability of fungi to survive in the presence of various xenobiotics that turn to be toxic to a number of other microorganisms.

Metabolism of aromatic compounds, and in particular phenol and its derivatives, has been extensively researched in prokaryotic microorganisms [2]. Particularly huge information have been piled up about bacterial species of *Pseudomonas* genus [3,4]. Few are the yeast species pointed out as capable of biodegrading mono-aromatic compounds. The parallel between the major stages of aromatic compounds` degradation by bacteria and fungi reveals that different enzymes are involved in the initial stages of biodegradation, but in both cases the process ends by the formation of a limited number of medial metabolites, like protocatechuates and catechols. All these hydroxylated intermediates are disintegrated by *ortho-* or *meta-* degradation pathways. Both degradation mechanisms lead to the formation of metabolites belonging to central cell metabolism. This general scheme of aromatic compounds` catabolism demonstrates that microorganisms have extended their range of substrates through the development of enzymatic systems capable of transforming untraditional substrates into some of the common central metabolites. The specified mechanism contributes to the metabolic adaptation of natures` widespread fungi, which are often isolated from soil, vegetable refuse, industrial and household waste. Literature sources describe a number of individual representatives of the genera *Candida*, *Rodotorula* and *Trichosporon*, which are capable of metabolizing aromatic compounds [5,6,7]. The specific enzymes responsible for biodegradation occupy an important place in these investigations. There are certain studies attesting the ability of strains from *Penicillium*, *Aspergillus*, *Fusarium*, *Graphium* and *Phanerochaete* genera to disintegrate aromatic compounds [8,9,10]. V. Santos & V. Linardi (2004) report about isolation and examination of 30 fungal strains with respect to their phenol tolerance. In 15 strains belonging to *Fusarium, Aspergillus, Penicillium* and *Graphium* genera the presence of phenol hydroxylase and catechol 1,2-dioxygenase activity in cells cultivated on a phenol-containing medium has been confirmed. These findings demonstrate that catechol oxidation follows the *ortho* – pathway of breaking the aromatic ring.

The task of the current study was to examine the interactions between two fungal species on media containing mixtures of phenol and phenolic derivatives under growing conditions that provide a greater degree of phenolic derivatives' biodegradation.

MATERIALS AND METHODS

Microorganisms

Aspergillus awamori NRRL 3112 was provided by the Department of Agriculture, Illinois, USA; *Thermoascus aurantiacus* belonged to the collection of the Department of Biotechnology, University of Food Technologies, Plovdiv. Both strains were selected by adaptive development on phenol-containing media and were kept on malt agar, with *Aspergillus awamori* developing for 72 hours and *Thermoascus aurantiacus* - for 144 hours at 30 °C.

Biodegradation media

Biodegradation was conducted on 4 combined media, containing the following ingredients as single carbon and energy sources: phenol + 2,4-dichlorophenol, phenol + 2,6-dimethoxyphenol, phenol + catechol, phenol + diphenylamine. The total concentration of phenolic compounds in each mixture was 0.4%, of which 0.2% phenol and 0.2% contributed by the second phenolic derivative. Media contained also Chapek-Dox salts (in %), as follows: $NaNO_3$ – 0.2, KH_2PO_4 – 0.1, KCI – 0.05, $MgSO_4.7H_2O$ – 0.05, $FeSO_4.7H_2O$ – 0.001. The starting pH of culture media was 5.5.

Microbial biodegradation

14-day cultures were applied, by which 50 cm^3 volumes of growing media were inoculated. On media comprised of phenol + 2,4-dichlorophenol, phenol + 2,6-dimethoxyphenol and phenol + diphenylamine the dominant strain was *Thermoascus aurantiacus,* of which $1.6x10^7$ conidia/cm^3 media were inoculated; $0.5x10^7$ conidia/cm^3 of *Aspergillus awamori* were introduced in the first two phenolic mixtures, while the third was inoculated with $1.5x10^7$ conidia/cm^3 of *Thermoascus aurantiacus* and $1.0x10^7$ conidia/cm^3 of *Aspergillus awamori.* For the medium containing phenol + catechol the dominant strain was *Aspergillus awamori*, inoculated at $1.4x10^7$ conidia/cm^3, while for *Thermoascus aurantiacus* inoculation rate was $1.0x10^7$ conidia/cm^3. The latter strain was introduced into the media 48 hours after inoculation with the dominant strain. Control samples were flasks inoculated with the respective monocultures. Cultivation of the cultures was carried out in 300 cm^3 Erlenmeyer flasks containing 50 cm^3 growing medium, at a temperature of 30 °C on a rotation shaker (220 rpm).

Analytical methods

The concentration of phenolic substrates was determined using Folin-Ciocalteu reagent, with Gallic acid (0.01%) as a standard. 1.0 cm^3 of the standard solution was added to 10 cm^3 deionized water, 1.0 cm^3 of Folin-Ciocalteu reagent and 2.0 cm^3 20% $NaCO_3$. After 1 hour the absorbance at 750 nm was read [12]. Residual phenol in the samples was determined

after centrifugation of the cultural medium for 15 min at 3500 rpm. Conidia count was assessed with the help of a Bürker chamber.

Enzyme analyses

Laccase – by spectrometric measurement at 430 nm wavelength with syringaldazine as a substrate [13];
Phenol hydroxylase - by spectrometry at 340 nm, measuring the oxidation of NADPH in the presence of phenol [14];
Catechol-2,3-dioxygenase – by spectrometry at 375 nm, quantifying the amount of 2-hydroximuconic semi-aldehyde formed [15];
Catechol-1,2-oxygenase – by spectrometry at 260 nm, measuring the levels of the emerging *cis, cis* – muconic acid [16].

RESULTS AND DISCUSSION

The cultivation of the two cultures in concern on the selected phenolic mixtures was preceded by a series of preliminary experiments aimed at the assessment of their ability to degrade 0.2% phenol and up to 0.4% of the other phenol derivatives (unpublished data). At the chosen concentration of the substrate, the growing pattern of both *A. awamori* and *T. aurantiacus* was by abundant sporulation and absence of mycelium biomass. The fact that *A. awamori* develops in its mycelium form at 0.06% phenol, while at concentrations above 0.1% sporogenesis is exclusively involved, clearly shows that sporogenesis is a means of survival for both strains in toxic substrate concentrations. Physiological activity by spores of other fungi causing various transformations of organic compounds has also been reported [17].

The types of interaction between co-cultivated microbial populations could be categorized as neutral, positive and negative, according to Stanier et al. (1986) and Goswami et al., 2005. Decisive criteria about the type of interaction are cell count or biomass amount in the mixed culture – whether found in higher, lower or equal concentrations than in the respective monocultures. In the current study, on the medium containing phenol + 2,4-dichlorophenol the concentration of conidia in the mixed population equaled the sum of conidia in the pure cultures (Fig. 1). Both cultures established a neutral type of interaction. According to Linton and Drozd (1982) the specified type of interaction is to be demonstrated solely in laboratory conditions with excess of substrate. The residual phenol content in the mixed culture was 0.18%, i.e. 55% degradation was achieved, compared to 47% and 46% for the pure cultures *T. aurantiacus* and *A. Awamori,* respectively. The bigger degree of biodegradation achieved was attributed to the greater concentration of conidia in the mixed culture.

The growth of *T. aurantiacus* and *A. awamori* on the medium containing phenol + 2,6-dimethoxyphenol revealed a positive type of interaction between the two strains (Fig. 2). Conidia count in the mixed culture exceeded that in the pure cultures. The residual phenols in the mixed culture were 0.145 % or 63.8% biodegradation occurred. As a consequence of

the positive interactions between the microbial populations in the mixed culture the biodegradation of phenols exceeded that of the pure *T. aurantiacus* culture by a factor of 1.72 and that of *A. awamori* - by a factor of 2.48.

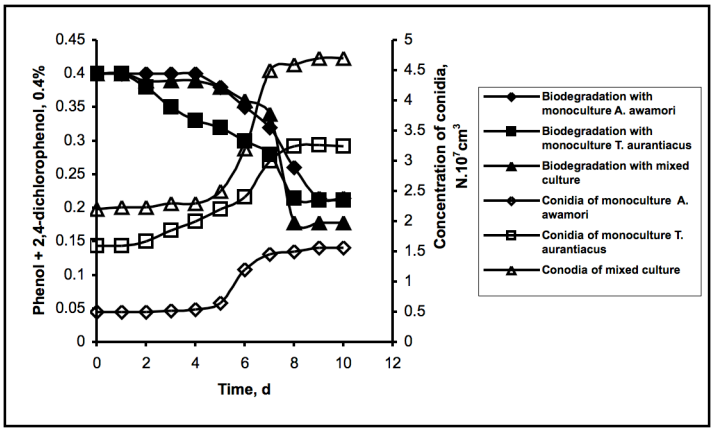

Fig. 1. Biodegradation of phenol + 2,4-dichlorophenol mixture with microbial association T. aurantiacus and A. awamori

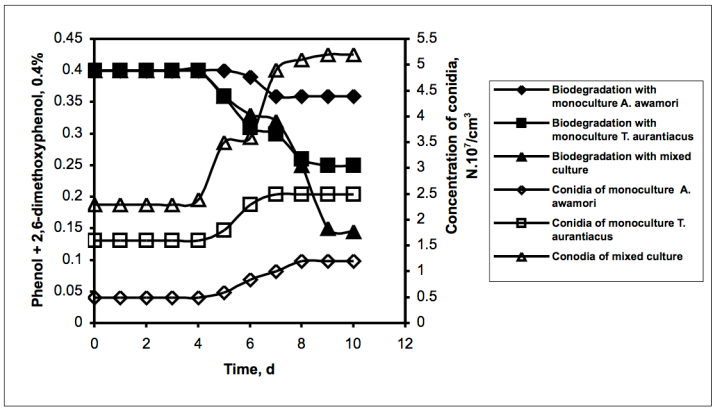

Fig. 2. Biodegradation of phenol + 2,6-dimethoxyphenol mixture with microbial association T. aurantiacus and A. awamori

The interactions between the studied species on phenol + diphenylamine medium were of certain interest (Fig. 3). For the specified medium, *T. aurantiacus* did not indicate any growth and no biodegradation of the phenol mixture occurred, although the strain was found capable of degrading both substances when used as single substrates (unpublished data).

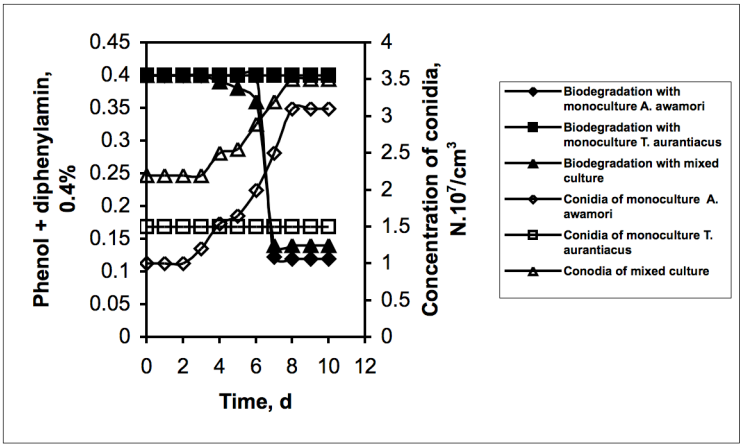

Fig. 3. Biodegradation of phenol + diphenylamin mixture with microbial association T. aurantiacus and A. awamori

Nevertheless, the strain probably exercised a negative impact on *A. awamori,* being the reason why the concentration of conidia in the mixed culture was approximately equal to that in *A. awamori* monoculture. Those negative interactions reflected in the lower degree of phenol biodegradation in the mixed culture – 65.0 % for the mixed culture as opposed to 70.0 % for *A. awamori* monoculture.

Negative were also strains' interactions monitored on phenol + catechol containing medium, due to which the concentration of conidia in the mixed medium were lower than the sum of conidia in the monocultures (Fig. 4). The biodegradation of the combined phenolics by the mixed culture was similar to that produced by *A. awamori* monoculture – 40% and 37.5%, respectively. *T. aurantiacus* monoculture caused only an insignificant biodegradation of the phenolic mixture – 6 %.

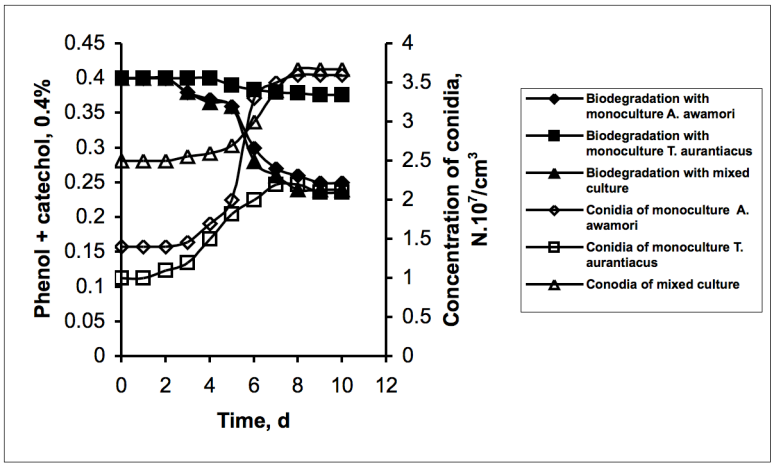

Fig. 4. Biodegradation of phenol + catechol mixture with microbial association A. awamori and T. aurantiacus

On all media studied, the growth of both pure and mixed cultures (with the exception of *T. aurantiacus* on phenol + diphenylamine medium) was marked by an extended period of adaptation to the medium – about 4-5 days. The stage of phenolic mixtures' biodegradation took about 4 days, after which the microbial species discontinued their

development without completing a full degradation of the phenolic mixtures – probably because of the toxicity of the substrates utilized.

Little is known about metabolism of phenols by fungi [9,10]. In most cases phenols' metabolism is accomplished via β-ketoadipate pathway, by *o*-fission of catechol. *Aspergillus awamori* NRRL possesses well-proven phenol-degrading activities, confirming the above-specified mode of biodegradation [21]. Our study established that *T. aurantiacus*, much alike *A. awamori,* did not possess catechol 2,3-dioxygenase, i.e. phenolic metabolism again followed the β-ketoadipate pathway.

The analyses for phenol hydroxylase detected traces of the enzyme in all mixed cultures, which might suggest that it was faintly secreted by conidia. Data concerning catechol 1,2-dioxygenase are presented in Table 1. *T. aurantiacus* monoculture produced highest catechol 1,2-dioxygenase activity on phenol + 2,4-dichlorophenol medium – 4.91 U/cm^3, while *A. awamori* demonstrated highest biosynthetic potential on the medium containing phenol + 2,6-dimethoxyphenol – 4.19 U/cm^3. On all mixed mediums enzyme's biosynthesis was less intensive than that of the pure cultures. That led to the conclusion that the selected conditions of cultivation – the quantitative proportions between the two strains and the moment of introduction of the second strain – were not adequate for achieving higher enough activity of the specified enzyme.

Table 1: Activity of catechol-1,2-oxygenase in the culture medium of the monocultures and in the microbial association of A. awamori amd T. aurantiacus on the 8-th day of their development

Phenol mixtures, 0,4%	**Monoculture A. awamori, U/cm^3**	**Monoculture T. aurantiacus, U/cm^3**	**Mixed culture, U/cm^3**
Phenol + 2,4-dichlorophenol	3.29	4.91	1.62
Phenol + 2,6-dimethoxyphenol	4.19	3.41	0.72
Phenol + diphenylamine	0.89	0	0.72
Phenol + catechol	2.39	0.89	0.70

With respect to laccase production by the two microbial species under study, enzymatic output confirmed data about the existence of neutral interactions on phenol + 2,4-dichlorophenol medium, of positive – on phenol + 2,6-dimethoxyphenol, and of negative relations between cultures on the two remaining media (Table 2). On the phenol + 2,4-dichlorophenol medium the two cultures collectively produced 17.2 U/cm^3, i.e. each species probably maintained its individual enzymatic synthesis even in joint cultivation. The positive interactions between the two strains taking place on phenol + 2,6-dimethoxyphenol medium found an expression in laccase biosynthesis as well – 30.2 U/cm^3 were achieved by the mixed culture, compared to 13.8 U/cm^3 by *A. awamori* monoculture and 10.8 U/cm^3 by *T. aurantiacus*. A number of researchers investigating enzymatic production in cases of mixed cultivation have concluded that such an accelerated enzymatic biosynthesis by a mixed culture is a result of synergistic interrelations between the co-cultivated species [22,23,24,25,26,27].

Table 2: Activity of laccase in the culture medium of the monocultures and in the microbial association of A. awamori amd T. aurantiacus on the 8-th day of their development

Phenol mixtures, 0,4%	**Monoculture A. awamori, U/cm^3**	**Monoculture T. aurantiacus, U/cm^3**	**Mixed culture, U/cm^3**
Phenol + 2,4-dichlorophenol	6.4	12.0	17.2
Phenol + 2,6-dimethoxyphenol	13.8	10.8	30.2
Phenol + diphenylamine	12.8	0	10.8
Phenol + catechol	14.2	4.6	13.4

The negative interactions between the studied species on media containing phenol + diphenylamine and phenol + catechol brought about a weaker enzyme activity of laccase produced by the mixed cultures as well.

The synergism demonstrated by the two microbial cultures under study suggests that different conditions for cultivation can be further explored – like proportions within the inoculation material or the moment of introduction of the second culture – which will accommodate synergism between the species in the phenolic mixtures with respect to catechol 1,2-dioxygenase enzyme, too, thus creating grounds for their more substantial catabolism.

CONCLUSIONS

Studied cultures' ability for detoxification of high concentrations (0.4%) of phenolic mixtures had been established – a finding that had no analogue in scientific literature.

The two cultures utilized in the study - *A. awamori* and *T. Aurantiacus* – were appropriate partners for a more efficient catabolism of phenol + 2,6-dimethoxyphenol mixture, which might be the grounds for conducting further research targeted at establishing positive interactions between them on other phenolic mixtures as well.

Data reported in the current study reveal the future of filamentous fungi application in environmental protection against phenolic contaminants and support the necessity of selection and further examination of similar strains, aiming at their use as biocatalysts in the detoxification of the environment.

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