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ORIGINAL PAPER

Growth of *Trametes versicolor* on phenol

H. Yemendzhiev · M. Gerginova · A. Krastanov · I. Stoilova · Z. Alexieva

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Abstract *Trametes versicolor* 1 was shown to grow on phenol as its sole carbon and energy source. The culture growth and degradation ability dependence on culture medium pH value was observed. The optimal pH value of a liquid Czapek salt medium was 6.5. The investigated strain utilized completely 0.5 g/l phenol in 6 days. The dynamics of the phenol degradation process was investigated. The process was characterized by specific growth rate μ_{max} 0.33 h^{-1}, metabolic coefficient $k = 4.4$, yield coefficient $Y_{x/s} = 0.23$ and rate of degradation $Q = 0.506$ h^{-1}. The intracellular activities of phenol hydroxylase (0.333 U/mg protein) and *cis*,*cis*-muconate lactonizing enzyme (0.41 U/mg protein) were demonstrated for the first time in this fungus. In an attempt to estimate the occurrence of gene sequences in *T. versicolor* 1 related to phenol degradation pathway a dot blot analysis with total DNA isolated from this strain was performed. Two synthetic oligonucleotides were used as hybridizing probes. One of the probes was homologous to the 5′end of *phy*A gene coding for phenol hydroxylase in *Trichosporon cutaneum* ATCC 46490. The other probe was created on the basis of *cis*,*cis*-muconate lactonizing enzyme coding gene in *T. cutaneum* ATCC 58094. The results of these investigations showed that *T. versicolor* 1 may carry genes similar to those of *Trichosporon cutaneum* capable to degrade phenol.

H. Yemendzhiev (✉) · M. Gerginova · Z. Alexieva
The Stephan Angeloff Institute of Microbiology,
Bulgarian Academy of Sciences,
Acad. G. Bontchev Str., bl. 26, 1113 Sofia, Bulgaria
e-mail: h_bio@yahoo.com

A. Krastanov · I. Stoilova
Department of Biotechnology, University of Food Technologies,
Maritza blvd., 26, 4002 Plovdiv, Bulgaria

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Introduction

Phenol and its various derivatives, as well as many other aromatic compounds, are known as hazardous pollutants. Major problems associated with phenolic wastes arise since these compounds are usually present as mixtures of different phenolics, with especially some substituted phenols known to be unfavorable for biodegradation. They can be detected in effluents from oil refineries, coal and chemical industries [1, 18].

Besides physical and chemical methods, biological methods of removal play an important role in waste water management. Aqueous phenolic wastes have been treated for many years by activated sludge processes which are known to be sensitive to fluctuations in the phenolic load [21]. Various phenol-degrading microorganisms have been extensively studied for the purpose to innovate and improve the technological processes of biodegradation. Most of the published investigations on the degradation of phenol have been done using bacterial strains and some yeast [5, 6, 16, 23]. The use of fungal strains for degradation is a relatively untouched area. Some strains of mycelia fungi such as *Fusarium floccieferum* [2], *Graphium sp.* [17] and *Aspergillus awamori* [19] have been cited for their potential for phenol degradation.

Recently, the ability of white-rot fungi to degrade a variety of toxic organo-pollutants has attracted the attention of scientists. This group of microorganisms is capable of completely degrading polymers of phenolic origin, including lignin. The biodegradation activity toward some wide

spread phenolic pollutants is studied together with the laccase production and expression of phenol oxidase activities in general [4, 20]. Lignin degradation by white-rot fungi is known as a cometabolic process, and growth of white-rot fungi on monoaromatics (constituents of the lignin macromolecule) have only rarely been described [3].

One of the members of the white-rot fungi family with proven bioremediation and degradation capacity is *Trametes versicolor*. The successful bioremediation of a phenolic wastewater by *T. versicolor* was found to be dependent on a fungal growth, enzyme (laccase) production and some inductors [14]. A strain of *T. versicolor* isolated from paper mill effluent has been described as capable of degrading phenolic compounds [22].

The aim of this study is to prove the ability of strain *T. versicolor* 1 to utilize phenol as a sole carbon and energy source and to find out the evidences for its catabolic activity.

Materials and methods

Microorganisms, growth conditions and analytical methods

The strains used in this study were as follows: *Trichosporon cutaneum* R57, *Trichosporon cutaneum* ATCC 46490, *Trametes versicolor* 1, *Burkholderia* sp. K1 strain, *Escherichia coli* JM 109. The microbial cultivation was carried out in accordance with common media and conditions typical for each one of the species [1, 8, 10, 13, 15].

The carbon free Chapek–Dox medium was used in the biodegradation experiments with a strain *T. versicolor* 1 [19]. The flasks containing 50-ml inoculated culture medium were agitated at 240 rpm, 26 °C. Samples were taken at interval of 24 h and centrifuged at 5,000 rpm for 20 min. The dry weight of the cells was determined by ULTRA X apparatus for drying. The phenol concentration in cell free supernatant was analyzed by HPLC performed on a reversed phase C18 column (Lichrosorb RP18, Perkin Elmer, Waltham, MA, USA) with methanol-water (50:50) liquid phase by using UV detector at 220 nm.

The phenol hydroxylase activity was performed spectrophotometrically (LKB UV–Vis Ultraspec 1000), measuring the oxidation of NADPH in the presence of substrate [12]. The activity of *cis*,*cis*-muconate lactonizing enzyme was analyzed spectrophotometrically at 260 nm, measuring the decrease of *cis*,*cis*-muconic acid concentration [11]. The total protein concentration was determined by Lowry's method [9].

DNA isolation procedure

The cells were frozen at −20 °C and then ground mechanically and collected in a plastic tube with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH = 7.5). The obtained mixture was treated in liquid nitrogen followed by instant incubation at 90 °C water bath. The following step presents a treatment with lysis buffer (50 µl 1-M Tris, 50 µl 0.5-M EDTA, 100 µl 5-M NaCl, 10% SDS, pH = 7.5). DNA extraction was accomplished by phenol–chloroform extraction [15]. The DNA samples were purified with GFX columns (GE Healthcare, UK).

DNA dot blot hybridization

The biotin labeling of a single stranded oligonucleotide was performed in accordance with the manufacture's protocol (The Biotin ULS Labeling Kit, Fermentas).

On positively charged nylon membranes for dot blot analysis (Boehringer, Mannheim, Germany) was fixed 1 µl (300 ng genomic DNA), following the recommendation of the manufacturer. A pre-hybridization was performed by incubation of the fixed on the membrane DNA for 1.5 h at 42 °C in a hybridization solution (Boehringer, Mannheim, Germany) in a hybridization chamber (Techne, Cambridge, UK). To the hybridization solution used in the process of the real hybridization, the biotin-labeled oligonucleotide was added (1 µl/ml).

The hybridization program for oligonucleotide "OligoD1" was as follows: 15 min at 66 °C, 15 min at 60 °C, 1 h at 55 °C, 1 h at 50 °C, 1 h at 45 °C, 18 h at 42 °C and 1 h at 35 °C. The hybridization conditions for oligonucleotide "OligoZA1" were as follows: 15 min at 72 °C, 15 min at 66 °C, 1 h at 58 °C, 1 h 50 °C, 18 h at 48 °C, 1 h at 42 °C.

After the hybridization the membranes were washed three times for 15 min each in 2 ∞x SSC, 0.1% SDS as follows: twice at 25 °C for 15 min with constant agitation and once at 42 °C for 15 min. The detection was done according to the instructions of the DNA Labeling and Detection kit (Boehringer Mannheim, Germany). Labeled Lambda DNA was used as a hybridization probe in a dot blot of homologous DNA.

Results and discussion

The experimental data proved that the strain *T. versicolor* 1 can grow and utilize phenol as a sole carbon and energy source. The influence of pH value on phenol biodegradation was investigated in the medium containing 0.5 g/l phenol. The experiments for determination of biodegradation capacity of the investigated strain were performed in triplicate. The optimal pH value established for the degradation process was 6.5. The results are demonstrated in Fig. 1a, b. At the optimal pH of the media, the investigated strain utilized 0.5 g/l phenol completely in 6 days. We could suppose that the acidity of the medium exerts a considerable

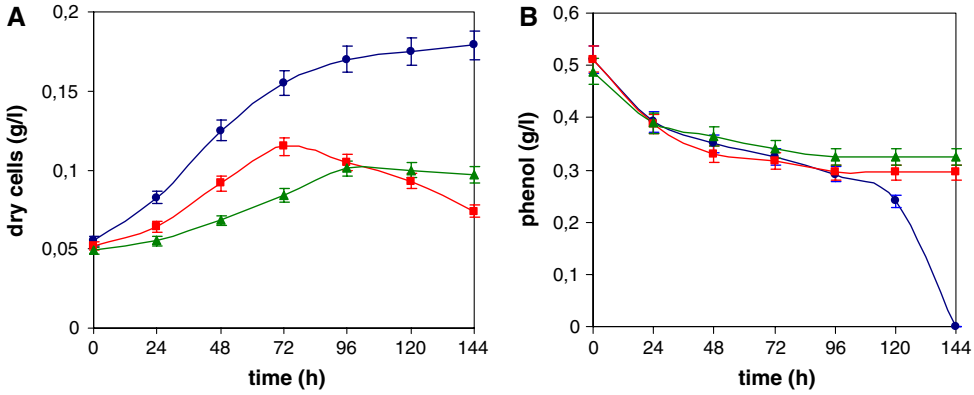

Fig. 1 Growth of *Trametes versicolor* strain 1 on phenol: **a** biomass accumulation; **b** Phenol degradation curves (pH 5.5, *filled square*; pH 6.5, *filled circle*; and pH 7.5, *filled triangle*)

effect on the strain ability to degrade phenol. In the process of HPLC determination of phenol concentration, decreasing small peaks indicating a presence of catechol in all of the analyzed probes were observed. These data indicate that phenol and catechol in *T. versicolor* were consecutively oxidized.

The dynamics of the phenol degradation process carried out in a medium with optimal pH = 6.5 was investigated according to Haldane equation. The process was characterized by specific growth rate $\mu_{max} = 0.33\ h^{-1}$, metabolic coefficient $k = 4.4$, $Y_{x/s} = 0.23$ and rate of degradation $Q = 0.506\ h^{-1}$.

If we compare our results with the scarce data in the literature, we should conclude that the studied strain showed a strong activity with respect to phenol degradation [22].

The species *T. versicolor* has been extensively investigated for its notable laccase activity [13]. In this study, the emphasis was laid on its ability to utilize phenol as a sole carbon and energy source. It has been reported that the fungal metabolism of aromatic compounds occur via ortho-fission pathway [1, 7]. The phenol hydroxylase (EC 1.14.13.7) and *cis*,*cis*-muconate lactonase (EC 5.5.1.1) are key enzymes in this catabolic process. The intracellular activities of phenol hydroxylase and *cis*,*cis*-muconate lactonizing enzyme in *T. versicolor* are demonstrated for the first time in this report. The determination of enzyme activities was performed within intracellular extracts from *T. versicolor* strain 1 cultivated in a medium (pH6.5) with 0.5 g/l phenol as sole carbon and energy source. The obtained phenol hydroxylase activity was 0.333 U/mg protein and c*is*,*cis*-muconate lactonizing enzyme activity was 0.409 U/mg protein.

In the present work we have investigated the occurrence of phenol hydroxylase and/or *cis*,*cis*-muconate cyclase-related gene sequences in *T. versicolor* 1 strain. For this purpose, we used two gene specific biotin-labeled oligonucleotides in dot blot hybridization experiments. Some previously obtained and described phenol-degrading microbial strains were used as controls in these experiments.

The strains of *Trichosporon cutaneum* are known as effective biodegraders, accomplishing phenol catabolism via ortho-pathway [1, 8]. The presence of *Burkholderia* sp. K1 strain in our investigation was motivated by the ability of such strains to degrade aromatic compounds through the meta-cleavage pathway [10, 13]. The used strain of *Escherichia coli* JM 109 was proven to be incapable to resist and degrade phenolic compounds. It was used as a strain not comprising any genes coding for phenol catabolizing enzymes in its genome.

The isolated total genome DNA from *T. versicolor* 1 was used together with the DNAs of all strains turned to account as controls in the dot blot hybridization experiments.

The first set of experiments was carried out with the oligonucleotide probe "Oligo D1" (5′-AAGTACTCCGAG TCCTACTGCGACGTCCTCATCGTCGG-3′) created to be homologous to the 5′ end of TORPHD locus [National Center for Biotechnology Information(NCBI)] coding phenol hydroxylase (*phyA*) in *T. cutaneum* ATCC 46490 [8]. The results from dot blot hybridization analyses are presented on Fig. 2. The results demonstrated that all of the examined strains DNAs showed positive signals with "Oligo D1" probe, i.e., they possessed *phyA*-like sequences. Naturally, *E.coli* JM 109 strain showed a negative signal.

The second oligonucleotide "OligoZA1" (5′-AGCTAT GATATTTTGATGGGCACGTTCCGCTCGCCCTACCT CTAC-3′) was designed earlier in our laboratory on the basis of TORCCMLE locus for *cis*,*cis*-muconate lactonizing enzyme (MLE) in *T. cutaneum* ATCC 58094 (NCBI) [10]. This oligonucleotide probe was used in the next set of

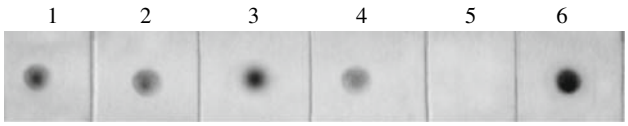

Fig. 2 Dot blot analysis of purified DNA samples hybridized by oligonucleotide probe for *phy*A gene like sequences. Total genomic DNAs from: *1 Trichosporon cutenum* ATCC 46490; *2 Trichosporon cutaneum* R57; *3Bulkhorderia* sp. K1; *4 Trametes versicolor* 1; *5 Escherichia coli* JM 109(negative control); *6* positive control, reversed "OligoD1"

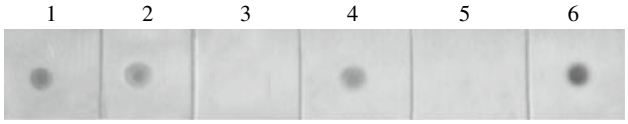

Fig. 3 Dot blot analysis of purified DNA samples hybridized by oligonucleotide probe for *cis*,*cis*-muconate lactonizing enzyme gene like sequences. Total genomic DNAs from: *1 Trichosporon cutenum* ATCC 46490; *2 Trichosporon cutaneum* R57; *3 Bulkhorderia* sp. K1; *4 Trametes versicolor* 1; *5 Escherichia coli* JM 109(negative control); *6* positive control, reversed "OligoZA1"

dot blot hybridizations. The obtained results are presented on Fig. 3.

The DNAs of both strains *T. cutaneum* and the investigated *T. versicolor* 1 displayed a positive hybridizing signal with "OligoZA1". These results corresponded to previously proved ability of both strains *T. cutaneum* ATCC 46409 and *T. cutaneum* R57 to utilize phenol via ortho-pathway [1, 12]. The DNA of *Burkholderia* sp. K1 strain did not hybridize with fOligoZA1, i.e., it did not contain any similarity with oligonucleotide probe for MLE of *T. cutaneum* ATCC 58094 because of meta-cleavage mechanism of phenol degradation typical for such species [13].

Escherichia coli JM 109 strain DNA used as negative controls in the experiments were not able to utilize phenol. Logically they did not reveal any sequence similarity to the both applied oligonucleotides.

The combined data received from both hybridization sets of experiments and the reported enzyme activities gave us a reason to presume that the mechanism of phenol biodegradation in *Trametes versicolor* 1 strain was *ortho*-cleavage type. The obtained ability of *Trametes versicolor* 1 to grow and develop in the medium containing phenol as a sole carbon source corresponded with such a conclusion.

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