Influence of Various Phenolic Compounds on Phenol Hydroxylase Activity of a *Trichosporon cutaneum* Strain

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The phenol-degrading strain *Trichosporon cutaneum* R57 utilizes various aromatic and aliphatic compounds as a sole carbon and energy source. The intracellular activities of phenol hydroxylase [EC 1.14.13.7] of a *Trichosporon cutaneum* R57 strain grown on phenol (0.5 g/l) were measured. Different toxic phenol derivatives (cresols, nitrophenols and hydroxyphenols) were used as substrates in the reaction mixture for determination of the enzyme activity. The data obtained showed that the investigated enzyme was capable to hydroxylate all applied aromatic substrates. The measured activities of phenol hydroxylase varied significantly depending on the aromatic compounds used as substrates. The rate of phenol hydroxylase activity with phenol as a substrate (1.0 U/mg total cell protein) was accepted as 100%.

Key words: Phenol Hydroxylase, Phenols, *Trichosporon cutaneum*

Introduction

Phenol and its various derivatives, as well as many other aromatic compounds, are known as hazardous pollutants. They can be detected in effluents from oil refineries, coal and chemical industries (Schie and Young, 2000). Some of the components, for example dissolved hydrocarbons, are highly toxic and not easily broken down in the environment. Physical and chemical treatments using flotation columns and centrifugation or filtration are traditionally used to remove the harmful compounds from the industrial waste waters. In addition, biological methods for purification of polluted waters and soils with relatively low processing costs are wide-spread in the field of bioremediation technologies (Aleksieva *et al.*, 2002; Yan *et al.*, 2005). Numerous microorganisms, namely bacteria, yeasts and fungi, which can grow using toxic organic compounds as carbon source, have been reported as hydrocarbon degraders (Li *et al.*, 2005; Bergauer *et al.*, 2005). Studying the specificity of microbial enzymes involved in the degradation and detoxification of various phenol derivatives is of much interest because of their broad occurrence. It contributes to a better understanding and application of new approaches to environment cleaning and protection.

Phenol hydroxylase [EC 1.14.13.7] hydroxylates phenol to catechol. This reaction is the first step of the degradation process permitting the utilization of aromatic compounds as a source of carbon and energy (Enroth *et al.*, 1998). The substrate specificity of phenol hydroxylases described in the literature includes a number of phenol derivatives (Ahuatzi-Chacon *et al.*, 2004; Cejkova *et al.*, 2005).

The sub-cellular localization of phenol hydroxylase is not yet known but most investigators speculate that the logical site would be the cell membrane, thereby avoiding penetration of phenol into the cytosol (Leonard and Lindley, 1999). The strong sensitivity of phenol hydroxylase to ultrasound is observed in experiments carried out with *Candida tropicalis* (Krug and Straube, 1986). The strictly aerobic soil-living yeast *Trichosporon cutaneum* uses flavin adenine dinucleotide (FAD)-containing enzymes to hydroxylate phenols. Our data for phenol hydroxylase obtained in cell-free extracts and in permeabilized cells show as well that the method of cell permeabilization is more favorable than cell disruption by ultrasonication for enzyme analyses in *Trichosporon cutaneum* (Alexieva *et al.*, 2004).

The objective of the present study is to investigate the influence of a variety of mono-substituted phenols on phenol hydroxylase activity in *T. cutaneum* strain R57.

Materials and Methods

Yeast strain and media

The basidiomycetes yeast strain *Trichosporon cutaneum* R57, registered in National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC) under number N 2414, Bulgaria (Ivanova and Alexieva, 1996), was used in all experiments. The cultivation was carried out on the carbon-free medium for yeast containing 6.7 g/l yeast nitrogen base without amino acids (YNB w/o AA, Fluka, Seelze, Germany). After autoclaving, 0.5 g/l phenol was added. Agar (1.5%) was used for solidification of the media. Yeast cells were transferred from solid medium to 10 ml of the liquid medium for preculture. The preculture was cultivated for 18 h. The cells were harvested and washed twice with sterilized salt solution (0.9% NaCl) by centrifugation (3000 rev/min) for 20 min. The cells' residues were re-suspended and appropriate aliquots of cell suspensions were transferred into a 500 ml-shaking flask containing 50 ml YNB w/o AA liquid medium. The initial optical density value at 610 nm (OD_{610}) was adjusted to 0.135 ± 0.02.

All experiments were done at pH 6.0 and at ambient temperature (28–30 °C) on a New Brunswick rotary shaker (200 rev/min).

Enzyme assay

Cells were harvested in the late logarithmic phase and washed twice in 50 mM tris(hydroxymethyl)methylamine-sulfate buffer, pH 7.6. Enzyme activities were determined in permeabilized cells. Permeabilization procedures were essentially similar to those described for *Yarrowia lipolytica* (Galabova *et al.*, 1996). In our experiments, maximal permeabilization of the cells was achieved with 0.1% of the non-ionic surfactant Triton X-100.

Phenol hydroxylase [EC 1.14.13.7] activity was assayed spectrophotometrically (LKB UV-Vis Ultraspec 1000), following NADPH absorbance at 340 nm (Neujahr and Gaal, 1973). All investigated phenolic compounds were added to the enzyme reaction mixture as single substrates in the concentration of 0.5 μM. One unit of activity is defined as the amount of enzyme transforming 1 μmol of substrate in 1 min under the assay conditions. Activities were expressed as units (U) per mg total cell protein.

Analytical methods

Cell density was monitored spectrophotometrically by measuring the optical density at $\lambda = 610$ nm (OD_{610}).

The phenol concentration was determined in cell-free medium by a residual calorimetric method using the reagent 3,4-dimethyl amino antipyrine (Hristov, 1997).

Determination of total protein content of the permeabilized cells was carried out with Folin-Ciocalteu reagent (Herbert *et al.*, 1971).

The experiments for determination of enzyme activity of the investigated strain were performed in triplicate.

Results and Discussion

The cells of *T. cutaneum* R57 were induced and cultivated in YNB w/o AA medium including no inhibitory concentration of phenol (0.5 g/l) as sole carbon and energy source. The measured activity of phenol hydroxylase with phenol as substrate was 1.0 U/mg total cell protein as distinguished from earlier reported experiments (0.8 U/mg total cell protein) carried out without preculture and corresponding cells' induction (Alexieva *et al.*, 2004). The established difference is in accordance with the well-known phenol hydroxylase inducibility (Fialova *et al.*, 2004). The rate of phenol hydroxylase activity with phenol was accepted as a basis (1.0 U/mg total cell protein = 100%) for comparing the results obtained with the other investigated substrates.

The analyses of data from experiments with *o*-, *m*- and *p*-cresols showed a high degree of similarity to the data obtained in experiments with phenol. The enzyme activities obtained were as follows: with both substrates *o*- and *m*-cresols 1.0 U/mg total cell protein; with *p*-cresol 0.93 U/mg total cell protein. The same effect could be observed in experiments done with *o*-nitrophenol (*o*-NP). The established enzyme activities with both *m*-nitrophenol (*m*-NP) and *p*-nitrophenol (*p*-NP) were rather lower, 0.43 U/mg total cell protein and 0.47 U/mg total cell protein, respectively (Fig. 1). It should be pointed out that *o*-cresol and *p*-nitrophenol are non-growth substrates for *T. cutaneum* R57. The similar effects have been observed in experiments with chlorophenols (Krug *et al.*, 1985). On the contrary, any hydroxylating activity with *o*-nitrophenol not degradable by *C. tropicalis* HP15
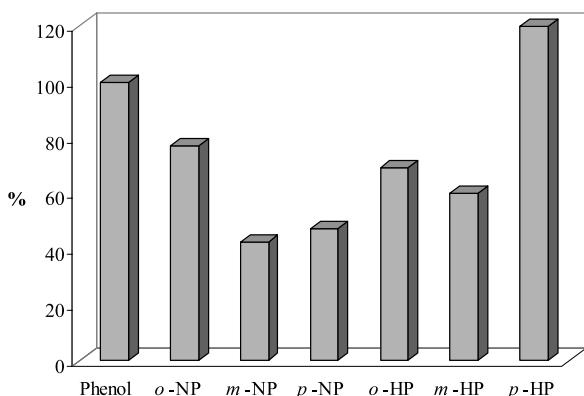
Fig. 1. Effect of nitro-substituted (NP) and hydroxylated (HP) phenols, used as substrates, on phenol hydroxylase activity in cells of *T. cutaneum* R57 grown on phenol. The rate of phenol hydroxylase activity with phenol as a substrate (1.0 U/mg total cell protein) was accepted as 100%.

has not been found (Krug *et al.*, 1985). The enzyme activities obtained in the experiments with non-growth substrates indicated the existence of different causes for cells' inability to assimilate them. As it was reported earlier we observed differences in the characteristics of the strain degradation ability maintained with the rest of the investigated substrates (Zlateva *et al.*, 2005).

The ability of the mono-hydroxylated aromatics compounds to affect the level of intracellular FAD-dependent phenol hydroxylase in *Trichosporon cutaneum* strain R57 was studied, as well. In these experiments the enzyme capacity to oxidize catechol (*o*-HP) (0.7 U/mg total cell protein) or resorcinol (*m*-HP) (0.6 U/mg total cell protein) was significantly lower compared to the data received with phenol as a substrate under the same conditions. On the contrary, the level of phenol hydroxylase activity obtained in experiments with hydroquinone was considerably higher – 1.2 U/mg total cell protein (Fig. 1). The results obtained in the present investigation with *T. cutaneum* R57 yeast strain showed some differences in phenol hydroxylase substrate specificity compared to other data published in the literature. For instance, in our experiments the most efficient substrate for hydroxylation was hydroquinone (*p*-HP). So far as in other phenol-degrading yeast strains the most efficient substrate is resorcinol as well (Neujahr and Gaal, 1973; Krug and Straube, 1986).

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Ahuatzi-Chacon D., Ordorica-Morales G., Ruiz-Ordaz N., Cristiani-Urbina E., Juarez-Ramirez C., and Galindez-Mayer J. (2004), Kinetic study of phenol hydroxylase and catechol 1,2-dioxygenase biosynthesis by *Candida tropicalis* cells grown on different phenolic substrates. World J. Microbiol. Biotechnol. **20**, 695–702.

Aleksieva Z., Ivanova D., Godjevargova T., and Atanasov B. (2002), Degradation of some phenol derivatives by *Trichosporon cutaneum* R 57. Proc. Biochem. **37**, 1215–1219.

Alexieva Z., Gerginova M., Zlateva P., and Peneva N. (2004), Comparison of growth kinetics and phenol metabolizing enzymes of *Trichosporon cutaneum* R57 and mutants with modified degradation abilities. Enz. Microbiol. Technol. **34**, 242–247.

Bergauer P., Fonteyne P.-A., Nolard N., Schinner F., and Margesin R. (2005), Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts. Chemosphere **59**, 909–918.

Cejkova A., Masak J., Jirku V., Vesely M., Patek M., and Nesvera J. (2005), Potential of *Rhodococcus erythropolis* as bioremediation organism. World J. Microbiol. Biotechnol. **21**, 317–321.

Enroth C., Neujahr H., Schneider G., and Lindqvist Y. (1998), The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis. Structure **15**, 605–617.

Fialova A., Boschke E., and Bley T. (2004), Rapid monitoring of the biodegradation of phenol-like compounds by the yeast *Candida maltosa* using BOD measurements. Int. Biodeter. Biodegr. **54**, 69–76.

Galabova D., Tuleva B., and Spasova D. (1996), Permeabilization of *Yarrowia lipolytica* cells by Triton X-100. Enz. Microbiol. Technol. **18**, 18–22.

Herbert O., Phipps P., and Strange R. (1971), Chemical analyses of microbial cells. In: Methods in Microbiology, Vol. 5B (Norris J. R. and Ribbons D. W., eds.). Academic Press INC, London, New York, pp. 244–249.

Hristov A. E. (1997), Change in the processes of microbial respiration in Black sea ecosystem in the presence of phenol. Comp. Rend. Acad. Bulg. Sci. **50**, 101–104.

Ivanova N. and Alexieva Z. (1996), Characteristic of biological peculiarities and content of amino acids in *Trichosporon cutaneum* R 57. Bulg. J. Agric. Sci. **2**, 761–766.

Krug M. and Straube G. (1986), Degradation of phenolic compounds by the yeast *Candida tropicalis* HP15. J. Basic Microbiol. **26**, 271–281.

Krug M., Ziegler H., and Straube G. (1985), Degradation of phenolic compounds by the yeast *Candida tropicalis* HP15. J. Basic Microbiol. **25**, 103–110.

Leonard D. and Lindley N. (1999), Growth of *Ralstonia eutropha* on inhibitory concentrations of phenol: diminished growth can be attributed to hydrophobic perturbation of phenol hydroxylase activity. Enz. Microbiol. Technol. **25**, 271–277.

Li Q., Kangb C., and Zhang C. (2005), Waste water produced from an oilfield and continuous treatment with an oil-degrading bacterium. Proc. Biochem. **40**, 873–877.

Neujahr H. Y. and Gaal A. (1973), Phenol hydroxylase from yeast. Purification and properties of the enzyme from *Trichosporon cutaneum*. Eur. J. Biochem. **35**, 386–390.

Schie P. M. and Young L. Y. (2000), Biodegradation of phenol: Mechanisms and applications. Bioremed. J. **4**, 1–18.

Yan J., Jianping W., Hongmeia L., Suliang Y., and Zongding H. (2005), The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*. Biochem. Eng. J. **24**, 243–247.

Zlateva P., Gerginova M., Manasiev J., Atanasov B., Peneva N., Dimova N., and Alexieva Z. (2005), Kinetic parameters determination of the phenolic derivatives assimilation by *Trichosporon cutaneum* R57. Biotechnol. Biotechnol. Eq. **19**, 93–97.