The level of phenol hydroxylase in *Candida maltosa* – the key activity for efficient aromatic compounds biodegradation

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**Abstract**

The yeast *Candida maltosa*, biodegrader of aromatic compounds, was obtained from a soil sample long-term contaminated with phenol-like and other aromatic compounds, using modified methodology of aerobic enrichment and consequent yeast (single species) isolation. We showed that the phenol hydroxylase in *C. maltosa* (phenol 2-monoxygenase, EC 1.14.13.7), the key enzyme for the decomposition of some aromatic compounds, is inducible.

This work is focused on the possibility to exploit the inducibility of phenol hydroxylase in order to enhance the biodegradation potential of this microorganism. The changes in the determined phenol hydroxylase activity in vivo are dependent on the substrate type (phenol, catechol, resorcinol, *p*-cresol benzoic acid), substrate concentration and on the growth phase of the microorganism. The substrate specificity and the influence of environmental conditions (pH, temperature, etc.) on activity of an isolated enzyme were determined.

The best inducer was phenol (ca 1 g/l). Enzyme activity was very low in the cultivation with *p*-cresol as a sole C-source. Optimization of phenol addition, as a growth substrate, allowed the successful biodegradation of *p*-cresol presented in a medium. We have shown that humic acids positively modulate the level of phenol hydroxylase activity. Application of these compounds in the reactor cultivation enhanced the total biodegradation activity of *C. maltosa* for phenol-like substances.

The reached results indicated that optimization of composition and concentration of C-sources and/or addition humic acids (non-toxic compounds)
Introduction

The capacity of microbial cell to degrade phenolic as well as other aromatic compounds is being studied with increasing interest to degrade efficiently their mixtures. The microbial catabolism of phenol and cresols has been studied in some detail and some catabolic sequences have been identified. Phenol is degraded by bacteria via catechol, whilst p-cresol is catabolized either via 4-methylcatechol or via protocatechuate [1], less is known concerning the metabolism of above pollutants by low eukaryotic (fungal) species. However, the aerobic degradation pathways in both cell types involve the hydroxylation of phenol (and some related compounds) to catechols, which are substrate of ring-cleaving enzymes, thus following the modified ortho- and meta- cleavage pathways, respectively [2]. Phenol hydroxylase (phenol 2-monooxygenase; EC 1.14.13.7) catalyses the initial step of microbial phenol mineralization by insertion of oxygen providing catechol. Potential degradative microorganisms differ largely in this enzyme substrate specificity as well as in their tolerance towards aforementioned compounds. In mixed substrate cultivations, the respective degradation of individual compounds proceeds in different phases suggesting regulatory effect of some substrates on phenol metabolism (phenol hydroxylase induction). This paper reports on investigation, which assessed the phenol degradation by Candida maltosa in the background of the induction of phenol hydroxylase and the effect of humic acids.

Material and methods

2.1 Microorganism and culture preparation

Candida maltosa was obtained via the modified aerobic enrichment of soil samples microflora according to Masák et al. [3], and consequent lower fungi (single species) isolations. Inoculum cultivations were performed aerobically using a rotary shaker (90 rpm, at 25 °C). Cell growth and the effect of additives were monitored using Bioscreen C analyzer (Labsystem Oy, Finland). The reactor cultivations were carried out in a bioreactor (Braun Biotech International, Germany) with an operating volume of 2.0 l, temperature controlled at 25 °C, and pH maintained at 6.4, using yeast nitrogen base medium (YNB, Difco Laboratories, USA). Rotation speed of the impeller was fixed at 240 rpm. Humic acids were isolated from oxihumolites by Dr. J. Novák (Institute of Inorganic Chemistry, Ústí nad Labem, Czech Republic).
2.2 Preparation of cell-free extracts

Cells were harvested by centrifugation (25 000 x g, 15 min, 4 °C), washed with and resuspended in phosphate buffer (50 mM, pH 7.4). Cells were disrupted by Bio-Neb® (Glas-Col Laboratories Products, USA) treatment. Cell debris was removed by centrifugation (35 000 x g, 15 min, 4 °C). The supernatant was designated as cell free extract.

2.3 Analytical methods

Protein quantity monitoring was performed according to Bradford [4] (1972). Phenol concentration in the cell-free medium was assayed using 4-amino-antipyrine colorimetric method [5]. Phenol hydroxylase (phenol 2-monoxygenase, EC 1.4.13.7) was measured assaying the oxidation of NADPH at 340 nm [U = 0.1 ΔA/min] [6].

3 Results and discussion

Enzyme activities were determined in C. maltosa cells during the cultivations on either phenol or catechol (Fig. 1). Specific phenol hydroxylase activity was found to be dependent on phenol concentration with a highest activity being associated with the concentrations in the range from 0.3 to 0.5 g/l; with further increasing phenol concentration the phenol hydroxylase activity declines. Increasing catechol concentration does not affect the starting enzyme level.

![Figure 1: Changes in phenol hydroxylase activity in C. maltosa cells growing, respectively, with phenol (●) and 1,2-catechol (Δ) as the carbon source.](image-url)
Figure 2: Changes in phenol hydroxylase activity [▲] in *C. maltosa* cells growing with resorcinol (0.5 g/l) as the carbon source. [○] - biomass concentration.

Figure 3: Time course of changes in phenol hydroxylase activity in non-growing *C. maltosa* cells (100 mg of cell protein/l) exposed to hydroxylated and non-hydroxylated substrates (0.3 g/l). [●] – *p*-cresol; [□] – salicylate; [▲] – benzoate.
The capacity of *C. maltosa* to utilize resorcinol as sole source of carbon and energy is shown in the Fig. 2. The time course of changes in phenol hydroxylase level in growing yeasts indicates that maximal level of the enzyme induced by resorcinol is not retained over all interval of exponential growth.

In order to investigate the phenol hydroxylase induction by substrates that are not utilized as sole source of carbon, the non-growing *C. maltosa* cells were exposed, respectively, to benzoic acid, *p*-cresol and salicylic acid. The time course of changes in the intracellular level of enzyme demonstrates (Fig. 3) that non-growing cells are able to produce effectively (after an adaptation phase) phenol hydroxylase only in the presence of *p*-cresol, suggesting different induction potential of hydroxylated compounds.

To obtain information concerning the co-metabolism of phenol and *p*-cresol the fed-batch (reactor) cultivation was performed to find out the profile of parallel utilization of both substrates. The apparent uniformity of both uptake profiles (Fig. 4) suggests the capability of *C. maltosa* cells to mineralize *p*-cresol in the presence of phenol.

On the other hand the lost capacity of *C. maltosa* to utilize phenol in the presence of salicylic acid indicates the effect of some non-hydroxylated substrates to suppress the induction of phenol hydroxylase, among others. In most phenol degrading microorganisms phenol hydroxylase activity is affected by substrate composition, strain tolerance to cytotoxic inhibition and by definite phenol concentration in the medium.

![Figure 4: Respective conversion of phenol and *p*-cresol by *C. maltosa* cells](image-url)
Figure 5: Growth of *C. maltosa* with phenol (1.0 g/l) and salicylic acid ([△] - 0.1 g/l; [▲] - 0.3 g/l); [●] - control

The generally observed oscillation of this enzyme specific activity potentially affects the biodegradation efficiency of any biological treatment system. In order to find out whether the presence of some additives could modulate (stabilize) phenol hydroxylase accumulation, the enzyme activity in *C. maltosa* cells growing on phenol in the presence of humic acid (Fig. 6) was investigated in view of the fact that some biological effects (binding capacity) of humic acids consist in their ability to aggregate on yeast (outer) cell wall [7]. The results obtained, comparing the phenol utilization in the absence and presence of humic acid (Fig. 7), support the idea that an additive layer on the cell surface that could control and stabilize the phenol concentration in this microenvironment could stabilize the phenol hydroxylase activity as well.

Figure 6: Hypothetical structure of a segment of humic acid (based on information from several sources)
Figure 7: Time course of changes in phenol hydroxylase activity in *C. maltosa* cells growing with phenol in the absence (A) and presence (B) of humic acid (0.05 g/l).

[■] – phenol hydroxylase activity; [◊] – biomass concentration; [▲] – phenol
4 Conclusions

In agreement with known data of comparable phenol hydroxylase induction in bacterial cells, the phenol degradation by yeasts revealed that similar detection of this enzyme activity is required to propose conditions optimizing the function of any (phenol, aromatic compounds) degrading yeast strain.

References