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Phenol degradation by yeasts isolated from industrial effluents

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Yeast strains of the genera *Aureobasidium*, *Rhodotorula* and *Trichosporon* were isolated from stainless steel effluents and tested for their ability to utilize phenol as the sole carbon source. Fourteen strains grew in the presence of up to 10 mM phenol. Only the strain *Trichosporon* sp. LE3 was able to grow in the presence of up to 20 mM phenol. An inhibitory effect was observed at concentrations higher than 11 mM, resulting in reduction of specific growth rates. Phenol degradation was a function of strain, time of incubation and initial phenol concentration. All strains exhibited activity of catechol 1,2-dioxygenase and phenol hydroxylase in free cell extracts from cells grown on phenol, suggesting that catechol was oxidized by the *ortho* type of ring fission. Addition of glucose and benzoate reduced the phenol consumption rate, and both substrates were used simultaneously. Glucose concentrations higher than 0.25% inhibited the induction of phenol oxidation by non-proliferating cells and inhibited phenol oxidation by pre-induced cells.

Key Words——catechol 1,2-dioxygenase; phenol; phenol hydroxylase; yeasts

Introduction

Phenol and other phenolic compounds are common constituents of the aqueous effluents from operations such as polymeric resin production, petrochemical plants, ceramic plants, oil refining, coking plants and stainless steel production. Although a variety of physicochemical techniques are available for the clean up of surface water, some interest in the use of microbial biodegradative activity is growing (Klein and Lee, 1978; Müller et al., 1996). The degradation or biotransformation of these phenolic compounds in aquatic and terrestrial environments by bacteria has been well documented. The most information on metabolic pathways comes from studies on bacteria. Some filamentous fungi (Jones et al., 1995), algae (Semple and Cain, 1996), and higher plants (Prasad and Ellis, 1978) have been reported to be able to degrade phenol. Reports on the decomposition of phenolic compounds by yeasts are scanty when compared to those on bacteria (Anderson and Dagley, 1980; Cook and Cain, 1974; Gaal and Neujahr, 1981; Hirayama et al., 1994; Hofmann and Vogt, 1987) and in all of the cases reported, the utilization of phenols was limited and slow. Middelhoven (1993) has made an extensive study on the metabolism of aromatic compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi, and demonstrated that the catechol branch of the 3-oxoadipate pathway and its hydroxyhydroquinone variant were involved in phenol and resorcinol catabolism of ascomycetes as well as of basidiomycetes.

As in every metabolic process, biodegradability can be affected by several factors, including, for example, the phenol concentration and the presence of alternative carbon sources. Phenol, in sufficiently high con-

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centrations, can be a toxicant for species capable of using it as a growth substratum (Alexander, 1985). Concentrations as low as 0.25 mM have been supported to inhibit growth of mesophilic microorganisms in waste water treatment plants (Gurujeyalakshmi and Oriel, 1989). In these environments, organic pollutants frequently occur in mixture with other synthetic as well as natural organic compounds. Therefore, it is necessary to understand how the biodegradation of the polluting compounds is affected by the presence of alternate substrates. Several studies have demonstrated the effectiveness of supplemental substrates for enhancing the rate of biodegradation of toxic chemicals (Hess et al., 1990; Scow et al., 1989). Zaidi and Mehta (1995) observed that the effects of glucose and phenol on the degradation of *p*-nitrophenol were dependent on the inoculated bacteria. Other authors have reported that several organic acids, such as succinate, acetate and benzoate have an inhibitory effect on the synthesis of phenol catabolic enzymes in bacteria, corresponding to a decrease or repression in phenol consumption (Ampe et al., 1998; Müller et al., 1996). Such findings have practical significance. It would be beneficial if these results could be applied to the operation of wastewater treatment systems to stimulate the breakdown of synthetic compounds.

The actual contribution of yeasts in the biodegradation of hydrocarbons in the environment may be more important than that previously expected, considering the metabolic diversity demonstrated for yeasts (Middelhoven, 1993; Sampaio, 1999). The use of naturally occurring yeasts represents a potentially promising means of destroying polluting chemical in waste water treatment systems or soil. In a screening program carried out in our laboratory, we isolated from stainless steel production effluents yeasts able to utilize phenol in up to 20 mM concentrations. The present paper describes: (I) isolation and the resistance of the isolates to phenol, (II) phenol degradation by yeasts and (III) some aspects of the synthesis of enzymes participating in phenol metabolism.

Materials and Methods

Microorganisms. Yeasts were isolated from effluents collected at different stages of operation in a stainless steel plant (Minas Gerais, Brazil) by an enrichment technique. Fifty milliliters of each sample was inoculated into flasks containing 50 ml of twice concentrated Yeast Nitrogen Base (YNB, Difco, Detroit, MI, USA) plus 2 mM phenol and incubated at 25°C for 72 h. The microorganisms were identified according to the keys Kreger-van Rij (1984) and Barnett et al. (1990). The yeasts were maintained on GYMP slant medium (w/v—2% glucose, 0.5% yeast extract, 1% malt extract, 0.2% NaH_2PO_4, and 2% agar) under a mineral oil layer and stored at 4°C, or in liquid nitrogen.

Culture conditions. The mineral medium used contained (g L^{-1}): 3.4 K_2HPO_4, 4.3 KH_2PO_4, 0.3 $MgCl_2 \cdot 2H_2O$, 1 $(NH_4)_2SO_4$, 0.05 yeast extract, plus 5 ml of a trace element solution (mg L^{-1}—1 $MnCl \cdot 4H_2O$, 0.6 $FeSO_4 \cdot 7H_2O$, 2.6 $CaCl_2 \cdot H_2O$, 6 $Na_2MoO_4 \cdot 2H_2O$). Phenol, glucose or benzoate as a carbon source was added to the sterilized mineral medium at the desired concentrations.

For the induction and phenol degradation studies using non-proliferating cells, *Trichosporon* sp. LE3 was grown in Erlenmeyer flasks containing 1 L mineral medium plus 0.5% glucose or 3 mM phenol, and was incubated in a rotary shaker (150 rpm) at 30°C. The cells were washed twice with sterile 50 mM potassium phosphate buffer, pH 7.6, and used as inoculum (3.6 g dry weight). The assays were conducted in 500 ml Erlenmeyer flasks containing 250 ml of sterile 50 mM potassium phosphate buffer, pH 7.6, plus 1 mM phenol or 1 mM phenol and 0.5% glucose placed in a rotary shaker (150 rpm) at 30°C. Twenty milliliter samples were harvested aseptically at 1 h intervals and centrifuged. Pellets were used for the preparation of cell-free extract and the supernatant for phenol and/or glucose determination.

Tests of phenol tolerance and degradation. The strains were grown in plates containing YNB (Difco) plus phenol at concentrations of 1 to 10 mM for 48 h at 30°C. The phenol degradation experiments were performed after inoculation of 20 mg (dry weight) cells grown in 3 mM phenol into 40 ml liquid mineral medium plus phenol (1 to 20 mM) and incubated in a rotary shaker at 30°C. To determine the effect of the addition of glucose or benzoate on phenol degradation, these carbon sources were also added to the YNB medium containing 4 mM phenol at concentrations of 0.5% and 5 mM, respectively. Growth was determined at 24 h intervals by measuring optical density (O.D. 440 nm) and substrate consumption.

Enzyme assays. For cell-free extracts, cells were harvested and washed twice in 100 mM Tris-HCl, pH

8.0, at 5°C. The cells were cooled in liquid nitrogen, disrupted and resuspended in the same buffer. The crude extracts were centrifuged at 5,000 rpm for 10 min at 5°C and used to assay enzyme activities. The enzyme assay as phenol hydroxylase (EC 1.14.13.7) was conducted spectrophotometrically by monitoring the decrease in absorbance at 340 nm, as described by Jones et al. (1995). Spectrophotometric measurements were recorded every 15 s for 2 min at ambient temperature. Catechol 1,2-dioxygenase (EC 1.14.13.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) activities were measured spectrophotometrically at 260 nm for the detection of *cis*,*cis*-muconic acid (Varga and Neujahr, 1970) and at 375 nm for the detection of 2-hydroxymuconic semi-aldehyde (Sala-Trepat and Evans, 1971), respectively. Readings were taken at 30 s intervals for 5 min at ambient temperature. Controls without substrate or extract were prepared for each assay. One enzyme unit is defined as the amount of enzyme that catalyzes the disappearance of 1 μmol co-substrate per minute or the formation of 1 μmol product per minute. Protein concentration in the cell extract was determined by the method of Bradford (1976) using bovine serum albumin as standard. Specific activity was defined in units mg protein^{-1}.

Analytical methods. Phenol concentrations were measured spectrophotometrically at 270 nm. The concentrations of substrates and products were determined by high performance liquid chromatography using an HPLC-Shimadzu, Model-10 AD apparatus, equipped with an integrator Model C-R7A Chromatopac (Shimadzu, Kyoto, Japan). Detection was at 210 nm with a UV-detector (SPD-MGA, Shimadzu). Separation was obtained with a reverse-phase column (C18 5 μm Lichrosorb RP 18, 125×4.0 mm, Merck, Darmstadt, Germany) and the operating conditions were as follows: ambient temperature; mobile phase, 20 mM H_3PO_4–MeOH–CH_3CN (65 : 15 : 20, v/v/v); and flow rate, 0.8 ml min^{-1}. Glucose concentration in the medium was measured by an enzymatic colorimetry method (Labtest, Brazil).

All experiments were repeated three times and the mean values obtained are reported. The values of standard deviations (SD) obtained were less than 10%.

Results and Discussion

Isolation and tests of phenol tolerance

Twenty-two yeast strains were isolated from effluents from the stainless steel industry. The steel is obtained by smelting iron ore with charcoal or mineral coal, in the presence of heat, and the effluents generated contained phenol concentrations from 5 to 326 ppm. The isolated strains were identified as belonging to the genus *Trichosporon* sp. (LE3, LE4), *Aureobasidium* sp. (FE6, FE7, FE9, FE13) and *Rhodotorula* sp. (FIB22, AF1, AF3, FE1, FE3, FE11, LE1, LE2). Among the yeasts isolated, 14 grew in YNB with phenol concentrations up to 10 mM, and were selected for the tests of phenol degradation in YNB liquid. Another study has cited the occurrence of yeasts in the stressed environment of a gold extraction liquid circuit, in which the cyanide concentration varies from 150 to 300 ppm, and the isolated yeasts belonged to the genera *Candida*, *Debaryomyces*, *Aureobasidium*, *Geotrichum*, *Pichia*, *Rhodotorula*, *Tremella*, *Hanseniaspora* and *Cryptococcus* (Rezende et al., 1999). It has been suggested that some yeasts isolated from all types of water, for example *Aureobasidium* and *Rhodotorula* that normally are associated with foliage and soil, probably enter water with run-off (Hagler and Ahearn, 1987). Various biotic and abiotic factors, including the availability of a suitable organic carbon source, are of importance for the distribution of yeast species in the environment. The phenol tolerance observed for the isolated yeasts was determinant to their occurrence in these effluents containing phenol.

Tests of phenol degradation

The extent of phenol degradation and the time for complete phenol degradation varied as a function of strain and the initial phenol concentration in the culture medium (Table 1). For concentrations lower than 6 mM, no significant decreases in percent degradation were observed. Concentrations above 6 mM reduced the percentage of phenol degradation to values up to 10%. *Trichosporon* strains showed complete phenol degradation when grown in 8–10 mM phenol during 120 h of incubation. *Trichosporon* sp. LE3 and LE4 and *Aureobasidium* sp. FE9 and FE13 presented the highest degradation percentage. LE2 and FIB22 strains of *Rhodotorula* sp. presented the highest degradation percentage, up to 8 mM of initial phenol concentration, and Fe11 and AF3 strains, the highest percentage at

Table 1. Phenol degradation by yeast strains.

Strain	Phenol degradation (%)[a]				
	Initial phenol concentration (mM)				
	2	4	6	8	10
Trichosporon					
LE3	97 (24)	96 (24)	95 (24)	93 (24)	95 (48)
LE4	94 (24)	93 (24)	96 (48)	85 (48)	94 (72)
Aureobasidium					
FE6	89 (48)	96 (96)	87 (96)	78 (120)	25 (120)
FE7	84 (48)	88 (96)	90 (96)	77 (120)	21 (120)
FE9	94 (48)	85 (72)	91 (96)	90 (96)	30 (120)
FE13	90 (48)	85 (72)	90 (96)	90 (96)	68 (120)
Rhodotorula					
FE11	99 (96)	99 (96)	87 (120)	56 (120)	21 (120)
FE1	98 (96)	99 (96)	91 (120)	20 (120)	10 (120)
FE3	97 (72)	92 (96)	87 (120)	63 (120)	19 (120)
AF1	89 (72)	85 (72)	79 (120)	56 (120)	18 (120)
AF3	94 (72)	84 (72)	92 (120)	56 (120)	20 (120)
LE1	99 (120)	98 (120)	87 (120)	77 (120)	16 (120)
LE2	87 (48)	81 (72)	91 (120)	23 (120)	10 (120)
FIB22	95 (48)	95 (96)	89 (120)	24 (120)	11 (120)

[a] The values between parentheses indicate the time (h) required for phenol degradation (%). The cultures were incubated for 120 h.

8–10 mM of phenol. The values of residual phenol, specific growth rate (h^{-1}) (μ) and phenol degradation rates ($g\,L^{-1}\,h^{-1}$) (Tx) of these *Trichosporon* sp. and *Aureobasidium* sp. strains are shown in Fig. 1. The increase in phenol concentration resulted in an increase of cell mass, degradation rate and specific growth rate. FE9 and LE4 strains showed increasing values of μ and Tx up to 5 and 9 mM, respectively (Fig. 1A and D). The values of μ and Tx for strains FE13 increased up to 7 mM (Fig. 1B). The LE3 strain showed increasing values of μ and Tx up to 10 mM (Fig. 1C), suggesting a higher phenol tolerance. This strain was used for degradation tests up to 20 mM in liquid YNB medium.

Trichosporon LE3 grew in the presence of up to 20 mM phenol, but showed a reduction in μ at concentrations higher than 11 mM, suggesting an inhibitory effect of phenol at these concentrations (Fig. 2). According to Monod (1949), the higher the concentration of the growth-limiting substrate, the higher will be μ and the closer it will approach a maximum value. However, if this substrate show inhibitory action, the continuous increment in μ should not happen, but rather a reduction in μ would occur increasing substrate concentration (Haldane and Briggs, 1925). The inhibitory effect on growth resulted in reduced phenol degradation rates and increased incubation times for phenol degradation (Fig. 3). Complete phenol degradation was not observed at concentrations higher than 18 mM during 120 h of incubation. The tolerance limit of phenol was relatively high in comparison to most yeasts and bacteria isolated in the environment. The strains of bacteria of genus *Pseudomonas*, with high tolerance limit of phenol already isolated, were able to utilize 10 mM phenol (Hinteregger et al., 1992; Kotturi et al., 1991; Masqué et al., 1992). Most recently, a strain of *Comamonas testosteroni* P15 and its mutant strain E23, tolerant up to 15 and 20 mM phenol, respectively, were cited by Yap et al. (1999). A strain of *Trichosporon cutaneum* reported by Neujahr and Varga (1970) showed a tolerance to phenol lower than 500 ppm (5.31 mM). A mutant of *Candida tropicalis*' high tolerance of phenol, 22 mM, was reported by Chang et al. (1995).

Activities of enzymes involved in phenol degradation

Table 2 shows that cells grown on 2 mM phenol presented phenol hydroxylase and catechol 1,2-dioxygenase activities, suggesting that phenol induces the synthesis of these enzymes. The 2-hydroxymuconic semi-

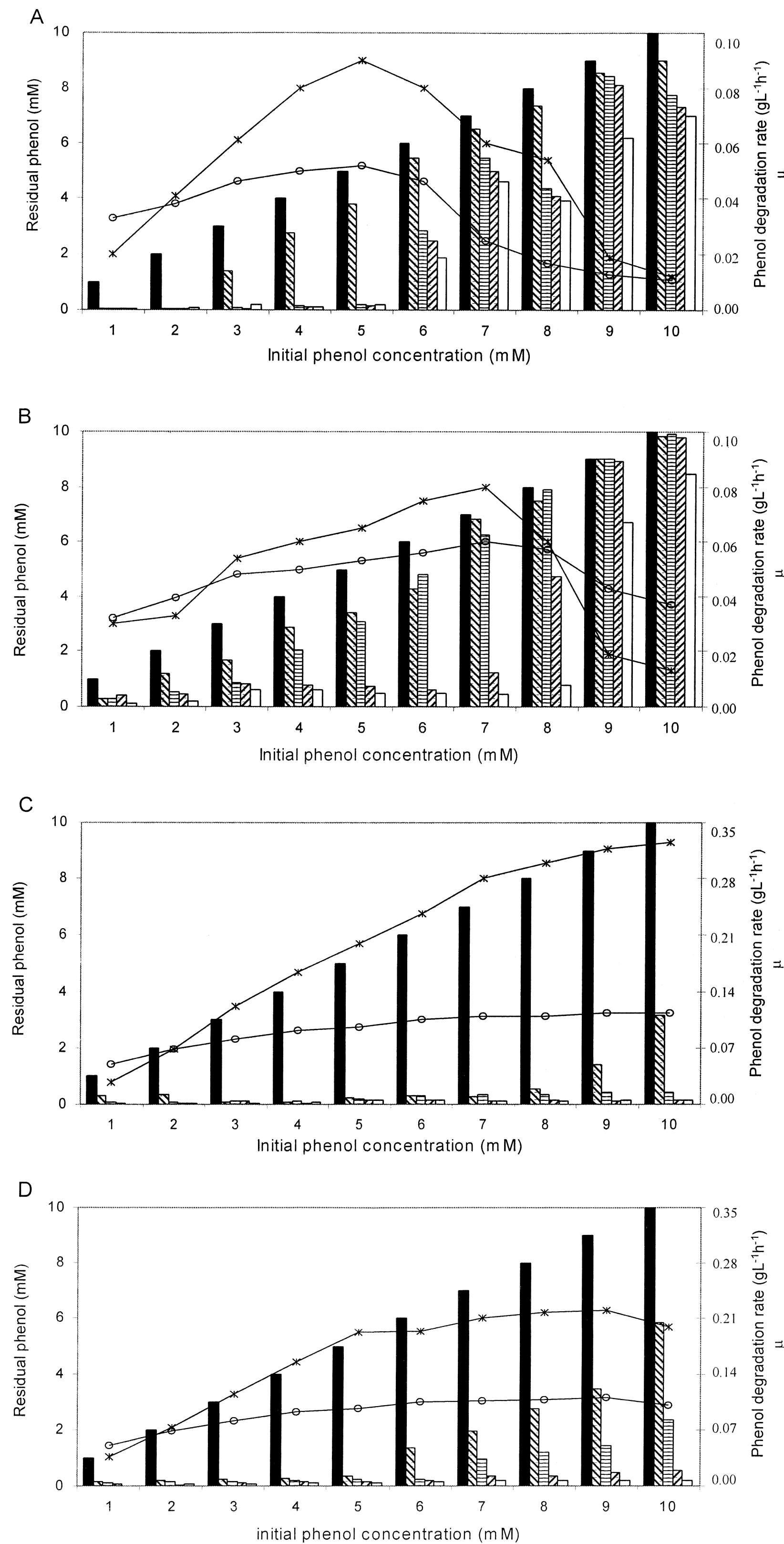

Fig. 1. Effect of initial phenol concentration on residual phenol values, on specific growth rate (μ) (h^{-1}) (○), and on the rates of phenol degradation (∗) for *Aureobasidium* FE9 (A) and FE13 (B), and *Trichosporon* LE3 (C) and LE4 (D), at 30°C during 120 h of incubation.

Phenol degradation rate values were multiplied ten times for legibility on the secondary *y* axis. ■ 0 h, ▧ 24 h, ▤ 48 h, ▨ 72 h, □ 120 h.

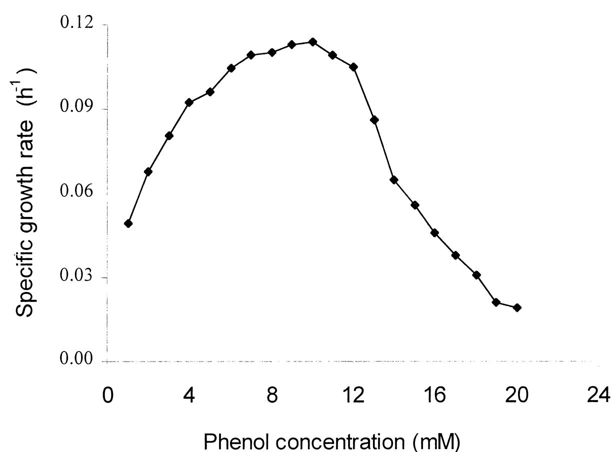

Fig. 2. Effect of initial phenol concentration on specific growth rates (μ) of *Trichosporon* sp. LE3.

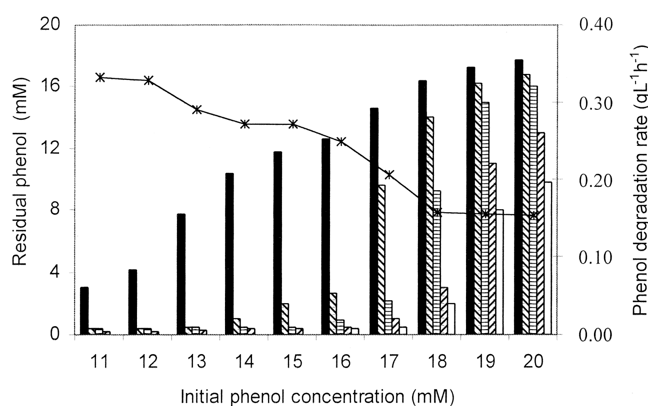

Fig. 3. Phenol degradation by *Trichosporon* sp. LE3 grown on 11 to 20 mM phenol during different incubation times, at 30°C.

Phenol degradation rates (∗). ■ 24 h, ▧ 48 h, ▤ 72 h, ▨ 96 h, □ 120 h.

Table 2. Enzymatic activity as phenol hydroxylase and catechol 1,2-dioxygenase activities from cell-free yeasts extracts.

Isolate	Specific activity (U mg protein^{-1})	
	Phenol hydroxylase[a]	Catechol 1,2-dioxygenase[b]
Trichosporon sp.		
LE3	0.132	1.395
LE4	0.052	0.516
Aureobasidium sp.		
FE6	0.021	0.216
FE7	0.026	0.244
FE9	0.032	0.300
FE13	0.028	0.267
Rhodotorula sp.		
FIB22	0.099	0.199
AF1	0.009	0.072
AF3	0.007	0.063
LE1	0.019	0.100
LE2	0.038	0.359
FE1	0.009	0.083
FE3	0.008	0.093
FE11	0.017	0.158

[a] One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *cis,cis*-muconic acid min^{-1} mg protein^{-1} at ambient temperature.

[b] One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADPH min^{-1} mg protein^{-1} at ambient temperature.

aldehyde, the product of catechol 2,3-dioxygenase action on catechol oxidation by cell extracts, was not detected by spectrophotometric methods utilized in this study. The highest enzyme activities were obtained with cells of *Trichosporon* sp. LE3 and LE4 strains, *Rhodotorula* sp. LE2 and FIB22 strains and *Aureobasidium* sp. FE9 and FE13 strains, which also showed the highest rates of phenol degradation. The activities of phenol-degrading enzymes of strain LE3 were three times higher than those of strain LE4. This difference could explain the higher values of Tx shown by strain LE3 grown in up to 10 mM phenol and resulting in an increase of phenol tolerance (Fig. 1C and D). The maximum values of Tx were 0.032 g L^{-1} h^{-1} for LE3 and 0.022 g L^{-1} h^{-1} for LE4. Catechol was oxidized by means of the *ortho* pathway as indicated by the increase in absorbance at 260 nm, probably due to yield of *cis,cis*-muconic acid (Varga and Neujahr, 1970). A product with an identical catechol peak was obtained from a sample of yeast strain cultures grown in phenol by chromatographic analysis (data not shown). This indicated that phenol was hydroxylated to catechol prior to ring cleavage. Authors have reported the potential of the yeasts *Trichosporon*, *Candida* and *Aureobasidium* for the degradation of aromatic compounds, such as phenol (Hofmann and Krüger, 1985; Middelhoven, 1993; Neujahr and Varga, 1970). These authors observed that phenol was metabolized by the β-ketoadipate pathway, through *ortho* fission of the catechol.

Effect of glucose and benzoate addition on phenol degradation by proliferating cells

Glucose addition had an inhibitory effect on phenol degradation by *Trichosporon* sp. LE3, mainly at con-

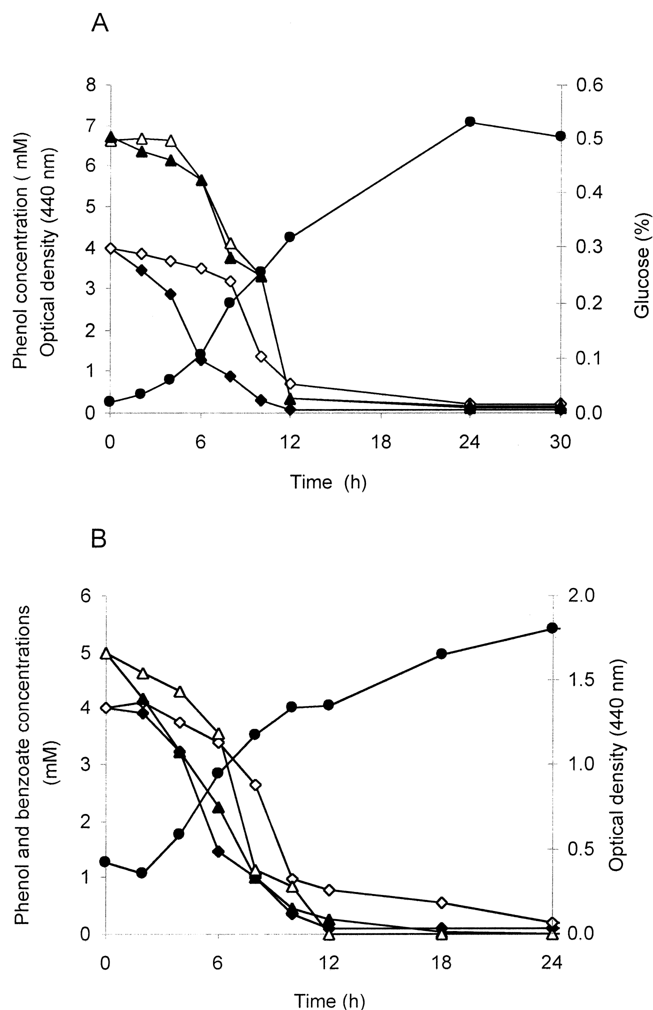

Fig. 4. Effect of glucose (A) and benzoate (B) on the rate of phenol degradation.

Concentration of glucose (A) or benzoate (B) in medium containing the two substrates (▲); phenol concentration in medium with phenol added (◆); phenol concentration in medium containing phenol and glucose (A) or phenol and benzoate (B) (◇); concentration of glucose (A) or benzoate (B) in medium without phenol (△); growth in medium supplemented with phenol and glucose (A) or phenol and benzoate (B) (●).

centrations above 0.28% (Fig. 4A). However, the inhibition was not complete, because phenol was degraded in the presence of glucose, although at a lower rate. Addition of benzoate reduced the rate of phenol consumption, but did not abolish it, both substrates being used simultaneously (Fig. 4B). The repression of the catabolism of the less favorable substrate for other carbon sources (catabolic repression) has been extensively described for enteric bacteria and some other genera such as *Bacillus* and *Pseudomonas* (Müller et al., 1996). Little is known about these mechanisms in filamentous fungi and yeasts. According to Skoda and Udaka (1980), phenol was preferentially utilized by *Trichosporon cutaneum* in growth medium containing phenol and glucose. Glucose prevents phenol utilization in resting cells of another strain of *T. cutaneum* (Gaal and Neujahr, 1981) and in growing batch cultures of *Candida maltosa* (Hofmann and Kruger, 1985). In carbon-limited chemostat cultures of *C. maltosa*, glucose and phenol are utilized simultaneously (Hofmann and Vogt, 1987). Hofmann and Schauer (1988) observed that *n*-hexadecane inhibited phenol degradation in a strain of *C. maltosa*, but not in *C. tropicalis* or *T. cutaneum*. It has been suggested that these substrates repress phenol catabolism by inhibiting the synthesis of phenol hydroxylase and of the enzymes of catechol fission.

Effect of glucose on induction and phenol metabolism by non-proliferating cells of Trichosporon LE3

Figure 5A shows that in the absence of glucose, phenol was consumed after 2 h by cells grown in phenol (induced for the synthesis of the enzymes of phenol metabolism). However, in the presence of glucose, phenol consumption by induced cells began after 2 h of reaction (Fig. 5B). Addition of glucose decreased the rate of phenol consumption, which occurred after 7 h. According to Gaal and Neujahr (1981), glucose or its metabolites could inhibit the transport of phenol or the synthesis of such a transport system.

The cells grown in glucose did not present phenol hydroxylase or catechol 1,2-dioxygenase activities (Fig. 5C and D). The synthesis of the enzymes began when the cells were added to the reaction mixture containing phenol (Fig. 5C). In the presence of glucose, the enzyme activities were observed after 3 h of reaction, when glucose concentration had decreased to about 0.25% (Fig. 5D). Glucose reduced the rate of phenol degradation, but did not fully inhibit its utilization. Hofmann and Krüger (1985) observed that in batch cultures of *C. maltosa*, phenol monooxygenase and catechol dioxygenase were induced by phenol, but these enzymes were not detected in cultures growing on glucose and *n*-hexadecane.

These yeast strains present a great potential for the biodegradation of phenol and possibly of other related phenolic or aromatic compounds. Such microorganisms can be further studied for use in industrial effluent treatment and decontamination of natural areas.

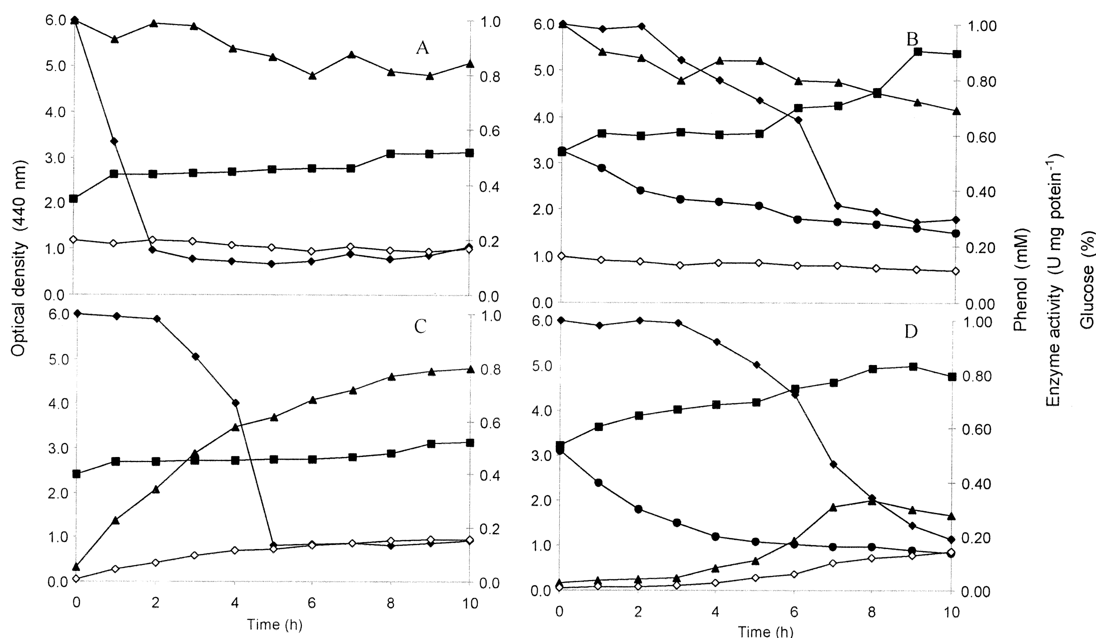

Fig. 5. Enzyme induction and phenol and glucose consumption in non-proliferating cells.

Cells grown in phenol: A) incubation mixture with 1 mM phenol, B) incubation mixture with 1 mM phenol and 0.5% glucose. Cells grown in glucose: C) incubation mixture with 1 mM phenol, D) incubation mixture with 1 mM phenol and 0.5% glucose. Catechol 1,2-dioxygenase activity (▲); phenol hydroxylase activity (◇); residual phenol (◆); glucose (%) (●); OD at 440 nm (■).

Acknowledgments

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