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Phenol metabolism by two microorganisms isolated from Amazonian forest soil samples

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Two microorganisms isolated from Amazonian forest soil samples and identified as *Candida tropicalis* and *Alcaligenes faecalis* were capable of degrading phenol (16 and 12 mM, respectively) at high salt concentrations (15% and 5.6%, respectively). Chromatographic and enzymatic studies revealed that each microorganism cleaved phenol at the *ortho* position with total phenol mineralization. ^{14}C-phenol mineralization assays showed that both microorganisms assimilated about 30% of the total label. No phenol degradation metabolite (i.e., catechol, *cis, cis*-muconic acid) was detected in the intercellular medium. The presence of phenol hydroxylase (EC 1.14.13.7) and catechol 1,2-dioxygenase (EC 1.13.11.1) extracellular activity suggested that these microorganisms may secrete these enzymes into the extracellular medium. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 403–409.

Keywords: phenol; soil; enzyme; extracellular

Introduction

Despite the toxicity of phenol and its derivatives, often present in effluents of many industrial processes [32], numerous microorganisms can degrade these compounds when they are present at low concentrations [15,36]. However, in media enriched with micronutrients, some *Pseudomonas* isolates can utilize phenol as the sole carbon source at concentrations up to 1500 ppm (16 mM) [2]. Some characteristics of these media, such as available nutrients and temperature, humidity, pH and aeration, may affect phenol degradation [30]. Determining the environmental parameters required for microbial growth and degradative activity will assist in appropriate strategies for implementing remediation processes. Among these processes [39], bioremediation may be one of the safest, least costly and most socially acceptable [11]. In biological remediation methods, microorganisms, especially bacteria, are commonly used and they normally utilize two different pathways of phenol degradation, based on *ortho* or *meta* aromatic ring cleavage [1]. Both pathways use phenol hydroxylase in the first step of degradation but they are different because generally in the second degradation step, eukaryotes and prokaryotes utilize catechol 1,2-(*ortho*) or 2,3-(*meta*) dioxygenase, respectively [26,27].

Despite the fact that phenol degradation has been studied, there are no studies comparing intra- or extracellular enzyme levels responsible for phenol metabolism. However, some fungi show that extracellular phenol oxidases are utilized in degradation of lignin, a complex phenolic compound [7,14]. An understanding of where the reaction takes place (intra- or extracellularly) is important since the secreted enzymes are highly dependent on the environmental physical–chemical conditions [21]. Of these conditions, high salinity in the medium results in osmotic stress and requires the presence of microorganisms capable of tolerating this environmental condition [38]. The salinity in intensively cultivated soils and in most industrial effluents is a selective factor, being in some situations more toxic or stressful than the main pollutant.

In the research presented, two microorganisms isolated from an Amazonian soil sample and adapted to high salt and phenol concentrations [5] were studied to determine their degradation pathways and intra- and extracellular phenol-degrading enzyme activities during phenol degradation.

Materials and methods

Chemicals

Phenol ($14_{C_6}H_5OH$, UL), agar, FAD (flavin adenine dinucleotide), POP (2,5 diphenyl-oxazole), POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene) and NADPH (β-nicotinamide adenine dinucleotide phosphate) (Sigma, St. Louis, IL, USA), catechol and ethyl acetate (Mallinckrodt, Chesterfield, Missouri, USA), salts (Fisher Scientific, Fairlawn, NJ, USA), BSTFA (*N*,*O*-bis trimethylsilyl-trifluoracetamide, Pierce, Rockford, IL, USA), malt, yeast extract, chloroform, methanol and toluene (Merck, Whitehouse Station, NJ, USA) were used.

Microorganisms, inoculum development, culture medium and conditions

Two microorganisms were isolated from Amazonian soils following phenol enrichment as described in Ref. [4]. Briefly, the Amazonian soil samples were stirred in sterile distilled water (1:1, w/v; 1 h, room temperature) and 5 ml aliquots were

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inoculated in saline media (5× M_9 media, g/l: Na_2HPO_4 34; KH_2PO_4 15; NaCl 2.5; NH_4Cl 5; ml/l: 1 M $CaCl_2$ 0.5; 1 M $MgSO_4$ 10; pH 7.0) containing increasing phenol concentrations (0.21–14 mM) over approximately 2 months with shaking at 150 rpm and at 30°C. The 7.45 mM phenol considered was the best phenol concentration to support microbial growth. Colonies were selected and purified on solid 5× M_9 medium containing 7.45 mM phenol, Two isolates showing rapid phenol degradation were identified. The bacteria were classified based on colony and cell morphology, physiological and biochemical characteristics, comparing these data with standard species as described Refs. [22,25]. Bacterial identification was conducted using the API 20 kit from Bio Merieux Laboratories [8]. The yeast was identified as described in [3,23]. Inoculum was prepared in DSM65 liquid media (g/l: glucose 4, yeast extract 4, malt extract 10, $CaCO_3$ 2, agar 18, pH 7.0). All experiments utilized 2.5 g of cells (wet weight) per liter medium and growth at 29°C, with shaking at 150 rpm.

Phenol concentration

Phenol concentration in the culture medium was determined spectrophotometrically (Hewlett Packard 8451A, Palo Alto, CA, USA). Aliquots (1 ml) were centrifuged (10 min at 12,000×*g*, room temperature), the supernatant was diluted 10 times in distilled water and the absorbance was read at 270 nm in 1 ml quartz cuvettes [5]. A standard curve (0–14 mM) was determined, plotting the phenol concentration *versus* 270 nm absorbance.

Phenol metabolism

All experiments used appropriate abiotic controls to determine any influence on phenol degradation from non-biological processes.

Gas chromatographic analysis: The isolates were inoculated in 300 ml 5× M_9 media containing 7.45 mM phenol. Thirty milliliters of culture medium were centrifuged (10 min at 12,000×*g*, room temperature) and either supernatant or cell (pellet) water solution pH was adjusted to 2. The supernatant and pellet were extracted three times with the same volume of ethyl acetate, dried under a N_2 atmosphere and submitted to the silylation reaction with 100 μl *N*,*O*-bis trimethylsilyl-trifluoracetamide (BSTFA) (method modified from Refs. [18,37]). The reaction was carried out in closed flasks containing the same volume of solvent and heated in a water bath at 70°C for 1 h. The samples were re-suspended in 3 ml ethyl acetate and analyzed by gas chromatography mass spectrometry (GC-MS; ion trap detector, Finnigan Mat GCQ, San Jose, CA, USA). An Rtx-5 ms (Restek, Bellefonte, PA, USA) 30 m column was used (inner diameter: 0.25 μm; film thickness; 0.25 μm). Analytical conditions were: 50°C for 1 min, 50–250°C at 10°C per min and 250°C for 9 min. The temperatures of the injector, ion source and transfer lines were 250°C, 180°C and 260°C, respectively. The helium flow was 30 cm/s. The mass spectrometer was adjusted to have the detector filament activated at 6.20 min into the run, with positive polarity, 1 scan/s and a mass range of 30–500 uma. The experiment was done with six replicates.

^{14}C incorporation: Flasks (250 ml) containing 100 ml 5× M_9 medium supplemented with 7.45 mM phenol received enough radiolabeled phenol (phenol-UL-^{14}C) to yield 16.700 Bq initial radioactivity. The microorganisms were incubated (29°C, 150 rpm) and during the phenol degradation process, 1.0 ml aliquots were sampled and centrifuged (5 min at 12,000×*g*, 25°C), generating the first supernatant. The cell pellet was re-suspended in water and centrifuged (second supernatant). All sample activities (first and second supernatants and cell pellet) were measured using liquid scintillation spectroscopy (Packard Tri-Carb 1600 TR, Palo Alto, CA, USA) after the samples had been added to 10 ml of scintillation cocktail (0.4% POP, 0.02% POPOP, 33% RENEX 95, made up to the final volume in toluene) and the radioactivity was measured after incubation in 2 ml methanol for 12 h in the dark. All experiments were conducted in triplicate.

Assays of extra-and intracellular enzymes: The enzymes assayed were phenol hydroxylase (EC 1.14.13.7), catechol 1,2-dioxygenase (EC1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.1.2). The microorganisms were inoculated into 1.6 l 5× M_9 medium with one of the following: 7.45 mM phenol or 25 mM lactate (bacterial growth) or 25 mM glucose (yeast growth). After 70% phenol consumption (18 and 90 h, respectively, for yeast and bacteria), the growth media were centrifuged. The cells were macerated with sand in the appropriate buffer [27,31,36] and the crude extract was analyzed for the three enzymes. The supernatant was concentrated to a volume of 4 ml using Amicon YM 10 (Grace, Boca Raton, FL, USA) with a cellulose membrane PM 10,000 (Millipore, Bedford, MA, USA), under an argon atmosphere at 6°C. Each 2 ml was added to a dialysis membrane (Visking, London, UK) kept in an appropriate buffer for 16 h (at 6°C) [dialysis buffers: phenol hydroxylase: KH_2PO_4 50 mM, β-mercaptoethanol 1 mM, EDTA 0.1 mM, FAD 2 μM, pH 7.6; catechol 1,2-dioxygenase: Tris–HCl 50 mM, β-mercaptoethanol 5 mM, $FeSO_4$ 20 μM, pH 8.3 and catechol 2,3-dioxygenase: KH_2PO_4 100 mM, acetone 10%, pH 7.5]. The protein concentration was determined as in Ref. [16]. One enzyme unit (U) corresponds to 1 μmol compound formation or consumption per minute. Phenol hydroxylase activity was determined as previously described [27] following disappearance of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and recording the change in absorbance at 340 nm. Catechol 1,2-dioxygenase was assayed by following the appearance of *cis*,*cis*-muconic acid at 260 nm [36] and modifying the Tris–HCl buffer pH to 7.5 and catechol 2,3-dioxygenase by following the appearance of 2-hydroxymuconate semi-aldehyde at 375 nm [31]. All enzymes were assayed spectrophotometrically at ambient temperature. Experiments were replicated three times.

Results

Identification

The microorganisms were identified as the yeast *Candida tropicalis* and the Gram-negative bacterial species *Alcaligenes faecalis* based on their morphology, physiological and biochemical characteristics, Gram-negative reaction and the API identification scheme. We verified this identification using Biolog plates (Biolog, Hayward, CA, USA), fatty acid profiles and 16*S*/18*S* RNA analyses (Bastos et al., unpublished results). Although *C. tropicalis* has been referenced many times in the literature as a phenol degrader [9,10,28], there is only one report of *A. faecalis* as a phenol degrader isolated from petroleum wastewater sediment samples [33]. Most works on *Alcaligenes* species show phenol degradation by *meta* cleavage

of the aromatic ring [20] while studies on *C. tropicalis* indicate phenol degradation by *ortho* cleavage [28].

Chromatographic studies

Analyses of the supernatant, from the growth of each microorganism, showed the presence of the same metabolites (Figures 1 and 2). A comparison of the mass spectra from sample-extracted compounds (Figure 2) with silylated standards (phenol, catechol) and literature data [18] showed that the peaks in Figure 1 are phenol (peak 1), catechol (peak 2) and *cis,cis*-muconic acid (peak 3), indicating that the phenol pathway for both microorganisms was via *ortho* cleavage of the aromatic ring.

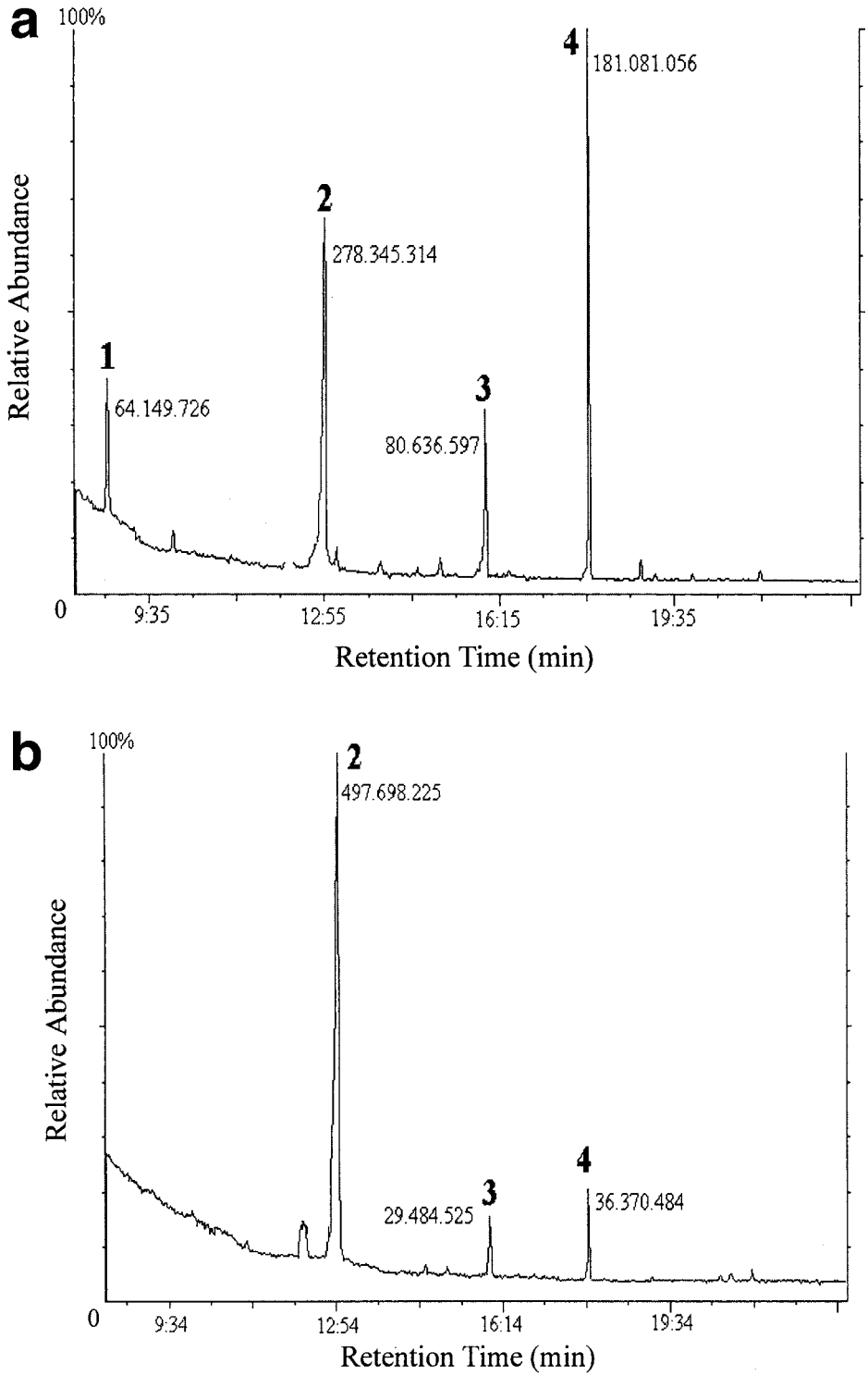

Figure 1 GC-Chromatogram from *Candida tropicalis* (1a) and *Alcaligenes faecalis* (1b) extracted supernatant during phenol degradation. The numbers next to the peaks represent their electronic areas. Peak 1 is phenol; Peak 2 is catechol; Peak 3 is *cis,cis*-muconic acid (Peak 4 is unknown).

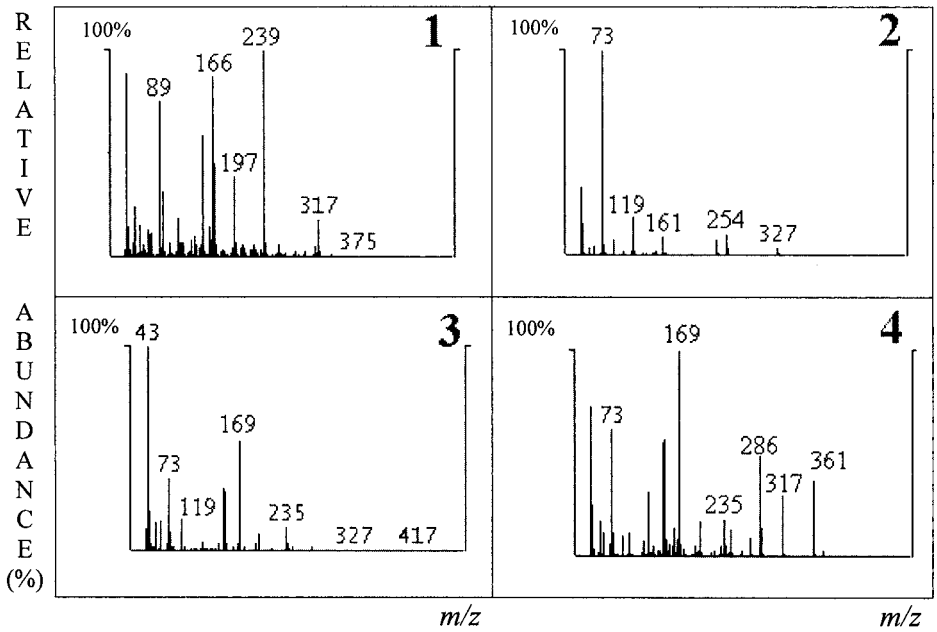

Figure 2 Mass spectrometry analysis (ion mass per charge; *m/z*) for the compounds 1 (phenol), 2 (catechol), 3 (*cis,cis*-muconic acid) and 4 (unknown) present in the supernatant during phenol degradation by *Candida tropicalis* and *Alcaligenes faecalis*.

All compounds analysed were totally metabolized by both microorganisms. *C. tropicalis* degraded all phenol present after 24 h and *A. faecalis* produced the same level of phenol degradation after 96 h. Peak number 4 (Figure 2) showed mass spectra similar to spectra of the reference *cis*,*cis*-muconic acid, but it has not been previously identified, to our knowledge. Although these compounds were detected in the supernatant, they were not found in the cell extracts from either microorganism.

^{14}C incorporation

Background radioactivity from control experiments, for both the supernatant and the cells, without radiolabeled phenol addition was about 1.0 Bq. Both the supernatant and cell activity are shown in Figure 3.

Approximately 85% of the initial media activity was not available in the supernatant after 30 and 150 h growth for *C. tropicalis* and *A. faecalis*, respectively (Figure 3). The final activity balance after phenol degradation by both microorganisms is shown in Table 1.

Despite final cell ^{14}C assimilation being approximately 30%, no metabolites were detected inside the cells. Thin layer chromatography (samples were chromatographed on silica plates [10×20 cm^2 plates, 5–17 μm particle size, dried 1 h, at 110°C; Sigma, St. Louis, MO, USA] using chloroform:ethyl acetate:acetic acid [49:49:2; v/v/v] as solvent and iodine and Scanner Trace Master 40 for detection) of cell extracts did not show any compounds related to phenol degradation (data not shown).

Enzyme assays

Phenol hydroxylase and catechol 1,2-dioxygenase activity for both microorganisms was detected in the presence of phenol. No catechol 2,3-dioxygenase activity, intra- or extracellular, was detected. These data confirm the results obtained by gas chromatography, showing *ortho* cleavage of phenol. Intra- and extracellular phenol hydroxylase activity from both microorganisms growing on phenol media is presented in Table 2. Activity was evident in both microorganisms from both intra- and extracellular measurements. Extracellular activity was three times lower for *C. tropicalis* and two times higher for *A. faecalis* compared to intracellular activity. The intra- and extracellular activity in *C. tropicalis* was 7 and 1.2 times higher, respectively, in comparison to *A. faecalis*.

The catechol 1,2-dioxygenase activity was analyzed from intra- and extracellular samples (Table 3). Activity in *C. tropicalis* was 9.5 times higher than extracellular and in *A. faecalis*, it was five times higher. The intracellular activity was two times higher in *C. tropicalis* than *A. faecalis*, while the extracellular activity was approximately the same for both. Intracellular catechol 1,2-dioxygenase activity for both microorganisms was higher than phenol hydroxylase activity.

Discussion

Most yeasts degrade phenol by *ortho* cleavage while bacteria degrade by *meta* cleavage [1,6]. Chromatographic and enzymatic results showed that *C. tropicalis* and *A. faecalis* degraded phenol by *ortho* cleavage. In aromatic compound degradation, the *meta* pathway is considered unproductive, especially in haloaromatic degradation [29]. Muller and Babel [26] determined that *ortho* cleavage is 9–23% more efficient than *meta* on a biomass production basis. This metabolic characteristic may be because both microorganisms were isolated from a large soil population and considered the best phenol degraders from each group selected (prokaryotic and eukaryotic) [4].

In all experiments (chromatographic studies and ^{14}C incorporation), there was no evidence of an intracellular metabolite related to initial phenol degradation. The cell extracts analyzed by GC-MS or thin layer chromatography (TLC) did not show any phenol or phenol metabolite (catechol or *cis*,*cis*-muconic acid)

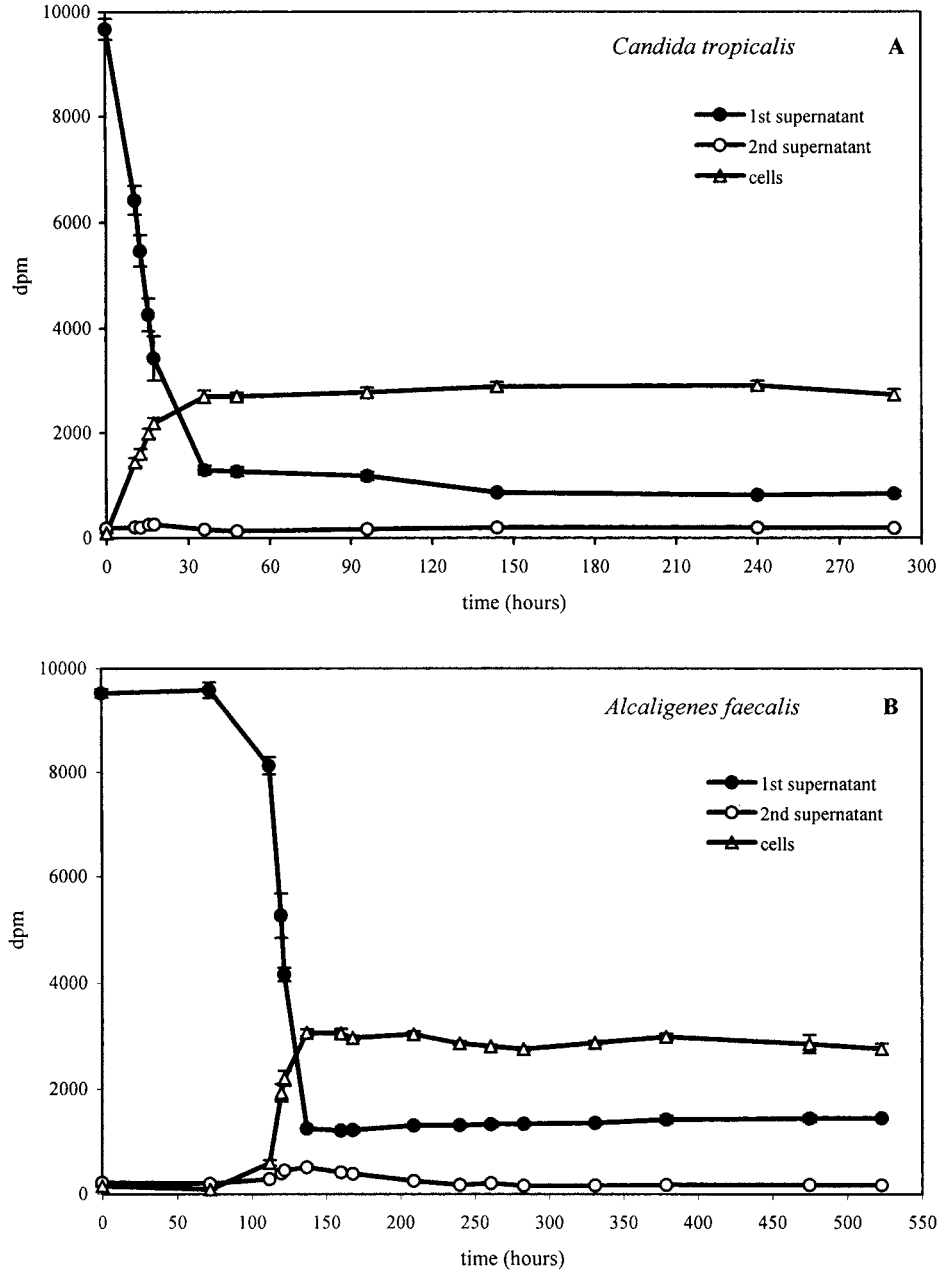

Figure 3 ^{14}C-Activity (Bq) determined from supernatants 1, 2 and cells during phenol degradation by *Candida tropicalis* (3A) and *Alcaligenes faecalis* (3B). Each value is the mean ± standard error (n = 3). Supernatants 1 and 2 refer to the supernatants after the first centrifugation and after washing the cell-pellet with distilled water, respectively.

but they were easily detected in the supernatant (extracellular). The increase in cell radioactivity was not related to phenol, catechol or *cis*,*cis*-muconic acid incorporation by cells. Leonard and Lindley [24] studied the *meta* phenol pathway from *A. eutrophus*. They determined that the 2-hydroxymuconate semi-aldehyde (2 hms) diffused passively through the membrane and their estimates showed an internal 2 hms concentration seven times higher than external. Thus, it is possible to analyze internal compounds during phenol metabolism. Our results also revealed that there were no internal compounds related to phenol metabolism, indicating there is a possibility of extracellular or membrane level phenol degradation, not permitting phenol or initial metabolites entrance in the cell. These results were confirmed by phenol hydroxylase and catechol 1,2-dioxygenase activities in the supernatant. Despite the higher activity in crude cell extracts, significant extracellular activity was observed, showing that even after an extended time to concentrate the supernatant fluid (8 h) and dialysis (12 h), the enzymes were still active. Previous work suggests that the phenol hydroxylase (PH) and catechol 1,2-dioxygenase (C1,2D) enzymes from crude cell extracts have been completely inactivated after extended dialysis, mainly in the presence of catechol derivatives and inorganic salts [17,27,36]. This condition was the same in the growth medium and the results showed a tolerance for both enzymes with a significant extracellular activity. Working with PH measurements on crude cell extracts, Spanning and Neujahr

Table 1 Final radioactivity balance (^{14}C-phenol) after phenol degradation by *C. tropicalis* and *A. faecalis*

Radio activity	*C. tropicalis* (after 290 h)	*A. faecalis* (after 523 h)
Initial activity (0 h) [I]	161±5.81 Bq	159±2.4 Bq
Supernatant activity (1+2) [S]	8.7±0.88%	15±0.33%
Cell radioactivity (final) [C]	28±1.84%	29±1.78%
Radioactivity liberated on degradation [I] − ([S]+[C])	63.3±1.41%	56±1.52%

The percentage is related to the initial activity (0 h). The data presented are the mean of triplicate experiments (±SD, n=3).

Table 2 Phenol hydroxylase activity in cell crude extracts (intracellular) and concentrated growth media (extracellular) after growth of *C. tropicalis* and *A. faecalis* (after 70% phenol consumption, 18 and 90 h, respectively, for the yeast and bacterium) in media (5× M_9 medium) enriched with phenol (7.46 mM)

Source	Total volume (ml)	Protein (mg/ml)	mU/ml[a]	mU/mg	Total activity[b] (mU)
C. tropicalis intracellular	4.5	1.02±0.02	36±1.5	35±3.6	163±7
C. tropicalis extracellular	2.0	0.97±0.02	30±2.5	32±3	61±3
A. faecalis intracellular	4.0	2.48±0.036	6±0.15	2±0.15	24±0.6
A. faecalis extracellular	2.0	3.21±0.045	24±3	8±0.32	47±0.6

The data presented are the mean of triplicate experiments (±SD, n=3).
[a]One enzyme unit (U) corresponds to 1 μmol compound formation or consumption per minute.
[b]Total activity is unit per ml (mU/ml) multiplied by total volume (ml).

Table 3 Catechol 1,2-dioxygenase activity in cell crude extract (intracellular) and concentrated growth media (extracellular) from *C. tropicalis* and *A. faecalis* (after 70% phenol consumption, 18 and 90 h, respectively, for the yeast and bacterium) growing in media (5× M_9 medium) enriched with phenol (7.46 mM)

Source	Total volume (ml)	Protein (mg/ml)	mU/ml[a]	mU/mg	Total activity[b] (mU)
C. tropicalis intracellular	5.2	0.65±0.027	472±19.7	731±8.5	2 456±102
C. tropicalis extracellular	2.0	2.11±0.085	138±4.3	68±5.8	276±9
A. faecalis intracellular	4.0	1.18±0.006	349±5.6	286±7.1	1 396±22.3
A. faecalis extracellular	5.0	1.93±0.05	53±6	25±5	267±31

The data presented are the mean of triplicate experiments (±SD, n=3).
[a]One enzymatic unit (U) corresponds to 1 μmol compound formation or consumption per minute.
[b]Total activity is unit per ml (mU/ml) multiplied by total volume (ml).

[35] found a specific activity (mU/mg) of 39 and Gaal [12] of 240, showing that the enzymatic activity depends on the specific microorganism and the phenol quantity in the growth medium during sample collection. Since phenol oxidation is an inductive process, enzyme production could vary under different growth conditions [13]. Some authors [12,34,36] working on C1,2D in crude extracts from *Trichosporon cutaneum* showed specific activities similar to ours of 105, 150 and 560 mU/mg, respectively. The higher PH and C1,2D activity in *C. tropicalis* compared to *A. faecalis* could be the reason for the better induction of the yeast during phenol degradation and could explain the shorter degradation time for the yeast for the same phenol concentration. The C1,2D activity was always in contrast to the conclusions of Ref. [27], showing once again differences in enzymatic expression. These results can be compared only for intracellular enzymatic activity, since there have been no previous studies on extracellular activity. The capacity to secrete phenol oxidases (i.e. lignin peroxidase, Mn-peroxidase and laccase) is known in filamentous fungi [14,19,21] but unknown to our knowledge in bacteria or yeast. Although these enzymes are different from the phenol-degrading enzymes in our study, previous studies show that the capability of selected microorganisms to produce extracellular enzymes during complex aromatic compound degradation, as well as lignin degradation, should be further researched.

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