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Utilization of nitriles by yeasts isolated from a Brazilian gold mine

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Yeast strains from the genera *Candida*, *Debaryomyces*, *Aureobasidium*, *Geotrichum*, *Pichia*, *Rhodotorula*, *Tremella*, *Hanseniaspora*, and *Cryptococcus* were isolated from samples of a gold mine from liquid extraction circuit. These strains were tested for their ability to utilize acetonitrile at 12 mM as the sole nitrogen source. The yeasts that grew using acetonitrile at 12 mM were tested in the presence of acetonitrile, isobutyronitrile, methacrylnitrile, and propionitrile at concentrations of 12, 24, 48, 97, and 120 mM. One strain was selected for each nitrile and the concentration of nitrile in which the best growth occurred. *Cryptococcus* sp. strain UFMG-Y28 had a better growth on 120 mM propionitrile and 97 mM acetonitrile, *Rhodotorula glutinis* strain UFMG-Y5 on 48 mM methacrylnitrile, and *Cryptococcus flavus* strain UFMG-Y61 on 120 mM isobutyronitrile. The utilization of different nitriles and amides by yeast strains involves hydrolysis in a two-step reaction mediated by both inducible and intracellular nitrile hydratase and amidase.

Key Words——amidase; nitrile hydratase; nitriles; yeasts

Nitrile compounds are numerous and fairly widespread in the environment. They are used in many industrial processes, i.e., the synthesis of plastics and polymers and the production of herbicides. Nitriles are highly toxic, and some are mutagenic, carcinogenic, and teratogenic. This toxicity represents a serious health hazard for humans and livestock, since the presence of nitrile compounds such as cyanogenic glycosides can result in food poisoning. The extensive use of these compounds may also lead to environmental problems (Legras et al., 1990).

Nitriles are used in organic synthesis by the chemical industry to produce compounds such as amides and organic acids (Jallageas et al., 1980). The bioconversion of nitriles has several advantages when compared with chemical conversion. The need for strong acidic or alkaline conditions, high energy consumption, and formation of unwanted by-products are the main drawbacks of chemical conversion (Mayaux et al., 1990). Microbial degradation of nitriles is becoming a convenient, cost-effective method for removing potential pollutants (Wyatt and Knowles, 1995).

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Enzymatic hydrolysis of nitriles by a wide variety of both gram-positive and gram-negative bacteria to their corresponding acids and metabolically available ammonia is well documented (Chapatwala et al.,1990; Nagasawa and Yamada, 1985). This transformation can be accomplished by either a one-step reaction by nitrilase (EC3.5.5.1), with the formation of ammonia and acid, or a two-step conversion mediated by nitrile hydratase (EC4.2.1.84) and amidase (EC3.5.1.4). The two-step reaction is applicable for the biotechnological production of both amides and acids (Asano et al.,1980). Few studies have been carried out on the use of nitriles by yeasts. Fukuda et al. (1973) reported a strain identified as *Torulopsis candida* (*Candida famata*), which utilized DL-α-hydroxynitrile compounds as the sole nitrogen source; van der Walt et al. (1993) examined nine strains of yeasts by screening their ability to utilize nitriles and their corresponding amides as the sole source of nitrogen; Brewis et al. (1995) reported the ability of ascomycetous yeasts to utilize aromatic, cyclic, and heterocyclic nitriles, and Linardi et al. (1996) isolated a strain of *Candida famata* from a gold mine effluent that was able to grow on several nitrile compounds and their corresponding amides as the only source of nitrogen. The present study describes the selection of yeast strains isolated from a

Table 1. Enzymatic assay with supernatant, whole cells, or cell extract.

Strain	Growth substrate	Assay substrate
Cryptococcus sp. UFMG-Y28	acetonitrile 97 mM	acetonitrile 97 mM acetamide 97 mM
	propionitrile 120 mM	propionitrile 120 mM propionamide 120 mM
Cryptococcus flavus UFMG-Y61	isobutyronitrile 120 mM	isobutyronitrile 120 mM isobutyramide 120 mM
Rhodotorula glutinis UFMG-Y5	methacrylnitrile 48 mM	methacrylnitrile 48 mM methacrylamide 48 mM

gold extraction liquid circuit capable of utilizing nitriles as the sole nitrogen source and the enzymatic path of nitrile degradation.

Materials and Methods

Yeast isolation. The yeasts used in this study were isolated from five different ore processing stages during gold extraction by cyanidation (Mineração Morro Velho, Nova Lima-Minas Gerais, Brazil) by an enrichment technique in which 25 ml of each sample was inoculated into flasks containing 25 ml of Yeast Nitrogen Base (YNB, Difco, Detroit, MI, USA) twice concentrated plus 1% glucose and 10 mg% chloramphenicol (Dias et al., 1996). The microorganisms were identified according to the keys of Kreger van Rij (1984) and Barnett et al. (1990) and maintained in liquid nitrogen.

The organic nitriles acetonitrile, propionitrile, isobutyronitrile, and methacrylnitrile were obtained from Merck (Darmstadt, Germany). These compounds were tested as the sole source of nitrogen for the yeasts. Acetamide, propionamide, methacrylamide, and isobutyramide were obtained from Aldrich, Milwaukee, WI, USA.

Selection of yeast strains. The strains were inoculated into Sabouraud agar at 25°C for 48 h. To avoid endogenous growth, samples were transferred to Erlenmeyer flasks containing 10 ml deionized water and incubated with shaking for 24 h; these suspensions served as preinoculum. All strains were inoculated with aliquots of 0.5 ml (A_{480} 0.500) into 125 ml Erlenmeyer flasks containing 10 ml of Yeast Carbon Base (YCB, Difco) plus 12 mM acetonitrile as the sole nitrogen source. Growth was estimated by optical density (OD_{480}) after 72 h of incubation with shaking at 28°C.

Nitrile utilization. Aliquots of 0.5 ml (preinoculum, A_{480} 0.500) of the strains selected by growth on 12 mM acetonitrile were inoculated into Erlenmeyer flasks containing 10 ml YCB plus acetonitrile, isobutyronitrile, methacrylnitrile, or propionitrile at concentrations of 12, 24, 48, 97, and 120 mM. The control samples were prepared by inoculating 0.5 ml of cell suspensions into Erlenmeyer flasks with YCB, without a nitrogen source. After incubation for 72 h at 28°C, growth was determined by measuring the optical density. Selected for each nitrile tested was a yeast strain and the concentration of nitrile in which the best growth occurred. These selected strains were inoculated into Erlenmeyer flasks containing 10 ml YCB and nitrile at predetermined concentrations. The flasks were incubated with shaking for 120 h at 28°C. During this period growth, substrate consumption, and production of ammonia were determined. The substrate consumption was determined by gas chromatography, the growth by measuring the optical density, and the production of ammonia by the method of Fawcet and Scott (1960).

Enzymatic assay using whole cells. At regular intervals of 48, 72, 96, and 120 h after growth of *Cryptococcus* sp. UFMG-Y28 on 97 mM acetonitrile and 120 mM propionitrile, *Cryptococcus flavus* UFMG-Y61 on 120 mM isobutyronitrile and *Rhodotorula glutinis* UFMG-Y5 on 48 mM methacrylnitrile, the cells were centrifuged to obtain supernatant and cells (intact). The supernatant and resting cells were investigated for enzymatic activity in assays (Table 1) of 30 and 60 min at 30°C under shaking by measuring the production of ammonia. The supernatant was assayed in tubes containing 1.0 ml solution of nitrile or amide in 25 mM sodium phosphate buffer, pH 7.0, and 1.0 ml of supernatant. Before these experiments, the presence of ammonia was measured in the growth supernatant as a control. The cells were washed twice in 25 mM sodium phosphate buffer, pH 7.0. The assay was prepared with 350 mg of resting cells (wet weight) in 10 ml solution (25 mM sodium phosphate buffer, pH 7.0) containing nitrile or amide. After assay, the yeast cells were harvested by centrifugation, and ammonia was assayed colorimetrically in the supernatant. To determine whether the enzyme system for nitrile metabolism was inducible or constitutive, the strains were grown in YCB medium containing 0.1% ammonium sulphate. One unit of enzyme activity as nitrile hydratase and amidase was defined as the amount of enzyme that catalyzed the formation of 1 μmol ammonia/min/mg dry weight at 30°C, pH 7.0.

Enzymatic assay using cell-free extract. The cell extract was prepared from cells (Table 1) cultured in nitriles. Yeast cells were harvested by centrifugation, washed twice in 25 mM phosphate buffer (pH 7.0). The packed cells were frozen in liquid nitrogen and ground. The debris was suspended in 5 ml cold 25 mM phosphate buffer and the unbroken cells and debris were separated by centrifugation. The cell-free supernatant fraction was used as the crude enzyme source. The reaction mixture consisted of nitrile or amide in 4 ml 25 mM phosphate buffer plus 1 ml of crude enzyme. The assays were carried out at 30°C in a water bath. After 60 min the reaction was stopped by adding 0.5 ml of 5 N HCl. One unit of nitrile or amide-degrading enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol ammonia/mg protein/min at 30°C, pH 7.0. The amounts of proteins were estimated by the method of Bradford (1976) with bovine serum albumin as the standard.

Substrate consumption and product formation. Substrate consumption and product formation were determined during growth and in enzymatic assay with cells and cell extract of *Cryptococcus* sp. UFMG-Y28 utilizing 120 mM propionitrile. The assay with whole cells was analyzed with the aid of gas chromatography using a GC 5890 (Hewlett-Packard, series II, Wilmington, DE, USA) equipped with a flame ionization detector. The column used in the determination was an HPFFAP (cross-linked polyethylene glycol, TPA) with 25 m×0.20 mm internal diameter×0.33 mm film thickness. The operational conditions were as follows: hydrogen as carrier gas, 15 : 1 split ratio; volume of injection 1 μl; temperature of injector 200°C; and temperature of detector 230°C. The assays with cell-free extract were analyzed by high-performance liquid chromatography by using an HPLC-Shimadzu Model-10 AD apparatus equipped with a UV-detector (SPD-M6A, Shimadzu, Kyoto, Japan) under UV light detection of 210 nm wavelength. The integration-calculation of the peak areas was performed with an integrator Model C-R7A Chromatopac (Shimadzu). All measurements were made on a Reverse-Phase column (C18 5 μm LiChrosorb RP 18, 125×4.0 mm, Merck). Elution was carried out under isocratic conditions for separation, and the composition of mobile phase was 0.1% MeOH in deionized water. All measurements were made at 28°C.

Oxygen consumption. Oxygen consumption was measured with a Yellow Springs Instrument oxigraph (YNI-model 53) containing a semipermeable membrane coated electrode attached to a water-jacketed cell and linked to a chart recorder. After culture of the selected yeasts in the selected nitrile, the cells were harvested by centrifugation at 5,000 rpm for 15 min. The pellet was washed twice in 25 mM sodium phosphate buffer, pH 7.0, and 75 mg (wet weight) was suspended in 3 ml of 25 mM sodium phosphate buffer. The suspension was injected into a cell chamber, and the substrate was added (97 mM acetonitrile, 120 mM propionitrile, for cells of *Cryptococcus* sp. UFMG-Y28; 120 mM isobutyronitrile, for cells of *Cryptococcus flavus* UFMG-Y61; 48 mM methacrylnitrile, for cells of *Rhodotorula glutinis* UFMG-Y5). Oxygen uptake was measured for 10 min. All polarographic activity determinations were carried out with the oxygraph cell sealed with a glass stopper.

Table 2. Yeast strains isolated from extraction circuit liquids of a gold mine and strains able to grow on 12 mM acetonitrile.

Strains	Number of strains
Aureobasidium-like[a]	1 (0)[b]
Aureobasidium pullulans	1 (1)
Candida boidinii	1 (1)
Candida citrea	1 (0)
Candida dattila-like	2 (0)
Candida famata	8 (3)
Candida intermedia	1 (1)
Candida krusei	1 (0)
Candida pseudointermedia	1 (1)
Candida sp.	9 (3)
Candida vinaria-like	1 (1)
Cryptococcus flavus	1 (1)
Cryptococcus-like	1 (0)
Cryptococcus sp.	1 (1)
Debaryomyces hansenii	2 (1)
Geotrichum sp.	4 (1)
Hanseniaspora guilliermondii	1 (0)
Hanseniaspora sp.	2 (2)
Hanseniaspora valbyensis	1 (0)
Pichia kluyveri	2 (1)
Rhodotorula glutinis	7 (2)
Rhodotorula rubra	4 (0)
Tremella sp.	1 (0)
Total	54 (20)

[a] Probable new species similar in characteristics to the species indicated.

[b] The number of strains that grew on 12 mM acetonitrile is given in parentheses.

Results

Starting with 54 yeast strains isolated from circuit liquids from a gold mine, 20 showed the ability of utilize acetonitrile at 12 mM (Table 2). These strains were screened in the presence of acetonitrile, isobutyronitrile, methacrylnitrile, or propionitrile as nitrogen source. For each nitrile tested, a strain and the correspondent concentration at which the best growth was obtained was selected (Table 3). The growth of *Cryptococcus* sp. UFMG-Y28 on 120 mM propionitrile and 97 mM acetonitrile is shown in Fig. 1A and B, respectively; the growth of *Cryptococccus flavus* UFMG-Y61

on 120 mM isobutyronitrile is shown in Fig. 1C; and the growth of *Rhodotorula glutinis* UFMG-Y5 on 48 mM methacrylnitrile is shown in Fig. 1D. The best growth was observed for *Cryptococcus* sp. UFMG-Y28 on 120 mM propionitrile (Fig. 1A). As shown by the results presented in Fig. 1, fast consumption of nitriles was observed during growth assays in which controls were prepared with uninoculated flasks. *Cryptococcus flavus* UFMG-Y61 showed the most rapid reduction of isobutyronitrile (Fig. 1C). Cells grown in ammonium sulphate showed no nitrile degradation. Enzyme activities increased with the increase of incubation time, with the maximum activity occurring for cells of *Cryptococcus* sp. UFMG-Y28 cultivated in the presence of 120 mM propionitrile or 97 mM acetonitrile harvested after 72 h of incubation (Fig. 2A and B). The enzyme activities of *Cryptococcus flavus* UFMG-Y61 cultivated in the presence of 120 mM isobutyronitrile and *Rhodotorula glutinis* UFMG-Y5 cultivated in the presence of 48 mM methacrylnitrile were higher with cells harvested after 120 h of incubation (Fig. 2C and D). Prolonging the incubation periods resulted in lower enzyme activities. Yeast cells also hydrolyzed amides, producing ammonia. Table 4 shows the specific activities of nitrile hydratase and amidase calculated by the ammonia concentration in the reaction mixture using intact cells and cell-free extracts. The highest enzymatic activity was obtained with cells of *Cryptococcus* sp. UFMG-Y28 by using propionitrile and propionamide as substrates. When the cell-free extract was

Table 3. Yeast strains selected and the concentration of nitriles at which best growth was obtained.

Strain	Substrate	OD (A_{480})[a]
Cryptococcus sp. UFMG-Y28	97 mM acetonitrile	0.772
Cryptococcus sp. UFMG-Y28	120 mM propionitrile	1. 205
Cryptococcus flavus UFMG-Y61	120 mM isobutyronitrile	1.000
Rhodotorula glutinis UFMG-Y5	48 mM methacrylnitrile	0.575

[a] Growth (A_{480}) determined after 72 h of incubation.

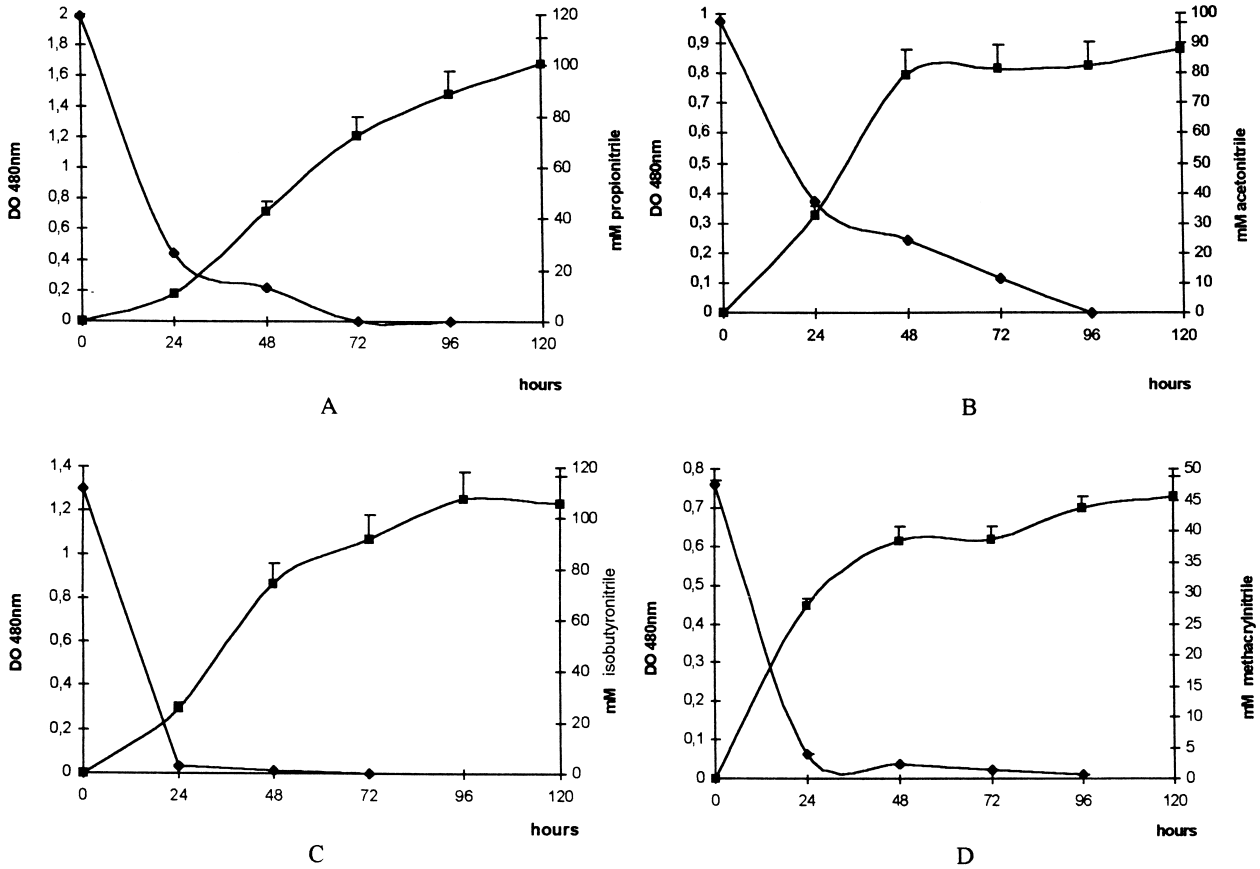

Fig. 1. Kinetics of growth (■) and substrate consumption during growth (◆) in media YCB of *Cryptococcus* sp. UFMG-Y20 utilizing 120 mM of propionitrile (A) and 97 mM acetonitrile (B); *Cryptococcus flavus* UFMG-Y61 utilizing 120 mM isobutyronitrile (C); and *Rhodotorula glutinis* UFMG-Y5 utilizing 48 mM methacrylnitrile (D).

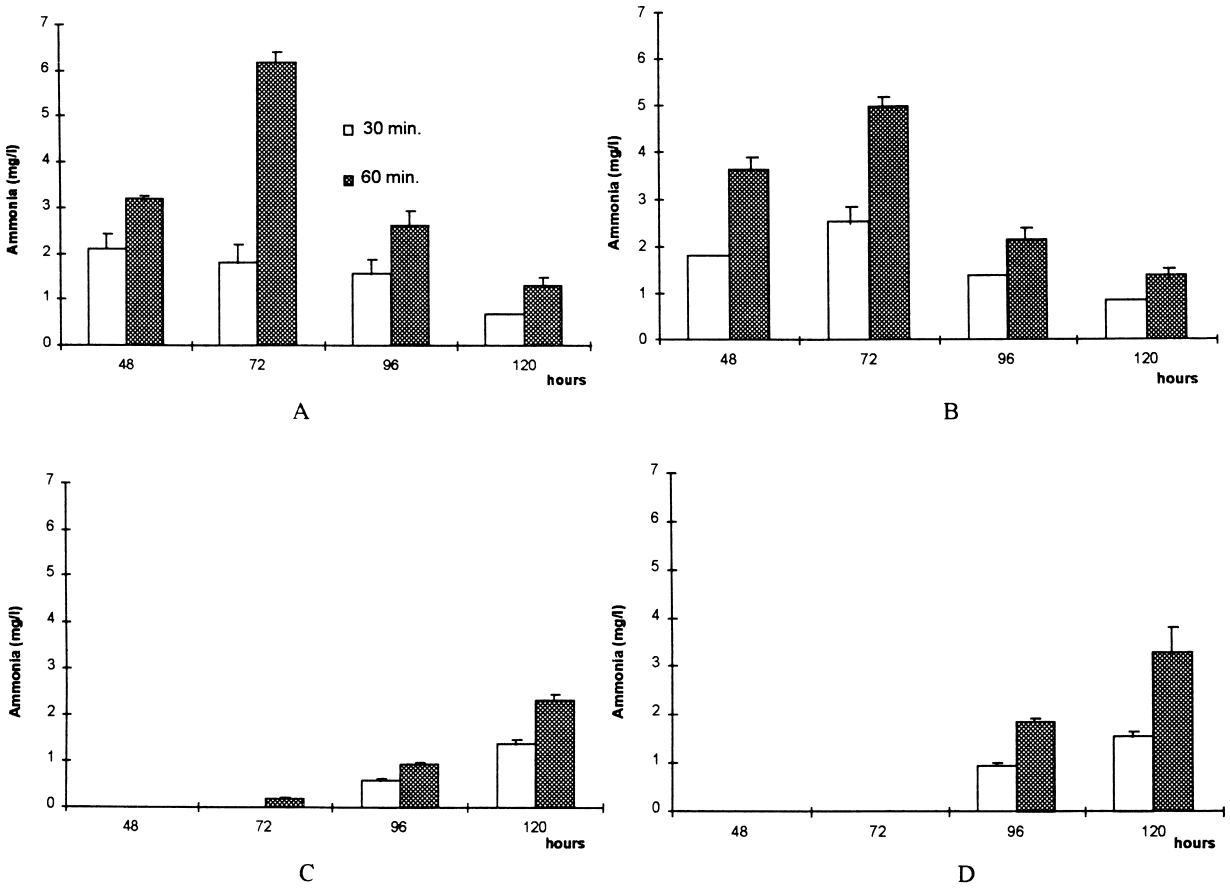

Fig. 2. Production of ammonia in different times of incubation by cells (harvested at different periods of yeast growth) of *Cryptococcus* sp. UFMG-Y28 from 120 mM propionitrile (A) and 97 mM acetonitrile (B); *Cryptococcus flavus* UFMG-Y61 from 120 mM isobutyronitrile (C); and *Rhodotorula glutinis* UFMG-Y5 from 48 mM methacrylnitrile (D).

Table 4. Enzymatic activity as nitrile hydratase and amidase by yeast cells and cell-free extract.

Strain	Substrate	S. A.[a] intact cells	S. A.[b] cell-free extract
Cryptococcus sp. UFMG-Y28	acetonitrile 97 mM	1.5	9.17
	acetamide 97 mM	0.6	11.30
Cryptococcus sp. UFMG-Y28	propionitrile 120 mM	2.00	22.34
	propionamide 120 mM	3.90	32.28
Cryptococcus flavus UFMG-Y61	isobutyronitrile120 mM	0.70	7.13
	isobutyramide 120 mM	1.90	76.01
Rhodotorula glutinis UFMG-Y5	methacrylnitrile 48 mM	1.00	8.88
	methacrylamide 48 mM	0.50	34.40

[a] S. A. (specific activity)=one unit of enzyme activity as nitrile hydratase and amidase was defined as the amount of enzyme that catalyzed the formation of 1 μmol ammonia/min/mg dry weight at 30°C, pH 7.0.

[b] S. A. (specific activity)=one unit of enzyme activity as nitrile hydratase and amidase was defined as the amount of enzyme that catalyzed the formation of 1 μmol ammonia/min/mg protein at 30°C, pH 7.0.

used as the crude enzyme source, it was observed as a higher value of specific activity if compared with the obtained specific activity with whole cells. Propionitrile was the preferential substrate for nitrile-degrading enzyme by cell-free extract of *Cryptococcus* sp. UFMG-Y28. Therefore the strain was chosen for chromatography assays. Assays with cell-free extract and intact cells showed that propionitrile breakdown yielded propionic acid, propionamide, and ammonia. Propionic acid and ammonia were found in the reaction mixture when propionamide was used as substrate for the enzyme system (data not shown). The maximum amount

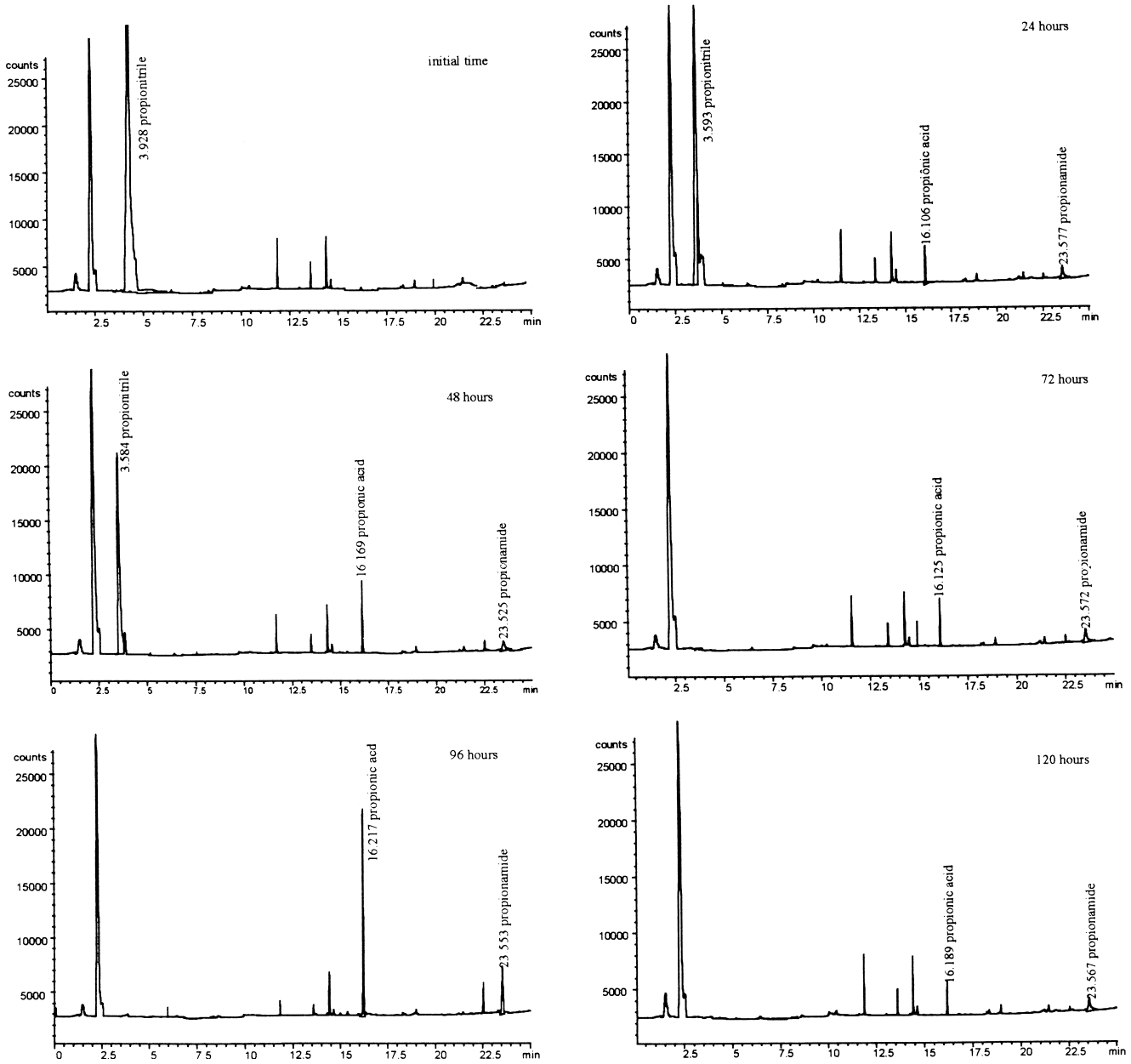

Fig. 3. Kinetics of substrate consumption and product formation during growth of *Cryptococcus* sp. UFMG-Y28, utilizing propionitrile as the sole nitrogen source.

of propionic acid formed (1.040 mM) during growth of *Cryptococcus* sp. UFMG-Y28 using propionitrile as nitrogen source was detected after 96 h of growth (Fig. 3). Propionamide was detected at low concentrations, and the maximum amount (343 μM) was detected after 96 h of growth (Fig. 3). Washed yeast cells in the presence of nitriles showed significant oxygen uptake, as shown in Table 5. *Cryptococcus* sp. UFMG-Y28 grown on propionitrile and acetonitrile showed the greatest oxygen consumption, followed by *Rhodotorula glutinis* UFMG-Y5 and *Cryptococcus flavus* UFMG-Y61 grown on methacrylnitrile and isobutyronitrile, respectively.

Table 5. Oxygen consumption by yeast cells on nitriles.

Strain	Substrate	Oxygen uptake (nmol)[a]
Cryptococcus sp. UFMG-Y28	acetonitrile 97 mM	31.2
Cryptococcus sp. UFMG-Y28	propionitrile 120 mM	46.8
Cryptococcus flavus UFMG-Y61	isobutyronitrile 120 mM	15.6
Rhodotorula glutinis UFMG-Y5	methacrylnitrile 48 mM	23.4

[a] Oxygen uptake after 10 min of incubation.

Discussion

Although the hydrolysis of nitriles by bacteria has been well documented (Harper, 1977; Kobayashi et al., 1989; Nawaz et al., 1989; Yamada et al., 1979), only a few reports are available with regard to the utilization of nitriles by yeasts (Brewis et al., 1995; Fukuda et al., 1973; Linardi et al., 1996; van der Walt et al., 1993). Yeasts are the organisms most frequently utilized in industry because of their capability of adaptation to industrial processes and to their relative safety. Yeast strains belonging to genera *Candida*, *Debaryomyces*, *Aureobasidium*, *Geotrichum*, *Pichia*, *Rhodotorula*, *Cryptococcus*, and *Hanseniaspora* were able to utilize acetonitrile as the sole nitrogen source. In a mine containing cyanide, most of these yeasts were probably carried from the ore to the gold extraction circuit and has the soil as origin substratum. The frequency of yeasts in these ore processing stages, in which the cyanide concentration varies from 150 to 300 ppm, depends on their source and its resistance to the conditions of the effluent. Homology exists among enzymes that catalyze the hydrolysis of cyanide and nitriles. Enzymes such as cyanide hydratase, rhodanase, oxygenases, and various nitrilases have been isolated and identified as active in cyanide metabolism (Westley, 1981). Watanabe et al. (1998) demonstrated a remarkable homology in the amino acid sequence between cyanidase and nitrilase.

Yeast strains grew on different aliphatic nitriles, and the greatest growth was obtained for *Cryptococcus* sp. UFMG-Y28, *Cryptococcus flavus* UFMG-Y61, and *Rhodotorula glutinis* UFMG-Y5. All showed basidiomycete affinity. In contrast, strains studied previously showed ascomycete affinity (Brewis et al., 1995; Fukuda et al., 1973; Linardi et al., 1996; van der Walt et al., 1993). The enzymatic activities, such as nitrile hydratase and amidase, changed as a function of yeast strain and substrate. According to Nawaz et al. (1989), the production of ammonia by washed (intact) cells for the determination of enzymatic activities depends on different periods of yeast growth. Nitrile-degrading enzyme activity was detectable from the exponential to the stationary phase and was highest at the exponential phase for cells of *Cryptococcus* sp. UFMG-Y28. The enzymatic pathway for nitrile hydrolysis is an inducible system of an intracellular nature. The hydrolysis of nitrile and its corresponding amides by the yeasts suggests that the breakdown of nitriles might occur in a two-step mechanism mediated by nitrile hydratase and subsequent hydrolysis of the resulting amides by amidase (Asano et al., 1980). The presence of propionic acid is a direct evidence of amidase activity. The decrease of propionic acid (120 h) during growth, just after its accumulate (96 h), can be due to its utilization as an additional carbon source. Babu et al. (1995) showed that acetic acid production during the growth of *Pseudomonas marginalis*, had disappeared within 72 h of incubation. Acharya and Desay (1997) attributed the acid disappearance to its utilization in the synthesis of cell material. The limited amide detected during the growth kinetics of *Cryptococcus* sp. UFMG-Y28 by using propionitrile may be due to a fast use of amide by the microorganism as a result of the action of amidase or to a slow release rate into the extracellular medium. Digeronimo and Antoine (1975) attributed the failure to detect amide to the direct conversion of propionitrile to its carboxylic acid and ammonia or to a slow turnover of propionitrile. Babu et al. (1995) and Langdahl et al. (1996), studying *Pseudomonas marginalis* and *Rhodococcus erythropolis*, respectively, rarely observed the presence of amide, attributing this fact to its fast utilization. The presence of amide was better confirmed in assays with cell-free extract and whole cells of *Cryptococcus* sp. UFMG-Y28 cultivated in the presence of propionitrile. Propionamide, propionic acid, and ammonia were found in the reaction mixture of the cell-free extract of *Cryptococcus* sp. UFMG-Y28 in the presence of propionitrile. The presence of propionamide confirms the action of nitrile hydratase. The amide detected in the present study is considered to be a true intermediate because of its conversion to propionic acid and ammonia in reactions starting with either propionitrile or propionamide. This fact strongly confirms the action of nitrile hydratase and amidase in propionitrile degradation by *Cryptococcus* sp. UFMG-Y28. Oxygen consumption is a measure of enzymatic activity (Nawaz et al., 1991) and a reliable tool for determining cell viability and physiology in the new environment, in this case utilizing organic solvents. The results obtained in the present study suggest that nitriles are biologically oxidizable substrate. The yeast strains may be useful for the detoxification or bioremediation of sites contaminated with synthetic nitriles.

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