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Methylotrophic extremophilic yeast *Trichosporon* sp.: a soil-derived isolate with potential applications in environmental biotechnology*

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A yeast isolate revealing unique enzymatic activities and substrate-dependent polymorphism was obtained from autochthonous microflora of soil heavily polluted with oily slurries. By means of standard yeast identification procedures the strain was identified as *Trichosporon cutaneum*. Further molecular PCR product analyses of ribosomal DNA confirmed the identity of the isolate with the genus *Trichosporon*. As it grew on methanol as a sole carbon source, the strain appeared to be methylotrophic. Furthermore, it was also able to utilize formaldehyde. A multi-substrate growth potential was shown with several other carbon sources: glucose, glycerol, ethanol as well as petroleum derivatives and phenol. Optimum growth temperature was determined at 25ºC, and strong inhibition of growth at 37ºC together with the original soil habitat indicated lack of pathogenicity in warm-blooded animals and humans. The unusually high tolerance to xenobiotics such as diesel oil (>30 g/l), methanol (50 g/l), phenol (2 g/l) and formaldehyde (7.5 g/l) proved that the isolate was an extremophilic organism. With high-density cultures, formaldehyde was totally removed at initial concentrations up to 7.5 g/l within 24 h, which is the highest biodegradation capability ever reported. Partial biodegradation of methanol (13 g/l) and diesel fuel (20 g/l) was also observed. Enzymatic studies revealed atypical methylotrophic pathway reactions, lacking alcohol oxidase, as compared with the conventional methylotroph *Hansenula polymorpha*. However, the activities of glutathione-dependent formaldehyde dehydrogenase, formaldehyde reductase, formate dehydrogenase and unspecific aldehyde dehydrogenase(s) were present. An additional glutathione-dependent aldehyde dehydrogenase activity was also detected. Metabolic and biochemical characteristics of the isolated yeast open up new possibilities for environmental biotechnology. Some potential applications in soil bioremediation and wastewater decontamination are discussed.

Keywords: methylotrophic yeast, autochthonous microflora, *Trichosporon* sp., non-conventional yeast, xenobiotic biodegradation, formaldehyde

INTRODUCTION

The use of non-conventional yeasts in biotechnology has attracted much interest in recent years (Spencer *et al.*, 2002). In environmental biotechnology, genera such as *Pichia, Hansenula, Candida, Yarrowia,* and *Trichosporon* are of special importance. Among these, several species have been applied for recultivation of polluted soil, bioremediation of heavy metal contamination, treatment of industry-generated wastewaters, and used as microbiological sensors (Walker 1998; Spencer *et al.*, 2002). Many useful strains for environmental projects have been isolated from natural habitats (water and soil),

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Abbreviations: AldD, aldehyde dehydrogenase; AO, alcohol oxidase; FDD, formaldehyde dehydrogenase; Fd, formaldehyde; FDR, formaldehyde reductase; FMD, formate dehydrogenase; ITS, internal transcribed spacer.

or from polluted areas (slurries and wastewaters) (Spencer *et al.*, 2002). The application potential of these strains is often a result of rare enzymatic activities which enable them to gain resistance to toxic agents, to biodegrade certain xenobiotics, to produce specific metabolites, to detoxify detrimental metal compounds, etc.

Within the genus *Trichosporon*, over thirty species have been identified so far (Fell *et al.*, 2000; Sugita *et al.*, 2002; Middelhoven *et al.*, 2004). Since the yeast is distributed in the environment very widely, the strains have been isolated from a variety of sources (Middelhoven *et al.*, 2004), including plant and animal material, human clinical patients, air, water, industrial effluents, and – preferably – soil (Middelhoven *et al.*, 2001). Six to eight species have been reported to be medically important as disease-causing pathogens (Sugita *et al.*, 1999; 2001; Middelhoven *et al.*, 2001; Schwartz, 2004). Characteristics of the other ones proved that the genus comprises robust yeasts with a wide variety of biochemical pathways. *Trichosporon* species were reported to utilize benzene, phenol, and many other low-molecular weight aromatics (Middelhoven *et al.*, 2004; Sampaio, 1999), biarylic compounds (Sietman *et al.*, 2002), polycyclic aromatic hydrocarbons (MacGillivray *et al.*, 1993), as well as other organic compounds, some of which are toxic (Sampaio 1999; Godjevargova *et al.*, 2003; Middelhoven *et al.*, 2004). However, there are only few reports on methanol assimilation capability (Aleksieva *et al.*, 2002; Godjevargova *et al.*, 2003) and formaldehyde biodegradation activity (Kato *et al.*, 1982; Glancer-Soljan *et al.*, 2001), revealed only by single strains of *T. cutaneum* and *T. pennicilatum*, respectively.

The described metabolic potential of *Trichosporon* species has made the application of these yeasts for environmental purposes very promising. Up to now, they have been used to treat industrial wastewater (Glancer-Soljan *et al.*, 2001; Godjevargova *et al.*, 2003) and applied as biosensors for determination of biological oxygen demand (BOD) (Yang *et al.*, 1996), ammonium ion and phenol (Reiser *et al.*, 1994). This paper describes a novel environmental yeast isolate of the genus *Trichosporon*. The unique biochemical and physiological features exhibited by this strain make it a microorganism of special biotechnological value.

MATERIALS AND METHODS

Strain identification methods. The isolated yeast was first identified by means of standard microbiological methods (Kurtzman, 2000) based on: (a) morphological features such as characteristics of growth in minimal rice medium, in which the tendency to form pseudomycelium and to produce chlamydospores and arthrospores was established, and (b) biochemical characteristics like carbohydrate fermentation capabilities as well as auxanography — assimilation of carbohydrate and nitrogen compounds.

Rapid molecular identification by means of PCR product analyses. Two independent molecular identification methods were performed: (a) amplification of a 170 bp region of small-subunit (SSU) rDNA, according to Sugita *et al.* (1998), and (b) amplification of the conserved regions of 18S, 5.8S, and 28S rDNA, according to Fujita *et al.* (2001). Several strains of the genus *Candida* served as negative controls.

DNA isolation and PCR amplification. Yeast strains were grown on 20 g/l glucose in optimal medium for 24 h at 25°C (for other growth conditions see the next section). After centrifugation of a 15 ml culture (4800 × *g*, 10 min, 4°C), the cell pellets were kept frozen at –80°C, then re-suspended in 500 µl of STET buffer and homogenized with 200 µl of 0.5 mm glass beads by vigorous vortexing for 8 min., and boiling in a water bath for 3 min. After cooling on ice the samples were centrifuged (28000 × *g* for 10 min at 4°C) and then to 300 µl of each supernatant, 700 µl of 7.5 M ammonium sulfate was added, incubated overnight at –20°C, and centrifuged as above. DNA was precipitated from supernatants with 96% ethanol. After centrifugation, the pellets were dried and dissolved in 50 µl of water. DNA was purified with the Wizard SV gel and PCR clean-up system (Promega).

For the amplification of a 170 bp region of small-subunit rDNA the following primers were used: TRF (5′-AGAGGCCTACCATGGTATC-3′) and TRR (5′-TAAGACCCAATAGAGCCCTA-3′) (Sugita *et al.*, 1998). For the amplification of ITS1 and ITS2 regions and the region of 5.8S rDNA the primers used were: C_ITS1_F (5′-TCCGTAGGTGAACCTGCG-3′), C_ITS3_F (5′-GCGTCGATGAAGAACGCAGC-3′), and C_ITS4_R (5′-TCCTCCGCTTATTGATATGC-3′). All primers were obtained from Generi Biotech (Czech Republic).

The amplification was done in a Mastercycler gradient thermocycler (Eppendorf). Total reaction volume was 15 µl, of which the master mixture was composed of 5 mM $MgCl_2$ (Qiagen), 1× buffer (Qiagen PCR Buffer), 0.25 mM dNTP mix (Fermentas), 0.25 µM of each primer pair, 0.075 U HotStraTaq Polymerase (Qiagen), 1.5 µl of DNA, and distilled water. Reaction conditions consisted of an initial step of 15 min at 5°C and 35 cycles of subsequent steps: (a) 92°C for 45 s, (b) 57°C for 45 s, and (c) 72°C for 2 min. The ending elongation was carried out at 72°C for 10 min.

Yeast cell cultures. Yeasts were grown either in optimal or minimal media containing 2% (v/v) (that is 1.58 g/l) methanol or other xenobiotics at desired concentrations, in 300 ml Erlenmeyer flasks in thermostated gyro-shakers (200 r.p.m., at 25°C). Optimal growth medium contained (per liter): 3 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.3 g $MgSO_4 \times 7H_2O$, 0.2 g $CaCI_2 \times 6H_2O$, 2 g yeast extract, and 2 g peptone. Minimal medium contained salt solutions as above, and 0.2 g/l yeast extract. To this medium 0.5 ml/l of microelement cocktail (H_3BO_3, 56 mg/l; $CuSO_4 \times 5H_2O$, 39.3 mg/l; $MnSO_4 \times 7H_2O$, 50.4 mg/l; $(NH_4)_6Mo_7O_{24} \times H_2O$, 120 mg/l; $ZnSO_4 \times 7H_2O$, 307.9 mg/l) was added. Iron (0.2 mg/l) was provided with Mohr salts.

Solid YPD media for yeast culture plating contained 20 g/l peptone, 10 g/l yeast extract, 20 g/l glucose and 20 g/l bacto-agar. Sabouraud agar with an appropriate antibiotic cocktail (see Results and Discussion) was used for yeast selection.

Biomass density was evaluated turbidimetrically, as optical density at 540 nm (OD_{540}). It was checked experimentally that $OD_{540} = 1.0$ corresponded to approx. 1.9×10^7 cells/ml. Cell culture viability was verified by plating appropriate culture decimal dilutions onto solid agar media and then by counting c.f.u. The methylotrophic yeast *Hansenula polymorpha* NCYC 2309 was used as a reference strain in the experiments.

Yeast growth and xenobiotic biodegradation tests. For growth tests, *Trichosporon* sp. was inoculated at a low density ($OD_{540} = 0.01$, that is 1.9×10^5 cells/ml) to media supplemented with particular sources of carbon.

In biodegradation experiments, high density *Trichosporon* sp. cultures (typically of $OD_{540} = 1.2$, corresponding to 2.3×10^7 cells/ml), pre-grown on 20 ml/l methanol up to the logarithmic growth phase, were centrifuged ($3000 \times g$ for 5 min) to remove the optimal medium and then the cells were re-suspended in media containing different xenobiotics.

Preparation of cellular extracts. Yeast cell cultures were centrifuged ($3000 \times g$ for 5 min) to remove the medium, then to each 1 g of the cellular pellet 1 ml buffer (25 mM Tris/HCl, pH 7.5, containing 50 mM EDTA and 10% dithiothreitol) was added. The tube with cell suspension was cooled by placing in an ice bath and then the cells were disrupted in four 15 min cycles with a laboratory sonicator UP 50H Ultrasonic Processor (dr. Hielscher GmbH) using an MS7 sonotrode (50% maximum output power, 0.5 s pulses).

Protein profiles of cellular extracts were obtained using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS/PAGE), according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250, and protein determination in cellular extracts was done according to the method of Lowry *et al.* (1951).

Determination of enzyme activities in kinetic tests. The activities of glutathione-dependent formaldehyde and aldehyde dehydrogenases in cellular yeast extracts were measured by means of spectrophotometric determination of the generated NADH at $\lambda = 340$ nm (Jasco V-530 UV/VIS spectrophotometer). The reaction mix consisted of 0.6 mM NAD^+, 0.1 mM glutathione (GSH), 5 mM KCl, and 10 mM formaldehyde or acetaldehyde as substrates. Yeast alcohol dehydrogenase and aldehyde dehydrogenase were used as reference controls. In brief, 1 ml of the reaction mix was placed in a standard quartz cuvette and then 0.3 ml of the tested extract was added to start the enzymatic reaction.

Zymographic analyses of the extracts were performed to reveal activities of unspecific aldehyde dehydrogenases. The reagent solution for native PAGE enzymatic staining contained 25 mg of NAD^+, 15 mg of *p*-nitroblue tetrazolium chloride (NBT), 1 mg of phenazine methosulfate (PMS), 10 ml of 0.2 M Tris/HCl, pH 7.5, 1 ml of 50 mM KCl, 35 ml of water, 1 ml of 10 mM cysteine as a donor of thiol groups, and 5 ml of 1 M acetaldehyde as a substrate.

All chemicals used were of analytical grade. The solutions and buffers were made using bidistilled water, and whenever required, fully sterile conditions were applied.

Analytical methods. Methanol and diesel-fuel constituents were determined by a gas chromatography analysis using Schimadzu GC- 17A ver. 3 gas chromatograph equipped with a capillary column Zebron ZB-5 (Phenomenex) 30 m/Ø 0.32 filled with 5% Phenyl Polysiloxane. A flame ionization detector (FID) was used. Temperature gradients were optimized for the compounds to be determined.

Formaldehyde concentration was determined by means of Nash reagent (Nash, 1953), as described earlier (Kaszycki & Koloczek, 2000).

RESULTS AND DISCUSSION

Isolation of the strain

The yeast strain was isolated from autochthonous microflora present in oil slurry-contaminated soil in a petroleum-refinery industrial area in southern Poland. An approx. 10 g sample of polluted soil was suspended in 90 ml of distilled sterile water and incubated for 3 h at room temperature in 500 ml flasks shaken in a laboratory rotary shaker (200 r.p.m.). Then the soil, sand and other solid particles were filtered out and the remaining suspension was enriched with a minimum amount of bacto-peptone

and yeast extract (0.5 g/l each) and treated with gradually increasing amounts of the original soil organic extract. This procedure enabled over several weeks the development of a consortium of microbial species capable of utilizing xenobiotics present in the soil. The obtained biocenosis consisted of several bacterial and yeast species. To isolate yeast, appropriate dilutions of the original suspension were spread onto Petri dishes with solid Sabouraud medium. After growth at 30°C for 2 days single colonies were picked based on their morphology and microscopic observations and re-plated onto solid media containing an antibiotic cocktail (40 mg/l streptomycin, 28 mg/l penicillin). The isolated yeast strains were tested for assimilation of xenobiotics, and one isolate that tended to form pseudomycelium was selected for the current study. The strain was then stored on solid beer-agar slants.

Description and identification of the yeast isolate

Optimal conditions for growth were determined. The cultures grew well under aerobic conditions, with aeration provided by vigorous rotary-shaking culture flasks (200 r.p.m.), at an optimal temperature between 25 and 26°C. The pH value of 8.1–8.2 was found to be physiological, and it was spontaneously maintained by the growing yeast, both on glucose and on methanol. The growth of the isolated *Trichosporon* sp. was strongly inhibited at temperatures above 30°C, which is an important feature that reduces any risk of pathogenicity for animals and humans.

Cells grown on solid Sabouraud medium after 5-day growth at 25°C formed slant, textured, creamy-coloured shiny colonies, as presented in Fig. 1a. During prolonged incubation (up to 3 weeks, Fig. 1b) the culture evolved into yellowish wrinkled pseudomycelium, elevated at the bottom and forming fimbriate-like margins. Three-day cultures (Fig. 1c) grown at 25°C in the liquid optimal medium containing 2% glucose, revealed relatively large, oval (approx. 5 μm diameter), ellipsoidal or elongate shapes (up to 15 μm), appearing as single cells or forming pairs and chain-like or branched aggregates. Budding was often visible.

The isolated yeast was multi-trophic as it was able to grow in minimal media containing either one of several organic compounds as sole carbon sources: methanol, ethanol, glycerol, diesel oil, all at the tested concentrations of 20 g/l, and phenol at 1.5 g/l. Cell culture appearance in liquid media varied depending on the carbon source, revealing an unusual substrate-based polymorphism (Fig. 1d–h).

The yeast was identified by means of the standard microbiological method (Kurtzman, 2000) in which the cell morphology, colony appearance, pseudo-true hyphae formation, substrate assimilation, physiological characteristics, and biochemical activities were considered. Such a phenotypic screening resulted in the species *Trichosporon cutaneum* (*Basidiomycota*, *Hymenomycetes*, *Tremelloidae*, *Trichosporonales*). However, several unique physiological attributes that seem to be characteristic of the novel strain suggested the need of a more thorough identification approach. Therefore, we performed PCR-based analyses of ribosomal DNA with two independent methods, both of which had been used for identification of *Trichosporonales* (Sugita *et al.*, 1998; Fujita *et al.*, 2001).

Both molecular analyses suggested the genus to be *Trichosporon;* however, the species identity could not be established at this level. It can be observed in Fig. 2 lane c that the first analysis revealed the amplification of a 170 bp region of small-subunit rDNA which served as a molecular marker for the genus. The negative result (Fig. 2 lanes a and b) obtained from the reference strain *Candida* sp. confirmed our conclusion. In the second test, as presented in Fig. 3, the primers ITS1-ITS4 were used to amplify the conserved regions of 18S, 5.8S, and 28S rDNA, and the ITS3 and ITS4 primer pair was used to amplify the 5.8S rDNA region and the adjacent ITS2 region. As seen in Fig. 3 A and B, the respective lanes d show the amplified products of approx. 530 and 350 bp, which match very well the expected range of ITS1-ITS4 (522, 536, 539 bp) and ITS3-ITS4 (349, 356, 354 bp), from which Fujita *et al.* (2001) were able to identify three *Trichosporon* species. Again, the negative controls obtained for three *Candida* strains supported the reliability of the analysis.

It should be noted here that the precise identification of basidiomycetous yeasts is very difficult, even with the use of combined standard and molecular techniques, as pointed out by Fell *et al.* (2000). Thus, our isolate still requires a non-traditional approach for definite species identification, which was also postulated for *Trichosporon* environmental isolates by Middelhoven *et al.* (2004).

Tolerance for selected xenobiotics

High density *Trichosporon* sp. cultures, pregrown on 20 ml/l methanol up to the logarithmic growth phase (OD_{540} within the range 1.2–2.0), were re-suspended in media containing different concentrations of methanol, phenol, diesel fuel, or formaldehyde. Cell culture viability was checked for 36 h by culture plating at 12-h time intervals. All the tested xenobiotics were found not to affect the cell survival at the concentrations applied up to 50 g/l, 2 g/l, 30 g/l (the highest tested), and 1.2 g/l, respectively. Methanol at 100 g/l led to a drop of cell

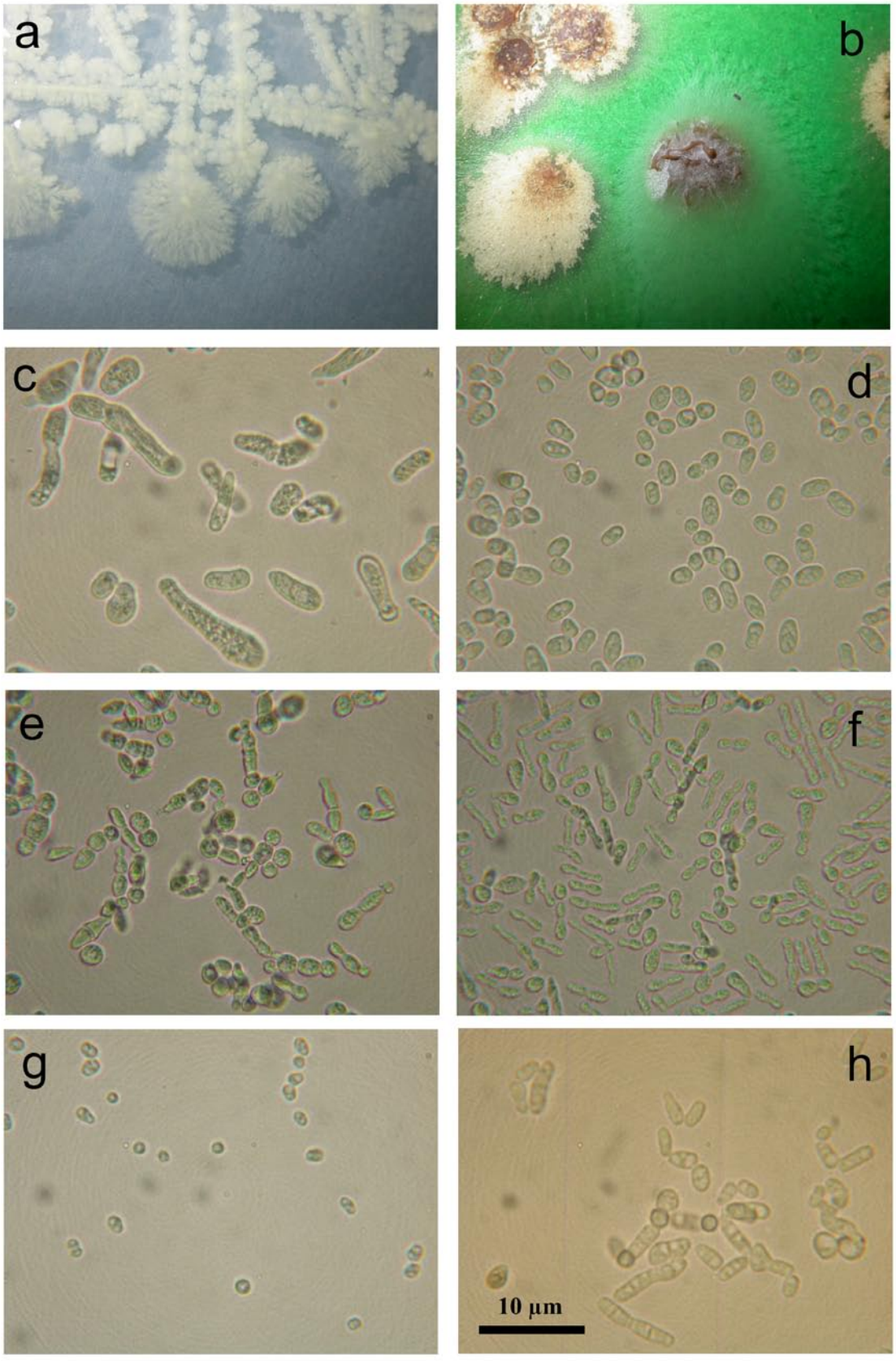

Figure 1. (a), (b) Macro-photographic morphology of colonies formed by *Trichosporon* sp. isolate on agar plates after 5-day (a) and 2-week (b) incubation; (c)–(h) Microscopic (magnification 600 ×) images of substrate-dependent 3-day cell culture polymorphism in liquid minimal media with glucose (c), methanol (d), ethanol (e), glycerol (f), diesel fuel (g), and phenol (h) as sole carbon sources. Growth substrates were applied at 20 g/l, except for phenol (1.5 g/l).

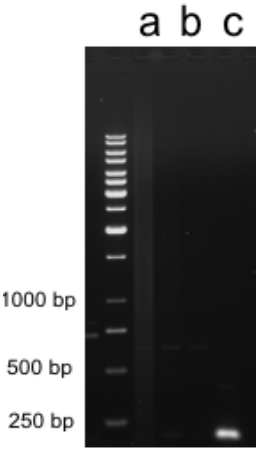

Figure 2. Molecular identification of the genus *Trichosporon* based on the amplification of a 170 bp region of small-subunit (SSU) rDNA, according to the method of Sugita *et al.* (1998).
Lanes (a) and (b), negative controls (genus: *Candida*); lane (c), the tested strain isolate – a positive result is represented by the amplified product of 170 bp.

survival by 74%, and 2.5 g/l phenol caused a 38% viability decrease.

The described high tolerance to phenol (above 2000 mg/l) placed the yeast among one of the microorganisms most resistant to this compound (Tsai *et al.*, 2005; Dzirba *et al.*, 2006). For the highly tolerant yeast *Candida albicans* TL3, Tsai *et al.* (2005) reported the culture growth capability at a phenol concentration up to 24 mM (approx. 2250 mg/l). Within the genus *Trichosporon*, however, the highest reported phenol resistance (1700 mg/l) was found for *T. cutaneum* (Santos & Linardi, 2001).

Formaldehyde (Fd) as a single xenobiotic in the medium proved to be lethal at concentrations above 0.2% (2000 mg/l). The observed tolerance is very high since Fd is an extremely toxic agent (Bardana & Montanaro, 1991; Gonzalez-Gil *et al.*, 2000). Fd presence negatively affects various microorganisms at much lower concentrations, and at levels above 1000 mg/l it cannot be tolerated even by most of preadapted biocenoses such as activated sludges (Qu & Bhattacharya, 1997; Gonzalez-Gil *et al.*, 2000). However, with our *Trichosporon* isolate, an even more striking result was obtained when 1.58 g/l (2%, v/v) methanol was added to the culture medium. The high density culture of the yeast gained tolerance up to 7500 mg/l of formaldehyde. To our knowledge, this is the first report of a microorganism exhibiting such a resistance to formaldehyde, which, together with the observed high survival in the presence of other xenobiotics makes it reasonable to consider the strain as extremophilic (Satyanarayana *et al.*, 2005). As the Fd resistance was due to an enhanced ability to biodegrade this compound, detailed data and further discussion are given in the biodegradation section below.

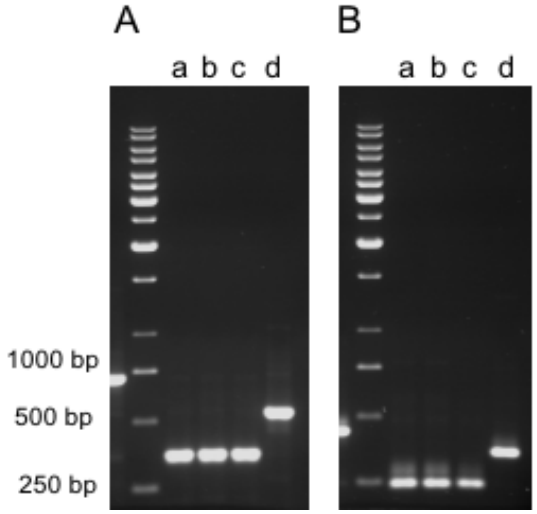

Figure 3. Molecular identification of the genus *Trichosporon* based on the amplification of the conserved regions of 18S, 5.8S, and 28S rDNA, following the method of Fujita *et al.* (2001).
PCR amplification products were obtained for the primers (A) ITS1-ITS4 and (B) ITS3-ITS4. Respective lanes (a)–(c), negative controls (genus: *Candida*); lanes (d), the tested strain isolate – a positive result is represented by the amplified product of 530 bp and 350 bp for (A) and (B), respectively.

Biodegradation potential

The strain was tested for biodegradation efficiency of xenobiotics such as diesel oil, methanol and formaldehyde. As described above, high-density cell suspensions were used (OD_{540} = 1.2, which corresponded to 2.3×10^7 cells/ml).

In the case of diesel fuel, which consists of a number of aliphatic and aromatic hydrocarbons, the biodegradation was gradual over time and led to a decrease of the total amount of the initial xenobiotic load (20 g/l) by approx. 50% within 11 days of the test. The initial and final stages of the experiment are shown in Fig. 4, where gas-chromatographic analyses revealed the concomitant degradation of practically all the constituents detected. The obtained biodegradation efficiency was lower than that observed for specialized bacterial consortia (Hamme *et al.*, 2003); however, it was comparable to the results obtained for monocultures of other autochthonous yeasts isolated from oil-contaminated soil (Koloczek *et al.*, 2004). The ability of the *Trichosporon* sp. isolate to utilize complex hydrocarbon mixtures indicated the presence of a rich enzymatic apparatus responsible for the metabolism of a variety of organic compounds.

Methanol was not removed from the medium completely, regardless of the initial concentration applied. This rather surprising result was not caused by any decrease in the culture viability. As presented in Fig. 5, although the initial level was not lethal to the yeast (13 000 mg/l), methanol decreased within 8 days over two-fold; past this time, its concentration remained constant. The mechanism of the partial degradation of methanol requires further studies, especially with growing, methanol-limited cultures. It

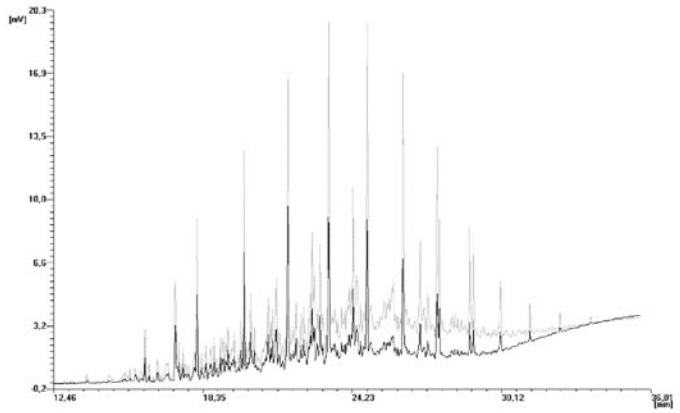

Figure 4. Gas-chromatography analysis of the degradation of diesel oil by *Trichosporon* sp. high-density culture.
Gray line, start of the process; black line, after 11 days.

should be pointed out here that other methanol-assimilating yeasts are capable of totally reducing the level of this compound (Kaszycki & Koloczek, 2000; Kaszycki *et al*., 2001).

The formaldehyde biodegradation kinetics obtained with the isolated strain is shown in Fig. 6. The dotted and solid lines represent maximum levels of Fd utilized under two experimental conditions studied: in the absence of methanol and in a mixture with 1.58 g/l methanol, respectively. As mentioned earlier, the strain tolerance toward formaldehyde was achieved by means of its effective metabolic removal, which is in agreement with our previous experiments with the methylotrophic model yeast *Hansenula polymorpha* (Kaszycki *et al*., 2001). The presence of methanol enhanced the ability to degrade Fd almost five-fold, by means of a mechanism which is yet to be explained. The possibility of co-metabolism of both substrates should be considered. A higher potential for Fd biodegradation in the presence of methanol has also been reported in other studies (Eiroa *et al*., 2004a; 2005a), including methylotrophic yeast (Kaszycki *et al*., 2001).

In our experiments we used several increasing Fd concentrations and the intriguing result was that the period of total degradation of formaldehyde never exceeded 24 h. The lack of a direct correlation of the biodegradation time with the concentration of formaldehyde applied was in contrast to the case of *H. polymorpha* (Kaszycki *et al*., 2001), where such a dependence was straightforward. Also, unlike *H. polymorpha*, before treatment with sub-lethal Fd levels, *Trichosporon* sp. required no pre-cultivation with smaller doses of the xenobiotic.

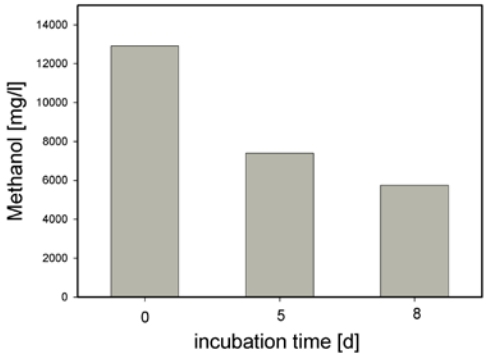

Figure 5. Degradation of methanol by *Trichosporon* sp. high-density culture (2.3×10^7 cells/ml).

Another important observation was that the isolated *Trichosporon* sp. could not grow on formaldehyde as a sole carbon source (not shown), although it was able to metabolize high doses of this xenobiotic. This, again, is in opposition to our results obtained for *H. polymorpha* which proved to gain biomass upon Fd assimilation (Kaszycki & Koloczek, 2000).

The described physiological differences between *Trichosporon* sp. and other methylotrophic strains might suggest the presence of diverse enzymatic mechanisms (see below).

On the other hand, upon Fd biodegradation, the studied yeast isolate tended to acidify the incubation medium from the initial value of pH 8.1 to a pH below 6.0, as shown in the lower section of Fig. 6. It was a very efficient process (a drop of pH was observed in a matter of seconds) and could be explained by the action of formate dehydrogenase (FMD, EC 1.2.1.2). This enzyme has been found in methylotrophic yeasts, and the culture medium acidification during Fd degradation was found to be similar (Maidan *et al*., 1997, and our observations, not shown).

The maximum biodegradation efficiency (7500 mg/l consumed within 24 h) revealed by the *Trichosporon* isolate and reported in this study is exceptionally high and has never been observed for any aerobic or anaerobic organism, microbial consortia or activated sludge.

Among *Trichosporonales,* only *T. penicillatum* was able to oxidize formaldehyde (Kato *et al*., 1982), and this species was used to treat industrial wastewater with an Fd concentratrion of 1000 mg/l (Glancer-Soljan *et al*., 2001). Our previous research on methylotrophic yeast (Kaszycki *et al*., 2001) proved that preadapted *H. polymorpha* culture was capable of biodegrading formaldehyde at concentrations up

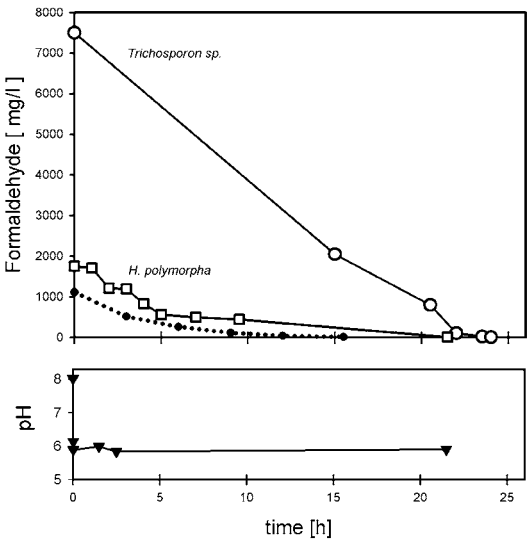

Figure 6. Biodegradation of formaldehyde.
Upper section: maximum Fd levels biodegraded with *Trichosporon* sp. (circles) and with *Hansenula polymorpha* (squares) in the presence of methanol (1.58 g/l). The dotted line represents maximum Fd biodegradation potential of *Trichosporon* sp. in the absence of methanol. Lower section: pH value of *Trichosporon* sp. culture medium during Fd biodegradation in the presence of methanol.

to 1750 mg/l (Fig. 6, squares). Recently, Mirdamadi *et al*. (2005) reported an aerobic bacterial strain of *Pseudomonas pseudoalcaligenes* that consumed 3700 mg/l of formaldehyde within 24 h. Extreme concentrations of formaldehyde were tested with sludges in wastewater-treatment model upflow reactors. The tests were performed under aerobic (up to 3168 mg/l of Fd, Eiroa *et al*., 2005b), anoxic (up to 5000 mg/l, Eiroa *et al*., 2004a), and anaerobic conditions (1110 mg/l, Qu & Bhattacharya, 1997), as well as in batch culture reactors (3890 mg/l, Eiroa *et al*., 2004a).

Methylotrophy

The ability to metabolize single-carbon compounds is essential for methylotrophy (Michalik, 1975; Michalik & Raczynska-Bojanowska, 1976; Gleeson & Sudbery, 1988; Sibirny *et al*., 1988). The isolate of study showed its ability to assimilate methanol and to utilize formaldehyde, proving to be a methylotrophic yeast. The growth of the *Trichosporon* sp. isolate can be observed in Fig. 7. The liquid optimal medium containing 2% (v/v) methanol was inoculated with the yeast at a low density, i.e. at approx. 10^5 cells/ml. Rapid growth was then observed, and the culture proliferated after a relatively short lag phase to gain the density of over 10^7 cells/ml within 2 days. Both the growth dynamics and the shape of the growth curve resembled that obtained for the methylotrophic yeast *H. polymorpha* (Sibirny *et al*., 1988; Kaszycki & Koloczek, 2000).

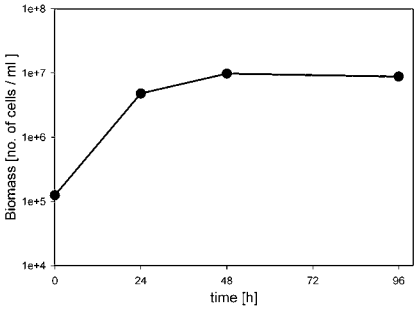

Figure 7. Growth kinetics of *Trichosporon* sp. culture on methanol (1.58 g/l) as a sole carbon source.

Preliminary enzymatic characteristics

For the extraordinary biochemical features of the isolated *Trichosporon* sp. it was of interest to search for the enzymatic background determining the physiological activities. In a preliminary study, some of the enzyme activities characteristic of the methylotrophic pathway reactions were examined and compared with a model methylotrophic strain of *Hansenula polymorpha* (Gleeson & Sudbery, 1988; Sibirny *et al*., 1988) for enzymatic characterization of the pathway.

SDS/PAGE electrophoresis performed for cellular extracts of methanol-grown cultures of *H. polymorpha* and *Trichosporon* sp. revealed that the newly isolated strain was lacking in the key enzyme of the conventional methylotrophic pathway — alcohol oxidase (AO, EC 1.1.3.13) (Fig. 8). Since this enzyme is responsible for methanol oxidation as the first step in assimilation reactions, the pathway for methanol utilization in *Trichosporon* sp. must be substantially different.

In order to reveal the activity of glutathione-dependent formaldehyde dehydrogenase (FDD, EC 1.2.1.1), kinetic activity tests were performed using *Trichosporon* sp. crude cellular extracts. This enzyme is also typical for methylotrophic yeast and is claimed to be mainly responsible for the Fd-detoxifying action of the methylotrophic pathway. Fig. 9 (solid line) gives proof of the presence of a significant FDD activity, which was comparable to the

Figure 8. SDS/PAGE electrophoretic analysis of cellular extracts of the isolated strain *Trichosporon* sp. (lane b) as compared with *Hansenula polymorpha* (lane a).
The arrow points to the protein band of 64 kDa, indicating the presence of alcohol oxidase (AO).

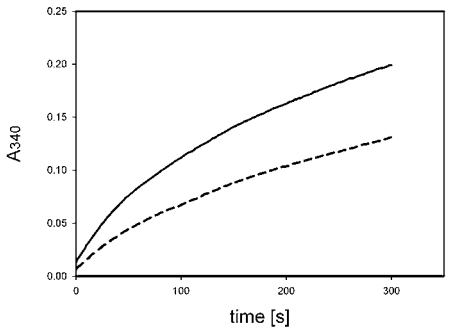

Figure 9. Demonstration of aldehyde dehydrogenase activities in cellular extracts of *Trichosporon* sp.
Solid line, glutathione-dependent formaldehyde dehydrogenase (FDD); dotted line, glutathione-dependent aldehyde dehydrogenase (AldD). To reveal activities of the two enzymes, formaldehyde and acetaldehyde were used as substrates, respectively.

activities obtained for *H. polymorpha* extracts (not shown). At the same time, by using glutathione and acetate aldehyde as substrates, a new activity of a glutathione-dependent aldehyde dehydrogenase was revealed (Fig. 9, dotted line). This interesting finding might indicate the existence of a novel, yet unidentified dehydrogenase. The observed activity was not due to FDD since the latter enzyme is substrate (i.e. formaldehyde) specific. Similar activity has never been detected in *H. polymorpha*.

Zymographic analyses were applied to check for the presence of unspecific aldehyde dehydrogenase (AldD, EC 1.2.1.5). Zymograms were obtained for *Trichosporon* sp. and *H. polymorpha* (Fig. 10b and a, respectively), revealing strong acetaldehyde-based activities for both strains, yet significant differences between the two lanes were visible. The pattern observed for the new *Trichosporon* isolate might suggest an involvement of different enzymatic proteins as compared to conventional methylotrophs. This constitutes a future case for detailed study since unspecific aldehyde dehydrogenases may play an important role in Fd dissimilation reactions, as pointed out by Maidan *et al*. (1997).

It would also be interesting to find out whether exogenous formaldehyde was reduced to methanol by the activity of NADH-dependent formaldehyde reductase, another enzyme that has

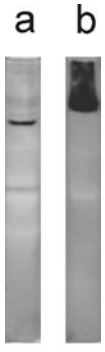

Figure 10. Zymographic analysis of cellular extracts of the isolated *Trichosporon* sp. strain (lane b) as compared with *Hansenula polymorpha* (lane a).
The reagent solution contained acetaldehyde as a substrate to reveal activities of unspecific aldehyde dehydrogenases.

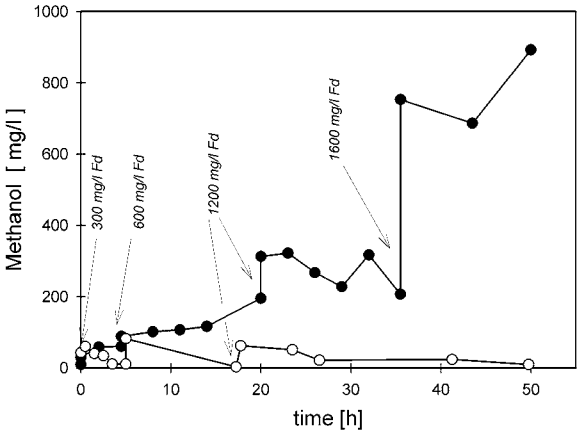

Figure 11. Generation of endogenous methanol upon formaldehyde biodegradation by the isolate *Trichosporon* sp. (solid circles) as compared with *Hansenula polymorpha* (open circles).
Subsequent doses of formaldehyde are indicated by arrows and they were added after total degradation of the preceding Fd portion.

been identified in methylotrophic yeasts. This is an NADH-dependent formaldehyde-reducing enzyme and might be important for Fd detoxification action as it generates the less toxic methanol. Indeed, when *Trichosporon* sp. culture was treated with increasing formaldehyde amounts, methanol was formed in a dose-dependent manner (Fig. 11, solid circles) and tended to cumulate in the medium (up to 900 mg/l in Fig. 11). This was unlike the test with *H. polymorpha*, where the endogenous Fd-generated pool of methanol was efficiently removed by methanol-utilizing enzymes (Fig. 11, open circles), never exceeding the level of 100 mg/l.

Regarding the observed physiological reaction to the presence of formaldehyde with *Trichosporon* sp., it is difficult to account for the discrepant results which prove efficient methanol-based growth and its assimilation, on one hand, and endogenous methanol accumulation on the other. However, when the activities of NAD^+-dependent Fd and aldehyde dehydrogenases were taken into account, the presence of a regulatory mechanism that triggers FDR might be suggested. Upon Fd administration the culture could react with an immediate dehydrogenase action, which would lead to the generation of excess NADH and to a deficit in the NAD^+ pool. Since it is known that NADH down-regulates FDD and FMD, the dissimilative Fd pathway would be inhibited whereas NAD^+ could be regenerated by means of FDR activity. The proposed enzymatic interaction mechanism in *Trichosporon* sp. needs to be further verified and elucidated, however.

Summing up, the newly isolated strain of *Trichosporon* sp. reveals a methylotrophic biochemical pathway, several enzymes of which could be

identified in a model methylotrophic yeast, as well. However, the presence of novel enzymes, the lack of AO, and the particularly efficient FDR activity imply a different, as yet unknown, regulatory mechanism for methanol assimilation as well as for endo- and exogenous formaldehyde biodegradation. Also, little is known about any possible link between Fd degrading enzymes and other xenobiotic-metabolizing activities. These topics appear to be challenging problems for future studies.

CONCLUSIONS – FUTURE PERSPECTIVES FOR BIOTECHNOLOGICAL APPLICATIONS OF THE ISOLATED STRAIN

Several biochemical and physiological features of the *Trichosporon* strain presented in this article indicate that the application potential of this yeast in biotechnology is very promising. In particular, there is growing interest in the use of microbial atypical enzymatic pathways for environmental biotechnology efforts. The isolated strain can adapt to extreme levels of various xenobiotics: phenol, formaldehyde, methanol and petroleum-derived hydrocarbons. In such environments this yeast induces metabolic potential enabling it to biodegrade at least some of these compounds. Thus, the strain seems to be a suitable microorganism for the treatment of heavy-load wastewaters originating from chemical installations. When grown as monoculture, it could be used as a biofilter to treat undiluted wastewaters since it can tolerate certain environmental contaminants at the highest concentrations generated by technological processes. For example, formaldehyde in industrial effluents can reach the level of 10 g/l (Gonzales-Gil *et al.*, 2000) and phenol, after chemical pretreatment of wastewaters, 0.4 g/l (Godjevargova *et al.*, 2003). The use of the strain as a component of activated sludge or as an organism integrated with specialized microbial consortia (Glancer-Soljan *et al.*, 2001) for wastewater treatment and bioremediation of oil-polluted soil should also be considered.

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