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Identification and Characterization of Yeast Isolates from Pharmaceutical Waste Water

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Summary

In order to develop an efficient an system for waste water pretreatment, the isolation of indigenous population of microorganisms from pharmaceutical waste water was done. We obtained pure cultures of 16 yeast isolates that differed slightly in colony morphology. Ten out of 16 isolates efficiently reduced COD in pharmaceutical waste water. Initial physiological characterization failed to match the 10 yeast isolates to either *Pichia anomala* or *Pichia ciferrii*. Restriction analysis of rDNA (rDNA-RFLP) using three different restriction enzymes: *Hae*III, *Msp*I and *Cfo*I, showed identical patterns of the isolates and *Pichia anomala* type strain. Separation of chromosomal DNAs of yeast isolates by the pulsed field gel electrophoresis revealed that the 10 isolates could be grouped into 6 karyotypes. Growth characteristics of the 6 isolates with distinct karyotypes were then studied in batch cultivation in pharmaceutical waste water for 80 hours.

Key words: COD, electrophoretic karyotyping, physiological test, *Pichia anomala*, rDNA –RFLP, waste water

Introduction

The pharmaceutical industry generates plenty of waste water, from chemical syntheses and fermentation processes. These waste waters often require a pretreatment before conventional waste water treatment can be used. For biological pretreatment, appropriate microorganisms are necessary to lower a COD load efficiently. Our recent work *(1)* revealed that, only 3 out of 60 yeast strains from the Culture Collection of Industrial Microorganisms (ZIM) Ljubljana, Slovenia, were able to grow in pharmaceutical waste water and reduce its COD efficiently (*i.e.* 60–64 %) after 72 hours. On the other hand, 10 out of 16 yeast isolates from pharmaceutical waste water were more efficient in COD reduction (78–81 %) under the same experimental conditions. The present study describes physiological and genetic characteristics of these 10 isolates.

Morphological, physiological and biochemical tests *(2,3)* have commonly been used for phenotypic characterization of yeast species. These methods are often unreliable, due to strain variability *(4)* and, therefore, do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular techniques provides more powerful means of strain identification and differentiation among strains *(5–7)*. Restriction fragment length polymorphisms of yeast ribosomal DNA (rDNA-RFLP) has been used successfully to show inter- and intraspecific relationships among different yeast *(8–11)*. Electrophoretic karyotyping using PFGE technique, has been reported to allow differentiation among strains of several yeast species: medically important *Candida* spp. *(12–14)*, *Saccharomyces cerevisiae (15)*, *Yarrowia lipolytica (16)*, *Hansenula polymorpha/*

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Pichia angusta (17), *Kluyveromyces* spp. *(18)* and *Saccharomycodes ludwigii (19).*

The aim of this study was to identify yeast isolates from pharmaceutical waste water by physiological and morphological tests, by rDNA-RFLP analysis and by PFGE karyotyping.

Material and Methods

Yeast strains

Sixteen yeast strains were isolated from pharmaceutical waste water derived from the ergot alkaloid production in the »Lek« Pharmaceutical & Chemical Company, Ljubljana, Slovenia. Selected 10 yeast isolates designated as ZIM 83, ZIM 85, ZIM 88, ZIM 92, ZIM 93, ZIM 94, ZIM 95, ZIM 96, ZIM 99 and ZIM 419, all showed efficient COD reduction in previous studies *(1)*. *Pichia anomala*^{T} ZIM 1769 (CBS 5759; Centraalbureau voor Schimmelcultures, Baarn and Delft, The Netherlands) and *Pichia ciferrii*^{T} ZIM 1835 (NRRL Y-1031; Nothern Regional Research Laboratory, Peoria, IL, USA) were used as type strains for rDNA-RFLP analysis. All these strains are maintained in the Culture Collection of Industrial Microorganisms (ZIM), Ljubljana, Slovenia.

Isolation and phenotypic characterization of isolates

Isolation and phenotypic characterization of yeast strains were done according to Barnett *et al*. *(2)* and Yarrow *(20).* Experimental results of physiological tests were analyzed by the software package of Barnett *et al*. *(21).* Growth assays were repeated 3 times in 3 replicates.

Restriction fragment length polymorphism of amplified rDNA

Total genomic DNA was isolated according to Smole Mozina *et al*. *(11).* The primers (NS1:5'GTAGTCATATGCTTGTCTC3' and ITS4:5'TCCTCCGCTTATTGATATG 3'), described by White *et al*. *(22)* were used to amplify the 18S-ITS1-5.8S-ITS2 region of rDNA. Amplification was done on the Perkin Elmer Gene Amp PCR System 2400 using the following thermal cycling parameters: initial denaturation at 95 °C for 2 min, followed by 35 denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 3 min and final step at 72 °C for 7 min. Aliquots (3 μL) of amplified rDNA were digested with the following restriction endonucleases: *Hae*III, *Msp*I, *Cfo*I and *Rsa*I. The restriction fragments were electrophoresed on a 1.5 % (w/v) agarose gel in 1x TAE buffer at 170 V for 1.2 h, stained with ethidium bromide and photographed using Polaroid MP-4 camera and Polaroid 667 film.

Electrophoretic karyotyping

Yeast biomass was embedded in low melting point agarose and sequentially digested with Novozyme and Proteinase K according to Carle and Olson *(23)* with some modifications as described in the Instruction Manual for Pulsaphor™ System *(24).* Yeast chromosomes were separated by the pulsed field gel electrophoresis CHEF (LKB Pharmacia) at 10 °C on 1.0 % agarose gel in 0.5 x TBE buffer. A constant voltage of 100 V was used for 68 hours with the following pulsing intervals: 150 s for 24 h, 300 s for 24 h and 600 s for 20 h in 1 % agarose gel. The gel was stained with ethidium bromide and photographed as described above.

Cultivation of selected strains in pharmaceutical waste water

Growth experiments were conducted in batches at 28 °C in 2 L airlift (1.8 L min^{-1}) bioreactors. Bioreactors were filled with 1.8 L of filtered pharmaceutical waste water from the ergot alkaloid production (»Lek« Pharmaceutical & Chemical Company, Ljubljana, Slovenia) and autoclaved at 121 °C for 15 min. Initial pH was adjusted to pH=5 with 98 % H_2SO_4. Inital chemical oxygen demand (COD) of filtered and sterilized waste water ranged from 30 500 to 35 800 mg/L and its detail composition was given elsewere *(1).* After sterilization and cooling bioreactors were inoculated with 90 mL of 18–20 h old broth cultures of individual strains. Samples were taken every 12 hours and analyzed for chemical oxygen demand, biomass dry weight, and total and ammoniacal nitrogen content, as described previously *(1).*

Results and Discussion

Phenotypic characterization

Pure cultures of 16 morphologically distinct yeast isolates from pharmaceutical waste water were selected on the basis of the colony morphology and color on the malt extract agar. Our previous study revealed that 10 out of 16 isolates, capable of growing in waste water, efficiently reduced its COD *(1).* All of these isolates were able to sporulate on 5 % malt extract agar after 2 weeks and produced hat-shaped ascospores characteristic for the genus *Pichia*. According to their ability of sugars fermentation and assimilation of carbon and nitrogen sources the 10 isolates generally matched the physiological characteristics of *P. anomala* and *P. ciferrii* (Table 1).

Comparing physiological tests of the 10 isolates among themselves there were a few exceptions. All of the isolates were able to ferment maltose, except for the isolate ZIM 95. Another exception was the isolate ZIM 419, which did not grow on lactose, whereas other isolates grew on this carbon source but to a variable extent. The differences, however, were not sufficient to classify the isolates as heterogeneous species. Furthermore, the isolates did not differ in morphological features of vegetative cells and number of ascospores. These results confirmed the previously reported *(5)* problems with identification, based on physiological tests and phenotypic characterization alone.

Restriction enzyme analysis of amplified rDNA

In our previous studies (data not shown) rDNA-RFLP analysis of 18S-ITS1-5.8S-ITS2 region of different strains of *P. anomala*, by 4 different endonucleases: *Hae*III, *Msp*I, *Cfo*I and *Rsa*I, showed stable species-specific restriction patterns. Restriction patterns obtained by 3 endonucleases (*Hae*III, *Msp*I, *Cfo*I) of the isolates ZIM 83, ZIM 94 and ZIM 99 were identical to that of *P. anomala* ZIM 1769 type strain but different from *P. ciferrii*

Table 1. Physiological characteristics of yeast isolates, *P. ciferrii* and *P. anomala*

	ZIM 83	ZIM 85	ZIM 88	ZIM 92	ZIM 93	ZIM 94	ZIM 95	ZIM 96	ZIM 99	ZIM 419	*P. ciferrii*	*P. anomala*
D-Glucose fermentation	+	+	+	+	+	+	+	+	+	+	V	+
D-Galactose ferment.	–	–	–	–	–	–	–	–	–	–	V	V
Maltose fermentation	+	+	+	+	+	+	–	+	+	+	V	V
Sucrose fermentation	+	+	+	+	+	+	+	+	+	+	+	+
Lactose fermentation	–	–	–	–	–	–	–	–	–	–	–	–
D-Glucose growth	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose growth	+	+	+	+	+	+	+	+	+	W	+	V
D-Ribose growth	–	–	–	–	–	–	–	–	–	–	+	V
L-Arabinose growth	V	V	V	V	V	V	V	V	V	V	V	–
D-Arabinose growth	+	+	+	+	+	+	+	+	+	+	V	–
L-Rhamnose growth	–	–	–	–	–	–	–	–	–	–	V	–
Sucrose growth	+	+	+	+	+	+	+	+	+	+	+	+
Maltose growth	+	+	+	+	+	+	+	+	+	+	+	+
Lactose growth	V	V	V	V	V	V	V	V	V	–	–	–
Raffinose growth	+	+	+	+	+	+	+	+	+	+	+	V
Melezitose growth	+	+	+	+	+	+	+	+	+	+	+	V
Starch growth	V	V	V	V	V	V	V	V	V	V	+	+
Glycerol growth	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol growth	+	+	+	+	+	+	+	+	+	+	+	V
Ribitol growth	–	–	–	–	–	–	–	–	–	–	V	V
Xylitol growth	+	+	+	+	+	+	+	+	+	W	+	V
D-Mannitol growth	+	+	+	+	+	+	+	+	+	+	+	+
Galactitol growth	–	–	–	–	–	–	–	–	–	–	–	–
Myo-Inositol growth	–	–	–	–	–	–	–	–	–	–	–	–
D-Glucuronate growth	–	–	–	–	–	–	–	–	–	–	–	–
Succinate growth	V	V	V	V	V	V	V	V	V	V	+	+
Citrate growth	+	+	+	+	+	+	+	+	+	+	V	+
Methanol growth	–	–	–	–	–	–	–	–	–	–	–	–
Ethanol growth	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate growth	+	+	+	+	+	+	+	+	+	+	+	+
Ethylamine growth	V	V	V	V	V	V	V	V	V	V	+	+
Growth w/o vitamins	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37 °C	V	V	V	V	V	V	V	V	V	V	V	V
0.01 % Cycloheximide g.	–	–	–	–	–	–	–	–	–	–	–	–
0.1 % Cycloheximide g.	–	–	–	–	–	–	–	–	–	–	–	–
1 % Acetic acid growth	–	–	–	–	–	–	–	–	–	–	–	–
Urea hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–

+ positive; – negative; V variable response; W weak positive response

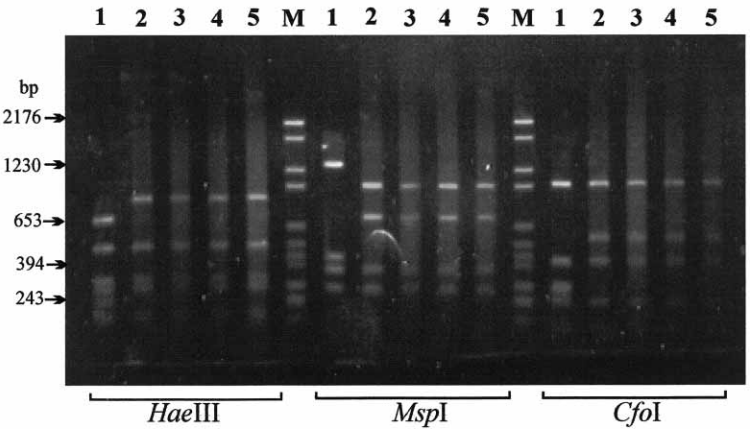

Fig. 1. rDNA-RFLP patterns of *Pichia ciferrii* ZIM 1835 (NRRL Y-1031), *P. anomala* ZIM 1769 (CBS 5759) type strains and selected yeast isolates. Lanes: 1. *P. ciferrii* ZIM 1835, 2. *P. anomal*a ZIM 1769, 3. isolate ZIM 83, 4. isolate ZIM 94 and 5. isolate ZIM 99; M-DNA molecular weight marker Boehringer VI; 18S-ITS1-5.8S-ITS2 restricted with *Hae*III, *Msp*I and *Cfo*I endonucleases. Identical patterns were obtained in 2 independent experiments.

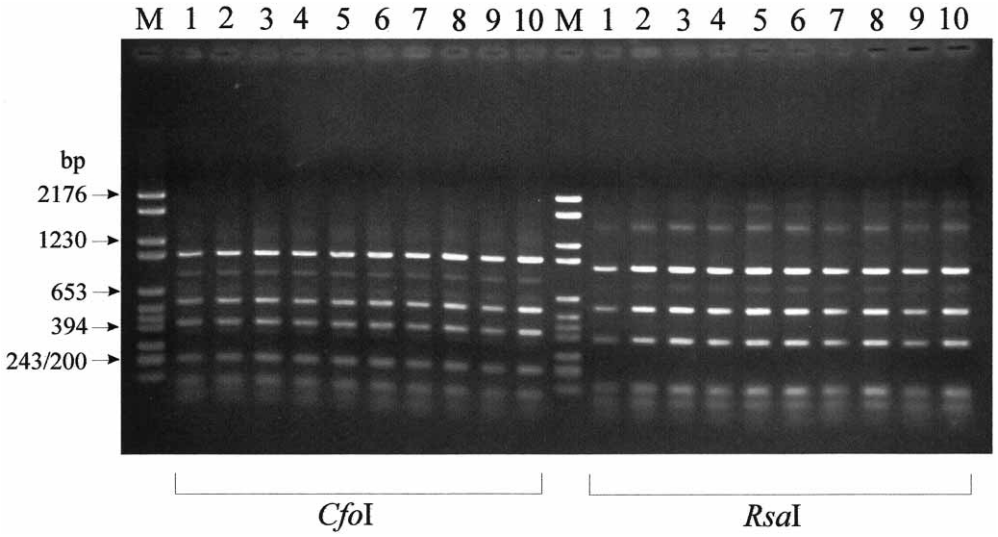

Fig. 2. rDNA-RFLP patterns of the 10 yeast isolates. Lanes: 1. isolate ZIM 83, 2. isolate ZIM 85, 3. isolate ZIM 88, 4. isolate ZIM 92, 5. isolate ZIM 93, 6. isolate ZIM 94, 7. isolate ZIM 95, 8. isolate ZIM 96, isolate ZIM 99 and 10. isolate ZIM 419; M-DNA molecular weight marker Boehringer VI; 18S-ITS1-5.8S-ITS2 restricted with *Cfo*I and *Rsa*I endonucleases. Identical patterns were obtained in 2 independent experiments.

ZIM 1835 type strain (Fig. 1). Restriction patterns of all 10 isolates were then compared among themselves, using 4 endonucleases: *Hae*III, *Msp*I, *Cfo*I and *Rsa*I by the same restriction protocol. Identical restriction patterns obtained by *Cfo*I and *Rsa*I (Fig. 2) and *Hae*III and *Msp*I (data not shown) suggest that all of the isolates belong to the same yeast species – *P. anomala*. These results confirm the potential of rDNA-RFLP method for identification of strain at the interspecies level *(8,10,11).*

Electrophoretic karyotyping

The isolates were then subjected to karyotype analysis by the pulse field technique, using contour clamped homogeneous electric field electrophoresis. The karyotype polymorphism was detected in a number of bands, which ranged from 5–9, and their positions, as shown in Fig. 3. By using the same protocol in repeated (3 times) experiments we could clearly identify 6 reproducible karyotype patterns among the 10 isolates. First common pattern can be observed for isolates ZIM 88 and ZIM 92 and the second common pattern can be seen among isolates ZIM 94, ZIM 96 and ZIM 419. The karyotype pattern of isolate ZIM 99 was similar to the latter group although we could not separate bands 5 and 6. The rest of the isolates ZIM 83, ZIM 85, ZIM 93 and ZIM 95 revealed the distinct karyotype patterns. The size of the bands of the isolates was estimated to range among 1.34 and 3.13 Mb, based on comparison with band sizes of laboratory marker strain. Despite the variability of electrophoretic patterns, which may be attributed to the instability of the karyotype or, to the presence of several strains in the original isolates *(15)*, electorphoretic karyotyping apparently permits differentiation among strains at the intraspecies level *(25,26)*.

Cultivation of yeasts in pharmaceutical waste water

Growth characteristics of the 6 isolates with distinct karyotypes ZIM 83, ZIM 85, ZIM 92, ZIM 93, ZIM 95

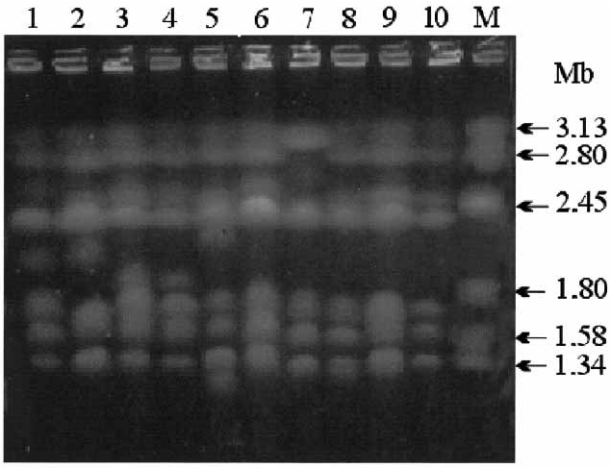

Fig. 3. Electrophoretic karyotype analysis of chromosomal DNAs of the 10 yeast isolates. Lanes: 1. isolate ZIM 83, 2. isolate ZIM 85, 3. isolate ZIM 88, 4. isolate ZIM 92, 5. isolate ZIM 93, 6. isolate ZIM 94, 7. isolate ZIM 95, 8. isolate ZIM 96, 9. isolate ZIM 99, 10. isolate ZIM 419 and M laboratory marker strain. Identical patterns were obtained in 3 independent experiments.

and ZIM 419 were studied in 2 L airlift bioreactors for 80 hours. The COD, pH, total and ammoniac nitrogen content and biomass accumulation in waste water were followed during the growth experiments.

Cell growth reached the end of the exponential phase after 48 hours and within this period most isolates achieved COD reduction in waste water by 62±4 % (Fig. 4a). Isolate ZIM 85 reduced COD by 72±3 % within this period of time. Maximum COD reduction (84±5% reduction of initial COD) was reached after 72 hours, in both independent cultivations, when the final COD values ranged between isolates from 3 800 to 5 300 mg/L in the first batch culture and 5 000 to 6 800 mg/L, in the repeated culture. By prolonging the culturing for addi-

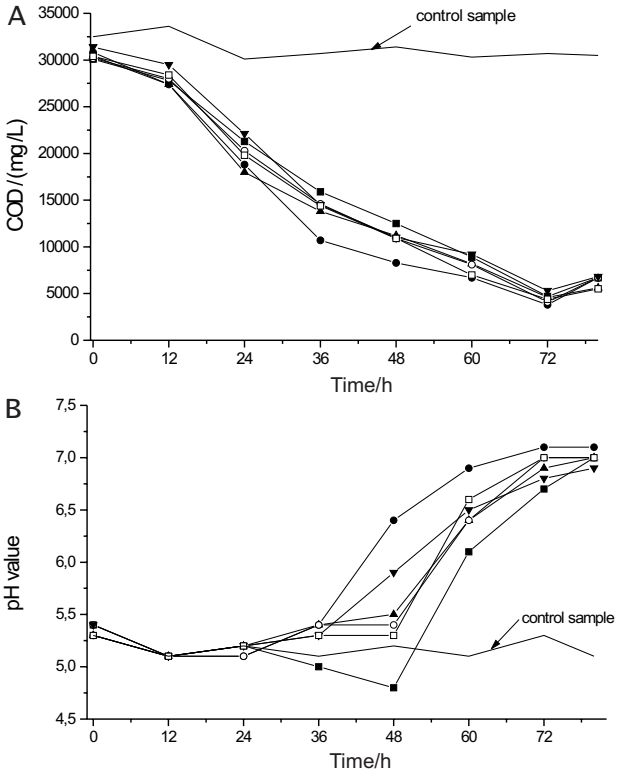

Fig. 4. COD (A) and pH values (B) of pharmaceutical waste water during cultivation of the 6 electrokaryotyped yeast isolates ZIM 83, ZIM 85, ZIM 92, ZIM 93, ZIM 95 and ZIM 419. Isolates were grown in 2 L airlift bioreactors, at 28 °C, for 80 hours, as described in Material and Methods section. Data represent means ± standard deviations of 2 independent cultivations.

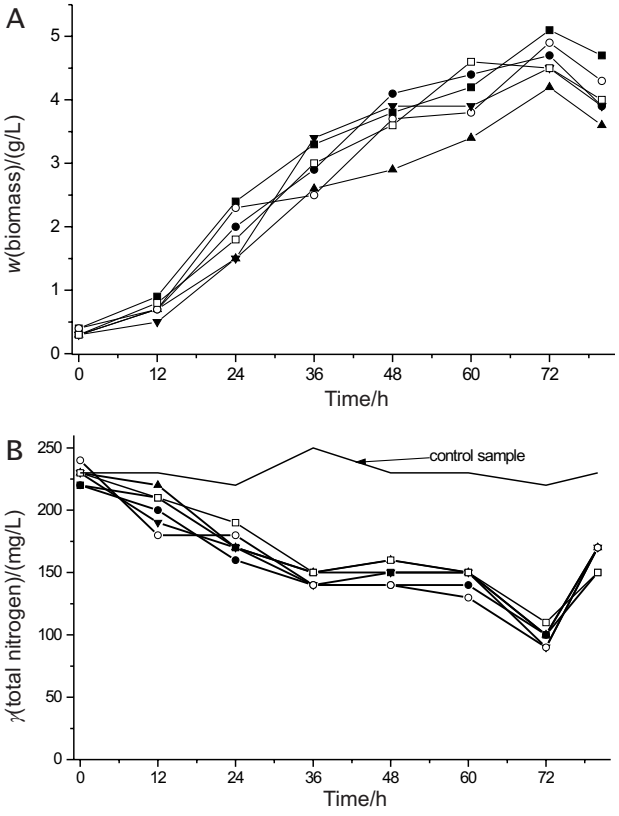

Fig. 5. Biomass accumulation (A) and total nitrogen assimilation (B) during growth of the 6 electrokaryotyped yeast isolates in pharmaceutical waste water (ZIM 83, ZIM 85, ZIM 92, ZIM 93, ZIM 95 and ZIM 419). Isolates were grown as described in Fig. 4. Data represent means ± standard deviations of 2 independent cultivations.
Legend of Figs. 4 and 5: –■– ZIM 83; –●– ZIM 85; –▲– ZIM 92; –○– ZIM 93; –▼– ZIM 95; –□– ZIM 419

tional 8 hours, COD value increased suggesting the beginning of cell autolysis. COD value was apparently related to pH changes of waste water, as shown in Fig. 4b. Initial pH of waste water was adjusted to 5, since it was found in preliminary experiment that this was the optimum for COD reduction.

Accumulation of yeast biomass increased through 72 hours except for the isolate ZIM 419. Biomass concentration ranged from (4.5±0.2) to (5.2±0.3) g/L, except for ZIM 92 which had lower biomass concentration (Fig. 5a). Utilization of nitrogen from waste water was similar in all strains. Ammoniac nitrogen was assimilated during the first 12 hours and decreased thereafter from 50 to 10 mg/L (data not shown). Total nitrogen assimilation was also similar in all isolates (Fig. 5b). Increased concentration of total nitrogen, accompanied by biomass decrease after 72 hours of cultivation can be attributed to cell death and autolysis, as they become deprived of nutrients.

Conclusions

Ten yeast isolates from pharmaceutical waste water could not be identified by physiological tests. Based on rDNA-RFLP analysis we concluded that all of the isolates belong to the same species – *Pichia anomal*a. Electrophoretic karyotyping with PFGE indicated that the isolates may be grouped into 6 strains. Despite the lack of correlation between phenotypic and genotypic characteristics, differences between strains were manifested in COD reduction and biomass accumulation during aerobic cultivation in pharmaceutical waste water.

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Identifikacija i karakterizacija izolata kvasca iz otpadne vode farmaceutske industrije

Sažetak

Da bi se unaprijedio djelotvoran sustav za prethodnu obradbu otpadnih voda, izolirana je nativna populacija mikroorganizama iz otpadne vode farmaceutske industrije . Dobivene su čiste kulture 16 izolata kvasca koje su se neznatno razlikovale po morfologiji kolonija. Deset od 16 izolata učinkovito je smanjilo KPK-vrijednost u otpadnoj vodi. Početnom fiziološkom karakterizacijom nije se uspjelo utvrditi jesu li 10 izolata kvasca *Pichia anomala* ili *Pichia ciferrii.* Restrikcijska analiza rDNA (rDNA-RFLP), koristeći tri različita restrikcijska enzima *Hae*III, *Msp*I i *Cfo*I, pokazala je identičnu strukturu izolata i soja tipa *P. anomala*. Separacija kromosomskih DNA u izolatima kvasca gel-elektroforezom u pulsirajućem polju otkrila je da se 10 izolata može svrstati u 6 kariotipova. Proučavane su značajke rasta 6 izolata određenoga kariotipa tijekom 80 sati šaržnog uzgoja u otpadnim vodama farmaceutske industrije.