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## Molecular taxonomy studies on some yeast strains with biodegrading abilities

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### Abstract

*Microorganisms (bacteria and yeasts) are subjects of many bioremediation studies, as an interesting and modern solution for restoring oil-polluted environments. Our study presents molecular taxonomy analysis of five yeast strains with biodegrading abilities, named D1, D2, D3, D4 and D6, and preliminary identified: D1, D2 and D4 as Rhodotorula glutinis, D3 as Candida parapsilosis and D6 as Candida tropicalis. The first step involved optimization of the strain specific cell wall lysis and deproteinisation conditions, for chromosomal DNA isolation. The parameters for % mol GC determination were set up, and the values obtained fitted within the ranges described in literature for the three species: D1 - 56.67 %, D2 - 61.04 %, D3 - 41.47 %, D4 - 58.54 %, D6 - 35.87%. Strain D3 showed an RNA plasmid, but no killer activity. DNA plasmids were present for D3 and D4. Although further investigation will be performed in order to establish the role of the RNA and DNA plasmids in D3 and, respectively, in D4, the final overall look, confirmed our previous taxonomical classification.*

Keywords: yeasts, oil-polluted environments, biodegradation, molecular taxonomy.

### Introduction

The industrial revolution changed the way of life and the environment as new urban communities appeared. The augmentation of the amount of industrial and house holds wastes and the accidental oil spills raised the problem of their storage and treatment with the aim of preserving the nature. Biologic treatment of house wastes has a long history, but studies concerning the possibility of applying this process for degrading organic compounds and industrial products or by-products, are performed only for the last three decades. Thus, bioremediation represents the stimulation of biodegradation, for minimizing the environmental impact of an oil spill [17]. Microorganisms (bacteria and yeasts) are subjects of many bioremediation studies, mainly due to their ability of assimilating a large range of organic compounds, such as the hydrocarbons, main components of oil.

So far there have been described at least 22 species of bacteria and approximately 14 species of yeasts [1]. The way that bacteria act in the biodegradation processes is relatively well known, while there are still many questions concerning the way yeasts participate in the same processes. The yeast species described in the literature as being able to use hydrocarbons as carbon sources belong especially to the genera *Candida* [8; 11; 15; 18], *Rhodotorula* [9] and *Yarrowia* [4]. The importance of bioderemediation as alternative technology for restoring the polluted environments, raised the problem of a better

taxonomical identification of new yeast strains with biodegrading abilities. Such studies begin with conventional taxonomy tests, and involve further molecular biology analysis, meant to achieve better knowledge of their chromosomal and extra-chromosomal genome.

Five yeast strains (named *D1*, *D2*, *D3*, *D4* and *D6*) have been isolated from oil-polluted soil and water, and have been studied by morphological and biochemical tests and by electrokaryotyping [6]. The results indicated a possible belonging of *D1*, *D2* and *D4* to *Rhodotorula glutinis*, of *D3* to *Candida parapsilosis* and of *D6* to *Candida tropicalis*. The outline of the present article is the analysis at molecular level of these strains, for a more accurate characterization and taxonomical classification.

## Materials and Methods

### Yeast strains and media

Studies were performed on five yeast strains named *D1*, *D2*, *D3*, *D4* and *D6* isolated from oil-polluted environment from Pitesti area (Romania). The following standard yeast strains were used: *S. cerevisiae* ATTC 26108, *S. cerevisiae* D649 MATa/MATa MAL2/mal2 trp1/TRP1 pet6/PET6 ade2/ADE2 ADE1/ade1 lys2/LYS2 HIS4/his4 LEU2/leu2 THR4/thr4, *C. boidinii* ICCF (Institute for Chemical and Pharmaceutical Research and Development, Bucharest), *C. parapsilosis* GM (MICROGEN, Faculty of Biology, University of Bucharest), *C. tropicalis* GM *Rh. glutinis* GM, *S. cerevisiae* S288c, *S. cerevisiae* 17/17 a his Ko (K<sup>+</sup>R<sup>+</sup>), *S. cerevisiae* 10701 C a thr 4 K1 (K<sup>+</sup>R<sup>+</sup>), *S. cerevisiae* SMR 4 K2 (K<sup>+</sup>R<sup>+</sup>).

Yeasts were maintained at -70°C on yeast peptone glucose (YPG) medium (yeast extract 5 g L<sup>-1</sup>, peptone 10 g L<sup>-1</sup>, glucose 20 g L<sup>-1</sup>) supplemented with 20% glycerol. Previous to taxonomical tests, yeast were cultured on YPG slants, and grown for 18 hours, at 30°C.

### Chromosomal DNA isolation and purification

The chromosomal DNA isolation and purification were performed using as starting point the method described by Ausubel [2]. Yeast strains were grown for 18 h on liquid YPG, centrifuged and the pellet was re-suspended in 400 µL TEG (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0). The cell walls were lysed with 2 µL β-mercaptoethanol (30 min at 37°C) and zymolyase in final concentration of 0.4 mg mL<sup>-1</sup> for strains *D3* and *D6* and of 0.8 mg mL<sup>-1</sup> for strains *D1*, *D2* and *D4*, and incubation 90 min. For total cell lysis the protoplasts were re-suspended in 300 µL TEG and 30 µL SDS 10%. Deproteinization was performed using protease K treatment (200 µg mL<sup>-1</sup>, 30 min at 37°C) and 2.5 M KCl. After centrifugation, the supernatant was mixed with fenol:cloroform:isoamylic alcohol (25:24:1 v/v/v), followed by RNase A treatment (100 µg mL<sup>-1</sup>, 30 min at 37°C). After a new deproteinization with cloroform:isoamylic alcohol (24:1 v/v), DNA was precipitated with isopropanol at room temperature and resuspended in 20 µL TE (10 mM Tris, 1 mM EDTA, pH 8.0).

### 2 microns/ 2microns – like DNA plasmid isolation

2 microns / 2 microns – like DNA plasmid isolation was performed using a technique adapted from J.F.T.Spencer [20]. *S. cerevisiae* D649 strain was used as positive control strain for the presence of 2 microns plasmid. Yeast strains were grown for 18 h on liquid YPG and the protoplasts were obtained using the same protocol as for chromosomal DNA. The protoplasts were then treated with 200 µL TEG for 15 min on ice. Then, 400 µL lysis solution (0.1 N NaOH, 1% SDS, final pH 12.5) was added and the mixture was kept for 10 min on ice. The chromosomal DNA and proteins were precipitated with 300 µL acetate solution (v/v:

11.5 % acetic acid, 60 % 5M potassium acetate, 28.5 % water, final pH 4.8). After incubation on ice the samples were centrifuged and the deproteinization of the supernatant was achieved using chloroform:isoamyl alcohol (24:1 v/v). Plasmid DNA was precipitated with cold ethanol (100%) and resuspended in 20  $\mu\text{L}$  TE.

#### **Nucleic acids electrophoresis**

The electrophoretic analysis of genomic DNA samples was performed by agarose gel electrophoresis using agarose gel 0.8% in TBE 1X (0.089 M Tris, 0.089 M boric acid, 0.005 M EDTA, pH 8.0) [13]. For 2 microns plasmid DNA extracts 0.85% agarose gel was used. In both cases, the electric field voltage was  $2.5 \text{ V cm}^{-1}$ , and DNA bands were stained with ethidium bromide  $1 \mu\text{g mL}^{-1}$ .

#### **Spectrophotometric analysis of nucleic acids extracts**

The spectrophotometric analysis of nucleic acids extracts was performed using a spectrophotometer ULTROSPEC 3000 (PHARMACIA). The nucleic acids absorbance spectra were obtained for wavelengths ranging from 200 nm to 320 nm. The contamination of extracts was determined by reading the absorbance values for DNA ( $A_{260}$ ), proteins ( $A_{280}$ ) and polysaccharides ( $A_{300}$ ). The contamination was considered minimal for values of the  $A_{260} / A_{280}$  ratio ranging between 1.8 – 2.0, and for the lowest values of  $A_{300}$ .

#### **Determination of guanine plus cytosine molar percentage (% mol GC)**

For the determination of % mol GC we used the thermal denaturation technique [7; 14; 19]. Yeast chromosomal DNA was isolated as described previously, re-suspended and then diluted to a value of  $A_{260}$  of approximately 0.5 in 0.1X SSC (prepared from 1X SSC stock solution – 0.15M NaCl, 0.015M trisodic citrate, pH 7.0), The DNA samples were then denatured by heating from  $20^{\circ}\text{C}$  to  $100^{\circ}\text{C}$ , using a spectrophotometer ULTROSPEC 3000 (Pharmacia), equipped with a heating Peltier unit. The hyperchromic shift was automatically recorded by reading the  $A_{260}$  values for each heating step of  $2^{\circ}\text{C}$ . The % mol GC was calculated using the melting point ( $T_m$ ) value from the hyperchromic shift, using two formula: (a) Owen [16]: % mol GC =  $2.08 \times T_m - 106.4$  and (b) Frank [10]:  $2.44 \times (T_m - 53.9)$ .

#### **Killer phenotype assay**

The killer test for strain *D3* was performed by patching colonies of fresh grown culture of *D3* and standard killer strains *S. cerevisiae* 10701 C and *S. cerevisiae* SMR 4, onto K medium plates (0.1M phosphate citrate buffer pH 4.8, 2% glucose, 1% yeast extract, 2% agar, 0.03% methylen blue) with an overlay of the sensitive yeast strain *S. cerevisiae* 17/17.

## **Results and Discussion**

#### **Optimization of the chromosomal DNA isolation parameters**

The parameters for chromosomal DNA isolation from the natural yeast strains were optimized with regard to: (a) the strain-specific conditions for cell wall lysis by using various concentrations of zymolyase and incubation periods, and (b) deproteinization of cellular lysate. After several tests, the conditions for protoplasts obtaining were established as follows: for strains *D3* and *D6* –  $0.4 \text{ mg mL}^{-1}$  zymolyase and 90 min incubation at  $37^{\circ}\text{C}$ ; for strains *D1*, *D2* and *D4* –  $0.8 \text{ mg mL}^{-1}$  zymolyase and 30 min alternative periods of incubation (heat shock) at  $37^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and, again,  $37^{\circ}\text{C}$ . Deproteinisation was performed using a high concentration of proteinase K ( $200 \mu\text{g mL}^{-1}$ ), and successive treatments with chemical solvents.

### Spectrophotometric analysis

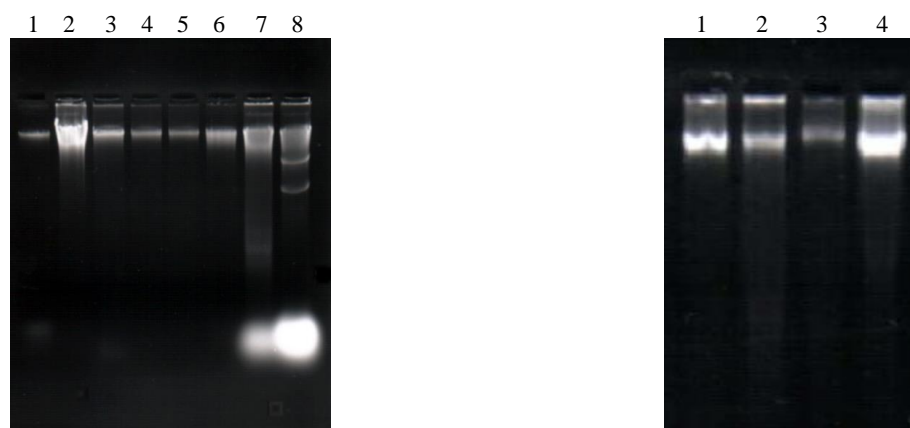
The spectrophotometric analysis of the integrity and purity of the DNA samples showed no contamination with proteins and polysaccharides. The calculation of the DNA concentration revealed differences between the values of the strains belonging to the same genera (*D1*, *D2* and *D4*, respectively, *D3* and *D6*).

Strain	DNA concentration [ $\mu\text{g } \mu\text{L}^{-1}$ ]
<i>D1</i>	6.475
<i>D2</i>	3.480
<i>D3</i>	4.240
<i>D4</i>	12.095
<i>D6</i>	8.140

Knowing that for all the strains we used the same protocol, we believe that these results are due to possible modifications in the structure of the cell wall, which are normally described for yeasts able to uptake hydrocarbons from media, and which could impair the efficiency of the lysing enzymes.

### Electrophoretic analysis

Based on the electrophoretic patterns (Figure 1a and Figure 1b) we concluded that for all the strains, the chromosomal DNA did not undergo any fragmentation during the isolation and purification procedures, since we obtained compact clear spots. Also, no RNA contamination was observed. An interesting case was observed for strain *D3*, for which the gel electrophoresis of the DNA extract obtained using a protocol that did not include RNase treatment, revealed the presence of 2 spots corresponding to RNA plasmids (Figure 1a – lane 8).



**Figure 1a** (lanes: 1 – *S. cerevisiae* 288 C; 2 – *C. boidinii*; 3 – *D6*; 4, 5 – *Y. lipolytica*; 6 – *D3* DNA extract with RNase A treatment, loaded in gel with RNase A; 7 – *D3* DNA extract without RNase A treatment, loaded in gel with RNase A; 8 – *D3* DNA extract without RNase A treatment, loaded in gel without RNase A)

**Figure 1b** (lanes: 1 – *Rh. glutinis*, 2 – *D1*, 3 – *D2*, 4 – *D4*)

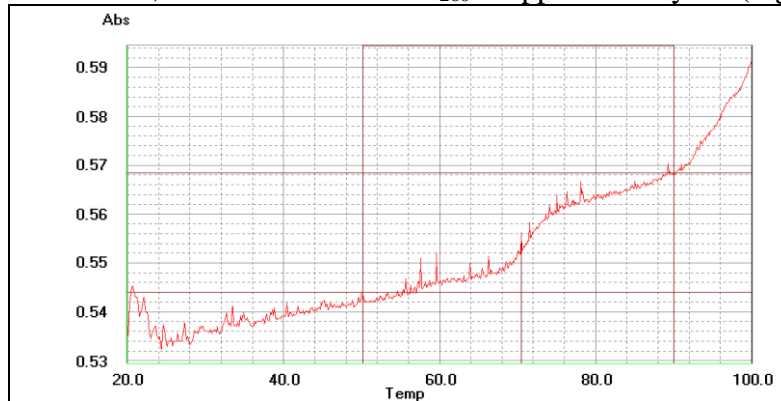
**Figure 1.** Agarose gel electrophoresis of yeast chromosomal DNA

### **Determination of % mol GC**

In order to obtain reliable data on the base composition of the new isolated strains, we had to set up first the technical parameters, using standard laboratory strains of *S. cerevisiae* ATCC 26108 and *C. boidinii* ICCF. We performed multiple tests varying: (i) the temperature

increment ( $1^{\circ}\text{C min}^{-1}$  and  $2^{\circ}\text{C min}^{-1}$ ), (ii) the range of temperature for which the  $T_m$  was calculated ( $20^{\circ}\text{C}$  to  $100^{\circ}\text{C}$ , and  $50^{\circ}\text{C}$  to  $90^{\circ}\text{C}$ ), (iii) the initial absorbance value ( $A_{260}$ ) of the DNA samples (from approximately 0.2 to 0.7). The hyperchromic shifts were recorded automatically by the spectrophotometer, and the different  $T_m$  values were introduced in the two formulas [10; 16] for % mol GC calculation.

The comparative analysis of the results with the ones from taxonomical references, pointed out as optimal conditions: the increment of  $2^{\circ}\text{C min}^{-1}$ , the  $T_m$  obtained for the manually setted range of  $50^{\circ}\text{C}$  to  $90^{\circ}\text{C}$ , and the initial DNA  $A_{260}$  of approximately 0.5 (Figure 2).



**Figure 2.** Hyperchromic shift for *S. cerevisiae* ATCC 26108  
(Temp = temperature; Abs = absorbance)

The % mol GC thus calculated fitted within the values described in literature [3; 12] as being characteristic for the two species (Table 1).

**Table 1.**  $T_m$  and % mol GC values for standard laboratory yeast strains

Strain	$T_m$	% mol GC (Owen's formula)	% mol GC (Frank's formula)	% mol GC Barnett [3]
<i>S. cerevisiae</i> ATCC 26108	70.4	40.03	40.26	38.3 - 42.0
<i>C. boidinii</i> ICCF	67.4	33.72	32.9	32.4 - 33.0

The same technique was then used for the determination of guanine + cytosine content of our five yeast strains (Table 2).

**Table 2.** Guanine+cytosine molar percentage (% mol GC) values for yeast strains *D1* – *D6*

Strain	$T_m$	% mol GC (Owen's formula)	% mol GC (Frank's formula)
<i>D1</i>	78.4	56.67	59.78
<i>D2</i>	80.5	61.04	64.9
<i>D3</i>	71.2	41.47	42.21
<i>D4</i>	79.3	58.54	61.98
<i>D6</i>	68.4	35.87	35.38

The values obtained by us matched those for *C. parapsilosis*, *Rh. glutinis* and *C. tropicalis* [3].

*C. parapsilosis* mol % GC: 39.3 - 41.2

*Rh. glutinis* mol % GC : 60.2 – 67.8

*C. tropicalis* mol % GC: 34.1 – 36.1

Anyhow, the small differences observed for *D1* and *D3*, were not surprising. The base composition is strain-specific, and the present study dealt with new isolated strains, fact which might involve some minor variation from the data already described and which might refer to other strains.

### 2 microns plasmid analysis

Only few yeast species or strains present 2 microns / 2 microns – like plasmid DNA [5]. For *Rh. glutinis* and *Candida* species this type of extrachromosomal DNA was not described until now. In our experiments *D1* and *D2* strains acted similarly to *Rh. glutinis*, and *D6* to *C. tropicalis*, i.e. did not show 2 microns plasmids, contrary to *D4* and *D3* for which DNA plasmids were isolated. (Figure 3). These observations, although surprising, could be explained by the natural variation of the 2 microns plasmid existence within the strains of the same species. Nevertheless, this suggested that the presence of plasmids should not be considered as singular criteria of yeast strains classification.

### The killer phenotype

As shown above, the electrophoretic analysis of the strain *D3*, revealed the presence of an RNA plasmid (Figure 1a – lane 8). Knowing that for some yeast species, the RNA plasmids are related to the killer phenotype, we wondered if this could also be our case. The tests performed didn't reveal any killer activity for strain *D3*. The result is normal for *C. parapsilosis* strains, which have no killer system. Nevertheless, further tests will be performed in order to elucidate the role of the RNA plasmids and the way in which this character, along with possible others, could possibly influence the taxonomical classification of strain *D3*.

## Conclusions

The aim of this study was an accurate identification and characterization of the yeast strains *D1*, *D2*, *D3*, *D4* and *D6*, isolated from oil-polluted environment. For this purpose we performed molecular level taxonomy analysis.

Previous preliminary morphology and biochemistry studies revealed a great similarity between *D1*, *D2*, *D4* and *Rhodotorula glutinis*, *D3* and *Candida parapsilosis*, *D6* and *Candida tropicalis*.

In a first step, we established the optimal conditions for chromosomal DNA isolation and purification. Thus, a double concentration of zymolyase ( $0.8 \text{ mg mL}^{-1}$ ) was used for cell wall lysis for strains *D1*, *D2* and *D4*, compared to *D3* and *D6*, and 90 minutes incubation period, with heat shock for *D1*, *D2* and *D4*. Spectrophotometric and electrophoretic analysis of the DNA extracts revealed their high purity and integrity.

For strain *D3*, spots corresponding to RNA plasmids were present, but were not related to a killer phenotype.

The values obtained for guanine+cytosine contents (% mol GC) were: for *D1* - 56.67 % mol GC ; for *D2* - 61.04 % mol GC; for *D3* - 41.47 % mol GC; for *D4* - 58.54 % mol GC; for *D6* - 35.87% mol GC. The values fitted within the ranges described for *Rh. glutinis*, *C. parapsilosis* and *C. tropicalis*.

Regarding the 2 microns / 2 microns – like plasmid DNA, *D1* and *D2* strains showed no 2 microns DNA, opposite to *D3* and *D4* strains for which plasmid DNA was observed.

The data obtained during the present study, together with the ones from conventional taxonomical tests and those on the electrophoretic karyotypes, allowed us to establish the strains *D1*, *D2* and *D4* as belonging to *Rhodotorula glutinis*, *D3* to *Candida parapsilosis* and *D6* to *Candida tropicalis*.

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