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Phylogenetic analysis and characterization of an alkane-degrading yeast strain isolated from oil-polluted soil

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Abstract: The isolation and phylogenetic analysis of new yeast strains represent an important basis for the development of practical applications in biotechnology, industry, and bioremediation. The yeast strain RG1 was isolated from oil-polluted soil and characterized using morphological observations and the API 20C AUX system. The amplified ITS1-5.8S rDNA-ITS2 region had 653 bp and the restriction profiles obtained with *Cfo* I, *Dde* I, *Hae* III, *Hinf* I, and *Hpa* II endonucleases showed high similarities with those from *Rhodotorula glutinis* reference strains. The phylogenetic tree based on the *Cfo* I patterns confirmed the classification of the strain RG1 as belonging to the *R. glutinis* species. The studies performed during the present work indicate the existence of different mechanisms of *n*-alkane assimilation and biodegradation in the RG1 cells and their correlation with the production of biosurfactants. An evaluation of the emulsifying activity revealed that biosurfactant-mediated assimilation of *n*-alkanes decreases in the order *n*-decane > *n*-tetradecane > *n*-hexadecane. Biodegradation and emulsification assays using culture media supplemented with 1% *n*-alkanes as the sole carbon source showed that *n*-tetradecane represented the optimal hydrocarbon substrate for the growth of the strain RG1 over 14 days and for obtaining biosurfactants at stable rates.

Key words: *Rhodotorula glutinis*, 5.8S-ITS, phylogenetic analysis, *n*-alkanes, biodegradation, biosurfactants

1. Introduction

The yeast strains belonging to the *Candida*, *Pichia*, *Rhodotorula*, and *Yarrowia* genera are successfully used in biotechnology domains related to the food and chemical industries, therapeutics, and bioremediation. The process of bioremediation is based on the ability of microorganisms to assimilate and biodegrade petroleum or crude oil by using the hydrocarbon compounds as main carbon sources in specific metabolic pathways (Vidali, 2001). The final products of this metabolism are sometimes represented by biosurfactants, surface-active molecules produced on the surface of cells whose diverse structure allows them to reduce surface and interfacial tension and to mediate the assimilation of hydrophobic substrates, including hydrocarbons, at the surface of cells (Muthusamy et al., 2008; Kaya et al., 2014). Therefore, the development of knowledge concerning the structure and production of biosurfactants is important for the improvement of bioremediation technologies in oil recovery, agriculture, cosmetics, therapeutics, and microbial lipase production (Ron and Rosenberg, 2001; Gautam and Tyagi, 2006; Mukherjee et al., 2006; Banat et al., 2010; Açikel et al., 2011; Sachdev and Cameotra, 2013).

Yeast strains capable of assimilating hydrocarbons have been isolated from microbial communities in polluted soil or water and were generally described as biodegrading mostly *n*-alkanes with long carbon chains C_{10}–C_{24} (Mauersberger, 1996). Biosurfactants produced by strains, particularly *Candida* and *Yarrowia*, growing on hydrocarbon substrates were studied and were found to be sophorolipids, mannosylerythritol lipids, carbohydrate-protein-lipid complexes, carbohydrate-protein complexes, or fatty acids (Van Bogaert et al., 2007; Amaral et al., 2008; Campos-Takaki et al., 2010). The *Rhodotorula* species have hydrocarbon-degrading abilities and produce sophorolipids, polyol lipids, and carbohydrate-protein complex types of biosurfactants (Mulligan, 2004; Nunez et al., 2004; Nerurkar et al., 2009; Chandran and Das, 2011). *R. glutinis* is one of the most representative species of the genus due mainly to the wide incidence of its strains in soil, seawater, atmosphere, fruits, and the human body (Barnett et al., 1984; Kurtzmann et al., 2011) and to its practical applications in obtaining lipids and biodiesel, in biocontrol, and in bioremediation (Van Hamme et al., 2003; Dai et al., 2007; Rizzo et al., 2008; Easterling et al., 2009; Sharma et al., 2009).

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The development of new strategies for biotechnology implies the taxonomical identification of yeast species or strains isolated from various environments comprising their morphological, phenotypic, and genetic characterization. The restriction analysis of the amplified ITS1-5.8S-ITS2 (5.8S-ITS) region of rDNA is one of the molecular techniques extensively used in yeast classification, allowing the interspecific differentiation of many species belonging to the *Candida*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Zygosaccharomyces*, and *Yarrowia* genera (Esteve-Zarzoso et al., 1999; Cirak et al., 2003; Clemente-Jimenez et al., 2004; Villa-Carvajal et al., 2006; Suárez Valles et al., 2007; Akpınar and Uçar, 2013).

The present article first deals with the phylogenetic identification, using morphophysiological tests and PCR-RFLP analysis of the 5.8S-ITS region, of a yeast strain named RG1 isolated from oil-polluted soil in Romania. Preliminary studies showed that the strain RG1 belongs to *Rhodotorula glutinis* and has the ability to grow on *n*-alkanes over a short period of time, producing biosurfactants (Csutak, 2002; Csutak et al., 2012b). Therefore, a second goal of our present work was to understand the assimilation mechanisms of different *n*-alkanes in the RG1 cells, the biodegradation of *n*-alkanes during 14 days of incubation, and their involvement in the production of biosurfactants.

2. Material and methods

2.1. Yeast strains

The yeast strain RG1 was maintained in a freezer (–70 °C) in Yeast Peptone Glucose (YPG) medium (g/L: yeast extract 5.0, peptone 10.0, glucose 2.0) supplemented with 20% glycerol. The reference yeast strains used during this study were *Candida boidinii* ICCF (National Institute for Chemical and Pharmaceutical Research and Development, Bucharest, Romania), *Candida* (*Pichia*) *guilliermondii* CMGB44 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania), *Candida tropicalis* CMGB165, *Candida krusei* CMGB94, *Hansenula polymorpha* NCYC495, *Kluyveromyces lactis* CBS2359/152, *Pichia pastoris* ICCF, *Rhodotorula glutinis* ICCF, *Rhodotorula glutinis* CMGB, *Saccharomyces cerevisiae* S288C, and *Yarrowia lipolytica* ICCF.

2.2. Morphophysiological identification

For the morphological characterization, yeasts from a fresh RG1 culture were cultivated in petri dishes on YPGA medium (YPG medium supplemented with 2% agar) and the appearance and colony type was observed using a stereomicroscope SZM-1 (OPTIKA Microscopes, Italy). Yeasts from the same fresh culture were inoculated in flasks with YPG medium and the shape of cells and type of vegetative reproduction were examined with an optical microscope (MICROS, Austria).

Physiological identification was performed using the API 20C AUX system (bioMérieux, France) according to the manufacturer's specifications.

2.3. PCR-RFLP analysis of the ITS1-5.8S-ITS2 region and phylogenetic tree construction

The genomic DNA of the yeast strains was isolated and then amplified using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, and the PCR products were separated by agarose gel electrophoresis according to Csutak et al. (2012a). The 5.8S-ITS amplicons from the strains RG1 and *R. glutinis* CMGB were digested with 5 restriction endonucleases: *Cfo* I (5'-GCG/C-3'), *Dde* I (5'-C/TNAG-3'), *Hae* III (5'-GG/CC-3'), *Hinf* I (5'-G/ANTC-3'), and *Hpa* II (5'-C/CGG-3') (10 U/µL, Promega, USA). For the rest of the yeast strains the amplicons were digested only with *Cfo* I. The size of amplicons and restriction fragments was evaluated using the program Quantity One (Bio-Rad, USA).

The data obtained from the *Cfo* I restriction profiles were used for the construction of a phylogenetic tree based on the unweighted pair group method with arithmetic mean method (UPGMA) from the Quantity One program.

2.4. Assessment of *n*-alkane biodegradation

The strain RG1 was grown overnight at 28 °C and 120 rpm on YPG. Five milliliters of culture was centrifuged for 6 min at 6000 rpm and the inoculum in a final concentration of 1% was cultivated in Bushnell-Haas mineral medium (g/L: KH_2PO_4 1.0, K_2HPO_4 1.0, NH_4NO_3 1.0, $MgSO_4 \times 7H_2O$ 0.2, $FeCl_3$ 0.05, $CaCl_2 \times 2H_2O$ 0.02). The carbon source was represented by each of the following *n*-alkanes in a final concentration of 1%: *n*-decane, *n*-tetradecane, or *n*-hexadecane (Sigma, USA). Yeast cultures were incubated for 14 days at 28 °C and 120 rpm. Samples were collected at time 0 and after 3, 6, 10, and 14 days. The biodegrading ability of the strain RG1 was evaluated by monitoring the pH and the cell counts on the Thoma counting chamber (de Cássia Miranda et al., 2007; Pirôllo et al., 2008).

2.5. Determination of emulsification activity

The emulsification activity was determined by calculating the emulsification index (E_{24}). For this purpose 3 mL of cell-free broth was mixed with 2 mL of hydrocarbon substrate and vortexed for 2 min at 2200 rpm. The samples were maintained at room temperature for 24 h and the E_{24} was calculated according to Cooper and Goldenberg (1987).

2.6. Biosurfactant assays

Two types of experiments were performed. First, in order to determine the involvement of the biosurfactants in *n*-alkane assimilation in yeast cells, the strain RG1 was grown for 48 h on YPG medium and the emulsification activity of the cell-free broth containing biosurfactants was determined against each of the 3 *n*-alkanes.

The production of biosurfactants in the presence of *n*-alkanes was evaluated by growing the strain RG1 on Yeast Peptone (YP) medium (g/L: yeast extract 10.0, peptone 10.0) supplemented with 1% *n*-decane, *n*-tetradecane, or *n*-hexadecane. The emulsification index of cell-free broth was determined after 48 and 72 h in the presence of petroleum (FLUKA, Germany).

2.7. Alkane assimilation on the surface of yeast cells

The strain RG1 was grown on YP medium with 1% *n*-hexadecane. After 5 h of incubation, samples were collected and the presence and aspect of the alkane droplets and yeast cells were observed with an optic microscope (Fickers et al., 2005).

3. Results

3.1. Morphophysiological and molecular identification

The study of the morphological aspects of strain RG1 revealed salmon-pink, smooth, glossy colonies, while microscopical observations showed small- and medium-sized round cells with budding and no filaments, characteristic of the *Rhodotorula glutinis* species (Figure 1) (Barnett et al., 1984; Kurtzmann et al., 2011).

Tests using the API 20C AUX system showed that the strain RG1 belongs to *Rhodotorula glutinis*, with a 94.7% match.

The genomic DNA purified from the strain RG1 and reference yeast strains was amplified using the ITS1 and ITS4 primers. The amplicons of the ITS1-5.8S rDNA-ITS2 region from the strains RG1 and *R. glutinis* CMGB had 653 bp and 645 bp, respectively, and were further digested with 5 endonucleases: *Cfo* I, *Dde* I, *Hae* III, *Hinf* I, and *Hpa* II. After the electrophoresis we obtained 3 restriction fragments for the 5.8S-ITS amplicons cut with *Cfo* I, *Dde* I, and *Hinf* I, ranging from 75 to 416 bp in size, and 2 fragments for *Hae* II and *Hpa* II, between 90 and 550 bp in size. The strains RG1 and *R. glutinis* CMGB showed highly similar restriction patterns. The results were also compared to those described for other *R. glutinis* strains (Table).

Since the best degree of similarity between various *R. glutinis* strains was observed by comparing the *Cfo* I restriction profiles, we decided to use them in the phylogenetic analysis of the strain RG1. The *Cfo* I digestion reactions of the 5.8S-ITS amplicons from the strain RG1 and all the reference strains were run in a single agarose gel (Figure 2).

The digitized image was used to construct a dendrogram based on the UPGMA method within the Quantity One computer program (Bio-Rad) (Figure 3). From this image we could identify a separate branch comprising the 3 *Rhodotorula* strains. The strains RG1 and *R. glutinis* CMGB presented about 95% similarity to each other and 87% similarity to *R. glutinis* ICCF.

3.2. Biodegradation of *n*-alkanes

The ability of the strain RG1 to biodegrade *n*-alkanes was evaluated by monitoring the cell growth and pH variation of a culture from the Bushnell-Haas medium supplemented with *n*-alkanes. The strain RG1 showed a constant growth during the first 6 days of the experiment (Figures 4a–4c). The growth was the most significant in the presence of *n*-tetradecane during the first 3 days of incubation and it continued at a slower rate until the end of the study (Figure 4b). The growth rates recorded after 6 days were 1.06×10^6 cells/mL in the presence of *n*-decane and 1.44×10^6 cells/mL on *n*-hexadecane. After this point a slow descending curve could be observed in the case of *n*-decane-grown

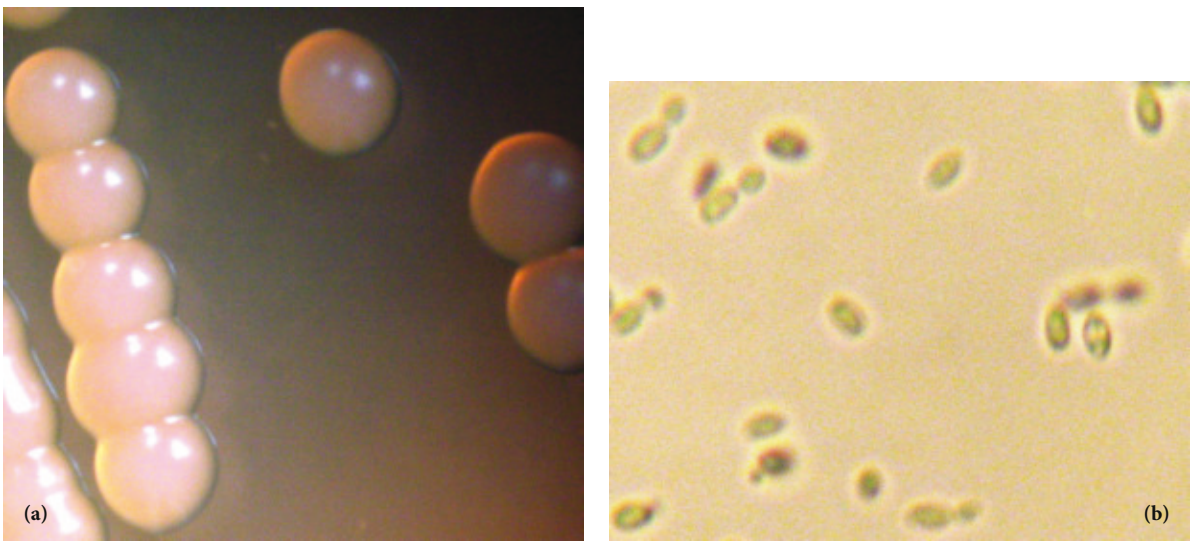

Figure 1. Colonies (a) and cells (b) of the strain RG1.

Table. Amplicon and restriction fragment size from ITS1-5.8S-ITS2 PCR analyses of the RG1 and *R. glutinis* CMGB strains.

Strain	Amplicon (bp)	Restriction fragments (bp)					Reference
		Cfo I	*Dde* I	*Hae* III	*Hinf* I	*Hpa* I	
RG1	653	310, 234, 96	394, 136, 95	416, 218	370, 225, 75	525, 107	ts*
R. glutinis CMGB	645	308, 233, 100	400, 136, 97	416, 218	380, 225, 73	520, 107	ts*
R. glutinis ECT1137	640	320, 240	-	430, 210	170, 150	-	Guillamon et al., 1998
R. glutinis ECT1137	640	320, 240, 80	-	430, 210	340, 225, 75	-	Esteve-Zarzoso et al., 1999
R. glutinis var. *glutinis*	-	300, 210, 100	-	600	210, 120, 110	600	Cadez et al., 2010
R. glutinis ICCF	650	300, 230, 80	380, 130, 80	400, 220	340, 220, 80	550, 90	Csutak et al., 2012a

*ts - this study.

cultures followed by a more significant reduction in cell numbers until day 14 (Figure 4a). When *n*-hexadecane was used, the cell number dropped by almost half from day 6 to day 10, followed by a relative plateau (Figure 4c).

The pH values ranged from 7.5 at the beginning of the experiment to 6.7 after 10 days of incubation. A slower decrease of pH was observed in the presence of *n*-tetradecane (Figure 4b) while for *n*-decane the medium reached a pH of 6.7 rapidly, after the first 6 days (Figure 4a).

3.3. Assimilation of *n*-alkanes in the yeast cells

We performed 2 sets of experiments in order to investigate the presence of specific mechanisms of assimilation of each *n*-alkane in the RG1 cells and to establish the involvement of *n*-alkanes in the production of biosurfactants.

First, the emulsification of the *n*-alkanes was determined using the cell-free broth containing biosurfactants from a 48-h RG1 culture on YPG medium. The best emulsification was achieved against *n*-decane (50%), followed by *n*-tetradecane (44%) and *n*-hexadecane (28%).

For the second set of experiments we used the YP medium with 1% *n*-alkanes and the biosurfactant production was evaluated by calculating the emulsification index (E_{24}) against petroleum. The best result (38%) was obtained for cell-free broth from *n*-tetradecane-grown cultures after 48 h of incubation. The biosurfactant production was stable and the 72-h samples showed approximately the same E_{24} value (37%) (Figure 5). A similar situation was observed when *n*-hexadecane was used. However, for *n*-decane after 72 h the emulsifying activity decreased from 31% to 24%. Comparable E_{24} values were obtained during the experiments performed using media supplemented with *n*-tetradecane and *n*-hexadecane, suggesting almost equal biosurfactant production rates. This affirmation was verified by analyzing the microscopical aspects of the interactions between the alkane droplets formed by emulsification due to biosurfactants and the yeast cells. Previous work comprised data concerning the aspect of *n*-tetradecane droplets in the presence of RG1 cells (Csutak et al., 2012b) and therefore we decided to further investigate the case of *n*-hexadecane-grown cultures. The active production of biosurfactants could be observed in the 5-h samples showing numerous small hydrocarbon droplets and scattered cells (Figure 6).

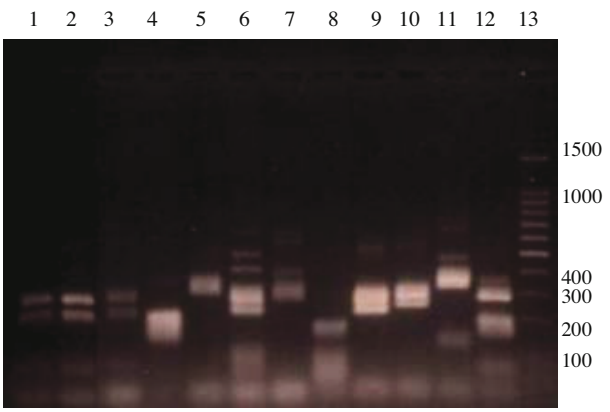

Figure 2. PCR-RFLP of the ITS1-5.8S-ITS2 region using *Cfo* I endonuclease (lanes: 1, RG1; 2, *R. glutinis* CMGB; 3, *R. glutinis* ICCF; 4, *Y. lipolytica* ICCF; 5, *P. pastoris* ICCF; 6, *H. polymorpha* NCYC495; 7, *C. boidinii* ICCF; 8, *C. krusei* CMGB94; 9, *C. tropicalis* CMGB165; 10, *C.* (*P.*) *guilliermondii* CMGB44; 11, *S. cerevisiae* S288C; 12, *K. lactis* CBS2359/152; 13, BenchTop 100-bp DNA Ladder (Promega).

4. Discussion

The strain RG1 has been preliminarily characterized as *R. glutinis* (Csutak, 2002) using methods and identification keys described by Barnett et al. (1983).

The results from the API 20C AUX tests performed during the study confirmed the previous identification of the strain RG1. Moreover, correlating the data with those described by Kurtzmann et al. (2011), the strain RG1 was physiologically identified as belonging to *R. glutinis* within the *R. glutinis* sensu stricto group.

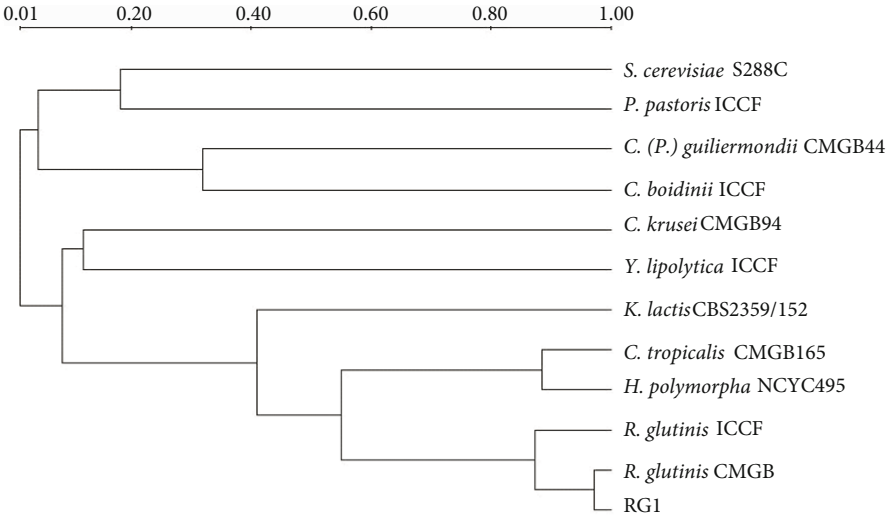

Figure 3. Phylogenetic analysis of the strain RG1 obtained by quantification of the *Cfo* I restriction profiles of the ITS1-5.8S-ITS2 regions.

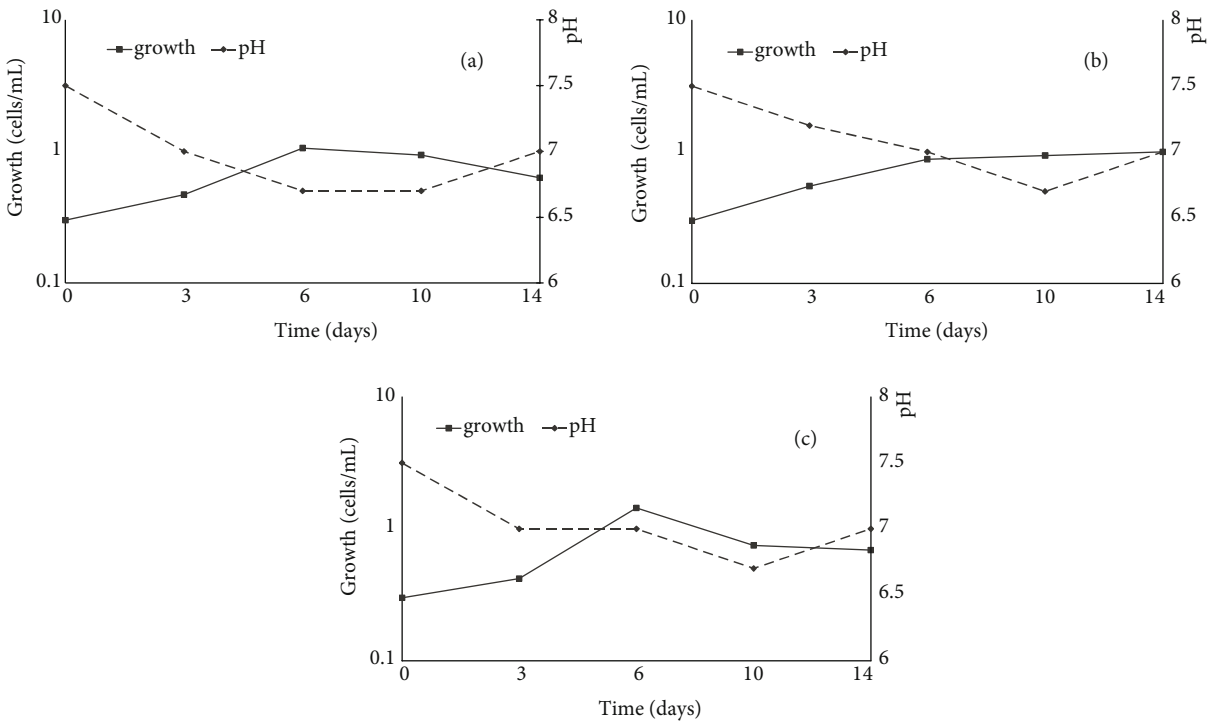

Figure 4. Growth curves and pH variation for the strain RG1 during 14 days of biodegradation of (a) *n*-decane, (b) *n*-tetradecane, and (c) *n*-hexadecane.

Molecular techniques are critical for taxonomical classification of newly isolated yeast strains. The analysis of the amplicons of the 5.8S rRNA gene and its neighboring internal transcribed spacers ITS1 and ITS2 is an important criterion used in yeast taxonomy, the patterns of the restriction bands being species-specific. Therefore, we used the PCR-RFLP technique on the ITS1-5.8S rDNA-ITS2 region for a more accurate identification of the strain RG1. The size of the amplicons and the restriction profiles obtained from the strain RG1 were highly similar to those described in the case of *R. glutinis* ICCF and *R. glutinis* CMGB for *Cfo* I, *Dde* I, *Hae* III, *Hinf* I, and *Hpa* II endonucleases; *R. glutinis* CECT1137 for *Cfo* I, *Hae* III, and *Hinf* I; and *R. glutinis* var. *glutinis* for *Cfo* I. The variations

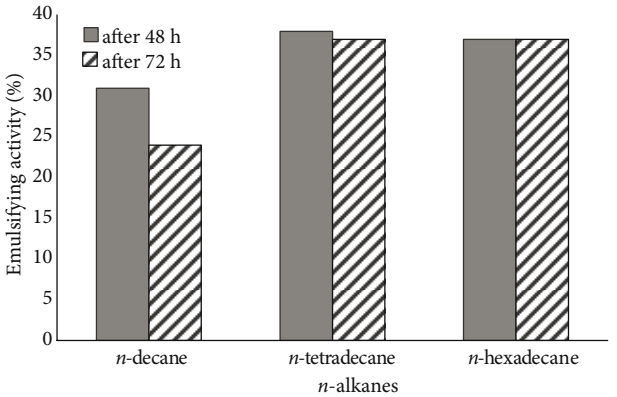

Figure 5. Emulsifying activity of cell-free broth of the strain RG1 grown in YP medium with 1% *n*-alkanes.

of the patterns are a consequence of the intraspecific variability due to the large range of strains used during the experiments in different laboratories. It is important to emphasize that, to our knowledge, these are the first reported data regarding the *Dde* I restriction profile of the 5.8S-ITS amplicon from a *R. glutinis* strain.

The *Cfo* I profiles were used to analyze the phylogenetic position of the strain RG1 in correlation with reference yeast strains belonging to *R. glutinis* and to other species. The UPGMA has been successfully used for the construction of dendrograms based on the ARDRA analysis, providing valuable results regarding the characterization and phylogenetic analysis of microbial strains and species isolated from natural environments (Freitas da Silva et al., 2011; Barbieri et al., 2013). Therefore, we decided to use the UPGMA from the Quantity One program for our phylogenetic study of the RG1 strain. The Quantity One program allows the construction of phylogenetic trees based on band matching, i.e. using as reference a band most commonly found within all the lanes in the analyzed image and setting up a minimum spacing (tolerance percentage) for creating a matching model between unique bands. In our case, the reference band corresponded to the *Cfo* I restriction fragment of approximately 230 bp. This explains the branches from Figure 3. The first branch is formed by the strains presenting a restriction fragment of 230–240 bp comprising the strains RG1, *R. glutinis* CMGB, and *R. glutinis* ICCF. The strains most closely related are *H. polymorpha* NCYC495 (Figure 2, lane 6) with a *Cfo* I restriction fragment of about 245 bp, *C. tropicalis* CMGB165 (Figure 2, lane 9) with a 250 bp fragment, and *K. lactis* CBS2359/152 (Figure 2, lane 12) showing a 260 bp fragment. Strains with restriction fragments of rather different sizes and therefore presenting a lower similarity are *C. krusei* CMGB94 and *Y. lipolytica* ICCF (forming a separate branch), *C. boidinii* ICCF, *C.* (*P.*) *guilliermondii* CMGB44, *P. pastoris* ICCF, and *S. cerevisiae* S288C.

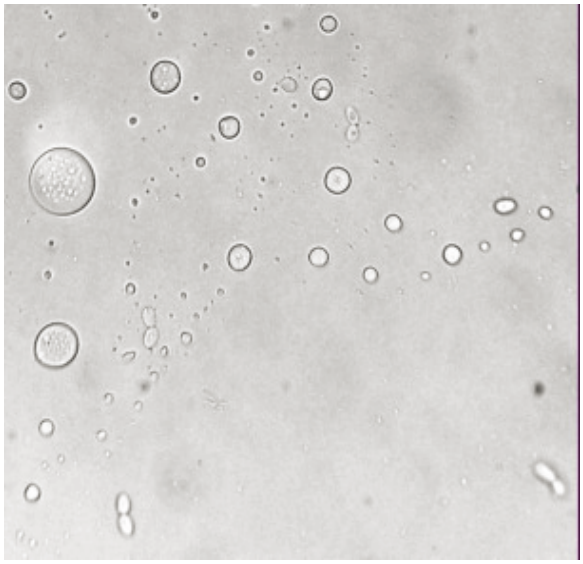

Figure 6. RG1 cells and *n*-hexadecane droplets after 5 h of incubation (40×).

The molecular characterization and phylogenetic analysis validate the morphophysiological identification of the strain RG1 as belonging to the *R. glutinis* species.

Compared to bacteria, there are not many studies concerning the assimilation and biodegradation of hydrocarbons in *Rhodotorula* cells. However, *Rhodotorula* strains were isolated from oil- and petroleum-polluted environments and as contaminants in stored products (Gaylarde et al., 1999; Yemashova et al., 2007) or have been described as being able to degrade oil compounds, with a preference for the saturated fractions represented by the aliphatic hydrocarbons and higher alkanes (Shailubhai et al., 1984; White et al., 1987; van Beilen et al., 2003; Omotayo et al., 2011).

The alkane metabolism involves complex processes, from uptake and transport in the yeast cells to the degradation pathways and production of biosurfactants. Since our previous work revealed the ability of the strain RG1 to grow on *n*-alkanes during the first 72 h of incubation, we considered that it is important to focus on the specificity of *n*-alkane degradation over a longer period of time. The biodegradation assays comprised monitorization of the RG1 culture in terms of cell growth and pH variation. Our studies revealed clear differences between the 3 growth profiles of the strain RG1 recorded during 14 days on Bushnell-Hass media supplemented with 1% *n*-alkanes. The continuous cell growth in the presence of *n*-tetradecane indicates that the RG1 culture adapts well to the presence of *n*-tetradecane and therefore its consumption from the medium involves metabolic processes that extend to a larger period of time when compared to *n*-decane and *n*-hexadecane. On the contrary,

it seems that the *n*-decane is actively consumed during the first 6 days of incubation, after which either it becomes less accessible for the yeast cells or the metabolic by-products released into the environment inhibit further cell growth. The most impressive case was that of *n*-hexadecane-grown cultures. After the maximum recorded in 6 days the cell number dropped by half, suggesting that *n*-hexadecane is the most rapidly metabolized alkane. However, the maintenance of a rather constant cell density from day 10 (0.75×10^6 cells/mL) to day 14 (0.69×10^6 cells/mL) could indicate a possible intermediate period or that the yeast culture is adapting to the new nutritional resources from the environment.

Variations of the growth rates as adaptive responses to different hydrocarbons used as substrates were also described by de Cássia Miranda et al. (2007) in the case of a *Rhodotorula aurantiaca* strain isolated from a petroleum-polluted environment and by Pirôllo et al. (2008) for a *Pseudomonas aeruginosa* strain isolated from hydrocarbon-contaminated soil. The strain RG1 presented a good rate of biodegradation of the *n*-tetradecane, showing an augmentation of 54% for the growth rate on Bushnell-Haas medium with 1% *n*-tetradecane, from 0.3×10^6 cells/mL to 0.55×10^6 cells/mL within 3 days. The *P. aeruginosa* LBI strain cultivated on Bushnell-Haas medium supplemented with 1% kerosene or diesel oil (Pirôllo et al., 2008) recorded almost the same growth rate, from 0.5×10^7 cells/mL to approximately 10^8 cells/mL, within 24 h. However, this is not surprising since bacteria generally have higher hydrocarbon-biodegrading abilities than yeasts. Moreover, these data reflect the ability of *P. aeruginosa* LBI to break down complex substrates (kerosene, diesel oil) using coordinated metabolic mechanisms involved in the degradation of each component of the substrate. In this case, we do not know if *P. aeruginosa* LBI would maintain a higher degradation rate than the strain RG1 in the presence of a single hydrocarbon source, for example one of the *n*-alkanes used during our research. Additionally, as a comparative study, it would be interesting to determine the degradation of kerosene or diesel oil by the strain RG1.

It seems that low pH values are more likely due to an intense metabolism of hydrocarbon substrate and the release of fatty acids in the environment (Oboh et al., 2006; Pirôllo et al., 2008) while higher pH values are related to biosurfactant production and emulsification activity (de Luna et al., 2009). The results can be correlated to the cell growth profile recorded on *n*-decane medium. Furthermore, the slight variation of pH during the first 6 days of RG1 incubation on *n*-tetradecane could suggest a more active biosurfactant production. The case of *n*-hexadecane is more complex. Although the biodegrading activity of the RG1 culture is obvious, the pH is maintained at 7.0 for 6 days. An explanation resides in the fact that *n*-hexadecane might also represent an important substrate for an active biosurfactant production, which determines a balanced pH value.

A comparative analysis of the pH variations recorded for the strain RG1 with those observed for *R. aurantiaca* and *Candida ernobii* strains from petroleum-polluted environments (de Cássia Miranda et al., 2007) reveals that the degradation of hydrocarbons in the strain RG1 is a slower process, more active from days 6 to 10 of the experiment. At this point, the values of the pH (6.7–6.8) and, by consequence, the biodegrading abilities are similar for the *Rhodotorula* strains (RG1 and *R. aurantiaca*). In contrast, the *C. ernobii* strain shows a different degrading profile, more intense during the first 3–6 days, with a pH of 6.8, followed by a period with reduced activity. On the other hand, the higher pH values obtained for the strain RG1 during the first 6 days might indicate increased production and involvement of biosurfactants in hydrocarbon assimilation as compared to the *R. aurantiaca* and *C. ernobii* strains.

It is interesting to notice that pH variation profiles resembling those obtained for the strain RG1 were described by Oboh et al. (2006) for *Pseudomonas stutzeri*, *Pseudomonas mullei*, and *Alcaligenes* sp. strains isolated from Nigerian bitumen deposits and grown on 1% kerosene, diesel, or naphthalene. The strain RG1 might be thus considered as having similar metabolic abilities and, possibly, biosurfactant synthesis rates as these bacterial strains. This is a very important observation, indicating a high potential for the application of the strain RG1 in the bioremediation technologies.

The alkanes can be assimilated in the yeast cells by direct adhesion and transport using channels within the cell wall by biosurfactant-mediated pseudosolubilization, emulsification, or both mechanisms (Tanaka and Fukui, 1989; Tanaka and Ueda, 1993; Kim et al., 2000). The biosurfactants augment the hydrophobicity of the cell surface, ensuring a better adhesion of the cells to the hydrocarbon substrate and therefore influencing its assimilation rate in the cells. Assessment of *n*-alkane emulsification by biosurfactants contained in the cell-free broth of the RG1 culture grown on YPG shows that the importance of biosurfactant-mediated assimilation of *n*-alkanes in the RG1 cells decreases in the order of decane > tetradecane > hexadecane.

During our experiments for the evaluation of biosurfactant production by the RG1 cultures from the YP media with 1% *n*-alkanes, we observed that the *n*-decane seemed to perform poorly in inducing the production of biosurfactants. On the contrary, the *n*-hexadecane represents a stimulating factor for obtaining biosurfactants easily released in the environment, a fact also described for other yeast species (Cavalero and Cooper, 2003; Amaral et

al., 2010). This explains the appearance of numerous small *n*-hexadecane droplets observed by optic microscope in the samples collected after 5 h of incubation. It also sustains the idea of *n*-hexadecane consumption from the medium by emulsification corroborated with an important cell growth.

The emulsifying activity of the cell-free broth from the RG1 cultures on *n*-tetradecane was relatively constant for 72 h. The E_{24} was similar to the one obtained on *n*-hexadecane and higher than the E_{24} from *n*-decane. The results are consistent with the pH variations from the biodegradation assays and confirm the connection between elevated pH values and the presence of biosurfactants. The large hydrocarbon droplets observed after 5 h in the samples from *n*-tetradecane-grown cultures (Csutak et al., 2012b) suggest that the biosurfactants are probably retained for a longer period of time on the surface of cells, facilitating the adhesion and transport of hydrocarbon compounds, rather than being immediately released in the environment and forming small droplets like those observed for *n*-hexadecane (Cardoso Fontes et al., 2010).

The ability of the strain RG1 to degrade *n*-alkanes producing biosurfactants might represent, in our opinion, an interesting finding because there are few studies detailing methods for obtaining biosurfactants from *R. glutinis* strains and those that do exist mention different carbon sources, such as glucose, starch, or soybean oil (Johnson et al., 1992; Oloke and Glick, 2005).

Biosurfactant-mediated assimilation of *n*-alkanes plays an important role during the first steps of alkane biodegradation in the RG1 cells. However, since *n*-decane does not determine high rates of biosurfactant production despite its rather rapid degradation from the culture medium, we suppose that in the case of the RG1 strain the alkanes are incorporated in the cells both by emulsification and by passive transport through cell wall channels. Moreover, each mechanism seems to increase its activity depending on the carbon sources available in the environment (glucose or *n*-alkanes).

In conclusion, the phylogenetic analysis of the yeast strain RG1 isolated from oil-polluted soil allowed its identification as belonging to *R. glutinis* species. In the present article we describe the existence of specific mechanisms for the assimilation and biodegradation of different *n*-alkanes in the RG1 cells and their correlation with the production of biosurfactants. The emulsification assays showed that the biosurfactant-mediated assimilation represents the preferred mechanism for an active uptake of *n*-decane from culture media. Alkane solubilization contributes also to *n*-hexadecane and *n*-tetradecane assimilation in the cells, although to a lower extent. Biosurfactant production and release is highly induced by *n*-hexadecane, but the decreasing cell number suggests that *n*-hexadecane might not represent the best substrate for maintaining the RG1 cultures over a long period of time. Finally, the strain RG1 proved to be well adapted to the presence of the *n*-tetradecane, which also represents the optimal hydrocarbon source for a stable production of biosurfactants at good rates. Therefore, we consider that the data acquired during the present work might constitute a starting point for using the strain RG1 in domains related to bioremediation or based on biosurfactant applications.

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