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ORIGINAL PAPER

Enrichment of a continuous culture of *Saccharomyces cerevisiae* with the yeast *Issatchenkia orientalis* in the production of ethanol at increasing temperatures

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Abstract A fermentation system was continuously fed with sugar-cane syrup and operated with recycling of *Saccharomyces cerevisiae* cells at temperatures varying from 30 to 47°C. The aim of the present work was to obtain and study the colonies of isolates showing elongated cells of yeasts which were sporadically observed at the end of this continuous process. Based on a sequence of assays involving methods of classical taxonomy and RAPD-PCR, two groups of isolates showing characteristics of non-*Saccharomyces* yeasts were identified in the yeast population where *S. cerevisiae* was the dominant yeast. The largest group of non-*Saccharomyces* yeasts, resulting from a slow proliferation over the 2 months, reached a final level of 29.6% at the end of the process. RAPD-PCR profiles obtained for the isolates of this dominant non-*Saccharomyces* yeast indicated that they were isolates of *Issatchenkia orientalis*. *Pichia membranifaciens* was the only species of non-*Saccharomyces* yeast detected together with *I. orientalis* but at a very low frequency. The optimum temperature for ethanol formation shown by the isolate 195B of *I. orientalis* was 42°C. This strain also showed a faster ethanol formation and biomass accumulation than the thermotolerant strain of *S. cerevisiae* used as the starter of this fermentation process. Some isolates of *I. orientalis* were also able to grow better at 40°C than at 30°C on plates containing glycerol as carbon source. Yeasts able to grow and produce ethanol at high temperatures can extend the fermentation process beyond the temperature limits tolerated by *S. cerevisiae*.

Keywords *Saccharomyces cerevisiae* · *Issatchenkia orientalis* · *Pichia membranifaciens* · Fermentation at high temperatures · Ethanol production

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Introduction

Changes in environmental stresses such as nutritional starvation, pH variation, osmotic and heat shocks, frequently occur in nature. Alterations in physiological functions are strategically adopted by yeast cell populations in order to slow growth and consequently to reduce cell death as was observed in a five-stage fermentation system operating at increasing temperatures [34]. Gradual increases in temperature of this fermentation system give rise to favorable conditions for the enrichment of a culture with organisms that are more tolerant to thermal stress. Yeast strains, tolerant to ethanol at temperatures above the limits usually tolerated by the cells of *Saccharomyces cerevisiae* (≤35°C), can be beneficial to the process during periods of elevated temperatures within industrial bioreactors. A thermotolerant strain of *Kluyveromyces marxianus* was able to produce ethanol

to a level of 6.0–7.2% (w/v) fermenting sugar-cane molasses at temperatures varying from 30–40°C [33]. Other non-*Saccharomyces* yeasts (*Pichia, Candida, Hanseniaspora, Issatchenkia*, and *Metschnikowia*) have been detected in breweries [36], spontaneous wine fermentation [9] and in the production of indigenous fermented foods and beverages in Africa and Asia [1, 12].

Issatchenkia orientalis has been detected in a variety of processes, such as the tumultuous or late stage of natural wine fermentation, in the tequila fermentation process [18], cooperating with *S. cerevisiae* in the conversion of the remaining sugar into ethanol [9]. This yeast can also be found in traditional sourdough fermentation producing flavor compounds [24]; in African indigenous fermented foods such as the *ogi* beverage [30] and *Kenkey* [13]; Chinese yeast cake [22], and yoghurt [23]. In addition, *I. orientalis* is more resistant to lactic acid [11] and acetic acid [7] than *S. cerevisiae*.

Different names have been attributed to the *I. orientalis* species such as *Candida krusei* [15], *Saccharomyces krusei*, or *Pichia kudriavzevii* [35], among other names. Recently, species assigned to the *Issatchenkia* genera have been included in the *Pichia membranifaciens* clade and have also been proposed for transfer to *Pichia*, based on the analysis of sequences from subunits of rRNA, mitochondrial DNA (mitDNA) and a section of the elongation factor -1α (EF-1α) gene [17].

Depending on growth conditions, cells of *S. cerevisiae* can show filamentous growth [8]. Yeast cells showing morphological alterations were eventually observed during the operation of a continuous fermentation system used in the present work to produce ethanol at high temperatures [19]. In this case, it was deemed necessary to know whether the observed elongated cells resulted from morphological alterations of the cells of *S. cerevisiae* or from a slow proliferation of the other thermotolerant yeasts still remaining in the sugar-cane syrup due to an insufficient sterilization. Yeast strains tolerant to high temperatures can be of great value for biotechnological applications.

Materials and methods

Yeast source and maintenance

The isolates of non-*Saccharomyces* yeasts (80 isolates shown in Table 1) studied in the present work were obtained from a continuous fermentation process carried out in the five-stage system as described below. The thermotolerant strain 63 M of *S. cerevisiae* was the starter yeast of the afore-mentioned system. The isolate 195B of *I. orientalis* was obtained in the present work and was selected for the fermentation studies carried out at 40 and 42°C. The two strains used as type strains in the identification procedures were strain Y01030 of *Issatchenkia orientalis* and strain Y01044 of *Pichia membranifaciens* (*Issatchenkia orientalis* Kudryavtsev and *Pichia membranaefaciens* Hansen as described by the NCAIM catalogue, National Collection of Agriculture and Industrial Microorganism, Budapest, Hungary). During the present study, stock cultures were periodically transferred to solid YPD medium (2% glucose, 2% peptone, 1% yeast extract, and 2% agar) every 3–4 months for growth prior to storage of the culture slants at 4°C.

Preparation of sugar-cane syrup for the feeding of the fermentation system

The raw material (58% total reducing sugar) was diluted to 15% (total reducing sugar, w/v) and sterilized for 60 min at 120°C in a 10-l feeding reservoir. The entire empty system was also sterilized for 60 min at 120°C and 1 atm pressure before the addition of the sterilized and diluted sugar-cane syrup. The diluted syrups were supplemented with the addition of 0.2% (w/v) ammonium sulfate and 2% (w/v) monobasic sodium phosphate with the pH being adjusted to 4.5 before sterilization. The efficiency of the sterilization was assayed by seeding the sterilized sugar-cane syrup on YPD medium and no microbial growth was observed after 3–4 days of incubation at 30°C. In order to be enriched with eventual contaminant cells not detected on YPD plates, the sterilized sugar-cane syrup was also incubated for 1 week at 30°C and no growth was also observed.

Inoculum propagation for the fermentation system

The starter strain 63 M used as the inoculum of the fermentation system was propagated in sugar-cane syrup at 30°C in a two-step procedure in order to adapt the cells to increasing sugar concentrations as follows: after inoculation with a full loop of cells, the propagation of the pre-inoculum took place in a test tube containing 5% sugar-cane syrup (w/v, total reducing sugar) for 16 h and the entire culture (6 ml) was added to 25 ml of 10% sugar-cane syrup (w/v, total reducing sugar) for a second propagation under agitation (125 rpm). The cells resulting from the last propagation were used as inoculum of the fermentation system as previously described.

Operation of the fermentation system

To study the effects of increasing temperatures, a continuous fermentation system made up of five reactors linked in series was used [19]. This system was continuously fed ($F_{feeding} = 0.12\ l\ h^{-1} = F_{out}$) with sterilized sugar-cane syrup containing 15% diluted sugar-cane syrup at pH 4.5.

The entire system was aerated at an airflow rate of 120 l h^{-1} so that the cells were maintained in suspension in each reactor (0.6 l medium in each reactor, which was 24.5 cm in height and 8.5 cm in diameter). When a constant biomass (steady state) was attained in all the five reactors for 3 days at 30°C, the temperature of each reactor was raised so that the temperature of the entire system ranged from 35°C in last reactor (where less nutrients were available) to 43°C in the first reactor or feeding reactor, such that a 2°C temperature difference was kept from one reactor to the next during the entire operating process. Once this gradient of temperature was achieved, the feeding of the entire system and cell recycling ($F_{recycling} = 0.10$ l h^{-1}) were both immediately started and the temperature of the entire system was controlled for 60 days as follows: 15 days operation at 35–43°C; 25 days at 37–45°C; and 20 days at 39–47°C.

Isolation and identification procedures

A total of 15 samples (three samples from each reactor) were collected from the five mini-reactors of the fermentation system [19] after 60 days of operation as described above. The samples were diluted and plated on YPD medium containing 0.035 g l^{-1} Rose Bengal and 0.192% (v/v) propionic acid to inhibit fungal proliferation as recommended by Booth [5]. After 3 or 4 days of growth on plates at 30°C, 240 colonies were selected based on their sizes and morphologies and also on the dye uptake from the Rose Bengal medium. A total of 240 isolates were isolated for studies. A positive growth on lysine agar medium indicated that the isolates were not *S. cerevisiae* [16, 36]. Yeast isolates initially resembling non-*Saccharomyces* yeasts, as suggested by methods of the classical taxonomy, had their identification confirmed at the species level using RAPD-PCR. The majority of the yeast colonies that did not show positive growth on lysine medium were identified as resembling *S. cerevisiae* [34].

Physiological characterization of non-*Saccharomyces* yeasts

Classical taxonomic assays as described by Barnett et al. [3] were applied to the non-*Saccharomyces* yeasts (total of 80 isolates) for an initial differentiation among the isolates (Table 1). Based on these assays, the non-*Saccharomyces* yeasts were divided into groups of isolates showing similar characteristics. Then, the groups of isolates resulting from the application of the classical taxonomy assays were re-analyzed using both the API-ID32C kit (BioMerieux, Marcy-L'Ètoile, France) and the set of assays proposed by the key of Middelhoven [27]. The identification of the groups of isolates showing similar results was validated using RAPD-PCR.

The maximal temperatures for growth of the isolates and their capacity to assimilate glycerol were determined using conventional YPD medium and the YNB-glycerol medium (0.67% yeast nitrogen base, 1.0% glycerol as carbon source

Table 1 Groups of non-*Saccharomyces* yeasts based on physiological assays

List of assays	Group I (71 isolates)	Group II (6 isolates)	Group III (3 isolates)
Sugar fermentation of:			
D-Glucose	(+)	(−)	(−)
D-Galactose	(−)	(−)	(−)
Sugar assimilation of:			
D-Glucose, glycerol, DL-lactate, N-acetyl-D-glucosamine	(+)	(+)	(+)
D-Galactose, sucrose, maltose, cellobiose, lactose, raffinose, melezitose, starch, D-xylose, L-rhamnose, L-arabinose, trehalose, glucitol, erythritol, *myo*-Inositol, mannitol, methanol, 2-keto-D-gluconate, hexadecane	(−)	(−)	(−)
Succinate, citrate	(+)	(−)	(−)
Nitrogen assimilation of:			
Nitrate	(−)	(−)	(−)
Ethylamine, lysine	(+)	(+)	(+)
D-glucosamine:	(−)	(+)	(+)
Growth at/with:			
1% Acetic acid	(−)	(−)	(−)
Cycloheximide (100 ppm)	(−)	(−)	(−)
10% NaCl	(+)	(−)	(−)
50% Glucose	(−)	(−)	(−)
Growth (°C)	44	44	37

(+) = positive assay within 2–7 days of growth; (−) = negative assay; Isolates of group I = 10B, 13B, 14B, 16A,16B, 22B, 25B, 26B, 29B, 31B, 33B, 49B, 54B, 55B, 56B, 57B, 59B, 62B, 63A, 63B, 69B, 73B, 75B, 78B, 79B, 80B, 82B, 84B, 95B, 96B, 101B, 102B, 105B, 106B, 110B, 112B, 113B, 114B, 116B, 118B, 119B, 122B, 128B, 130B, 136B, 142B, 144B, 145B, 146B, 148B, 151B, 154B, 156B, 157B, 159B, 161B, 162B, 164B, 166B, 167B, 169B, 170B, 171B, 172B, 173B, 181B-1, 192B, 194B, 195B, 198B, 199B; Isolates of group II: 32A-1a, 42A-1a, 42A-2, 51A, 98B, 34A; Isolates of group III: 32A-1b, 32A-2, 42A-1b

plus 2% agar) in which the glycerol concentration was raised from 0.1% (as originally recommended by Barnett et al. [3]) to 1.0%. Loops of cells from the stock cultures were suspended in 0.5 ml of water to obtain the concentrated cell suspensions used in the replication of the isolates on the solid medium. After replication, the plates were incubated for 2 days at 30 and 40°C. Plates were incubated in a plastic box containing water-soaked paper to maintain the humidity. The yeasts showing growth after 3 days of incubation at 40°C or above were designated as thermotolerant yeasts.

Color and colony morphologies of the non-*Saccharomyces* isolates were observed on YM medium (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% D-glucose and 2% agar) after 3–5 days of growth at 30°C using stereomicroscopy (Quimis model Q734ZT, connected to a Quimis Digital Color Camera SDC-312). Cell morphologies were determined by phase-contrast microscopy (model DMR connected to a Leica MPS 60 camera from Leica Microsystems, Wetzlar, Germany) of 3-day-old cultures grown in liquid YM medium at 30°C. The liquid YM medium was also used to detect the pellicles formed on the surface of the cultures after 3 days of growth at 30°C.

DNA isolation for RAPD-PCR

DNA was extracted using hexadecyltrimethylammonium bromide or CTAB and purified following procedures as described by Messner et al. [25]. The purified DNA preparation was diluted with TE buffer to a concentration of approximately 5 ng μl^{-1} and stored at −20°C.

RAPD-PCR assay

Conditions and procedures for the separation of the RAPD-PCR fragments were carried out as described by Messner et al. [25]. The primers were selected based on the procedure used by Prillinger et al. [31] for the identification of yeasts in cheese. The amplification reaction was prepared as follows: a few microliters of samples containing 0.1 μg of genomic DNA was subjected to amplification in 50 μl of a solution containing 10 mmol l^{-1} KCl, 10 mmol l^{-1} $(NH_4)_2SO_4$, 20 mmol l^{-1} Tris–HCl (pH 8.8), 4.5 mmol l^{-1} $MgSO_4$, 0.1% (w/v) Triton X-100, each primer (nucleotide) at a concentration of 0.2 mmol l^{-1}, (Invitrogen-GIBCO), 0.2 μg of bovine serum albumin fraction V per ml, and 2.5 U of *Taq* DNA polymerase (Amersham, Pharmacia Biotech, n° 27-0799-61). The mixture was overlaid with 50 μl of light mineral oil (Sigma Chemie GmbH) and processed with a thermocycler (Programmable Thermal Controller, model PTC-100, MJ Research Inc). The primers M13 (5′-GAGGGTGGCGGTTCT-3′) as proposed by Meyer et al. [26] and D2 (5′-TGCCGAGCTG-3′) as proposed by Caetano-Anolles et al. [6] were synthesized by Invitrogen, Carlsbad, CA, USA. Using primer M13, the cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation (98°C for 15 s), annealing (60 s at 50°C), extension (100 s at 72°C) and a final extension of 72°C for 5 min. For primer D2, the cycling program was the same except that 90 s at 40°C was used for annealing.

Analysis of RAPD-PCR products

The products of the amplification reactions were separated and analyzed by electrophoresis (100 V for 1 h) on 1.0% (w/v) agarose gel in 1X TAE buffer (40 mmol l^{-1} Tris–acetate, 1 mmol l^{-1} EDTA, pH 8.0), which was stained with ethidium bromide (0.5 μg ml^{-1}). A DNA molecular size marker (1-kb Plus DNA Ladder from Invitrogen-GIBCO BRL, no. 10787-018) was used. Gel images were captured and digitized on a computer using a Fluor-STM MultiImager (Multi-Analyst/PC 1.1 Version, Bio-Rad, Hercules, California). Dice coefficients of similarity were determined using the RAPD-PCR profiles while the dendrograms were generated by the GelCompar II Program, Version 2.0 (Applied Maths, Kortrijk, Belgium) based on the UPGMA method.

Growth and fermentation in liquid medium and analytical assays

Each inoculum was propagated under agitation (125 rpm) in the YPD medium (2% glucose) for 12 h at 30°C and used to inoculate the Erlenmeyer flasks containing 25 ml of medium to assay both the growth and ethanol production at temperatures varying within the range of 30–47°C. Fermentation experiments were carried out in YPD medium containing 10% (w/v) glucose and this medium was inoculated with the yeast strains to obtain an initial low cell density of 0.2 mg ml^{-1}. The following analytical assays (carried out in triplicate) were used in the growth and fermentation experiments: cell viability using the methylene blue method as described by Laluce et al. 2009 [20]; total residual sugar using the 3,5-dinitrosalicylic acid method [28]; ethanol using a gas chromatograph (model CG-37; Instrumentos Científicos, São Paulo, Brazil) and expressed as g l^{-1}; biomass assays using the cells washed by vacuum filtration (Millipore membrane DAWP02500, 0.65-μm pore size) and dried at 105°C until constant weight and expressed as mg ml^{-1}.

Results

The abnormal elongated cells were occasionally observed after 2 months of operation of the fermentation system and this seemed to be related to the changes in the sugar-cane

syrup donated by the alcohol plants. Colonies of the isolates showing elongated cells were obtained at the end of the continuous fermentation process when these elongated cells were clearly dominating the process as observed under the microscope. Based on the lysine assimilation assay on plates, the yeast population was made up of 80 isolates (33.3% or 80 isolates out of 240 total isolates) of non-*Saccharomyces* yeasts plus 160 isolates (66.7%) resembling *S. cerevisiae*. The colonies showing elongated cells on YM-medium were able to grow on lysine medium and they were initially divided into three groups of isolates showing similar physiological properties as equally indicated by the common physiological assays [3], the API-ID32C kit and the key of Middelhoven assay set [27]. To finalize, RAPD-PCR was used to analyze the groups of non-*Saccharomyces* yeasts that showed growth on lysine medium.

Grouping of the isolates identified based on physiological and morphological phenotypes

Non-*Saccharomyces* yeasts (positive growth on lysine medium as sole nitrogen source) that showed the same physiological properties [3] were divided into three groups as described in Table 1: group I having 71 isolates; group II having six isolates, and group III having three isolates. Groups II and III differed from the isolates of group I by showing negative fermentation of glucose and negative assimilation for citrate and succinate. Isolates from group I and group II showed growth at 44°C while maximum temperature for growth was 37°C for isolates from group III. The growth temperature is a strain-dependent property.

Concerning morphologies, pseudohyphae was observed for all isolates of the three groups after 3 days of growth on liquid YM medium at 30°C: white and opalescent colonies were observed for isolates from groups I and III after 3 days of growth on solid YM medium and pinkish hue color (not shown) was observed for some colonies of isolates in group II after growth for 5 days. Figure 1 illustrates the cell and colony morphologies shown by the isolates of group I: elongated cells along with pseudohyphae formation (Fig. 1a) were seen in the opalescent colonies of isolate 181B-1 (Fig. 1b); elongated cells without showing pseudohyphae formation (Fig. 1c) were observed in the colonies of isolate 110B (Fig. 1d) that showed a brilliant cream color after 3 days of growth.

Differentiation between the groups of isolates

After the morphological assays, all the isolates were analyzed using the API-ID32C kit and the key of Middelhoven assay set [27]. Based on these two methods, all isolates located in group I were considered as being isolates resembling the species of *I. orientalis* due to the following sequence of assays of the key of Middelhoven [27]: negative assimilation of nitrate, positive assimilation of ethylamine, white colony formation, bud formation, negative

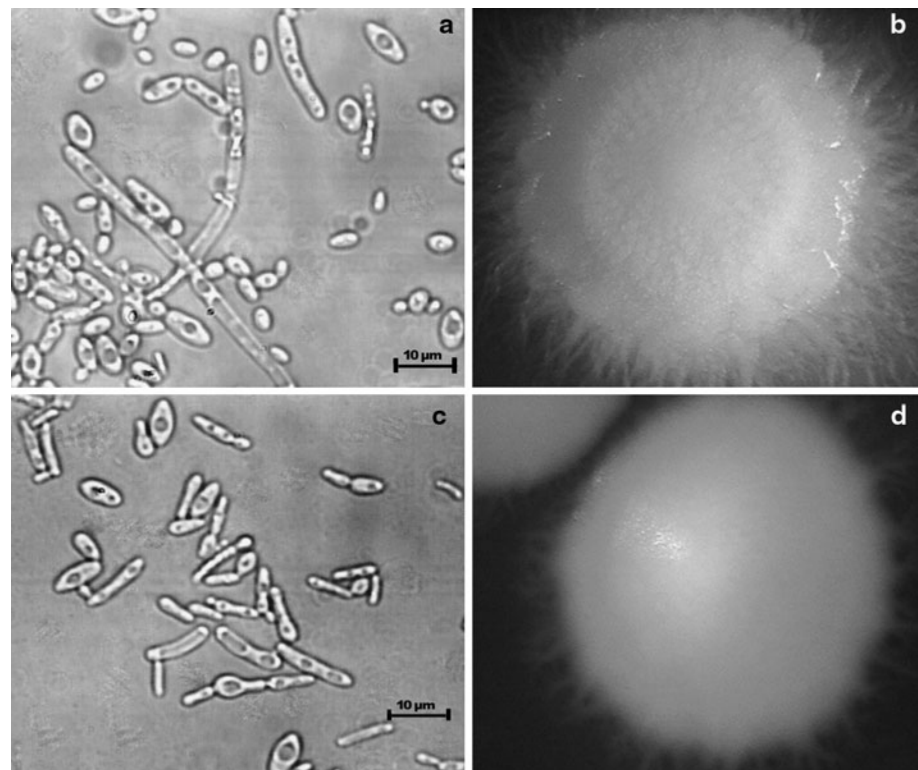

Fig. 1 Cell (phase contrast microscopy, 1000X) and colony (phase-contrast stereoscopy, 40X) morphologies of *I. orientalis* isolates are as follows: cells of isolate181B-1 (**a**) and 110B (**c**) after 3 days of growth on liquid YM medium and colonies of isolate of 181B-1 (**b**) and 110B (**d**) after 3 days of growth on solid YM medium

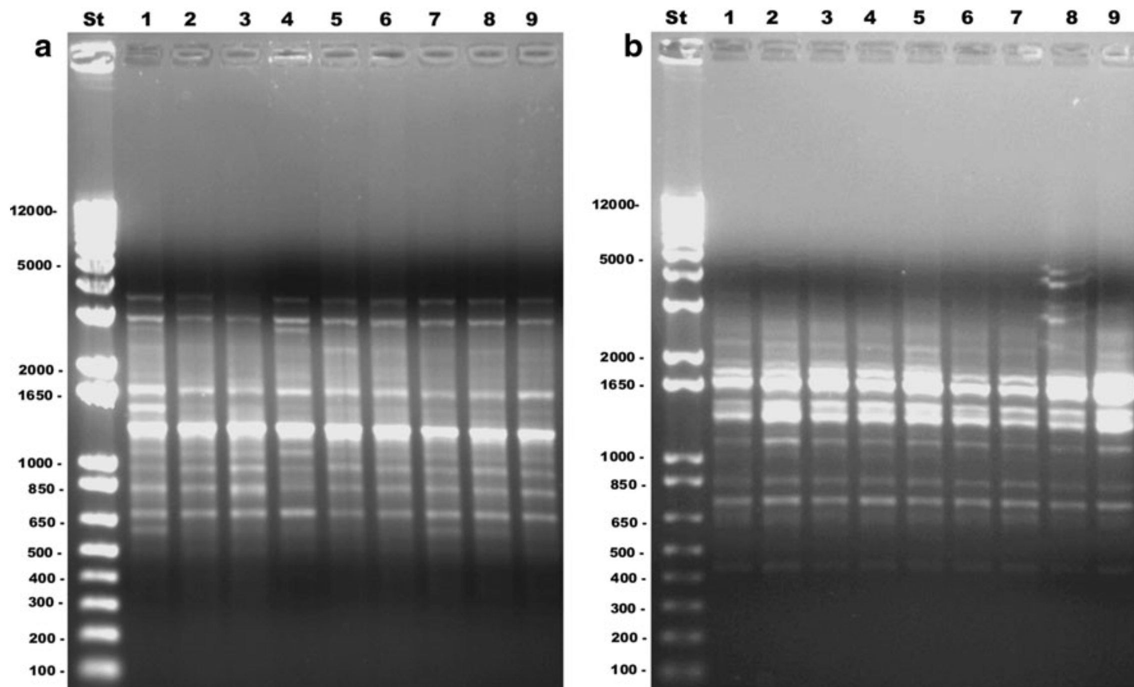

Fig. 2 RAPD-PCR patterns of the reference strain of *I. orientalis* and isolates primed by the oligonucleotide M13 (**a**) and the strains are: type strain Y01030 (1); 101B (2); 105B (3); 110B (4); 195B (5); 113B (6); 162B (7); 170B (8); 181B-1 (9). The same yeasts were primed by oligonucleotide D2 (**b**). *Lane 1* St contains the DNA molecular size marker

assimilation of inositol, 2-keto-D- gluconate, starch, sucrose, positive assimilation of N-acetyl-D-glucosamine, negative assimilation of glucitol and cellobiose, positive fermentation of glucose and negative assimilation of xylose. Isolates from groups II (strains 32A-1a, 42A-1a, 42A-2, 51A, 98B, 34A) and III (strains 32A-1b, 32A-2, 42A-1b) resembled species of *P. membranifaciens*. The negative fermentation of glucose observed for isolates in groups II and III was the only difference between strains of *P. membranifaciens* and *I. orientalis*. The proportion of the cells resembling *P. membranifaciens* was very low (3.75% out of a total of 240 isolates) when compared to the number of isolates resembling *I. orientalis* (29.6%).

RAPD-PCR profiles for isolates resembling *Issatchenkia orientalis* and *Pichia membranifaciens*

Figure 2 describes the RAPD-PCR profiles obtained using eight isolates of *I. orientalis* from group I (strains 101B, 105B, 110B, 195B, 113B, 162B, 170B, and 181B-1, which were taken from a total of 71 isolates of this group) including the type strain Y01030, using both primers, namely M13 (Fig. 2a) and D2 (Fig. 2b). The RAPD-PCR profiles obtained for the eight isolates of group I were similar to those shown by the type strain Y01030 of *I. orientalis* except that a band observed below the 1,650-bp band in the profile of type strain Y01030 is very weak or hardly seen in the profiles of the isolates analyzed as shown in Fig. 2. Using oligonucleotide D2 as primer (Fig. 2b), no difference was observed between the profiles of type strain Y01030 and those obtained for the isolates of *I. orientalis*. In Fig. 3, isolates from group II and group III, (strains 32A-1a, 42A-1a, 42A-2, 51A, 98B, 34A, 32A-1b, 32A-2, 42A-1b, totalizing together nine isolates) described in Table 1 showed RAPD-PCR profiles similar to those shown by type strain Y01044 of *P. membranifaciens* using primers M13 (Fig. 3a) and D2 (Fig. 3b). Dendrograms generated by RAPD-PCR (supplementary material) showed a 79–97% similarity between the type strain Y01044 and nine isolates of *P. membranifaciens* using the oligonucleotide M13, while a 93–100% similarity was obtained for the same strains using oligonucleotide D2. Low homology levels (58–80%) have been reported for strains of *P. membranifaciens* and *P. fermentans* isolated from cheese compared to the data reported in literature for DNA sequences [2]. Similarity of 88–100% between the isolates of *I. orientalis* (strains 110B, 105B, 170B, 162B, 195B, 101B, 113B, 181B-1) and the type strain Y01030 was observed using oligonucleotide M13 as primer while 87–100% similarity was obtained using the oligonucleotide D2.

Isolates of *Issatchenkia orientalis* fermenting at high temperatures

The growth and fermentation of isolates resembling species of *I. orientalis* (strain 14B, 25B, 56B, 63A, 69B, 95B,

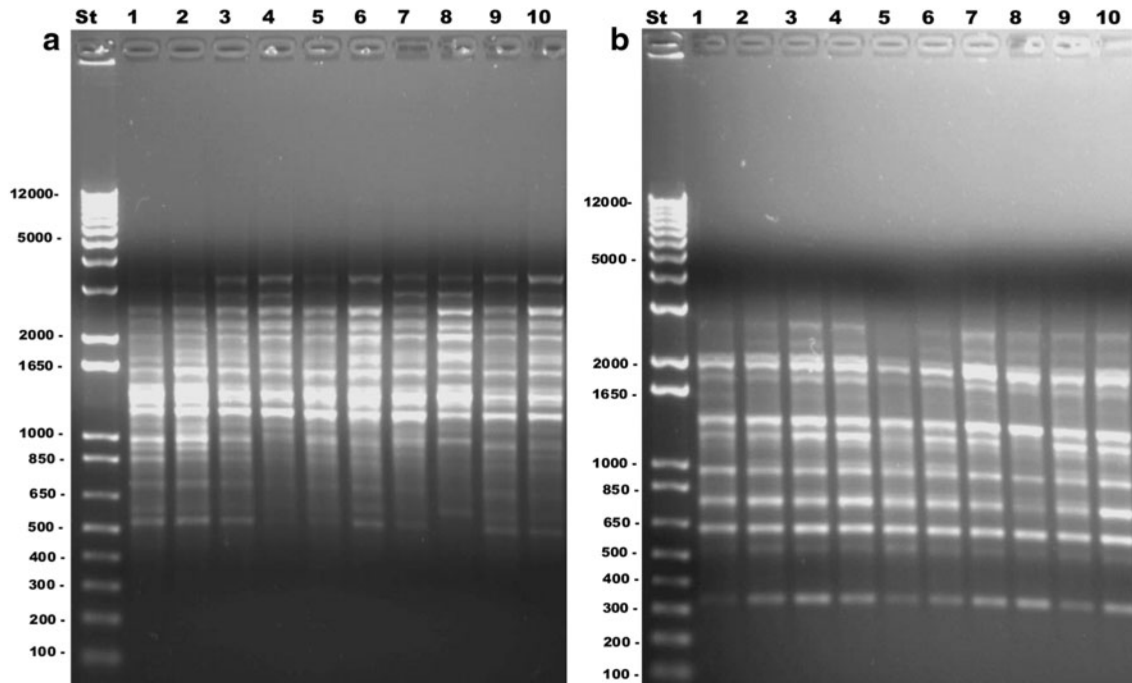

Fig. 3 RAPD-PCR patterns of the reference strain of *P. membranaefaciens* and isolates primed by the oligonucleotide M13 (**a**) and the strains are: type strain Y01044 of *P. membranaefaciens* (1); 32A-1a (2); 42A-1a (3); 42A-2 (4); 51A (5); 98B; 34A (7); 32A-1b (8); 32A-2 (9); 42A-1b (10). The same yeasts were primed by oligonucleotide D2 (**b**). Lane St contains the DNA molecular size marker from Invitrogen-GIBCO BRL (n° 9 10787-018)

110B, 162B, 170 B, 172B, 192B, 195B) were compared at 30°C using YPD medium containing 10% (w/v) glucose (data not shown). Some of these isolates (strain 162B, 170B, and 195B) identified as *I. orientalis* by RAPD-PCR gave the highest biomass yields (data not shown) and for this reason the isolate 195B was selected for the fermentation studies. The effects of high temperatures on ethanol production by isolate 195B of *I. orientalis* and strain 63 M of *S. cerevisiae* are shown in Figs. 4 and 5. Figure 4 shows the effect of the temperature on the ethanol production by strain 195B of *I. orientalis* and strain 63 M. Under these assayed conditions, the best temperature for ethanol formation by this isolate was 42°C. Isolates of *I. orientalis* fermented glucose but not sucrose as sole carbon source (see Table 1). Isolate 195B of *I. orientalis* converted glucose into ethanol (Fig. 5c) and accumulated biomass (Fig. 5a) faster than strain 63 M of *S. cerevisiae* on YPD medium. Due to its faster capacity to produce ethanol, isolate 195B of *I. orientalis* lost more viability than strain 63 M (Fig. 5b) when the fermentation time was prolonged to after the time required for the complete exhaustion of the sugar from the medium (Fig. 5d).

Another relevant property shown by a few isolates including isolate 195B was the ability to assimilate glycerol faster (only 2 days of incubation) at 40°C rather than at 30°C on solid YNB medium having 1% glycerol as the sole carbon source (data not shown). The starter strain 63 M of *S. cerevisiae* was not able to grow on this solid medium under the same conditions.

Discussion

As yeast strains tolerant to high temperatures can be of great value for biotechnological applications, it was decided to identify the elongated cells that were sporadically proliferating within a continuous fermentation system when temperatures as high as 39–47°C were reached within the bioreactors [34]. Based on this, it was concluded that this continuous system was capable of enriching its culture with yeasts able to grow at temperatures higher than those tolerated by conventional strains of *S. cerevisiae.* So, colonies of the non-*Saccharomyces* yeasts were identified using a sequence of classical and molecular methods of yeast taxonomy.

The association of assays for the determination of morphological and physiological phenotypes to molecular methods of taxonomy has been described in the literature [14, 36]. Yeast colonies showing distinct phenotypes on differential media (differences in morphologies, sizes, and intensity of dye uptake by the colonies) is an indication of the heterogeneity of the yeast population in the industrial bioreactors as in the case of the production of the Brazilian beverage named *aguardente*, in which samples withdrawn

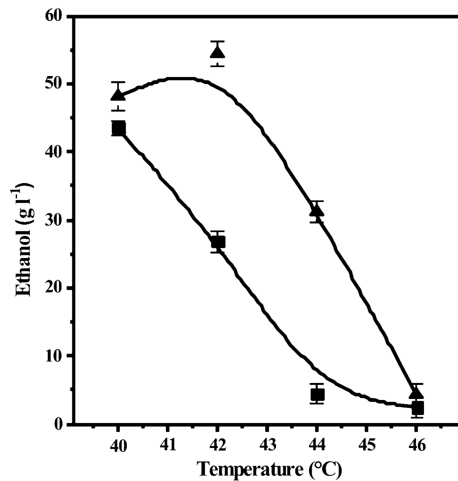

Fig. 4 Effect of temperatures on the ethanol production curves by isolate 195B of *I. orientalis* (*filled triangle*) and strain 63 M of *S. cerevisiae* (*filled square*): measurements obtained after a fermentation period of 12 h in Erlenmeyer flasks containing 25 ml YPD medium (100 g l^{-1} glucose) and started at an initial cell density of 0.2 mg ml^{-1}

from the bioreactors were plated on WLN medium for an initial differentiation of the isolates [29]. In the present work, the Rose Bengal medium was used as a differential medium [5]. The total isolates showing a diversity of morphological phenotypes were divided into two groups based on the growth assay on lysine medium. Starter strain 63 M of *Saccharomyces* was the predominant isolate within the same fermentation system when operated at increasing temperatures. Due to the increasing temperatures, a great diversity of variants made up of haploid cells and recombinant and mutant strains of *S. cerevisiae* were obtained from this system [34].

In the present work, the non-*Saccharomyces* yeasts were initially divided into three different groups of isolates showing similar characteristics of classical taxonomy (Table 1). Despite the fact that the commercial identification kits and the key of Middelhoven assay set [27] were based on a limited number of classical assays, it was possible to identify two groups of yeast resembling *I. orientalis* and *P. membranifaciens* before the application of RAPD-PCR to some isolates representing each group. Commercial identification kits, such as the API-ID32C kit, have been used to distinguish *S. cerevisiae* from other yeasts in large brewery samples [36]. Differences in colony and cell morphology have not always been able to differentiate yeasts at the species level, but they might be useful as a preliminary screening of a large number of industrial isolates. Changes in color of *Candida* colonies were observed on CHROMagar and used for differentiation and rapid presumptive identification of the *Candida* species [35]. In the present work, the change of the white color to the pinkish hue observed for some isolates after growth for 5 days on YM medium did not represented an indication of a different yeast species. These isolates (three isolates of pinkish hue colors) showed maximal temperature for growth at 37°C, physiological assays

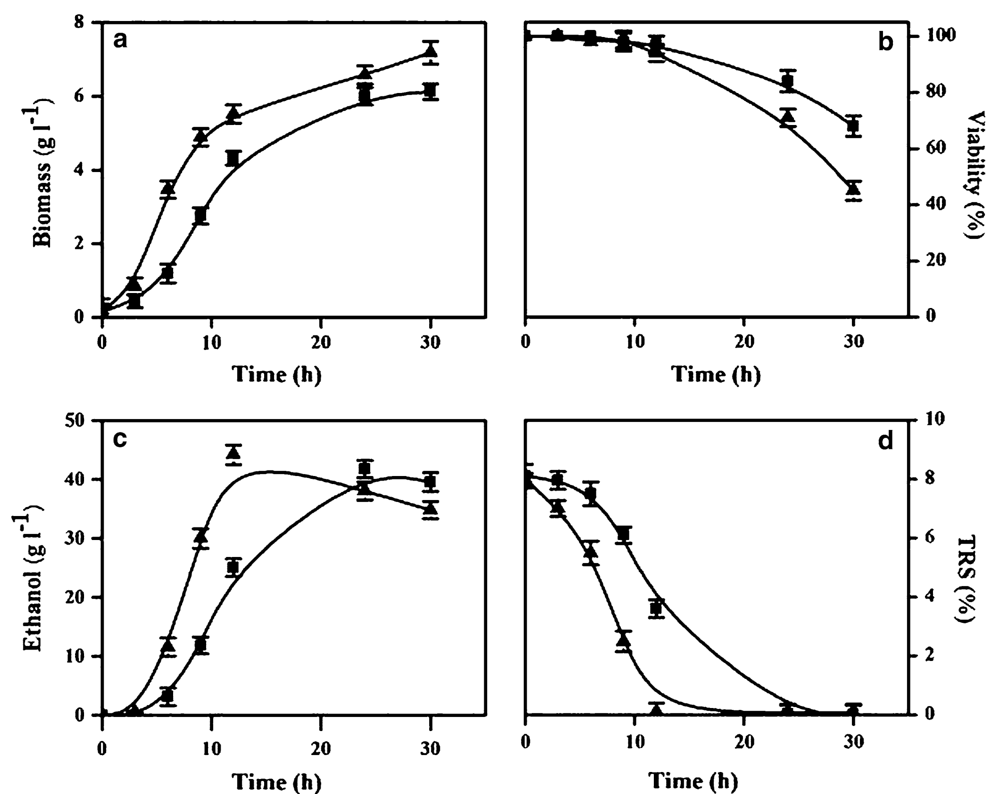

Fig. 5 Time courses of biomass formation (**a**), viability (**b**), ethanol production (**c**), and total residual sugar (**d**) obtained at 42°C for strain 195B of *I. orientalis* (*filled triangle*) and the strain 63 M of *S. cerevisiae* (*filled square*): fermentations were carried out in Erlenmeyer flasks containing 25 ml YPD medium (100 g l^{-1} glucose) and started at an initial cell density of 0.2 mg ml^{-1}

and RAPD-PCR profiles similar to *P. membranifaciens.* Isolates from another group were identified by RAPD-PCR as *I. orientalis*. *Issatchenkia orientalis*, and *P. membranifaciens* were not able to ferment sucrose and have sporadically been found at low frequency in the sugar-cane juices and molasses used in the production of fuel ethanol and the distilled beverage named *cachaça* [4, 10]. However, these two sporogeneous yeasts are able to ferment fructose and glucose as described in the literature [3]. It seems that cells and/or spores of these two yeasts, which were not detected in the contamination-control assays (see "Materials and methods"), were not killed during the sterilization of the sugar-cane syrup. The proliferation of theses yeasts occurred due to the high tolerance of these non-*Saccharomyces* yeasts at temperatures beyond the temperature limits tolerated by *S. cerevisiae.* The present work shows that *I. orientalis* is capable of growing at 42°C (Fig. 5) on YPD medium and this was the reason of the tolerance of *I. orientalis* to the thermal conditions that are lethal to strains of *S. cerevisiae* (>40ºC).

Rapid and accurate identification methods are needed to monitor and control industrial biotechnological processes. Ready information regarding the composition and dynamics of the yeast flora will help to control of the ethanol production process and the quality of beverages in the food industry. It is not always possible to analyze all colonies obtained from a great number of samples withdrawn from an industrial bioreactor due to the great microbial diversity and size of the yeast population. However, molecular methods are crucial for the taxonomic validation of an organism. On the other hand, it is possible to reduce costs and time during the control of an industrial process by reducing the number of isolates that shows similar physiological characteristics in each group to be analyzed by RAPD-PCR. Despite the less discriminatory ability in differentiating yeast strains, the use of both the key of Middelhoven assay set [27] and the API-ID32C kit was seen to be efficient in indicating that the two groups of non-*Saccharomyces* yeast were similar to the type strains of *I. orientalis* and *P. membranifaciens*. *Issatchenkia orientalis* and *Pichia membranifaciens* were the only non-*Saccharomyces* yeasts resembling the two species that proliferated at increasing temperatures in the present work.

The use of *I. orientalis* for ethanol production has some advantages when it is cooperating with *S. cerevisiae* in a mixed culture. In the present work, *I. orientalis* was able to perform cell division and to produce ethanol at temperatures in which the cell division and ethanol production by *S. cerevisiae* (>40°C) were increasingly constrained. As *P. membranifaciens* and *I. orientalis* are not able to assimilate sucrose [3], the propagation of these two yeasts in sugar-cane syrup having sucrose as the main carbon source depends on the production of invertase by *S. cerevisiae* [32]. *Saccharomyces cerevisiae* is known to produce the highest levels of ethanol within the range of 30–35°C, while the capability of producing ethanol from glucose by *I. orientalis* was maximal at 42°C as shown in the present work. The capacity of a few strains of *I. orientalis* to better assimilate glycerol at 30°C than at 40°C is an interesting characteristic. As glycerol is the largest by-product of the biodiesel industry, the use of glycerol by yeasts can be of a great advantage to the biodiesel industry. Genes involved in the synthesis and degradation of trehalose and glycerol are stress induced, and trehalose and glycerol were synthesized simultaneously during the initial stages of diverse stress treatments [21].

Conclusions

The predominance of an organism in a mixed population is dependent on the process conditions and its competition with other microbial cells. Based on the identification procedures used in the present work, it can be inferred that *I. orientalis* was the dominant non-*Saccharomyces* yeast in the continuous fermentation system operated at temperatures as high as 39–47°C. The present results also demonstrate that the continuous system used for ethanol production at increasing temperatures was enriched by the species of *I. orientalis,* which remained viable due its maximal growth observed above 40°C. This system seems to be promising for the selection and adaptation of yeasts to high temperatures. Keeping temperature down in large-scale industrial bioreactors can be a serious problem in tropical climates due to the high cost of an efficient refrigeration system. Yeasts able to grow and produce ethanol at high temperatures can extend the fermentation process beyond the temperature limits tolerated by *S. cerevisiae.*

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