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MOLECULAR IDENTIFICATION AND METABOLIC SCREENING OF SOME YEAST STRAINS FROM FOODS

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Abstract

Yeast strains from Saccharomyces, Kluyveromyces and Candida genera are present in most foods representing the basis for various industrial and biotechnological processes. The strains CMGB79 and CMGB159 were identified using PCR-RFLP on the ITS1-5.8S-ITS2 region as belonging to Candida parapsilosis, respectively, to Kluyveromyces marxianus. The primer OPA03 yield the highest degree of intraspecific RAPD polymorphism for the strains Saccharomyces cerevisiae CMGB59, CMGB121and ATCC201583. Lipase production was observed in the presence of Tween 80 in concentration of 0.1 and 0.5% for Candida parapsilosis CMGB79, respectively, 0.1 to 0.8% for Candida parapsilosis CBS604. The oleic acid represented the best substrate for lipase induction and cell growth for Kluyveromyces marxianus CMGB159. All the yeast strains tested positive for lipase synthesis in the presence of tributyrin. The antagonistic interactions between the studied strains were assessed using killer assays against Candida parapsilosis CMGB79 and CBS604. The killer activity was high for Kluyveromyces marxianus CMGB159 and good for the Saccharomyces cerevisiae strains, the toxin representing a stress factor which determined modifications in the sensitive cells. The results obtained during the present work showed that the characterized yeast strains present an important potential for applications in food industry, in obtaining probiotic compounds or as therapeutic agents of biomedical interest.

Key words*: Candida, Kluyveromyces, Saccharomyces, killer activity, lipases.*

INTRODUCTION

The intensive development of numerous industry and biotechnology domains and the advances of knowledge on the molecular and metabolic characteristics of the microorganisms, revealed that many genera and species present strains with multiple and various abilities. The ubiquitous presence of yeasts in natural and industrial environments and their ability to assimilate a wide range of substrates and to produce proteins or specific metabolites, represent the basis for their important practical applications.

The lipases are a family of enzymes involved in catalyzing the hydrolysis of triglycerides producing mono- or diglycerides, glycerol and fatty acids. Yeast lipases produced by *Candida*, *Kluyveromyces* and *Saccharomyces* species are intensively studied and used in food industry (for taste and flavour improvement, preservation of food products, hydrolysis of milk fat), in production of cosmetics, detergents, leather, pharmaceutics, in biomedicine and in bioremediation of polluted environments (Saxena et al., 1999; Vakhlu and Kour, 2006; Lock et al., 2007; Karigar and Rao, 2011).

Strains belonging to *S. cerevisiae*, *K. lactis* and *K. marxianus* produce extracellular killer toxins which induce the death of cells from the same species or even belonging to different genera and species. While the killer systems and toxins are well studied for *S. cerevisiae* (Marquina et al., 2002) and *K. lactis* (Schraffrath and Breunig, 2000), the knowledge concerning *K. marxianus* is still poor (Abranches et al., 1997). Even though the killer toxins are of main interest in beer, wine or bakery products, they gain a growing importance in obtaining monoclonal killer toxin-like antibodies with candidacidal activity (Magliani et al., 1997).

In the present work yeast strains isolated from foods are identified and characterized at molecular level and screened for the lipolytic activity, with emphasis on the influence of carbon and nitrogen sources on lipase induction and cell growth. Also, the killer activity was

used to evaluate the antagonistic relations between the strains and to assess the effect of the killer toxin on *Candida* sensitive cells.

MATERIALS AND METHODS

1. Yeast strains

The yeast strains CMGB79, CMGB159, *Saccharomyces cerevisiae* CMGB59 and *S. cerevisiae* CMGB121 were previously isolated from fermented food products (diary), food industry and fodder and maintained in the Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania (CMGB). For the experiments were used fresh cultures grown for 20 hours on Yeast Peptone Glucose (YPG) medium (0.5% yeast extract, 1% peptone, 0.2% glucose).

2. Analysis of the ITS1-5.8S-ITS2 amplicons

The genomic DNA of the strains CMGB79, CMGB159, *S. cerevisiae* CMGB59, *S. cerevisiae* CMGB121 and *S. cerevisiae* ATCC201583 was isolated (Csutak et al., 2014) and then amplified using the PCR program: initial denaturation 5 min at 94^{o}C, 40 cycles of 1 min at 94^{o}C, 30 sec at 55^{o}C, 2 min at 72^{o}C, and a final extension 5 min at 72^{o}C. The reaction mix comprised 1 µl genomic DNA, 25 µl Thermo Scientific DreamTaq Green PCR Master Mix (2X) and 1.2 µM ITS1 (5'TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) primers. The amplicons were digested with 0.5 µl of each endonuclease *Cfo* I (5'-GCG/C-3'), *Hae* III (5'-GG/CC-3'), *Hinf* I (5'-G/ANTC-3') and *Msp* I (5'-C/CGG-3') (10U/µl, Promega) and vizualized by gel electrophoresis using 1.7% agarose and Tris-Borate-EDTA (TBE) 0.5X. The sizes of the amplicons and restriction fragments were determined using the program Quantity One (Bio-Rad).

3. PCR-RAPD

The PCR-RAPD analysis of the strains *S. cerevisiae* CMGB59, *S. cerevisiae* CMGB121 and *S. cerevisiae* ATCC201583 was performed in a total volume of 25 µl using GoTaq Green Master Mix 2X (Promega), 1 µM of OPA01 (5'CAGGCCCTTC-3'), OPA03 (5'-AGTCAGCCAC-3'), OPB17 (5'-AGGGAACGAG-3') or M13 (5'-AGGGTGGCGGTTCT-3') and the amplification program: initial denaturation 5 min at 94^{o}C, 45 cycles of 1 min at 94^{o}C, 1 min at 36^{o}C and 2 min at 72^{o}C, and a final extension of 10 minutes at 72^{o}C. The RAPD fragments were analysed in 1.5% agarose gels in TBE 0.5X.

4. Screening for lipase production

Lipase production was evaluated using two types of tests. In a first place, we used the Tween opacity test with six different media: S (1% BactoPeptone, 0.5% NaCl, 0.01% $CaCl_2$, 2% agar-agar, 0.5% Tween 80), S w/o Ca (S without $CaCl_2$), S-0.4Ca (S with 0.04 % $CaCl_2$), S-1T (S with 0.1% Tween 80), S-8T (S with 0.8% Tween 80) and S-10T (S with 1% Tween 80) (Slifkin, 2000). Lipase production resulted in a white halo of precipitate of calcium salts surrounding the colonies of the tested strains. In parallel, lipase production was also determined by growing the yeast strains for five days on YPTA medium (0.3% yeast extract, 0.5% peptone, 1% tributyrin, 2% agar) (Shirazi et al., 1998; Darvishi, 2012). The qualitative evaluation of the lipolytic activity was based on the ratio between the measured diameters of the tributyrin hydrolysis halo (TH) and those of the cell colonies (CC). Thus, if the TH/CC ratio is 1, the yeast strain is not able to hydrolyse the tributyrin and does not produce lipases. On the contrary, a high TH/CC ratio indicates active lipase secretion.

5. Influence of the growth media on the lipolytic activity

The studied strains (2 x 10^7 cells/ml) were grown on liquid media: T20 (0.7% YNB, 2.5% Tween 20), T80 (0.7% YNB, 2.5% Tween 80) and D (1% olive oil, 0.2% yeast extract, 0.05 % KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4$ x 7 H_2O, 0.01% $CaCl_2$, 0.01% NaCl) (Tsuboi et al., 1996; Darvishi et al., 2009). The ability of the yeast strains to produce lipase for hydrolyzing Tween 20, Tween 80 and olive oil was estimated by monitoring cell growth after 24, 48 and 72 hours with a Thoma counting chamber. The aspect of the cells was also microscopically observed.

6. Antagonistic activity

The antagonistic activity was assessed using the killer assay. Colonies from overnight YPG grown cultures of CMGB159, *S. cerevisiae* CMGB59, *S. cerevisiae* CMGB121 and *K. lodderae* CMGB64 were spotted onto Petri

dishes with killer medium (0.1 M phosphate citrate buffer pH 4.8, 2% glucose, 1% yeast extract, 2% agar, 0.03% methylene blue) inoculated with 10^7 cells/ml of potential sensitive yeast strains: CMGB79 and *C. parapsilosis* CBS604. The plates were checked daily during seven days of incubation at 22°C. A strain was considered as killer positive when an inhibition halo or a zone with reduced growth of the sensitive strain appeared surrounding the colonies (Vassu et al., 2001). The killer toxin effect was also microscopically analyzed (40X) by observing the modified sensitive yeast cells.

RESULTS AND DISCUSSIONS

Molecular identification

Accurate identification of yeasts using molecular approaches represents the basis for the development of new research strategies aimed to improve the metabolic abilities of yeast strains for various practical applications such as food industry. Besides sequencing, the PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region is one of the techniques extensively used in interspecific differentiation of yeast species from food industry (Clemente-Jimenez et al., 2004; Naumova et al., 2004).

The genomic DNA obtained from the strains CMGB79 and CMGB159 was amplified using the primers ITS1 and ITS4 and the amplicons were digested with four endonucleases. The PCR products had 540 bp for CMGB79 and 740 bp for CMGB159 (Table1).

The restriction profiles obtained with *Cfo* I and *Hinf* I for CMGB79 and CMGB159 (Table 1, Figure 1) were highly similar with those obtained for other strains from the species *C. parapsilosis* (Guillamon et al., 1998; Esteve-Zarzoso et al., 1999; Jeyaram et al., 2008), respectively, *K. marxianus* (Bockelmann et al., 2008; Pham et al., 2011; Verdugo Valdez et al., 2011).

No restriction sites were found for *Msp* I for both CMGB79 and CMGB159. Our results were identical with those obtained during previous studies for *C. parapsilosis* (Mirhendi et al., 2001; Mirhendi et al., 2006; Basilio et al., 2008; Ayatollahi Mousavi et al., 2012) and for *K. marxianus* for which, in present, only few data can be found (Pérez-Brito et al., 2007).

Table 1. The size of the amplicons and restriction fragments of ITS1-5.8S-ITS2 region for the strains CMGB79 and CMGB159

Yeast strain	Amplicon (bp)	Fragment size (bp)			
		***Cfo* I**	***Hae* III**	***Hinf* I**	***Msp* I**
CMGB79	540	270, 240	420, 110	260, 255	520
CMGB159	740	300, 210, 180, 90	660, 80	285, 190, 120, 70	720

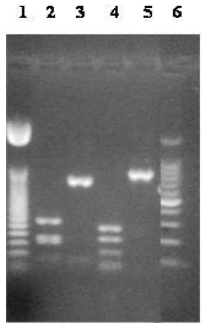

Figure 1. The restriction profile of the ITS1-5.8S-ITS2 amplicon of the strain CMGB159 obtained with: 1 -50 bp DNA Step ladder (Promega), 2- *Cfo* I, 3 - *Hae* III, 4 - *Hinf* I, 5 - *Msp* I, 6-Benchtop 100 bp DNA ladder (Promega)

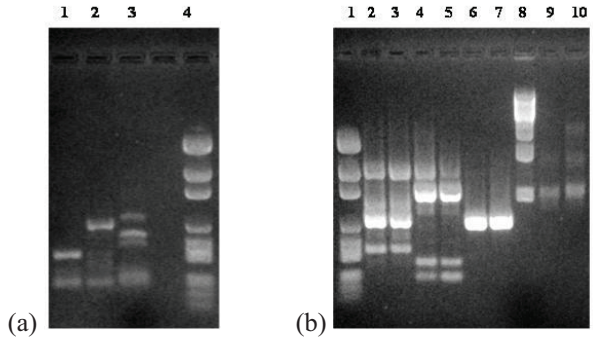

Figure 2. The PCR-RAPD amplicons obtained for: (a) *S. cerevisiae* ATCC201583 with the primers: 1 - OPA03, 2 - OPB17, 3 - M13; (b) *S. cerevisiae* CMGB59 (2, 4, 6, 9) and *S. cerevisiae* CMGB121 (3, 5, 7, 10) with the primers: 2, 3 - M13; 4, 5 - OPB17; 6, 7 - OPA01; 9, 10 - OPA03. Molecular markers used: (a) 4 and (b) 1 - Benchtop pGEM marker (Promega), 8 - 1 kb DNA ladder (Promega)

On the contrary, the variations observed in the restriction patterns obtained with *Hae* III for our strains compared to strains from the works mentioned above, are probably the result of the intraspecific variability related to the different environments from which the strains were isolated. The data from the analysis of the ITS1-5.8S rDNA-ITS2 amplicons allowed us to conclude that the strain CMGB79 belongs to *C. parapsilosis* and the strain CMGB159 to *K. marxianus*.

PCR-RAPD analyses were performed in order to determine intraspecific polymorphisms within the *Saccharomyces* specie using four primers: OPA01, OPA03, OPB17 and M13. Only two amplified fragments were obtained in the case of OPA01, while OPB17 and M13 yield the highest number of bands, followed by OPA03 (Figure 2b). The profiles were identical for *S. cerevisiae* CMGB59 and *S. cerevisiae* CMGB121 except when the OPA03 primer was used (Figure 2b). Therefore, in order to obtain a more accurate discriminatory characterization of the *S. cerevisiae* strains, we decided to also use a reference strain: *S. cerevisiae* ATCC201583. The compared analysis of the RAPD profiles (Figure 2a and b) showed that, although the OPA03 primer yield a total of only seven bands for all three strains, it provided a maximum degree of polymorphism - 100% (Table 2). Our results can be correlated with similar studies using the primers OPA03, OPB17 and M13 (Gomes et al., 2003; Andrade et al., 2006; Zhang et al., 2015).

Table 2. Polymorphic frequency of *S. cerevisiae* strains generated with primers M13, OPB17 and OPA03

Primer	Total amplified lanes	Common lanes	Polymorphic lanes	Polymorphic frequency (%)
M13	10	6	4	40
OPB17	10	3	7	70
OPA03	7	0	7	100

Lipolytic activity

The strains *C. parapsilosis* CMGB79, *C. parapsilosis* CBS604 and *K. marxianus* CMGB159 were tested for lipase production, since they belong to genera described as having lipolytic activity (Vakhlu and Kour, 2006). The strain *C. parapsilosis* CBS604 showed best results on S-1T medium with 0.1% Tween 80. Good results were also obtained for Tween 80 at 0.5% (S and S-0.4 Ca media) and 0.8% (S-8T medium) (Table 3).

Table 3. Lipase production on media with Tween 80 and $CaCl_2$ after seven days of incubation

Growth media	Opacity halos (mm)	
	C. parapsilosis CMGB79	*C. parapsilosis* CBS604
S	2	3
S w/o Ca	-	-
S-0.4Ca	2	3
S-1T	2	5
S-8T	-	3
S-10T	-	2

(-) no halo

C. parapsilosis CMGB79 was able to hydrolyze Tween 80 when added in small concentrations (0.1 and 0.5%). Moreover, it seems that lipase production was not influenced by the amount of Tween 80 from the growth media, or the variation of the activity was reduced and therefore could not be detected using the Tween opacity test even in the presence of higher concentrations of $CaCl_2$ (S-0.4Ca medium). In contrast, no lipase synthesis could be observed for the strain *K. marxianus* CMGB159 under the same conditions.

Since *C. parapsilosis* CBS604 showed the best screening results from the two *Candida* strains tested, we decided to investigate the factors that influence its lipolytic activity. We observed the ability of the yeast strain to use as sole carbon source for growth the oleic acid (C_{18}) and the lauric acid (C_{12}) liberated by the hydrolysis of Tween 80 (polyoxyethylene (20) sorbitan monooleate), respectively, Tween 20 (polyoxyethylene (20) sorbitan monolaurate), added in various concentrations.

In general, *C. parapsilosis* CBS604 preferred Tween 20 (E20-T20) as source for lipase production. The growth was reduced in the presence of 0.1% Tween. Best results with

similar curve profiles were obtained using 2.5% Tween. The lipolytic activity was high within the first 24 hours, followed by a plateau phase at 48 hours and a slower ascending curve at 72 hours (Figure 3). This might indicate that, in the first day of incubation, only part of the Tween was hydrolyzed forming fatty acids. Once these carbon sources exhausted, the growth was reduced. In the next hours, a possible enhancement of the lipolytic activity could take place due to residual Tween from the environment. New quantities of fatty acids were liberated, providing thus a basis for the cell growth. It was reported previously that Tween 20 and 80 from the culture media activate lipase production in *Candida* and promote synthesis of multiple lipase isoforms (Aravidan et al., 2007). On the other hand, the ethylene oxide (oxyethylene) groups present in Tween, are successfully used in production of copolymers aimed to increase lipase activity (Park et al., 2006).

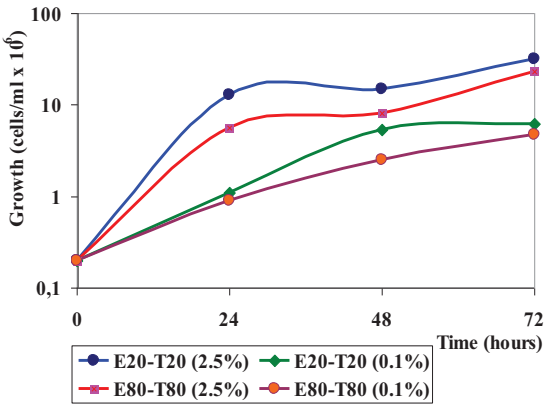

Figure 3. Effect of carbon source on cell growth of *C. parapsilosis* CBS604

The influence of carbon and nitrogen substrates on lipase synthesis of the strain *K. marxianus* CMGB159 was determined by monitoring the cell growth in liquid media. Cell multiplication was fast within the first 24 hours similar to *C. parapsilosis* CBS604, then it maintained a constant rate or decreased rapidly in the case of Tween 80 (E80), respectively, Tween 20 (E20) (Figure 4). The lipolytic activity was enhanced in the presence of olive oil (medium D). The olive oil and the oleic acid, component of the olive oil and Tween 80, were determined as best carbon source for lipase production also for other *K. marxianus* strains (Lock et al., 2007; Stergiou et al., 2012).

The nitrogen source played an important role in the process. In D medium, the yeast extract provided a complex nutritional basis for the cell growth, comparatively to the YNB (Yeast Nitrogen Base) used in E20 and E80 cultures. This could be observed also at microscopic level, *K. marxianus* CMGB159 presenting numerous cells and developed pseudohyphae after 48 hours of incubation on D medium (Figure 5).

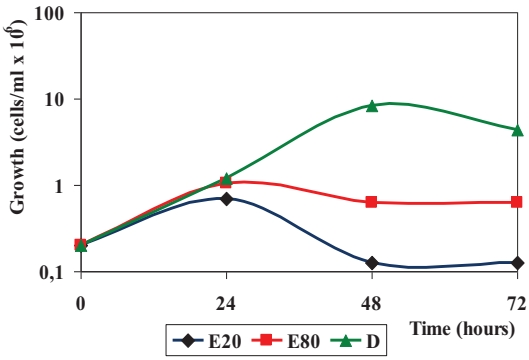

Figure 4. The influence of media on the cell growth of *K. marxianus* CMGB159

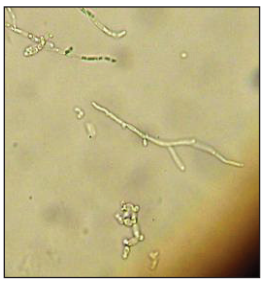

Figure 5. *K. marxianus* CMGB159 pseudohyphae on D media with olive oil

When tested on media with tributyrin, *K. marxianus* CMGB159 showed the highest lipolytic ability, followed closely by the *S. cerevisiae* strains CMGB59 and CMGB121 and, finally, by *C. parapsilosis* CMGB79 (Table 4). All three species can be found in fermented food, such as various dairy products, in which the butyric acid released by tributyrin hydrolysis is one of the main chemical compounds. Recent studies showed that the butyric acid inhibits the growth of potential pathogenic bacteria such as *Helicobacter pylori* (Yonezawa et al., 2012), has a major positive impact on the energy metabolism of the host when produced by the intestinal microbiota and is involved in gene regulation processes (Kasabuchi et al., 2015).

Therefore, the ability to hydrolyze trybutirin represents an important characteristic that recommend our strains for potential probiotic use.

Table 4. Tributyrin hydrolysis after seven days of incubation

Yeast strains	*K. marxianus* CMGB159	*C. parapsilosis* CMGB79	*S. cerevisiae* CMGB59	*S. cerevisiae* CMGB121
Lipolytic activity (HT/CC)	1.8	1.5	1.7	1.7

Antagonistic interactions

The antagonistic interactions between yeast strains are widespread not only in nature, but also in industry, having a major importance on the quality of the product and on the rate of success of the process.

S. cerevisiae and *K. marxianus* are species with antimicrobial activities related to the presence of killer toxins acting both intra and interspecific, including against *Candida* species (Marquina et al., 2002; Hernández et al., 2008). *C. parapsilosis* can be found in fermented food such as the fruit juice (Arias et al., 2002), but the most important aspect is represented by its pathogenicity, being one of the most known opportunistic pathogens which causes nosocomial infections (van Asbeck et al., 2009).

In fact, in present, the killer toxins present a growing importance not only for food industry, but also as therapeutic agents (Magliani et al., 2008).

Therefore, it was interesting to determine the potential interactions between *Candida*, *Kluyveromyces* and *Saccharomyces* strains. Besides the strains described during this work, we also tested the strains *C. parapsilosis* CMGB79 and *K. lodderae* CMGB64 from the pharmaceutical industry (Csutak et al., 2014).

K. marxianus CMGB159 showed good killer activity against *C. parapsilosis* CMGB79 while for *C. parapsilosis* CBS604 the results were less important (Figure 6 a, b). *S. cerevisiae* CMGB59 and *S. cerevisiae* CMGB121 formed growth inhibition halos for both *Candida* strains tested (Figure 6 c, d). When observed at the microscope, the presence of the killer toxin from *K. lodderae* CMGB64 determined modifications in the *C. parapsilosis* CBS604 culture: the appearance of pseudohyphae with large vacuoles and dead cells which absorbed the methylene blue (Figure 7).

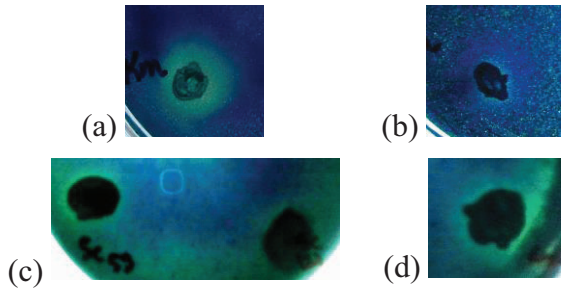

Figure 6. Killer activity of: *K. marxianus* CMGB159 against *C. parapsilosis* CMGB79 (a) and *C. parapsilosis* CBS604 (b); *S. cerevisiae* CMGB59 and *S. cerevisiae* CMGB121 against *C. parapsilosis* CMGB79 (c); *S. cerevisiae* CMGB59 against *C. parapsilosis* CBS604 (d)

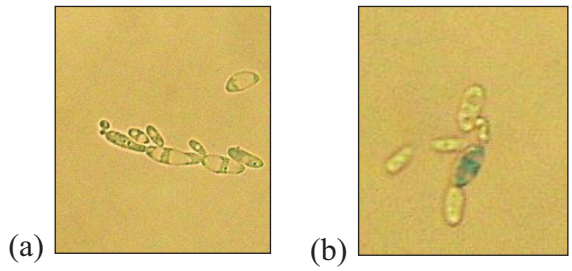

Figure 7. Modifications of *C. parapsilosis* CBS604 cells due to *K. lodderae* CMGB64 killer toxin: pseudohyphae with large vacuoles (a) and dead cells coloured with methylene blue (b)

Until present, the zymocin produced by *K. lactis* is the best known toxin of the *Kluyveromyces* genera. It acts in G1 before DNA replication causing cell-cycle arrest in the sensitive cells, similar to the K28 toxin from *S. cerevisiae* (Schaffrath and Breunig, 2000). It has been suggested that the presence of low concentrations of killer toxins in the environment determine caspase-like apoptosis and accumulation of reactive oxygen species in the susceptible cells (Schmitt and Breinig,

2006). The vacuoles play an important role in cell adaptation to these processes. Moreover, emergence of pseudohyphae at *Candida* is accompanied by appearance of numerous large vacuoles (Palmer et al., 2005). This might explain our observations regarding the mechanism of action observed for *K. lodderae* CMGB64 toxin on *C. parapsilosis* CBS604.

CONCLUSIONS

Yeast strains isolated from foods were characterized at molecular level and screened for specific metabolic abilities. The strains CMGB79 and CMGB159 were identified as belonging to *C. parapsilosis*, respectively, to *K. marxianus*. The primer OPA03 assured the highest degree of intraspecific polymorphisms within the *S. cerevisiae* speciae. When tested for the lipolytic activity, *C. parapsilosis* CBS604 hydrolyzed Tween 20 and Tween 80 in concentration up to 2.5%, while the presence of oleic acid and nitrogen substrates had an important impact on lipase induction and cell proliferation in *K. marxianus* CMGB159. All the strains showed good ability to hydrolyze tributyrin which might represent a basis for studies concerning a potential probiotic use. Moreover, the antagonistic interactions revealed high killer activity for the *Kluyveromyces* and *Saccharomyces* strains, an important characteristic for future studies aiming not only the food industry, but also biomedical applications.

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