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Molecular identification and antimicrobial activity of two new *Kluyveromyces lodderae* and *Saccharomyces cerevisiae* strains

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ABSTRACT

The main objectives of the present research are the identification and characterization of two yeast strains Y-CMGB 64 and Y-CMGB 168 from the pharmaceutical industry. The strains Y-CMGB 64 and Y-CMGB were identified using the analysis of the ITS1-5.8S-ITS2 amplicons and restriction profiles as belonging to *Kluyveromyces lodderae*, respectively, to *Saccharomyces cerevisiae*. A simple and rapid technique for the isolation of genomic DNA was optimized using a solution of LiCl 0.8 M and 1% SDS for obtaining yeast spheroplasts and a TE solution with RNase A for reducing the RNA contamination of the samples. The analysis of the RAPD patterns obtained with the primer M13 showed a total number of 15 bands with 100% polymorphism, thus allowing a discriminatory characterization of our strains. The antimicrobial activities of the strains Y-CMGB 64 and Y-CMGB 168 were tested using the killer assays. The strain Y-CMGB 64 was more active at 28^{o}C, while the incubation temperature seemed to have no influence on the activity of the strain Y-CMGB 168. The two yeast strains were re-named and included in the CMGB collection as *K. lodderae* CMBG 64 and *S. cerevisiae* CMGB 168. Our results represent an important basis for further studies and practical applications of the two strains in medicine and production of probiotic compounds.

Keywords: *Kluyveromyces lodderae, Saccharomyces cerevisiae, ITS1-5.8S-ITS2, RAPD, antimicrobial activity.*

1. INTRODUCTION

The yeasts are microorganisms with a widespread occurence in natural and industrial environments, many species including strains isolated from various sources and presenting addapted metabolic pathways [1]. The development of industry and the human population growth raised the problem of the taxonomic and phylogenetic identification of yeast species [2; 3] as basis for applications in bioremediation [4], production of biosurfactants [5; 6], biocontrol [7; 8], human and animal health, including the production of probiotic compounds [9; 10; 11; 12]. The analysis of the 5.8S rDNA gene and the neighbouring regions ITS1 and ITS2 are intensively used for the interspecific differentiation of many yeast species belonging to various genera such as *Candida*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Metschnikowia*, *Saccharomyces* and *Yarrowia* [13, 14; 15; 16]. Although used expecially for intraspecific identification of the yeast strains, the RAPD-PCR is another technique used in taxonomy studies [17; 18].

Besides these molecular approaches, the assays aimed to determine the production of extracellular products such as killer toxins, represents an important criteria not only for the taxonomic characterization, but also for further practical applications in biotechnology, food industry, medicine and agronomy [19; 20].

The present work deals with the molecular identification of two yeast strains Y-CMGB 64 and Y-CMGB 168 from the pharmaceutical industry and the characterization of their antimicrobial activity against strains belonging to yeast species with pathogenic potential.

2. EXPERIMENTAL SECTION

2. 1.Yeast strains and media

The two yeast strains Y-CMGB 64 and Y-CMGB 168 from the pharmaceutical industry (ICCF – National Institute for Chemical and Pharmaceutical Research and Development, Bucharest, Romania) were mentained at CMGB (the Collection of Microorganisms of the Center for Research, Consulting and Training in Microbiology, Genetics and Biotechnology, Department of Genetics, Faculty of Biology, University of Bucharest, Romania). Other strains used during this study were: *Candida albicans* ATCC 10231, *Candida glabrata* CMGB 35, *Candida guilliermondii* CMGB 44, *Candida krusei* CMGB 94, *Candida parapsilosis* CBS 604, *Candida tropicalis* CMGB 165, *Kluyveromyces lactis* CBS 2359/152, *Metschnikowia pulcherrima* CMGB-SG1, *Saccharomyces cerevisiae* ATCC 201583, *Saccharomyces cerevisiae* 17/17 (Kil-K0, *a his* $K^{-}R^{-}$). The yeasts were grown and mentained on yeast peptone glucose (YPG) medium (yeast extract 5 g/l, peptone 10 g/l, glucose 20 g/l).

2. 2. Genomic DNA isolation and purification

Genomic DNA isolation was performed using a technique addapted and optimized after [21]. Yeast strains were grown o/n on YPG medium at 28^{o}C, 180 rpm, then 100 µl culture (aproximately 10^{8} cells/ml) were centrifuged and the cell pellet was resuspended in 100 µl solution with 1% SDS and different concentrations of lithium chloride (LiCl): 0.2 M, 0.4 M and 0.8 M. The suspension was incubated for 15 min at 70^{o}C and 300 µl of 95% ethanol was added. The mixture was vortexed briefly and

centrifuged for 5 min at 13000 rpm. The sediment was washed with 500 µl of 70% ethanol, followed by a new centrifugation. The sediment comprising DNA was resuspended in 40 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with RNase A (final concentration 40 µg/ml). The debris was removed by centrifugation and the supernatant containing genomic DNA was used for PCR amplification.

The genomic DNA samples were analysed by agarose gel electrophoresis using an 0.8% agarose gel in TBE 0.5X. After electrophoresis, the gel was stained with ethidium bromide, visualized under UV light (UV-VIS Spectrophotometer) and digitalized.

2. 3. PCR-RFLP analysis of ITS-5.8S rDNA

The ITS1-5,8S-ITS2 region was amplified in a Biometra TGradient cycler using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers and the program described by [22]: 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 amplification was performed in a total volume of 50 µl PCR mixture using: 2 µl genomic DNA, 25 µl GoTaq Green Master Mix 2X (Promega) and 1,2 µM of each primer (ITS1 and ITS4).

The PCR products were digested for 90 min in a total volume of 12 µl with 0.5 µl of each of the following restriction endonucleases: *Cfo* I (5'-GCG/C-3'), *Hae* III (5'-GG/CC-3') and *Hinf* I (5'-G/ANTC-3') (10U/µl, Promega). The amplicons and the restriction fragments were separated by agarose gel electrophoresis using 1.2% agarose and TBE 0.5X. The size of amplicons and restriction fragments was evaluated using the program Quantity One (Bio-Rad).

2. 4. RAPD assay

The RAPD assay was performed in a total reaction volume of 25 µl l using GoTaq Green Master Mix 2X (Promega), 1 µl genomic DNA and 1 µM primer M13 (5'-AGGGTGGCGGTTCT-3'). The amplification program was addapted after [23] and comprized: 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 1.2 % agarose gels in TBE 0.5X.

2. 5. Antimicrobial assays

The strains Y-CMGB 64 and Y-CMGB 168 were tested for antimicrobial activity using the killer assays. Colonies from o/n grown culture of Y-CMGB 64 and Y-CMGB 168 were spotted onto killer medium plates (0.1 M phosphate citrate buffer pH 4.8, 2 % glucose, 1 % yeast extract, 2 % agar, 0.03 % methylene blue) inoculated with an overlay of 10^6 cells/ml of potential sensitive yeast strains. The killer activity of the strains Y-CMGB 64 and Y-CMGB 168 was tested against potential sensitive strains: *C. albicans* ATCC 10231, *C. glabrata* CMGB 35, *C. krusei* CMGB 94, *C. tropicalis* CMGB 165 and *S. cerevisiae* 17/17. The plates were incubated for five days at 25 and 28 ^{o}C and checked daily. A positive response was considered when the colonies were surrounded by a clear blue zone or a zone with reduced growth of the potential sensitive strain.

3. RESULTS SECTION

The technique we used for the isolation of the genomic DNA is simple and rapid compared with the one based on β-mercaptoethanol and specific enzymes like zymolyase, lyticase, a.s.o. [24]. Both lithium acetate (LiAc) and lithium chloride (LiCl) are intensively used in yeast transformation protocols with heterologous DNA due to their chaotropic effect which consists in the disruption of the hydrogen bonds at the level of the proteic structures within the yeast cell wall and membrane. As a consequence, the lithium cations increase the permeability of the yeast cells [25].

Since it seems that LiAc is aproximately 1.7 times more effective than LiCl [26], we tested three different concentrations of LiCl in combination with 1% SDS. As expected, the best results were obtained using a 0.8 M LiCl solution instead of the 0.2 M LiAc solution mentioned by [21]. Also, the DNA was resuspended in TE solution supplemented with RNase A reducing the RNA contamination of the samples. The DNA extracts obtained were further successfully used for PCR amplifications.

The analysis of the region ITS1-5.8S-ITS2 by enzymatic digestion of the amplicons obtained by PCR represents one of the most reliable techniques used in yeast taxonomy, the dimension of the amplicons, the number and dimension of the restriction fragments being species-specific.

The genomic DNA isolated from the yeast strains Y-CMGB 64 and Y-CMGB 168 was amplified using the primers ITS1 and ITS4. The amplicons had 680 bp for Y-CMGB 64, respectively, 830 bp for Y-CMGB 168 and were further digested with three endonucleases (Figure 1). The size of the restriction fragments was determined using the Quantity One program (Bio-Rad) (Table 1).

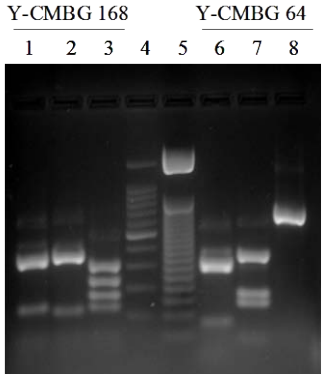


Figure 1. The PCR-RFLP profiles of the ITS1-5.8S-ITS2 regions of the strains Y-CMGB 168 and Y-CMBG 64 obtained with: 1, 6 - *Cfo* I; 2, 7 – *Hinf* I; 3, 8 – *Hae* III; 4 – 100 bp DNA Ladder (Promega); 5 – 50 bp DNA Step Ladder (Promega)

The restriction fragments number and size were compared with those from the scientific literature. As a result, high similar profiles were observed for the strain Y-CMGB 168 and *S. cerevisiae* strains, respectively, for the strain Y-CMBG 64 and strains from *Kluyveromyces* genus, especially *K. lodderae* [27; 28; 29].

Table 1. The size of the amplicons and restriction fragments of the ITS1-5.8S-ITS2 regions of the strains Y-CMGB 168 and Y-CMBG 64

Strain	Amplicon (bp)	Restriction fragments (bp)		
		Cfo I	*Hinf* I	*Hae* III
Y-CMGB 168	830	120, 320	110, 330	120, 170, 230, 280
Y-CMBG 64	680	80, 280, 290	130, 180, 330	670

In order to verify these results, we performed a comparative analysis using *Hinf* I endonuclease and amplicons from the strains Y-CMGB 168, Y-CMBG 64 and reference strains *K. lactis* CBS 2359/152 and *S. cerevisiae* ATCC 201583. The restriction patterns from Figure 2 confirmed our previous molecular identification: the strain Y-CMGB 168 belongs to *S. cerevisiae*, while the strain Y-CMBG 64 belongs to the *Kluyveromyces* genus.

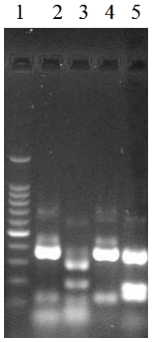


Figure 2. The comparative PCR-RFLP analysis of the strains Y-CMGB 168, Y-CMBG 64 and reference strains using *Hinf* I: 1 - 100 bp DNA Ladder (Promega); 2 - *S. cerevisiae* ATCC201583; 3 - *K. lactis* CBS2359/152; 4 - Y-CMGB 168; 5 - Y-CMBG 64

Both strains CMBG 64 and CMGB 168 belong to species with high potential of antimicrobial activity. Therefore, we performed a RAPD assay in order to obtain a discriminatory characterization of our strains in the presence of other yeast strains with similar abilities. The primer M13 was chosen due to its frequent utilization in RAPD analyses for a large range of yeast species from *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula* and *Saccharomyces* genera [30; 31; 32; 33; 34].

From the analysis of the RAPD-PCR profiles (Figure 3) we could observe the appearance of a total number of 15 bands presenting 100% polymorphism. The species *M. pulcherrima* and *R. glutinis* are frequently used for biocontrol [35; 36; 37; 38]. Moreover, the strain *M. pulcherrima* CMGB-SG1 was reported as having high antimicrobial abilities against strains of fungi and yeasts with pathogenic potential for humans [39, 40]. Also, it is interesting to outline that the polymorphic patterns obtained by RAPD were observed not only for strains belonging to different species, but also for conspecific strains: Y-CMGB 168 and *S. cerevisiae* ATCC 201583.

As a consequence, we consider that the M13 primer can be successfully used not only for inter- but also for intraspecific identification of our strains.

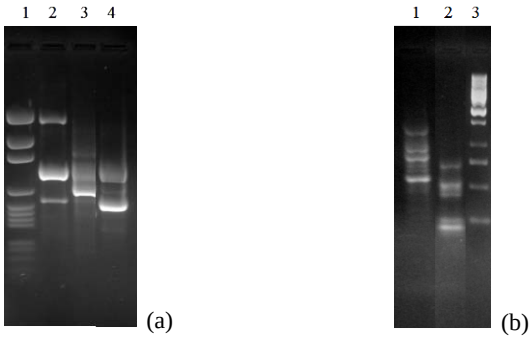


Figure 3. The RAPD profiles obtained with the primer M13 for:
(a) 1 – pGEM DNA Markers (Promega); 2 - Y-CMBG 64; 3 - Y-CMGB 168; 4 – *R. glutinis* RG4 ; (b) 1 – *M. pulcherrima* CMGB-SG1; 2 – *S. cerevisiae* ATCC201583; 3 - Gene Ruler 1Kb DNA Ladder (Fermentas)

Since the two yeast strains Y-CMGB 64 and Y-CMGB 168 were isolated from pharmaceutical industry, it was interesting to test their potential antimicrobial activity. The antimicrobial activity, both for *S. cerevisiae* and *Kluyveromyces* species is due to the presence in the cytoplasm of the killer system [41; 42; 43] and production of extracellular proteins (killer toxins).

The strain Y-CMGB 168 showed medium killer activity against *C. glabrata* CMGB 35 in contrast to the strain Y-CMGB 64 which exhibited no killer action in the same conditions. When plated against *C. krusei* CMB 94 and *S. cerevisiae* 17/17, the strain Y-CMGB 64 reduced the growth of the sensitive cells (Figure 4a and 4b).

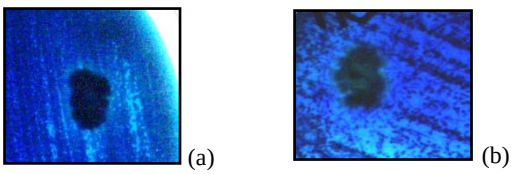
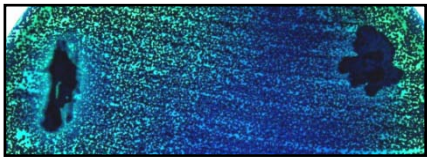


Figure 4. The killer activity of the strain Y-CMBG 64 after eight days in the presence of (a) *C. krusei* CMGB 94 at 28°C, (b) *S. cerevisiae* 17/17 at 25°C


(a)

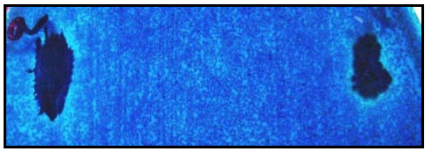

(b)

Figure 5. The killer activity of the strain YCMGB 168 (left) and Y-CMBG 64 (right) against *C. tropicalis* CMGB 165 after eight days of incubation at (a) 25°C and (b) 28°C

Both strains had good killer activity in the presence of *C. tropicalis* CMGB 165 forming clear inhibition zones sorrounding the colonies (Figure 5).

It is interesting to emphasize the influence of the incubation temperature on the killer activity. Thus, the strain Y-CMGB 64 exhibited a higher activity when incubated at 28°C compared to the strain Y-CMGB 168. On the other hand, the incubation temperature seemed to have no influence on the activity of the strain Y-CMGB 168 (Figure 5). An explanation might reside in the fact that the killer toxins described until present for *K. lactis* strains have chitinase and glucanase activity [45; 46] being thus more active on *Candida* cells making the transition from the yeast form to the pseudohyphae form [47; 48; 49]. Also, the *K. lactis* killer toxins are active and stable at a high range of temperature from 22 to 40°C [50], while the *S. cerevisiae* killer toxins are more active and stable at lower temperatures (20-25°C). The results of the killer assays are presented in Table 2.

The identification of killer activity for the strain Y-CMGB 64 identified as *K. lodderae* represents an important results of our study. Although *K. lodderae* is known as being a yeast species found in dairy products like kefir [51; 52], until present we could not find any data regarding its antimicrobial / killer activity.

Table 2. The killer activity after eight days of incubation for the strains Y-CMBG 64 and Y-CMGB 168

Potential sensitive strain	Incubation temperature (°C)	Y-CMBG 64	Y-CMGB 168
C. albicans ATCC 10231	25	-	-
	28	-	-
C. krusei CMGB 94	25	-	-
	28	+	-
C. glabrata CMGB 35	25	-	+
	28	-	+
C. tropicalis CMGB 165	25	+	++
	28	++	++
S. cerevisiae 17/17	25	+	-
	28	-	-

- = no activity, + = medium, ++ = good activity

4. CONCLUSIONS

Two yeast strains Y-CMGB 64 and Y-CMGB 168 were identified using the analysis of the ITS1-5.8S-ITS2 region as belonging to *Kluyveromyces lodderae*, respectively, to *Saccharomyces cerevisiae*. The genomic DNA for the PCR amplifications was isolated using a rapid and simple technique. The comparative RAPD analysis with the primer M13 allowed a successful molecular identification of our strains both at inter- and intraspecific level. The strain Y-CMGB 64 presented a more active killer activity at 28°C against *Candida* strains, probably due to the thermal stability of the *Kluyveromyces* killer toxins and their chitinase and glucanase activity. To our knowledge, this is the first report concerning the killer activity of a *K. lodderae* strain, which might represent an important starting point for its applications in medicine, obtaining probiotic compounds and biocontrol. Finally, the two yeast strains were re-named and included in the CMGB collection as *K. lodderae* CMBG 64 and *S. cerevisiae* CMGB 168.

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