ANTI-*CANDIDA* ACTIVITY OF *KLUYVEROMYCES LACTIS* CMGB 226 FROM DAIRY PRODUCTS

Ortansa CSUTAK*, Ioana CÎRPICI, Robertina IONESCU, Ileana STOICA, Tatiana VASSU

Department of Genetics, Faculty of Biology, University of Bucharest, 060101 Bucharest, Romania

*Corresponding author: cs_ortansa@hotmail.com

Abstract: Yeast killer toxins represent an interesting field of study not only for food preservation, but also for development of new bioproducts with biomedical applications. The yeast strain *Kluyveromyces lactis* CMGB 226 from dairy products was identified using PCR-RFLP on ITS1-5.8S rDNA-ITS2 region. The *Hae* III and *Hinf* I restriction profiles of the 675 bp amplicon were similar to those from reference yeast strains. The RAPD analysis using the primer M13 assured the best discriminatory inter and intraspecific characterization of our strain. Killer screening tests were positive against five *Candida* species, the highest response being observed against *C. parapsilosis* CBS 604. The 10-fold concentrated killer toxin had a good thermostability and was more active when resuspended in citrate-phosphate buffer with pH from 5.0 to 6.2. The correlation of pH with temperatures of 22, respectively, 28^{o}C represented an important fungistatic factor. Longer treatments with the toxin caused a decline in the viability of *C. parapsilosis* CBS 604 sensitive cells, with an augmented effect at 28^{o}C after 24 hours. The combination of conditions near to human body favors the killer toxin activity, representing a potential benefit for its future biomedical use against pathogenic *Candida* strains.

Key words: *Kluyveromyces*, *Candida*, killer toxin, viability, 5.8S rDNA, RAPD

Introduction

The yeast species from diary products belong to the genera *Candida*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Pichia*, *Saccharomyces* and *Yarrowia*, their presence depending on the type of product analyzed. In general, these species are able to ferment or to assimilate lactose and galactose using the Leloir pathway and to grow at low temperatures or high concentrations of lactic acid due, mainly, to their co-existance with the lactic acid bacteria (Jacques and Casaregola, 2008). Most of these species present antimicrobial activities, can be used for obtaining probiotic products and some of the food additives derived from their metabolism are considered as GRAS (*Generally Recognized As Safe*).

Kluyveromyces lactis, initialy known as *Saccharomyces lactis* then *Zygosaccharomyces lactis* and finally *Kluyveromyces lactis* (Dombrowski) (Kurtzman *et al.*, 2011), can be found in milk, yoghourt, cheese or kefir. Due to the high verstatility of its metabolic pathways and the recent

 Received November 1, 2015 / Revised November 16, 2015/Accepted November 17, 2015

information on the nuclear and extranuclear genome structure, *K. lactis* became one of the most important non-conventional yeast species used in biotechnology as host for production of heterologous proteins of industrial and therapeutic interest such as α-amylase, celullase, xylanase, cow prochymosine, growth hormone, human insuline precursor, lysozyme and seric albumine (van Ooyen *et al.*, 2006).

The nuclear genome of *K. lactis* has been studied by the Génolevures Group (http://www.genolevures.org/klla.html) and data concerning the nucleotide sequence of the mitochondrial genome was published (Zivanovich *et al.*, 2005). Although it is not characteristic, the nuclear pKD1 2 μm-like plasmid from *K. drosophilarum* can be transfered and maintained in *K. lactis* cells.

The killer toxin (zymocine) produced by *K. lactis* is a glycoproteic complex which affect the sensitive cells by arresting the cell cycle in G1. The killer phenotype is determined by the presence in the cytoplasm of two types of DNA plasmids named pGKL1 (8.8 kbp) and pGKL2 (13.4 kbp), isolated and described for the first time by Gunge *et al.* (1981). The two plasmids code for the killer toxin and the immunity factor, respectively, for DNA replication, transcription and mRNA modification (Schikel *et al.*, 1996; Schraffrath and Breunig, 2000; Schraffrath and Meinhardt, 2005).

The killer activity of *K. lactis* cover a wide spectrum of yeast species (*Saccharomyces*, *Candida*, *Kluyveromyces*) from various environments, from plants and food products to human infections. Therefore, the identification of new strains producing killer toxins and the study of their mode of action is important both for industrial and medical use. The present article deals with the molecular identification of a new yeast strain *Kluyveromyces lactis* CMGB 226 from dairy products and the characterization of its killer activity. We also investigate the corroborated effect of pH and temperature on the anti-*Candida* activity of the killer toxin.

Material and methods

Yeast strains

The yeast strain CMGB 226 (the Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania) was isolated from raw cheese and milk. Other strains used during this study were: *Candida albicans* ATCC 10231, *Candida glabrata* CMGB 35, *Candida glabrata* CMGB 62, *Candida guilliermondii* CMGB 44, *Candida krusei* CMGB 94, *Candida parapsilosis* CBS 604, *Candida parapsilosis* CMGB 79, *Candida tropicalis* CMGB 165, *Candida zeylandoydes* CMGB 166, *Kluyveromyces lactis* CMGB 230, *Kluyveromyces lactis* CBS 2359/152 (derived from *K. lactis* CBS 2359: K^+R^+ a met^- [K1, K2]^{2359}) (Wesolowski *et al.*, 1982) and *Kluyveromyces lodderae* CMGB 64. The yeasts were grown and maintained on yeast peptone glucose (YPG) medium (yeast extract 5 g/L, peptone 10 g/L, glucose 20 g/L).

PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region

The genomic DNA of the strains CMGB 226, *K. lactis* CBS 2359/152, *K. lactis* CMGB 230 and *K. lodderae* CMGB 64 was isolated according to Csutak *et al.* (2012) and used further for RFLP-PCR and RAPD analyses.

The ITS1-5,8S rDNA-ITS2 region was amplified (Biometra TGradient cycler) in a total volume of 50 μL PCR mixture using GoTaq Green Master Mix 2X (Promega) and 1,2 μM of each ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as decribed previously (Csutak *et al.*, 2014). The program used comprised: initial denaturation 5 min at 94°C, 40 cycles of 1 min at 94°C, 30 sec at 55°C, 2 min at 72°C, and a final extension 5 min at 72°C.

The PCR products were digested for 90 min with 0.5 μL of *Hae* III (5'-GG/CC-3'), respectively, *Hinf* I (5'-G/ANTC-3') (10U/μL, Promega). The amplicons and the restriction fragments were separated by electrophoresis using 1.2% agarose and TBE 0.5X. The size of the amplicons and restriction fragments was evaluated with the computerized program Quantity One (Bio-Rad), using as reference the size of the fragments from BenchTop 100 bp DNA Ladder (Promega).

RAPD assay

The RAPD assay was performed in a total reaction volume of 25 µL using GoTaq Green Master Mix 2X (Promega), 9 to 12 ng genomic DNA and 1 µM of each of the following primers: OPA03 (5'-AGTCAGCCAC-3'), OPB17 (5'-AGGGAACGAG-3') and M13 (5'-AGGGTGGCGGTTCT-3'). The amplification program was: initial denaturation 5 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, and a final extension of 10 minutes at 72°C (Csutak *et al.*, 2014). The RAPD fragments were separated in 1.2 % agarose gels using TBE 0.5X.

Screening for the killer activity

For the screening assays colonies from overnight (o/n) grown culture of the strain CMGB 226 and was spotted on Petri plates with agar K medium (0.1 M phosphate citrate buffer pH 4.8, 20 g/L glucose, 10 g/L yeast extract, 20 g/L agar, 0.3 g/L methylene blue) inoculated with an overlay of 10^7 cells/mL of potential sensitive yeast strains: *C. albicans* ATCC 10231, *C. glabrata* CMGB 35, *C. glabrata* CMGB 62, *C. krusei* CMGB 94, *C. guilliermondii* CMGB 44, *C. parapsilosis* CBS 604, *C. parapsilosis* CMGB 79, *C. tropicalis* CMGB 165 and *C. zeylandoydes* CMGB 166. The plates were incubated for seven days at 22°C (Vassu *et al.*, 2001). The response was considered positive when the colonies were surrounded by a clear blue zone or a zone with reduced growth of the potential sensitive strain.

Production of the killer toxin concentrate

The killer toxin concentrate was obtained using a method addapted after Baeza *et al.* (2008) and Parveen and Begum (2010). Thus, the yeast strain CMGB 226 was grown o/n at 28°C and 150 rpm, and centrifuged for 10 min at 8000 rpm at 4°C. The supernatant was filtered through 0,22 µm size pore filter (Millipore). The filtrate was incubated for 1 hour at 4°C for the precipitation and crystalization of the killer toxin and then centrifuged for 20 min at 12000 rpm at 4°C. The sediment was re-suspended in small volumes (in order to obtain a 10-fold concentrated killer toxin) of citrate-phosphate buffer with different pH values: 4.4, 5.0, 5.6 and 6.2. The toxin was stored at 4°C until used.

Influence of pH and temperature on the killer activity

Petri plates were prepared using agar K medium with various pH values (4.4, 5.0, 5.6 and 6.2) seeded with 10^7 cells/mL from an o/n YPG cultures *C. parapsilosis* CBS 604. For the well test assays, 80 µL of concentrated killer toxin were loaded into 8 mm-diameter wells cut in the agar plates K medium. The plates were incubated for seven days at 22, 28 and 37°C.

The killer toxin activity was measured according to De Igeniis *et al.* (2009) and Ígzü *et al.* (2006). Thus, one unit of enzymatic activity (AU) was identified as the amount of toxin contained in 80 µL forming a growth inhibition halo of 8 mm.

Growth rate reduction assay

The strain *C. parapsilosis* CBS 604 was grown o/n in YPG medium. Sensitive *C. parapsilosis* CBS 604 cells (10^7 cells/mL) were mixed with 10, 20 or 35 µL killer toxin with pH 4.4, 5.0, 5.6 and 6.2 and placed in microtitre wells in a final volume of 150 µL. The microplates were incubated at 22 and 28°C for 48 hours. The cell growth was evaluated at 0 h and at 2-hours intervals up to 8 hours, then at 24, respectively 48 hours, by reading the OD at 480 nm using an automatic plate reader (Multi-mode reader Synergy HTK, BioTek) (after Hodgson *et al.*, 1995; Ciani and Fatichenti, 2001).

Results and discussions

Molecular characterization

The molecular identification of the strain CMGB 226 was done using the PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region. The DNA extracts from CMGB 226 and other *Kluyveromyces* reference strains were amplified with the primers ITS1 and ITS4 and the amplicons were digested with *Hae* III and *Hinf* I (Figure 1).

The amplicon of the strain CMGB 226 had 675 bp and the restriction profile obtained with the endonuclease *Hae* III was identical with *K. lactis* CBS2539/152 (Table 1). A high degree of similarity was obtained when *Hinf* I was used for digestion of CMGB 226, *K. lactis* CMGB230 and *K. lactis* CBS2539/152 amplicons. We could also observe the

 Received November 1, 2015 / Revised November 16, 2015/Accepted November 17, 2015

RESEARCH ARTICLE

resemblance between the sizes of the amplicons, respectively, the *Hinf* I patterns from CMGB 226 and *K. lodderae* CMGB 64, two taxonomically related species.

Table 1. *The size of the amplicons and restriction fragments of the ITS1-5.8S-ITS2 regions*

Strain	Amplicon (bp)	Restriction fragments (bp)	
		***Hae* III**	***Hinf* I**
CMGB 226	675	70, 620	60, 115, 180, 270
K. lactis CMGB 230	710	710	60, 120, 185, 270
K. lactis CBS 2539/152	700	70, 620	70, 120, 190, 305
K. lodderae CMGB 64	680	670	130, 180, 330

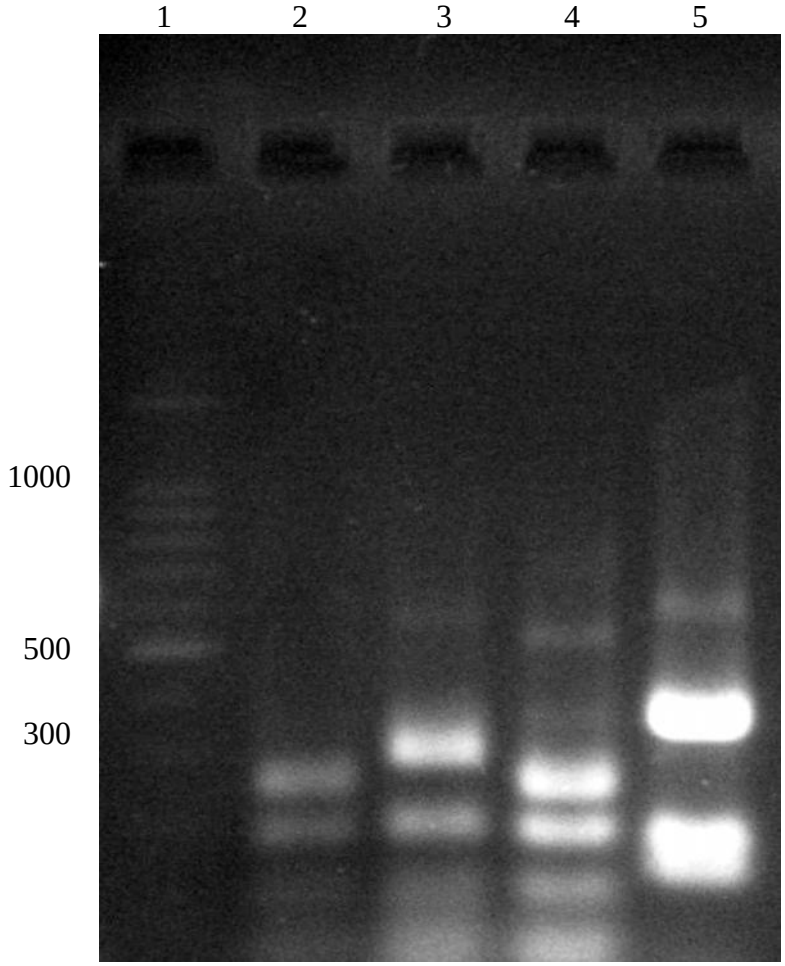


Figure 1. *The PCR-RFLP profiles of the ITS1-5.8S-ITS2 amplicons digested with Hinf I*

(1– BenchTop 100 bp DNA Ladder (Promega); 2- CMGB 226;

3- *K. lactis* CBS 2359/152; 4 – *K. lactis* CMGB 230; 5 – *K. lodderae* CMGB 64)

Moreover, our results matched with those from other studies (Esteve-Zarzoso *et al.*, 1999; Alvarez-Martin *et al.*, 2007; Bockelmann *et al.*, 2008), confirming thus the belonging of our strain CMGB 226 to *Kluyveromyces* genera, specifically, to *Kluyveromyces lactis*.

In order to achieve a discriminatory characterization of newly identified *K. lactis* CMGB 226, we

performed a RAPD analysis using three primers OPA03, OPB17 and M13, frequently mentioned in similar works (Prillinger *et al.*, 1999; Bujdosó *et al.*, 2001; Binetti *et al.*, 2013; Gomes *et al.*, 2003).

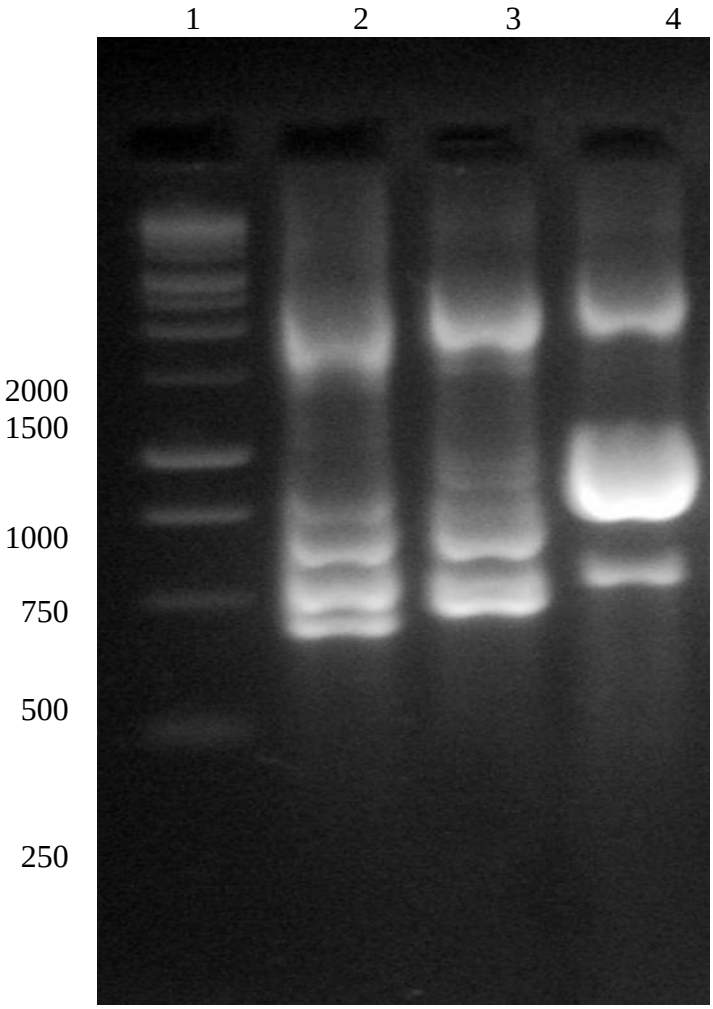


Figure 2. *The RAPD amplicons obtained using the M13 primer*

(1- BenchTop 1 kb DNA Ladder (Promega); 2 - *K lactis* CMGB 226; 3- *K lactis* CBS 2359/152; 4- *K. lodderae* CMGB 64)

In the case of *K lactis* CMGB 226 and *K lactis* CBS 2359/152 we obtained a total number of 30 bands corresponding to amplification products, from which 12 were obtained with OPA03 and 9 with each primer OPB17 and M13 (Figure 2, lanes 2 and 3). Although the polymorphic frequency was identical for OPB17 and M13 (78%) (Table 2), we decided to use M13 for further comparative interspecific characterization due to its more often application in RAPD analyses on strains from various yeast species or genera (Latouche *et al.*, 1997; Wuczowski and Prillinger, 2004; Andrade *et al.*, 2006).

Table 2. *Number of amplified bands and polymorphic frequency for PCR-RAPD studies*

Primer	Amplified bands	Common bands	Polymorphic frequency (%)
OPA03	12	4	67
OPB17	9	2	78
M13	9	2	78

From a total of 12 bands (Figure 2) no common amplified bands could be identified for *K lactis* CMGB 226 and the reference strain *K. lodderae* CMGB 64. This allows us to conclude that the primer M13 yields a 100% polymorphic frequency and can be successfully used for both inter and intraspecific characterization of *Kluyveromyces* strains.

 Received November 1, 2015 / Revised November 16, 2015/Accepted November 17, 2015

Screening for the killer activity. Effect of pH and temperature on the activity of the killer toxin

The screening assays revealed a high killer activity of the strain *K. lactis* CMGB 226 against most *Candida* species tested after seven days of incubation, except for *C. albicans* ATCC 10231, *C. guilliermondii* CMGB 44 and *C. zeylandoydes* CMGB 166. The most significant activity represented by 6 mm wide halos was observed in the case of *C. parapsilosis* strains CBS 604 and CMGB 79.

After the first three days, a weak halo of approximately 2 mm surrounding *K. lactis* CMGB 226 colonies appeared on the plates seeded with *C. parapsilosis* CBS 604 (Table 3), indicating a rapid killer action. Therefore, our subsequent studies regarding the fungistatic / fungicidal mechanism of action of *K. lactis* CMGB 226 killer toxin were performed using *C. parapsilosis* CBS 604 cells as sensitive substrate.

Table 3. *The killer activity of the strain K. lactis CMGB 226 recorded after three, respectively, seven days of incubation at 22°C*

Sensitive strains	Killer activity after 3 days of incubation	Killer activity after 7 days of incubation
C. albicans ATCC 10231	-	-
C. glabrata CMGB 35	-	+
C. krusei CMGB 94	-	+
C. guilliermondii CMGB 44	-	-
C. parapsilosis CBS 604	+	+++
C. parapsilosis CMGB 79	-	+++
C. tropicalis CMGB 165	-	+
C. zeylandoydes CMGB 166	-	-

+++ significant, 6 mm halo; ++ good, 4 mm halo; + weak, 2 mm halo; - no activity

The toxin isolated and concentrated from *K. lactis* CMGB 226 showed a significant activity at pH 6.2 and 22°C (AU 94) and best results (AU 125) at 28°C (Table 4). In fact, using citrate-phosphate buffer with pH 6.2, 5.6 and 5.0 seemed to assure, in general, a better activity of the toxin, compared with the pH 4.4 buffer.

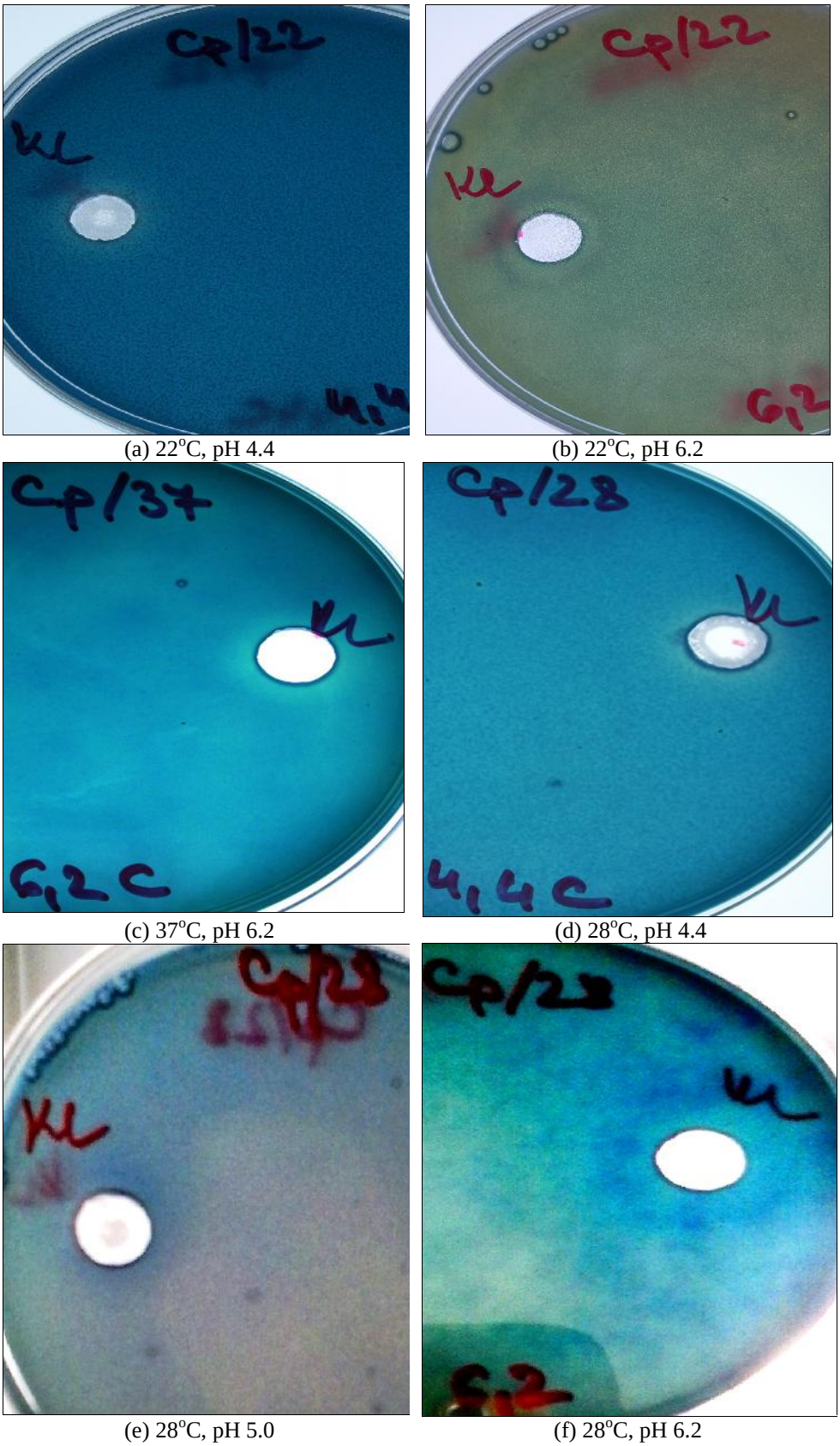
Table 4. *Influence of pH and incubation temperature on the activity of K. lactis CMGB 226 killer toxin against C. parapsilosis CBS 604 after 7 days of incubation*

pH	Temperature (°C)	Killer activity
4.4	22°C	++
	28°C	+++
	37°C	-
5.0	22°C	+++
	28°C	+++
	37°C	-
5.6	22°C	+++
	28°C	+++
	37°C	+
6.2	22°C	+++
	28°C	++++
	37°C	++

++++ high, 8 mm halo; +++ significant, 6 mm halo; ++ good, 4 mm halo; - no activity

It is interesting to notice that the correlation of the pH with the temperature represented an important factor for the activity of the toxin (Table 4, Figure 3). While the incubation at 28°C lead to similar killer activities for pH 5.0 and 5.6 (AU 94), the temperature of 37°C had a clear positive influence when correlated with more alkaline pH values, 5.6 and 6.2.

According to these results, the killer toxin from *K. lactis* CMGB 226 was more active for the pH range 5.0 to 6.2, with a good thermostability, fact also described during studies on other *K. lactis* strains (Sugisaki *et al.*, 1984; Panchal *et al.*, 1985; Meinhardt and Klassen, 2009). Although, the killer activity of other yeast species against *Candida* strains at 37°C has been previously investigated (Buzzini and Martini, 2001), to our knowledge, this is the first work to present the correlated effect of the pH and temperature on the activity of the *K. lactis* toxin against *C. parapsilosis*.



(a) 22°C, pH 4.4 (b) 22°C, pH 6.2

(c) 37°C, pH 6.2 (d) 28°C, pH 4.4

(e) 28°C, pH 5.0 (f) 28°C, pH 6.2

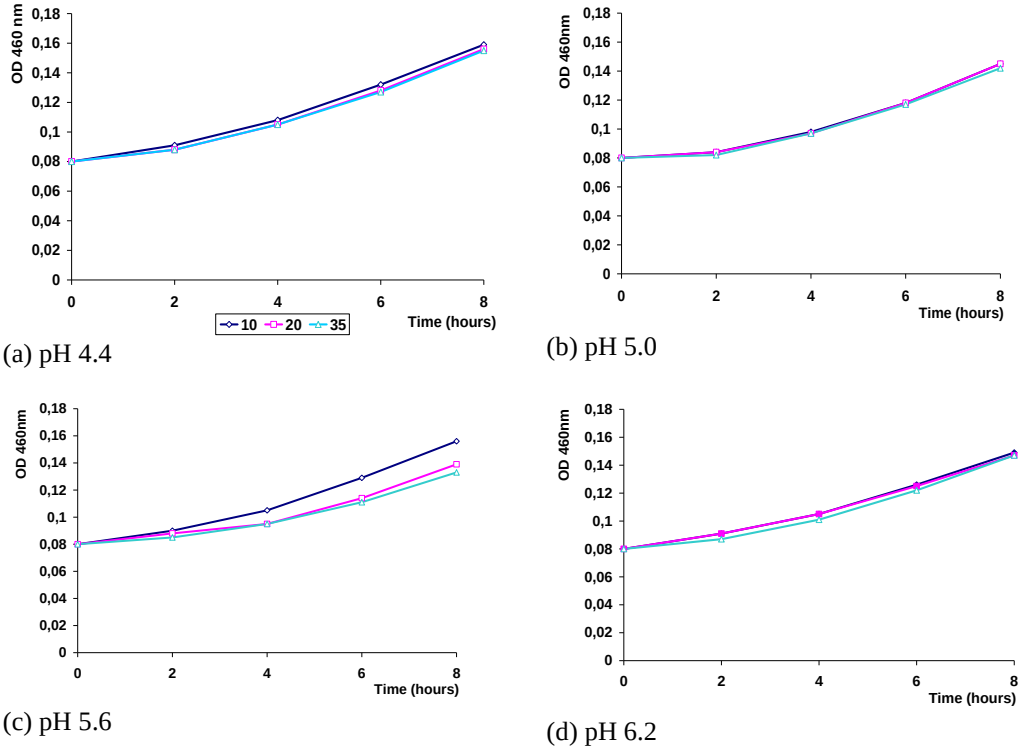
Figure 3. Halos produced by *K. lactis* CMGB 226 killer toxin against *C. parapsilosis* CBS 604, after seven days of incubation

 Received November 1, 2015 / Revised November 16, 2015/Accepted November 17, 2015

Growth kinetics of C. parapsilosis CBS 604 in presence of K. lactis CMGB 226 toxin

The screening tests showed that the *K. lactis* CMGB 226 toxin had a visible action against *C. parapsilosis* CBS 604 beginning with the third day of incubation at 22°C. From Table 4 we can also see that good results were obtained for all the pH values tested for the concentrated killer toxin, both at 22 and 28°C. Therefore, our further experiments aimed to determine the mode of action and lethal effect of *K. lactis* CMGB 226 toxin on *C. parapsilosis* CBS 604 sensitive cells within the first 48 hours of incubation, using toxin concentrates with pH from 4.4 to 6.2 and temperatures of 22 and 28°C.

The toxin was mixed with the *C. parapsilosis* CBS 604 culture in three concentrations: 10, 20 and 35 µL. The cell growth was reduced especially in the presence of 35 µL toxin with pH 5.6 and 5.0, the OD recorded after eight hours being 0.133, respectively, 0.142 (Figure 4 b and c, Figure 5). The fungistatic action of different toxin concentrations on the growth curve of *C. parapsilosis* CBS 604 was obvious for pH 5.6: OD 0.156 for 10 µL toxin, 0.139 for 20 µL and 0.133 for 35 µL (Figure 4 c). A good inhibitory effect was also detected at pH 6.2, although the influence of different amounts of toxin on the growth kinetics was more difficult to detect (OD 0.149 for 10 µL toxin, 0.147 for both 20 and 35 µL toxin).



(a) pH 4.4 (b) pH 5.0

(c) pH 5.6 (d) pH 6.2

Figure 4. Effect of different pH values and concentrations of *K. lactis* CMGB 226 killer toxin on the viability of *C. parapsilosis* CBS 604 cells at 22°C

The toxin concentrate had a reduced inhibitory effect on *C. parapsilosis* CBS 604 within the first eight hours at 22°C at pH 6.2 compared with pH 5.6 or 5.0 (Figure 5). However, the halos observed at 22°C after seven days (Table 4) indicated similar activities for all these three pH values, while an augmented activity was obtained at a pH 6.2 by incubation at 28°C. This can be explained by a comparative experiment. Thus, although a reduced viability of *C. parapsilosis* CBS 604 cells was observed at 22°C in the first eight hours (Figure 6 a and b), the situation began to change after 24 hours when the fungistatic effect was slightly augmented at 28°C (OD 0.715) compared to 22°C (OD 0.755) (Figure 6 b). The shift between the profiles of the growth curves continued to be maintained during the next period, so that after 48 hours the OD was 1.02 at 28°C, respectively, 1.12 at 22°C.

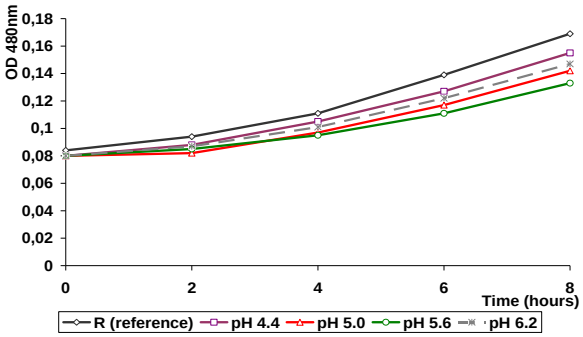


Figure 5. Growth inhibition of *C. parapsilosis* CBS 604 by *K. lactis* CMGB 226 killer toxin at 22°C

In their study regarding the response of sensitive cells from various *S. cerevisiae* strains to the killer toxin of *K. lactis* IFO 1267, Butler *et al.* (1991a) showed that the activity of the purified toxin was high at 22 and 35°C and good, having similar values at pH 5.5 and 6.5. Also, the experiments revealed that the viability of the sensitive cells continued to decline after longer treatments with the killer toxin.

Our results are in agreement with these findings, with the exception of the incubation temperature of 28°C which seems to represent a favorable condition for the activity of *K. lactis* CMGB 226 toxin against *C. parapsilosis* CBS 604 cells. We assume that this is due to modifications that affect the composition of the cell wall of *Candida* species in time under various environmental conditions. As stated in previous studies (Butler *et al.*, 1991b; Magliani *et al.*, 1997; Magliani *et al.*, 2008) chitin acts as first receptor for the *K. lactis* toxin on the surface of the sensitive cells causing cell cycle arrest in G1. According to Chaffin *et al.*, (1998) *Candida* hyphal cells contain almost three times more chitin as yeast cells. Also, pH near the neutral value and higher temperatures are favorable to pseudohyphal / hyphal transition in *Candida* cells (Sudbery, 2011). Therefore, the *Candida* cells making the transition from yeast to pseudohyphae are more susceptible on the activity of the *K. lactis* killer toxin, fact that might explain the results obtained during the present work.

In conclusion, the yeast strain *K. lactis* CMGB 226 from dairy products was characterized at molecular level and the killer assays revealed a good activity against five *Candida* species. The concentrated killer toxin was tested using *C. parapsilosis* CBS 604 sensitive cells and the fungistatic effect was high at pH values from 5.0 to 6.2. This is the first work to present the correlated action of the pH and the incubation temperatures on the killer activity of a yeast strain. Moreover, we noticed a good fungicidal action of the *K. lactis* CMGB 226 toxin against *C. parapsilosis* in conditions close to those of the human body, i.e. almost neutral pH (6.2) and high temperature (37°C). This might represent an important benefit for future applications of the toxin as fungistatic/fungicidal agent against potential pathogen strains belonging to the *Candida* genus.

Acknowledgments

This work was supported by CNFIS-UEFISCDI project PCCA number 77/2012.

References

Alvarez-Martin P., Belén Flórez A., López-Diaz T., Mayo B. (2007) Phenotypic and molecular identification of yeast species associated with Spanish blue-veined Cabrales cheese, International Dairy Journal, 17, 961–967.

Andrade M.J., Rodríguez M., Sanchez B., Aranda E., Córdoba J.J. (2006) DNA typing methods for differentiation of yeasts related to dry-cured meat products, International Journal of Food Microbiology, 107, 48 – 58.

Baeza M.E., Sanhueza M.A., Cifuentes V.H. (2008) Occurrence of killer yeast strains in industrial and clinical yeast isolates, Biological Research, 41, 173-182.

Binetti A., Carrasco M., Reinheimer J., Suárez V. (2013) Yeasts from autochthonal cheese starters: technological and functional properties, Journal of Applied Microbiology, 115, 434-444.

Bockelmann W., Heller M., Heller K.J. (2008) Identification of yeasts of dairy origin by amplified ribosomal DNA restriction analysis (ARDRA), International Dairy Journal, 18, 1066–1071.

Bujdosó G., Egli C.M., Henick-Kling T. (2001) Characterization of *Hanseniaspora* (*Kloeckera*) strains isolated in Finger Lakes wineries using physiological

 Received November 1, 2015 / Revised November 16, 2015/Accepted November 17, 2015

and molecular techniques**,** Food Technology and Biotechnology, 39, 83–91.

Butler A., White J.H., Stark M.J.R. (1991a) Analysis of the response of *Saccharomyces cerevisiae* cells to *Kluyveromyces lactis* toxin, Journal of General Microbiology, 137, 1749-1757.

Butler A.R., O'Donnell R.W., Martin V.J., Gooday G.W., Stark M.J.R. (1991b) *Kluyveromyces lactis* toxin has an essential chitinase activity, European Journal of Biochemistry, 199, 483-488.

Buzzini P., Martini A. (2001) Large-scale screening of selected *Candida maltosa*, *Debaryomyces hansenii* and *Pichia anomala* killer toxin activity against pathogenic yeasts, Medical Mycology, 39, 479-482.

Chaffin W. L., López-Ribot J.L., Casanova M., Gozalbo D., Martínez J.P. (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression, Microbiology and Molecular Biology Reviews, 62, 130-180.

Ciani M., Fatichenti F. (2001) Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine yeasts, Applied and Environmental Microbiology, 67, 3058-3063.

Csutak O., Ghindea R., Stoica I., Tanase A-M., Vassu T. (2012) Identification of two yeast strains from oil-polluted environment by RFLP on ITS-5.8S rDNA and RAPD analysis, Roumanian Biotechnology Letters, 17, 6913-6920.

Csutak O., Stoica I., Vassu T. (2014) Molecular identification and antimicrobial activity of two new *Kluyveromyces lodderae* and *Saccharomyces cerevisiae* strains, Biointerface Research in Applied Chemistry, 4, 873-878.

De Ingeniis J., Raffaelli N., Ciani M., Mannazzu I. (2009) *Pichia anomala* DBVPG 3003 secretes a ubiquitin-like protein that has antimicrobial activity, Applied and Environmental Microbiology, 75, 1129–1134.

Esteve-Zarzoso B., Belloch C., Uruburu F., Querol A. (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers, International Journal of Systematic Bacteriology, 49, 329–337.

Gomes L.H., Echeverrigaray S., Conti J.H., Laurenço M.V.M., Duarte K.M.R. (2003) Presence of the yeast *Candida tropicalis* in figs infected by the fruit fly *Zaptionus indianis* (Dip: *Drosophilidae*), Brazilian Journal of Microbiology, 34, 5-7.

Gunge N., Tamaru A., Ozawa F., Sakaguchi K. (1981) Isolation and characterization of linear deoxyribonucleic acid plasmids from *Kluyveromyces lactis* and the plasmid-associated killer character, Journal of Bacteriology, 145, 382-390.

Hodgson V.J., Button D., Walker G.M. (1995) Anti-Candida activity of a novel killer toxin from the yeast *Williopsis* mrakii, Microbiology, 141, 2003-2012.

Ígzü F., Altınbay D., Acun T. (2006) Killer toxin of *Pichia anomala* NCYC 432; purification, characterization and its *exo*-β-1,3-glucanase activity, Enzyme and Microbial Technology, 39, 669–676.

Jacques N., Casaregola S. (2008) Safety assessment of dairy microorganisms: The hemiascomycetous yeasts, International Journal of Food Microbiology, 126, 321–326

Kurtzman C.P., Fell J.W., Boekhout T. (2011) The Yeasts. A Taxonomic Study. 5th Edition, Elsevier, USA.

Latouche N.G., Daniel H-M., Lee O.C., Mitchell T.G., Sorrell T.C., Meyer W. (1997) Comparison of use of phenotypic and genotypic characteristics for identification of species of the anamorph genus *Candida* and related teleomorph yeast species, Journal of Clinical Microbiology, 35, 3171-3180.

Magliani W., Conti S., Gerloni M., Bertolotti D., Polonelli L. (1997) Yeast killer systems, Clinical Microbiology Reviews, 10, 369–400.

Magliani W., Conti S., Travassos L.R., Polonelli L. (2008) Minireview. From yeast killer toxins to antibiobodies and beyond, FEMS Microbiology Letters, 288, 1–8.

Meinhardt F., Klassen R.. (2009) Chapter 6: Yeast killer toxins: Fundamentals and applications, In: Anke T., Weber D. (Eds.): Physiology and genetics, 1st Edition The Mycota XV, Springer Verlag, Berlin, Heidelberg.

Panchal C.J., Meacher C., Van Oostrom J., Stewart G.G. (1985) Phenotypic expression of *Kluyveromyces lactis* killer toxin against *Saccharomyces* spp., Applied and Environmental Microbiology, 50, 257-260.

Parveen M.R, Begum A.J. (2010) Production and effect of killer toxin by *Saccharomyces cerevisiae* on sensitive yeast and fungal pathogens, International Journal of Pharmaceutical Sciences Review and Research, 3, 126-129.

Prillinger H., Molnar O., Eliskases-Lechner F., Lopandic K. (1999) Phenotypic and genotypic identification of yeasts from cheese, Antonie van Leeuwenhoek, 75, 267–283.

Schaffrath R., Breunig K.D. (2000) Genetics and molecular physiology of the yeast *Kluyveromyces lactis*, Fungal Genetics and Biology, 30, 173–190.

Schaffrath R., Meinhardt F., 2005, *Kluyveromyces lactis* zymocin and other plasmid-encoded yeast killer toxins, In: Microbial Protein Toxins, Manfred J. Schmitt, Raffael Schaffrath (Eds.). Springer-Verlag Berlin Heidelberg, germany, p.133-157

Schickel J., Helmig C., Meinhardt F. (1996) *Kluyveromyces lactis* killer system: analysis of cytoplasmic promoters of the linear plasmids, Nucleic Acids Research, 24, 1879–1886.

Sudbery P.E. (2011) Growth of *Candida albicans* hyphae, Nature Reviews Microbiology, 9, 737-748.

Sugisaki Y., Gunge N., Sakaguchi K., Yamasaki M., Tamura G. (1984) Characterization of a novel killer toxin encoded by a double-stranded linear DNA plasmid of *Kluyveromyces* lactis, European Journal of Biochemistry, 141,.241 -245.

van Ooyen A.J.J., Dekker P., Huang M., Olsthoorn M.M.A., Jacobs D.I., Colussi P.A., Taron C.H. (2006) Heterologous protein production in the yeast *Kluyveromyces lactis.* FEMS Yeast Research, 6, 381–392.

Vassu T., Stoica I., Csutak O., Musat F. (2001) Genetics of microorganisms and microbial genetic engineering. Theoretical aspects and laboratory techniques, Petrion, Bucharest.

Wésolowskil M., Algeri A., Go ffrini P., Fukuhara H. (1982) Killer DNA plasmids of the yeast *Kluyveromyces lactis.* I. Mutations affecting the killer phenotype, Current Genetics, 5, 191-197.

Wuczkowski M., Prillinger H. (2004) Molecular identification of yeasts from soils of the alluvial forest national park along the river Danube downstream of Vienna, Austria (‘‘Nationalpark Donauauen’’), Microbiological Research, 159, 263–275.

Zivanovich Y., Wincker P., Vacherie B., Bolotin-Fukuhara M., Fukuhara H. (2005) Complete nucleotide sequence of the mitochondrial DNA from *Kluyveromyces lactis.* FEMS Yeast Research, 5, 315–322.