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Pichia (Kodamaea) ohmeri CMGB-ST19 - A NEW STRAIN WITH COMPLEX BIOTECHNOLOGICAL PROPERTIES

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Abstract

The yeast species Pichia ohmeri has been described as far, as biocontrol agent. The present work deals with the complex characterization of a new yeast strain CMGB-ST19 isolated from the surface of jam roses (Rosa damascena) petals. The conventional taxonomy tests and the BiologMicroLog system allowed the identification of CMGB-ST19 as Pichia ohmeri. The results were confirmed using ITS-PCR, the strain being renamed as Pichia ohmeri CMGB-ST19. Various tests were performed for determination of its biotechnological properties. P. ohmeri CMGB-ST19 presented good lipolytic activity and hydrolysed tributyrin releasing the butyric acid with positive impact on human health. Moreover, the strain showed antimicrobial activity against 11 Candida and Saccharomyces strains in presence of low pH values. During the preliminary assays for bioethanol and biosurfactants synthesis, significant growth rates were recorded after 24 hours on molasses, respectively, after 72 hours on D-xylose when morphological changes were also observed related to cell stress. In conclusion, P. ohmeri CMGB-ST19 represents an interesting newly characterized yeast strain with high potential for a wide range of biotechnological applications.

Key words*: Pichia ohmeri, lipase, antimicrobial activity, molasses, D-xylose.*

INTRODUCTION

Kodamaea ohmeri belongs to the *Kodamaea* genus (class *Ascomycetae, Saccharomycetaeae* family) which includes species isolated both from environment and entomopathogenic organisms. *K. ohmeri* is well integrated in the new *Kodamaea* genus which forms both hat-shaped and spherical spores depending on the mating type. Subsequent, other *Kodamaea* species were described (*K. anthophila*, *K. kakaduensis*, *K. laetipori*, *K. nitidulidarum, K. transpacifica*) proving the necessity to form a different genus, derived from *Yamadazyma* which accommodate *Pichia* species that can form hat-shaped spores (Freitas et al., 2013; Kurtzman et al., 2011). *Kodamaea ohmeri*, formerly known as *Pichia ohmeri*, is isolated mostly from food industry being involved in fermentation of pickled products (Vivas et al., 2016). This species was also reported to be found in sand, marine environment and part of the microbiota of different living animals (Chakrabarti et al., 2014). Although some strains of *K. ohmeri* are known as being responsible for different types of opportunistic infections there are many studies regarding its biotechnological potential (Al-Sweih, 2011; Distasi et al., 2015; Kanno et al., 2017). In 2013, Kitcha et al reported good level of lipid production when *K. ohmeri* was grown on crude glycerol. *K. ohmeri* was also reported as presenting high degradation levels of patulin and recommended it for patulin detoxification of apple derived products. A new *K. ohmeri* strain isolated from fish gut had high phytase activity which can be used as supplement for animal and human nutrition to liberate the inorganic phosphate from phytic acid from grains, increasing thus their nutritional value (Hirimuthugoda et al., 2006; Li et al., 2007). Also, *K. ohmeri* is known as being able to convert glucose to D-arabitol. Zhu et al. (2010) reported a *K. ohmeri* strain isolated from honeybee hives and from osmotic natural environment able to produce high yields of D-arabitol from glucose.

The present study focuses on the preliminary identification of a new strain *K. ohmeri* strain (CMGB-ST19) isolated from jam roses (*Rosa damascena*) petals and on the investigation of its biotechnological potential.

MATERIALS AND METHODS

Biological material

The yeast strain CMGB-ST19 was isolated from the surface of jam roses (*Rosa damascena*) petals from Bucharest Botanical Garden (Romania) and maintained in a Revco LegaciTM Refrigeration System (Copeland, UK) at -70%, on Yeast Peptone Glucose (YPG) medium (0.5% yeast extract, 1% peptone, 0.2% glucose) supplemented with 20% glycerol. The other yeast strains used in this study are: *Candida albicans* ATCC 10231; *C. albicans* CMGB-Y1 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest); *C. parapsilosis* CMGB79; *C. parapsilosis* CBS 604; *C. parapsilosis* CMGB-Y3; *C. catenulata* CMGB-Y7*; C. krusei* CMGB-Y8; *C. krusei* CMGB 94; *C. tropicalis* CMGB165; *C. tropicalis* CMGB 114; *Saccharomyces cerevisiae* 17/17; *Yarrowia lipolytica* CMGB32 and *S. cerevisiae* CMGB-RC.

Previous to testing, yeasts were grown for 24 hours at 28°C on YPGA (YPG with agar-agar 2%) slants.

Morphological observations. Ascospores formation

The morphological aspect of CMGB-ST19 colonies was observed after 48 hours growth on YPGA medium using a stereomicroscope SZM-1 (Optika Microscopes, Italy). The shape of the yeast cells and the budding type were observed with an optical microscope (MICROS, Austria).

Ascospores formation was assessed using two different media with $CaCO_3$ 10% and Malt extract 5%, followed by staining with fuchsine 1/10 (for ascospores) and methylene blue 1% (for asci). The preparations were analysed at optical microscope (MICROS, Austria) using immersion oil (Vassu et al., 2001). As positive control we used *S. cerevisiae* CMGB-RC.

Physiological and biochemical analyses were performed according to Barnett et al. (1983) and Kurtzman et al. (2011). The assimilation of carbon sources was tested on Yeast Nitrogen Base with aminoacids (YNB-Sigma Aldrich) (6.7g/L) supplemented with 50mM of raffinose, D-galactose or xylitol. The growth was assessed using the card method (Kurtzman et al., 2011). As positive control we used test tubes with 50 mM glucose. For the negative control no carbon source was added.

The ability to assimilate nitrogen sources was tested using a similar protocol. The 10X Yeast Carbon Base (YCB) (Sigma Aldrich) medium was supplemented with 1% stock solution: 7.8% KNO_3, 2.6% $NaNO_2$, 5.6% L-lysine or 5.08% $(NH_4)_2SO_4$.

The strain CMGB-ST19 was assessed for growth under osmotic stress conditions in presence of 50 and 60% glucose, at 28°C.

The ability to grow at different temperatures (20, 28, 37 and 42°C) was recorded for 3 weeks.

Urease test was performed using as positive control *Yarrowia lipolytica* CMGB 32 and as negative control *Saccharomyces cerevisiae* CMGB-RC (Corbu et al., 2018).

Resistance to various cycloheximide concentrations was determined on YNB medium supplemented with 50 mM glucose and 0.01%, respectively, 0.1% cycloheximide. The results were recorded every day for a week by the card method, using *Candida boidinii* CMGB 95 as positive control.

Phenotypic phylogeny

The Biolog Microbial ID System was used for more accurate taxonomic identification following manufacturer`s instructions. The results were recorded after 48 and 72 hours of incubation.

Genomic DNA isolation

Genomic DNA was isolated using a protocol described by Csutak et al. (2014). The DNA samples were observed by agarose gel electrophoresis using 0.8% agarose, 0.5X Tris-Boric acid-EDTA (TBE).

The concentration of the DNA samples was determined at OD=260nm with a NanoVue Plus spectrophotometer.

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

The ITS1-5.8S-ITS2 region was amplified in a total reaction volume of 50 µL comprising 600 ng/µL genomic DNA, 0.3 µL of each primer ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (200 pM) and Dream Taq Green PCR Master Mix (2X) (Thermo Scientific). The PCR program included: initial denaturation 5 min at 94°C, 40 cycles which consist of 1 min at 94°C, 30 secs at 55°C and 2 min 72°C followed

by a final extension 5 min at 72°C (Biometra T Gradient Thermocycler).
The amplicons were digested with 4 restriction endonucleases: *Cfo* I (5'-GCG/C-3'), *Hinf* I (5'-G/ANTC-3'), *Hae* III (5'-GG/CC-3'), *Msp* I (5'-C/CGG-3') (10 U/µl, Promega). The restriction fragments were observed by gel electrophoresis (1.7% agarose, TBE 0.5X). The size of the amplicons and restriction fragments was determined using Quantity One program (Bio-Rad).

PCR-RFLP of the ITS1-18SrDNA region

ITS1-18S region was amplified using the following program: initial denaturation 2 min at 95°C, 36 cycles which consist of 30 secs at 95°C, 30 secs at 57°C and 3 min 72°C followed by a final extension 7 min at 72°C (EppendorfMasterCycle Nexus Gradient Thermal Cycler). The total volume of 20 µL comprised: 300 ng/µL DNA, 0.5 µL of each primer ITS2 (5`TCCGTAGGTCCTGCGG) and NS1 (5`GTAGTCATATGCTTGTCT) (50pM) and Dream Taq Green PCR Master Mix (2X)(Thermo Scientific). The amplicons were digested with *Hae* III and *Msp* I (10 U/µL, Promega). The amplicons and the restriction fragments were observed by gel electrophoresis (1.5% agarose in TBE 0.5X). The size of the amplicons and restriction fragments were determined with Quantity One program (Bio-Rad).

Screening for lipase production

Evaluation of lipase production was performed as described previously (Corbu et al., 2017; Corbu et al., 2018). After inoculation the Petri dishes were incubated at 3 different temperature values (20, 28, 37°C). The result was considered positive if a clear halo appeared surrounding the yeast colonies.

Growth on molasses and D-xylose

Evaluation of growth on media with non-conventional carbon source was tested on YP medium (1% yeast extract, 1% peptone) supplemented with 5% D-xylose or 0.5% molasses, using 1% yeast inoculum (OD600nm = 1). After inoculation at 28°C, 150 rpm the growth was determined spectrophotometrically (OD600nm) for 24, 48, 72 and 144 hours. The aspect of the cells was also microscopically observed.

Antimicrobial activity

The strain CMGB-ST19 was tested for antimicrobial activity against potential pathogenic and pathogenic *Candida* and *Saccharomyces* strains: *Candida albicans* ATCC 10231; *C. albicans* CMGB-Y1 *C. parapsilosis* CMGB 79; *C. parapsilosis* CBS 604; *C. parapsilosis* CMGB-Y3; *C. catenulata* CMGB-Y7*; C. krusei* CMGB-Y8; *C. krusei* CMGB 94; *C. tropicalis* CMGB165; *C. tropicalis* CMGB 114, and *S. cerevisiae* 17/17. We used two different assays: the first based on nutrient competition and the second based on killer activity at low pH values (Corbu et al., 2018). The results were recorded daily for one week. The result was considered positive if a clear halo, respectively, an inhibition zone appeared surrounding the yeast colonies.

RESULTS AND DISCUSSIONS

Morpho-physiological characterization

After 48 hours of growth on YPGA media at 28°C, the strain CMGB-ST19 formed white-cream colonies of 2-3 mm with folded surface and fringed margins (Figure 1-A). Cells are ovoid with unipolar budding (Figure 1-B). *Pseudohyphae* were observed after two days.

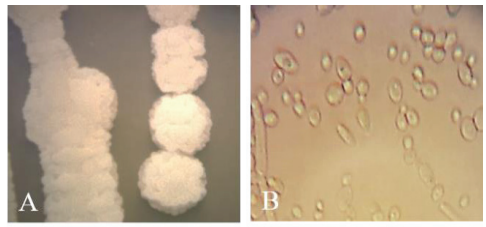

Figure 1. Colonies (A) and cells (B) formed by CMGB-ST19 strain (40x)

Morphological and physiological characterization of yeast strains represents a useful tool for prediction of their behaviour when tested for biotechnological applications. According to Table 1, CMGB-ST19 isolated from the surface of plants petals, had an optimal growth temperature between 20°C and 37°C and a high resistance to osmotic stress conditions. The strain could not produce urease. Since urea hydrolysis is generally absent in ascogenous yeast species being present mostly in basidiomycetous taxa, the

result indicated the belonging of CMGB-ST19 to the Ascomycota yeast taxa.

The cycloheximide (acti-dione) resistance test is based on the inhibitory action of this compound. According to the test, cycloheximide limits growth of the eukaryotes by inhibiting protein synthesis in the 80 S ribosomes. Since 1948 when Whiffen reported for the first time the inhibitory activity of cycloheximide, this test was used intensively in yeast taxonomy being able to divide this group into three categories depending on cycloheximide concentration: markedly sensitive - inhibited by 1 µg/mL; moderately sensitive - inhibited by 25 µg/mL and tolerant - yeast species that can resist to higher concentration than 1000 µg/mL. The strain CMGB-ST19 was not able to grow at concentrations higher than 100 µg/mL which suggests its possible belonging to *Saccharomyces*, *Pichia*, *Schizosaccharomyces* genera (Kurtzman, 2011). Assessing the ability to assimilate specific carbon or nitrogen source for aerobic growth can be a use as identification keys to distinguish between yeast species (Barnett et al., 1983). In this case we determined growth in presence of D-raffinose, xylitol, D-galactose as carbon sources and potassium nitrate, sodium nitrite, L-lysine as nitrogen sources. The strain CMGB ST-19 was able to assimilate D-raffinose. D-galactose and L-lysine but not xylitol. Potassium nitrate was also tolerated but growth was recorded as delayed. Although, some growth was also recorded in the test tube corresponding to sodium nitrite and negative control, the results were registered as negative according to the indication suggested in Kurtzman et al. (2011). The low rate growth observed is certainly due to the dissolution of ammonia from the atmosphere into the medium (Barnett et al., 1983).

Table 1. Results of conventional taxonomy tests

Strain/Species	Urease	Cycloheximide		Temperatures				Osmotic stress growth	
		0.01%	0.1%	20°C	28°C	37°C	42°C	50% glucose	60% glucose
CMGB-ST19	-	-	-	+	+	+	D	+	+
***Pichia ohmeri* CBS 1950 (Kurtzman et al., 2011)**	-	-	-	+	+	+	+/W	+	+/D

In order to complete the conventional taxonomy tests we used the BiologMicrobial ID system, a methodology used for the metabolic fingerprinting of microorganisms. This system can be used both for identification of pure cultures by comparing the results obtained with the database comprising over 267 yeast species or for characterization of mixed populations (DeNittis et al., 2010). In this case we used Biolog Microbial ID System to narrow the number of species suitable for our isolate. According to Figure 2, the Biolog Microbial ID system generated a phylogenetic tree based on phenotypic properties of CMGB ST19 strain indicating the belonging to *Pichia ohmeri* species in a proportion of 97%. We further compared the results obtained from the conventional taxonomy tests with the profile described in Barnett et al., (1983) for *Pichia ohmeri* species and (as shown in Table 1) the results were similar.

Sexual reproduction is an important taxonomical criterion for yeasts. In this study we used two different types of media recommended by Kurtzman et al. (2011) in order to determine the presence of ascopores. The strain CMGB-ST19 did not formed ascospores after ten days of growth. The possible explanation for this fact is that *Kodamaea ohmeri* strains are usually isolated as haploids not diploids and it requires conjugation of complementary mating types to induce sporulation.

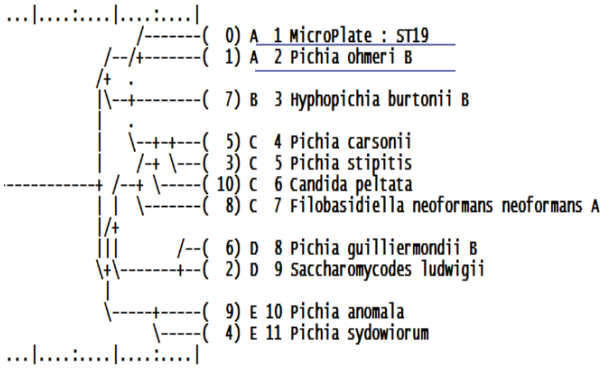

Figure 2. Phenotypic phylogeny obtained using the Biolog Microbial ID System

Molecular characterization

To confirm the results obtained from previous tests, we used PCR-RFLP to analyse the ITS1-

5,8S-ITS2 and ITS1-18S rDNA regions. Both regions analysed are of particular importance for phylogeny studies as they have a highly conserved nucleotide sequence. The ITS1-5.8S rDNA-ITS2 region was amplified using ITS1 and ITS4 primers and subsequently the amplicons were digested with four different endonucleases. The amplicons obtained had approximately 415 bp and after digestion with *Cfo* I, *Hae* III and *Msp* I no digestion fragments were obtained. However, the *Hinf* I digestion generated 2 fragments of 165 and 220 bp (Figure 3). The restriction patterns obtained were compared with the results from literature (Villa-Carvajal et al., 2006; Basilio et al., 2008) and with theoretical digestion of sequences from NCBI nucleotide database (JN183446.1; EF190229.1; KY792622.1) (Table 2).

Although the FASTA sequence corresponding to the accession numbers mentioned above contains also small fragments of 18S/26S genes and there is no annotation made, using the RestrictionMapper-free molecular biology resources we managed to obtain a theoretical profile of digestion. The results obtained showed that the pattern of restriction of CMGB-ST19 strain is highly similar to the pattern of other *K. ohmeri* strains (Table 2).

Table 2. Amplicons and restriction fragments from ITS-5.8S rDNA -ITS2 region

Strain/ Species	Amplicon (bp)	Restriction fragments (bp)			
		Cfo I	*Hae* III	*Hinf* I	*Msp* I
CMGB-ST19	415	415	415	220; 165	415
P. (K.) ohmeri ***CBS 5367***	420	420	420	210;175	**ND**
P.(K.) ohmeri GDB-JPCM(2)	430	**ND**	**ND**	**ND**	430
JN183446.1	383	383	383	218;142; 15	383
EF190229.1	432	432	432	208;201; 15	432
KY792622.1	419	419	419	210;186; 15;	419

Legend: ND-no data

The ITS1-18S rDNA region was amplified using the NS1 and ITS2 primers which allow complete amplification of 18S coding region and internal transcribed spacer (ITS1). After the PCR reaction the amplicons of approximately 2600 bp were digested with *Hae* III and *Msp* I endonucleases (Figure 3). The *Hae* III digestion resulted in 5 fragments ranging from 180 to 800 bp, while for *Msp* I we obtained 4 fragments with sizes between 100 and 820 bp. According to our knowledge this is the first study regarding the restriction map of ITS1-18S coding region of *K. ohmeri*.

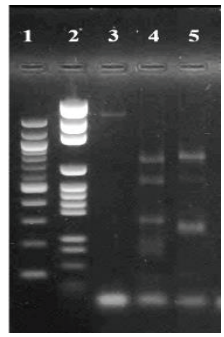

Figure 3. PCR-RFLP of the ITS1-18S region: Legend: 1-100-bp DNA Ladder (ThermoFisher Scientific); 2-BenchTop-pGEM-DNA Ladder (Promega); 3-undigested amplicons; 4-*Hae* III; 5-*Msp* I

We theoretically digested the 18S partial sequences downloaded from NCBI database (NG061102.1, EF428120.1, EF413018.1) and compared the results (Table 3). The differences between the restriction pattern of CMGB-ST19 and the theoretical restrictions are determined by the fact that we could not find any complete ITS1-18S sequences uploaded on NCBI nucleotide Database.

Table 3. Amplicons and restriction fragments from ITS1-18S- rDNA region

Strain/species	Amplicon (bp)	Restriction fragments (bp)	
		Hae III	*Msp* I
CMGB-ST19	2600	800; 580; 300; 220; 180	820; 600; 270; 100
NG061102.1	1698	759; 293; 269; 175; 151	799; 271; 267; 238; 99; 44
EF428120.1	1648	759; 274; 238; 175; 151; 43	799; 271; 236; 219; 99; 24
EF413018.1	1659	759; 277; 246; 175; 151	799; 271; 244; 99; 22

From this point forward we renamed the strain CMGB-ST19 as *K. ohmeri* CMGB-ST19.

Biotechnological assays. The second part of our study aimed to investigate the biotechnological potential of *K. ohmeri* CMGB-ST19.

Lipase production

Lipases or triacylglycerol acylhydrolase (EC 3.1.1.3) are enzymes that catalyse the hydrolysis of long-chain triglycerides. Although many organisms, including humans, can produce lipases, microbial lipases have important industrial potential due to their thermostability, stability at variable pH values and in presence of organic solvents (Verma et al., 2012). Therefore, microbial lipases have been successfully used in biodiesel production, pharmaceutic industry, as part of agrochemical compounds or in food industry for obtaining flavour compounds. Even though some yeast species were already characterised as good lipase producers (*C. rugosa, C. antarctica*), there is a growing interest for the isolation of new yeast strains with lipolytic properties (Bussamara et al., 2010). *K. ohmeri* CMGB ST19 was tested for lipase production by determination of tributyrin hydrolysis to glycerol and butyric acid. The strain was incubated at three different temperatures corresponding to those used in industry (20°C), the optimal temperature for yeast growth (28°C) and the temperature of the human body (37°C). The positive result was considered the appearance of clear halo surrounding the yeast colony. According to Figure 4, our strain was able to synthetize lipase and therefore to hydrolyse tributyrin. The aspect of the halo is similar at all three temperature proving that the synthesized lipases are thermostable. Also, there are no significant changes between the results obtained after three, respectively, seven days indicating that the yeast strain has a high ability to synthetize lipases in the first part of growth interval. This is very important for industry since short synthesis time involves low production costs. In present, there are few studies concerning the lipolytic activity of *K. ohmeri*. Bussamara et al. (2010) reported two *K. ohmeri* strains isolated from *Hibiscus rosa-sinensis* leaves as being ableto grow in a medium with Tween 20. In the same study, the researchers determined the lipase activity on basal medium supplemented with bovine fat and soy oil, lipase activity being noticed only in the case of bovine fat. Also, according to Gana et al. (2014), *K. ohmeri* strains are not able to produce lipase on Sierra`s medium with Tween 80.

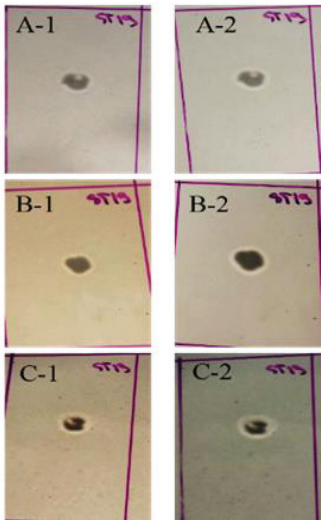

Figure 4. Aspect of culture spot on YPTA medium (A: 20°C-1: 4 days; 2: 7 days; B: 28°C-1: 4 days; 2- 7 days; C: 37°C-1: 4 days; 2: 7 days)

Although our strain has a rather low level of lipase production it can be used in biomedicine since it is able to hydrolyse tributyrin known as a precursor of butyric acid. Butyric acid is known to serve as nutrient for colonocytes and as an important mediator of gene expression, immune modulation and oxidative stress reduction (Bedford and Gong, 2018).

Xylose and molasses assimilation

Xylose is, after glucose, the second most abundant carbohydrate in nature being the building block of xylan biopolymer. Xylose can be extracted from corn cobs through a chemical process consisting of acid hydrolysis, condensation and crystallization. Although this process is optimized for xylose extraction, large amount of waste still containing large amount of xylose, L-arabinose, glucose and galactose are thrown away (Jagtap and Rao, 2018). The industrial potential of xylose is huge. Thus, it is used in food industry for sweetener production, mainly xylitol which has the best sweetening capacity and also is used in pharmaceutical and cosmetic products such as toothpaste or mouthwash (Ping et al., 2013). Xylitol is also important in energy industry for biofuels production (Aditiya et al., 2016), in chemistry for obtaining isopropanol, the most used industrial solvent and a potential precursor for propylene (Collas et al., 2012), for furfural production an intermediate for the production

of chemicals used as solvents (Liu et al., 2018). The microorganisms have the enzymatic equipment necessary for the xylose metabolism more precisely the pentose phosphate pathway. This pathway is involved in producing intermediates involved in nucleic acid biosynthesis (D-ribose); synthesis of aromatic aminoacids (D-erythrose 4-phosphate) and cofactors for anabolic reactions (NADPH). The pentose phosphate pathway has two mainly phases: oxidative (when hexose-6-phosphate is converted in D-ribulose-5-phosphate, carbon dioxide and NADPH) and nonoxidative phase (when D-ribulose-5-phosphate is converted into D-ribose-5-phospate; D-xylulose-5-phosphate; D-sedoheptulose-7-phosphate a.s.o). In yeasts, D-xylose is reduced and oxidate under catalytic action of xylose reductase and xylitol dehydrogenase enzymes to form D-xylulose and this compound enters the pentose phosphate pathway (Jeffries, 2006).

Xylose assimilation and fermentation by yeasts has a huge biotechnological potential since xylose can be transformed in so many important chemicals. Yet, not all yeasts have the same yield in converting xylose into useful compounds. Therefore, is important to identify new species with natural abilities in utilizing xylose.

Annually large amounts of molasses are produced as a co-product of sugar obtaining process from sugar cane and sugar beet. Regardless of its origin, molasses contains large quantities of sugar (47-48%), water, minerals such as magnesium, manganese, iron, zinc and aluminium and aminoacids and proteins. A number of studies described molasses as a useful waste that can be used as a sole source of full nutrients necessary for microbial production of lipids (Taskin et al., 2016), biosurfactants (Makkar and Cameotra, 2002), succinic acid used as a precursor for pharmaceuticals, feed additives and green solvents (Liu et al., 2008) and citric acid used in food, pharmaceutical and cosmetic industries as acidulant, preservative or emulsifier (Ali et al., 2002; Ciriminna et al., 2017).

In the present work, we evaluated the ability of *K. ohmeri* CMGB-ST19 to grow on two special media supplemented with 0.5% molasses, respectively, 5% D-xylose as a preliminary step to determine the potential of this strain as a producer of biotechnological interest compound like bioethanol or biosurfactants. According to Figure 5, our strain was able to grow on both media. However, the dynamic of growth was completely different. While *K. ohmeri* CMGB-ST19 was able to assimilate rapidly molasses in the first day after inoculation, on D-xylose supplemented medium, significant growth was recorded only after three days of incubation.

We also observed the microscopically aspect of the culture. Although no significant changes were observed on molasses medium, the cells presented large vacuoles and some of them started to form pseudohyphae. In the case of D-xylose medium, we could observe significant morphological changes in the aspect of the cells starting with the third day, most probably induced by stress conditions (Figure 6).

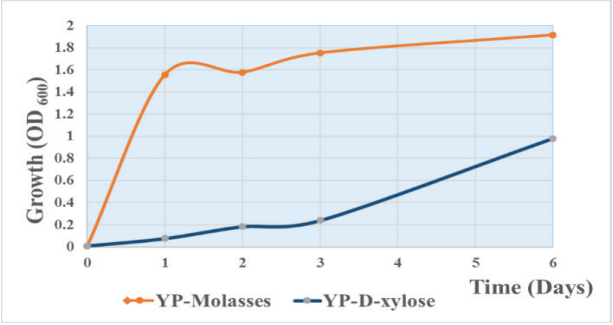

Figure 5. Growth dynamics of *K. ohmeri* CMGB-ST19 on molasses and D-xylose supplemented media

It is well known that some yeast strains are able of developing filamentous-like growth under starvation conditions (Kim and Rose, 2015). In this case, it seems that the limiting factor was the carbon source.

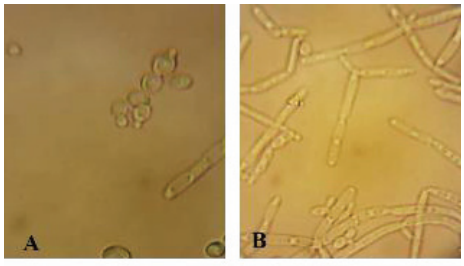

Figure 6. Aspect of the culture after 6 days of incubation in presence of: A- molasses; B- D-xylose

Until now there are no important studies regarding the ability of *K. ohmeri* to assimilate xylose. Zhu et al. (2010) showed that *K. ohmeri* is able to produce xylitol through a different

strategy, by converting D-glucose into D-arabitol which can be oxidized to D-xylulose and subsequently reduced to xylitol. According to our study, this species can use xylose as carbon source, although with a slower rate compared to other sugar substrates.

Since in biotechnological processes time is essential for lowering the costs, we cannot recommend *K. ohmeri* CMGB-ST19 as an important xylose assimilating yeast for biotechnology. Nevertheless, the results obtained on molasses, encourage us to perform further studies regarding its ability to assimilate this substrate.

Antimicrobial activity

The antimicrobial activity of yeasts has implications both in the biomedical field for control of diseases-causing microorganisms and in agriculture, for the control of plant pathogens. Until now, different mechanisms involved in antagonistic action of yeasts have been described: production of antimicrobial compounds (killer toxins, cell wall lytic enzymes, large quantities of ethanol, organic acids) and space or nutrient competition (Csutak, 2014; De Ingeniis et al., 2009). *Candida* genus species, that colonize the mucosal surface of the gastrointestinal tract, vaginal mucosa and oral mucosa, can adhere to different tissues, cause a variety of infections and, in case of immune compromised patients, causing death. Recent studies reported that many yeast species can inhibit growth or even kill pathogens like *Candida albicans* (Liu et al., 2015). In this study we evaluated the ability of our strain to inhibit the growth of pathogenic and potential pathogenic yeast strains from *Candida* and *Saccharomyces* genera by nutrient competition and killer activity at low pH values.

Nutrient competition assay is based on determining the ability of *K. ohmeri* CMGB-ST19 to inhibit the growth of other yeasts by immobilizing large amounts of iron ions. Previous study reported *K. ohmeri* as being able to produce small amount of siderophore-like compounds (data not shown) that can involve the existence of a highly effective mechanisms of iron uptake. The results showed that our strain was not able to inhibit growth of the tested yeasts through iron depletion.

According to Table 4, *K. ohmeri* CMGB-ST19 inhibited the growth of different *Candida* and *Saccharomyces* strains at low pH values. Best results were observed against *C. parapsilosis* CMGB Y3, *C. catenulata* CMGB Y7 and *C. krusei* CMGB Y8 (Figure 7). Therefore, it is possible that the antimicrobial action of our strain is due to the killer toxin production, similar results being reported during other studies.

Table 4. Antimicrobial activity of *K. ohmeri* CMGB-ST19 at low pH values

Potential pathogenic/ pathogenic strain	Size of inhibition zone	
	4 days	7 days
C. albicans ATCC10231	-	-
C. albicans CMGB-Y1	-	-
C. parapsilosis CBS604	+	+
C. parapsilosis CMGB 79	+	+
C. parapsilosis CMGB Y3	++	++
C. catenulata CMGB Y7	++	++
C. krusei CMGB Y8	++	++
C. krusei CMGB 94	+	+
C. tropicalis CMGB 165	-	-
C. tropicalis CMGB 114	-	-
S. cerevisiae 17/17	+	+

Legend: (+) inhibition zone 2-3 mm; (++) inhibition zone >4 mm

The antagonism mechanism based on killer toxin production was first reported *in Saccharomyces cerevisiae* but later was extended to *Kluyveromyces, Pichia* and *Zygosaccharomyces* yeast species.

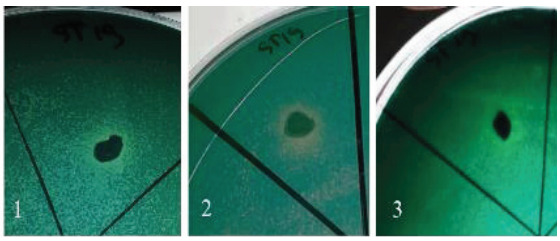

Figure 7. Aspect of the inhibition halo after 7 days of incubation (1-*C. parapsilosis* CMGB Y3; 2-*C. krusei* CMGB-Y8; *3-C. catenulata* CMGB-Y7)

In general, the killer toxin acts by interfering with the cell wall (directly or through specific receptors), by destabilizing the cell membrane, or by interfering with DNA replication or mRNA translation (Schaffrath et al., 2018). Although there are some studies regarding the killer toxin produced by *K. ohmeri* strains, the

mechanism of action is not yet fully understood. However, the toxin seems to be encoded by nuclear genes (Fuentefria et al., 2006). Coelho et al. (2009) reported a killer toxin of proteic nature smaller than 3000 Da, able to inhibit the growth of *S. cerevisiae* NCYC1006, *Pichia kluyveri* CAY-15 yeast strains and *Penicillium expansum* fungal strain by causing loss of cellular integrity. The ability to inhibit both conidia germination and hyphal growth of *P. expansum*, recommend *K. ohmeri* as an important tool for preventing post-harvested spoilage of fruits. Also, the excretion of killer toxin was stable for a long period of time suggesting that the toxin might be encoded by chromosomal genes (Coelho et al., 2009; Belda et al., 2017).

Another study conducted by Fuentefria et al. (2006) regarding killer activity of *K. ohmeri* reported that two strains isolated from leaves of *Hibiscus rosa-sinensis* were able to inhibit growth of three different varieties of *Cryptococcus neoformans* (*neoformans, grubii, gottii*) and different *Candida* species (*C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata*). The results, in this case, were highly dependable on the pH, but both strains preserved their killer phenotype after serial cultures at their optimal temperature value for more than 1 month. As a conclusion, the antimicrobial activity of *K. ohmeri* CMGB-ST19 is highly related to production of killer toxins.

CONCLUSIONS

The present work deals with preliminary identification and characterization of the new *K. ohmeri* CMGB-ST19 strain isolated from jam roses petals (Bucharest Botanical Garden, Romania). The strain has good lipolytic activity being able to produce lipases that hydrolase tributyrin to butyric acid and glycerol. *K. ohmeri* CMGB-ST19 presented a high growth rate on molasses. Further studies will aim understanding the mechanism of molasses assimilation and finding new strategies for improving its biotechnological potential. Finally, *K. ohmeri* CMGB-ST19 can inhibit growth of different pathogenic *Candida* strains, representing thus a base for biomedical applications.

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