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*Original paper*

# *New yeast strains from Black Sea and oil polluted soil with lipolytic potential*

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**Abstract** Lipases are enzymes with importance in different biotechnological domains. There is a growing interest for identification of new microbial lipases of greater efficiency at industrial scale and cheaper in terms of production costs. The aim of this paper is to determine the lipolytic potential of two new yeast strains isolated from polluted environments and to optimize a new protocol of non-specific mutagenesis aimed to increase lipase synthesis. The yeast strains CMGB-YR-P1 and CMGB-G1 were identified using conventional and molecular taxonomy techniques and the results were compared to the taxonomic identification generated by Bruker MALDI-TOF system. The strains were renamed *C. famata* CMGB-YR-P1, and *R. mucilaginosa* CMGB-G1 respectively. The lipolytic potential was determined at three different temperatures (20, 28 and 37°C). The influence of nitrogen/carbon source on the growth of *R. mucilaginosa* CMGB-G1 was determined for optimizing lipaze production yield. *R. mucilaginosa* CMGB-G1 strain showed high lipolytic potential being able to hydrolyze tributyrin and to release butyric acid. Since the strain *C. famata* CMGB-YR-P1 showed low rates of lipase synthesis, N-methyl-N`-nitro-N-nitrosoguanidin (NTG) mutagenesis was used to improve its lipolytic potential. Moreover, three mutants were isolated and will be further tested for different biotechnological applications.

**Keywords** Lipase, NTG mutagenesis, *Candida famata*, *Rhodotorula mucilaginosa*, chemical industry.

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# Introduction

Lipases or triacylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyze the cleavage of ester bonds from triacylglycerol to form free fatty acids and glycerol (MELANI, 2019 [35]). These enzymes act at the interface between two immiscible systems mainly due to a phenomenon entitled interfacial activation (SANTOS, 2013 [46]). Studies focused on X-ray crystallography proved that in most cases, in the tertiary structure of lipases is identified a flexible lid domain that covers the catalytic triad. In the presence of hydrophobic compounds this domain moves allowing the substrates to reach the catalytic triad and, implicitly, the hydrolysis of ester bonds (KUANG, 2020 [30]). Lipases along with amylases and proteases are considered very important biocatalysts being used in food industry, environmental biotechnology, for cosmetics and pharmaceuticals development, for detergents and as additives in products for sustainable agriculture (GUPTA, 2015 [22]).

Many of the commercialized lipases are of animal or vegetable origin and involve high costs for extraction and purification steps. Also, nowadays, using lipases of animal origin in food industry is regarded with skepticism as they cannot be used to process foods for Jews or vegetarians and some researchers have revealed the idea that the use of such extracts can be associated with the transfer of viruses from animals to humans (MELANI, 2019 [35]; VAKHLU, 2006 [56]). Microorganisms have simple nutritional requirements and involve low cultivation costs being thus an economical alternative for lipase production. In addition, many microbial lipases are secreted into the culture media facilitating, thus, the extraction and purification processes. Many yeast species belonging to *Candida*, *Rhodotorula*, *Yarrowia* and *Trichosporon* genera are already known as being able to produce large amounts of lipases in a short period using rather simple growth substrates (KUMAR, 2019 [31]). Many of these lipases are already commercialized.

According to Business Communication Company, Inc in 2016 the global lipase market was evaluated at 464.2 million dollars and it is supposed to reach 797.7 million dollars until 2025. The main competitors that occupy almost 75% of the lipase market are Novozymes, Dupont and DSM and among their products, yeast lipases occupy an important position being commercialized as Lipozyme® TL IM, Novozym®435 or Novozym®40086 (SUN, 2018 [48]; WANG, 2016 [59]). These products are successfully used in many industrial fields: for textile processing, for the development of detergents and balms active in cold water, to reduce the fat content of foods for people with metabolic deficiencies, to enrich the flavor of dairy products, for the synthesis of anti-inflammatory or analgesics and even for the production of biofuels. (KUMAR, 2019 [31]; MELANI, 2019 [35]). Although this is an intensely studied field, there is a growing interest for isolation of new yeast species able to synthetize efficient lipases and for improving their ability either by varying the cultivation conditions or by non-specific mutagenesis techniques using chemical or physical mutagens (THABET, 2012 [54]; TAN, 2016 [50]).

The aim of the present work is to determine the lipolytic potential of two new yeast strains from polluted environments and to optimize a new protocol of non-specific mutagenesis.

# Materials and Methods

### *1. Yeast strains*

The yeast strains used in this study, CMGB-YR-P1 (isolated from petroleum polluted soil- Berca-Albanesti area from Buzau County) and CMGB-G1 (isolated from Black Sea near Constanta harbor) were maintained on Yeast Peptone Glucose (YPG) medium (0.5% yeast extract, 1% peptone, 0.2% glucose) supplemented with 20% glycerol in a Revco LegaciTM Refrigeration System (Copeland, UK) at -70°C. Prior to any experiment, each yeast strain was cultivated on solid YPG medium for 24 hours at 28°C.

### *2. Morpho-physiological preliminary characterization*

Preliminary characterization of the newly isolated strains was performed using different conventional taxonomy tests.

The morphological aspect of the colonies formed by each strain after cultured on YPGA media for 48 hours at 28°C was observed using a stereomicroscope SZM-1 (Optika Microscopes, Italy). The aspect and the budding type of the cells were analyzed with an optical microscope (MICROS, Austria).

In order to determine the resistance to osmotic stress, the yeast strains were grown on YPGA medium (with 50 or 60% glucose) or on YPG medium and incubated at 20, 28, 37 and 60°C to evaluate the tolerance to temperature variation. The results were observed daily for 3 weeks (KURTZMAN, 2011 [32]).

The urea hydrolysis test was performed using a special medium containing phenol red and urea (0.1% peptone; 0.1% glucose, 0.5% sodium chloride; 0.2% potassium phosphate monobasic, 0.0012% phenol red; 2% agar; 2% urea added after sterilization). The strain *Yarrowia lipolytica* CMGB32 was used as positive control and *Saccharomyces cerevisiae* CMGB-RC as negative control. The change of the color of the medium from yellow to pale pink is determined by the hydrolysis of urea under the action of urease and is considered the positive result (CORBU, 2018 [13]).

### *3. Bruker MALDI-TOF identification*

For MALDI-TOF MS analysis we used fresh yeast cultures grown on YPGA media for 24 hours at 28°C. The samples were prepared according to the manufacturer instructions and applied to a coded plate. MALDI-TOF MS measurements were acquired using a Microflex III instrument (Bruker Daltonik, Bremen, Germany) and the results were compared to the reference mass spectra using MALDI BioTyper Software (Bruker Daltonics). The final results were expressed using scores ranging from 0 to 3 and only scores higher than 2 were considered reliable for yeast identification.

### *4. Genomic DNA isolation*

Yeast cultures grown for 24 hours on YPG medium at 28°C were used to prepare the cells suspension for genomic

DNA isolation according to (CSUTAK, 2014 [14]). The concentration and purity of the DNA extracts were determined using a NanoVue Plus spectrophotometer at λ 260 nm respectively 280 nm.

### *5. PCR-RFLP of the ITS1-5.8S rDNA-ITS2 region*

The amplification of ITS1-5.8S-ITS2 region was performed using ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) primers. The restriction profiles of the amplicons strains were determined using four enzymes: *Cfo* I (5'-GCG/C-3'), *Hinf* I (5'-G/ANTC-3'), *Hae* III (5'-GG/CC-3'), and *Msp* I (5'-C/CGG-3') (10U/µl, Promega). The restriction fragments were separated by 1.7% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA buffer and the size of the fragments was calculated using Quantity One program offered by BioRad.

### *6. Screening for lipase activity*

Qualitative determination of lipolytic properties of the yeast strains studied was performed using a previously described assay (CORBU, 2018 [13]) based on tributyrin hydrolysis. Fresh yeast cultures grown for 24 hours on YPGA media at 28°C were inoculated on YPTA (0.7% Yeast Nitrogen Base with amino-acids, 0,5% ammonium sulphate $(NH_4)_2SO_4$ 0.5% tributyrin, 0.0125% Tween 80 and 2% agar, pH-6.8) Petri plates. The plates were incubated at 20, 28 and 37°C for seven days and analyzed daily. The result is considered positive when a clear halo appears surrounding the yeast colony. The lipolytic activity is quantified by determination of the ratio between the size of the halo and the size of the yeast colony. In this case, if the ratio is equal to 1, the yeast strain has no lipolytic potential and a ratio higher than 1 indicates that the tested yeast strain has the ability to produce lipases.

### *7. Influence of the growth media on the lipolytic activity*

Lipase production is strongly influenced by the culture media used for testing. In order to optimize the production of lipases we evaluated the ability of CMGB-G1 yeast strain to grow using tributyrin as carbon source on three different liquid media: L-SC (yeast peptone 0.3%, peptone 0.5%, 0.1% glucose, 0.5% monopotassium phosphate pH-6); L-SC-TY (L-SC tributyrin: L-SC medium supplemented with 1% tributyrin pH-6) and YPT (YPTA medium w/o agar). Fresh cultures of the selected yeast strains were used for obtaining cell suspensions ($1X10^8$ cells/mL) inoculated (final concentration of 1%) on the culture media mentioned above. Yeast growth was determined using Thoma counting chamber at 24, 48, 96 and 168 hours of incubation at 28°C.

### *8. Optimization of N-methyl-N`-nitro-N-nitrosoguanidine (NTG) mutagenesis protocol for improving the lipolytic potential of the yeasts*

Non-specific mutagenesis with N-methyl-N`-nitro-N-nitrosoguanidin was optimized using CMGB-YR-P1 strain. 20 mL of CMGB-YR-P1 culture grown on YPG media at 28°C, at 150 rpm, were centrifuged for 7 min at 7000 rpm. The cell pellet was washed twice with phosphate saline buffer (pH 7.4). After the final centrifugation, the cell pellet was resuspended in phosphate saline buffer and brought to OD600nm=1. Subsequently, NTG was added in a final concentration of 50 µg/mL. The mutagenesis mixture was incubated for 30 minutes (28°C, 150 rpm). At the end of the incubation time, the mutagen was removed by centrifugation (10 minutes, 5000 rpm) and the cell pellet was resuspended in 5 mL phosphate saline buffer. The suspension was used to inoculate 24 spots of 10 µL on Petri dishes with 5 different solid culture media: YPTA medium; YPTA medium supplemented with 1% glucose; T80-0.5 (1% peptone, 0.5% sodium chloride, 0.01% calcium chloride, 1.5% agar, 0,5% Tween 80); T80-0.1 (1% peptone, 0.5% sodium chloride, 0.01% calcium chloride, 1.5% agar, 0.1% Tween 80); T20-0.5 (1% peptone, 0.5% sodium chloride, 0.01% calcium chloride, 1.5% agar, 0,5% Tween 20). The negative controls were performed similarly, except for the exposure to the mutagen.

In the case of the media containing Tween 80 or Tween 20, a positive result is indicated by the appearance of white precipitate crystals around the culture spot due to the reaction between the fatty acids released following the lipolytic action and the salts present in the culture medium. In the case of the YPTA medium the positive result is indicated by the presence of the clear halo surrounding the culture spot. At the end of the incubation time, parts of the Petri dishes were stained with Sudan Black solution 0,08% in 96% ethanol for 5 minutes.

## Results and Discussion

### *1. Taxonomic identification*

Conventional taxonomy tests allow both preliminary taxonomic classification and characterization of strains from a morpho-physiological point of view. A first step is to describe the appearance of the colonies and cells of our strains. After 48 hours of growth on YPGA media at 28°C, the strain CMGB-G1 formed pale red, smooth colonies of 2-3 mm (Fig.1-A.1) and medium size round cells (Fig.1-A.2). The colonies formed by the strain CMGB-YR-P1 had similar size but with a white-crème color and a smooth surface (Fig.1-B.1). The CMGB-YR-P1 cells were also round but smaller (Fig.1-B.2). The microscopical analysis revealed that both strains present multipolar budding.

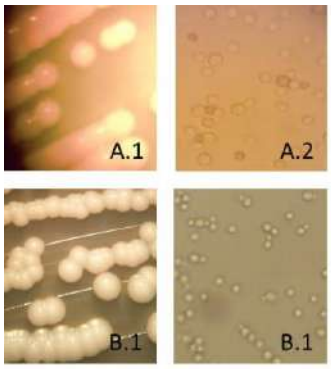



**Figure 1.** Morphological aspect of the colonies and cells formed by CMGB-G1(**A**) and CMGB-YR-P1 (**B**): (**A1/B1**) colonies; (**A2/B2**) cells (40X).

The behavior of yeasts in different culture conditions represents an useful tool for taxonomic identification and also, for establishing their possible use in biotechnology. Both strains, being isolated from natural environments, presented an optimal growth temperature between 20 and 37°C. The strains had no significant growth in presence of high concentrations of glucose. CMGB-YR-P1, in presence of 50/60% glucose had a long lag period needed for metabolic adjustment in these osmotic stress conditions. The strain CMGB-G1 strain recorded only weak growth on 50% glucose during all three weeks of incubation (Table 1). Both strains are able to hydrolyze urea in less than a week of growth on the specific media.

**Table 1.** Conventional taxonomy tests results

| Physiological features | CMGB-G1 | CMGB-YR-P1 |
|---|---|---|
| Growth at 20°C | +++ | +++ |
| Growth at 28°C | +++ | +++ |
| Growth at 37°C | +++ | +++ |
| Growth at 42°C | - | - |
| Growth in the presence of 50% D-Glucose | W | D |
| Growth in the presence of 60% D-Glucose | - | D |

Codes in table: **+++** rapid growth after 24 hours; **-** No growth after 2 weeks of incubation; **W** – week growth; **D** – delayed growth (after at least one week)

The preliminary characterization of CMGB-G1 strain indicated its possible belonging to *Rhodotorula* or *Rhodosporidium* genera. In the case of CMGB-YR-P1 strain, the preliminary characterization was not able to establish the possible belonging to a certain genera since many yeasts with this physical appearance from natural environments have this metabolic pattern (BARNETT, 1988 [5]; KURTZMAN, 2011 [32]). Therefore we used Bruker MALDI-TOF Identification System to narrow down the number of species suitable for our strains. MALDI-TOF MS is a modern, powerful, cost effective and rapid identification method based on determining the unique protein profile of each strain that is compared to a previously available database (LACROIX, 2014 [33]; RAHI, 2016 [43]). The result generated represents a similarity index determined by analyzing peak mass-to-charge ratio, peak intensities and peak frequencies (BEL, 2011 [15]; AGUSTINI, 2014 [3]). This technique is mainly used for differentiation of yeasts from clinical sample but recently, it has been used for identification of yeasts with industrial or environmental use (GUTIÉRREZ, 2017 [23]). Since it is only at the beginning, sometimes misidentifications might occurs and this is why we must always correlate the results of different taxonomy techniques. According to MALDI-TOF MS measurement, the strain CMGB-G1 strain belongs to *Rhodotorula mucilaginosa* species while CMGB-YR-P1 is a *Candida famata* strain.

Molecular confirmation of the results obtained through conventional taxonomy and Bruker MALDI-TOF was performed using PCR-RFLP on the ITS1-5.8S-ITS2 region. This technique is easier to achieve compared to other molecular techniques and generates reproducible results (RUSU, 2015 [44]). We amplified the analyzed region using specific primers and we obtained a 625 bp amplicon for CMGB-G1 respectively a 650 bp amplicon for CMGB-YR-P1. Restriction patterns specific for each strain were obtained by digesting the amplicons using four restriction enzymes (*Cfo* I, *Hae* III, *Hinf* I and *Msp* I) (Figure 2-A; Figure 2-B).

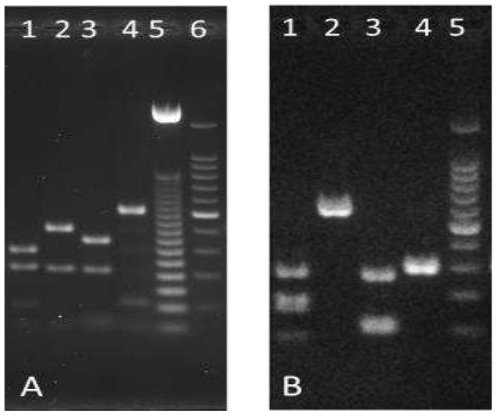



**Figure 2.** PCR-RFLP of the ITS1-5.8S-ITS2 region for CMGB-G1-(**A**); CMGB-YR-P1-(**B**) strains.

We compared the results obtained for our strains with those from the scientific literature and in case of the strain CMGB-G1 strain we were able to confirm the belonging to *R. mucilaginosa* species (Table 2).

The taxonomy of *Candida famata* species is intensely debated and as far as we know there are no studies regarding similar RFLP profiles. Moreover, most of the commonly used taxonomic techniques lead to the wrong classification of its members as *Meyerozyma guilliermondii* (previously known as *Candida/Pichia guilliermondii).* Therefore, we compared the restriction pattern obtained for our strain CMGB-YR-P1 with the profile of *M. guilliermondii*. Also, *C. famata* is considered the anamorph of *Debaryomyces hansenii*. Since many studies consider the two species as synonyms we also compared our results with those described for different *D. hansenii* strains (DESNOS-OLLIVIER, 2008 [16]; CASTANHEIRA, 2013 [12]).

**Table 2.** Comparative analysis of the amplicons and restriction fragments of the ITS1-5.8S rDNA-ITS2 regions (ND- not determined)

| Strain | Amplicon (bp) | *Cfo* I (bp) | *Hae* III (bp) | *Hinf* I (bp) | *Msp* I (bp) |
|---|---|---|---|---|---|
| CMGB-G1 | 625 | 300;235;125 | 425;235 | 365;235 | 525;125 |
| *R. mucilaginosa* M109 (Carvalho, 2005) | 640 | 320;240;80 | 425;215 | 340;225;75 | ND |
| *R. mucilaginosa* CECT 11010 (Esteve Zarzoso, 1999 ) | 640 | 320;240;80 | 425;215 | 340;225;75 | ND |
| *R. mucilaginosa* CECT 11016 (Guillamon, 1998) | 640 | 320;240 | 425;215 | 340;225 | ND |
| *R.mucilaginosa* CCY 20-1-32 (Suranska) | 650 | 300;220; 100 | 420;220 | 250;100 | 500;120 |
| *R.glutinis* (Aroyo Lopez, 2006) | 640 | 320;240 | 430;210 | 340;225;75 | ND |
| *R. glutinis (*Bockelman, 2008) | 610 | ND | ND | 275;125;110 | ND |
| *R. glutinis (*Guillamon, 1998) | 640 | 320;240 | 430;210 | 170;150 | ND |
| CMGB-YR-P1 | 650 | 300;235;100 | 350;150;85 | 325 | 650 |
| *D. hansenii* (Granchi, 1999) | - | 295;280 | 400;120;75 | 315;315 | ND |
| *D. hansenii* 6075 (Bochelman, 2008) | 640 | ND | ND | 315; 315 | ND |
| *D. hansenii* CBS 767 (Pham, 2011) | 639 | 295;284;50;10 | 421;138;180 | 315;316;8 | ND |
| *D. hansenii* CECT 10286 (Pham, 2011) | 650 | 300;300;50 | 420;150;90 | 325;325 | ND |
| *P. guilliermondii* (Bezzera, 2013) | 650 | 300;270 | 400;120;70 | 310;290 | ND |
| *P. guilliermondii* CECT1021 (Esteve-Zarzoso, 1999) | 625 | 300;265;60 | 400;115;90 | 320;300 | ND |
| *M. guilliermondii* UAF214(Pham, 2011) | 607 | 293;255;49;10 | 390;116;79;17;5 | 314;285;8 | ND |
| *M. guilliermondii* CECT 1019 (Pham 2011) | 625 | 300;265;60 | 400;110;90 | 320;300 | ND |
| *M. guilliermondii* NCYC 443 (Pham, 2011) | 625 | 300;265;60 | 400;115;90 | 320;300 | ND |
| *M. guilliermondii* CBS 2030 (Pham, 2011) | 605 | 320;270 | 380;120;80 | 340;300 | ND |
| *P. guilliermondii* (Valles, 2007) | 650 | ND | ND | ND | 460;90 |
| *P. guilliermondii* (Jeyarm, 2008) | 630 | 260;220;50 | 450;90 | 265 | ND |
| *C. guilliermondii* ATCC 9058 (Mirhendi, 2006) | 608 | ND | ND | ND | 371;155;82 |

As it is shown in Table 2, the strain CMGB-YR-P1 shared similarity with both *M. guilliermondii* and *D. hansenii*, and it cannot be accurately determined as belonging to any of them. Since Bruker MALDI-TOF proved to be a reliable method to identify *C. famata* species (CASTANHEIRA, 2013 [16]) and since the RFLP profile of our strain did not revealed an accurate result, we decided to consider it, from this point forward, as belonging to *C. famata* species.

### *2. Qualitative evaluation of lipolytic potential*

*R. mucilaginosa* along with *R. minuta* and *R. glutinis* are famous among *Rhodotorula* species with biotechnological potential. Members of this species are known for their use as enzymes and carotenoids producers (MOLINÉ, 2012 [38]; LARIO, 2015 [34]; VARMIRA, 2018 [58]), for their oleaginous properties (KHOT, 2017 [28]) and in biodiesel production. (HOF, 2019 [26]). *C. famata* is a flavinogenic yeast being able to produce riboflavin when is exposed to iron starvation (DMYTRUK, 2012 [18]; DMYTRUK, 2014 [17]). Also, members of this species are known for their ability to produce glucoamylases when grown on soluble starch as carbon source and urea as nitrogen source (MOHAMED, 2007 [37]).

In this study, we determined the lipolytic potential of our strains using a simple solid media which contains tributyrin as carbon source. The ability to hydrolize tributyrin in glycerol and butyric acid has high biomedical potential since the butyric acid is known for its role as mediator of gene expression, as oxidative stress reducer and immune modulator (BEDFORD, 2018 [7]; AALAMIFAR, 2020 [1]). In addition, this compound is involved in the well-functioning of colonocytes and some studies reported its possible use in relieving constipation (WANG, 2020 [60]). The importance of butyric acid exceeds the biomedical field being also used for aqua feeds improvement (ABDEL-LATIF, 2020 [2]). Glycerol or propane-1,2,3-triol is also a valuable by-product being used for pharmaceutical and cosmetics development, in food industry and for biodiesel production (TAN, 2013 [51]).

We tested the ability of our strains to hydrolyse tributyrin at three temperatures. Thus, 28°C, is considered the optimal growth temperature of yeasts, while the temperature of 20°C, and has double significance: is characteristic for industrial processes and assures the optimal growth of yeast strains isolated from natural environments such as soil or water. The value of 37°C was chosen as specific for the human body. Since neither of the strains grew well at temperatures above 37°C we considered as unnecessary to test lipase production at these values. According to Figure 3-A, *R. mucilaginosa* CMGB-G1 strain showed good lipolytic potential at all three temperatures. There were no significant differences between the aspects of the halos observed at four, respectively, seven days suggesting that lipase occurs during the first four days of incubation. This fact is very important for the industrial use since a short synthesis period involves lower production costs. Best lipolytic activity was recorded at 20°C when the ratio between the size of the halo and the size of the culture spot equals 3.6. An explanation might reside in the fact that this strain was isolated from the Black Sea which has an average annual temperature of about 12°C.

Microbial lipase production improvement can be achieved through manipulation of the medium for cultivation or the physical factors such as temperature, aeration, pH.

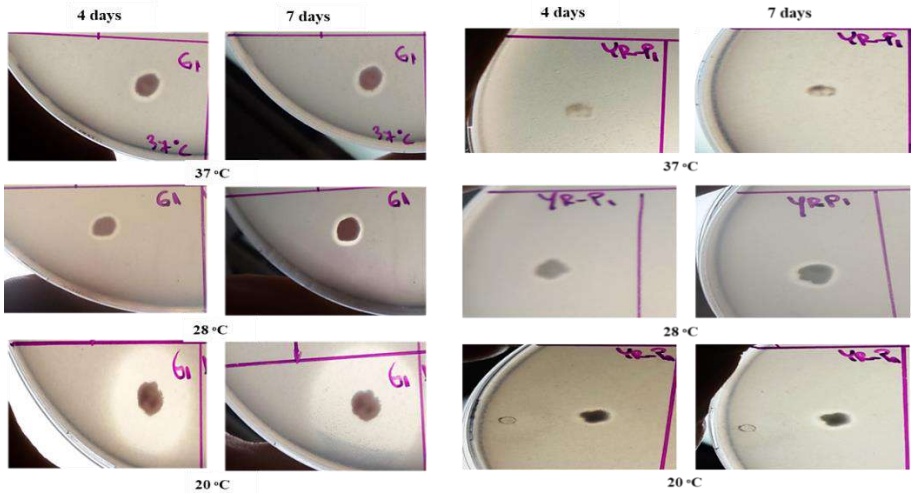



**Figure 3.** Aspect of culture spots on YPTA medium for (**A**)- *R. mucilaginosa* CMGB-G1; (**B**)-*C. famata* CMGB-YR-P1.

Since lipase production is highly influenced by the nature of nitrogen/carbon source we chose to determine the growth rate of *R. mucilaginosa* CMGB-G1 on three different media. L-SC and L-SC-TY media in which the organic nitrogen source was represented by peptone and the carbon sources by glucose, respectively, glucose and tributyrin; the YPT medium contained inorganic nitrogen source represented by ammonium sulphate and tributyrin as carbon source.

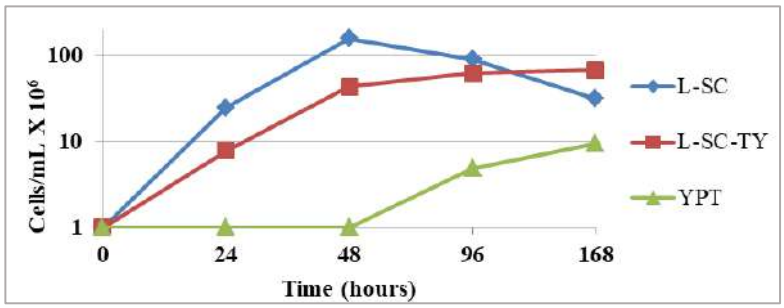
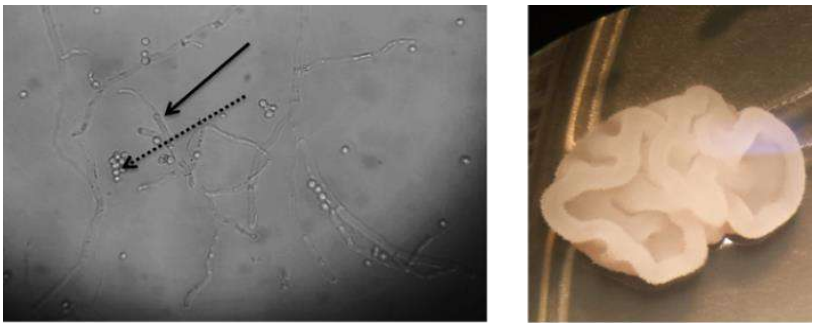



**Figure 4.** Cell growth of *R. mucilaginosa* CMGB-G1 on L-SC; L-SC-TY and YPT media.

The strain *R. mucilaginosa* CMGB-G1 strain presented high growth on L-SC and L-SC-TY medium (Figure 4). In the first case, the growth curve showed an exponential growth in the first 48 hours followed by a significant decrease in the cell population, which is probably due to the consumption of the carbon source represented by glucose. A similar growth was also observed on L-SC-TY medium with the mention that in this case, after the exponential growth, the cell population enters the plateau phase. It is very possible that this phenomenon is due to the presence of tributyrin as an alternative source of carbon. Taking into account the influence of the nitrogen source, it can be observed that the presence of an organic source facilitates cell growth. In the case of the third analyzed medium (YPT), significant increase was registered only after 48 hours, thus reflecting the need to adapt to the stringent cultivation conditions marked both by the lack of an organic nitrogen source and by the absence of glucose as a carbon source. The correlation of cell growth and screening tests performed previously revealed that the presence of tributyrin in the culture medium may be associated with increased expression of genes involved in lipase synthesis. These results suggest the need to conduct in-depth studies in order to optimize the culture parameters to increase the lipase synthesis capacity. Similar results were also reported by Nuylert (2013) who used Taghuci method to determine the influence of four factors (carbon and nitrogen source, pH value of the beginning of the experiment and the nature of the surfactant used for uniform distribution of medium components) on the production of lipaze by *R. mucilaginosa* P11I89 isolated from oil-contaminated soil. According to this study, the best carbon source tested for lipaze production was soybean with high content of oleic and linoleic acid, while peptone was chosen as optimal nitrogen source. Also, the author recommends using Tween 80 as surfactant. Another study regarding the lipolytic potential of *R. mucilaginosa* was performed by Hammamchi (2017) and revealed that using maltose and olive oil as carbon source, respectively, peptone as nitrogen source, induced highest rates of lipolytic activity.

On the contrary, the strain *C. famata* CMGB-YR-P1, even though was isolated from oil polluted soil showed

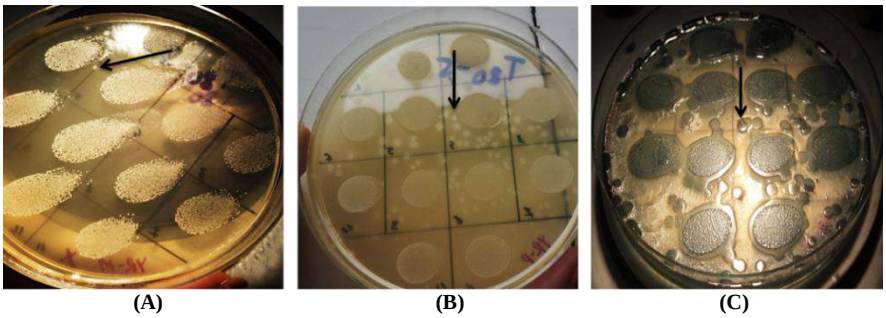
minimum lipolytic activity at all three temperatures (Figure 3-B). In order to improve the lipase production yield we used the nonspecific mutagenesis technique using NTG. This chemical mutagen is frequently used to improve the biotechnological potential of yeast strains by inducing spontaneous mutations in DNA structure. The mechanism of action involves the attachment of an alkyl group to O4 in the case of thymine or O6 in the case of guanine causing A / T to G / C and G / C to A / T transitions (HARPER, 2011 [25]; TURKI, 2013 [55]). The NTG was successfully used for improving resistance to stress conditions induced by exposure to high concentrations of salts or organic solvents (PETSAS, 2002 [40]), for obtaining higher amounts of different compounds of industrial use such as torularhodin, torulene, β-carotene (MOLINÉ, 2012 [39]) , lipids (KATRE, 2017 [28]) and even lipases (TAN, 2003 [52]). We exposed our strain to NTG in a final concentration of 50 µg/mL for 30 minutes and subsequently we inoculated the mutant cells on five different solid media frequently used in screening test for lipase producers. After seven days of growth no significant differences were recorded plates with T20-0,5 medium. The white precipitate crystals were absent in the negative control as well as in the exposed samples. Macroscopic analysis of the spots revealed the existence of different isolated colonies developed on the surface of the spot. Microscopic analysis of cells from these spots showed the existence of two cell types: normal and pseudohyphae, corresponding probably to the atypical colonies (Figure 5-A). The atypical colonies (Figure 5-B) were isolated and selected for further studies regarding the mutagenic effect of NTG on cell morphology in *C. famata* species and for screening for lipase production under different culture conditions. Also, the appearance of pseudohyphe is usually associated with pathogenicity. Therefore, further studies will concern analyzing the isolated mutants for virulence or pathogenicity factors. The use of Tween 20 as carbon source for lipase production is not as frequent compared to Tween 80 or trybutyrin due to its composition based mainly on lauric acid (over 40%), myristic, palmitic and stearic. However, some studies mentioned Tween 20 as potential lipase inducer (NOOR, 2006 [39]; SILVA, 2003 [47]).



**(A)** **(B)**

**Figure 5.** (**A**) – Aspect of the cells from samples grown on T20-0,5 medium at seven days after inoculation (black arrow-normal cells; dotted arrow-pseudohyphal cells). (**B**) – Aspect of the atypical colony selected for further studies.

On T80-0,1 and T80-0,5 media the aspect of the culture spots in the control and samples were similar. We observed the appearance of ice-like crystals around the colonies both in control and samples and, more frequently, in the samples. This might indicate that the mutagenesis influenced the ability of *C. famata* CMGB-YR-P1 strain to produce higher quantities of lipases when grown on medium supplemented with Tween 80. There was no differences between the amount of crystals observed on T 80-0,5% respectively on T 80-0,1%. Using Sudan Black for lipid staining, we observed an intensely stained ring surrounding every colonies, that might indicate the presence of higher concentration of free fatty acids and their salts, most probably due to the presence of a cellular bound lipase (Figure 6-C). The crystals observed previously to the Sudan Black staining, might suggest the possibility of a massive release of extracellular lipases in the culture medium. Tween 80 is frequently used as lipase production inducer due to its high content of oleic acid (over 58%) and in lower concentrations, linoleic, palmitic and stearic oleic acid and in lower concentrations linoleic, palmitic and stearic acids (TAOKA, 2011 [53]; BYREDDY, 2017 [10]; SALIHU, 2011 [45]).

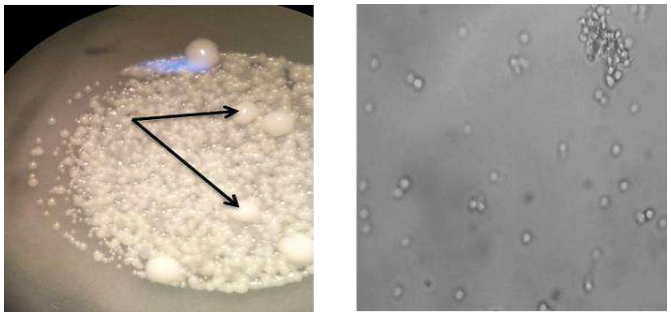


**(A)** **(B)** **(C)**

**Figure 6.** Aspect of the samples grown on T80-0,5 medium at seven days after inoculation; **A** – negative control; **B** – sample; **C** – Sudan Black stainned sample; black arrow-ice-like crystals.

Similar to the first medium described with Tween 80 as carbon source we also isolated an atypical colony with white rough and folded surface and pseudohyphal cell forms. This will be subject to future advanced studies aimed to determine the effect of the mutagenesis on *C. famata* cell morphology.

Since the screening test revealed a low ability of the strain *C. famata* CMGB-YR-P1 to hydrolyze tributyrin (YPTA medium), another set of tests were performed using YPTA-glucose medium. Adding glucose in the culture media can stimulate cells growth and facilitate metabolic adaptation to the new culture conditions. Thus, a stronger growth of the colonies was observed, although the halo remained poorly developed.



**(A)** **(B)**

**Figure 7.** (**A**) – Aspect of a spot from samples grown on YPTA-glucose medium at seven days after inoculation (black arrow-atypical colony); (**B**) – Aspect of the cells from the atypical colony.

This indicates that the mutagenesis did not influenced the ability to hydrolyze tributyrin and determined the appearance of atypical colonies, bigger than normal (Figure 7-A). Microscopical analysis of the cells from these colonies did not revealed significant morphological changes.

## Conclusions

This study allowed the identification of two new yeast strains isolated from polluted environment. The strain *R. mucilaginosa* CMGB-G1 proved to be highly valuable for lipase production with potential for biomedicine. Further work will be done in order to improve the growth conditions for stimulation of lipase production. Using NTG mutagensis, we succeeded the isolation of *C. famata* CMGB-YR-P1 mutants that will be later tested for their lipolytic ability and other potential biotechnological applications. Since, mutagenesis might affect genes involved in virulence and pathogenic factors, the mutants will be analyzed for lack of human pathogenicity and virulence factors.

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