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UV-INDUCED YEAST LIPASE PRODUCER WITH A WIDE SUBSTRATE SPECIFICITY — SELECTION, CHARACTERIZATION AND PRODUCTION OF THE ENZYME

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Abstract

Lipases are capable of changing the quantitative and/or qualitative characteristics of fat-containing raw materials and widely used for various tasks in modern food and agricultural industries. To meet the growing demand for these enzymes, highly effective producers are needed, especially those exhibiting multiple activity to lipids of different structure and origin. The aim of the study was to search for a new yeast strain with high production of lipase with broad substrate specificity, and to optimize its fermentation conditions. This work objectives also included obtaining an enzyme with a high grade of hydrolysis of various oils, and the study of its technological properties. Lipolytic characteristics were studied in 110 yeast isolates obtained from natural sources and the collections of Moscow State University of Food Production and State Research Institute of Genetics and Selection of Industrial Microorganisms. Qualitative assay of lipase activity was carried out using a differential nutrient medium with tributyrin and dye methyl red; quantitative analysis was carried out in accordance with the modified Y. Ota & K. Yamada method at pH 5.5. Of the 23 strains with lipolytic activity (LA), 12 had sufficiently high LA indices from 2.5 to 7.5 U/cm^3, of which M10 isolate with maximum activity was selected. For this isolate, morphological, cultural, physiological and biochemical properties were studied and molecular genetic identification was performed. The strain was identified as *Candida parapsilosis* (99 % of homology) using phylogenetic analysis and deposited in the All-Russian Collection of Industrial Microorganisms under the number Y-4055. After UV mutagenesis, a highly active mutant *C. parapsilosis* M10-10 was obtained. Using mathematical planning methods, the optimal nutrient medium for its growth and lipase production was determined as, %: mustard oil — 2.6, yeast extract — 1.8, soy flour — 1, glucose — 0.5, Tween 80 — 0.42, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02. An enzyme with a purification grade of 20× and lipolytic activity of 30630 U/g was obtained after culturing the strain M10-10 at a 30-40 °C temperature and pH of the nutrient medium 5.5-6.5. It was found that by 48 hours of fermentation, lipase reaches the highest activity in the culture medium when inoculum M10-10 is in an amount of 5%. Optimal conditions for the enzyme were determined as 37 °C and pH 5.5. In terms of activity, the resulting product is not inferior to commercial domestic and foreign enzymes, including Novozym 435 (Sigma-Aldrich, USA) with a lipolytic activity of 24020 U/g. The fatty acid specificity of the new lipase was determined by enzymatic treatment of various vegetable oils and gas chromatography of lipid products using a Shimadzu GC 2010 (Shimadzu, Japan). Modification of vegetable oils with M10-10 lipase in an oil/water emulsion significantly reduced 2.4, 4.6, 2.9 and 1.5 times the saturated fatty acids fraction (including palmitic and stearic acids) and increased 1.5, 1.6, 1.1 and 12 times the polyunsaturated fatty acids fraction (including ω-3 linolenic and ω-6 linoleic acids) for olive, mustard, sunflower and coconut oils, respectively.

Keywords: *Candida parapsilosis* M10-10, lipase producer, enzyme preparation, UV mutagenesis, fermentation conditions, lipolytic activity, vegetable oils, modification, saturated fatty acids, unsaturated fatty acids

Modification of raw materials or alteration of the original raw material components is used in order to improve quality of food and agricultural products. One of the objectives of modern food industry is to obtain fats and oils with required physical and chemical properties (consistence, plasticity, hardness) by modification thereof [1, 2], which is achieved by changing the fatty acid composition of lipids. Transformation of vegetable oils by lipases is the most perspective method of its modification [3, 4].

In recent years, lipases (EC 3.1.1.3) are actively used in food industry, namely in bakery, fermentation, and milk enrichment products, in processing of vegetable oil, and in production of margarine [2, 5]. Lipase treatment fosters better taste and texture of food products, extended shelf life, and increased softness of several products. Besides, lipases are used for creation of new functional ingredients and functional food products such as equivalents of cacao oil or equivalents of human milk fat [5]. As additives or biocatalysts, for instance, in production of coffee additives, lipases are used to modify taste and to obtain aromatic notes due to synthesis of compound ethers of short-chain fatty acids and spirits [6]. At processing of eggs such enzymes improve emulsifying properties of egg yolk lipids [5]. While demand for improved quality of meat grows at the global market, use of lipase in production of feed is actualized [7]. In dairy industry such enzymes are used for lipolysis of fats, improvement of taste and acceleration of cheese maturing, and production of cheese-like products [8]. Various cheese types could be produced by using lipases from various sources, for instance, pregastric lamb enzyme for Romano cheeselipase from *Penicillium camemberti* for Camembert cheese, enzymes from *P. roqueforti* for blue cheese, and *Aspergillus niger* or *A. oryzae* enzymes for Cheddar cheese [9, 10].

The perspectives of lipase use in food and agricultural industries [11, 12] necessitate studying practically significant characteristics of such enzymes, i.e. substrate specificity, optimum activity, and stability at alteration of external parameters such as, for instance, acidity of environment and temperature.

Known lipolytic enzyme preparations (EP) of microbial origin in Russia and abroad usually have fungal origin (mainly, representatives of genus *Candida C. rugosa*, *C. lipolytica*, actinomycetes *Streptomyces* and *Thermoactinomyces*) and bacterial origin (members of *Pseudomonas*, *Bacillus* genus) and optimum activity at neutral and alkali pH. Food industry needs new producers of such enzymes preserving functionality at acid pH and having significant lipolytic activity (LA) [13, 14].

Although recombinant genetically engineered enzymes are recently often proposed to food productions, issues on the safety of use of such strains and their metabolites in foods are still unsolved. Thus, the use of induced mutagenesis method with further sampling by target traits remains relevant. The main advantage of such method is that it is relatively simple and practically does not require detailed information both on genetics and metabolism of producer strain, as well as on properties of desired product.

In this work we have isolated yeast strain secreting lipase with high activity and wide substrate specificity, having characterized its technological properties and properties of enzyme preparation as compared to known Russian and foreign analogues.

Our purpose was to get highly effective lipase producer by UV-mutagenesis and selection, to determine terms of cultivation required for maximum enzyme biosynthesis, and to create enzyme preparation on its basis ensur-

ing high level of hydrolysis of various oils.

Techniques. Lipolytic properties were analyzed in 110 yeast isolates of taxa *Candida interace*, *C. maltose*, *C. tropicalis*, *Debaryomyces huansenii*, *Rhodotorula rubra*, *Zygosaccharomyces bailii*, *Z. rouxii*, *Saccharamyces cerevisiae*, *Yarrowia lipolytica*. Part of strains was received from collection of microorganisms at the Faculty of biotechnology and technology of bioorganic synthesis products of Moscow State University of Food Productions and the GosNIIgenetica, while other cultures were isolated from products with high content of lipids (samples of oils, meat and dairy products, fruits), and natural habitats (soil).

Cultural and morphological traits of strains and isolates were described using agar and liquid Saburo mediums (a thermostat TSO-1/80 SPU, OJSC Smolensk Special Design and Technological Bureau of Programmed Control Systems, Russia); light microscopy was used to study morphological characteristics (MIKMED 5, LOMO JSC, Russia).

The following basic nutrient media (NM, ingredients in percentage) were used. NM No. 1 (selective medium for strains capable of olive oil utilization as the only source of carbon) contained olive oil — 1, Na_2HPO_4 — 0.6, KH_2PO_4 — 0.3, NH_4Cl — 0.1, NaCl — 0.05, agar — 2 (pH = 7.2±0.1). NM No. 2 differential medium with tributyrin and methyl red contained Na_2HPO_4 — 0.6, KH_2PO_4 — 0.3, NH_4Cl — 0.1, tributyrin — 0.3, NaCl — 0.05, agar — 2, methyl red — 0.2 (pH = 6.5±0.1). NM No. 3, the Saburo medium, liquid or with 2% agar (HiMedia Laboratories Pvt., Ltd, India, included peptone — 1, glucose — 4, (pH = 5.6±0.1). NM No. 4 liquid medium for yeast fungi culture contained yeast extract — 2.0, glucose — 2.0, $CaCO_3$ — 0.5, KH_2PO_4 — 0.05 (pH = 5.4±0.1). NM No. 5 supplemented with lipase synthesis inducers included mustard oil — 2, yeast fungi extract — 2, soya flour — 1, glucose — 0.5, Tween 80 — 0.2, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02 (pH = 5.5±0.1). For optimization of NM composition, we used the Gausse-Seidel method of successive displacements implying an experimental search for function extremum of many variables presupposing stepwise finding of private extremums of target function for each factor. Mediums were sterilized by autoclaving during 30-40 minutes at 0.25 MPa.

Samples taken for isolation of potential lipase producers were kept for at least 1 month at 4 °C. Afterwards, samples were suspended in sterile 0.9% sodium chloride (ratio 1:10), with addition of 4% gentamicin sulphate (0.1% final concentration) to exclude development of related bacteria, and incubated for 1 hour at 30 °C. Next, enriched cultures were obtained using Saburo medium (NM No. 3), then the cultures were re-plated on the selective NM No.1 and incubated for 72 hours at 30 °C. Obtained pure yeast cultures, after control microscopy, were plated on differential NM No. 2 with indicator to reveal lipolytic property. Lipase synthesis potential of strain was assessed after growing for 48 hours at 30 °C by transparent areas around colonies and by changing the color of solid NM from yellow to orange and in furtherance to red. Pure cultures of lipolytically active microorganisms were re-plated on Saburo agar for further storage.

To quantify LA in CF centrifugates and to select the most lipolytically active strains, east isolates were grown in Erlenmeyer 750 cm^3 flasks with 100 cm^3 NM No. 4 for 48 hours at 30±2 °C and shaking. Water suspension of yeasts (5×10^6 cells/cm^3) from NM No. 3 agar slants grown for 48 hours at 30 °C was an inoculum. The inoculum made 5 % of NM No. 4 volume per flask. At the end of culturing, CF was clarified for 15-20 min at 8000 rpm (laboratory centrifuge Dastan, OJSC TNK Dastan, Russia). LA was assessed by titrimetry using olive oil as a substrate as per modified method of Ota and Yamada [15] at pH 5.5. The method is based on the rate of olive oil fermentolysis quantified by titration

of resultant organic acids with 0.05 N NaOH. Lipase activity unit means quantity of enzyme hydrolyzing 40% emulsion of olive oil to produce 1 μmol oleic acid for 1 hour at 37 °C and pH 7.0.

Selected producer strain was identified by sequencing variable region of gene encoding 18S rRNA [16]. Sequences encoding 5.8S rRNA and internal transcribed spacers ITS1 and ITS2, as well as nucleotide sequences of D1/D2 domain of 26S rRNA were compared to establish phylogenic homology [17, 18]. Biomass for DNA extraction was grown on Saburo agar. Standard PCR kits (Applied Biosystems, Inc., USA) were used to extract chromosomal DNA according to producer's instruction. Conservative primers NS1 and NS4; ITS1 and ITS4; NL1 and NL4 were used for 18S rRNA, primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for 5.8S-ITS region, and CTB6 (5′-GCATATCAATAAGCGGAGGAAAG-3′) with TW13 (5′-GGTCCGTGTTTCAAGACGG-3′) for D1/D2 domain [16]. Fragments were amplified as per the following protocol: 3 minutes at 95 °C (1 cycle); denaturation for 30 sec at 95 °C, primer annealing for 30 sec at 57 °C, elongation for 30 sec at 72 °C (35 cycles); 5 minutes at 72 °C (1 cycle) (a Mastercycler gradient, Eppendorf, Germany). Obtained fragments were subjected to gel electrophoresis (5 V/cm^3, 30 min; Bio-Rad Laboratories, Inc., USA). Amplicons stained with 10 mg/ml ethydium bromide were UV-visualized. For DNA sequencing (an automatic sequencer ABI 373A, Applied Biosystems, USA), fragments were purified using DNA Extraction kit (Thermo Scientific, Lithuania).

Specialized BLAST server software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and GenBank NCBI (https://www.ncbi.nlm.nih.gov/) were used to analyze sequencing data and to construct phylogenetic trees. Homology of nucleotide sequences of at least 97% served as criteria for taxonomic classification.

UV-mutagenesis was carried out in Petri dishes on NM No. 2 with tributyrin. Cell suspension was spread on plates in sterile conditions. Open Petri dishes were exposed for maximum 50 minutes to UV-lamp (Mineralight, USA; 30 W, 1 m distance from the lamp to plate surface). Samples were taken with 10 min interval and incubated in tubes on Saburo agar (NM No. 3) slants during 48 hours at 30 °C for culture enrichment.

Lipase-producing strain was cultured in semi-industrial conditions (OJSC Biohimmash, Moscow) in a 16 l lab fermenter, operating volume 11 l (Bioengineering AG, Switzerland) with NM No. 4 for 48 hours at 30±2 °C, pH 5.5 and airflow of 16 dm^3/min. Yeast suspension containing 5×10^6 cell/cm^3 was used as an inoculum in amount of 5 % (volume/vilume). Culture fluid was separated by centrifuging (SL 40, Thermo Scientific, USA). To purify supernatant from low-molecule admixtures and to concentrate EP, ultrafiltration (UVMT-5-20, polymer membrane with cut off 10 kDa, temperature 10-15 °C, input pressure 0.3 MPa, output pressure 0.2 MPa) was used followed by sterilizing filtration at 15-17 °C. The obtained residue was lyophilized. In pursuance of sterilizing filtration, stainless steel filter support of 142 mm in diameter (Milipore, USA) was used. Hydrophilic polyester-sulfone membrane filters with 0.22 μm pores (Pall Corp., USA) were used as filtering element. The assembled filter support was sterilized in autoclave for 30 min at 121 °C. Operating pressure at sterilizing filtration of EP concentrate comprised 0.1 MPa. Lyophilization (USP-20, FRG) was carried out at 1 cm layer depth, freezing temperature comprised –50 °C, coolant temperature was –70 °C in the first 1.5 h with temperature increase up to –20-30 °C during the next 1.5 h, and final drying during 3-4 hours at heat carrier temperature of 40 °C.

Experimental EP of lipase was compared to commercial Russian and foreign analogues, i.e. the preparations based on strains *Y. lipolytica* Pold, *S. cere-*

visiae FDS101, *Y. lipolytica* RNCIM Y-3600, *Candida* spp. L3170, *Pichia pastoris* DVSA-PLC-004, as well as with Pancreatin (PJSC Biosintez, Russia), Creon®10000 (Abbott Products GmbH, Germany), Mezym® forte 10000 (Berlin-Chemie AG, Germany), and Novozym 435 (Sigma-Aldrich, USA). In these tests, 1% solutions of studied preparations in distilled water were made and their LA was determined by modified method of Ota and Yamada [15].

To clarify fatty-acid specificity of the enzyme and to obtain lipid products rich in polyunsaturated fatty acids (PUFAs), olive, mustard, sunflower, and coconut oils were hydrolyzed [19]. To determine conditions of effective fermentolysis of vegetable oils, the effect of EP amount, oil to water ratio, temperature, and time of exposure were assessed. Free fatty acids (FA) were extracted by cold refining method. Hydrolysates were rinsed by water (1:3), and cooled up to 7±1 °C. NaOH water solution (2.5%) also cooled up to 7±1 °C was gradually added to a hydrolysate (10:1 volume/volume). The mixture was separated during 30 min at 7±1 °C. Resultant sodium salts of fatty acids were rinsed by water. The isolated neutral fat was neutralized with 5 % H_2SO_4, the remaining salts were separated. The obtained FA methyl ethers were analyzed by liquid gas chromatography (Shimadzu GC 2010, Shimadzu, Japan) with mass-detector GCMS-QP 2010 and column MDN-1 (l = 30 m, ∅ = 0.25 mmN; solid-phase-bound methyl silicone filler). Operating parameters: injector temperature 200 °C, interface temperature 210 °C, detector temperature 200 °C; helium carrier, flow rate of 1 cm^3/min, split ratio of 1:5. Mass-detector parameters: registration mode — TIC, with m/z range of mass analyzer 45-500. Sample components were identified using the mass-spectrum library NIST 02 (https://www.nist.gov/srd). Identification validity comprised over 93 %.

1. Lipolytic activity (LA) **in culture fluid centrifugates of strains and isolates** (nutrient medium No. 4)

Strain, isolates	LA, U/cm^3
Candida interace[1]	2.5±0.13
C. maltosa[1]	2.5±0.13
C. tropicalis[1]	5.0±0.25
Rhodotorula rubra[1]	2.5±0.13
Yarrowia lipolytica[2]	5.0±0.25
П-РОС[3] (P-ROS[3])	5.0±0.25
П-М[3] (P-M[3])	5.0±0.25
М10[3] (M10[3])	7.5±0.38
П5[3] (P5[3])	2.5±0.13
П8[3] (P8[3])	2.5±0.13
Ж1[3] (J1[3])	5.0±0.25
Ол[3] (Ol[3])	5.0±0.25

N o t e. [1] — collection of the Moscow State University of Food Productions, Moscow, [2] — RNCIM collection of GosNIIgenetica, Moscow; [3] — strains isolated from high-lipid products and natural sources.

Results are presented as means (*M*) at 3-fold replication with standard error of mean (±SEM). Confidence interval of arithmetic mean for p = 0.05 was determined using Statistica 6 software (StatSoft, Inc., USA) and Microsoft Excel 2010. The difference between two mean values was statistically significant if their confidence intervals were not overlapped.

Results. It was established that only 23 out of 110 yeast isolates plated on NM No. 1 can utilize olive oil as a single carbon sourcea. A total of 12 yeast culture isolates (Table 1) were selected by activity, where CF of M10 isolated from the surface of dairy butter brick had the highest LA. The M10 isolate was selected for further studies. As per morphological, cultural, physiological and biochemical traits (Table 2), the M10 strain was referred to *Candida* genus.

2. Morphocultural and physio-biochemical characterization of *Candida* sp. M10 isolate

Trait	Description
M o r p h o l o y	
Culture morphology on the 1st day	Round or oval cells, 2.5-4.5 rm in width and 2.5-9.0 rm in length
Culture morphology on the 2nd day	Budding of most cells; formation of pseudo mycelium.

Continued Table 2

Cultural traits	
Growth on Saburo agar	Round colonies with ruffled border; 1-7 mm in diameter; large-wrinkled surface, flat profile built in substrate; shiny and non-transparent colonies; light dingy-beige color; wave-shaped edges; coarse-grained structure; tight-leathery consistence
Growth in liquid Saburo medium	Opaqueness; residue and rink on tube walls; no medium pigmentation; no gas formation; yeast odor
Spore formation	Absent
Physio-biochemical traits	
Nitrate assimilation	Negative
Growth ability in vitamin free medium	Positive (biotin serving as a strong growth stimulator)
Gelatin liquefaction	Positive
Urea hydrolysis	Positive
NaCl concentration	Intensive growth at 8%, 10%, and 12% of NaCl
pH	Good growth at pH from 3 to 9
Temperature	Weak growth at 20 °C; intensive growth at 25-37 °C; weak growth at 50 °C

The nucleotide sequence of variable 18S rRNA gene region of M10 strain genome was as follows:

CATACATGTCTAAGTATAAGCAATTATACAGTGAAACTGCGAATGGCTCATAAA-
TCAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCT-
AGAGCTAATACATGCTTAAAATCCCGACTGTTTGGAAGGGATGTATTTATAGA-
TAAAAAATCAATGCCTTCGGGCTCTTTGATGATTCATAATAACTGAATCACAT-
GGCCTTGTGCTGGCGATGGTTCATTCAAATTTCTGCCCTCGATGGTAGGATA-
GTGGCCTACCATGGTTTCAACGGGTAACGATAAGGGTTCGATTCCGGAGAG-
GGAGCCTGAGAAACGGCTACACATCCAAGGAAGGCACAGGCGCCAAATTAC-
CCAATCCCGACACGGGGAGGTAGTGACAATAAATAACGATCAGGGCCCTTTC-
GGGTCTTGTAATTGGAATGAGTACAATGTAAATACCTTAACGAGGAACAATT
GGAGGGCAAGTCTGGTGCGCAGCCGCGGTAATTCCAGCTCCAAAACGTATA-
TTAAAGTTGTTGCAGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTTGGC-
CTGCCCGG.

Initially, the primary screening of nucleotide sequence using GenBank databases and BLAST server had shown that M10 strain belongs to groups: *Eukaryota*, *Fungi*, *Ascomycota*, *Saccharomycotina*, *Saccharomycetes*, *Saccharomycetales*, *mitosporic Saccharomycetales*, *Candida*. Analysis of variable DNA region of 18S rRNA gene allowed this strain to be referred to several species of *Candida* genus with probability of less than 97 %. Thus, to specifically identify the strain, we carried out a comparative analysis of DNA encoding 5.8S rRNA and internal transcribed spacers ITS1 and ITS2, and also comparative analysis of 26S rRNA gene sequences encoding D1/D2 domain.

For DNA region of 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 the following nucleotide sequence was obtained:

GCGGGGTAGTCTACCTGATTTGAGGTCGAATTTGGAAGAAGTTTTGGAGTTTG-
TACCAATGAGTGGAAAAAACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAA-
GCAAACCCAGCGTATCGCTCAACACCAAACCCGAGGGTTTGAGGGAGAAATG-
ACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAA-
AGATTCGATGATTCACGAATATCTGCAATTCATATTACTTATCGCATTTCGCTG-
CGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTATT-
AAATAATCGGTTGACATTAAATAAAATTTGGTTGAGTTTATCTCTGGCAGGC-
CCCATATAGAAGGCCTACCAAAGCAAAGTTTTCAAAAAAAGAAAAACACATG-
TGTAAGAAAAAATGCAGTTAAGCACTTTTCATTCTGTAATGATCCTTCCGCAG-
GTTCACCAGGAAGAATATTAAGAATGAAAAGTGCTTACTGCATTTTTTCTAAC-
ATGTGTTTT.

The sequence for region of 26S rRNA gene was as follows:

GGATTGCCTTAGTAGCGGCGAGTGAAGCGCAAAAAGCTCAAATTTGAAATCT-
GGCACTTTCAGTGTCCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGTCTTGC-
TCTTGTCTATGTNTCTTGGAACAGAACGTCACAGAGGGTGAGAATCCCGTGC-
GATGAGATGTCCCAGACCTATGTAAAGTTCCTTCGAAGAGTCGAGTTGTTGG-
GAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGA-

GAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAA-
GAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGA-
CTTGGTATTTTGTATGTTACTCTCTCGGGGGTGGCCTCTACAGTTTACCGG-
GCCAGCATCAGTTTGAGCGGTAGGATAAGTGCAAAGAAATGTGGGACTGCT-
TCGGTAGTGTGTTATAGTCTTTGTCGATACTGCCAGCTTAGACTGAGGACTG-
CGGCTTCGGCCTANGA.

Therefore, according to clarified data, M10 strain is close to *Candida parapsilosis* (homology level 99 %) [20]. Based on the results of molecular identification, strain *C. parapsilosis* M10 was deposited to All-Russia Collection of Industrial Microorganisms (GosNIIgenetica) under accession number RNCIM Y-4055.

Enzyme biosynthesis by a producer mainly depended on NM composition, physiological state of microorganism, terms of culturing, and the amount of inoculum [21, 22]. At submerged culturing of *C. parapsilosis* M10 strain, we have gradually varied concentrations and types of carbon, nitrogen, phosphor, and microelement sources in nutrient medium No. 5, since such factors may affect lipase production [23].

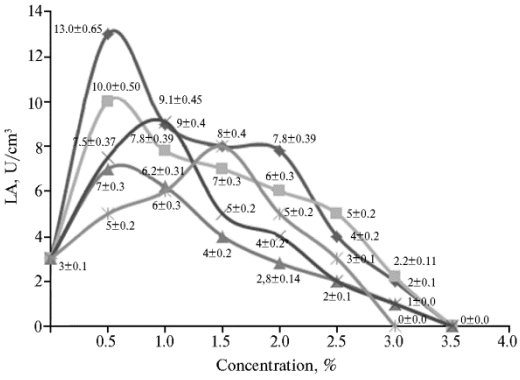

Fig. 1. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 at different concentrations of carbon sources in nutrient medium:** — glucose, — saccharine, — fructose, — maltose, — galactose.

Different sugars, which concentrations were changed at culturing of M10 strain on NM No. 5, were used as carbon source (Fig. 1). Maximum LA occurred in variant with 0.5% glucose concentration as yeast fungi prefer glucose out of sugars.

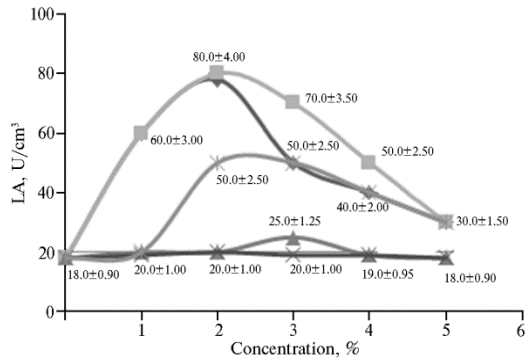

Fig. 2. Lipolytic activity (LA) **of producer strain C*andida parapsilosis* M10 depending on inducer (oil) type and concentration ////:** — olive, — mustard, — pure sunflower, — refined sunflower, — flax seed.

It is known that various vegetable oils are often used to induce lipase synthesis and as an additional carbon source. It has been reported that Y. *lipolytica* DSM 3286 strain produced 34.6±0.1 U/cm^3 lipase on medium with olive oil with addition of yeast extract [24].

It was established in previous work [25] that maximum LA in 48 hours of culturing *C. rugosa* (DSM 2031) strain comprised 4.43; 3.29; 2.86; 1.74; 1.23, and 1.03 U/cm^3 for sesame, peanut, sunflower, palm, coconut, and castor oils, respectfully. In our study we have also tested various oils and varied concentrations thereof (Fig. 2). Thus, upon addition of 2% mustard oil LA of M10 strain comprised 80 U/cm^3. According to results, mustard oil contains FA inducing lipase synthesis in *C. parapsilosis* M10.

Fickers et al. [26], having studied the ability of various mineral nitrogen

sources, for instance, NH_4Cl and $(NH_4)_2SO_4$, and organic substrates (casamino acids, trypton, urea, yeast extract, various peptones) to maintain lipase growth and production in *Y. lipolytica* LgX64 81 strain, had shown that enrichment of the medium by mineral nitrogen did not significantly affect cell growth or enzyme biosynthesis, whilst lipase production was notably increased at addition of several sources of organic nitrogen. The highest yield (166-fold yield increase up to 484.7±59.1 U/cm^3) was noted at presence of Hycase SF with trypton N1.

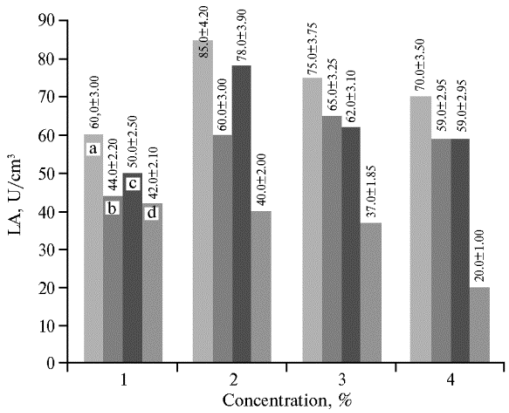

Fig. 3. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 depending on nitrogen type and concentration in nutrient medium:** a — yeast extract, b — soya flour, c — peptone, d — $(NH_4)H_2PO_4$.

As a nitrogen source, we used yeast extract, soya flour, peptone, and $NH_4H_2PO_4$ in different concentrations (Fig. 3). The highest lipase activity of producer strain was noted on nutrient medium No. 5 with 2 % yeast extract. Possibly, it was due to the fact that yeast extract contains essential amino acids and B group vitamins required for enzyme biosynthesis.

3. Lipolytic activity (LA) **of *Candida parapsilosis* M10 depending on soya flour concentration in nutrient medium**

Concentration, %	LA, U/cm^3
0.5	80±4.0
1.0	90±4.5
1.5	85±4.3
2.0	70±3.5

Soya flour containing up to 30% fat could render positive effect on lipase biosynthesis [27]. During the additional experiment on NM No. 5 with yeast extract (2.0 %), we have introduced different quantity of soya flour (Table 3). The most activity of producer was obtained on medium with 1.0% soya flour.

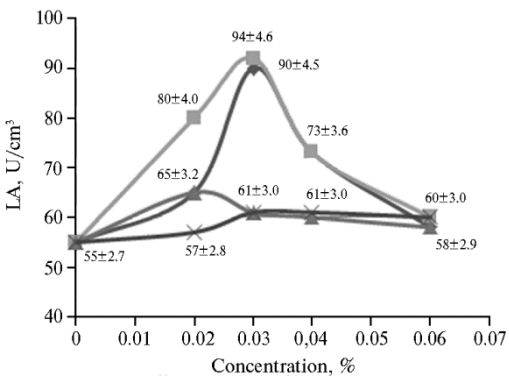

Fig. 3. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 depending on P type and concentration in nutrient medium:** —•— — K_2HPO_4, —■— — KH_2PO_4, —▲— — $(NH_4)_2HPO_4$, —×— — $(NH_4)H_2PO_4$.

Phosphorus is required for microorganism activity and enzyme biosynthesis [28]. We have studied the effect of K_2HPO_4, KH_2PO_4, $(NH_4)_2HPO_4$, and $(NH_4)H_2PO_4$ on LA of CF centrifugate of *C. parapsilosis* M10 strain (Fig. 4). The best indices of lipase activity were on medium No. 5 with 0.03% KH_2PO_4. The graph also allows tracing such fact that potassium ions are required for lipase biosynthesis.

Due to optimization by mathematical method of experiment design the following NM (No. 6), % was established: mustard oil — 2.6, yeast extract — 1.8, soya flour — 1, glucose — 0.5, Tween 80 — 0.42, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02, pH 5.5±0.1. Due to optimized NM, LA of *C. par-*

apsilosis M10 strain CF increased up to 204 U/cm^3.

Enzyme concentration in microbial cell could be significantly increased by changing the growth conditions, by mutagenesis or genetic manipulations [22, 23, 32]. Mutant *C. parapsilosis* M10-10 with LA in CF of 235 U/cm^3 was obtained due to UV-mutagenesis of *C. parapsilosis* M10 strain (Fig. 5). UV exposure lasting over 10 min rendered negative effect on enzyme biosynthesis. Similarly, Chen et al. [29] had isolated *Trichosporon fermentans* WU-C12 strain from soil showing maximum yield (nearly 30 U/cm^3) at culturing during 4 days at 30 °C. Afterwards 2PU-18 strain with higher enzyme activity (up to 70 U/cm^3) was obtained by UV-induced mutagenesis and by changing the medium composition. Other authors, when working with *Aspergillus niger*, have used nitrogen acid in addition to UV-treatment as a mutagen (both methods used twice) and have obtained the 20.7 and 39.1 % increase in lipase production, respectively, after double UV and nitrogen acid treatment as compared to parent strain having yield of 34.8 U/ml [30].

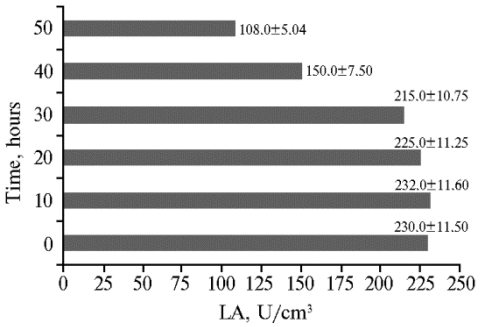

Fig. 5. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on UV-exposure.**

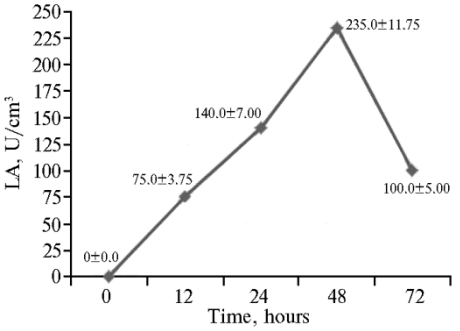

Fig. 6. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on time of culturing.**

4. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on pH**

pH	LA, U/cm^3
4.0	145±7.25
4.5	170±8.5
5.0	220±11.0
5.5	235±11.75
6.0	230±11.5
6.5	228±11.4
7.0	200±10.0
7.5	150±7.5
8.0	40±8.0

We have experimentally established that the highest lipase activity in CF is attained in 48-hour M10-10 culture, 5% inoculum volume to growth medium volume (Fig. 6), 30-40 °C and pH 5.5-6.5 (Table 4). Production of dry EP in fermenter culture comprised 7.92 g/cm^3 with LA 30630 U/g.

Comparison of LA in Russian and foreign lipase preparations and obtained EP implies (Table 5) that M10-10 G20× lipase was not inferior to other preparations. We have experimentally established the following optimal conditions for enzyme hydrolysis of oil using of M10-10 G20× lipase EP, the 0.1% enzyme (61.2 U/g lipids), oil to water ratio 1:1, 37 °C, pH 5.5, and 4-hour incubation.

5. Comparison of lipolytic activity (LA) **of the developed *Candida parapsilosis* M10-10-based lipase preparation and known commercial preparations**

Preparation	Collection, producer	LA, U/g
Lipase M10-10 G20×	Obtained in this work	30630±18.5
Preparation based on *Yarrowia lipolytica* Po1d strain [30]	Collection of Moscow State University of Food Productions	2400±8.5
Preparation based on *Saccharomyces cerevisiae* FDS101 strain [31]	Collection of Moscow State University of Food Productions	1800±6.7

Continued Table 5

Preparation based on *Y. lipolytica* strain RNCIM Y-3600 [32]	RNCIM of GosNIIgenetica, Russia	2700±6.2
Lipase from *Candida* spp. L3170	Sigma-Aldrich, USA	5000
PLC lipase BD16449 (based on *Pichia pastoris* DVSA-PLC-004 strain) [33]	Collection of Moscow State University of Food Productions	205±0.7
Pancreatin	Biosintez, Russia	4300
Kreon® (10000 U)	Abbott Products GmbH», Germany	10000
Mezym® forte 10000	Berlin-Chemie AG, Germany	10000
Novozym 435	Sigma-Aldrich, USA	24020±10.2

Identification of fatty-acid specificity of enzyme had shown that maximum yield of free FA in 4 hours comprised 30.0% for olive oil, 30.5% for mustard oil, 32.2% f sunflower oil, and 35.6% for coconut oil (of total acid content in oils). Thus, it was inferred on lipase enzyme specificity to the residues of saturated FA. Hydrolysis by lipase M10-10 G20× and further refining of vegetable oils allowed us validly to reduce the relative content of saturated FA 2.4; 4.6; 2.9 and 1.5 times and to increase PUFAs 1.5; 1.6; 1.1 and 12 times for olive, mustard, sunflower, and coconut oils, respectively (Table 6). Accordingly, modification of vegetable oils by lipase preparation M10-10 G20× in oil:water emulsion allows us to obtain lipid products enriched with ω-3 and ω-6 linoleic and linolenic PUFAs valuable for human health.

6. Fat components and fatty acid composition of vegetable oils and modified lipid products derived from M10-10 G20× lipase hydrolysis

Component	Olive		Mustard		Sunflower		Coconut	
	O	M	O	M	O	M	O	M
Mass content of fat components, %								
FA	20.0±4.00	8.3±1.66	14.3±2.86	3.1±0.62	12.0±2.40	4.1±0.82	89.1±17.82	60.8±12.16
PUFAs	11.4±2.28	17.5±3.50	37.3±7.46	58.1±11.62	58.5±11.70	65.4±13.08	1.6±0.32	19.4±3.88
PUFAs ω-6	6.9±1.38	7.9±1.58	24.0±4.80	27.9±5.58	58.5±11.70	65.4±13.08	1.6±0.32	19.4±3.88
PUFAs ω-3	4.5±0.90	9.7±1.94	13.3±2.66	19.0±3.80	–	–	–	–
Mass content of fatty acids, %								
Capric $C_{10:0}$	–	–	–	–	–	–	7.3±1.46	2.3±0.46
Lauric $C_{12:0}$	–	–	1.3±0.26	–	–	–	45.3±9.06	35.2±7.04
Myristic $C_{14:0}$	–	–	–	–	–	–	21.2±4.24	18.3±3.66
Palmalite $C_{16:0}$	15.5±3.10	6.8±1.36	9.4±1.88	1.4±0.28	7.7±1.54	1.3±0.26	12.3±2.46	10.7±2.14
Stearic $C_{18:0}$	4.2±0.84	1.6±0.32	3.1±0.62	1.8±0.36	4.0±0.80	2.7±0.54	3.0±0.60	2.8±0.56
Oleic $C_{18:1}$	61.3±12.26	64.7±12.94	34.5±6.90	38.3±7.66	26.3±5.26	27.6±5.52	9.3±1.86	19.9±3.98
Linoleic $C_{18:2}$	6.9±1.38	7.9±1.58	24.0±4.80	27.9±5.58	58.5±11.70	65.4±13.08	1.6±0.32	10.9±2.18
Linolenic $C_{18:3}$	4.5±0.90	9.7±1.94	13.3±2.66	19.0±3.80	–	–	–	–
Arachic $C_{20:0}$	0.3±0.06	–	0.6±0.12	–	0.4±0.08	–	–	–
Gondoinic $C_{20:1}$	0.3±0.06	–	5.5±1.10	2.8±0.56	–	–	–	–
Erucidic $C_{22:1}$	–	–	4.8±0.96	3.2±0.64	–	–	–	–

Note. FA — fatty acids, PUFA — polyunsaturated fatty acids; O — original, M — modified products. Dashes mean trace amounts or absence of components.

During the last years, yeast genus *Candida* (for instance, *C. antarctica*, *C. rugosa*, *C. tropicalis*, *C. curvata*, *C. parapsilosis* strains), as well as *Galactomyces geotricum*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Trichosporon fermantans*, *T. asahii*, *Rhodotorula mucilaginosa*, *Aureobasidium pullulans* are deemed the most perspective microbial producers of lipase with high enzyme activity [35, 36]. However, only some of lipases are commercially used for mass production purposes [37]. Such enzymes have high activity and wide temperature and pH optimums.

Activity of new lipase M10-10 G20× is close to such in Novozym 435 preparation (Sigma-Aldrich, USA) obtained based on *C. antarctica* strain. Literature sources confirm that lipase synthesized by *C. antarctica* strains manifest high LA. Lipase CALB with activity of 117 U/g of dry weight [38], as well as genetically engineered strain of *Pichia pastoris* synthesizing lipase of *C. antarctica*

CALB with activity of 220 U/g of dry weight are known [39]. Few more commercially successful EP such as Chirazyme® L-2 (Boehringer Mannheim GmbH, Germany) and SP 525 (Novo-Nordisk A/S, Denmark) are obtained based on the same microorganism [37].

In 2004, *Y. lipolytica* Pold (CLIB 139) and *Y. lipolytica* Polf (ATCC MYA-2613) widely used for metabolic engineering were derived from *Y. lipolytica* W29 (ATCC 20460) strain [40]. Moreover, genetically engineered *Saccharomyces cerevisiae* strains producing lipases Lip7 and Lip8 of *Y. lipolytica* with activity of 283 and 121 U/g (per dry weight) are also known [41]. In experiments, optimal pH for hydrolysis of olive oil by *Y. lipolytica* lipase comprised nearly 8.0. At temperature below 37 °C and pH 4.5-8.0, these enzymes remain stable for 20 minutes, at 5 °C — for 22 hours. Lipases synthesized by *C. rugosa* are one of mostly used in the industry due to high enzyme activity. Indian researchers have extracted and characterized three different *C. rugosa* lipase forms [4]. Purified isoforms of extracellular lipase (lipA, lipB and lipC) had molecular weights of 64, 62, 60 kDa and temperature optimum within the range of 35-40 °C. *Geotrichum candidum* lipases are of commercial interest due to high specificity to long-chain cis-9-unsaturated fatty acids in substrates. *G. candidum* is known as producer of extracellular lipase stable at pH 6.5-8.5 [42]. Ciafardini et al. [43] had established that yeast strain *Williopsis californica* 1639 isolated from virgin olive oil produces extracellular lipase with activity optimum at pH 6. Böer et al. [44] had cloned AL1P1 gene encoding lipase of yeast *Arxula adeninivorans*. It was shown that the enzyme is a 100 kDa dimmer with amino acid sequence similar to such in lipases from *C. albicans* and *C. parapsilosis*. Its maximum activity is at 30 °C and pH 7.5.

Commercial lipase preparations often are a mix of various isoforms. Thence, low reproducibility of biocatalytic processes when using commercial lipases, for instance, *C. rugosa* (CRL), could be due to presence of different isoforms, which complicates the interpretation of findings [45].

Our study of thermal- and pH-stability of lipase M10-10 G20× EP had shown that preparation, unlike the abovementioned, completely preserves its high activity in the range of 30-40 °C and pH 4.0-7.0, which is the advantage for industry and agricultural application. In addition, the potential of modern metabolic engineering of lipase producers, which we discussed above, can be applied to strain M10-10.

Thus, the following conclusions could be drawn based on performed studies. A new yeast lipase producer *Candida parapsilosis* M10-10 with lipolytic activity (LA) in cultural fluid (CF) of 235 U/cm3 was derived from UV-exposure of an isolate from surface of butter brick. Culture conditions for the optimal enzyme biosynthesis have been developed. Production of enzyme preparation (EP) involves submerged culturing of *C. parapsilosis* M10-10 strain in fermenter with further separation of cultural fluid, ultrafiltration, sterilizing filtration, and lyophilization. The technology allows 7.92 g/dm^3 yield of dry EP with LA of 30630 U/g. The lipolytic activity of M10-10 G20× EP is enough to ensure high hydrolysis of various vegetable oils. The EP LA is inferior to that of domestic and foreign analogues, i.e. Novozym 435, Chirazyme® L-2, SP 525, CALB and *C. antarctica* CALB lipases. The developed preparation can be used for modification of raw material components in agricultural production.

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