CHARACTERISATION OF ALKALINE LIPASE FROM AN ARCTIC YEAST STRAIN *RHODOSPORIDIUM BABJEVAE* BD19

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Abstract:

The yeast strain isolated from freshwater from Arctic Archipelago of Svalbard was identified biochemically by API C 20 aux test, genetically by 18S rRNA and ITS sequencing as *Rhodosporidium babjevae* BD19. *R. babjevae* secreted into the culture medium lipase with the activity 67.7 U/ml after 144 hours of growth on Tween 80 as a sole of carbon source and 115.5 U/ml after 144 hours on inorganic ammonium sulphate as a sole nitrogen source. The molecular mass of purified lipase was 37.6 kDa and optimum esterasic activity was at pH 9.0 and 30 ^{o}C. The chain length specificity of the enzyme was determined using p-nitrophenyl fatty acids esters as substrates and the highest hydrolysis rate was with p-nitrophenyl caprylate. The *R. babjevae* BD19 lipase seems to have unique 1-position specificity when hydrolyzing triolein.

Key Words: *Rhodosporidium babjevae*, Arctic yeast, lipase, esterase

Introduction

Lipases are capable of catalyzing a large number of reactions with high regio- and enantioselectivity (Schmid and Verger, 1998). They catalyze not only hydrolysis, but also esterification, acylation or transesterification reactions (Reis, et al., 2009). For example, hydrolytic reactions of lipases showed potential applications in degrading oil and fats in wastewater generated by dairy industries, producers of edible oils and fat refineries (Mendes and Castro, 2005). Acylation of carbohydrates is used for production of non-ionic surfactants for food and pharmaceutical industries (Nakamura, 1997). Transesterification reactions catalyzed by lipases have a prominent significance in the oil industry for tailoring vegetable oils (Gupta, et al., 2003).

The majority of lipases used for biotechnological purposes are isolated from bacteria and fungi (Schmid and Verger, 1998, Arpigny and Jaeger, 1999). Compared to other microorganisms, yeast enzymes are less used in commercial applications and only some species have been studied for potential industrial applications (Brizzini, 2006).

Extreme environments can serve as a reservoir of microorganisms secreting enzymes with novel, useful properties. Recent investigations showed many important qualities of lipases secreted by psychrophilic microorganisms (Joseph, et al., 2008). While the Antarctic yeast *Candida antarctica* is the most extensively studied microorganism due to its lipase (Kirk and Christensen, 2002), Brizzio and collaborators (Brizzio, et al., 2007) isolated new basidiomycetous yeasts characterized by their cold-active enzymes from glacial and subglacial waters. Among other yeast strains, they found *Rhodosporidium babjevae* with lipolytic activity.

Lipases isolated from different sources have a wide range of properties in terms of positional and substrate specificity, thermostability or pH optimum, and catalyze a number of reactions used for development of specific industrial applications such as organic synthesis, detergent additive or food processing. Thus, isolation of new strains secreting lipases which catalyze unique reactions will enable chemical industries to catalyze new compounds (drugs) or reduce the costs of existing technologies.

Screening of 132 morphologically distinct bacteria and yeasts isolated from freshwater from Arctic Archipelago of Svalbard for lipase secretion resulted in the isolation of ten species having high

lipase activity (Krasowska, et al., 2007, Krasowska and Łukaszewicz, 2011). In previous works we have identified *Pseudomonas fluorescens* strain producing novel biosurfactants (Janek, et al., 2011) and characterized their properties (Janek, et al., 2012). In this report we have identified a novel yeast strain *Rhodosporidium babjevae* BD19, optimized medium and growth conditions for secretion of extracellular lipase, partially purified the enzyme and characterized its specificity and activity.

Materials and methods

Isolation of yeast strain and culture conditions

The strain BD19 was isolated from freshwater from the Arctic Archipelago of Svalbard.

The strain was grown in 300 ml flasks containing 50 ml LB medium (5 g/l yeast extract, 10 g/l bacto-tryptone, 10 g/l NaCl). Flasks were inoculated with 5 ml of overnight pre-culture and incubated for 24 h at 28 °C with agitation (180 rpm).

The effect of different oils as carbon sources on lipase production by BD19 strain was investigated on a mineral medium (Papaparaskevas, et al., 1992) supplemented with 2 g/l of tested carbon source (glucose, Tween 80, rape, sunflower, flax or olive oil). For optimization of nitrogen source, BD19 strain was cultivated on the same mineral medium containing 20 g/l Tween 80 and supplemented with 10 g/l of tested substrate (yeast extract, bacto peptone, ammonium sulfate, urea or ammonium chloride).

Identification of the yeast BD19 strain

The pure culture of strain BD19 was identified by API C 20 aux test (BioMérieux, Marcy l'Etoile, France).

Moreover, the 18S rRNA gene was sequenced to support the biochemical identification. Genomic DNA was extracted with UltraClean ™ Microbial DNA Isolation Kit (MoBio®) according to manufacturer's instructions and 18S rRNA gene fragment was amplified with the primers: EucA 5'-AACCTGGTTGATCCTGCCAGT-3' and EucB 5' TGATCCTTCTGCAGGTTCACCTAC3'. The amplification conditions were as follows: 94 °C for 10 min., 30 cycles, 94 °C for 1 min., 59 °C for 1 min., 72 °C for 2 min., and final synthesis at 72˚C for 5 min. ITS fragments were amplified with the primers: ITS1 5' TCCGTAGGTGAACCTGCGG 3'and ITS4 5' TCCTCCGCTTATTGATATGC 3'. The PCR conditions were as follows: 94 °C for 10 min., followed by 30 cycles, 94 °C for 1 min., 48 °C for 1 min., 72 °C for 1 min., and final synthesis at 72 °C for 5 min. The PCR products were separated by agarose gel electrophoresis and purified for sequencing using a GeneMATRIX PCR/DNA Clean-up Purification kits (EURx, Gdansk, Poland). The sequences obtained were compared to rDNA sequences from the GeneBank (http://www.ncbi.nlm.nih.gov/BLAST/). ITS fragments obtained from GeneBank database were aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the phylogenetic tree was computed with Jalview 2.4.0.b2 using the neighbor-joining method.

Purification of lipase

The *R. babjevae* BD19 was cultured with 20 g/l of Tween 80, which enabled phase separation (Albertsson, 1986). Cells were removed by centrifugation at 4000 g for 10 minutes. To 100 ml of supernatant 37.6 g ammonium sulphate was added (50% saturation at 20 °C). After 30 min of gentle agitation ensuring solubilization and equilibrium of the partition, phase separation was accomplished by centrifugation at 25 200 g for 20 minutes 4 °C. The upper phase was collected and dialyzed against distilled water overnight. Samples were withdrawn for lipase assay (Gupta, et al., 2002) and total protein determination (Bradford, 1976).

Esterase assays

Lipase activity (Gupta, et al., 2002) was assayed spectrophotometrically with modifications using p-nitrophenyl palmitate (p-NPP) as substrate. Briefly, the reaction buffer contained Tris HCl 50 mM pH=9.0, arabic gum 0,9 mg/ml and Triton X100 4 mg/ml. To 10 ml of the reaction buffer 1 ml of p-NPP solution (3 mg/ml) in isopropanol was added before the test and 230 µl aliquots were dispensed on a 96-well plate. Reaction was started by addition of 20 µl of enzyme solution. The reaction was conducted at 23 °C for 30 minutes, and the absorbance at 410 nm was measured every 5 minutes. One unit was defined as the amount of the enzyme releasing 1 nmol of free p-nitrophenol per minute.

The chain length specificity of the enzyme was determined using p-nitrophenyl esters (Sigma) of acetate (C_2), butyrate (C_4), caproate (C_6), caprylate (C_8), decanoate (C_{10}), laurate (C_{12}), palmitate (C_{16}) and stearate (C_{18}) as substrates at pH 8.8 and at 23 °C.

Effect of pH and temperature on enzyme activity

The impact of pH was determined by measuring lipase activity at 30 °C in 0.5 M Tris HCl buffer at pH ranging from 7 to 10. The optimal temperature of the enzyme was determined by measuring its activity in a temperatures range (25-50 °C) in 0.05 M Tris HCl buffer, pH 9.0.

Positional specificity

Positional specificity was determined by analyzing lipolytic products of triolein by thin layer chromatography (TLC) on silica gel 60 plate (Merck, Darmstadt, Germany). The total reaction mixture (1.5 ml) containing 33 □l of 22.6 mM
incubated with 33 □l (10 units) of lipase at 30 °C with shaking at 800 rpm. Aliquots of 200 µl were removed at 0, 2, 4 and 24 hours and the products were extracted with 0.5 ml of diethyl ether. The extracted samples were applied to the silica gel-60 TLC plates for product analysis. The plate was developed in a chamber with the solvent mixture chloroform/ acetone/acetic acid (95:4:1). The hydrolysis products were visualized using saturated iodine vapor and compared with standards (oleic acid, 1-oleylglycerol, 1,2-sn-dioleylglycerol, 1,3-dioleylglycerol and triolein) from Sigma-Aldrich Sp. z o.o., Poland.

Results and Discussion

Identification of *Rhodosporidium babjevae* BD19

Cells of the isolated strain were oval, yeast-shaped, redish and aerobic. The strain BD19 was arabinose-, xylose-, idonitol-, galactose-, sorbitol-, metyl-glucopyranoside-, sucrose-, and raffinose utilization-positive and xylitol-, inositol-, cellobiose-, lactose-, maltose-, trehalose-, and melesitose utilization-negative. These biochemical results were not sufficient for classification to the genus. Comparative sequence analysis of the 18S rDNA gene (968 bp) in the GeneBank database revealed that the strain BD19 had 99% homology to *Rhodotorula glutinis, Rhodotorula graminis* and *Rhodosporidium babjevae.*

Rhodotorula species are ubiquitous in human environment and have been isolated from different environments such as soil (Mok, et al., 1984), fruit juice (Tournas, et al., 2006) or sea water (Nagahama, et al., 2001, Loureiro, et al., 2005). In 1967 Banno first described *Rhodosporidium toruloides* as a telomorph in the life cycle of *R. glutinis* (Banno, 1967). Several following studies indicated phenotypic and genetic heterogeneity in *Rhodotorula* gender (Yamazaki and Komagata, 1981, Hamamoto, 1987, Vancanneyt, 1992, Gadanho and Sampaio, 2002).

To further classify strain BD19 the Internal Transcribed Spacer (ITS) region of the rDNA from BD19 was PCR amplified, sequenced and compared with GeneBank database. The ITS regions are adequate to resolve the relationship between closely related genera (Libkind, et al., 2008). Neighbor-joining analysis of the data was carried out with the program Neighbor of the PHYLIP package.

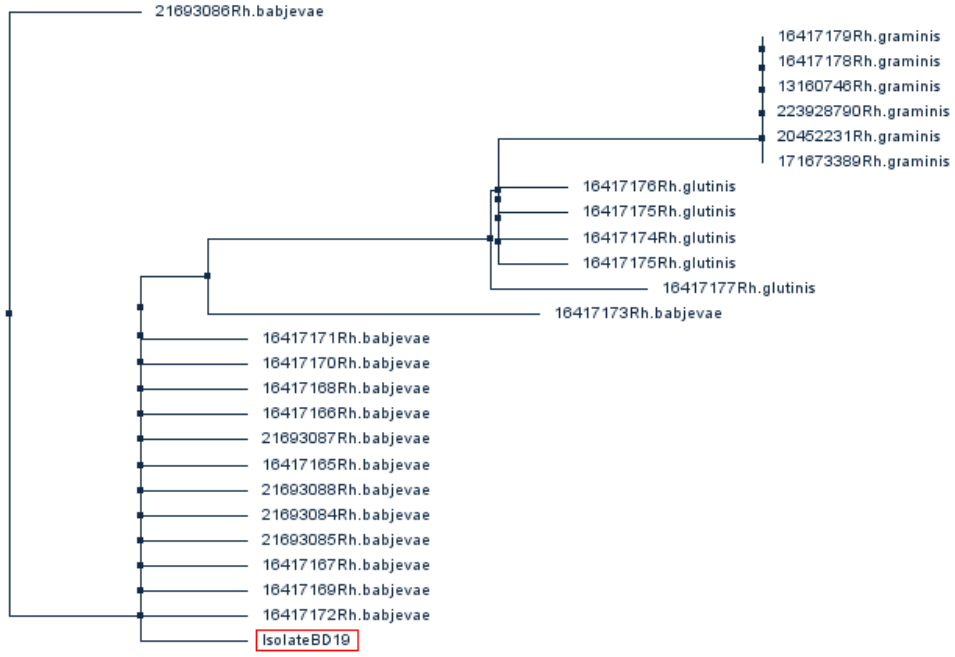

Fig.1 Phylogenetic tree of *R. glutinis*, *R. graminis*, and *R. babjevae* obtained by neighbor-joining analysis of ITS region of rDNA. The GI number of sequence is placed before the species name

The sequence obtained from BD19 strain (GeneBank JN003620) was placed together with sequences from microorganisms classified as *R. babjevae* (Fig. 1). The nucleotide sequence of ITS region from BD19 strain was identical to twelve other *R. babjevae* sequences included in the phylogenetic tree.

The influence of carbon and nitrogen sources on lipase production

In general, extracellular lipase production by yeast depends greatly on the medium composition (Papaparaskevas, et al., 1992, Darvishi, 2009, Kim, et al., 2009). With *R. babjevae* BD19 strain, the highest lipase activity (67.7 U/ml) was obtained after 144 hours of growth on Tween 80 as a sole of carbon source. The activity was 18, 45.5, 41 and 55% smaller when BD19 strain was grown on carbon sources such as olive, sunflower, rape and palm oils, respectively (Table 1). The lowest lipase activity was obtained using glucose as the sole carbon source (10.5 U/ml after 144 h incubation). In contrast to BD19 strain induced to lipase production by water-soluble emulsifier (Tween 80), Papaparaskevas and collaborators (Papaparaskevas, et al., 1992) found the highest activity of lipase secreted by *R. glutinis* cultured on olive oil (29.3 U/ml). In addition, the activity dramatically decreased when other substrates such as palm oil (2.4 U/ml), sunflower oil (2.0 U/ml) or Tween 80 (1.8 U/ml) were used (Papaparaskevas, et al., 1992). In this respect *R. babjevae* BD19 lipase production was much less influenced by different carbon sources such as esters of fatty acids.

Table 1. Effect of carbon source on lipase secretion by *R. babjevae* BD19. The strain was grown in a medium with yeast extract as a nitrogen source and different carbon sources at neutral pH. The lipase activity in culture supernatants was measured after 72 and 144 hours of incubation at 30 °C ±SD, n=3.

Carbon source	**Activity [U/ml]**	
	72 hours	**144 hours**
Glucose	1.16 ± 0.24	10.5 ± 1.16
Tween 80	47.69 ± 4.91	67.7 ± 16.9
Olive oil	41.57 ± 10.12	55.7 ± 9.06
Sunflower oil	43.96 ± 7.14	36.9 ± 9.74
Rape oil	51.9 ± 3.78	39.9 ± 0.07
Palm oil	17.79 ± 3.62	30.2 ± 4.51

The highest lipase yield (115,4 U/ml) produced by *R. babjevae* BD19 was obtained after 144 hours of incubation at 30 ^{o}C on inorganic nitrogen source (ammonium sulphate as a sole nitrogen source). When the strain was grown 144 hours on yeast extract, bacto peptone, ammonium chloride or urea as nitrogen sources, lipase activities were 65, 32, 27 and 99% lower, respectively (Table 2). Lipase secreted by *R. babjevae* BD19 after 72 hours of incubation on yeast extract, bacto peptone, ammonium sulphate, ammonium chloride or urea as carbon sources was accordingly 29.4-, 22-, 31-, 37- and 8-fold more active than lipase secreted by *R. glutinis* after 6 days of incubation (Papaparaskevas, et al., 1992).

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, with the exception of *R. glutinis* which requires inorganic nitrogen source such as ammonium phosphate for high lipase production (Papaparaskevas, et al., 1992).

Table 2. Effect of nitrogen source on lipase production. *R. babjevae* BD19. The strain was grown in cultivation medium with Tween 80 as carbon source and different nitrogen sources at neutral pH. The lipase activity in culture supernatants was measured after 72 and 144 hours of incubation at 30 °C ±SD, n=3.

Nitrogen source	**Activity [U/ml]**	
	72 hours	**144 hours**
Yeast extract	44.10 ± 2.77	40.73 ±4.71
Bacto peptone	26.57 ± 0.85	37.1 ± 2.71
Ammonium sulphate	37.66 ± 1.35	115.4 ± 5.44
Ammonium chloride	37.23 ± 4.54	84.0 ± 2.57
Urea	12.38 ± 4.75	1.3 ± 0.14

Purification of the enzyme

A two-phase-system purification procedure was used to characterize extracellular lipase from B19 strain. This procedure increased the specific activity of lipase 3.84-fold with a yield of 88.38% (Table 3). The lipase secreted into the medium had a molecular mass of 37.6 kDa by SDS-PAGE.

Table 3. Purification of the *R. babjevae*. BD19 extracellular lipase.

	Total activity [U]	Total protein [mg]	Specific activity [U/mg]	Purification fold	Yield [%]
Culture filtrate	5575.46	4305.40	1.29	1.00	100.00
Partitioning in two-phase system	4927.36	991.79	4.97	3.84	88.38

Effect of pH and temperature on enzyme activity

Lipases secreted by microorganisms mostly have optimum of activity in a weakly alkaline environment and at temperatures ranging from 30 to 40 ^{o}C (Choo, et al., 1998, Yu, et al., 2007). Lipase secreted by *R. glutinis* had optimum activity at 35 ^{o}C and pH 7.5 (Papaparaskevas, et al., 1992).

The highest activity of lipase from *R. babjevae* BD19 was obtained with pH 9.0 at 30 ^{o}C; it decreased by 30% when pH was increased to 10.0 and by 70% when pH decreased to 7.0 (Fig.2).

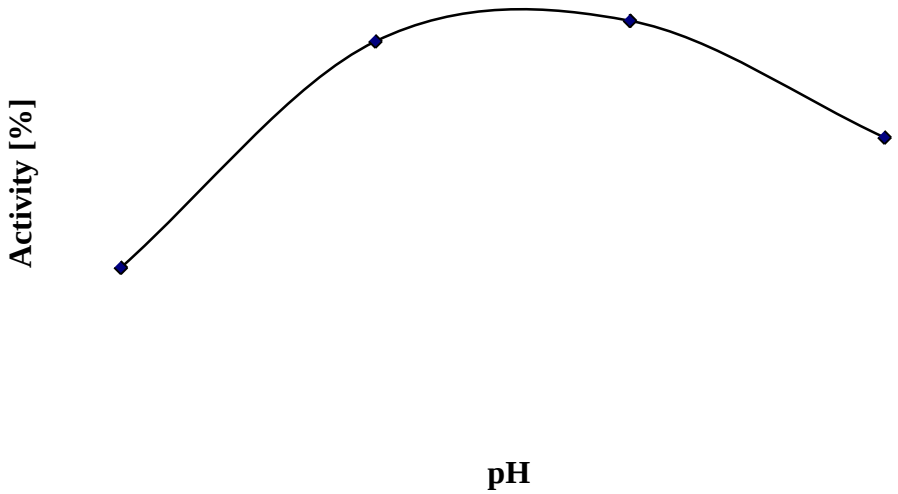

Fig. 2. Influence of pH on lipase activity. Data are given as means ±SD, n=3.

The optimum temperature for enzyme activity was 30 ^{o}C; it decreased with rising temperature, with 80% loss of original activity at 50 ^{o}C (Fig 3). Fungal lipases are generally active at 40-50 ^{o}C (Hiol, et al., 2000). The optimum pH was higher and optimum of temperature was lower than that for lipase-producing *R. glutinis* (pH 8.0 at 35 ^{o}C) (Papaparaskevas, et al., 1992), *R. mucilaginosa* (pH 7.0-8.0 at 40 ^{o}C) (Zimmer, et al., 2006) and. *R. pilimanae* (pH 4.0 and 7.0 at 45 ^{o}C) (Muderhwa, et al., 1986).

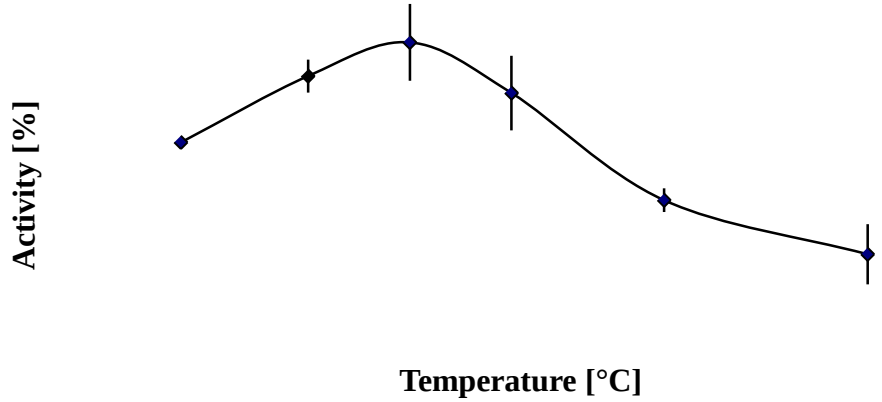

Fig 3. Influence of temperature on lipase activity. Data are given as means ±SD, n=3.

Substrate specificity with p-nitrophenyl esters

The substrate specificity of partially purified *R. babjeviae* BD19 lipase towards p-nitrophenyl esters with alkyl chain length ranging from C2 to C18 was determined (Fig. 4). The lipase showed the highest activity with the use of p-nitrophenyl caprylate ester (C_8). Specific activity decreased gradually as the alkyl chain was shortened or lengthened. When p-nitrophenyl acetate (C_2) was used as substrate no significant activity was measured.

Grognux and collaborators (Grognux and Reymond, 2004) classified lipases according to their specificity as dependent on the chain length. They suggested classification into 3 groups: the short-chain (C4±C6), long-chain (C10±C16), and middle-chain (C8±C10) reactivity. According to such classification *R. babjeviae* BD19 lipase has the highest specificity towards middle chain fatty acid esters. Lipase of *R. glutinis* was very active with nitrophenyl butyrate (C4) and nitrophenyl laurate (C12) (Hatzinikolaou, et al., 1999); *Rhodotorula minuta* lipase had high activity towards p-nitrophenyl butyrate (C4) in lecithin emulsion (Cinelli, et al., 2006). Lipases which utilized natural methylthioesters of volatile fatty acids (C_3-C_8) have considerable importance for flavor industry; thus, e. g. S-methyl butanetioate is an important constituent of dairy aromas (Rajendran, et al., 2009).

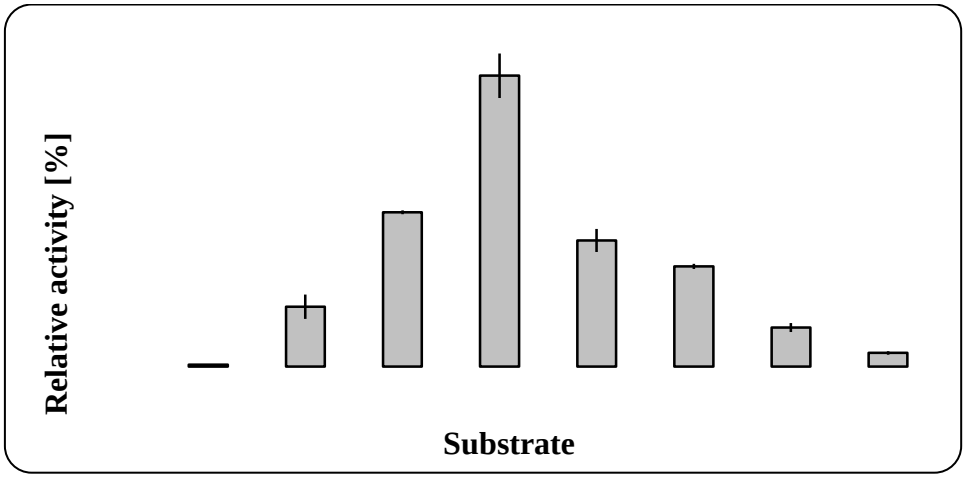

Fig. 4. *R. babjevae* BD19 lipase substrate specificity. Activity towards different p-nitrophenyl esters was determined at 30 °C and pH=9.0. Data are given as means ±SD, n=3

Positional specificity

Marcae and Hammond (Macrae, 1985) systematized lipases according to their regio-specificity. The first group of lipases is not specific and released fatty acids from all three positions. The second group released the fatty acids specifically from the outer 1 and 3 positions of acylglycerols. Microbial lipases show usually 1,3-positional specificity, releasing three products from triolein: 2-monoacylglycerol, 1,2- (or 2,3) – diacylglycerol and free fatty acid (Zimmer, et al., 2006,

Aloulou, et al., 2007). Lipases with specificity towards 2-position are very rare in nature. Only few lipases have 2-position specificity, such as lipase from *Geotrichum* sp. FO401B (Ota, et al., 2000), lipases from *Geotrichum candidum* ATCC 34614 (Sugihara, et al., 1994), or lipase from *Aspergillus niger* NCIM 1207 (Mhetras, et al., 2009).

Triolein was used as a substrate to investigate positional specificity of purified lipase. The separation of triolein hydrolysis products was performed by thin-layer chromatography (Fig. 5). The major product end product of the *R. babjevae* BD19 lipase was 1,2-diolein and prolonged incubation for 24 h strongly indicates that the enzyme has 1-position specificity.

Lipase from *R. babjevae* BD19 seems to be unique in its positional specificity because it cleaved triolein only once in 1-position, releasing only two products: 1,2 (or -2,3)-diolein and free fatty acid.

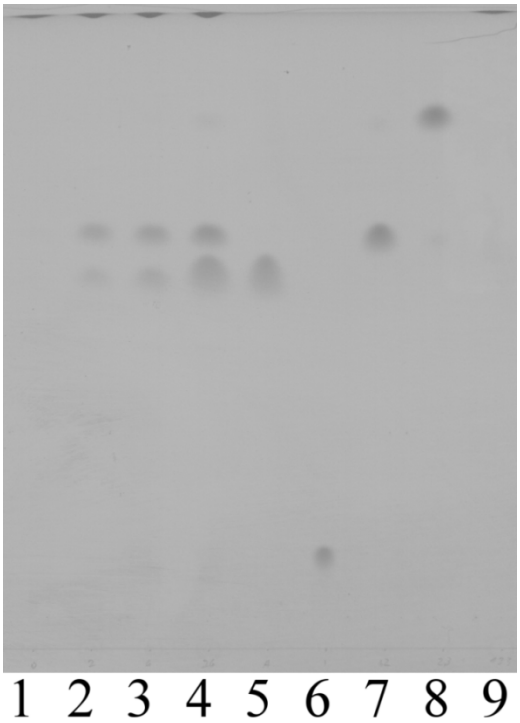

Fig. 5 Thin-layer chromatography of the hydrolysis products of triolein catalyzed by *R. babjevae* BD19 lipase. Lane 1: 0 h, Lane 2: 2h, Lane 3: 4 h, Lane 4: 24 h, Lane 5: oleic acid, Lane 6: 1-oleylglycerol, Lane 7: 1,2-sn-dioleylglycerol, Lane 8: 1,3-dioleylglycerol, Lane 9: triolein.

Conclusions

Biochemical and genetic characterization of the BD19 strain from Arctic fresh water led to its classification as *R. babjevae*.

The highest concentration of the *R. babjevae* BD 19 extracellular lipase in the medium was obtained after 144 hours on Tween 80 as a sole of carbon source and after 144 hours on inorganic nitrogen source (ammonium sulphate as a sole nitrogen source).

The purified lipase had a molecular mass of 37.6 kDa.

The maximum activity of the lipase was obtained at pH 9.0 and at 30 ^{o}C.

The lipase had the highest hydrolytic activity towards 8-carbon alkyl chain substrate - p-nitrophenyl caprylate.

The lipase is unique in comparison to other described enzymes since it hydrolyzed triolein to 1,2-diolein and in this respect.

Acknowledgments

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