VILNIUS UNIVERSITY

RENATA GUDIUKAITĖ

# ENGINEERING AND BIOCATALYTIC PROPERTIES OF LIPASES AND ESTERASES PRODUCED BY *GEOBACILLUS* BACTERIA

Summary of doctoral dissertation

Biomedical Science, Biology (01 B)

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VILNIAUS UNIVERSITETAS

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# *GEOBACILLUS* GENTIES BAKTERIJŲ SINTETINAMŲ LIPAZIŲ IR ESTERAZIŲ INŽINERIJA IR BIOKATALIZINIŲ SAVYBIŲ ĮVERTINIMAS

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# CONTENT

# INTRODUCTION

Microbial lipolytic enzymes are classified into two major groups: lipases (EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1) (Arpigny and Jaeger 1999; Bornscheuer 2002). Because of their regio- and stereospecifity and activity in organic solvents lipases and esterases have been recognized as very useful biocatalysts in industrial applications. Lipolytic enzymes are widely applied in the production of pharmaceuticals, leather, detergents, foods and medical diagnostics (Bornscheuer 2002; Hasan et al. 2010; Sharma et al. 2011). They are also important in the cosmetic and perfume industry for the synthesis of flavor esters or mono- and di-acylglycerols (Hasan et al. 2006), as well as nowadays – relevant synthesis of biopolymers, biodiesel and lipid modification (Bornscheuer 2008; Anobom et al. 2014; Yan et al. 2015).

During the last fifteen years since *Geobacillus stearothermophilus* L1 lipase was described (Kim et al. 1998) the focus on the lipases and esterases produced by *Geobacillus* bacteria strongly increased. These microorganisms synthesize lipolytic enzymes which can be active at extreme conditions (alkaline pH, high temperature, organic solvents or detergents in reaction environment) and have a great potential for application in bioconversion and ecotechnology (Sangeetha et al. 2011; Guncheva and Zhiryakova 2011; Dror et al. 2015). Industrial processes often take place under harsh conditions that are hostile to microorganisms and their biocatalysts. For these reasons enzymes which possess high thermoactivity and thermostability are necessary for such industrial processes (Elleuche et al. 2015).

One of the strategies to improve the thermoactivity and thermostability of industrial enzymes is protein engineering. The potential of this technique giving the target biocatalysts new properties depends on the knowledge about the role of individual domains and amino acids. Scanning mutagenesis' strategies have proven to be a useful approach to structure-function and protein evolution studies (Maynard et al. 2002; Moreina et al. 2007). In these experiments, one or several amino acid residues are changed (i.e., to alanine) and the resulting mutant proteins are analyzed for changes in function (Morrison and Weiss 2001).

Directed evolution is a common technique to achieve improved physicochemical properties of various enzymes and overcome the limitations of natural biocatalysts. It is a natural evolutionary process mimicked at lab bench and does not require prior knowledge of the primary sequence of proteins and their function or structure (Joshi and Satyanarayana 2015). The most popular methods of directed evolution are homologous recombination through DNA shuffling (Stemmer et al. 1994) and error-prone PCR (epPCR) (Pritchard et al. 2005). Only several epPCR studies were performed with *Geobacillus* lipases (Dror et al. 2014; Shi and Pan 2011; Kauffmann and Schmidt-Dannert 2001).

Another strategy to improve the physicochemical and kinetic properties of industrial enzymes is development of fused chimeric enzymes. Fusion proteins are a class of proteins with two or more different protein domains integrated into one molecule and have been developed as a class of novel biomolecules with multi-functional properties (Chen et al. 2013; Yu et al. 2015). Only several publications exists describing fused microbial lipases (Gustavsson et al. 2001; Rotticci-Mulder et al. 2001; Qin et al. 2014; Ahn et al. 2004).

**The aim of this study was:** to identify new regions important for the activity of *Geobacillus* lipases, design of new thermoactive and thermostable lipase and / or esterase using protein engineering strategies and evaluate physicochemical and kinetic characteristics of these enzymes.

**The following tasks have been formulated to achieve this aim:**

1. To investigate the influence of Asp371, Phe375 and Tyr376 amino acids located at C-terminal end on functionality of GD-95-10 lipase.
2. To perform screening, cloning, expression, purification and physicochemical and kinetic analysis of new carboxylesterase produced by *Geobacillus* bacteria.
3. To evaluate the influence of additional homodomain on the activity and physicochemical and kinetic properties of the esterase and / or lipase produced by *Geobacillus* sp. 95 strain.
4. To design new chimeric fusion biocatalyst composed of *Geobacillus* sp. 95 strain esterase and lipase and evaluate biocatalytic potential of this fusion enzyme.

5. Design of new lipolytic enzymes using DNA shuffling and epPCR strategies and analysis of its activity, catalytic and physicochemical properties.

**Scientific novelty and practical value:**

Lipases and esterases produced by *Geobacillus* bacteria is a promising and important area in basic research and industrial applications. This work demonstrates that 10 and 20 C-terminal amino acids of GD-95 lipase significantly affect lipolytic activity and thermostability of this enzyme, which has never been reported before. Also in this work the significance of Asp371, Phe375 and Tyr376 from C-terminal region for the efficient functionality of *Geobacillus* sp. 95 lipase (GD-95) was showed for the first time.

New carboxylesterase produced by *Geobacillus* sp. 95 strain with molecular size of 55 kDa was identified in this work. GDEst-95 esterase is the first carboxylesterase produced by *Geobacillus* bacteria with 55 kDa molecular size analysed in-depth. GDEst-95 esterase can be grouped into VII family of bacterial lipolytic enzymes and is a new member of this family. Because of its physicochemical and kinetic properties GDEst-95 esterase has high potential for application in various industrial areas.

In further experiments GDEst-95 esterase together with GD-95 lipase were used for construction of fused lipolytic chimeric biocatalyst GDEst-lip. GDEst-lip esterase / lipase possessed high lipolytic activity, a broad pH range, thermoactivity, thermostability and ability of efficient hydrolysis after treatment with various organic solvents or detergents. Because of these features GDEst-lip biocatalyst has high potential for application in various industrial areas. In this work the influence of additional domain on monomeric GDEst-95 esterase and GD-95 lipase activity, thermostability, substrate specificity and catalytic properties was investigated for the first time. It has been showed that usage of several fused domains can modulate the activity and physicochemical characteristics of target enzymes for industrial applications.

In this study three new *Geobacillus* lipases (GD-28, GD-66 and GD-76) were also identified. These proteins expanded the existing information about physicochemical properties of *Geobacillus* lipases. Furthermore, genes of GD-28, GD-66 and GD-95 lipases

were subjected to DNA shuffling and epPCR experiments. After direct evolution two new lipolytic enzymes (GDlip43 and GDlip30) were obtained. GDlip43 lipase possessed improved physicochemical properties compared to parental lipases, while further analysis of GDlip30 lipase might help to answer the question about minimal structure required for the lipolytic activity of *Geobacillus* lipases.

**Thesis statements:**

1. At C-terminal end located amino acids are important for the *Geobacillus* lipases' activity and functionality.
2. Addition of the same domain changes temperature activity and thermostability of GD-95 lipase and GDEst-95 esterase from *Geobacillus* sp. 95.
3. The fusion of lipase and esterase produced by *Geobacillus* bacteria is a strategy, that allows the creation of new more thermoactive and more thermostable lipolytic biocatalysts.
4. Even a small sequence variation of *Geobacillus* lipases can lead to different physicochemical properties of these enzymes.

# MATERIALS AND METHODS

**Bacterial strains, plasmids and culture conditions**

*Geobacillus* spp. strains 28, 66, 76 and 95 were selected from collection of microorganisms (Department of Microbiology and Biotechnology, Faculty of Natural Sciences, Vilnius University, Lithuania). For DNA manipulation, *Geobacillus* spp. strains were grown in Luria-Bertani (LB) broth (Sambrook and Rusell 2001) at 55–60 $^{o}$C with agitation (180 rpm). The *Escherichia coli* strains used in DNA manipulations were: *E. coli* DH5α (φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 phoA supE44 gyrA96 λ-thi-1 relA1) (Invitrogen) and *E. coli* BL21(DE3) (λcIts857 indl Sam7 nin5 lacUV-T7 genas 1) (Novagen). *E. coli* transformants were grown in LB medium supplemented with 100 μg/ml ampicillin (Applichem) at 30–37 $^{o}$C with agitation (150–180 rpm). Vector pTZ57R/T (Thermo Fisher Scientific) was used as a cloning vector for the target genes in *E. coli* DH5α strain and pET-21c(+) (Novagen) for enzyme expression in *E. coli* BL21 (DE3). LB agar plates containing 100 μg/ml ampicillin, 0.5 mM isopropyl-β-D thiogalactopyranoside (IPTG) (Thermo Fisher Scientific), 20 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) (Thermo Fisher Scientific) and 0.5 % emulsified tributyrin (TCI Europe) was used to screen the recombinant clones harboring pTZ57R/T plasmids with inserted genes. The *E. coli* BL21 transformants harboring pET-21c(+) plasmids with target inserts were screened on the same LB agar medium with ampicillin and tributyrin.

**DNA manipulation**

Genomic DNA of *Geobacillus* spp. strains was extracted with GeneJET DNA Purification Kit (Thermo Fisher Scientific). Plasmid DNA from *E. coli* transformants was isolated with the GeneJET$^{TM}$ Plasmid Miniprep Kit (Thermo Fisher Scientific) and Zyppy™ Plasmid Miniprep Kit (Zymo Research). PCR products after amplification were purified using GeneJET$^{TM}$ PCR Purification Kit (Thermo Fisher Scientific). GeneJET$^{TM}$ Gel Extraction Kit (Thermo Fisher Scientific) was used to recover DNA fragments from agarose

gels. Electrocompetent *E. coli* DH5α and *E. coli* BL21(DE3) cells were prepared using the protocol from Sambrook and Rusell (2001).

**Primers used in this work**

The primers used in this work are listed in Table 1. Also for the amplification of genes, inserted into pTZ57R/T and / or pET-21c(+) vectors M13 and T7 primers, respectively, were used. All primers were synthesized by Metabion.

**Polymerase chain reaction (PCR) and agarose gel electrophoresis**

The amplification reaction mixture (all components except primers were purchased from Thermo Fisher Scientific) contained 1.5 mM $MgCl_2$, 1 × *Taq* DNA Polymerase buffer without $MgCl_2$, 2 mM dNTP mix (0.2 mM of each dNTP), 2.5 U of recombinant *Taq* DNA Polymerase, 0,5 μM of each forward and reverse primers and genomic DNA (10 ng) / plasmid DNA or biomass of transformant. The conditions for the PCR amplification were as follows: 1) pre-denaturation at 95 $^{o}$C for 1–5 min; 2) denaturation at 95 $^{o}$C for 1 min; 3) annealing at 48–65 $^{o}$C (based on the primers) for 2 min; 4) extension at 72 $^{o}$C for 3–4 min; 5) final extension at 72 $^{o}$C for 7 min and preservation at 4 $^{o}$C. Stages 2–4 stages were repeated 29–49 times based on the experiment. The amplified products were detected by electrophoresis of target PCR product through 0.8 % agarose (ROTH) gel with ethidium bromide (1 μg/μL) (Sigma-Aldrich) and visualized with UV. The fragments after hydrolysis by DNase I (Thermo Fisher Scientific) were separated through 2 % agarose gel. The PCR products were purified and sequenced at the Institute of Biotechnology (Lithuania). As DNA size markers were used MassRuler DNA ladder mix, GeneRuler DNA Ladder Mix (Thermo Fisher Scientific) and SORPOsize$^{TM}$ DNA Ladder (SORPO).

**Cloning target genes into cloning pTZ57R/T vector**

Amplificated target genes were ligated into pTZ57R/T vector used T4 ligase following the manufacturer's ligation protocol (Thermo Fisher Scientific). The ligated products were transformed into *E. coli* DH5α electrocompetent cells using a standard electroporation protocol (Sambrook and Rusell 2001). The positive transformants were selected as described previously.

**Construction of expression plasmid**

For expression, gene fragments were cleaved from pTZ57R/T plasmid with the *Nde*I and *Not*I restriction enzymes (Thermo Fisher Scientific) (for the lipases), *Nde*I and *Sal*I (Thermo Fisher Scientific) (for the esterase) and others in Table 1 listed restriction endonucleases (for the fused genes). Then target gene fragments were purified from agarose gel and ligated in pET-21c(+) expression vector digested with the same restriction enzymes and dephosphorylated with alkaline phosphatase (Thermo Fisher Scientific). The ligation was carried out as described above. The resulting pET-21c(+) plasmid with inserted target gene was transformed into *E. coli* DH5α and then retransformed into *E. coli* BL21 (DE3) competent cells for gene expression.

**Identification of positive clone**

Positive clones were identified by lipolytic activity on plates supplemented with tributyrin and double restriction digestion of plasmids with *Not*I and *Nde*I or others restriction endonucleases listed in Table 1 based on the target construct. Also the size of the target insert was determined by performed linearization of resulting plasmid with *Xba*I restriction endonuclease (Thermo Fisher Scientific) and colony PCR.

**Alanine mutagenesis**

The mutated lipase variants R1–R6 were constructed using primers (Table 1) designed according to GD-95-10 lipase sequence (GD-95 lipase without 10 C-terminal amino acids). These mutated primers were used to substitute the nucleotide sequence coding Asp371, Phe375 and Tyr376 with GCT, coding Ala. After amplification each mutation was confirmed by sequencing. The cloning and positive clone detection procedures were carried out as described in sections “Cloning target genes into cloning pTZ57R/T vector; Construction of expression plasmid and Identification of positive clone “.

**Table 1. List of the primers used for the target gene amplification and design of new gene variants. Sequences underlined indicate restriction sites and sites of mutations are marked in square. Primers were synthesized by Metabion (Germany).**

| Gene or protein name | Primer | Sequence | Inserted restriction site | Amplicon size (bp) | Primer $T_m$ (°C) |
|---|---|---|---|---|---|
| Screening of *Geobacillus* lipase | GelipF-59 | 5'- TTG CCG GCA TTG AAT AGC CTG AT-3' | - | 1200 | 63 |
| | GelipR+179 | 5'-CTC CCA CCG TCC GTC CCA CTT G-3' | - | | 70 |
| Cloning of GD lipases into expression vector | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Gelip95R | 5'-TAG CGG CCG CAG GCC GCA AAC TCG C-3' | *NotI* | | 76 |
| R1 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Tyr376Ala+Asp371Ala | 5' –TAG CGG CCG CCC GCA AAG CAA AGG CGC GAA TAG CAA ATG-3' | *NotI* | | 82 |
| R2 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Tyr376Ala+Phe375Ala | 5' –TAG CGG CCG CCC GCA AAG CAG CGG CG- 3' | *NotI* | | 79 |
| R3 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Asp371Ala | 5'- TAG CGG CCG CCC GCA AAT AAA AGG CGC GAA TAG CAA ATG 3' | *NotI* | | 80 |
| R4 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Phe375Ala | 5' –TAG CGG CCG CCC GCA AAT AAG CGG CGC GAA T 3' | *NotI* | | 79 |
| R5 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Tyr376Ala | 5'- TAG CGG CCG CCC GCA AAG CAA AGG CGC G- 3' | *NotI* | | 79 |
| R6 | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 1200 | 73 |
| | Rev-10-Phe375Ala+Asp371Ala | 5'- TAG CGG CCG CCC GCA AAT AAG CGG CGC GAA TAG CAA ATG-3' | *NotI* | | 82 |
| Screening of esterases | GSLE-F-41 | 5' –GAC GTG GGA GGG GTG GTG GTT TAT-3' | - | 1500 | 69 |
| | GSLE-R+64 | 5'-TTG GCC GTT CCT TTG TTG GTT TAG-3' | - | | 64 |
| Cloning of GDEst-95 esterase into expression vector | GEStp-31F | 5'-GGG ATA AAG CAT ATG GAA CAA ACC GAT GTT G-3' | *NdeI* | 1500 | 70 |
| | GEStp-23R | 5'-TAG TCG ACG CGT CCT TGC CAT GC-3' | *SalI* | | 68 |
| GDEst-est | GEStp-31F | 5'-GGG ATA AAG CAT ATG GAA CAA ACC GAT GTT G-3' | *NdeI* | 3000 | 70 |
| | Est95-Rev-SacI | 5'-TAC GAG CTC GCG TCC TTG CCA TGC-3' | *SacI* | | 70 |
| | Est95-Forv+SacI | 5'-TAG CGA GCT CAT GGA ACA AAC CGA TGT TG-3' | *SacI* | | 70 |
| | GEStp-23R | 5'-TAG TCG ACG CGT CCT TGC CAT GC-3' | *SalI* | | 68 |
| GDLip-lip | Gelip95-43-F | 5'-TGA AGC GCA TAT GGC AGT TTC ACG CGC CAA-3' | *NdeI* | 2400 | 73 |
| | Lip95-Rev+SacI | 5'-TAG AGC TCA GGC CGC AAA CTC GC-3' | *SacI* | | 68 |
| | Lip95-Forv-SacI | 5'-TAG GAG CTC ATG GCA GTT TCA CGC GC-3' | *SacI* | | 71 |
| | Gelip95R | 5'-TAG CGG CCG CAG GCC GCA AAC TCG C-3' | *NotI* | | 76 |
| GDEst-lip | GEStp-31F | 5'-GGG ATA AAG CAT ATG GAA CAA ACC GAT GTT G-3' | *NdeI* | 2700 | 70 |
| | Est95-Rev-SacI | 5'-TAC GAG CTC GCG TCC TTG CCA TGC-3' | *SacI* | | 70 |
| | Lip95-Forv-SacI | 5'-TAG GAG CTC ATG GCA GTT TCA CGC GC-3' | *SacI* | | 71 |
| | Gelip95R | 5'-TAG CGG CCG CAG GCC GCA AAC TCG C-3' | *NotI* | | 76 |

## Construction of fused chimeric biocatalysts

Parental GD-95 lipase and GDEst-95 esterase were amplified with primers listed in Table 1 and Fig. 1.

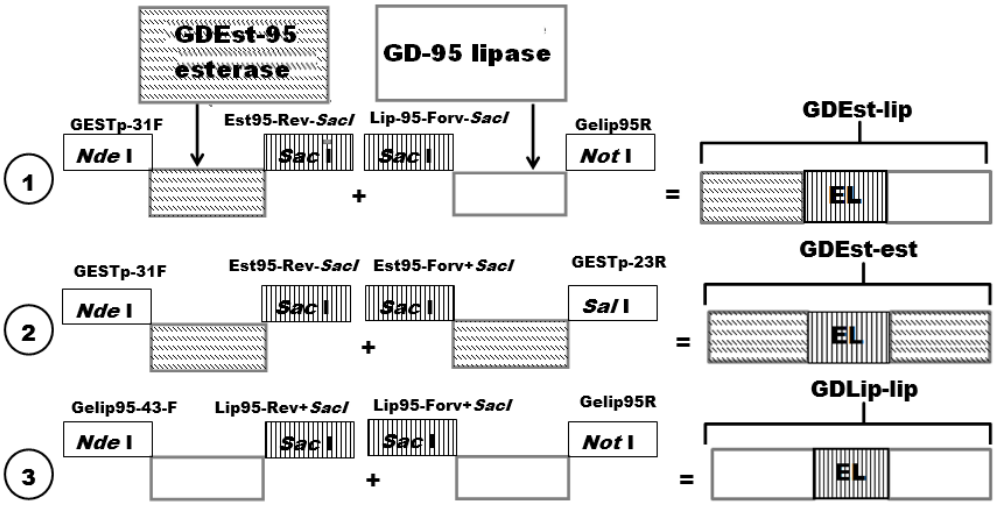


**Fig. 1. Schematic presentation of generated new fused biocatalysts. Same restriction endonuclease sites are marked and Glu-Lys (EL) amino acids represent linker formed after restriction with *Sac*I.**

The resulting PCR products were cloned into pTZ57R/T vector and transformed into *E. coli* DH5α cells. From positive transformants plasmids were isolated and restricted with restriction endonucleases (Table 1). After restriction target gene fragments were purified from agarose gel and ligated with each other using T4 ligase as specified by manufacturer. The chimeric gene variants were cloned into pET-21c(+) vector for expression as described previously.

## DNA shuffling

After amplification by PCR obtained lipase gene fragments were mixed in equal volume and used for fragmentation with DNase I. The fragmentation reaction mixture contained 1x digestion buffer (100 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) (Thermo Fisher Scientific), 10 mM $MnCl_2$ (Thermo Fisher Scientific), various amounts of parental DNA and DNase I (diluted in 1x digestion buffer) in reaction volume (10–50 µl). In this work reaction conditions (reaction time, amount, temperature and activity units of DNase I) were optimized. Hydrolysis was performed at 20–22 $^{o}$C and terminated by adding

optimized amount of EDTA. Then the digested fragments were separated by 2 % agarose gel electrophoresis and 50–400 bp fragments were purified by GeneJET™ Gel Extraction Kit.

Then purified lipase gene fragments were resuspended in PCR mixture without primers. The PCR products were analyzed by 0.8 % agarose gel electrophoresis and used as template in second PCR with primers (Gelip95-43-F and Gelip95R).

**Production on new GD lipase gene variant by error-prone PCR (epPCR)**

pTZ57R/T plasmid containing DNA shuffling – evolved GD lipase gene variant served as a template in epPCR experiments to introduce more mutations. Reaction conditions and primers (Gelip95-43-F and Gelip95R) were the same as described above, except that 4 mM and 6 mM $MgCl_2$, in first and second epPCR, respectively, were used. Equimolar mixture of positive variants from the first generation of random mutagenesis served as template for the second round of epPCR.

**Establishment of the conditions for expression of target enzymes**

A single colony of *E. coli* BL21(DE3) harboring pET-21c (+) plasmids with inserted gene of target lipolytic enzymes was incubated at 37 ºC overnight with agitation (180 rpm) in LB broth containing 100 µg/ml ampicillin. The culture was transferred using 1 % inoculum to a fresh LB broth containing the same concentration of ampicillin. The culture was incubated at 37 ºC with agitation (180 rpm) until the $OD_{600}$ reached 0.4 and IPTG was added to a final concentration of 0.5–1 mM. For protein detection by SDS-PAGE, cell samples obtained at different times after induction were adjusted so that their $OD_{600}$ value was 0.4, harvested by centrifugation, mixed with 100 µl 4x SDS-PAGE sample loading buffer and subjected to SDS-PAGE analysis. Negative control was prepared by the same method using cells not subjected to IPTG induction. SDS-PAGE was carried out on 12 % running gel using the Laemmli method (1970). PageRuler™ Unstained Protein Ladder and Pierce Unstained Protein MW Marker (Thermo Fisher Scientific) were used as a molecular mass marker. After electrophoresis, gels were stained with PageBlue™ Protein Staining Solution (Thermo Fisher Scientific).

**Purification of recombinant target enzymes**

The His-tagged target lipolytic enzymes were purified to homogeneity by one-step purification protocol using immobilized $Ni^{2+}$ affinity chromatography (Profinity ™ IMAC Resins, BIO-RAD) following the manufacturer's purification protocols for native or denaturing conditions with minimal modification. For purification under native conditions binding buffers (50 mM Tris-HCl, pH 8.0 / 20 °C) with 10 and 250 mM imidazole (Sigma-Aldrich) were used as wash and elution buffers, respectively. For purification under denaturing conditions the same binding buffer with 6M urea was used as binding buffer and the same buffer with urea and 10 or 250 mM imidazole – as wash and elution buffers, respectively. Protein concentration was determined using biophotometer (Eppendorf) and homogeneity of purified enzymes was analyzed by SDS-PAGE and zymography. The renaturation of target lipolytic enzymes after purification under denaturing conditions was performed by dialysis. After dialysis the protein sample was concentrated with PEG 35000 (Sigma-Aldrich).

GDlip30 and GDlip43 lipases obtained by DNA shuffling and epPCR were separated using elution from SDS-PAGE gel. Elution buffer was composed of 50 mM Tris-HCl (pH 8 / 20 °C), 150 mM NaCl (Applichem), 0.1 mM EDTA (Applichem) (pH 8). The elution process was carried out as presented in purify proteins from polyacrylamide gels protocol (Thermo Scientific).

**Zymography**

Lipolytic activity was determined on SDS-PAGE gels using tributyrin as substrate. Gel renaturation procedure was carried out following Levisson et al. (2007).

**Assay of lipase activity**

Lipase activity was measured spectrophotometrically (Winkler and Stuckmann 1979) *p*-nitrophenyl (*p*-NP) dodecanoate (Sigma-Aldrich) used as substrate. One unit of lipase activity was defined as 1.0 µmol of *p*-nitrophenol released per minute for 1 mg enzyme. The assay mixture contained 890 µL of glycine (Applichem)-NaOH (Barta a CIHLAR) buffer (50 mM, pH 9), 100 µL of substrate solution (2.5 mM *p*-NP dodecanoate in DMSO

(Merck)) and 10 µL of enzyme solution. Before reaction, the assay mixture was preincubated for 10 min at 55 °C and then the enzyme solution was added. After 5 min of incubation at 55 °C, the reaction was stopped by the addition of 1 mL 96 % ethanol. Reaction time (5 min) was chosen after calculation of the initial velocity, when the release rate of *p*-NP is linear. The released *p*-NP was monitored at 420 nm, using a standard curve of *p*-NP (Alfa Aesar).

**Physicochemical characterization of target lipolytic enzymes**

The optimal temperature was determined by carrying out the enzyme assay at temperatures ranging from 5 to 90 °C. The effect of temperature on target enzymes stability was investigated by measuring the residual activity at 55 °C after incubation for 30 min at 30–90 °C. The pH effect on desired enzymes was evaluated by testing pH range of 5–12 under assay conditions. Various buffer systems, including 50 mM acetate (sodium acetate (Merck); pH 4–6 / 55 °C), potassium phosphate (pH 7 / 55 °C) ($KH_2PO_4$; AnalaR), Tris-HCl (pH 8 / 55 °C), glycine-NaOH (pH 9–11 / 55 °C) and $Na_2HPO_4$ (Merck)-NaOH buffers (pH 11–12 / 55 °C) were used. Due to stability differences of *p*-NP dodecanoate at various pH's and at different temperatures, the reaction mixture used as a control was incubated parallelly at the given temperature or buffers without enzyme solution. The activity of the purified recombinant lipolytic enzymes was studied also by following incubation at room temperature for 30 min with 25 % (v/v) various organic solvents (dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) (ROTH), acetone (Merck), ethanol, methanol (Merck), *n*-butanol (ROTH), isopropanol) and 0.1 % surfactants (Tween-20, 40, 60, 80 (Merck), Triton X-100, urea). 100 % lipolytic activity was measured without the treatment with metal ions, inhibitors, surfactants or organic solvents.

Acyl chain length preference was determined by hydrolysis of different *p*-NP esters (Sigma-Aldrich) ranging from C2 to C18. In detection of substrate specificity instead of glycine-NaOH buffer (50 mM, pH 9, 55 °C) a potassium phosphate buffer (50 mM, pH 7, 55 °C) was used to reduce the instability of short acyl chain- length *p*-NP substrates.

**Kinetic analysis of target lipolytic enzymes**

The Michaelis constant ($K_m$) and $V_{max}$ as well as $K_{cat}$ were calculated using *p*-NP dodecanoate (C12) as substrate at concentrations ranging from 1.25 to 50 mM. Lineweaver-Burk plots were used to determine $V_{max}$ and $K_m$ parameters, assuming that the reactions followed a simple Michaelis-Menten kinetics. $K_{cat}$ was calculated using the following equation ($K_{cat}=V_{max} / [E]$). The catalytic efficiency was calculated using the equation $K_{cat}/ K_m$ (Eisenthal et al. 2007). Energy of activation ($E_a$) was calculated with the help of the Arrhenius plot.

**Statistical analysis**

All experiments were repeated three times and the average means were derived. Standard deviation from the mean is showed in Tables 2, 4–5, 8–9 and Figs. 2, 4–6, 8, 10. Significant differences between target recombinant lipolytic enzymes were calculated using two-sample T test (http://insilico.net/tools/statistics/ttest), $\alpha = 0.05$. Only results with two-tailed *p* value less or equal to 0.02 are presented in this thesis.

**Analysis *in silico***

Gene and protein sequences obtained by sequencing were analyzed using Lasergene v7.1 (DNASTAR, JAV), NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) and Mega 4.0.2 programs and tools. For detection of signal peptide SignalP tool (http://www.cbs.dtu.dk/services/SignalP/) and PrediSi (http://www.predisi.de/predisi/start) online server were used. Three-dimensional (3D) structures of target lipolytic enzymes were predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/ (Zhang 2008)). *G. thermocatenulatus* (2W22) lipase (Carrasco-Lopez ir kt., 2009) was used as template. Possible interactions of catalytic amino acids with other amino acids in protein structure model were analyzed using CAD-score (http://bioinformatics.ibt.lt/cad-score/ (Olechnovič et al. 2013)) server. Image of the resulting 3D model was generated using RasMol.

# RESULTS AND DISCUSSION

## 1. Detection of Asp371, Phe375 and Tyr376 influence on GD-95-10 lipase functionality using Ala scanning mutagenesis

Despite the fact that lipolytic enzymes produced by *Geobacillus* bacteria are being investigated for more than twenty years, data about the effect of C-terminal end of lipolytic enzymes on their activity and functionality is insufficient. Therefore, one of the tasks of this thesis was to evaluate the influence of several amino acids located at C-terminal region on the activity and functionality of GD-95 lipase (GenBank Accession No. KC609753; 388 amino acids (43 kDa), optimal activity at pH 9 and 55 ºC). New knowledge about lipases from thermophilic *Geobacillus* bacteria is important for *de novo* design of new lipolytic biocatalysts or for synthetic biology studies.

### 1.1 Analysis of GD-95-10 and GD-95-20 lipases

GD-95-10 and GD-95-20 lipases are modified GD-95 lipase variants, which lack 10 and 20 C-terminal amino acids, respectively. Physicochemical analysis showed that GD-95-10 lipase not only retained its functionality, but also had similar lipolytic activity to GD-95 lipase (415 U/mg), while activity of GD-95-20 lipase strongly decreased (14 U/mg). Removal of C-terminal amino acids significantly altered thermostability of GD-95 lipase (Fig. 2b), while the temperature activity profile of modified lipases was similar to GD-95 lipase (Fig. 2a). Thermostability analysis showed that GD-95-10 and GD-95-20 lipases completely lost their activity even after 30 min incubation at 65 ºC (Fig. 2b).

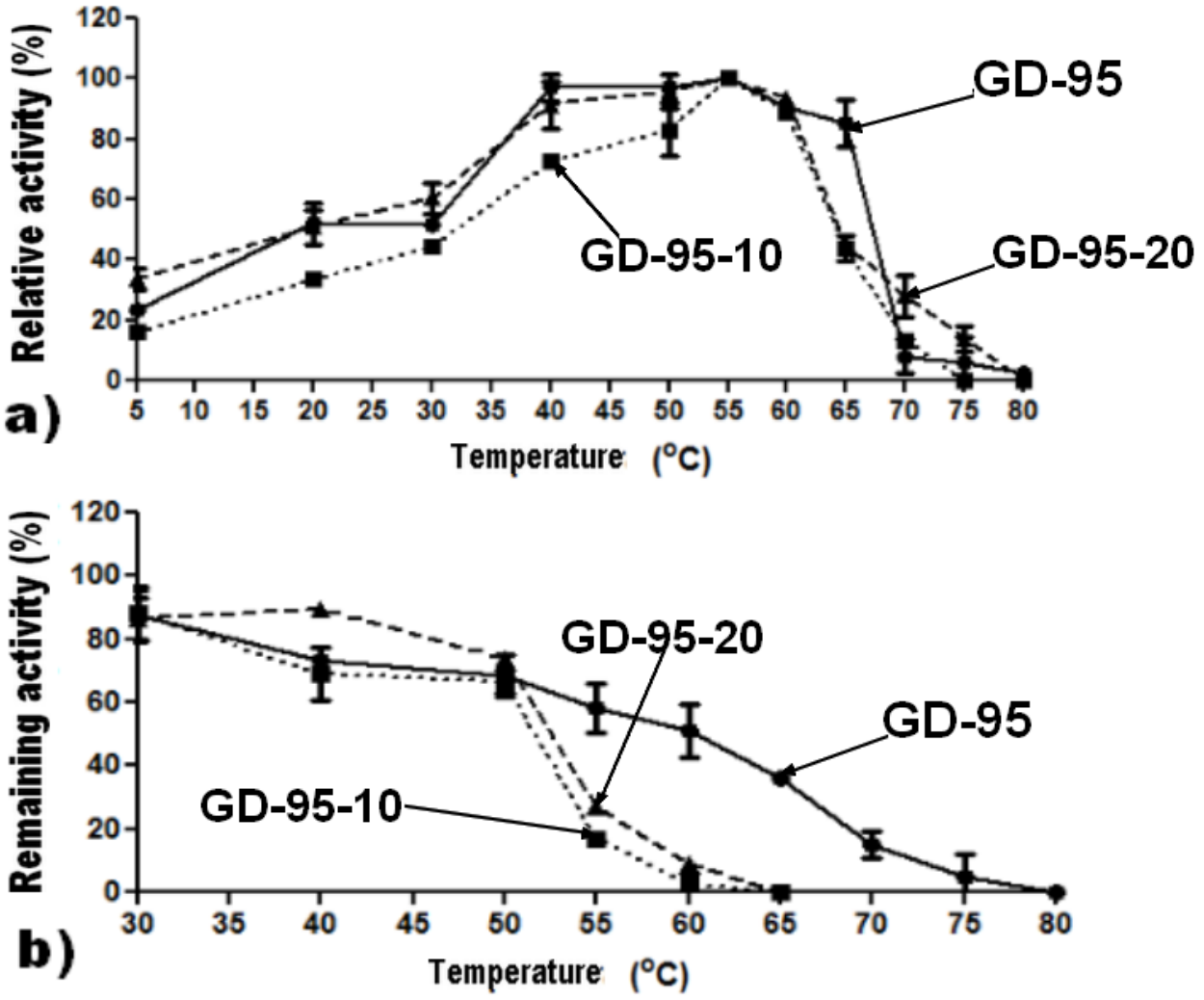


**Fig. 2. Effect of temperature on the activity (a) and stability (b) of recombinant GD-95 lipase and its modified GD-95-10 and GD-95-20 variants. Straight line with a filled circle – GD-95 lipase; dotted line with a filled square – GD-95-10 lipase; dashed line with a filled triangle – GD-95-20 lipase.**

Structural and sequence alignments of bacterial lipases revealed that the C-terminus forms an α13 helix, which is a conserved structure in lipases from S*taphylococcus*, *Deinococcus, Pseudomonas*, *Clostridium* and other microbial lipases. The bioinformatics analysis of the GD-95 lipase revealed that α13 helix of this lipase has six conserved residues (Asp371, Phe375, Tyr376, Leu377, Ala378 and Leu383). The functionality of GD-95 lipase without 10 C-terminal amino acids and loss of activity in GD-95-20 suggested the hypothesis that the most important amino acids from C-terminal-deleted region are located at the region from 369 to 378 amino acids. At this region three conservative amino acids (Asp371, Phe375 and Tyr376) are located. Analysis *in silico* also showed that these amino

acids have direct contacts with residues involved in substrate binding, stabilization of the serine loop or form oxyanion hole. It was decided to create single and double Ala mutants of Asp371, Phe375 and Tyr376 amino acids and evaluate the impact of these amino acids on the functionality of GD-95-10 lipase.

### 1.2 Design, expression and purification of GD-95-10 lipase mutants

Based on the GD-95-10 lipase gene sequence and used primers listed in Table 1 six GD-95-10 lipase Ala mutants (R1-R6) were constructed. The scheme of designed mutants is shown in Fig. 3.

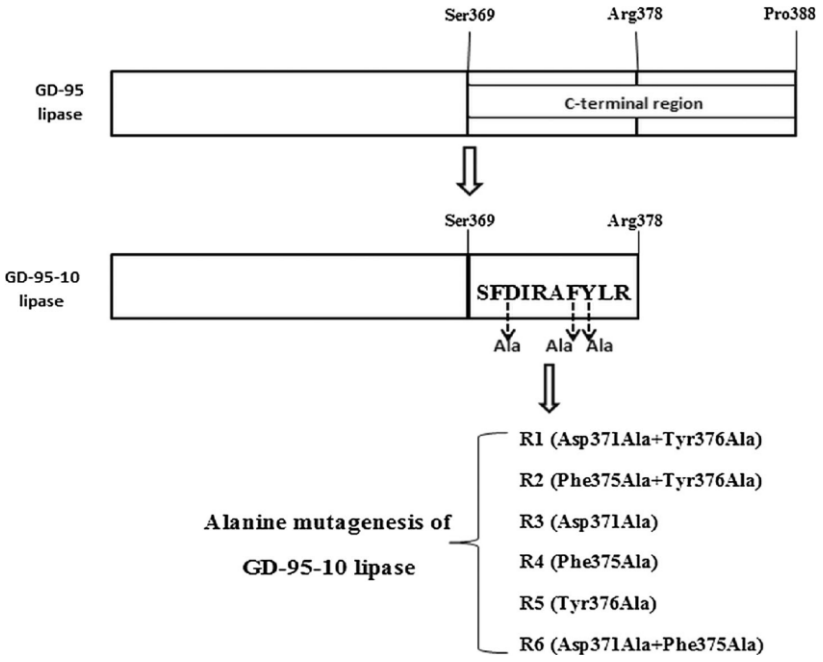


**Fig. 3. Schematic presentation of generated R1-R6 constructs.**

The optimum expression of R1-R6 mutated lipases in *E. coli* was detected at 2–3 h post-induction with 1.0 mM IPTG. All mutated lipases were purified under native conditions from soluble protein fractions and used for analysis of physicochemical and kinetic properties.

## 1.3 Influence of Asp371, Phe375 and Tyr376 on the lipolytic activity and kinetic characteristics of GD-95-10 lipase

All mutations strongly decreased the yield and activity of GD-95-10 lipases (Table 2). This might be explained by the fact that mutations significantly changed the distances and possible contacts between amino acids, which are responsible for the catalytic activity or binding of substrate (Gudiukaitė et al. 2016). Double Asp371Ala+Tyr376Ala (R1 lipase) and Phe375Ala+Asp371Ala (R6 lipase) mutations resulted in the most significant changes not only in the yield of GD-95-10 lipase but also in the lipolytic activity (Table 2).

**Table 2. Yield and activity of recombinant purified GD-95-10 and GD-95-20 lipases and GD-95-10 lipase mutants R1–R6.**

| Protein | Yield (mg) of expressed protein from 200 mL culture (soluble fraction) | Specific activity (U/mg) |
|---|---|---|
| **GD-95-10** | **9.52** | **415** |
| **GD-95-20** | **3.95** | **14** |
| **R1 (Asp371Ala+ Tyr376Ala)** | **3.20** | **12.52** |
| **R2 (Phe375Ala +Tyr376Ala)** | **5.33±1.7** | **11.67±7.41** |
| **R3 (Asp371Ala)** | **9.79±2.3** | **15.5±5.5** |
| **R4 (Phe375Ala)** | **5.43±0.5** | **42.5±2.5** |
| **R5 (Tyr376Ala)** | **6.05±0.5** | **32.5±2.5** |
| **R6 (Asp371Ala +Phe375Ala)** | **2.93** | **20** |

Since R3 mutant had the lowest lipolytic activity among all single mutants it was predicted that Asp371 has decisive influence in double mutants. R3 mutant retained only 4 % of lipolytic activity compared to GD-95-10 lipase. According to analysis *in silico*, Tyr376 makes the most important contacts with other amino acids in GD-95-10 lipase, but single Tyr376 mutant possessed 32.5 U/mg specific activity and this activity was higher than all double mutants as well as Asp371Ala.

Catalytic constants ($V_{max}$, $K_{cat}$, $K_m$, and catalytic efficiency) were calculated in order to evaluate the effect of mutations on lipolytic activity of GD-95-10 mutants. R1, R2 and R4 mutants clearly distinguished from all GD-95-10 lipase derivatives as they possessed very high $K_m$ values when *p*-NP dodecanoate was used as a substrate (Table 3).

**Table 3. Kinetic properties of recombinant purified GD-95-10 and GD-95-20 lipases and mutated GD-95-10 lipase variants. Values were determined at 55 °C, pH 9 using *p*-NP dodecanoate as substrate. ND – not detectable.**

| Kinetic parameters / Protein | $V_{max}$ (µmol/min mg protein) | $K_m$ (mM) | $K_{kat}$ ($min^{-1}$) | Catalytic efficiency $K_{cat}/K_m$ $min^{-1}$ $mM^{-1}$ |
|---|---|---|---|---|
| **GD-95-10** | **50.00** | **8.33** | **$2.83*10^3$** | **$3.40*10^2$** |
| **GD-95-20** | **40.00** | **7.70** | **$1.67*10^2$** | **2.16*10** |
| **R1 (Asp371Ala+ Tyr376Ala)** | **3.03** | **16.67** | **$1.26*10^2$** | **8.00** |
| **R2 (Phe375Ala +Tyr376Ala)** | **5.26** | **12.50** | **$2.08*10^2$** | **16.64** |
| **R3 (Asp371Ala)** | **5.61** | **6.67** | **$2.34*10^2$** | **35.00** |
| **R4 (Phe375Ala)** | **20.00** | **25.00** | **$8.33*10^2$** | **33.00** |
| **R5 (Tyr376Ala)** | **1.43** | **8.33** | **60.00** | **7.20** |
| **R6 (Asp371Ala +Phe375Ala)** | **ND** | **ND** | **ND** | **ND** |

R2 and R4 mutants have Phe375Ala mutation, suggesting that Phe375 is also important for GD-95-10 functionality. Kinetic analysis also showed that GD-95-10 derivates had very low $V_{max}$ compared with GD-95 and GD-95-10 lipases in the case with *p*-NP dodecanoate. Both R1 lipase, which is Asp371 and Tyr376 mutant and R5 (Tyr376Ala) mutant, possessed the lowest $V_{max}$, $K_{cat}$ and catalytic efficiency values in these experiments. Detailed analysis of kinetic parameters of mutated lipases is presented in Table 3. Taken together, it was demonstrated that Asp371, Phe375 and Tyr376 are very important for control of lipase activity, binding of substrate and catalytic efficiency.

## 1.4 Influence of Asp371, Phe375 and Tyr376 on the thermoactivity and thermostability of GD-95-10 lipase

The effect of a single amino acid mutation on GD-95-10 lipase temperature activity and thermostability was investigated using R3, R4 and R5 mutants (Fig. 4a and 4b).

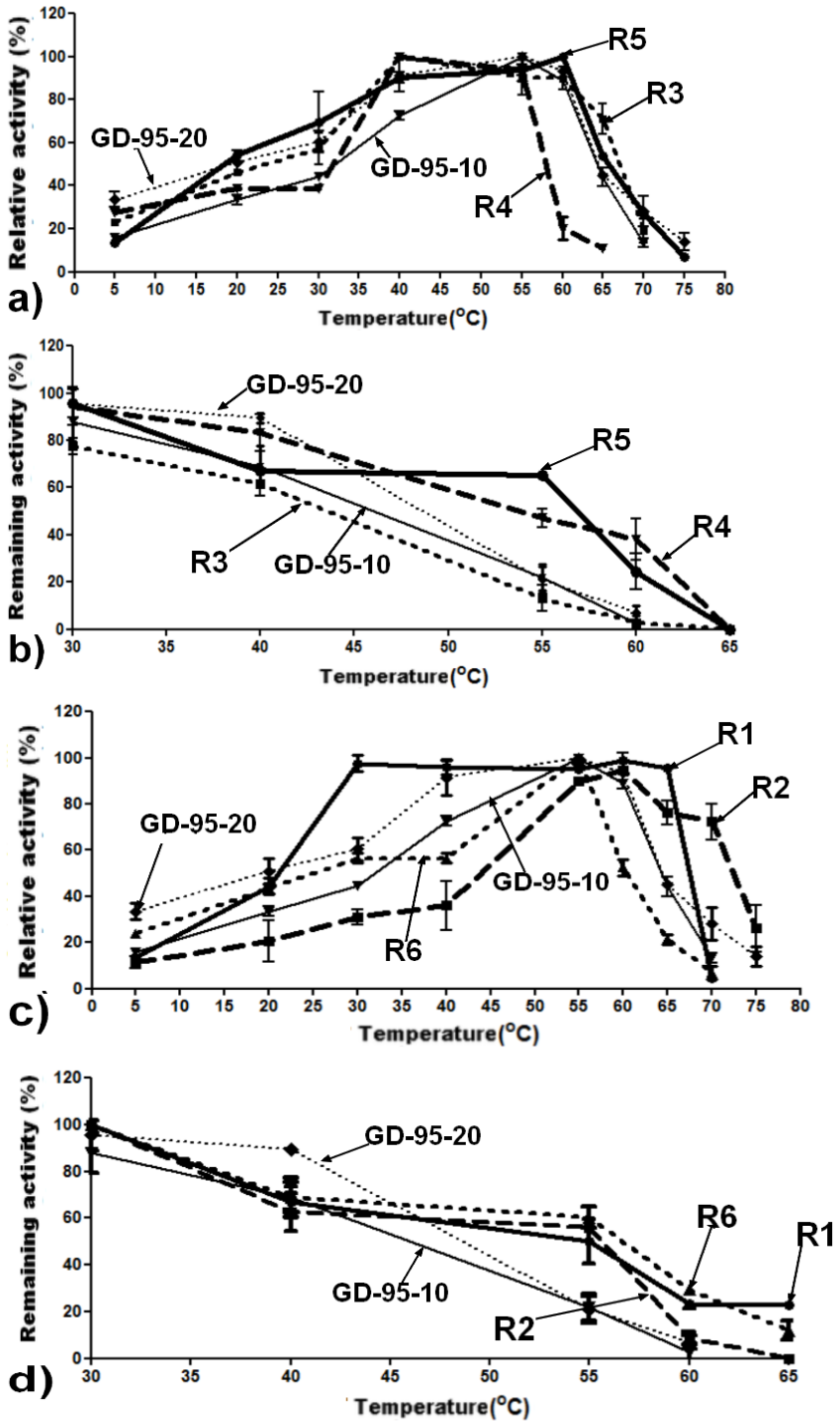


**Fig. 4. Effect of temperature on the activity (a, c) and stability (b, d) of purified GD-95-10 and GD-95-20 lipases and GD-95-10 lipase mutants R1-R6. Thin straight line with a triangular (a–d) – GD-95-10 lipase; thin dotted line with a rhombus (a–d) – GD-95-20 lipase; thick straight line with a filled circle (c, d) – R1 lipase; thick dashed line with a filled square (c, d) – R2 lipase; thick dotted line with a filled square (a, b) – R3 lipase; thick dashed line with a filled triangle (a, b) – R4 lipase; thick straight line with a filled circle (a, b) – R5 lipase; thick dotted line with a triangle (c, d) – R6 lipase.**

Analysis showed that Asp371Ala mutant (R3) displayed a very close temperature profile to GD-95-20 and GD-95-10 lipases. Also, the temperature optimum for R3 lipase activity was lower than GD-95-10 and GD-95-20 lipases (40 °C) (Fig. 4a), but R3 lipase retained its lipolytic activity up to 70 °C. R4 lipase mutant had the same temperature optimum, but the lipolytic activity of R4 lipase mutant strongly decreased at 60 °C (Fig. 4a and 4b). R3 mutant showed the lowest thermostability compared to both GD-95-10 and GD-95-20 lipases and other R mutants (Fig. 4b). This suggests that Phe375 might be responsible for lipase activity at higher temperature, while Asp371 plays the major role in stabilization. The analysis of double mutants suggested that more than a single amino acid is responsible both for the functionality of GD-95-10 lipase and for the inactivation of GD-95-20 lipase, which rather is a consequence of the loss of several amino acids, leading to the changes in the main structure of GD-95-10 lipase.

### 1.5. Influence of Asp371, Phe375 and Tyr376 on the main structure of GD-95-10 lipase

To understand the effects of mutated residues to overall structure of GD-95-10 lipase and explain the experimental results, we used *in silico* methods to obtain structures of mutants and evaluate their contact differences with neighbouring amino acids. The results showed that even changes of a single amino acid can lead to refolding of the main structure of *Geobacillus* lipases. Analysis *in silico* suggested that among amino acids analyzed in this work Tyr376 formed the most important contacts (Gudiukaitė et al. 2014). In GD-95-10 lipase, this amino acid has a direct connection with Leu12 (residue of oxyanion hole (Chakravorty et al. 2011)), His112 (in the closed state helps packing catalytic serine and is involved in stabilization of the serine loop (Carrasco-Lopez et al. 2009)), and Ile362 (involved in formation of active site cleft and acyl chain-binding pocket (Carrasco-Lopez et al. 2009)). Tyr376 mutation to Ala resulted in complete loss of these contacts (data not shown). This might explain the activity differences between R1 and R3 mutants since R1 lacks contacts between amino acids in active site cleft and oxyanion hole. The oxyanion hole is an important structure for stabilization of the tetrahedral intermediate during the hydrolysis reaction (Carrasco-Lopez et al. 2009). The situation is the same in case of R2

mutant. R1 and R2 mutants also lack contacts between Arg34, which interacts with Leu33 (involved in formation of channel leading toward the active site (Tyndall et al. 2002)) and Asp371. Contact analysis may also explain the low stability of R2 lipase. It is possible that this lipase lacks contact between the Ala240, which is involved in formation of active site cleft and Arg62, which plays stabilizing role during lid opening. These rearrangements of tertiary structure influence the binding of substrate and stability / activity of *Geobacillus* lipases.

Analysis *in silico* also showed that mutation of Asp371, Phe375 and Tyr376 to Ala dramatically changed the distances between catalytic Ser113, Asp317 and His358 (data not shown). Distances in GD-95-10 lipase between Asp317/His358 and His358/Ser113 catalytic amino acids are 5.72 and 2.69 Å, respectively. These values are similar to the literature data (Carrasco-Lopez et al. 2009; Goodarzi et al. 2014). Analysis of mutated GD-95-10 lipase variants showed that in all mutants the distance between catalytic Ser113 and His358 increased nearly twofold. The highest differences were observed in R2 and R4 mutants, which have Phe375 mutation. This correlated with experimental results, which showed that lipolytic activity of R4 lipase mutant significantly decreased at 60 °C. These mutants also had a higher $K_m$ for *p*-NP dodecanoate. Analysis *in silico* confirmed the hypothesis that Phe375 is important for modulation of lipase activity at higher temperature (data not shown). Abdul Rahman with colleagues (2012) in their study found that distances between catalytic Ser113 and His358 in T1 lipase are affected by the change in temperature. We found a similar trend in distance changes after Asp371, Phe375 and Tyr376 mutation to Ala. Thus, mutation of C-terminal amino acids affects the general structure of *Geobacillus* lipases. Although changes are minimal, it may be related to the functionality of lipases.

## 2. Construction of a novel lipolytic fusion biocatalysts GDEst-lip, GDEst-est and GDLip-lip

The key tasks of this experimental part was construction of new lipolytic fusion biocatalyst with improved properties. GD-95 lipase and GDEst-95 esterase produced by *Geobacillus* sp. 95 were chosen for the design of the first lipolytic chimeric enzyme. Also

effect of fusion with the same additional domain on the activity and physicochemical as well as kinetic properties of the monomeric GD-95 lipase and GDEst-95 esterase was performed.

### 2.1 Amplification, cloning, expression and purification of GDEst-95 esterase

Esterase gene from *Geobacillus* sp. 95 strain was amplificated for the purpose of design of the GDEst-est and GDEst-lip fusion proteins. Esterase as a fusion partner was selected for its ability to hydrolyze short acyl chain substrates, since lipases prefer medium or long acyl chain substrates. In this work was obtained a 1497-bp fragment, containing an open reading frame (ORF) which encodes 498 amino acids (GenBank Accession No. KX013768). The sequence alignment in GenBank showed high homology (94–98 %) with other known *Geobacillus* carboxylesterases (data not shown). This novel esterase was named GDEst-95 and the molecular weight of new esterase corresponded to 54.8 kDa. Only three publications exist regarding *Geobacillus* carboxylesterases with 55 kDa (Ewis et al. 2004; Liu et al. 2007; Soliman and Gaballa 2010). Therefore, in this work GDEst-95 esterase was cloned, expressed and purified using IMAC methodology under native conditions. This lipolytic enzyme was purified using Tris-HCl (pH 8, 20 $^{o}$C) buffer with 10 mM imidazole under native conditions (data not shown). These conditions are more attractive for industrial application compared to the GD-95 lipase, which was purified using the same binding buffer with 250 mM imidazole.

### 2.2 Biochemical characterization of GDEst-95 esterase

GDEst-95 esterase displayed better hydrolytic abilities at higher temperature than GD-95 lipase (Fig. 5a). The recombinant GDEst-95 esterase had an optimum activity at 60 °C and retained about 80 % lipolytic activity at 70–75 $^{o}$C, while the GD-95 lipase possessed less than 20 % activity at these temperatures (Fig. 5a). The temperature range of GDEst-95 activity was from 5 to 85 $^{o}$C and this feature gives it advantage over the GD-95 lipase, which at 80 $^{o}$C completely lost its activity (Fig. 5a). GDEst-95 esterase also retained higher lipolytic activity after incubation at high temperatures than GD-95 lipase (Fig. 5c). The

significant differences between GDEst-95 esterase and GD-95 lipase were detected at 65 ºC and at 70 ºC. These results suggested that GDEst-95 esterase is not only thermoactive, but also thermostable and has a great biotechnological potential. It provides the basis for fusion of both lipolytic enzymes produced by *Geobacillus* sp. 95.

When the effect of pH on the GDEst-95 esterase activity was analyzed it came out that the enzyme exhibits highest activity at pH 9–10. About 50 % of the enzyme activity was observed at pH 8 (Fig. 5b).

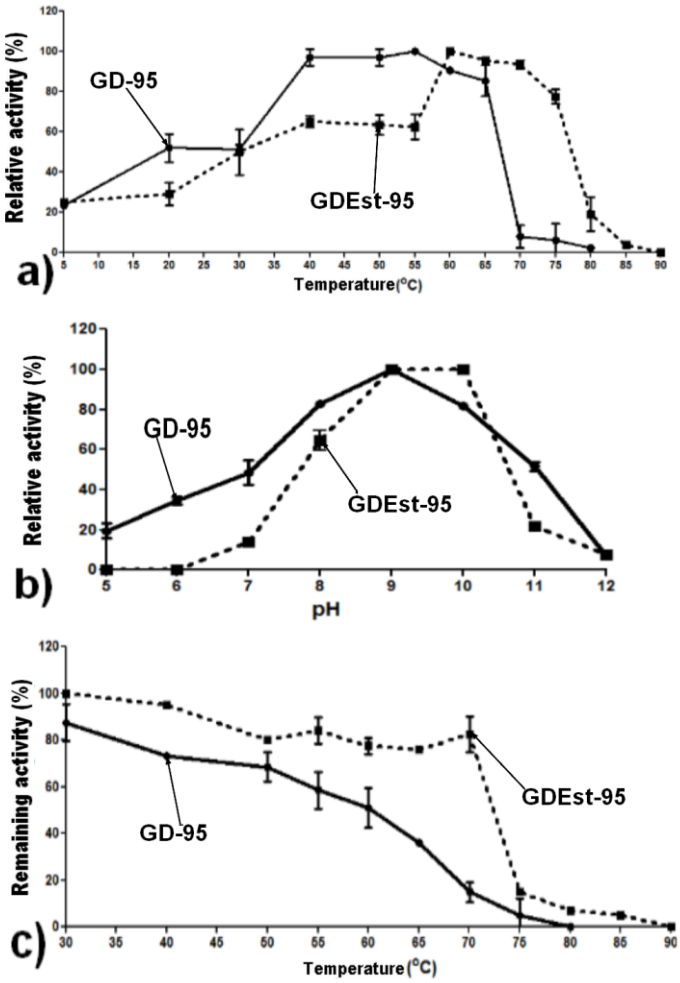


**Fig. 5. Effect of temperature on the activity (a) and stability (c) and pH influence (b) on the activity of purified GDEst-95 esterase and GD-95 lipase. Straight line with a circle – GD-95 lipase; dotted line with a square – GDEst-95 esterase. Assays were performed at various temperatures (5–90 ºC) (a) or various pH (b) under enzyme assay conditions. The remaining activity was assayed under enzyme assay conditions after the purified recombinant enzymes had been incubated at the indicated temperature (30–90 ºC) for 30 min (c).**

The substrate preference (Table 4) and influence of various organic solvents and detergents (Table 5) on the enzymatic activity of GDEst-95 was also examined in this research.

**Table 4. Activity of GD-95, GDEst-95 and fused GDEst-est, GDLip-lip and GDEst-lip toward various acyl length *p*-NP esters. Analysis was carried out at 55 ºC, pH 7. ND – not detectable. The substrate specificity was determined for each protein individually.**

| Substrate | Relative activity (%) of target enzymes | | | | |
|---|---|---|---|---|---|
| | GD-95 | GDEst-95 | GDEst-lip | GDLip-lip | GDEst-est |
| ***p*-NP acetate (C2)** | 87 ±1.41 | 83 ±1.41 | 100 | 53 ±4.24 | 49 ±7.07 |
| ***p*-NP butyrate (C4)** | 100 | 100 | 97 ±2.24 | 75 ±1.41 | 100 |
| ***p*-NP hexanoate (C6)** | 95 ±4.07 | 96 ±3.66 | 100 | 80 ±12.02 | 52 ±0.71 |
| ***p*-NP octanoate (C8)** | 88 ±4.95 | 82 ±3.54 | 62 ±4.24 | 100 | 52 ±4.95 |
| ***p*-NP decanoate (C10)** | 49 ±9.19 | 27 ±2.83 | 31 ±2.12 | 26 ±1.04 | 44 ±3.54 |
| ***p*-NP dodecanoate (C12)** | 80 ±1.41 | 18 ±0.71 | 52 ±1.41 | 40 ±4.95 | 34 ±3.54 |
| ***p*-NP myristate (C14)** | 43 ±7.78 | 7 ±1.41 | 24 ±0.71 | 16 ±2.12 | 17 ±1.41 |
| ***p*-NP palmitate (C16)** | 35 ±2.83 | 3 ±2.12 | 22±1.41 | 14 ±2.12 | 11 ±2.12 |
| ***p*-NP stearate (C18)** | 26 ±2.12 | ND | 25 ±1.41 | 7 ±1.41 | ND |

**Table 5. Effect of various organic solvents (25 %) and surfactants (0.1 %) on the enzyme activity of purified GD-95 lipase, GDEst-95 esterase and fused GDLip-lip, GDEst-est and GDEst-lip lipolytic enzymes. Lipase activity without addition of organic solvents and detergents was set as 100 %. ND – not detectable. The analysis was carried out at 55 ºC, pH 9, using *p*-NP dodecanoate as substrate.**

| Effect | Relative activity (%) of target enzymes | | | | |
|---|---|---|---|---|---|
| | GD-95 | GDEst-95 | GDEst-lip | GDLip-lip | GDEst-est |
| **Organic solvents** | | | | | |
| **DMSO** | 60±4.4 | 39±2.12 | 92±4.24 | 71±1.32 | 31±7.78 |
| **DMF** | 113±6.8 | 81±8.49 | 63±1.41 | 82±9.90 | 86±0.71 |
| **Ethanol** | 122±6.5 | 67±3.54 | 70±7.78 | 78 | 69±2.12 |
| **Methanol** | 80±2.6 | 82±12.02 | 81±0.71 | 90±9.90 | 11±4.24 |
| **Acetone** | 90±1.8 | 87±9.90 | 70 | 59±12.02 | 99±0.71 |
| **Isopropanol** | 104±3.5 | 129±12.02 | 105±0.71 | 74±2.12 | 85±2.12 |
| ***n*-Butanol** | 104±4.3 | 114±2.12 | 113±4.24 | 111 | 128±2.12 |
| **Surfactants** | | | | | |
| **Triton X-100** | ND | 113±2.12 | 63±6.36 | 12±0.71 | 123±2.83 |
| **Tween-20** | ND | 90±4.24 | 26±7.78 | ND | 111±5.66 |
| **Tween-40** | ND | 126±7.07 | 46±2.12 | ND | 164±7.07 |
| **Tween-60** | ND | 136±6.36 | 59±1.41 | ND | 100 |
| **Tween-80** | ND | 111±5.66 | 38±8.49 | ND | 100 |
| **Urea** | 110±3.3 | 89±1.41 | 100 | 74±2.12 | 124±4.24 |

GDEst-95 esterase displayed higher hydrolase activity toward *p*-NP esters with acyl group chain lengths between C2 and C8, with an optimal activity on C4 (*p*-NP butyrate) and C6 (*p*-NP hexanoate) (Table 4). This corresponds to the literature data stating that esterases prefer short acyl chain substrates.

In this thesis the influence of various organic solvents and detergents on the enzymatic activity of GDEst-95 was also determined (Table 5). These results are important if the target enzymes are to be applied in biofuel production or synthesis of industrial flavor and fragrance esters. GDEst-95 esterase displayed increased activity after 30 min treatment with isopropanol, *n*-butanol and various surfactants (Tween 20, 40, 60, 80) (Table 5). It is important to note that various Tweens completely inhibit the lipolytic activity of GD-95 lipase. It was hypothesized that after fusion of GD-95 lipase and GDEst-95 esterase resulting new chimeric lipolytic biocatalyst might have higher resistance to effect of detergents than GD-95 lipase and retain high activity after treatment with organic solvents.

## 2.3 Design, expression and purification of fused GDEst-est, GDLip-lip and GDEst-lip lipolytic biocatalysts

Significant differences between GDEst-95 esterase and GD-95 lipase in terms of thermoactivity, thermostability, resistance to organic solvents, detergents, substrate specificity and catalytic properties generated the idea of fusion of both these lipolytic enzymes. After cloning and expression GDEst-lip esterase / lipase was successfully purified using Tris-HCl (pH 8, 20 $^{o}$C) buffer with 10 mM imidazole – same situation as was in the case of GDEst-95 esterase. GDEst-est and GDLip-lip lipolytic enzymes were purified using 10 and 250 mM imidazole, respectively (data not shown). The yields of purified recombinant GDLip-lip, GDEst-est and GDEst-lip fusion biocatalysts and parental GD-95 lipase as well as GDEst-95 esterase were 13.26, 10.35, 38.56, 44.16 and 24.18 mg, respectively, from 200 ml culture. In this stage GDEst-lip demonstrated attractive features for further experiments and production on a large scale (it was easy to purify and showed high yield of recombinant protein).

### 2.4 Physicochemical analysis of fused GDEst-est and GDLip-lip lipolytic enzymes

After fusion with additional homodomain the temperature optimum of GD-95 lipase changed from 55 ºC to 65 ºC and in the case of GDEst-95 esterase – from 60 ºC to 70 ºC (Fig. 6a). Also GDLip-lip displayed better ability to work at higher temperature (70–85 ºC) than monomeric GD-95 lipase. GDEst-est showed no significant differences from GDEst-95 esterase. Different response of GD-95 lipase and GDEst-95 esterase to the fusion with additional homodomain might be explained by the amino acids and tertiary structure differences of both these lipolytic enzymes.

Thermostability analysis of GDLip-lip and GDEst-est fusion proteins suggested that fusion with esterase homodomain strongly decreased thermostability of GDEst-95 esterase. At 70 ºC GDEst-est lost more than 50 % its lipolytic activity (Fig. 6b). GDLip-lip lipase showed higher stability at mesophilic temperature, but at 60–75 ºC the remaining lipolytic activity was similar to the GD-95 lipase (Fig. 6b).

It is worth noting that lipolytic activity of GDEst-est esterase increased at pH 11 by 40 % compared to GDEst-95 esterase. GDLip-lip showed sizable difference from monomeric GD-95 lipase at pH 10–11 (Fig. 6c).

Analysis of substrate specificity and influence of organic solvents and detergents suggested that addition of the same domain decreased the ability of GDEst-95 to hydrolyze short acyl chain substrates, but improved lipolytic activity toward *p*-NP myristate and *p*-NP palmitate by 10 % (Table 4). This could be a consequence of the changes in three-dimensional structure of fused enzymes and the availability of substrate. GDLip-lip lipase from monomeric GD-95 lipase differed in its substrate specificity, when reaction was carried out at pH 7. Dimeric lipase displayed a 2–3 fold lower acitivity toward long acyl chain length (C12–C18) *p*-NP esters with an optimal substrate being *p*-NP octanoate (Table 4).

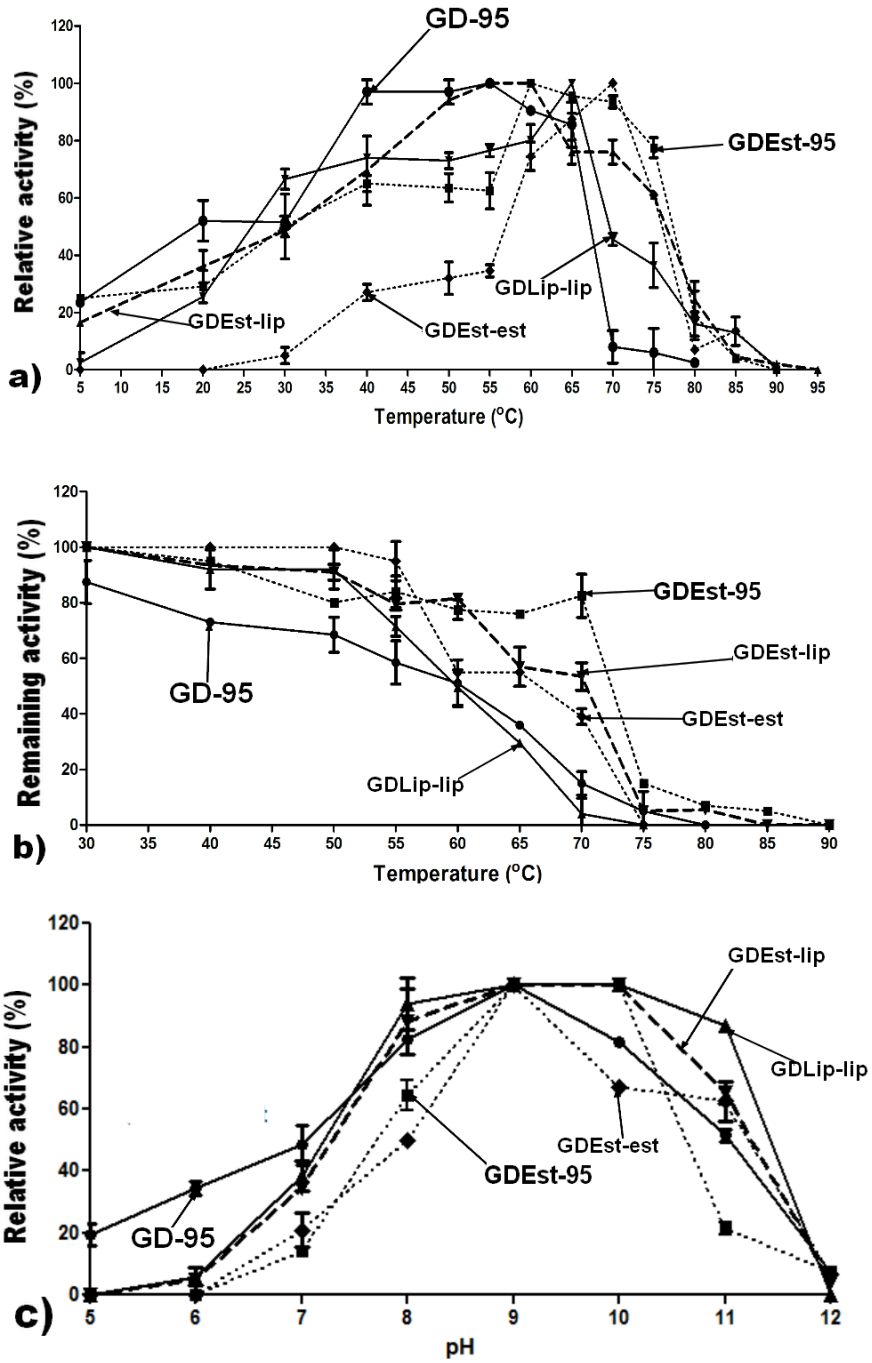


**Fig. 6. Effect of temperature on the activity (a) and stability (b) as well as pH influence (c) on the activity of purified GD-95 and GDLip-lip lipases, GDEst-95 and GDEst-est esterases and fused GDEst-lip esterase / lipase. Straight line with a circle – GD-95 lipase, straight line with a triangular – GDLip-lip lipase, dotted line with a square – GDEst-95 esterase, dotted line with rhombus – GDEst-est esterase, dashed line with triangular – GDEst-lip fused enzyme. Assays were performed at various temperatures (5–95 ºC) (a) or various pH (c) under enzyme assay conditions. The remaining activity was assayed under enzyme assay conditions after the purified recombinant enzymes had been incubated at the indicated temperature (30–90 ºC) for 30 min (b).**

GDEst-est esterase possessed a similar lipolytic activity after treatment with various organic solvents as GDEst-95 esterase (Table 5). The notable difference was observed only in the case of methanol. GDEst-est retained only 11 % its lipolytic activity after 30 min treatment with methanol while GDEst-95 demonstrated 82 % specific lipolytic activity. Also GDEst-est esterase was not activated by isopropanol (Table 5). Significant differences between GDLip-lip and GD-95 lipases were detected after incubation of lipases with DMF, ethanol and isopropanol (Table 5). It is important that GDLip-lip retained 12 % lipolytic activity after treatment with Triton X-100 (Table 5). Triton X-100, Tween-20, Tween-40 and urea activated GDEst-est esterase by 23 %, 11 %, 64 % and 24 %, respectively.

### 2.5 Kinetic analysis of fused GDLip-lip, GDEst-est and GDEst-lip lipolytic enzymes

Kinetic parameters were measured using a spectrophotometric activity assay with *p*-NP dodecanoate. GD-95 lipase showed the highest $V_{max}$ value, GDEst-95 esterase – the lowest (Table 6). This was not surprising, as *p*-NP dodecanoate is not an optimal substrate for GDEst-95, but it allows a comparison of both enzymes.

**Table 6. Comparative kinetic properties of purified GD-95, GDEst-95, GDLip-lip, GDEst-est and GDEst-lip lipolytic enzymes. The values were determined at 55 ºC, pH 9 using *p*-NP dodecanoate as substrate.**

| Protein | $V_{max}$ (µmol/min mg protein) | $K_m$ (mM) | $K_{cat}$ ($min^{-1}$) | Catalytic efficiency $K_{cat}/K_m$ ($Min^{-1}$ $mM^{-1}$) | Activation energy ($E_a$) (kJ/mol) | Specific activity (U/mg) |
|---|---|---|---|---|---|---|
| **GD-95** | **40.82** | **4.35** | **$1.78*10^6$** | **$4.10*10^5$** | **24.00** | **400** |
| **GDEst-95** | **5.88** | **5.88** | **$3.27*10^5$** | **$5.60*10^4$** | **50.30** | **100** |
| **GDEst-lip** | **20.00** | **14.29** | **$2.00*10^6$** | **$1.40*10^5$** | **68.34** | **600** |
| **GDEst-est** | **6.32** | **5.88** | **$7.02*10^6$** | **$1.19*10^6$** | **46.05** | **74.29** |
| **GDLip-lip** | **10.00** | **16.67** | **$8.33*10^6$** | **$5.00*10^5$** | **32.16** | **200** |

The new fused GDEst-lip enzyme showed threefold higher $V_{max}$ compared to GDEst-95 esterase, though this value was twofold lower than GD-95 lipase. Nevertheless the $V_{max}$ of GDEst-lip was the second highest value among all five proteins. These results confirmed hypothesis that chimeric GDEst-lip enzyme could demonstrate intermediate characteristics.

The positive influence of fusion of two GDEst-95 esterase domains was detected in $V_{max}$, $K_{cat}$, catalytic efficiency and activation energy, but the specific activity (74.29 U/mg) and yield (10.35 mg) of recombinant protein were almost 1.5 and twofold lower, respectively, than GDEst-95 esterase (Table 6).

Interestingly, the GDEst-est esterase showed positive changes of $K_m$, $K_{cat}$ and catalytic efficiency constants with *p*-NP dodecanoate as a substrate. The GDLip-lip did not demonstrate significant positive changes (Table 6). The new GDEst-lip lipolytic enzyme showed one of the best catalytic characteristics among all in this research analyzed lipolytic enzymes. GDEst-lip possessed 600 U/mg specific activity when *p*-NP dodecanoate was used as a substrate. This specific activity was 1.5 fold higher than parental GD-95 lipase and sixfold higher than GDEst-95 esterase (Table 6). GDEst-lip esterase / lipase also displayed the $K_{cat}$ similar to GD-95, high catalytic efficiency, but its activation energy was higher than GD-95 lipase, GDEst-95 esterase and fused GDEst-est variant (Table 6). Small activation energy indicates a good relationship between enzyme and the substrate (Ghori et al. 2011). Parent GD-95 lipase showed the lowest value of activation energy.

### 2.6 Physicochemical properties of the new synthetic lipolytic GDEst-lip biocatalyst

It was hypothesized that novel synthetic enzyme named GDEst-lip composed of *Geobacillus* sp. 95 strain lipase (GD-95) and esterase (GDEst-95) should have improved features compared to both parental proteins. It was assumed that GDEst-lip esterase / lipase should possess physicochemical and kinetic properties of both parental enzymes or demonstrate new unique characteristics if the features of GD-95 lipase and GDEst-95 esterase are very different, for example influence of detergents on lipolytic activity.

Experimental data suggested, that at 5–40 $^{o}$C GDEst-lip displayed an activity profile similar to GDEst-95 esterase. Then at 50–55 $^{o}$C it was similar to GD-95 lipase and temperature optimum of GDEst-lip was at 55–60 $^{o}$C (Fig. 6a). In temperature range of 65–75 $^{o}$C GDEst-lip biocatalyst showed significant differences from both parental enzymes. At 80–85 $^{o}$C temperature this enzyme displayed high similarity to GDEst-95 again and at 90 $^{o}$C it retained 2 % of its lipolytic activity. At this temperature both GD-95 lipase and GDEst-95

esterase have lost their lipolytic activity completely (Fig. 6a). These results confirm the hypothesis that GDEst-lip biocatalyst can demonstrate improved characteristics, which are more attractive for industrial applications.

One of the reasons for GDEst-lip design was the improvement of thermostability of GD-95 lipase. GDEst-lip completely lost its lipolytic activity after 30 min incubation at 85 $^{o}$C. This temperature is intermediate between temperatures, at wich GD-95 lipase and GDEst-95 esterase were inactivated (Fig. 6b). GDEst-lip showed unique intermediate thermostability characteristics at 65–70 $^{o}$C (Fig. 6b).

Purified GDEst-lip esterase / lipase displayed a broad pH activity range of pH 6–12, with an optimum pH of 9–10 in 50 mM glicine-NaOH buffer. At pH 6 and 12 GDEst-lip had 5 % its lipolytic activity (Fig. 6c). Significant differences between GDEst-lip and both parental enzymes at various pH values were not predicted.

Another reason for design of chimeric GDEst-lip biocatalyst was a creation of new enzyme, which was able to hydrolyze both short and long acyl chain *p*-NP esters. GDEst-lip possessed 100 % activity toward *p*-NP acetate, *p*-NP butyrate and *p*-NP hexanoate (Table 4), but significant differences from parental enzymes were not detected. The ability of GDEst-lip to hydrolyze *p*-NP decanoate, *p*-NP dodecanoate, *p*-NP myristate and *p*-NP palmitate was intermediate between GDEst-95 esterase and GD-95 lipase. Thus GDEst-lip has advantages over GDEst-95 esterase.

GDEst-lip demonstrated 20–70 % of its lipolytic activity after 30 min incubation with Triton X-100 and various Tweens (Table 4). GDEst-lip chimeric lipolytic biocatalyst confirmed the expectations and showed higher resistance to detergents than GD-95 lipase. After incubation with various organic solvents GDEst-lip possessed 60–90 % relative lipolytic activity (Table 4). It was activated only by isopropanol and *n*-butanol. Biochemical analysis of GDEst-lip esterase / lipase suggested that this novel fused synthetic lipolytic enzyme is a potentially wonderful new biocatalyst for industrial applications and possible to replace chemical catalysts.

Information presented in this work about GDEst-est, GDLip-lip and GDEst-lip fused enzymes shows that enzyme engineering by protein fusion is a powerfull tool to improve

biochemical and kinetic features of desired industrially important enzymes. After further detailed investigation new GDEst-lip lipase / esterase might be used as a commercially available lipolytic biocatalyst.

## 3. Design of new lipolytic enzymes using DNA shuffling and epPCR

Literature data lack research associated with *Geobacillus* lipases' and / or esterases' engineering experiments performed using DNA shuffling and epPCR strategy. In this study three new lipases from *Geobacillus* strains 28, 66, 76 were analyzed and used in direct evolution experiments. GD-28 and GD-66 lipases together with GD-95 lipase were applied in DNA shuffling and epPCR. In this work DNA shuffling was applied for improvement of *Geobacillus* lipases for the first time.

### 3.1 Cloning and purification of GD-66, GD-76 and GD-28 lipolytic enzymes

Lipases from *Geobacillus* spp. strains 28, 66 and 76 were amplified by PCR, sequenced and named GD-28, GD-66 and GD-76, respectively. These three lipases and in previous stage analyzed GD-95 lipase showed 96–98 % amino acid sequence similarity with each other. The alignment is present in Fig. 7. GD-76 lipase is not showed because after further physicochemical analysis this lipase had not been used in protein engineering experiments. Sequences of GD-28 and GD-66 lipases were submitted to GenBank (NCBI) with accession numbers KX258756 and KX258755, respectively. The variability of the GD-28, GD-66 and GD-76 lipases' sequences led to hypothesis, that these lipases may possess different physicochemical properties.


**Fig. 7. Comparison of the amino acid sequences of GD-28, GD-66, GD-95, after DNA shuffling obtained Chim and in further experiments after epPCR separated GDlip43 lipases. The sequences are presented without signal peptide. The squares marked differences in lipases sequences, the asterisks – the catalytic amino acids and the dashes indicate sites where sequencing was not successful.**

After sequencing, cloning into pTZ57R/T and pET-21c (+) vectors and expression analysis of GD-28, GD-66 and GD-76 was performed. Then expressed GD-28, GD-66 and GD-76 lipases were purified by nickel affinity chromatography under native conditions. All three *Geobacillus* lipases were purified using 250 mM imidazole. Zymogram analysis confirmed the presence of active lipases (data not shown).

### 3.2 Partial physicochemical characterization of GD-28, GD-66 and GD-76 lipases

The hydrolysis ability at higher temperature is a distinguishing factor between GD lipases. The new purified GD-66 and GD-76 lipases showed lipolytic activity in the temperature range of 5–90 ºC and GD-28 lipase – in the range of 5–75 ºC. The optimum activity was found to be at 55 ºC, 60 ºC and 55 ºC, respectively and these values are similar

to literature data. GD-28 lipase as well as GD-95 lipase lost 90 % of its lipolytic activity at 70 $^{o}$C. Meanwhile GD-66 and GD-76 lipases showed higher than 50 % lipolytic activity at this temperature (Fig. 8a). Thermostability analysis suggested that GD-28 lipase possessed the highest stability at higher temperature compared to GD-66 and GD-76 lipases (Fig. 8c). The stability of GD-28 lipase significantly differed from other GD lipases at temperature range of 65–85 $^{o}$C (Fig. 8c). After incubation at 80–90 $^{o}$C temperature range GD-28 lipase retained about 10 % of its lipolytic activity, while other GD lipases at these temperatures were completely inactivated. The lowest thermostability was demonstrated by GD-66 lipase (Fig. 8c). Based on this analysis, GD lipases can be divided into two groups. The first group of GD lipases is formed of GD-28 and GD-95 lipases and the second – of GD-66 and GD-76 lipases. GD-28 and GD-95 lipases possessed higher thermostability while GD-66 and GD-76 lipases demonstrated higher thermoactivity. These differences led to choosing GD-28, GD-66 and GD-95 lipase genes for DNA shuffling and epPCR experiments.

All in this report analyzed GD lipases possessed maximal lipolytic activity at pH 9, but the activity at pH 5–7 differs (Fig. 8b). At this pH range GD-76 and GD-95 lipases showed the highest relative lipolytic activity. GD-66 lipase demonstrated the lowest relative activity. These results came in agreement with literature data (Kim et al. 1998; Guncheva and Zhiryakova 2011) stating that *Geobacillus* lipases generally prefer alkaline conditions.

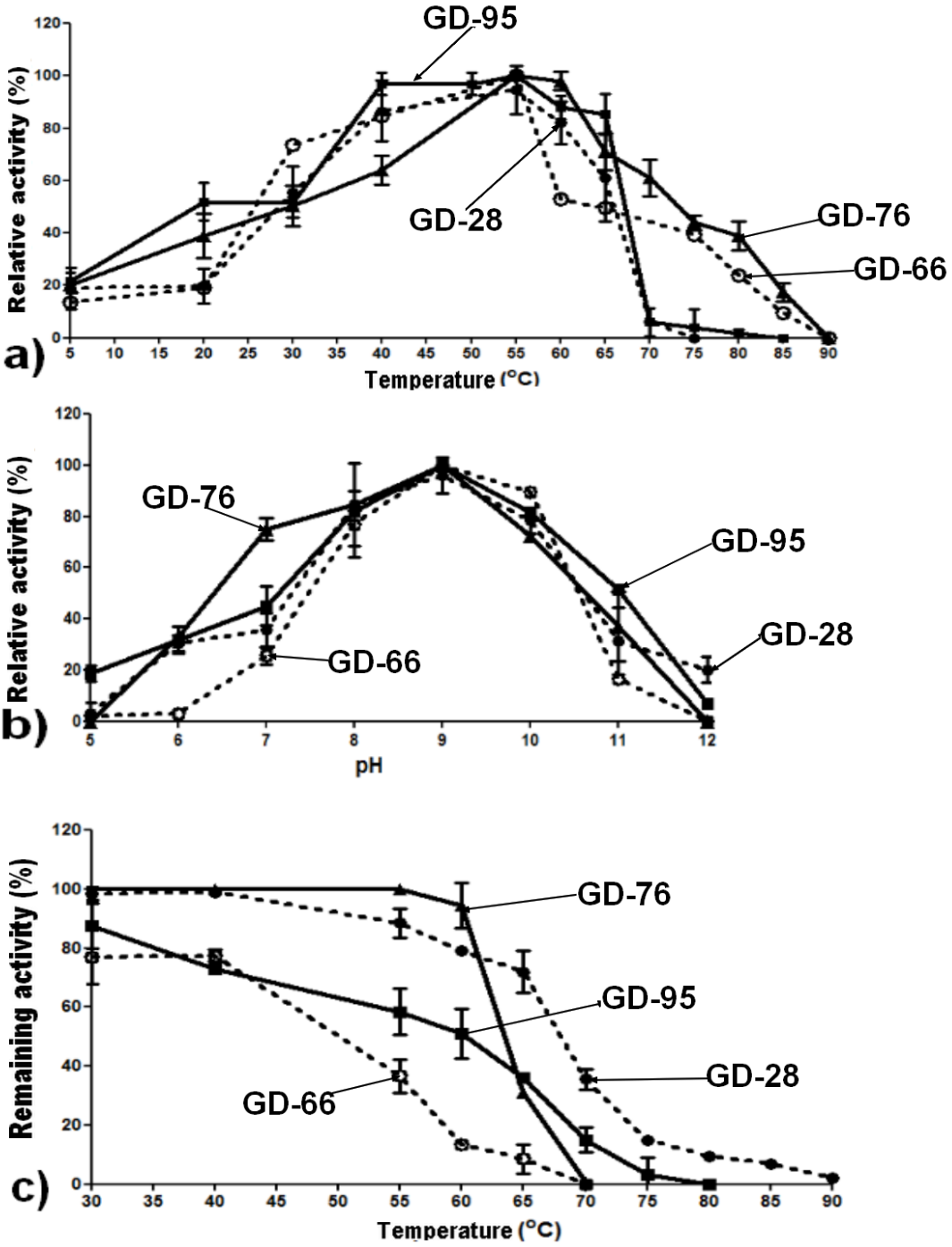


**Fig. 8. Effect of temperature on the activity (a) and stability (c) and pH influence (b) on the activity of purified GD-28, GD-66, GD-76 and GD-95 lipases. Straight line with a square – GD-95 lipase; straight line with a triangle – GD-76 lipase, dotted line with a white circle – GD-66 lipase, dotted line with a black circle – GD-28 lipase. Assays were performed at various temperatures (5–95 ºC) (a) or various pH (b) under enzyme assay conditions. The remaining activity was assayed under enzyme assay conditions after the purified recombinant enzymes had been incubated at the indicated temperature (30–90 ºC) for 30 min (c).**

### 3.3 Design of new lipolytic biocatalysts by DNA shuffling and epPCR

For the purpose of creation of new lipolytic enzyme with improved physicochemical properties and activity the first step of DNA shuffling was optimized (data not shown). Subsequently, the hydrolysis reaction of *Geobacillus* lipase genes obtained by PCR with DNase I was performed at room temperature (20–22 $^{o}$C) for 5 min using 0.04 U of DNase I in 20 µl reaction mixture with 2.5 mM $MgCl_2$ and 10 mM $MnCl_2$. Reaction was stopped by adding 7 mM EDTA in reaction mixtures.

For DNA shuffling GD-28, GD-66 and GD-95 lipase genes were amplified by PCR (Fig. 9a) and hydrolyzed with DNase I under optimized reaction conditions (Fig. 9b). Then 50–400 bp fragments were purified from 2 % agarose gel and used in self-priming PCR. In second PCR with primers 1.2 kb product was reassembled, which corresponds to the size of the parental lipase genes (Fig. 9c).

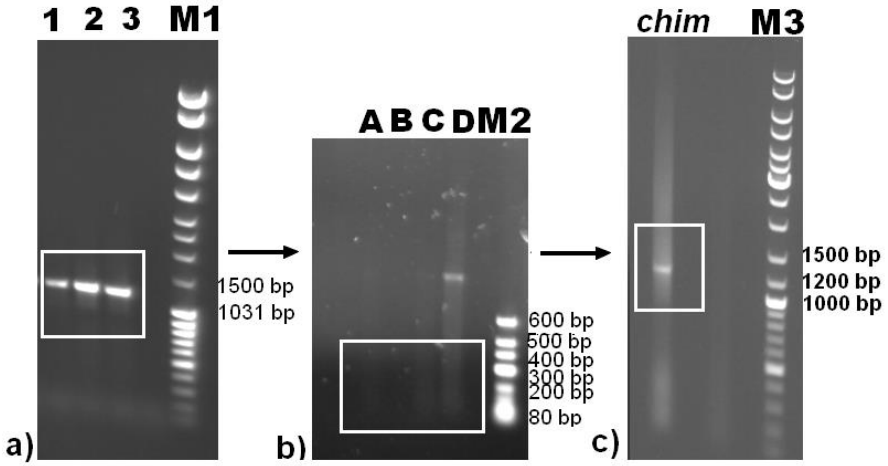


**Fig. 9. Amplification of parental GD-28, GD-66 and GD-95 lipase genes by PCR (a), hydrolysis of these fragments with DNaseI (b) and second PCR with primers resulting full length size gene product (c). The white rectangles marked the target fragments. Amplification of target lipase genes (a): lane M1 – MassRuler DNA Ladder Mix, lane 1 – *lip66* gene product, lane 2 – *lip28* gene product, lane 3 – *lip95* gene product. PCR products digestion with DNase I (b): lane M2 – SORPOsize ™ DNA Ladder, lane A – hydrolysis reaction with 0.1 U of DNaseI, lane B – hydrolysis reaction with 0.08 U of DNaseI; lane C – hydrolysis reaction with 0.04 U of DNaseI, lane D – hydrolysis reaction with 0.02 U of DNaseI. The reaction was performed for 5 min at room temperature in the presence $Mn^{2+}$. The amplification of full-length size gene with primers (c): M3 – GeneRuler DNA Ladder Mix; *chim* – during PCR amplificated gene fragment.**

Then 1.2 kb size gene fragment (Fig. 9c) was sequenced and sequence was compared with parental lipase sequences (Fig. 7). The sequence analysis showed that *chim* gene

variant displayed very high similarity to parental lipases. For this reason, epPCR was applied to introduce more mutations and increase the diversity of *chim* gene.

Lipase obtained after two rounds of epPCR showed 98 % sequence similarity to GD-95, 96 % to GD-66 and 97 % to GD-28 lipase. Despite high percentage similarity of *Geobacillus* lipases the introduced differences may be sufficient for lipases to demonstrate new improved physicochemical and kinetic properties or lipolytic activity. Purification analysis in SDS-PAGE and zymogram showed that after epPCR obtained lipase was nonhomogeneous and in zymogram two clear zones of lipolytic activity were observed (data not shown). This indicates two lipolytic enzymes in protein sample. According to the molecular size of clear zones in zymogram the two proteins were named GDlip30 and GDlip43. Interestingly, both GDlip30 and GDlip43 lipases were expressed during translation of one and the same sequence. This fact raised hypothesis that during translation process ribosome recognized another AUG located in the new lipase gene transcript as start codon. Truncated fragments of target protein can be also the result of limited proteolysis or irregular initiation of translation (Jennings et al., 2016). Detailed information about amino acid sequence of GDlip30 lipase can help to identify the minimal structure required for the lipase activity. GDlip30 is the smallest *Geobacillus* lipase variant which has lipolytic activity. In further steps GDlip30 and GDlip43 lipases were separated by elution from SDS-PAGE gel (data not shown).

## 3.4 Physicochemical and kinetic analysis of GDlip30 and GDlip43 lipases

### *3.4.1 Kinetic analysis of GDlip30 and GDlip43 lipases*

The highest specific lipolytic activity and the highest kinetic values were showed by GD-95 lipase. In contrast, GD-66 lipase demonstrated the lowest activity (Table 7). Both new GDlip30 and GDlip43 lipases showed 40 U/mg specific lipolytic activity (Table 7). This activity is twofold higher than parent GD-66 lipase, but tenfold lower than GD-95 lipase.

GDlip43 and GDlip30 lipases demonstrated very low $V_{max}$ values (Table 7). This was not surprising, because one of parental lipases (GD-66) also showed low $V_{max}$. Meanwhile,

GDlip43 lipase possessed $K_m$ value similar to GD-95 lipase when *p*-NP dodecanoate was used as a substrate (Table 7). $K_{cat}$ and catalytic efficiency values of new lipases also are similar to GD-66 lipase (Table 7).

**Table 7. Comparative kinetic properties of purified GD-95, GD-28, GD-66, GDlip30 and GDlip43 lipolytic enzymes. The values were determined at 55 ºC, pH 9, using *p*-NP dodecanoate as substrate.**

| Protein | Specific activity (U/mg) | $V_{max}$ (µmol/min mg protein) | $K_m$ (mM) | $K_{cat}$ ($min^{-1}$) | Catalytic efficiency $K_{cat}/K_m$ ($Min^{-1}$ $mM^{-1}$) |
|---|---|---|---|---|---|
| **GD-95** | **400** | **40.82** | **4.35** | **$1.78*10^6$** | **$4.10*10^5$** |
| **GD-66** | **22** | **9.05** | **14.29** | **$3.93*10^5$** | **$2.75*10^4$** |
| **GD-28** | **60** | **28.01** | **11.74** | **$1.22*10^6$** | **$1.04*10^5$** |
| **GDlip30** | **40** | **11.15** | **7.14** | **$3.38*10^5$** | **$4.73*10^4$** |
| **GDlip43** | **40** | **8.65** | **6.67** | **$3.76*10^5$** | **$5.64*10^4$** |

Experimental results showed that GD-95 and GD-66 lipases possessed the highest differences in lipolytic activity. GDlip43 lipase demonstrated higher similarity to GD-66 than GD-95 lipase. These results led to hypothesis that amino acids which differ in these three lipases can be significant for the lipolytic activity of *Geobacillus* lipases. Candidate amino acids are Arg153 and Phe154. These amino acids are present in GD-95 lipase, but in GD-66 and GDlip43 lipases instead of these amino acids are localized His and Val (Fig. 7). Other potential candidate amino acid is Leu247, which is present in GDlip43, but GD-66 and GD-95 lipases at this site possess Ser and Pro, respectively. Close to this amino acid are localized amino acids, which are involved in the substrate binding pocket (Trp234, Ala240, Leu244 (Carrasco-Lopez et al. 2009)). GD-66 lipase also demonstrated lower ability to hydrolyze long acyl chain substrates (Table 8) compared to GD-95 and GDlip43 lipases. For this reason, it was assumed that at 153, 154 and 247 positions localized amino acids can be involved in the regulation of substrate specificity of *Geobacillus* lipases.

### *3.4.2 Temperature activity, thermostability and pH tolerance of GDlip30 and GDlip43 lipases*

One of the main objectives of this research was design of new *Geobacillus* lipase variant with improved thermoactivity and thermostability. The optimum temperature for new GDlip43 lipase was 50 $^{o}$C, but this lipase also showed 99 % activity at temperature range 55–60 $^{o}$C (Fig. 10a). The significant positive difference from parental lipases was detected at 70 $^{o}$C. GDlip43 lipase possessed about 66 % relative lipolytic activity at this temperature. At 75–85 $^{o}$C temperature range GDlip43 lipase was similar to GD-66 parent lipase and its activity was higher than other parental lipases (GD-95 and GD-28 lipases) (Fig. 10a). GDlip43 lipase also demonstrated increased thermostability compared with parental GD-66 and GD-95 lipases. After 30 min of incubation at 65 $^{o}$C GDlip43 retained 50 % of its lipolytic activity compared to 36 % (GD-95 lipase) and 9 % (GD-66 lipase) These results confirmed that DNA shuffling and epPCR can help to improve physicochemical properties of important industrial biocatalysts. Subsequently, the new GDlip43 lipase displayed improved thermoactivity and thermostability properties compared to parental GD lipases.

The temperature optimum of the second lipase obtained in this research (GDlip30) was at 40–50 $^{o}$C and higher than 25 % relative lipolytic activity was retained up to 75 $^{o}$C. This feature was similar to GDlip43 and parent GD-66 lipases (Fig. 10a). Thermostability properties of GDlip30 lipase showed similar pattern to parent GD-66 lipase as it was already inactivated after 30 min incubation at 70 $^{o}$C (Fig. 10c).

New GDlip43 lipase distinguished from other GD lipases by higher activity at pH 11 (Fig. 10b). The optimal pH for both GDlip30 and GDlip43 lipases was determined to be at pH 8 and 8–9, respectively.

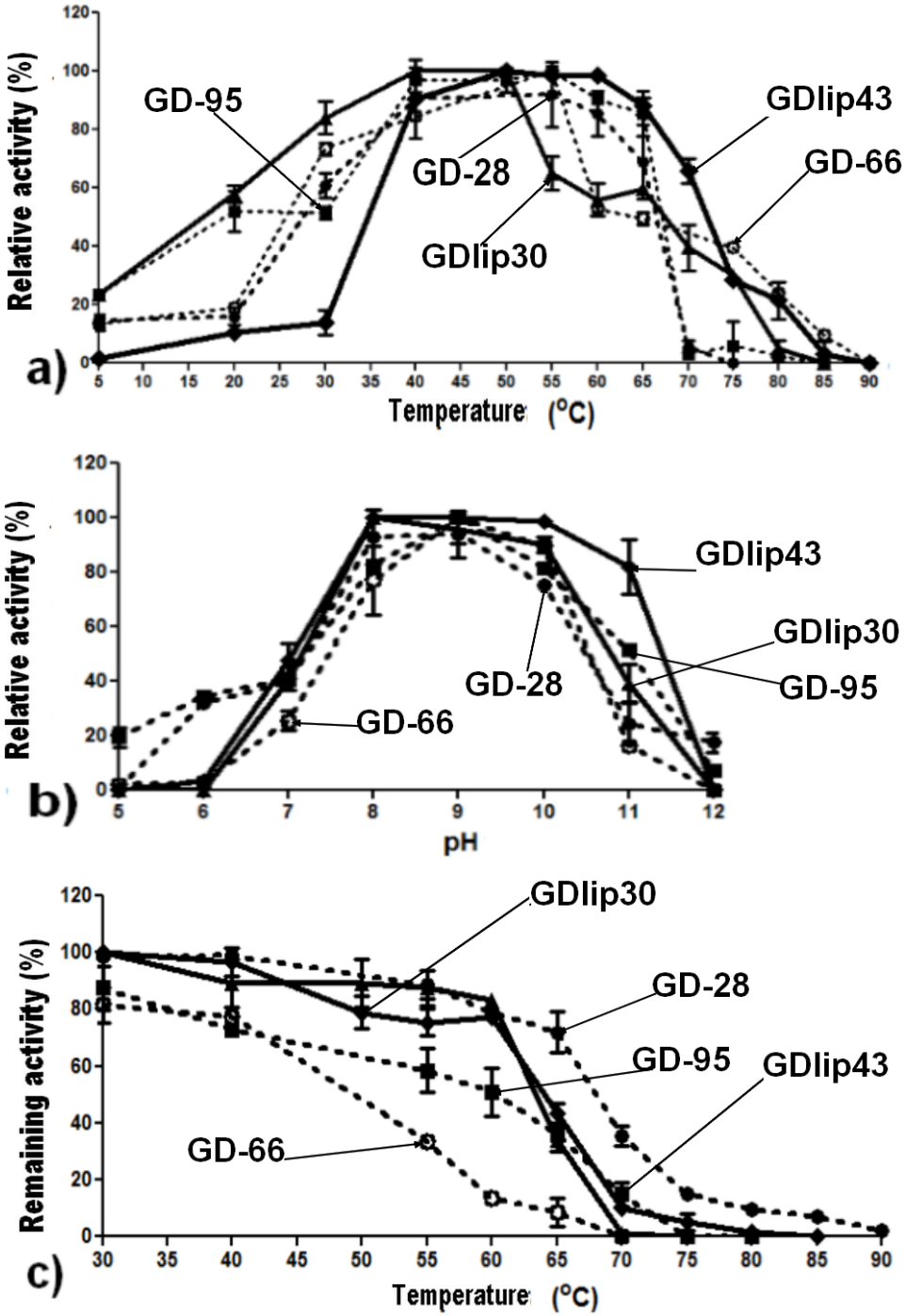


**Fig. 10. Effect of temperature on the activity (a), stability (c) and pH influence (b) on the activity of purified GD-28, GD-66, GD-95 lipases and after DNA shuffling and epPCR experiments obtained GDlip30 and GDlip43 lipolytic enzymes. Straight line with a filled rhombus – GDlip43 lipase; straight line with a filled triangle – GDlip30 lipase; dotted line with a filled circle – GD-28 lipase; dotted line with a empty circle – GD-66 lipase; dotted line with a filled square – GD-95 lipase. Assays were performed at various temperatures (5–95 ºC) (a) or various pH (b) under enzyme assay conditions. The remaining activity was assayed under enzyme assay conditions after the purified recombinant enzymes had been incubated at the indicated temperature (30–90 ºC) for 30 min.**

### *3.4.3 Substrate specificity and tolerance to organic solvents of GDlip30 and GDlip43 lipases*

The new lipolytic enzyme (GDlip43) preferred *p*-NP acetate and *p*-NP dodecanoate (Table 8). The substrate specificity profile of GDlip43 of long-chain length substrates (C14–C18 substrates) hydrolysis was similar to GD-95 lipase (Table 8). Meanwhile, GDlip30 lipase showed more esterase activity (Table 8).

**Table 8. Activity of new GD-28, GD-66, GDlip30, GDlip43 and GD-95 lipases toward various acyl length *p*-NP esters. The analysis was carried out at 55 ºC, pH 7. ND – not detectable. The substrate specificity was determined for each protein individually.**

| Substrate | Relative activity (%) of target enzymes | | | | |
|---|---|---|---|---|---|
| | GD-95 | GD-66 | GD-28 | GDlip43 | GDlip30 |
| ***p*-NP acetate (C2)** | 87 ±1,41 | 50 ±1,41 | 81±1,41 | 87±6,36 | 15±0,71 |
| ***p*-NP butyrate (C4)** | 100 | 100 | 100 | 61±1,41 | 100 |
| ***p*-NP hexanoate (C6)** | 95 ±4,07 | 57 ±2,66 | 45±1,41 | 47±4,24 | 63±2,12 |
| ***p*-NP octanoate (C8)** | 88 ±4,95 | 21 ±1,41 | 65±1,41 | 78±0,71 | 54±5,66 |
| ***p*-NP decanoate (C12)** | 80 ±1,41 | 4±1,41 | 76±0,71 | 92±2,12 | 33±2,12 |
| ***p*-NP myristate (C14)** | 43 ±7,78 | 4±1,41 | 71±1,41 | 39±1,41 | 26±0,71 |
| ***p*-NP palmitate (C16)** | 35 ±2,83 | 4±1,41 | 5±2,12 | 37±1,41 | ND |
| ***p*-NP stearate (C18)** | 26 ±2,12 | ND | ND | 18 | ND |

All organic solvents used except isopropanol increased lipolytic activity of GDlip43 lipase (Table 9). The GDlip30 lipase was activated by DMSO, DMF and methanol, but isopropanol decreased its activity by 22 %. Significant differences between GDlip30 and GDlip43 lipases were detected only for acetone and n-butanol (Table 9).

**Table 9. Effect of various organic solvents (25 %) on the enzyme activity of purified GD-28, GD-66, GD-95, GDlip30 and GDlip43 lipases. Lipase activity without addition of organic solvent was set as 100%. The values were determined at 55 ºC, pH 9, using *p*-NP dodecanoate as substrate.**

| Effect | Relative activity (%) of target enzymes | | | | |
|---|---|---|---|---|---|
| | GD-95 | GD-66 | GD-28 | GDlip43 | GDlip30 |
| **DMSO** | 60±4.4 | 90±3.54 | 97 | 134±7.07 | 110±3.43 |
| **DMF** | 113±6.8 | 106±4.95 | 98±1.82 | 117±4.95 | 123±5.65 |
| **Ethanol** | 122±6.5 | 87±6.26 | 103±0.71 | 115±3.5 | 96±6.36 |
| **Methanol** | 80±2.6 | 88±2.12 | 98±2.12 | 147±4.95 | 139±3.54 |
| **Acetone** | 90±1.8 | 100±0.71 | 96±1.41 | 147±4.95 | 62±3.54 |
| **Isopropanol** | 104±3.5 | 91±2.83 | 94±2.82 | 76±1.41 | 78±5.66 |
| ***n*-Butanol** | 104±4.3 | 90 | 100±2.82 | 107±9.90 | 62±3.54 |

The physicochemical and kinetic analysis of GDlip43 and GDlip30 lipases suggested, that GDlip43 lipase displayed improved thermostability, thermoactivity and resistance to treatment with various organic solvents. These features made this enzyme a potential biocatalyst for industrial application, although improvement of lipolytic activity is a good object for further experiments. Further analysis of GDlip30 lipase can help to answer the question about minimal structure required for the functionality of *Geobacillus* lipases.

# CONCLUSIONS

1. At C-terminal region located amino acids are significant for the functionality of *Geobacillus* lipases: Asp371 is important for thermostability, Phe375 is responsible for thermoactivity and Tyr376 helps maintain the tertiary structure of GD-95-10 lipase.

2. New GDEst-95 esterase from *Geobacillus* sp. 95 was active at broad pH and temperature range, displayed thermostability, resistance to various organic solvents and detergents. Due to these properties it is an atractive potential biocatalyst for industrial application.

3. The fusion of GD-95 lipase and GDEst-95 esterase with additional homodomain increased the optimal temperature of both enzymes and modified their thermostability profile as well as changed hydrolysis efficiency of various *p*-NP esters. This strategy can be applied for modulation of various characteristics of target enzymes.

4. New fused GDEst-lip enzyme possessed high specific lipolytic activity (600 U/mg), ability to operate at wide temperature and pH ranges. Also this enzyme hydrolyzed both short and long acyl chain substrates and retained activity after treatment with various organic solvents, surfactants and high temperatures. Thsese features suggest a potential of GDEst-lip for application in various industrial processes.

5. GDlip43 lipase obtained by direct evolution experiments showed higher thermoactivity and thermostability than parental lipases. Meanwhile, GDlip30 lipase is a new and unique *Geobacillus* lipase variant with a lower molecular weight and is a potential object for future research in order to identify the minimal structure required for the functionality of *Geobacillus* lipases.

# LIST OF PUBLICATIONS

**The thesis is based on the following original publications:**

1. **Gudiukaitė R**., Gegeckas A., Kazlauskas D., Citavicius D. Influence of N- and/or C-terminal regions on activity, expression, characteristics and structure of lipase from *Geobacillus* sp. 95. *Extremophiles*, 2014, 18(1), 131– 145.
2. **Gudiukaitė R.,** Gegeckas A., Sadauskas M., Citavičius D. Detection of Asp371, Phe375 and Tyr376 influence on GD-95-10 lipase using alanine scanning mutagenesis, *Appl Biochem Biotechnol*, 2016, 178(4), 654 – 669.

# CONFERENCE PRESENTATIONS

**Dissertation theme was presented in 4 conferences (3 of them – international):**

1. **Gudiukaitė R**., Gegeckas A., Čitavičius D. Cloning, purification and esterification capability determination of lipase produced by *Geobacillus* sp, 76 (poster presentation), FEMS 2013 : 5th Congress of European Microbiologists, Leipzig, Germany, 2013 07 21–25.
2. **Gudiukaitė R.**, Gegeckas A., Čitavičius DJ. Variability evaluation of physicochemical properties of recombinant lipases from *Geobacillus* spp, strains (poster presentation), VIII Gamtos mokslų fakulteto konferencija "Mokslas Gamtos Mokslų Fakultete", Vilnius, Lithuania, 2014 10 03.
3. **Gudiukaitė R.**, Gegeckas A., Čitavičius D. GD-95 lipase – new biocatalyst in wide industry areas (poster presentation), 2nd Congress of Baltic Microbiologists, Tartu, Estonia, 2014 10 16–19.
4. **Gudiukaitė R.**, Gegeckas A., Malūnavičius V., Čitavičius D. Functionality analysis of structural domains from GD-95 lipase by site specific and random mutagenesis (poster presentation), FEMS 2015: 6th Congress of European Microbiologists, Maastricht, The Netherlands, 2015 06 07–11.

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# REZIUMĖ

*Geobacillus* genties bakterijų sintetinami lipoliziniai fermentai (lipazės ir karboksilesterazės) – vis dar aktuali ir perspektyvi tyrimų sritis. Šiame darbe sujungus bioinformatinę analizę, genų inžinerijos, enzimologijos ir biochemijos principus bei pritaikius gana naujos srities – baltymų inžinerijos metodus, tokius kaip dviejų skirtingais aktyvumais pasižyminčių baltymų jungimas, vietai savita mutagenezė, kuri persipynė su alanino (Ala) skenuojančia mutageneze bei DNR maišymo ir klaidingosios PGR metodikas, pavyko aptikti ar naujai sukurti, klonuoti, išgryninti bei išanalizuoti aštuoniolika lipoliziniu aktyvumu pasižyminčių fermentų. Taikant Ala skenuojančią mutagenezę buvo pirmą kartą nustatyta Asp371, Phe375 ir Tyr376 svarba *Geobacil*lus lipazių funkcionalumui. Tyrimai parodė, kad Asp371 yra svarbi palaikant šių fermentų temperatūrinį stabilumą, Phe375 užtikrina fermentų veiklą aukštoje temperatūroje, o Tyr376 palaiko svarbius ryšius tretinėje baltymo struktūroje. Struktūros ir funkcijos tyrimai yra svarbūs sintetinėje biologijoje ar baltymų kūrimui *de novo*, kada žinant svarbius regionus ar aminorūgštis galima kurti sintetinius fermentus, pasižyminčius pramonei patraukliomis savybėmis ir galinčius pakeisti cheminius katalizatorius ar ne tokius efektyvius biokatalizatorius.

Šiame darbe sukurtas efektyvus ir potencialus sulietas lipolizinis fermentas GDEst-lip – pirmasis tokio pobūdžio lipolizinis chimerinis biokatalizatorius, sudarytas iš *Geobacillus* sp. 95 kamieno sekretuojamų GD-95 lipazės ir GDEst-95 esterazės domenų. GDEst-95 esterazė – nauja 55 kDa dydžio *Geobacillu*s genties bakterijų karboksilesterazė. Šio fermento fizikinių, cheminių ir kinetinių savybių analizė papildo negausią informaciją apie tokio dydžio *Geobacillus* esterazes.

GDEst-lip sintetinis fermentas pasižymi aukštu lipoliziniu aktyvumu, termoaktyvumu, temperatūriniu stabilumu, atsparumu įvairių organinių tirpiklių ir detergentų poveikiui. Šios savybės daro šį fermentą potencialiu komerciniu produktu. Taip pat įvertinus sulietų, iš dviejų vienodų domenų (GDEst-est ir GDLip-lip) sudarytų lipoliziniu aktyvumu pasižyminčių fermentų savybes buvo nustatyta, kad to paties domeno priliejimas gali padėti modeliuoti tikslinių fermentų charakteristikas.

Atlikta trijų naujai išskirtų *Geobacillus* genties bakterijų sintetinamų lipazių (GD-28, GD-66 ir GD-76) analizė praplėtė turimą informaciją apie šių bakterijų lipolizinių fermentų fizikines, chemines bei katalizines savybes. Tolimesniuose eksperimentuose GD-28, GD-66 ir GD-95 lipazės pritaikytos tiesioginės evoliucijos eksperimentuose, kuriant naujus *Geobacillus* lipazių variantus, kurie pasižymėtų didesniu temperatūriniu stabilumu ir aktyvumu. Po DNR maišymo ir klaidingosios PGR sukurtos GDlip43 ir GDlip30 lipazės praplečia natūralių fermentų įvairovę, papildo turimą informaciją apie *Geobacillus* bakterijų lipazes bei turi potencialo būti taikomos kaip biokatalizatoriai pramoniniuose procesuose.

# REFERENCES


1. Abdul Rahman MZ, Salleh AB, Abdul Rahman RN, Abdul Rahman MB, Basri M, Leow TC. 2012. Unlocking the mystery behind the activation phenomenon of T1 lipase: a molecular dynamics simulations approach. *Protein Sci* 21:1210–21
2. Ahn JO, Choi ES, Lee HW, Hwang SH, Kim SC, Jang HW, Haam SJ, Jung JK. 2004. Enhanced secretion of *Bacillus stearothermophilus* L1 lipase in *Saccharomyces cerevisiae* by translational fusion to cellulose-binding domain. *Appl Microbiol Biotechnol* 64: 833–839
3. Anobom CD, Pinheiro AS, De-Andrade RA, Aguieiras ECG, Andrade GC, Moura MV, Almeida RV, Freire DM. 2014. From structure to catalysis: recent developments in the biotechnological applications of lipases. *BioMed Res Int* doi: 10,1155/2014/684506
4. Arpigny JL, Jaeger KE. 1999. Bacterial lipolytic enzymes: classification and properties. *Biochem J* 343:177–83
5. Bornscheuer UT. 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol Rev* 26:73–81
6. Bornscheuer UT. 2008. Alteration of lipase properties by protein engineering methods. *OCL* 15:184–188
7. Carrasco-Lopez C, Godoy C, de las Rivas B, Fernandez-Lorente G, Palomo JM, Guisan JM, Fernandez-Lafuente R, Martinez-Ripoll M, Hermoso JA. 2009. Activation of bacterial thermoalkalophilic lipases is spurred by dramatic structural rearrangements. *J Biol Chem* 284:4365–72
8. Chakravorty D, Parameswaran S, Dubey VK, Patra S. 2011. *In silico* characterization of thermostable lipases. *Extremophiles* 15:89–103
9. Chen XY, Zaro JL, Shen WC. 2013. Fusion protein linkers: property, design and functionality. *Adv Drug Deliver Rev* 65:1357–69
10. Dror A, Kanteev M, Kagan I, Gihaz S, Shahar A, Fishman A. 2015. Structural insights into methanol-stable variants of lipase T6 from *Geobacillus stearothermophilus*. *Appl Microbiol Biotechnol* 99:9449–61
11. Dror A, Shemesh E, Dayan N, Fishman A. 2014. Protein engineering by random mutagenesis and structure-guided consensus of *Geobacillus stearothermophilus* lipase T6 for enhanced stability in methanol. *Appl Environ Microbiol* 80:1515–27
12. Eisenthal R, Danson MJ, Hough DW. 2007. Catalytic efficiency and $k_{cat}/K_M$: a useful comparator. *Trends Biotechnol* 25:247–9
13. Elleuche S. 2015. Bringing functions together with fusion enzymes-from nature's inventions to biotechnological applications. *Appl Microbiol Biotechnol* 99:1545–56
14. Ewis HE, Abdelal AT, Lu CD. 2004. Molecular cloning and characterization of two thermostable carboxyl esterases from *Geobacillus stearothermophilus*. *Gene* 329:187–95
15. Ghori MI, Iqbal MJ, Hameed A. 2011. Characterization of a novel lipase from *Bacillus* sp. isolated from tannery wastes. *Braz J Microbiol* 42:22–9
16. Goodarzi N, Karkhane AA, Mirlohi A, Tabandeh F, Torktas I, Aminzadeh S, Yakhchali B, Shamsara M, Ghafouri MA. 2014. Protein engineering of *Bacillus thermocatenulatus* lipase via deletion of the α5 helix. *Appl Biochem Biotechnol* 174:339–51
17. Gudiukaitė R, Gegeckas A, Kazlauskas D, Citavicius D. 2014. Influence of N- and/or C-terminal regions on activity, expression, characteristics and structure of lipase from *Geobacillus* sp. 95. *Extremophiles* 18:131–45
18. Gudiukaitė R, Gegeckas A, Sadauskas M, Citavicius D. 2016. Detection of Asp371, Phe375 and Tyr376 influence on GD-95-10 lipase using alanine scanning mutagenesis. *Appl Biochem Biotechnol* 178:654–69
19. Guncheva M, Zhiryakova D. 2011. Catalytic properties and potential applications of *Bacillus* lipases. *J Mol Catal B-Enzym* 68:1–21
20. Gustavsson M, Lehtio J, Denman S, Teeri TT, Hult K, Martinelle M. 2001. Stable linker peptides for a cellulose-binding domain-lipase fusion protein expressed in *Pichia pastoris. Protein Eng* 14:711–5
21. Hasan F, Shah AA, Javed S, Hameed A. 2010. Enzymes used in detergents: lipases. *Afr J Biotechnol* 9:4836–44
22. Hasan F, Shah AA, Hameed A. 2006. Industrial application of microbial lipases. *Enzyme Microb Technol* 39:235–251
23. Jennings MJ, Barrios AF, Tan S. 2016. Elimination of truncated recombinant protein expressed in *Escherichia coli* by removing cryptic translation initiation site. *Protein Expr Purif* 121:17–21
24. Joshi S, Satyanarayana T. 2015. In vitro engineering of microbial enzymes with multifarious applications: prospects and perspectives. *Bioresour Technol* 176:273–83

25. Kauffmann I, Schmidt-Dannert C. 2001. Conversion of *Bacillus thermocatenulatus* lipase into an efficient phospholipase with increased activity towards long-chain fatty acyl substrates by directed evolution and rational design. *Protein Eng* 14:919–28
26. Kim HK, Park SY, Lee JK, Oh TK. 1998. Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Biosci Biotech Bioch* 62:66–71
27. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–5
28. Levisson M, van der Oost J, Kengen SWM. 2007. Characterization and structural modeling of a new type of thermostable esterase from *Thermotoga maritima*. *Febs J* 274:2832–42
29. Liu P, Ewis HE, Tai PC, Lu CD, Weber IT. 2007. Crystal structure of the *Geobacillus stearothermophilus* carboxylesterase Est55 and its activation of prodrug CPT-11. *J Mol Biol* 367:212–23
30. Maynard JA, Chen G, Georgiou G, Iverson BL. 2002. In vitro scanning-saturation mutagenesis. *Methods Mol Biol* 182:149–63
31. Moreira IS, Fernandes PA, Ramos MJ. 2007. Computational alanine scanning mutagenesis: an improved methodological approach. *J Comput Chem* 28:644–54
32. Morrison KL, Weiss GA. 2001. Combinatorial alanine-scanning. *Curr Opin Chem Biol* 5:302–7
33. Olechnovic K, Kulberkyte E, Venclovas C. 2013. CAD-score: a new contact area difference-based function for evaluation of protein structural models. *Proteins* 81:149–62
34. Pritchard L, Corne D, Kell D, Rowland J, Winson M. 2005. A general model of error-prone PCR. *J Theor Biol* 234:497–509
35. Qin XL, Huang HH, Lan DM, Wang YH, Yang B. 2014. Typoselectivity of crude *Geobacillus* sp. T1 lipase fused with a cellulose-binding domain and its use in the synthesis of structured lipids. *J Am Oil Chem Soc* 91:55–62
36. Rotticci-Mulder JC, Gustavsson M, Holmquist M, Hult K, Martinelle M. 2001. Expression in *Pichia pastoris* of *Candida antarctica* lipase B and lipase B fused to a cellulose-binding domain. *Protein Expr Purif* 21: 386–392
37. Sambrook J, Russel DW. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, NY
38. Sangeetha R, Arulpandi I, Geetha A. 2011. Bacterial lipases as potential industrial biocatalysts: an overview. *Res J Microbiol* 6:1–24
39. Sharma D, Sharma B, Shukla AK. 2011. Biotechnological approach of microbial lipase: a review. *Biotechnol* 10:23–40
40. Shih TW, Pan TM. 2011. Substitution of Asp189 residue alters the activity and thermostability of *Geobacillus* sp. NTU 03 lipase. *Biotechnol Lett* 33:1841–6
41. Soliman NA, Gaballa AA. 2010. Molecular studies on a thermostable carboxyl esterase and dual function acylaminoacyl-peptidase from *Geobacillus thermoleovorans* YN. *J Biotechnol* 150:390
42. Stemmer WP. 1994. Rapid evolution of a protein in vitro by DNA shuffling. *Nature* 370:389–91
43. Tyndall JDA, Sinchaikul S, Fothergill-Gilmore LA, Taylor P, Walkinshaw MD. 2002. Crystal structure of a thermostable lipase from *Bacillus stearothermophilus* P1. *J Mol Biol* 323:859–69
44. Winkler UK, Stuckmann M. 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J Bacteriol* 138:663–70
45. Yan J, Yan Y, Madzak C, Han B. 2015. Harnessing biodiesel-producing microbes: from genetic engineering of lipase to metabolic engineering of fatty acid biosynthetic pathway. *Crit Rev Biotechnol* 2:1-11
46. Yu K, Liu CC, Kim BG, Lee DY. 2015. Synthetic fusion protein design and applications. *Biotechnol Adv* 33:155–64
47. Zhang Y. 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40