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Alisa
GRICAJEVA

Search and analysis of novel bacterial lipolytic enzymes

SUMMARY OF DOCTORAL DISSERTATION

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Academic supervisor - Prof. Dr. Lilija Kalėdienė (Vilnius University, Natural Sciences, Biology, N 010).

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Dr. Inga Matijošytė (Vilnius University, Natural Sciences, Biochemistry, N 004);
Doc. Dr. Jolanta Sereikaitė (Vilnius Gediminas Technical University, Technological Sciences, Chemical Engineering, T 005).

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VILNIAUS UNIVERSITETAS

Alisa
GRICAJEVA

Naujų bakterinių lipolizinių fermentų paieška ir analizė

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Mokslinė vadovė – prof. dr. Lilija Kalėdienė (Vilniaus universitetas, gamtos mokslai, biologija, N 010).

Gynimo taryba:

Pirmininkas – **prof. habil. dr. Algimantas Paulauskas** (Vytauto Didžiojo universitetas, gamtos mokslai, biologija, N 010).
Nariai:
prof. dr. Nomeda Kuisienė (Vilniaus universitetas, gamtos mokslai, biologija, N 010);
dr. Saulius Kulakauskas (Prancūzijos Nacionalinis žemės ūkio tyrimų institutas, gamtos mokslai, biologija, N 010);
dr. Inga Matijošytė (Vilniaus universitetas, gamtos mokslai, biochemija, N 004);
doc. dr. Jolanta Sereikaitė (Vilniaus Gedimino technikos universitetas, technologijos mokslai, chemijos inžinerija, T 005).

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INTRODUCTION

Hydrolases (EC 3) catalyze cleavage of chemical bonds in aqueous media. In the presence of excess water, equilibrium of these reactions is shifted towards product formation and, consequently, under physiological conditions, hydrolysis reactions are irreversible. Hydrolases may act on different types of substrates such as polysaccharides, DNA, proteins and lipids. The homeostasis of these biological macromolecules is crucial for all living organism, therefore, hydrolases are ubiquitous and are found in large quantities in all three domains of life[1].

Carboxylester hydrolases (EC 3.1.1.X) form a very diverse group of hydrolases which catalyze conversion of ester bonds – their cleavage and formation. Many of these enzymes are characterized by a wide range of possible substrates suggesting that their evolution has enabled organisms to access various carbon sources and ensured the presence of these enzymes in catabolic pathways[2]. One of the most important groups of carboxylester hydrolases are lipolytic enzymes, i.e. carboxylesterases or true/non-specific esterases (carboxylester hydrolases, EC 3.1.1.1) and lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) (hereinafter "lipolytic enzymes") which are of high physiological importance to all living organisms and make up a vast practical interest in biotechnology. Bacterial lipolytic enzymes are involved in various functions of these microorganisms: from external lipid degradation in order to obtain energy to membrane adaptations of native cells and pathogenesis[3].

Esterases and lipases are distinguished based on the differences in the nature of the substrates they use: these enzymes catalyze, respectively, the hydrolysis of water-soluble and insoluble fats such as mono-, di- and triacylglycerols. Another unique feature used for discrimination lipases and esterases is interfacial activation that has been attributed to lipases. However, lipolytic enzymes having a broad and overlapping substrate specificity are known, moreover, there are lipases that have lid domain but do not possess

interfacial activation as well as there are esterases which undergo interfacial activation. Therefore, criteria that have been widely used for the discrimination of lipases and esterases have to be revised. Although some new criteria are suggested, there are propositions to combine these and some other related enzymes into a single group of "universal lipases" (EC 3.1.1.x)[4].

Although bacterial lipolytic enzymes possess low amino acid sequence homologies, they have similar three-dimensional structures. Based on properties of amino acid sequences and fundamental biological characteristics, 20 years ago Arpigny and Jaeger[5] (1999) for the first time classified known bacterial lipolytic enzymes into 8 (I-VIII) families and 6 subfamilies (I.1-I.6). Currently, classification of bacterial lipolytic enzymes was updated and consists of 35 (I-XXXV) families and 12 subfamilies (I.1 – I.12)[5, 6].

Although structures of some lipolytic enzymes have been extensively studied, and numerous convenient methods have been developed to increase their yield and activity (protein engineering, chemical modification, immobilization, etc.),[7,8,9,10] the demand for these new enzymes is not diminishing[11]. The current amount of scientific literature on bacterial lipolytic enzymes leaves no doubt that these enzymes are among the most sought after in biotechnology. Because of their particular set of biocatalytic properties bacterial lipolytic enzymes are extremely attractive for the sustainable industry applications. They are valued not only for their natural ability to catalyze hydrolysis of carboxylic esters, but also for the *in vitro* promiscuity and ability to catalyze carboxylic ester bond synthesis and other non-specific activities. Easy extraction of bacterial lipolytic enzymes, their stability in various reaction conditions (extreme temperatures, pH, and chemical agents), wide range of substrates enable their applications in the fields of oleochemistry, polymer, textile, biodiesel production, detergent, food, cosmetic industries and bioremediation. With one of the most exceptional properties of enantioselectivity, bacterial lipolytic enzymes are distinguished as one of the most important biocatalysts

used to produce optically pure chemical compounds. Their biotechnological significance is reflected by the fact that certain microbial lipases and esterases are commercialized by the global biotechnology companies such as Fluka, Novozymes, Biocatalysts Ltd and other[12, 13].

Despite the current high number of identified lipolytic enzymes (~ 5000), only a small percentage (<10 %) of them are cloned, expressed and functionally characterized[1]. Present study provides experimental evidence on existence and functional characteristics of two lipolytic enzymes – LipBST and unusual EstAG1 coded in *Bacillus stratosphericus* and *Staphylococcus saprophyticus* genomes, respectively.

Aim of the study:

Search and identification of lipolytic enzymes by employing bacterial genome mining approach, heterologous synthesis of selected enzymes, their detailed functional characterization and analysis.

The following tasks were set in order to achieve the aim:

1) To mine and analyze genomes of the selected bacterial lipolytic strains in order to identify novel carboxylesterases (EC 3.1.1.1) and triacylglycerol acylhydrolases (EC 3.1.1.3).
2) To create suitable heterologous expression systems of the selected LipBST and EstAG1 lipolytic enzymes.
3) To perform biochemical characterization of LipBST and EstAG1 lipolytic enzymes.
4) To investigate hydrolytic potential of free LipBST, immobilize the lipolytic enzyme and evaluate the ability of the obtained enzymatic preparation to catalyze transesterification reactions.
5) To identify EstAG1 active site amino acid residues related to the catalytic function of this lipolytic enzyme.

6) To qualitatively evaluate *estAG1* expression at the transcription level in the native *S. saprophyticus* AG1 cells.

Scientific novelty and practical significance

With the significant improvement of genome sequencing over the past decades, large amounts of data have become available in the public databases, analysis of which allows identification of new enzymes and helps to fill in the gap of knowledge regarding certain enzymes and other biological molecules. Moreover, genome-mining approach allows easy discovery of new biocatalysts more purposefully and without some expenses needed by utilizing classical microbiological enrichment culture approach or metagenomic analysis and protein engineering.

In the present study, recombinant, putative lipolytic enzymes of *Bacillus* sp. L1 (the set of encoded lipolytic enzymes identified was specific and identical to those found in *B. stratosphericus*) and *S. saprophyticus* AG1 were identified utilizing genome analysis of the strains and functionally characterized in detail. Recombinant mesophilic LipBST lipase which was naturally encoded in bacteria identified as *B. stratosphericus* was characterized as a highly active enzyme having a minimal α/β-hydrolase domain without a lid. Enzyme possessed functional characteristics suitable for biodegradation of different fatty compounds and was successfully tested (using free form of the enzyme) for the ecologically-friendly cleaning of fish-fat contaminated polyethylene surfaces which were further suitable for the reuse. Immobilized preparation of the LipBST lipase was also analyzed and was shown to be suitable for the transesterification reactions such as enzymatic synthesis of biodiesel in water-free reaction media. Immobilization of the LipBST has also improved some key properties of the enzyme: temperature/pH optima and thermostability.

Moreover, this dissertation thesis covered identification and comprehensive analysis of an unusual putative EstAG1

carboxylesterase coded in *S. saprophyticus* AG1 genome which was determined to be distantly related to bacterial hormone-sensitive lipase-like family (bHSL) homologues. It was determined that EstAG1, in terms of its amino acid sequence, is a unique bacterial lipolytic enzyme, having atypical conservative sequence motives. It was discovered that in the amino acid sequence of EstAG1, instead of a highly conservative GX**S**XG (X – any amino acid) pentapeptide, a GD**G**TG pentapeptide lacking conservative nucleophilic Ser amino acid residue is found. GD**G**TG motive have not been described in any up to date known bacterial lipolytic enzyme family and was found to be intrinsic to EstAG1 enzyme and some other putative, uncharacterized or hypothetical enzymes/proteins coded in the genomes of other *Staphylococcus* sp. Furthermore, for the first time it was shown that catalytic Ser in the sequence of EstAG1 (and possibly in the sequences of mentioned uncharacterized homologues coded in the genomes of other *Staphylococcus* sp.) is located closer to the C-terminus of the enzyme in a previously unspecified **S**PLL sequence motive. Peculiarities of the primary sequence characteristics of EstAG1 and ≤30 % sequence identities with functionally and structurally characterized bHSL family homologues, allowed the assignation of EstAG1 to a novel family of bacterial lipolytic enzymes that, according to the latest classification by Hitch and Clavel (2019), can be classified as a new XXXVIth bacterial lipolytic enzyme family. Natural existence of *estAG1* gene transcript in a native *S. saprophyticus* AG1 strain has been also experimentally demonstrated in the present work.

Therefore, this dissertation thesis provides experimental existence evidence and some insights (biochemical characterization, *in silico* structure analysis, identification of catalysis related amino acids, *etc.*) into a new unusual carboxylesterase EstAG1 of *S. saprophyticus* AG1 and LipBST lipase of *B. stratosphericus*, and most importantly widens knowledge about the diversity of esterases and lipases known up to date, creating and expanding general concept of understanding of their evolution.

MATERIALS AND METHODS

Chemical reagents and enzymes. All chemical reagents used in this work were of analytical/molecular biology grade and were purchased from Roth, Sigma-Aldrich, AppliChem (Germany) and Thermo Fisher Scientific (Lithuania) unless otherwise stated. *Taq* DNA Polymerase (recombinant), *Pfu* DNA Polymerase (High-Fidelity), *Phusion* High-Fidelity DNA Polymerase, PCR Master Mix (2X) with *Taq* polymerase; FastDigest *Nde*I, *Xho*I restriction endonucleases (REases) (used for cloning, double restriction and linearization of plasmids); T4 DNA ligase (used for ligation and recirculization of DNA fragments); FastAP Thermosensitive Alkaline Phosphatase, dsDNase (Thermo Scientific dsDNase) were purchased from Thermo Fisher Scientific (Lithuania), unless otherwise stated. All listed enzymes were used according to the manufacturer‘s recommendations and protocols, unless otherwise stated.

Molecular biology kits. GeneJet Genomic DNA Purification Kit, GeneJet Plasmid Miniprep Kit, GeneJet PCR Purification Kit, GeneJet Gel Extraction Kit, InsTAclone PCR Cloning Kit, Verso 1-Step RT-PCR Kit, with Thermo-Start Taq (Hot Start) were purchased from Thermo Fisher Scientific (Lithuania) and used according to the manufacturer‘s protocols. RNA extraction / purification and concentration kits *Quick*-RNA Fungal/Bacterial Miniprep Kit and RNA Clean & Concentrator-25 Kit, respectively, were purchased from Zymo Research (USA) and used according to the manufacturer‘s protocols.

Strains, plasmids and oligonucleotides. Bacterial cultures were isolated from soil contaminated with fatty substances, oil and fat-rich food (spoiled cheese in the refrigerator). For the propagation of plasmids, gene overexpression and optimization of the production of recombinant proteins *E. coli* DH5α, *E. coli* BL21 (DE3), *E. coli* C41 (DE3) and *E. coli* Rosetta (DE3) (Novagen) bacterial strains were used. Electrocompetent *E. coli* cells were prepared according to

Sambrook and Russell (2011) recommended protocols[14]. Detailed information about all the bacterial strains used in this work is provided in Table 1.

Table 1. Bacterial strains used in this work.

Strain	Properties / purpose of the use	Source
Bacillus sp. L1	Mesophilic culture isolated from fat-rich food; a donor of LipBST enzyme gene	This work
Staphylococcus saprophyticus AG1	Mesophilic culture isolated from soil contaminated with fats; a donor of EstAG1 enzyme gene	This work
Staphylococcus aureus ATCC 25923^{T}	Strain used for comparative novobiocin resistance and coagulase tests	A gift from LSMU*
Staphylococcus epidermidis ATCC 12228	Strain used for comparative novobiocin resistance and coagulase tests	A gift from LSMU
E. coli DH5α genotype: ϕ80*lac*ZΔM15Δ(*lac*ZYA-argF) U169 recA1 endA1 hsdR17 *pho*A *sup*E44 *gyr*A96 *λ*-thi^{-1} *rel*A1	Plasmid propagation / gene cloning strain	Novagen
E. coli BL21 (DE3) genotype: *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)*	Classical strain used for the overexpression of the target recombinant proteins	Novagen
E. coli C41 (DE3) genotype: F^{-}*ompT hsdSB* (r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3)	Variety of *E. coli* BL21 (DE3) strain used for the expression of toxic proteins; target enzyme expression optimization	Gift from P. Kavaliauskas
E. coli Rosetta (DE3) genotype: F^{-}*ompT hsdS*$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3) *pRARE* (Cam^{R})	Variety of *E. coli* BL21 (DE3) strain which has a pRARE that supplies tRNA for rarely used codons; target enzyme expression optimization	Gift from P. Kavaliauskas

* - abbreviation of Lithuanian University of Health Sciences (lt., ***L**ietuvos **S**veikatos **M**okslų **u**niversitetas*).

Plasmid vectors and plasmid constructs used / created during this work and their purpose of the use are described in Table 2.

Table 2.2. Plasmid vectors and plasmid vector constructs.

Plasmid vector	Purpose of the use	Source
pTZ57R/T	*lipBST* TA cloning vector, Amp^R	TFS*
pET-26b(+)	IPTG-induced expression of the target genes obtaining recombinant enzymes with C-terminal 6-His-Tag, Kan^R	Invitrogen
pTZ57R/T_*lipBST*	*lipBST* cloning, propagation	This work
pET-26b(+)_*lipBST*	Heterologous expression of *lipBST*	This work
pET-26b(+)_*estAG1*	Heterologous expression of *estAG1*	This work
pET-26b(+)_*estAG1*_S179G	Heterologous expression of gene coding for mutated (S179G) EstAG1	This work
pET-26b(+)_*estAG1*_S179A	Heterologous expression of gene coding for mutated (S179A) EstAG enzyme	This work
pET-26b(+)_*estAG1*_T152A	Heterologous expression of gene coding for mutated (T152A) EstAG1 enzyme	This work
pET-26b(+)_*estAG1*_E246A	Heterologous expression of gene coding for mutated (E246A) EstAG1 enzyme	This work
pET-26b(+)_*estAG1*_H276A	Heterologous expression of gene coding for mutated (H276A) EstAG1 enzyme	This work
pET-26b(+)_*estAG1*:S179A:E246A:H276A	Heterologous expression of gene coding for mutated (S179A:E246A:H276A) EstAG1 enzyme	This work

* - Thermo Fisher Scientific.

Primers that were used in this work were synthesized at METABION (Germany). List of used primers is given in Table 3.

Table 3. List of oligonucleotides.

Name	Sequence (5'→3')	Target
27F	GAGAGTTTGATCCTGGCTCAG	16S rDNA
1495R	CTACGGCTACCTTGTTACGA	
M13/pUC (-20)F	GTAAAACGACGGCCAGT	pTZ57R/T_*lipBST* sequencing
M13/pUC (-26)R	CAGGAAACAGCTATGAC	
LipBPF	ATGCTCTAACTATCCTCTGCA	LipBP
LipBPR	TTAATATGGAATATCTCCATG	

LipBSF	ATGAGCGAAAACATG	LipBS
LipBSR	CTCGAGCCTGCTTTTTTCAC	
LipBSIF	ATGAGAGAAGGGCATTG	LipBSI
LipBSIR	TTACAACCGTAACCATCTG	
LipBST1F	GCGC**CATATG**AAAGTGATTCG	LipBST
LipBST1R	GCGC**CTCGAG**ATTCGTATTCTGTC	
EstAG1F	GCGC**CATATG**AAATCAAGACTG	EstAG1
EstAG1R	GCGC**CTCGAG**TTCATCTTTTAACG	
EstAG1_S179G_F	**p**GGCCCGTTACTAGATGCCCAA	EstAG1 mutation (S179G)
EstAG1_S179G_R	**p**AAACAAATATAATTTACTTGG	
EstGA1_S179A_F	**p**GCGCCGTTACTAGATGCCC	EstAG1 mutation (S179A)
EstAG1_S179A_R	**p**AAACAAATATAATTTACTTGG	
EstAG1_T152A_F	**p**GCGGGTGCTGCTTTAGCACT	EstAG1 mutation (T152A)
EstAG1_T152A_R	**p**GCCATCTCCCATAATGACAAT	
EstAG1_G151S_F	**p**AGCACTGGTGCTGCTTTAGC	EstAG1 mutation (G151S)
EstAG1_G151S_R	**p**ATCTCCCATAATGACAATAG	
EstAG_E246A_F	**p**GCGATTTACCTCCCAGACATG	EstAG1 mutation (E246A)
EstAG_E246A_R	**p**GTATGCACCGCCAAACATAT	
EstAG1_H276A_F	**p**GCGGCTTTCCCCTTATTACC	EstAG1 mutation (H276A)
EstAG1_H276A_R	**p**AATCATTCTTTTATATTCAT	
T7P	TAATACGACTCACTATAGGG	pET plasmid vector analysis and sequencing
T7T	GCTAGTTATTGCTCAGCGG	

p- oligonucleotide phosphorilation at 5‘-end.
Bold and underlined oligonucleotide parts designate restriction targets of *Nde*I and *Xho*I REases.

Culture media. TBA (Tributyrin agar)[15] and RBA (Rhodamine B agar)[16] media were used for the qualitative selection of lipolytic bacteria cultures isolated from soil contaminated with fatty compounds and spoiled fat-rich food. Isolates were grown at 35 °C and 60 °C, selected isolates were further grown at 35 °C. Native and recombinant microorganisms were cultured on standard commercial CASO/TSB, Luria-Bertani (LB), M9 media (agar or liquid), respectively. LB used for the growth of recombinant *E. coli* strains was supplemented with appropriate antibiotics (100 mg/mL Amp, 50 mg/mL Kan and 30 mg/mL Cam stock solutions were used). M9 medium modified (mM9) by adding standard 1000× vitamins and/or tributyrin was used for the *estAG1* transcription study. SOC and GYT media were used for the regeneration of the transformed bacteria and preparation of electrocompetent cells[14]. *E. coli* strains were grown at 37 °C / 27 °C / 17 °C, 200 rpm, unless otherwise stated.

Purification of gDNA and plasmid DNA. Genomic DNA and plasmids from target bacterial strains were purified with GeneJet Genomic DNA Purification Kit and GeneJet Plasmid Miniprep Kit, respectively.

DNA/RNA analysis. DNA/RNA electrophoresis was performed in 1 % agarose (TopVision Agarose) gels. Agarose gels were prepared using 1×TAE buffer and supplemented with ethidium bromide to a final concentration of 0.5 mg / mL. Electrophoresis was ran in 1×TAE buffer, 8–10 V / cm. MassRuler DNA ladder Mix or GeneRuler DNA Ladder Mix DNA molecular markers were used. Gels were analyzed under UV (DNA Bio-Imaging Systems MiniBIS Pro).

Identification of microorganisms: 16S rRNA phylogenetic analysis, whole-genome sequencing, digital DNA-DNA hybridization and biochemical tests. 16S rDNA was amplified from chromosomal DNA of the target bacteria by PCR using 27F and 1495R primers (Table 3). Thermocycling program used according to Kuisienė (2008)[17]. PCR products were purified by GeneJet PCR Purification Kit and sequenced in the Vilnius University, Institute of Biotechnology (Vilnius, Lithuania). Sequences were analyzed using DNASTAR LaserGene 7.1 / SnapGene software tools, and aligned using BLASTn (Basic Local Alignment Tool Nucleotide) algorithm available in the NCBI database (ncbi.nlm.nih.gov)[18]. The 16S rDNA sequences used for the phylogenetic analysis of 16S rDNA were selected in the Silva database (arb-silva.de/projects/living-tree/). Evolutionary relationship was analyzed in MEGA 7.0[19] using Clustal W algorithm[20]. Evolutionary history was determined by the Neighbor-Joining method[21] with a bootstrap value equal to 1000 replicates[19,22].

Staphylococcus sp. AG1 gDNA was sequenced in BaseClear (The Netherlands). Genome was annotated by Prokka (*Prokka: rapid prokaryotic annotation*)[23] and additionally using RAST server (rast.nmpdr.org/rast.cgi).

Comparative digital DNA-DNA hybridization (dDDH) was done in http://ggdc.dsmz.de/home.php using Genome-to-Genome Distance Calculator (GGDC)[24]. For the comparative analysis genomes of *S. saprophyticus* subsp. *saprophyticus* ATCC 15305-NCTC 7292^T (AP008934.1), *S. saprophyticus* NCTC 7666 (SAYP00000000.1), *S. saprophyticus* 1A (LR134089.1), *Staphylococcus xylosus* ATCC 29971 (LT963439.1), *Staphylococcus equorum* NCTC 12414^T (UHDI00000000.1) and *Staphylococcus succinus subsp. succinus DSM* 14617^T (LCSH00000000.1) were used.

For the identification of *Staphylococcus* sp. AG1 coagulase and novobiocin resistance tests were performed as well[25,26]. *S. aureus* ATCC 25923^T and *S. epidermidis* ATCC 12228 were used as control strains.

Enzyme activity assay. The standard reaction was carried out with the appropriate amount of purified LipBST/EstAG1 in 1 mL of mixture containing 50 mM Briton-Robinson buffer (pH 7.5)[27] and 10 mM *p*-NPC. For the determination of substrate specificity and kinetic constants of the enzyme, reactions were carried out using different carbon atom chain length *p*-NP-esters. Substrates with the chain length shorter than $C_{10:0}$ were dissolved in 2-propanol; $C_{10:0}$ - $C_{18:0}$ *p*-NP-esters were emulsified using sodium deoxycholate and gum Arabic[27]. The activity of the enzyme was determined by measuring the amount of released *p*-NP at 35 - 45 °C, 410 nm using UV–Vis spectrophotometer with Peltier system (Perkin-Elmer). All samples were measured in triplicate and corrected for autohydrolysis of the substrates. The molar extinction coefficient of *p*-NP: 14,770 M^{-1} cm^{-1} at 410 nm.

Primary analysis and amplification of lipolytic enzyme genes coded in selected bacterial strains. For the search and analysis of the lipolytic enzyme genes, genomes of *B. stratosphericus* available in NCBI and sequenced genome of *S. saprophyticus* AG1 were used. The genes / CDS's encoding for the target lipolytic enzymes were translated to amino acid sequences and

their secretion fate was predicted using SignalP-5 (http://cbs.dtu.dk/services/SignalP/) and Secretome2.0 (http://cbs.dtu.dk/services/SecretomeP/) online servers. ProP 1.0 (http://cbs.dtu.dk/services/ProP/) tool was used for the prediction of pro-peptides[28,29].

For the determination and amplification of the target lipolytic enzyme genes of *Bacillus* sp. L1 (*B. stratosphericus* L1) and *S. saprophyticus* AG1, primer pairs (listed in Table 3) complementary to the conservative regions of the genes were used. PCR reaction conditions for the amplification of *Bacillus* sp. L1 lipolytic enzyme genes were similar to those used for 16S rDNA PCR. Primer annealing temperatures were determined empirically. For the amplification of *S. saprophyticus* AG1 lipolytic enzyme genes, high-fidelity *Phusion* polymerase was used. PCR was done according to standard protocols and recommendations of the manufacturer of the polymerase. Amplicons were analyzed by DNA electrophoresis.

Development of heterologous system for the expression of genes encoding target lipolytic enzymes. ***1) Bacillus sp.*** **L1.** *Taq* polymerase-amplified target *lipBST* gene with *Nde*I/*Xho*I restriction sites and dA overhangs was cloned into pTZ57R/T applying TA cloning strategy. Recombinant pTZ57R/T was transformed into *E. coli* DH5α cells by electroporation (1800 mV, 5 ms). Positive *E. coli* DH5α clones were selected using blue-white screening technique and further analyzed by colony PCR with M13/pUC (-20) and M13/pUC (-26) primers (Table 3). To confirm the insert of required size, purified recombinant plasmids were tested by carrying out the restriction analysis with *Nde*I/*Xho*I. LipBST gene insert was confirmed by sequencing. Further, to establish a system for the overexpression of the target lipolytic enzyme, it was subcloned into pET-26b(+) expression vector. For this purpose, double restriction (*Nde*I/*Xho*I) of pTZ57R/T with target gene insert and pET-26b(+) was performed. Double-digested pET-26b(+) and target gene were subjected to preparative DNA electrophoresis: fragments were gel-extracted according to GeneJet Gel Extraction Kit manufacturer‘s

protocol and ligated by T4 DNA ligase. Obtained plasmid construct was transformed into *E. coli* DH5α by electroporation. In order to confirm insertion, transformants were analyzed by colony PCR with plasmid-specific T7P/T7R primers (Table 3). Purified plasmids were analyzed by double-digesting with herein previously mentioned REases. After the confirmation of accuracy of the gene sequence by sequencing, construct was transformed into *E. coli* expression strains. ***2) S. saprophyticus* AG1.** For the cloning of *estAG1* lipolytic enzyme gene of the latter strain classical molecular cloning technique was applied: amplified gene fragments and pET-26b(+) were double-digested with *Nde*I/*Xho*I. Restriction targets were introduced into the gene during PCR amplification. Double-digested gene fragment and pET-26b(+) were subjected to preparative DNA electrophoresis, gel-purified and ligated. Obtained plasmid vector construct was transformed into *E. coli* DH5α by electroporation. Further analysis steps of the transformants and purified recombinant plasmid vectors were the same as described previously in this section for *Bacillus* sp. L1. After the confirmation of accuracy of the gene sequence by sequencing, construct was transformed into *E. coli* expression strains.

For the multiple alignments of LipBST and EstAG1 amino acid sequences of the nearest homologues obtained from BLASTp NCBI, Protein Data Bank (PDB), Uniprot/SwissProt databases were used. Multiple sequence alignment was performed using Clustal W algorithm (in MEGA 7 and other web servers). Similarities between aligned sequences and information on the formation of secondary structures were reproduced using ESPript3.0 (Easy Sequencing in PostScript) (espript.ibcp.fr/ESPript/ESPript/).

Heterologous overexpression. For the determination of optimal expression conditions, recombinant pET-26b(+) plasmids with LipBST/EstAG1 gene inserts were transformed into different expression strains (not only *E. coli* BL21 (DE3), but also *E. coli* C41 (DE3), Rosetta (DE3) stains) by electroporation. Transformants were cultured in LB containing 50 μg/mL kanamycin (for *E. coli* Rosetta

(DE3) medium is additionally supplemented with 30 μg/mL chloramphenicol), clones were analyzed by colony PCR. Further, positive *E. coli* transformants were inoculated into LB medium with appropriate antibiotics and cultured overnight at 37 °C, 180 rpm. 1 % of the overnight culture was inoculated into a fresh LB medium and bacteria were grown in the same conditions. Upon OD_{600} reached 0.6, suspension was supplemented with the gene expression inductor IPTG (until its final 1 mM concentration). Uninduced growing bacterial cells were used as a control. During 1-7 h of induction, both flasks were analyzed by collecting cell samples (bacterial OD in every sample was adjusted to be equal). Collected cells were centrifuged 1 min, 9000 x *g*, 4 °C, supernatant discarded and pellet resuspended in 50 μL of 4× protein loading buffer (40 % glycerol, 8 % sodium dodecylsulfate (SDS), 0.04 bromphenol blue). Protein profiles of induced and uninduced cells were analyzed by SDS-PAGE.

After determination of optimal expression strain, synthesis of the recombinant proteins was further optimized by changing growth media (LB/M9) of the selected strains, OD_{600} (0.4, 0.6 and 0.8) of the cells to be induced, final IPTG concentration in the medium (0,1 mM, 0.4 mM and 1 mM) and growth temperature (37 °C, 27 °C, 17 °C). Localization of the recombinant proteins in the recombinant cells was determined according to Pet System Manual protocols (kirschner.med.harvard.edu/files/protocols/Novagen_petsystem.pdf).

SDS-PAGE and zymography. Proteins were analyzed using glycine SDS-PAGE with 5 % concentrating and 12 % separating gels. Gels and buffers were prepared according to Laemmli[30]. For the zymographic analysis, one part of the gel was stained with Page Blue and other part processed in order to renature the proteins (i.e. the target enzyme). Renaturation was performed by washing the SDS-PAGE gel with renaturation buffer (50 mM Briton-Robinson buffer containing 1 % Triton X-100, pH 8) 3 × 15 min and 3 × 10 min with 50 mM Briton-Robinson buffer (pH 8) without Triton X-100. Further, gel was transferred into a sterile Petri dish and poured with

substrate solution (50 mM Briton-Robinson buffer, agar and 1 % emulsified tributyrin). After the solidification of the substrate-containing gel, Petri dish was transferred into 35-37 °C for 12-24 h. Upon the occurrence of clearance zone (a consequence of the tributyrin lipolysis) in the Petri dish poured with substrate solution, zymogram was overlaid with the gel stained with Page Blue, determining preliminary molecular mass of the target protein.

Purification of recombinant proteins. LipBST was purified in two steps: ammonium sulphate precipitation (60 % degree of saturation) and immobilized Ni^{2+} ion affinity chromatography (IMAC) in non-denaturing conditions. LipBST dissolved in IMAC binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 2 mM imidazole, pH 8) was loaded on the column with IMAC resin (Profinity IMAC Resins, Bio-Rad) and after wash step eluted with the buffer containing 250 mM of imidazole. Fractions were analyzed by measuring UV absorbance change at 280 nm and SDS-PAGE. Protein concentration was also analyzed by Bradford method with bovine serum albumin (BSA) as a standard[31].

EstAG1 enzyme forming inclusion bodies was solubilized in urea-based buffer (50 mM NaH_2PO_4, 300 mM NaCl, 2 mM imidazole, 8 M urea, pH 8) and purified in denaturing conditions using IMAC. Enzyme was eluted in a buffer: 50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, 6 M urea, pH 8. In order to renature the enzyme and eliminate residual imidazole and other impurities, protein was further purified by gelfiltration using Sephadex G-25 in a gel filtration buffer (25 mM Tris- HCl, 100 mM NaCl, pH 7,6) and then dialyzed against 25 mM Tris-HCl, pH 7.6. The purity of the protein was confirmed by SDS-PAGE and concentration was determined by the method of Bradford and at Abs_{280}.

Response surface methodology (RSM). In order to determine the mutual temperature and pH effects which are optimal for the performance of LipBST/EstAG1, central composite design (CCD) was employed. The experimental design and data were analyzed using Design Expert 11 software. Analysis of variance, a regression

analysis, and the plotting of response surface were performed to evaluate significance of the methodology and establish optimum conditions for the activity of LipBST/EstAG1 in different temperatures and pH. Intervals of the variables were established (A) to be from 5 °C (15 °C for EstAG1) to 80 °C and (B) from 3 to 12, for temperature and pH, respectively.

Biochemical characterization of LipBST and EstAG1. For biochemical characterization of the LipBST and soluble renatured EstAG1, activity at each characterization stage was measured as it was indicated above (Enzyme assay). Thermostability of the enzymes was analyzed by measuring the residual activity at the optimal temperatures after incubating enzymes for 60–240 min at 25, 35, 45, 55 and 65 °C for LipBST and 25, 30, 35, 40, 45 and 50 °C for EstAG1 in the absence of the substrate. The substrate specificity was determined with various *p*-NP esters: *p*-NPA ($C_{2:0}$), *p*-NPB ($C_{4:0}$), *p*-NPC ($C_{8:0}$), *p*-NPD ($C_{10:0}$), *p*-NPL ($C_{12:0}$), *p*-NPM ($C_{14:0}$), *p*- NPP ($C_{16:0}$) and *p*-NPS ($C_{18:0}$). Kinetic parameters (constants) of LipBST were analyzed with *p*-NPB, *p*-NPC and *p*-NPD using 40 mM stock solutions; EstAG1 kinetics were determined with different *p*-NPC concentrations. Michaelis-Menten constant (K_M) and the maximum velocity of the reaction (V_{max}) were analyzed based on Michaelis-Menten equation using GraphPad Prism 6 Software (GraphPad Inc., USA). Enzyme turnover number (k_{cat}) and kinetic efficiency (k_{cat}/K_M)[34] were calculated using V_{max} / [E_0] and k_{cat} / K_M formulas, respectively. Conditions of the reactions: 35 °C, 50 mM Briton-Robinson buffer, pH 9 for LipBST and 45 °C, 50 mM Briton-Robinson buffer, pH 8 for EstAG1.

The influence of various metal ions (Ca^{2+}, Mg^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, K^+, Li^+, 5 mM), inhibitors (EDTA, Pefabloc-SC, 5 mM), detergents (Tween 40, Tween 60, Tween 80, Triton X-100, SDS, 1 % v/v), chaotropic agent urea (1 %, v/v), organic solvents (*2*-propanol, ethanol, methanol, acetone, *n*-hexane, dimethyl sulfoxide (DMSO), 50 % v/v) on the activity of LipBST was determined by incubating the enzyme with mentioned chemicals for

30 min, 35 °C, pH 9 and measuring the residual activity in standard enzymatic assay conditions (35 °C, pH 9) with *p*-NPB and *p*-NPC substrates.

In the case of EstAG1, influence of different chemical agents on the activity of the enzyme was examined in the presence of 1 or 5 mM Ca^{2+}, Mg^{2+}, Ni^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, K^{+} and the chelating agent EDTA. The effect of the detergents was determined in the presence of 0.02, 0.05, 0.1 and 0.5 % (v/v) Tween 20, Tween 60, Tween 80, Triton X-100, SDS or cetyl trimethylammonium bromide (CTAB). The effect of organic solvents on the enzyme activity was studied in the presence of 0.5, 1, 5, 10, 20 % (v/v) ethanol, methanol, DMSO, dioxane, *n*-hexane, *tert*-butanol, acetone or 2-propanol, respectively. All measurements were done in standard reaction conditions (45 °C, pH 8) with *p*-NPC as a substrate. Enzyme activity without addition of chemical compounds was considered as 100 % activity.

Determination of positional selectivity of LipBST and EstAG1. For the determination of the positional selectivity (regiospecificity) of the enzymes, linseed oil (for the analysis of LipBST) and 1,3-dipalmitoyl-2-oleoylglycerol (for the analysis of EstAG1) were used. Reactions were carried out in water media: substrate with the enzyme solution in ratios from 1:1 to 1:10, respectively, were mixed with further incubation and stirring at 25 °C from 1 to 24 h. As a control, substrate was incubated with known regiospecificity commercial enzyme Lipolase L 100. Reactions were stopped by dissolving samples in diethyl ether (1:1) and stored at −20 °C until thin-layer chromatography (TLC) analysis.

LipBST hydrolysis of natural fats. Ability of LipBST to catalyze the hydrolysis of natural fatty substrates was analyzed employing triolein, rapeseed, linseed olive oils and animal fat (lard). Samples were prepared by mixing 0.4 mL of the lipid substrate with different quantities of LipBST (from 0.05 mg/mL to 0.5 mg/mL) with further incubation and stirring at 35 °C for 24 h. After incubation, 50 μL samples collected at different times of the reaction

were dissolved in diethyl ether (1:1) and analyzed by TLC. Control samples of triolein, canola, flaxseed oils, lard and oleic acid were prepared as the assayed samples without the addition of enzyme.

Immobilization of LipBST. For the immobilization of LipBST, octylsepharose and pyrolyzed sugar industry waste product (PSIWP) were used. Octylsepharose suspension was first prepared: 2 g of hydrophobic particles were suspended in 50 mM Tris-HCl (pH 7.5) and centrifuged at 4000 × *g*, 4 °C. Then octylsepharose was mixed with 5-10 mL of recombinant LipBST (0.1-0.5 mg/mL) suspended in 50 mM Tris-HCl (pH 7.5) and incubated at room temperature. Further, supernatant of the incubated enzymatic preparation was subjected to activity assay and protein concentration measurements by Bradford method. In the case of successful immobilization, mentioned parameters (lipolytic activity and protein concentration) decrease. Proper preparation was centrifuged and is analyzed: optimal catalysis conditions (temperature and pH), thermostability and the influence of various chemical agents are evaluated as it was described in previous sections. The preparation is also used for the evaluation of ability to catalyze transesterification of rapeseed oil by methanol (biodiesel production reaction).

LipBST immobilization onto PSIWP was done analogously to the described immobilization of the enzyme on octylsepharose. Also, to evaluate the ability of LipBST to catalyze transesterification reactions, immobilization of the enzyme on CFAPP was accomplished utilizing other technique that has been used in previous work[33]. For this purpose, 2 g of carrier were mixed with cooled at -20 °C acetone. After homogeneous distribution of the carrier particles, 5-10 mL of 0.1-0.5 mg/mL recombinant LipBST were mixed with the carrier and incubated for 30 min. The LipBST-coated carrier precipitate was dried at 4 °C.

Transesterification of rapeseed oil by methanol. For this purpose, reaction mixture was established by mixing 0.4 mL of rapeseed oil, 0.2 mL of methanol, 5 mL of *n*-hexane and 1 g of immobilized LipBST. Identical reaction mixtures without

immobilized enzyme were used as controls. Enzymatic reactions were carried out at 25 °C, 250 rpm. Samples (50 µL) were collected from 1 to 96 h of incubation every 2-10 h. Reactions were stopped by adding diethyl ether (1:1) and kept at -20 °C until TLC analysis.

Thin-layer chromatography. From 2 to 10 µL of each sample were applied on a TLC Silica Gel G-25 UV-254 plates (MACHERY-NAGEL). Mixture of petroleum ether, diethyl ether and acetic acid mixed in a ratio of 80:20:1 (v/v/v), respectively, was used as a solvent system for the separation of hydrolysis products[34]. Solvent system made of the same solvents as herein described and mixed in ratios of 85:15:2 was used for the separation of transesterification products. For the detection of the hydrolysis/transesterification products, chromatograms were visualized in iodine-saturated chambers.

Modeling of the three-dimensional structures of LipBST and EstAG1. Modeling studies on LipBST/EstAG1 three-dimensional structures were performed by employing a variety of automated web-server tools specializing in protein structure homology modeling such as SWISS-MODEL and I-TASSER. Model structures were created based on templates derived from previously determined structures that exhibited lipolytic and other activities. Models predicted in I-TASSER were selected according to C and TM prediction reliability scores (C-score represents the confidence of the achieved model and TM-score is a measure of the global structural similarity between the predicted LipBST/EstAG1 structure and template proteins). PyMOL (PyMOL molecular graphics system, Schrödinger, LLC) was used to visualize predicted models.

Phylogenetic analysis of EstAG1. In order to perform phylogenetic analysis of EstAG1, dataset of amino acid sequences consisting of EstAG1, top 104 hits obtained via NCBI BLASTp and 6 BLASTp hits of complete proteins with evidence at the protein/transcript levels were retrieved via the Uniprot (https://www.uniprot.org/blast/). Primary investigations in ESTHER database revealed that EstAG1 may belong to bHSL (family IV) of

esterases. Based on this, additional proteins (accession numbers: AB029896, SC07131, FN985096, BAB05967, AAW62260, AAZ67909, 4Q3O.1, 3K6K.1, 3DNM.1, 4Q05, 4XVC.1) were included in the analysis. The final dataset consisted of 122 sequences. The alignment used for tree reconstruction was generated using Clustal W. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) model. All positions with less than 95 % site coverage were eliminated. The tree was generated in MEGA7 and the tree with the highest log likelihood was selected.

Identification of active site amino acids related to catalytic function of EstAG1: whole-plasmid mutagenesis. Constructed pET-26b(+)_*estAG1* was used as a template for mutagenesis of *estAG1*. For the introduction of the target mutations (Gly151Ser, Thr152Ala, Ser179Gly, Ser179Ala, Glu246Ala, His276Ala) site-directed mutagenesis was applied. For the amplification of the recombinant plasmids, *Pfu* DNA polymerase and mutagenic primers (Table 3) were used. Mutagenic primers complementary to the *estAG1* gene were constructed using SnapGene 1.1.3 software tools.

Mutations were introduced into the *estAG1* by performing whole-plasmid (pET-26b(+)_*estAG1)* PCR. After the PCR, methylated primary DNA matrix was hydrolyzed by *Dpn*I REase. PCR-amplified linear plasmids were ligated and propagated in *E. coli* DH5α as it was described previously. The correct mutations of the mutant plasmids containing the *estAG1* gene mutated at the desired positions were identified by DNA sequencing. For the heterologous expression of mutated EstAG1 variants *E. coli* BL21 (DE3) were used. Recombinant mutated enzymes were purified from the inclusion bodies, renatured and analyzed applying activity assay in standard in standard conditions as it was described before. Native EstAG1 was used as a control.

Qualitative analysis of *estAG1* expression in *S. saprophyticus* AG1 at the transcription level. *Extraction of total RNA*. For the purification of the total RNA, *S. saprophyticus* AG1

was cultivated in CASO / LB / mM9 (with or without 1 % of emulsified tributyrin) at 35 °C, 180 rpm. Bacterial cell samples used for the total RNA purification were collected from different growth phases: early, middle, late exponential, stationary and death. Total RNA samples were purified and concentrated using *Quick*-RNA Fungal/Bacterial Kit and RNA Clean and Concentrator-25 Kit, respectively. 1 % agarose gel electrophoresis was performed in order to analyze integrity of isolated RNA. ***RT-PCR (reverse transcription PCR).*** Before RT-PCR, total RNA samples were treated with dsDNase to remove any possible contaminative gDNA. DNA impurity in the total RNA samples was analyzed by performing 16S rDNA PCR using dsDNase treated total RNA samples. RT-PCR with *estAG1* gene specific primers was performed using Verso One-Step RT-PCR Kit according to manufacturer‘s protocol.

RESULTS AND DISCUSSION

This dissertation thesis covered analysis of two lipolytic bacterial strains – *Bacillus* sp. L1 (initially designated as L1 isolate) and *S. sparophyticus* AG1 (initially designated as AG1) and their respective lipolytic enzymes – LipBST and EstAG1.

1. Identification of L1 bacterial lipolytic isolate and lipolytic enzymes coded in the target bacteria genome

16S rDNA sequencing (GenBank: KP065495.1) of L1 isolate and further phylogenetic analysis revealed that L1 was evolutionary most closely related to *B. stratosphericus*, *Bacillus altitudinis*, and *Bacillus aerophilus* species which are the members of so-called *B. pumilus* group (evolutionary relationship depicting phylogenetic tree not shown)[35]. Genomes of the mentioned bacterial strains available at NCBI were mined in order to identify lipolytic enzyme genes and design complementary primers for the detection of particular gene products in the genome of the target *Bacillus* sp. L1. PCR with

designed primers resulted in a detection of three DNA products of theoretically expected size (Table 4).

Table 4. Lipolytic enzyme genes identified in the genome of *Bacillus* sp. L1.

Detected product / Characteristics	*lipBST*	*lipBS*	*lipBSI*
Gene size, bp	648	816	1490
Putative product	triacylglycerol acylhydrolase / esterase (EC 3.1.1.3 / 1)	secreted lipase (EC 3.1.1.3)	Class 3 lipase (EC 3.1.1.3)
Precursor length (a.a)	215	271	486
Signal sequence	Sec system recognized N-terminal signal which is cleaved by SPI at $AKA_{34-35}AA$	Sec system recognized N-terminal signal which is cleaved by SPII at $LSA_{36-37}CS$	No signal peptide or non-classical
Length of mature enzyme (a.a)	181	235	486
M_m of precursor and mature protein, kDa	22,89 /19,16	30,72 / 26,53	53,96
Putative localization	extracellular	membrane	unclear
Superfamily	α/β – hydrolase	α/β/α-hydrolase	α/β – hidrolase
Putative family	I (I.4 superfamily)	Family II	Family XI

Sequencing of the products revealed that amplified lipolytic enzyme genes were identical to the genes encoded in the genome of *B. stratosphericus.* Furthermore, the set of three detected lipolytic enzymes was determined to be characteristic only to *B. stratosphericus*, although some homologues of the detected genes were separately found in other *Bacillus* sp. genomes. Genes were confirmed by sequencing. At this point, target strain was preliminary assigned to belong to *B. stratosphericus* species. Some characteristics of the detected lipolytic enzyme genes are provided in Table 4.

Further, at this point of work, for the creation of heterologous protein production system and analysis, *lipBST* gene (GenBank: KP331444.1) encoding putative ~ 19 kDa lipolytic enzyme

belonging to the family of true lipases (I.4) was selected. Structure modeling (I-TASSER) of LipBST revealed that enzyme putatively has a compact structure consisting only from the catalytic α/β-hydrolase domain and has no lid, therefore the active site of the LipBST is open to surrounding solution (Figure 1).

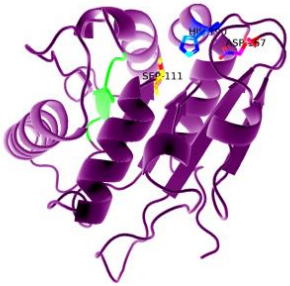

Fig. 1. The modeled three-dimensional structure of LipBST. Purple color depicts minimal α/β-hydrolase domain, green arrow depicts oxyanion hole forming amino acid motive. In the upper part of the model putative catalytic triad-forming residues are shown.

Multiple sequence alignment of LipBST with characterized homologues revealed that the catalytic triad of the enzyme is formed by Ser / His / Asp amino acid residues with catalytic nucleophile Ser located in Ala-X-Ser-X-Gly pentapeptide.

2. Heterologous overexpression and purification of LipBST

lipBST was cloned using TA strategy and further subcloned into pET26(+)b expression vector which, in order to achieve best protein synthesis, was transformed into *E. coli* BL21 (DE3), C41 (DE3) and Rosetta (DE3). Classical *E. coli* BL21 (DE3) heterologous expression strain revealed to be the optimal. Other optimal overexpression conditions were determined to be as follows: LB medium, 37 °C, induction of recombinant cells at OD_{600}=0.4 to the final 0.1 mM IPTG concentration. After the first hour of induction following optimal conditions, ~ 19 kDa target protein band, analyzing the total amounts of proteins in recombinant cells, was detected. The biggest amount of the recombinant LipBST was detected during 2 h of induced synthesis. LipBST enzyme was

purified from the extracellular medium in two steps: 1) precipitation by ammonium sulphate and 2) Ni^{2+} IMAC in non-denaturing conditions. Purity and preliminary molecular mass of the LipBST was evaluated by SDS-PAGE and zymography (Figure 2).

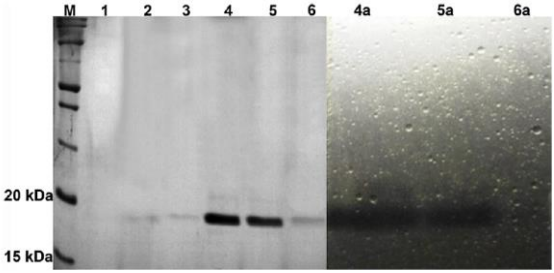

Fig. 2. SDS-PAGE (on the left) and zymogram of purified LipBST. 1, 2, 3–unbound protein fractions, 4, 5, 6–elution fractions. In a zymogram visible clear zones of the tributyrin (4a, 5a, 6a) correspond to 4, 5, 6 protein bands on the level of 19 kDa molecular mass marker in SDS-PAGE.

SDS-PAGE shower that LipBST was sufficiently purified and proper for the subsequent characterization and immobilization.

3. Characterization of LipBST

Using response surface methodology (RSM), mutual effect of temperature and pH on the activity of LipBST was tested. Results showed that the optimal conditions for the activity of LipBST are 35 °C, pH 7.5 (response surface plot is shown in Figure 3). ANOVA of the results revealed that F- and p-values were equal to 643.66 and <0.0001, respectively. This demonstrated that the model is significant, i.e., statistically suitable for describing the relationship between the independent variables and response. Nevertheless, after evaluation of the effect of temperature and pH separately, pH was specified to be pH 9.

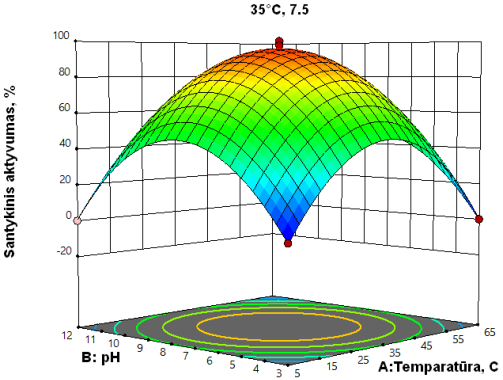

Fig. 3. Response surface plot describing mutual effect of temperature and pH on optimal LipBST activity.

Mesophilic LipBST enzyme was determined to be sufficiently thermostable in a range of 35-55 °C: it maintained more than 60 % residual activity over the incubation period (240 min).

In order to investigate LipBST lipolytic activity, substrate specificity using various *p*-NP ($C_{2:0}$ to $C_{18:0}$) in standard conditions, catalytic parameters of LipBST-mediated hydrolysis and regiospecificity were determined. LipBST was active towards wide range of *p*-NP substrates with highest activity towards *p*-NPC ($C_{8:0}$). Maximal specific activity of LipBST in optimal conditions (35 °C, pH 9, *p*-NPC) was determined to be 6244.5 U/mg. Affinity towards the medium chain *p*-NP substrate was confirmed by the kinetic study. Concentration of *p*-NPC needed to achieve the half of the reaction's maximal velocity (V_{max}) was 1.5 and 57 times lower than it was needed when measuring with *p*-NPB and *p*-NPD, respectively (Table 5).

Table 5. Kinetic constants of LipBST for different *p*-NP substrates.

Substrate	V_{max} (mM min^{-1})	K_M (mM)	k_{kat} (min^{-1})	k_{kat}/K_M ($mM^{-1}min^{-1}$)
p-NPB	1,1	5×10^{-2}	$4{,}6\times10^{3}$	$9{,}2\times10^{4}$
p-NPC	**2,5**	$\mathbf{3{,}4\times10^{-2}}$	$\mathbf{9{,}9\times10^{3}}$	$\mathbf{30{,}0\times10^{4}}$
p-NPD	0,1	194×10^{-2}	$0{,}5\times10^{3}$	$0{,}2\times10^{4}$

Determined affinity towards middle chain *p*-NP substrate could be attributed to the specific structure of the LipBST. It is known that enzymes having the minimal α/β-hydrolase domain and no lid domain are more easily positioning substrates with shorter carbon chains, while substrates with longer carbon atom chains protrude from the surface of the enzyme and are therefore less efficiently hydrolyzed[36].

Positional selectivity of lipolytic enzymes is the ability to distinguish between primary (i.e., *sn*-1,3) and secondary (*sn*-2) ester functionalities in a molecule. In comparison to *sn*-1,3-regiospecific Lipolase 100 L which released 1, 2-diacylglicerols (DAGs), LipBST exhibited no regiospecificity for the investigated substrate: it was able to hydrolyze the three positions (*sn*-1,2 and 3) on the triacylglycerol backbone (Figure 4)[37].

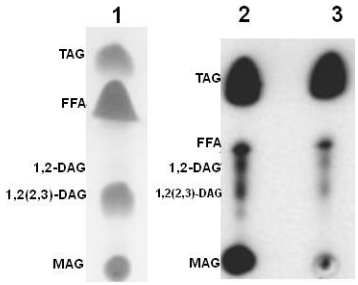

Fig. 4. TLC of linseed oil hydrolysis products: 1 – hydrolysis products by Lipolase 100 L; 2 – hydrolysis products by LipBST, 3– linseed oil not affected by the enzymes. TAG – triacylglycerols, FFA-free fatty acids, DAG – diacylglycerols, MAG – monoacylglycerols.

Lipolytic enzymes with *sn*-1,3 positional selectivity can be important in manufacture of structured lipids, although non-regiospecific lipases/esterases can be useful as well, for example, in enzymatic ethanolysis of oil[38].

Various metal ions can have stimulating as well as suppressing effect on the activity of lipolytic enzymes. Investigation of their effect on enzymes is important for understanding their action and for industrial applications. LipBST was determined to be inhibited by metal ions and EDTA when the activity was measured using *p*-NPB

substrate. Nevertheless, in standard reaction conditions using *p*-NPC, ~ 100 % residual activity after the incubation with various metal ions (except Fe^{3+}) and EDTA was measured. The lower residual activity of LipBST towards *p*-NPB in the mentioned conditions could be explained by the fact that the positively charged metal ions bind to the negatively charged amino acid residues or interact with the protein in other way and alter the conformation and / or proper ionization of the enzyme. As a result, the hydrolysis of the *p*-NPB became ineffective, although the catalysis of *p*-NPC was not obviously affected. Based on the results of the study of the effects of metal ions and EDTA on LipBST residual activity, it was concluded that the enzyme is not a metal ion activated enzyme.

Determination of the effect of various detergents on the activity of enzymes such as lipases is important from the scientific and an applied point of view. Detergents can increase the water-lipid area, stabilize the open conformation of the enzyme (this factor is not applied for the lipases lacking the lid domain), increase substrate solubility and prevent lipases from aggregation what leads to higher activity[39]. LipBST was strongly inhibited by all tested detergents with the only exception of ionic detergent SDS. In the presence of SDS, LipBST retained 70 ± 6 % of activity comparing with the control. This may be attributed to the ability of the detergent to prevent enzyme aggregation by weakening hydrophobic interactions and ameliorating substrate accessibility to the enzyme's active site. Negative effect of the surfactants on LipBST was probably due to their ability to block substrate entrance to the active site.

Enzymatic reactions in organic solvents are an emerging area of research and regarded as an extra valuable for the achievement of various industrial products. Screening of solvent-tolerant bacteria is usually cheaper than modifying known enzymes. Therefore, the evaluation of the LipBST stability in different organic solvents was performed as well. It was revealed that LipBST was stable in acetone and *n*-hexane (131 ± 2 % and 116 ± 1 %, respectively). In the most of the transesterification reactions for biosynthesis of new esters

hexane is the most favorable solvent[39,40]. Hydrophobic solvents are regarded as more superior in the enzyme-solvent interactions, mainly by maintaining the solvation carcass of the protein. Hydrophilic solvents are known to be more destabilizing as they remove water molecules off the protein surface. This can explain inactivation of LipBST by 2-propanol and ethanol. Stability of lipases in organic solvents correlates with distribution of surface charged residues and surface property of the enzyme. However, the mechanism of enzyme interactions and adaptation towards different organic solvents is yet poorly understood[40].

In this study LipBST ability to catalyze the hydrolysis of chosen fatty substances (triolein, canola, flaxseed oils, and lard) was also defined. Thin-layer chromatogram of the hydrolysis products is shown in Figure 5 (A, B).

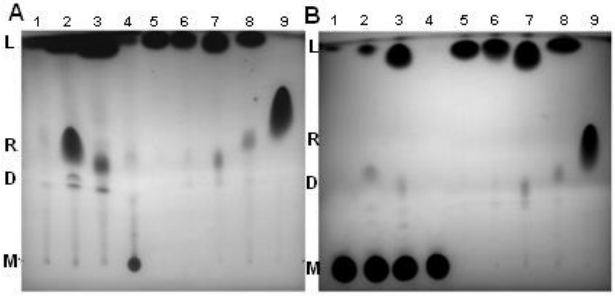

Fig. 5. (A). Recombinant LipBST specificity towards natural lipid substrates: 1–hydrolysis of triolein, 2–canola oil, 3–flaxseed oil, 4–lard; 5, 6, 7, 8–triolein, canola, flaxseed oils and lard hydrolysis control tracks, 9–oleic acid control. (B). Control chromatogram of fatty substrates undissolved in diethyl ether (aqueous layers of the samples): 1–9 corresponds to the numbers of Fig. 4 (A). L − triacylglycerols, F − fatty acids, D − diacylglicerols, M − monoacylglycerols.

Lard hydrolysis products appeared blurry in the chromatogram because saturated fatty acids that are found in the lard are poorly stained by iodine vapor. Figure 5 (B) depicts triolein, canola, flaxseed oils, and lard which were not dissolved in diethyl ether (the water fraction). Based on the ability of LipBST to catalyze the

hydrolysis of natural long-chain TAG's, it was assigned as a lipase (EC 3.1.1.3). Also, due to its high hydrolytic activity and efficient degradation of different oils and animal fats, LipBST has been successfully tested for the removal of fish fat from contaminated polyethylene surfaces that were further reused (MITA: Applied Research and Works Contract Innovation Check No. VP2-1.3-ŪM-05-K, 2014).

4. Immobilization of LipBST

Impossible reuse of free biocatalysts often determines high cost of application of enzymes. Such drawback can be overcome by immobilization that can also improve some important properties of enzymes and modulate their action. Therefore, studies of LipBST as well included its adsorption immobilization on a hydrophobic octylsepharose support. Immobilized LipBST exhibited new optimal operating conditions: temperature and pH conditions shifted from 35 °C, pH 9 to 50 °C, pH 10. Enzyme also exhibited higher thermostability at 35-65 °C compared to free LipBST. However, activity of the immobilized enzyme was inhibited by all tested metal ions, detergent and organic solvents. Immobilized preparation was also determined to be promising for the catalysis of the transesterification reactions in anhydrous media (Figure 6).

LipBST was determined to catalyze rapeseed oil transesterification by methanol or the reaction of synthesis of biodiesel. Rapeseed oil methyl esters were detected on TLC 2 h after the initiation of the transesterification reaction.

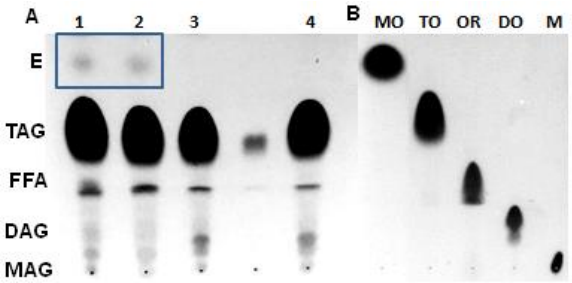

Fig. 6. Chromatogram of products obtained after transesterification of rapeseed oil by methanol catalyzed by LipBST immobilized on octylsepharose. 1 - profile of transesterification products by immobilized LipBST (iLipBST) preparation (2 h. of the reaction), 2 – profile of transesterification products by iLipBST (4 h of the reaction); 3, 4 – control samples without iLipBST (2 and 4 h); (B) Chromatographic image (control) of pure chemical compounds as potential lipase-catalyzed fat conversion reaction products. MO-oleic acid methyl ester; TO - triolein; OR - oleic acid; DO - mixture of 1,3, 1,2 - dioleins; M – monoolein.

Unfortunately, LipBST immobilized on PSIWP preparation was not suitable for the analysis.

5. Identification of AG1 bacterial lipolytic isolate

Phylogenetic analysis of the sequenced 16S rDNA revealed that AG1 was most closely related to *Staphylococcus saprophyticus* AP008934.1, as it is shown in the Fig. 7 (A). Therefore, AG1 was assigned as *Staphylococcus* sp. AG1.

Biochemical approaches applied in order to distinguish some staphylococci by coagulase and novobiocin tests, showed that *Staphylococcus* sp. AG1 was coagulase-negative and exhibited resistance to novobiocin (inhibition zone on the novobiocin plates was less than 12 mm (Fig. 7, B, C) while reference *S. aureus* ATTC 25923 and *S. epidermidis* ATTC 12228 were coagulase-positive (negative control) and coagulase-negative (positive-control), respectively, and both exhibited bigger than 16–27 mm inhibition zones on novobiocin plates.

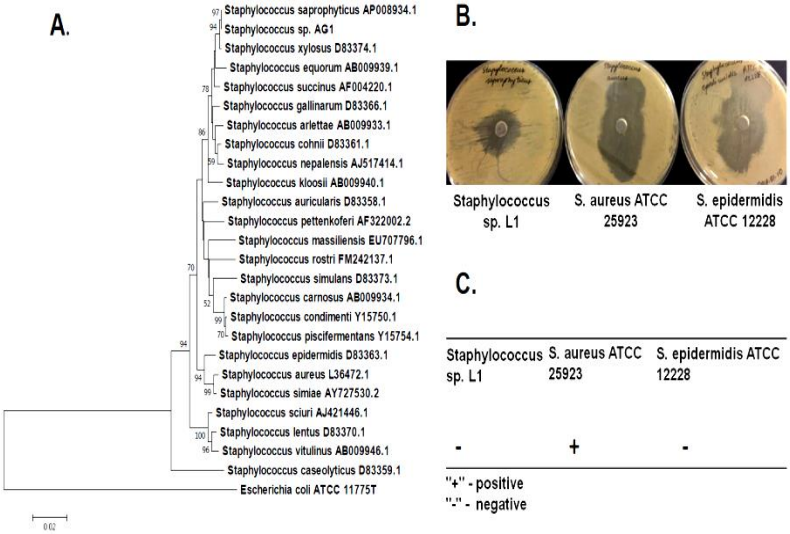

Fig. 7. (A). Phylogenetic tree illustrating evolutionary relationships of *Staphylococcus* sp. based on partial 16S rDNA sequences of the type strains of the genus. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Each code written after the species name indicates its accession number in GenBank. Phylogenetic tree was reduced according to far phylogenetic relatedness of some *Staphylococcus* species type strains which did not affect the phylogeny of the studied strain. Results of the novobiocin **(B)** and coagulase test **(C)**.

Phylogenomic analysis using (Genome-to-Genome Distance Calculator) GGDC revealed that the highest probability (DDH >70 %) to belong to the same species was between the studied *Staphylococcus* sp. AG1 strain and *S. saprophyticus* subsp. *saprophyticus* ATCC 15305. Results indicated that the query and ATCC 15305 belong to the same species. Differences of G+C % content between the *Staphylococcus* sp. AG1 and strain ATCC 15305 were 0.26. According to the GGDC, the difference between two strains belonging to the same species cannot be >1 %.

Genome-to-genome distance between *Staphylococcus* sp. AG1 and reference genomes according to DDH probability was less than 25 % (Table 6).

Table 6. Results of digital DNA-DNA hybridization (according to the Formula 2).

Query genome	Compared genomes	DDH	DDH > 70 %	G + C differences
***Staphylococcus* sp. AG1**	***S. saprophyticus* subsp. *saprophyticus* ATCC 15305-NCTC 7292^T**	**97,7**	**97,79**	**0,26**
	S. saprophyticus NCTC 7666	92,1	96,46	0,19
	S. saprophyticus 1A	92,1	96,46	0,25
	S. xylosus ATCC 29971^T	24,2	0,01	0,29
	S. equorum NCTC 12414^T	23,3	0	0,13
	S. succinus DSM 14617^T	22,5	0	0,04

6. Identification of lipolytic enzymes, heterologous expression and purification of EstAG1

After the genome sequencing of *S. saprophyticus* AG1 and its annotation by Prokka[23], six putative lipolytic enzyme (carboxylesterases (esterases) and lipases) coding genes were identified (Table 7).

Table 7. Putative lipolytic enzymes identified to be coded in the genome of *Staphylococcus saprophyticus* AG1.

Detected gene / Properties	*lip_1*	***est_1***	*est_2*	*est_3*	*est_4*	*MIMI_R526*
Gene size, bp	2259	903	975	735	741	1032
Putative product	lipase	esterase or lipase	esterase or lipase	esterase	esterase	α/β-hydrolase
Precursor size (a.a)	753	300	324	245	246	343
Mature protein (a.a)	716 / ?*	300 / ?	324 / ?	245 / ?	246 / ?	343 / ?
Signal peptide	Sec/SPI $AQA_{37-38}TE$	no or non-classical	no or non-classical	no or non-classical	no or non-classical	non-classical
M_m of precursor and mature protein, kDa	83,2 / ?**	35 / ?	36,7 / ?	27, 4 ?	28 / ?	39,6 / ?
Putative localization	Extrace-llular	Possibly intracellular	Possibly intracellular	Possibly intracellu-lar	Possibly intracellu-lar	Extrace-llular
Superfamily	α/β – hidrolases					
Putative family	I.6	IV	IV	XIII	XIII	IV

* - sequence length / molecular mass of the of mature proteins are unknown, since the exact signal sequence is unknown; ** - most probably 46 kDa, since it is a lipase having pre-pro-peptide structure, although ProP1 did not reveal a pro-peptide.

Because of the determined unusual sequence features, for the further analysis *est_1* (named *estAG1*) gene coding for the putative esterase was selected. According to the ESTHER database[41], EstAG1 was predicted to be a member of IV/bHSL family, although its similarity with the family's closest homologues was only ≤30 %. Furthermore, in the sequence of EstAG1, conservative GXSXG pentapeptide with nucleophilic Ser residue was substituted by GDGTG[149-153] motive without Ser. Based on the homology analysis of EstAG1, it was preliminary assumed that two of the catalytic residues of the EstAG1 could be formed by Glu-246 and His-276. More detailed primary sequence and three-dimensional model (Figure 7) analysis of EstAG1 revealed that a putative catalytic Ser residue could be located more closely to C-terminus in the **S**PLL[179-182] amino acid motive. The latter, as a possible place of localization of catalytic Ser, was never described before. Hypothesis, that the catalytic Ser residue is located in **S**PLL[179-182] was prompted by the fact that in the three-dimensional model of EstAG1, putative catalytic residue Ser-179 was revealed to be located near the mentioned presumably catalytic Glu-246 and His-276 residues.

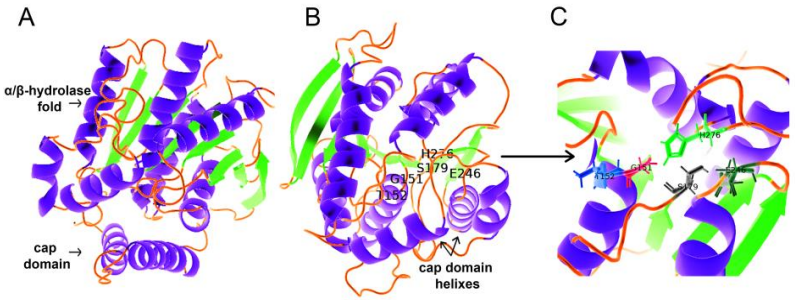

Fig. 7. (A) The modeled three-dimensional structure of EstAG1 with separately visible cap and catalytic (α/β-hydrolase) domains, **(B)** EstAG1 modeled molecule visualized with putative active and other amino acid residues, **(C)** zoomed amino acid residues of the active site.

Multiple sequence alignment of EstAG1 is shown in Figure 8.

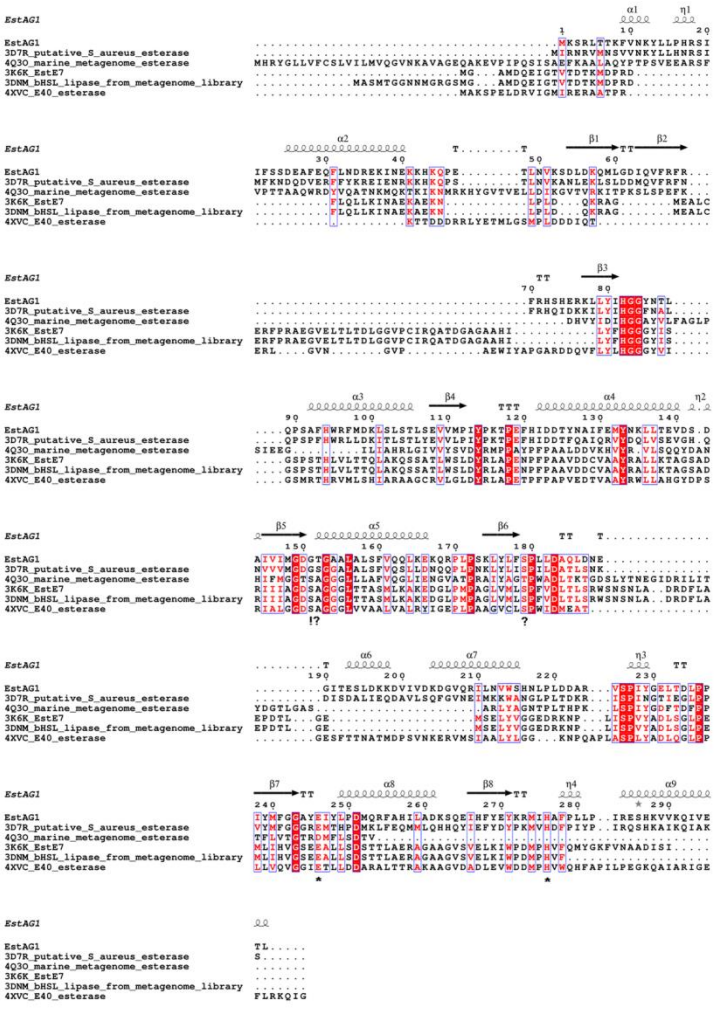

Fig. 8. Multiple sequence alignment of EstAG1 with structurally homologous enzymes, putative esterase (PDB: 3D7R), marine metagenome esterase (PDB: 4Q3O), HSL homologue EstE7 (PDB: 3K6K), bHSL lipase from metagenome library (PDB: 3DNM), E40 esterase (PDB: 4XVC). The alpha helix, beta sheet, random coil and beta turn correspond to α, β, η and T, respectively. Identical and highly conserved residues are colored in red and white, respectively. Black star (*) represents catalytic residues at the corresponding positions of Glu-246 and His-276. Black (!) indicates the site where normally Ser residue in the GXSXG conservative pentapeptide is found, (?) – indicates Ser-179 – possible catalytic Ser residue and Thr-152 – less possible amino acid residue which hypothetically could participate in the catalysis but most likely is an oxyanion hole-forming residue.

Recombinant EstAG1 was achieved by cloning the corresponding gene sequence into pET-26b(+). For the initial expression of the EstAG1, *E. coli* BL21 (DE3) cells transformed with pET-26b(+)_*estGA1* were used. During the synthesis in heterologous system, EstAG1 was determined to form inclusion bodies. Therefore, the process was optimized by employing *E. coli* C41 (DE3) and Rosetta (DE3) strains. Medium, temperature, OD_{600} of the inducible cells and final IPTG concentrations were optimized as well. However, all attempts to obtain soluble EstAG1 enzyme were not successful, therefore, the conditions for the expression were used as follows: *E. coli* BL21 (DE3), LB medium, 37 °C, OD_{600}=0.8 and 0.4 mM IPTG.

Functional, renatured enzyme was obtained after its purification by IMAC in denaturing conditions and further gelfiltration and dialysis. SDS-PAGE of the purification fractions by IMAC and gelfiltration are shown in Figure 9 (A, B).

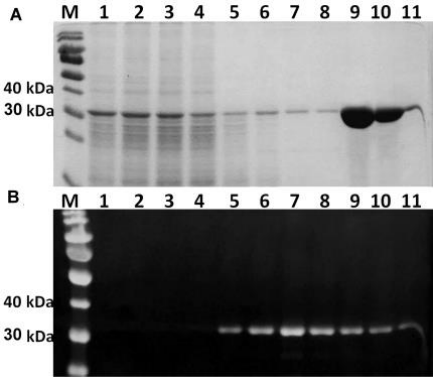

Fig. 9. SDS-PAGE of IMAC (A) and gelfiltration fractions (B). (A) 1–2 fractions – unbound protein; 3–6: wash fractions; 7–11: elution fractions. (B) 1–4: unbound protein, 5–11: elution fractions of EstAG1. M in both, (A) and (B), designates protein molecular weight marker.

Enzyme was purified sufficiently for the further biochemical characterization.

7. Characterization of EstAG1

First, EstAG1 performance was optimized considering mutual effect of two variables: temperature and pH. For the optimization, RSM central compositional experimental plan was selected and established statistical model was designed to evaluate mutual influence of the selected variables. According to the obtained F- and p-values, the model was determined to be statistically significant for describing the relationship between the independent variables (temperature and pH) and response. It was determined that chosen variables had a considerable influence on the activity of EstAG1. Optimal temperature and pH for the optimal activity of EstAG1 were determined to be 47.5 °C and 7.5, respectively. Response surface plot of the optimization is shown in Figure 10.

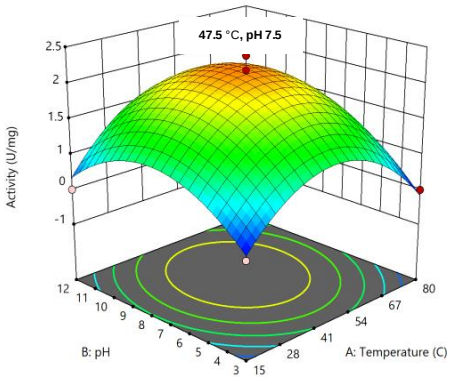

Fig. 10. Response surface plot describing mutual effect of temperature and pH on optimal EstAG1 activity. Results of optimization: 47.5 °C, pH 7.5. Substrate used: *p*-NPC, activity 2 U/mg.

When exposed to different temperatures for time period of 240 min, EstAG1, as determined by measurements of residual activity every 60 min, was sensitive to incubations at elevated temperatures. Thus, at 50–55 °C EstAG1 activity dropped dramatically during the first 60 min of incubation, with enzyme retaining only ~10 % of its activity for the subsequent 3 h of incubation. However, at 25 °C, EstAG1 did not lose activity and maintained it over the incubation period.

In order to investigate EstAG1 lipolytic activity, substrate specificity using various *p*-NP esters with acyl chain lengths from $C_{2:0}$ to $C_{18:0}$, catalytic constants of EstAG1-mediated hydrolysis and regiospecificity were determined. As it is shown in Figure 11 (A),

EstAG1 exhibited activity towards quite wide range of substrates (from $C_{2:0}$ to $C_{18:0}$) with the highest activity towards *p*-NPC ($C_{8:0}$). Evaluation of EstAG1 activity towards tributyrin and olive oil, showed that enzyme catalyzed the hydrolysis of tributyrin, but was not active towards olive oil. Thus, EstAG1 was assigned as carboxylesterase (EC 3.1.1.1).

Based on TLC analysis of products, in comparison with *sn*-1,3-regiospecific Lipolase 100 L that releases 1,2(2,3)-diacylglicerols (DAGs), EstAG1 had no regiospecificity for the investigated substrate: it was able to hydrolyze the three positions (*sn*-1,2 and 3) on the triacylglycerol backbone (Figure 11, B), which is rather common phenomenon among lipolytic enzymes, although some staphylococcal pre-pro-peptide structure lipases are known to be *sn*-1,3-regiospecific[37].

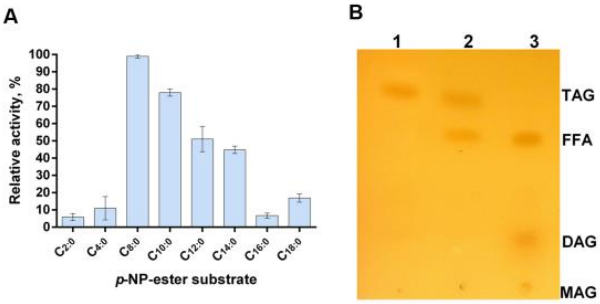

Fig. 11. Substrate specificity (A) and (B) TLC of 1,3-dipalmitoyl-2-oleoylglycerol (1,3-DP-2-OG) hydrolysis products: 1 – 1,3-DP-2-OG unaffected by enzymes (control); 2 – 1,3-DP-2-OG hydrolysis products by EstAG1, 3– 1,3-DP-2-OG hydrolysis products by Lipolase 100 L. TAG – triacylglycerols, FFA-free fatty acids, DAG – diacylglycerols, MAG – monoacylglycerols.

Michaelis-Menten kinetics were observed for the hydrolysis of the substrate that was rapidly hydrolyzed by EstAG1. The K_M values were determined by analyzing the slope of the Michaelis–Menten equation using GraphPad software, which revealed a linear response over the concentration range of the substrate. In accordance with the initial substrate profiling study described above, EstAG1 exhibited

high catalytic rates (k_{cat}) and catalytic efficiency (k_{cat}/K_M) for the middle chain fatty acid ester *p*-NPC. It was found that V_{max}, K_M, k_{cat} and k_{cat}/K_M values for this substrate were 2.068 ± 0.64 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, $2.054 \times 10^{-10} \pm 0.056$ μM, 1.969×10^3 s^{-1} and 9.586×1012 s^{-1} μM^{-1}, respectively.

Study of the effect of different metal ions, inhibitors, detergents and organic solvents revealed that EstAG1 is hyperactivated by some divalent metal ions (Mg^{2+}, Ba^{2+}) and is not inhibited by EDTA. The latter findings could preliminary indicate that EstAG1 is a metal activated enzyme. Although EstAG1 activity was inhibited by all tested detergents (with the exception of CTAB), enzyme exhibited high tolerance towards different organic solvents (ethanol, dioxane, DMSO and acetone). Pefabloc-SC, a sulfonylating agent that acts by reacting with the hydroxyl group of the active site Ser residue, inhibited EstAG1 (residual activity of 26 ± 2.2 %), indicating that the catalysis of EstAG1 is mediated by Ser residue.

8. Identification of active site amino acid residues related to the catalytic function of EstAG1

For the identification of EstAG1 amino acid residues essential to the catalytic function of EstAG1, Ser-179, Glu-246, His-276 and Gly-151, Thr-152 amino acid residues of the GDGTG[149-153] pentapeptide were chosen as targets for the whole plasmid (pET-26b(+)_*estAG1*) site directed mutagenesis. Introduced mutations were confirmed by sequencing. Mutant EstAG1 enzymes were expressed in *E. coli* BL21 (DE3) following optimal conditions as it was determined for the wild type (*wt*) / native EstAG1. Mutant enzymes were purified as it was indicated for the *wt*EstAG1.

Experiments confirmed that the mutation of Ser-179 by Ala / Gly amino acids completely inactivated the enzyme. Mutations of Glu-246 and His-276 have also significantly abolished the activity of EstAG1. These results indicate that each of three amino acids is crucial to the lipolytic activity of EstAG1 and the catalytic triad of

the enzyme consists of Ser-179 / His-276 / Glu-246. Therefore, it can be also stated that catalytic EstAG1 Ser-179 is located in an unusual SPLL[179-182] amino acid motive that was not previously described as a site of the location of catalytic Ser (Table 8).

Table 8. Specific activities of mutant EstAG1 carboxylesterases by each substitution of amino acid residues.

Carboxylesterase	Relative activity, %
*wt*EstAG1	100
EstAG1_S179G	**0**
EstAG1_G151S	80 ± 5.9
EstAG_T152A	56,76 ± 1.7
EstAG1_S179A	**0**
EstAG1_E246A	6,45 ± 2.4
EstAG1_H279A	23,15 ± 1.42
EstAG1_S179A:E246A:H279A	0

EstAG1 having Gly151Ser mutation, which was introduced into *estAG1* in order to restore the classical GXSXG pentapeptide, did not exhibit increased activity. The activity of Gly151Ser mutant did not drop significantly as well, indicating that Gly-151 is an amino acid residue that is not essential for the catalysis. It is known that mutations of amino acids which compose the oxyanion hole of the enzyme, lead to almost complete/complete activity abolishment[42], however EstAG1 with Thr152Ala mutation led to drop of the activity to 56,76 ± 1,7 %, indicating that the Thr-152 function can be partially substituted by Ala.

9. Phylogenetic analysis of EstAG1

Phylogenetic analysis revealed that EstAG1 and closest homologues form a deep branch (separate clade) (Figure 12). GDGTG pentapeptide-having EstAG1 and together clustered homologous sequences were rather evolutionary distant from IV / bHSL family of bacterial lipolytic enzymes. All of the sequences that clustered with EstAG1 were of bacterial origin belonging to the

genus *Staphylococcus* and have no assigned functions, and corresponded to either predicted uncharacterized α/β-hydrolases, or sequences with putative carboxylesterase/ lipase activity.

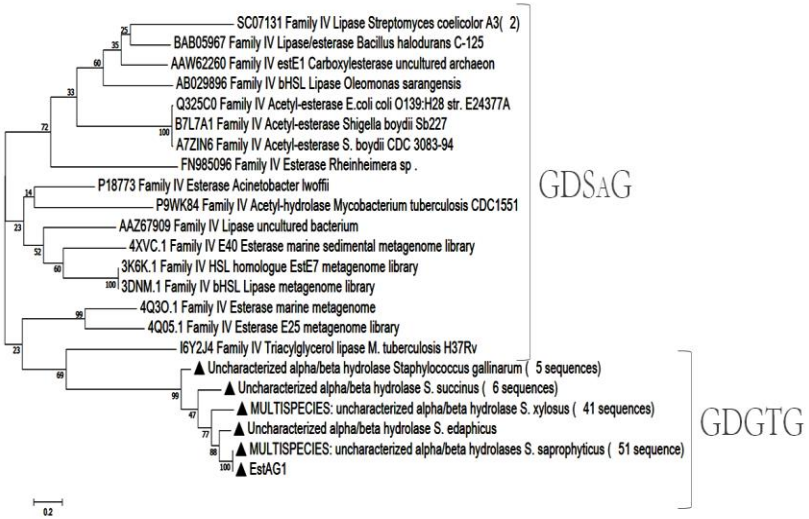

Fig. 12. Unrooted Maximum-Likelihood tree of EstAG1 and its close homologues with the esterase family IV. The tree with the highest log likelihood (−4100.42) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Black triangles (▲) denote the clade of enzymes consisting of EstAG1 and uncharacterized EstAG1 homologues which are proposed to be assigned to a new XXXVI family of bacterial lipolytic enzymes.

Low sequence identities of EstAG1, unusual GDGTG pentapeptide, catalytic Ser located in previously uncharacterized SPLL motive and phylogenetic analysis results, altogether indicate that EstAG1 represents a bacterial lipolytic enzyme that, according to the latest classification, must be assigned to a new XXXVIth family of bacterial lipolytic enzymes.

10. *estAG1* transcript detection in *S. saprophyticus* AG1

Transcription level expression study of *estAG1* gene in *S saprophyticus* AG1 revealed that the *estAG1* naturally exists on the transcript level. *estAG1* gene transcript was detected by RT-PCR using total RNA samples which were purified from bacterial biomass samples harvested from CASO and LB media, from different growth phases of the investigated bacteria (Figure 13).

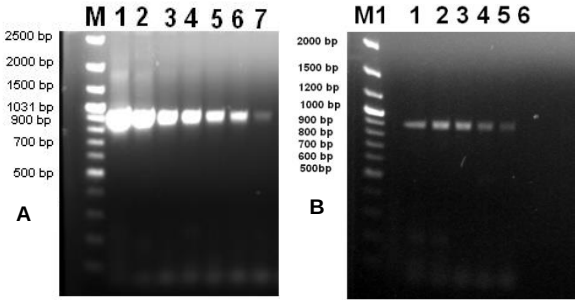

Fig. 13. DNA electrophoresis depicting *estGA1* gene fragment (903 bp) amplified by RT-PCR using total RNA samples harvested from biomass of *S. saprophyticus* AG1 (A): 1- 6 h; 2- 10 h; 3- 12 h (early, middle, late exponential phases, respectively); 4- 22 h; 5- 32 h; 6- 42 h (stationary phase), 7- 48 h (death phase) of growth in CASO medium (B): 1- 6 h; 2- 8 h; 3- 10 h (early, middle, late exponential phases, respectively) 4- 14 h; 5- 20 h (stationary) 6- 26 h (death phase) of growth in LB medium M, M1 –MassRuler DNA Ladder Mix and GeneRuler DNA Ladder Mix, respectively.

Unfortunately, the nature of the transcription, whether it is inducible or constitutive, could not be determined since *S. saprophyticus* AG1 did not grow in minimal or other medium enriched with tributyrin.

CONCLUSIONS

1. Genome-mining of identified lipolytic bacterial strains *Bacillus* sp. L1 and *Staphylococcus saprophyticus* AG1 enabled the discovery of putative, uncharacterized lipolytic enzymes coding genes, two of which – *lipBST* and *estAG1* were subjected to heterologous production and comprehensive characterization.

2. LipBST – is a mesophilic metal cofactor-independent lipase (EC 3.1.1.3) exhibiting high activity towards a wide range of synthetic and natural fatty substrates and is promising for applications in biocatalysis of hydrolysis processes important in biotechnology.

3. Adsorption-immobilization of LipBST allowed to improve enzyme's optimal temperature, pH parameters and thermostability; immobilized LipBST preparation was also preliminary evaluated as suitable for the catalysis of transesterification reactions in anhydrous media.

4. EstAG1 - is a thermolabile, mesophilic, Mg^{2+} ion activated and organic-solvent tolerant lipolytic enzyme which according to its substrate hydrolysis specificities can be classified as carboxylesterase (true esterase, EC 3.1.1.1).

5. EstAG1 – a novel, unique bacterial lipolytic enzyme having no close described homologues and is characterized by an unconventional GDGTG conservative pentapeptide and catalytic Ser residue located in a sequence previously unspecified for bacterial lipolytic enzymes.

6. Based on the low EstAG1 amino acid sequence identities, unique amino acid sequence peculiarities and phylogenetic analysis results, enzyme belongs to a new XXXVIth family of bacterial lipolytic enzymes.

7. Detection of *estAG1* gene expression in native *S. saprophyticus* AG1 cells allowed confirmation of *estAG1* existence at the transcription level.

LIST OF PUBLICATIONS

Dissertation is based on the following original publications:

1. **Gricajeva A**., Bendikienė V., Kalėdienė L. Lipase of *Bacillus stratosphericus* L1: cloning, expression and characterization. *International Journal of Biological Macromolecules*. 92 (2016) 96-104.
2. **Gricajeva A**., Bikutė I., Kalėdienė L. Atypical organic solvent tolerant bacterial hormone sensitive lipase-like homologue EstAG1 from *Staphylococcus saprophyticus* AG1: synthesis and characterization. *International Journal of Biological Macromolecules.* 130 (2019) 253-265.

Other publications:

1. Gudiukaitė R., **Gricajeva A**. Microbial lipolytic fusion enzymes: current state and future perspectives. *World Journal of Microbiology and Biotechnology*. 33(12) (2017) DOI: 10.1007/s11274-017-2381-8.
2. **Gricajeva A**., Kazlauskas S., Kalėdienė L., Bendikienė V. Analysis of *Aspergillus* sp. lipase immobilization for the application in organic synthesis. *International Journal of Biological Macromolecules*. 108 (2018) 1165-1175.

Conference presentations:

1. Gricajeva A., Kalėdienė L. Biochemical characterization of novel *Bacillus stratosphericus* L1 lipase, *Vita Scientia*, Vilnius, Lithuania, 2016.01.04.
2. Gricajeva A., Kalėdienė L., Lipase-secreting *Bacillus* species: from soil bacteria to promising strains for a variety of applications in biotechnology, *European Biotechnology Congress 2016*, Riga, Latvia, 2016.05.05-2016.05.07.

3. Gricajeva A., Kareivaitė G., Kalėdienė L. GTC1 lipase of the urinary tract infections-causing *Staphylococcus saprophyticus*, *3rd Congress of Baltic Microbiologists*, Vilnius, Lithuania, 2016.10.18-2016.10.21.
4. Gricajeva A., Kalėdienė L. Modulation and Analysis of the Activity of *Staphylococcus saprophyticus* GTC1 Lipolytic Enzymes, *7th Congress of European Microbiologists (FEMS 2017)*, Valencia, Spain, 2017.07.09-2017.07.13.
5. Gricajeva A., Gudiukaitė R., Malūnavičius V., Kalėdienė L. Biochemical analysis of *Staphylococcus saprophyticus* GTC1 natively secreted lipase. *46 th World congress on Microbiology*, Dublin, Ireland, 2017.09.18-2017.09.19.
6. Gricajeva A., Kalėdienė L.Biochemical characterization of novel *Staphylococcus saprophyticus* AG1 esterase belonging to prokaryotic hormone sensitive lipase-like enzyme family, *4th Congress of Baltic Microbiologists 2018 (CBM 2018)*, Gdansk, Poland, 2018.09.10-2018.09.12.

Other conference presentations:

1. Gricajeva A., Kazlauskas S., Kalėdienė L., Bendikienė V. Analysis of immobilized *Aspergillus* sp. lipase for the application in organic synthesis, *12th International Conference on Protein Stabilization (ProtStab2018)*, Vilnius, Lithuania, 2018.05.16-2018.05.18.
2. Malunavicius V., Gricajeva A., Lastauskiene E., Sadauskas M., Gegeckas A., Gudiukaite R. Lipolytic enzymes of *Geobacillus* sp. 95: potential for industrial application. *46 th World congress on Microbiology*, Dublin, Ireland, 2017.09.18-2017.09.19.

CURRICULUM VITAE

Name, surname	**ALISA GRICAJEVA**
Date of birth	1989-07-18
Work address	Institute of Biosciences, Life Sciences Center, Vilnius university, Saulėtekio av. 7, LT-10257 Vilnius, Lithuania
E-mail	alisa.gricajeva@gmc.vu.lt

EDUCATION

Institutions	Degrees and studies	Year
Vilnius university	PhD in Biology	2014-2018
Vilnius university	M.Sc in Microbiology	2012-2014
Vilnius university	B.Sc in Biology	2008-2012

WORK EXPERIENCE

Workplace	Position	Period(s)
Individual activity under a business certificate	Lecturer and supervisor at the educations of microbiology/ molecular biology for pupils	Since 2018 09
Vilnius university	Junior Assistant (Enzymology lectures and seminars; Food Microbiology and Biotechnology lectures and seminars	Since 2016 09
	Laboratory exercises in Biotechnology of Prokaryotic and Eukaryotic Cells	2014 – 2016
Vilnius university	Junior Research Associate	Since 2016 09
Vilnius university	Junior Research Associate	2012 09 - 2014 06
Vilnius university	Specialist	2013 05 – 2015 03
PE STEP	Scientific supervisor at Mobile Scientific Laboratory (*lt. abbr.*, MoMoLab)	Since 2014 09
Vilnius university	Lab. assistant	2012 09 – 2015 09

PROJECTS	
Duration	Name of the project and position
2019 07 – 2019 08	European social fund under the No 09.3.3-LMT-K-712 "Development of Competences of Scientists, other Researchers and Students through Practical Research Activities" measure, Grant No. 09.3.3.-LMT-K-712-15-0048, Project "Screening of new producents of esters hydrolyzing biocatalysts", **Head of project**
2014 09 – 2015 06	Guidance of pupils' scientific studies and conduction of scientific lectures, workshops, experiments in scientific mobile laboratory visits to schools of a state project: "Disclosure and Education System Development for Pupils as Young Researchers – Stage II" Nr. VP1-3.2-ŠMM-02-V-03-001, **Scientific supervisor**
2013 05 – 2015 03	High-level international research promotion "Selection and development of the biocatalysts for biogas production and utilization of biomass conversion processes control - BIOKONVERSA", Nr. VPI-3.1-MES-10-V, **Specialist**
2012 09 – 2014 06	National integrated program project "Biotechnology and Biopharmacy: Fundamental and Applied Research", Nr. VP1-3.1-ŠMM-08-K-01-005, **Junior Research Associate**

Scholarships, grants and other important achievements	
Activity	Year
FEMS (Federation of European Microbiological Societies) grant for participation in scientific conference	2018
Merit-based scholarship (Research Council of Lithuania)	2018
Merit-based scholarship (Vilnius university)	2018
Nominal K. Jankevičius scholarship (established by JSC "Grunto valymo technologijos" / "Soil remediation technology") award for the best defended Master thesis „Purification and determination of physical and chemical properties of *Bacillus* sp. recombinant lipase"	2014

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Vilniaus universiteto leidykla
Saulėtekio al. 9, LT-10222 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 33 egz.