

Yeast *Kluyveromyces lactis* as host for expression of the bacterial lipase: cloning and adaptation of the new lipase gene from *Serratia* sp.

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Abstract Many microbial lipases have been successfully expressed in yeasts, but not in industrially attractive *Kluyveromyces lactis*, which among other benefits can be cultivated on a medium supplemented with whey—cheap and easily available industrial waste. A new bacterial lipase from *Serratia* sp. was isolated and for the first time expressed into the yeast *Kluyveromyces lactis* by heterologous protein expression system based on a strong promoter of *Kluyveromyces marxianus* triosephosphate isomerase gene and signal peptide of *Kluyveromyces marxianus* endopolygalacturonase gene. In addition, the bacterial lipase gene was synthesized de novo by taking into account a codon usage bias optimal for *K. lactis* and was expressed into the yeast *K. lactis* also. Both resulting strains were characterized by high output level of the target protein secreted extracellularly. Secreted lipases were characterized for activity and stability.

Keywords Lipase · *Serratia* sp. · de novo synthesis · *Kluyveromyces lactis* · Whey

Introduction

Lipases are very versatile enzymes for application in various fields of industry: detergent, food and flavor, biocatalytic resolution of pharmaceuticals, fine chemicals, agrochemicals, biosensors, bioremediation, cosmetics and perfumery. Therefore, lipases are receiving much attention alongside with rapid development of enzyme technology. Lipases are produced by animals, plants and microorganisms. Microbial enzymes are often more useful than enzymes derived from plants or animals, because of a great variety of catalytic activities available, absence of seasonal fluctuations, rapid growth of microorganisms on inexpensive media and ease of genetic manipulations [12, 30]. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors [10].

The expression of functional proteins in heterologous hosts is a cornerstone of modern biotechnology and biocatalysis [11]. Yeast species like *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and others have been popular industrial hosts for recombinant protein production, because they combine the advantages of unicellular organisms (i.e., ease of genetic manipulations and rapid growth) with the ability to perform eukaryotic post-translational modifications [35]. Unlike more complex eukaryotic organisms, yeast expression systems are economically favorable, show high protein concentrations and do not contain pathogens, viral inclusions or pyrogens [6]. The yeasts are also considered to be easy to handle and grow, in comparison to bacteria, which makes them an attractive tool for expression of heterologous lipases [6]. The expression of various lipases, including those of bacterial origin, has been successfully performed in a series of yeast strains

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[15, 20]. However, the promising yeast host *Kluyveromyces lactis* has only been studied for expression of bacterial esterase gene, so far [25]. This prompted us to accomplish the expression of the true microbial lipase in this particular yeast.

Kluyveromyces lactis has been used in the food industry for the production of lactase since 1950s and for heterologous expression of bovine chymosin. A number of merits have contributed to the popularity of *K. lactis* for recombinant protein production: conventional genetic manipulations; the ability to use episomal as well as integrative expression vectors; availability of a fully sequenced genome; controlled recombinant protein glycosylation; the ability to utilize cheap substrates like lactose or whey. It is approved as a GRAS strain [7, 23]. By this study, we were aiming to express a new bacterial lipase from *Serratia* sp. into the heterologous protein expression system of the yeast *K. lactis*, which can be cultivated on the medium supplemented with a waste product of the food industry, cheap and easily available whey. We wanted to accomplish a comparative analysis of the lipase production capacity by applying two possibilities: by expression of the bacterial lipase gene composed of native codons and expression of de novo synthesized lipase gene which possesses codons adapted to *K. lactis* yeast.

Materials and methods

E. coli K-12 DH10B was used as donor for *Serratia* sp. lipase cloning. The plasmids pUC19, pLATE11, pJET1.2 (Thermo Fisher Scientific Baltics) and pKDU8R [3] (Vilnius University, LT) and yeast *Kluyveromyces lactis* MD2/1 *lysA argA ura3* strain (obtained from Dr M. Bianchi) were used for *Serratia* sp. lipase gene expression. Luria Broth (LB) was purchased from Fisher BioReagents. Buffer salts, tributyrin and Rhodamine B were purchased from Acros Organics. The origin of other components is indicated nearby.

Lipase from *Serratia* sp. purification

Serratia sp., isolated from soil, was kindly provided by JSC Biocentras (Cat. N° B-91–7A; www.biocentras.lt) for investigation. Cells were decanted from supernatant by centrifugation for 20 min at 14,000 rpm and 4 °C temperature. The purification procedure was adopted from Gupta et al. [9]. Before adsorption, Accurel-MP-1000 (Membrana GmbH) was wetted with ethanol in a proportion of 200 mg Accurel-MP-1000 with 2 ml ethanol. To this, 17 ml dH₂O and 3 ml 50 mM phosphate buffer (pH 7.5) were poured. Then, the supernatant was added at a proportion of 1 g of sorbent (dry, before wetting) per 20 mg of protein. The

suspension was shaken at 400 rpm and 25 °C for 12 h for adsorption to occur.

Afterward, the support was filtered off and washed twice with 50 mM phosphate buffer (pH 7.0) to remove unbound protein. The filtrate was assayed for lipase activity and protein content. The lipase-loaded Accurel was added to 20 ml of a 0.2 % Triton X-100 (Sigma-Aldrich) solution in 50 mM phosphate buffer (pH 7.5) and shaken at 400 rpm speed at 25 °C temperature. After 3.5 h, the support was filtered out and the filtrate was tested for lipase activity and protein content. 50 mM phosphate buffer (pH 7.0) was used for 25 ml volume gel-filtration Sephacryl S-200 column at 1 ml/min rate. Impurities of other proteins and remaining detergent (Triton) were removed during this purification step. Fractions containing pure lipase, based on activity and SDS-PAGE results, were pooled and concentrated using ultrafiltration with 30 kDa cutoff (Millipore). Lipase activity test was performed as described in [33]. 1 U is defined as 1 nmol of *p*-nitrophenol ($\lambda = 400$ nm) formed per 1 min at 25 °C temperature and pH = 8.0. Protein concentration was measured by using standard BCA™ (bicinchoninic acid) protein assay kit (ThermoFisher Scientific). SDS-PAGE gel electrophoresis was performed, according to the methods of Laemmli [17].

N-terminal sequencing of purified lipase from *Serratia* sp.

The N-terminal of the purified lipase was sequenced using 49X PROCISE Protein Sequencing Systems.

Cloning and monitoring of lipase expression

The lipase gene was cloned from *Serratia* sp., isolated from soil. All PCR primers and plasmids used in this study are described in Table 2. Recombinant DNA techniques were performed according to standard procedures [27]. All enzymes for the recombinant DNA experiments were obtained from the Thermo Fisher Scientific Baltics and used according to the manufacturer's recommendations. For the routine PCR analysis, DreamTaq DNA Polymerase (Thermo Fisher Scientific Baltics) was used according to the manufacturer's recommendations. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Baltics) was used for the precise DNA fragments amplification. Generated by PCR, DNA amplicons were cloned into pJET1.2 vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific Baltics). The DNA library of *Serratia* sp., created by partial hydrolysis of chromosome DNA by Bsp143I and ligated to vector pUC19, was used for the screening by PCR with pairs of primers, mentioned in Table 2. The plasmid pLATE11 with *Serratia* sp. lipase gene was transformed into *E. coli* BL21 (DE3) for lipase

expression. Recombinant bacteria clones were screened on solid LB medium containing 2.5 % rapeseed oil, 3 % Rhodamine B and 0.1 mM IPTG at 37 °C. The plasmids pKDU8RmG1 with native *Serratia* sp. lipase gene and pKDU8RsG1, containing synthetic lipase gene with codons adapted to *K. lactis*, were transformed into *Kluyveromyces lactis* MD2/1 *lysA argA ura3* for mG1 and sG1 lipase expression. Yeast clones were screened on solid YNB medium containing: 0.7 % YNB (with ammonium sulfate, Amresco), 2 % D (+) glucose (AppliChem), 2 % tryptone (Fisher Chemical) 2 % agar, 2 % rapeseed oil and 3 % rhodamine B at 30 °C. The results indicated the production of extracellular lipase.

Cultivation of mG1 and sG1 lipases

Initially, before each experiment, mG1 and sG1 (*Kl.lactis* with lipase genes) were subcultured onto agar medium consisting of 0.7 % of YBN with ammonium sulfate (without amino acids), 2 % D (+) glucose (Acros Organics), 2 % casamino acids (Amresco) and 2 % agar–agar (purchased from Merck). The set of optimal growth medium was prepared in a 100 ml volume Erlenmeyer flasks containing 30 ml of medium consisting of 2 % D (+) glucose (purchased from Acros Organics), 10 % dried whey (purchased from JSC Rokiskio suris), 1 % sunflower oil and 100 mM concentration of CaCl₂ in 100 % YNB. The final pH of the medium was 6.2. The medium, omitting CaCl₂ and sunflower oil, was autoclaved at 0.8 atm for 30 min. YNB was prepared as described in [33]. CaCl₂ solution was autoclaved separately at 1 atm for 20 min. Sunflower oil was added when the rest of the medium was autoclaved separately. Cultures were incubated at 30 °C in a rotary shaker at 200 rpm.

Effect of temperature on activity and stability

A thermostatically controlled spectrophotometer Spekol 2000 (Analytic Jena) was used to determine the temperature optimum for maximal activity for both lipases (mG1 and sG1). The enzyme assay was performed at pH 8.0 and incubating at a temperature range of 10–45 °C, gradually increasing in steps by 5 °C degrees. Thermostability was analyzed in triplicate by measuring the relative activity after incubating the enzymes for 30 min in a temperature range of 10–45 °C in the absence of substrate. Afterward, the samples were assayed for lipolytic activity by the p-NPP standard method, at 25 °C and pH 8.0.

Effect of pH on stability

Stability assays were done by adjusting the pH value of 200 µl of enzyme solution to a certain pH value, from

pH 6.0 to 9.5. The solutions were incubated at 25 °C for 10 min. Afterward, the pH values of solutions were standardized to pH 8.0 and lipase activity for each sample was measured by the p-NPP method.

Effect of organic media on stability

A number of organic solvents were chosen for the experiment: ethanol, acetone, methanol, 2-ethyl-1-hexanol, diethyl ether, tert-butanol, oleic acid and 2-butanol. Each solvent was prepared at a concentration of 50 % in 0.05 M phosphate buffer (pH 7.0). Enzyme and solvent solutions were mixed at a 1:1 ratio. A mixture of enzyme with buffer was prepared as a control reaction. The resulting mixtures were incubated for 30 min in a rotary shaker at 200 rpm at 30 °C temperature. After incubation, the pH value of the samples was standardized to pH 8.0 and samples were assayed for lipolytic activity by the p-NPP standard method, at 25 °C and pH 8.0. In addition, the performed control experiments for a solvent effect on enzyme activity assay were negative.

Effect of various compounds on enzyme stability

For the study, the following compounds were used: EDTA, CaCl₂, ZnCl₂, PMSF, Triton X-100, Tween 80, MgCl₂·6H₂O and CoCl₂·6H₂O. Each compound was prepared of 2 mM and 5 mM concentrations in 50 mM phosphate buffer (pH 7.0). A mixture of enzyme with buffer was prepared as a control reaction. Enzyme and solvents solutions were mixed at a 1:1 ratio. The resulting mixtures were incubated for 60 min in a rotary shaker at 200 rpm at 25 °C temperature. After incubation, the pH value of samples was standardized to pH 8.0 and samples were assayed for lipolytic activity by the p-NPP standard method, at 25 °C. In addition, the results of the performed control experiments for a compound effect on activity assay were negative.

Results and discussion

Purification of lipase from *Serratia* sp.

Cultivated culture of *Serratia* sp. was obtained from JSC Biocentras. Initially, the traditional multi-step chromatographic purification scheme, consisting of several ion exchanges and gel-filtration columns, was applied; however, the amount of purified enzyme was too low for further experiments. Therefore, atypical purification scheme was employed which was adopted from Gupta et al. [9]. The method is based on a single-step purification, the principles of which are adsorption and desorption of lipase using Accurel—a hydrophobic,

macroporous polypropylene matrix with a large surface area for absorption. It is inexpensive, rapid, high yielding and amenable to large-scale operations. The purification using matrices is a very easy and convenient method. Literature suggests that the use of a one purification step was enough to get pure protein, for example for lipase from *Burkholderia multivorans*, *Mucor meihei*, etc. [9]. However, the applied single-step purification was not enough for our lipase from *Serratia* sp. After desorption from the polypropylene matrix, impurities were still present (Fig. 1). Additional protein polishing by the use of gel-filtration column Sephacryl S-200 led to a purified enzyme with one band on SDS-PAGE corresponding to 66.2 kDa. Lipase from *Serratia* sp. was purified with 6.93-fold and 1.28 % purification yield. The summarized purification data are presented in Table 1. We also have tested other eluents for lipase desorption from Accurel-MP-1000, such as 1 % Tween 80, 1 % Tween 21, 1 M NaCl solution, 40 % $(\text{NH}_4)_2\text{SO}_4$, and 1 and 5 % of Triton X-100. However, the obtained results were not more efficient than those when 0.2 % Triton X-100 solution was used. Thus, the constituted lipase purification scheme using single-step purification enabled to reduce costs and consumption of reagents and energy, which are needed for purification procedures in a large scale.

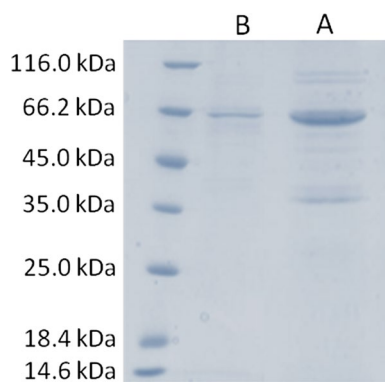


Fig. 1 SDS-PAGE of extracellular lipase from *Serratia* sp. Lane a, after desorption from Accurel-MP-1000; lane b, after Sephacryl S-200

Cloning of *Serratia* sp. lipase gene

For identification and subsequent cloning of *Serratia* sp. lipase, gene functional analysis of the *Serratia* genome DNA library in *E. coli* was performed. In parallel, the lipase gene sequence identification and cloning was implemented based on data obtained through protein N-end sequencing. *Serratia* sp. DNA was partially digested with Bsp143I and fragments ranging from 3 to 15 kb were ligated into a BamHI-linearized pUC19 vector. The DNA library was transformed into *E. coli* DH10B. In vivo screening for lipolytic activity was performed by the expression of clones onto LB agar medium containing tributyrin or Rhodamine–olive oil agar medium [5]. Unfortunately, even statistically reliable amount of clones (average size of heterologous DNA fragments was 5.000 bp in approx. 5.000 recombinant clones [19, 21]) did not reveal an appropriate clone. Subsequent results confirmed intracellular accumulation of recombinant *Serratia* lipase protein into exploited *E. coli* host strains. The N-terminal sequence of *Serratia* sp. lipase protein has been determined by Edman degradation method. The obtained sequence possesses 15 amino acid residues. A BLAST search against the NCBI non-redundant protein database [1] was performed and sequences with similar motifs in N-terminal regions were selected. Reported sequences are classified as lipases and belong mainly to bacteria of genus *Serratia*. The selected sequences were aligned by protein alignment tool COBALT (NCBI) [24] which revealed a number of well-conserved amino acid blocks. The degenerated DNA primers were designed in consonance with selected blocks and N-terminal sequence, using codon usage bias appropriate for *Serratia marcescens* (Table 2: Primers P294 and P296). The DNA amplicon of the anticipated length was obtained by PCR with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Baltics) from genomic DNA of *Serratia* sp. The obtained fragment was cloned into pJET1.2 vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific Baltics) and sequenced. A BLAST analysis showed that the obtained sequence was highly similar to known *Serratia* lipases. To define flanking sequences, a type of anchored-PCR—RAGE (rapid amplification of gene inserts)—with DNA primers specific for expected and vector sequences was performed [14]. A DNA library of

Table 1 Purification of lipase from *Serratia* sp.

Purification step	Volume (ml)	Protein (mg)	Activity (U ^a)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	450	81	575730	7107.8	1	100
Desorption from Accurel-MP-1000	60	5	48780	9756.0	1.37	8.47
Sephacryl S-200	0.2	0.15	7390	49266.7	6.93	1.28

Values shown are the average from five independent cultures and the percentage error in each set of reading was within 3 %

^a 1 U (unit) is defined as 1 nmol of *p*-nitrophenol ($\lambda = 400$ nm) formed per 1 min at 25 °C temperature and pH = 8.0

Table 2 Primers used for cloning

The primers
P294-5'-d(GCGATCTTCAACTACCAGGGCCT)-3'
P296-5'-d(ACCGGRTCGTTYTCGTAGCC)-3'
P301-5'-d(AGTCAGGGTTCAGGGAATAC)-3'
P307-5'-d(TATGTCGCCTTCGCTTCTCCTACCC)-3'
N-end-5'-d(ATGGGAATCTTCAATTACCAAGG)-3'
C-end-5'-d(TTACGCCAGCACCACTGG)-3'
M13/pUCdir-5'-d(GCCAGGGTTTTCCAGTCACGA)-3'
M13/pUCrev-5'-d(GAGCGGATAACAATTTACACAGG)-3'

Serratia sp., created by partial hydrolysis of genome DNA by Bsp143I and ligated to vector pUC19, was used for the screening by PCR with different pairs of primers, which included one of standard M13pUC primers and one of the specific, prepared, based on previously obtained sequence. (Table 2: P301 with M13/pUC reverse sequencing primer or P301 with M13/pUC sequencing primer, P307 with M13/pUC reverse sequencing primer or P307 with M13/pUC sequencing primer). PCR amplicons whose length corresponded to theoretically expected—not less than about 226 nucleotides long—were selected for ATG direction, and not shorter than about 1849 nucleotides for transcription termination direction. We managed to get a number of abundant DNA amplicons. As was approved by the sequencing, not nearly each of them was composed from quest sequences, but, nevertheless, the “good” DNA amplicons, containing the fragments of lipase, allowed us to get accurate nucleotide sequences of 5 and 3'- ends of the lipase gene. The rest of the sequencing data supported the assumption that the bacteria belongs to genus *Serratia*. New DNA primers, created in conformity with obtained data, have allowed amplification of complete coding sequence of the *Serratia* sp. lipase gene (Table 2: primers used N-end and C-end). The nucleotide sequence of the *Serratia* sp. lipase gene is 1848 nucleotides long and encodes a protein of 614 amino acids with a predicted molecular mass of 64.8 kDa and theoretical *pI* of 4.35. A GenBank accession number for the *Serratia* sp. E13 lipase gene was provided: KJ868240. Considering the sequences of nucleotides and deduced amino acids, the lipase from *Serratia* sp. was assigned as a new lipase. Almost 4 % of amino acids (23 aa of 616 aa) and 8 % of nucleotides (148 nt of 1848 nt) were substituted on the corresponding level in comparison to the well-characterized lipase of *SILipA* of *S. liquefaciens* S33 DB-1 (EF202840) [34]. The novelty of the cloned lipase may be attributed after an extensive study of the enzymological features. The coding sequence of the lipase gene was cloned into a vector pLATE11 (aLICator Ligation Independent Cloning (LIC) and Expression System, Thermo Fisher Scientific Baltics) and transformed into the *E. coli* BL21 (DE3) strain.

Despite the fact that lipase is secreted from the wild strain of *Serratia* and the system pLATE11/*E. coli* BL21 (DE3) provides efficient expression, only intracellular lipase activity was observed in 1-day-old culture. A typical halo around the colonies on solid media containing tributyrin or Rhodamine B began to form slightly only after a few days of incubation. Thus, results confirmed the intracellular accumulation of recombinant *Serratia* lipase protein into exploited *E. coli* host strains. Therefore, to get larger amount of protein secreted into the cultural medium, it was decided to express lipase-encoding gene into the yeast *Kluyveromyces lactis* heterologous protein expression system [2]. The native lipase-coding sequence was placed under the signal sequence of endopolygalacturonase (EPG1) and triosephosphate isomerase (TPI1) promoter of *K. marxianus* in the vector pKDU8R. The final construction was called mG1. Furthermore, the sequence of lipase coding gene was synthesized de novo in the laboratory of GenScript (GenScript USA Inc., USA) by the algorithm OptimumGeneTm, with codon usage bias appropriate for *K. lactis* [gbpln]. Codon usage bias in terms of codon adaptation index (CAI) for *K. lactis* has shifted from 0.48 to 0.87 for the de novo synthesized *Serratia* sp. lipase sequence. The new sequence was placed analogously to the previous into the expression vector pKDU8R. The final construction was called sG1. In both cases, lipase activity was found in the cultural medium and intracellular activity was not detected; thus, the bacterial lipase protein was efficiently secreted out of the yeast cells.

The deduced protein sequence of cloned lipase showed remarkable similarity to lipase *SILipA* of *S. liquefaciens* S33 DB-1 (EF202840) [34], with an identity of 96 % over the entire length of the sequence. The latter was successfully expressed in *Pichia pastoris*. Herewith, deduced amino acid sequence of our lipase is identical to polyurethanase A of *Serratia liquefaciens* (WP_020826684), the sequence of which has appeared in the NCBI database during the preparation of this article, as well as to sequences of lipases from *Serratia proteamaculans* 568 (YP_001478438S), *Serratia* sp. AS12 (YP_004500584), *S. plymuthica* (WP_006324946), *S. plymuthica* 4Rx13 (YP_008138205), polyurethanases A of *Serratia* sp. S4 (WP_017892670), *S. plymuthica* S13 (YP_008159034), *S. liquefaciens* ATCC 27592 (YP_008230139). However, the functions of all enumerated sequences were predicted from high-throughput DNA sequencing data and, to our knowledge, none of them has been cloned yet. Pfam analysis revealed significant matches for lipase active site (137–264 aa) typical for lipase from class 3 and for three hemolysin-type calcium-binding repeats in the carboxy terminal of the predicted protein. This feature allows assigning this protein to the RTX (repeats-in-toxin) protein family [32]. The RTX family proteins are characterized by a unique

mode of secretion via the type I secretion system (TISS) [19].

Cultivation of mG1 and sG1

After introduction of native *Serratia* sp. lipase gene (mG1) and de novo synthesized lipase gene (sG1) into the yeast *K. lactis* MD2/1, optimal conditions for lipase production were estimated. The influence of casamino acids, glucose, lysine, arginine, peptone, tryptone, yeast nitrogen base and ammonium sulfate and their concentrations was tested, although *K. lactis* can utilize lactose and thus grow on whey, the disposal of which is a major problem for the dairy industry. For example 9 kg of whey is produced for every 1 kg of cheese. Moreover, the replacement might reduce the costs, since significant concentrations of yeast extract, peptone or casamino acids which are not only relatively expensive, but also can increase the costs for enzyme recovery after growth in flasks or after fermentation [4]. After screening for the best growth medium composition, it was estimated that the most efficient medium composition consisted of 2 % D (+) glucose, 10 % whey, 1 % sunflower oil and 100 mM of CaCl₂ prepared in 100 % of own prepared YNB [13]. It should be noted that the solution of CaCl₂ must be autoclaved separately. Growth parameters such as optical density, protein concentration and lipase activity were followed over time, cultivating both mG1 and sG1 lipases at the defined optimal conditions (Online Resource: Fig. ESM1, Fig. ESM1, Fig. ESM3). The maximum cell density for both cultures was reached approximately at the same time after 27–29 h (Fig. ESM1); however, the cell density for mG1 was determined to be about 20 % higher than for sG1. Comprehensively, the maximum lipolytic activity for sG1 was determined in 29 h and for mG1 in 31 h of cultivation (Fig. ESM2). The maximum concentration of total protein in cultural medium was determined to be similar for both cultures in the range of 0.12–0.15 mg/ml (Fig. ESM3). Eventually, the calculated specific activity (Fig. 2) indicated that sG1 lipase was produced in lag phase (specific activity of sG1 was higher by almost twice in 3–7 h—about 5500 U/mg), meanwhile mG1 was produced in stationary phase (specific activity of mG1 reached about 3000 U/mg only in 27–32 h of cultivation). It is noteworthy that slightly higher optical density of mG1 cells next to the low specific activity of the lipase and the higher specific activity with a lower optical density of the sG1 show that codon optimization influences the expression of heterologous proteins. The culture of sG1 for the production of lipase is of interest because cultivation of *K. lactis* can be suspended after 4–8 h of growth. The comparison of expression level of our and other lipase genes in various eukaryotic protein expression systems is rather complicated due to diverse presentation of the results. The expression of heterologous

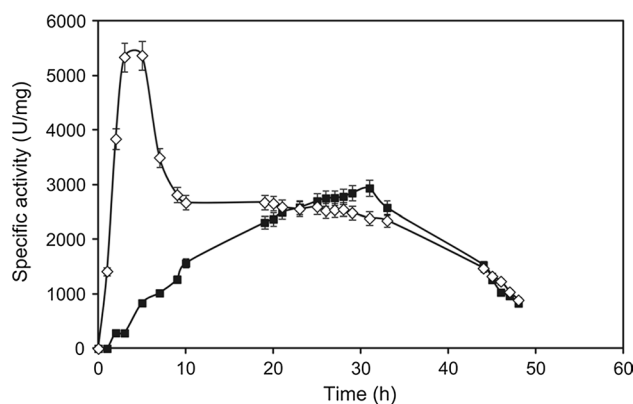


Fig. 2 Extracellular production of mG1 and sG1 lipases over time. mG1 and sG1 were cultivated in a 100 ml volume Erlenmeyer flasks containing 30 ml of medium consisting of 2 % D (+) glucose, 10 % dried whey, 1 % of sunflower oil and 100 mM concentration of CaCl₂ in 100 % YNB. The final pH of the medium was 6.2. Cultures were incubated at 30 °C in a rotary shaker at 200 rpm. Specific activity was calculated and followed up to 48 h of cultivation. Filled square mG1; open rhombus sG1

Rhizopus niveus lipase and *Geotrichum candidum* lipase in *S. cerevisiae* yielded 270 µg/ml and 200 U/ml of lipase, respectively [15]. The expression of lipase from *Candida antarctica*, known as CAL-A lipase, in *P. pastoris* provided 0.1 g/L of lipase [28]. *K.marxianus* produced its own lipase with enzyme activity of 80 U/ml [8] and 0.175 U/ml [29]. The production of *Thermus thermophilus* esterase achieved 600 U/l activity in *K. marxianus* and 3000 U/l activity in *K. lactis* after 36 h of cultivation [25]. 7 mg/l of pure CAL-A lipase of *Candida antarctica* was obtained from *S. cerevisiae*, 13.3 mg/l from *H. polymorpha*, and 237.5 mg/l from *K. lactis* [22]. Notwithstanding, a common method to evaluate different protein production hosts is to compare the expression levels of the desired protein. The expression system explored by us has produced up to 150 g/l of lipase, which was consistent with other studies of microbial- or yeast-originated lipase production in *S. cerevisiae* and *P.pastoris*.

Basic characteristics of mG1 and sG1

Disclosure of basic characteristics of activity and stability of enzymes at various temperature and pH values, in the presence of various organic solvents and metal ions, allows evaluation of enzyme potential to be applied as biocatalyst. The lipolytic activity was followed by using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate; nevertheless, activity determination beyond 50 °C was not performed because inaccuracies emerged by the spontaneous hydrolysis of *p*-NPP. In general, it is reported that microbial lipases are not very stable at temperatures above 40 °C without the addition of stabilizers, although there have been few reports

of moderately thermostable fungal lipases [10, 26]. However, temperature stability study showed that both lipases retained stability at elevated temperatures (Fig. 3). After 30 min incubation at various temperatures, sG1 lipase had maximum relative activity at 25 °C, while at 40 °C and 45 °C there was 47 and 29 % of relative activity, respectively. Conversely, mG1 lipase retained its activity at these temperatures. For example, 85 % of relative activity was obtained after incubation at 35 °C, and 75 and 60 % of relative activity were indicated after incubation at 40 and 45 °C, respectively. Inferior temperatures, from 10 °C up to 25 °C, were more favorable for sG1 lipase.

Extremely high or low pH values generally result in complete loss of activity for most enzymes; pH is also an essential factor in the stability of enzymes. Ordinarily, there is a pH region for enzyme stability, which has to be determined for each enzyme individually. It is known that lipase activity is stable at neutral or slightly basic pH values [18, 31]. Further, it is reported that fungal lipases are, in general, quite stable in the pH range from 4 to 7 and unstable at alkaline pH values [18, 26]. Experiments performed for assessment of enzyme pH stability indicated that the maximum stability of mG1 lipase was obtained at pH 7.5 and of sG1 lipase at pH 7.0 after 10 min incubation at 25 °C (Fig. 4). Both lipases were unstable when incubated at alkaline pH. The overall pH profile comparison of both enzymes demonstrated that mG1 lipase had more enhanced stability in a broader pH range, from 6.0 up to 8.0. A deeper comparison of pH stability among lipases from different species reported in the literature is complicated and not possible because of different methods, substrates and purification of enzymes given in different reports.

The stability in organic solvents is an important characteristic of lipases for their application in biocatalytic reactions. It is necessary to study the influence of organic solvents for lipase stability, which depends on the nature of both enzyme and solvent [16], because it is believed that hydrophilic solvents such as ethers and acetone are usually incompatible with enzyme activity, while water-immiscible solvents, such as alkanes or haloalkanes, retain their catalytic activity as they do not release the bound water from the enzyme surface which is important for enzyme activity and stability. In our study, not only common solvents (Fig. 5), but also 2-ethyl-1-hexanol and oleic acid were assayed for enzyme stability as they are components of interest for performance of 2-ethyl-1-hexyl oleate synthesis [16]. The settled relative activities of mG1 and sG1 lipases confirmed their good stability in water-insoluble oleic acid (Fig. 5). 2-Ethyl-1-hexanol, very little miscible with water solvent, gave compatible results only with sG1 lipase. The increased activity in oleic acid and 2-ethyl-1-hexanol can be explained by the phenomenon known as interfacial activation [5]. One-third of mG1 lipase activity was lost

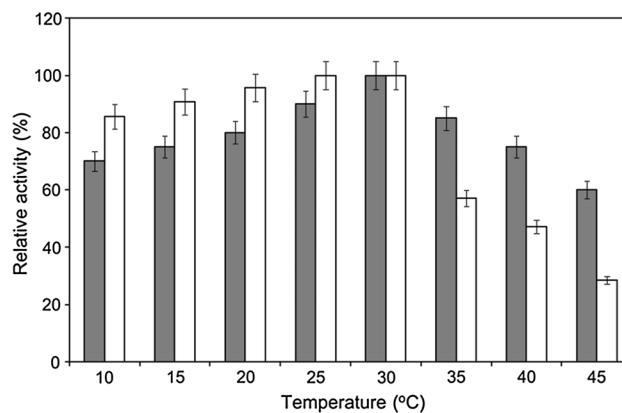


Fig. 3 Effect of temperature on the stability of mG1 and sG1 lipases. Thermostability was analyzed in triplicate by measuring the relative activity after incubating the enzymes for 30 min in a temperature range of 10–45 °C, gradually increasing in steps by 5 °C. Afterward, the samples were assayed for lipolytic activity by the pNPP standard method, at 25 °C and pH 8.0. Filled square mG1, open square sG1

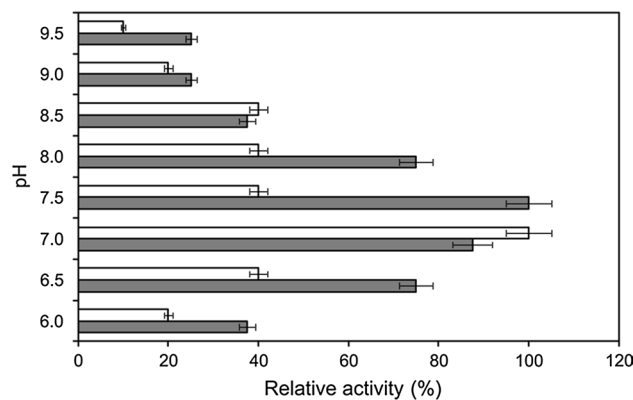


Fig. 4 pH stability of mG1 and sG1 lipases. Stability assays were done by adjusting the pH value of the enzyme solution to a certain pH value, from 6.0 to 9.5. The solutions were incubated at 25 °C for 10 min. Afterward, the pH value of the solution was standardized to pH 8.0 and lipase activity for each sample was measured by the pNPP method. Filled square mG1, open square sG1

in other water-soluble and miscible solvents such as tert-butanol, diethyl ether, methanol and acetone. The sG1 lipase was most resistant in tert-butanol; however in other solvents, its relative activity was not retained by more than 50 %.

The role of metal and detergents for activity and stability for lipases has been shown to be important [5]. Many bacterial lipases contain a Ca^{2+} -binding site; therefore these ions are important for stability. The influence of various organic compounds on lipase stability was examined by adding each compound at a concentration of 2 and 5 mM to the incubation mixture (Table 3). The incubation was performed for 1 h at 25 °C

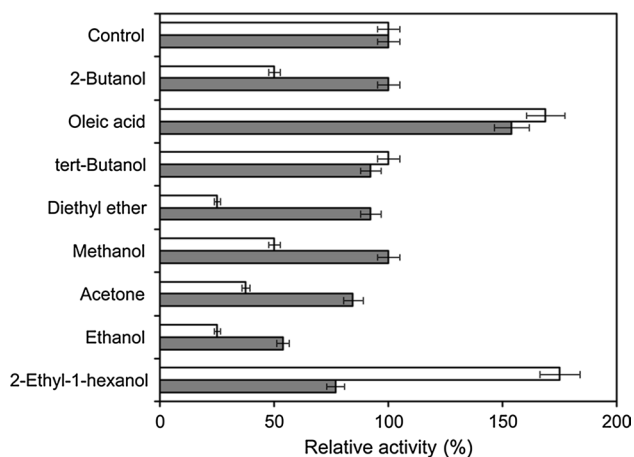


Fig. 5 Stability of mG1 and sG1 lipases in organic solvents. Each solvent was prepared at a concentration of 50 % in 0.05 M phosphate buffer (pH 7.0). Enzyme and solvent solutions were mixed at a 1:1 ratio. A mixture of enzyme with buffer was prepared as a control reaction. The resulting mixtures were incubated for 30 min in a rotary shaker at 200 rpm at 30 °C temperature. After incubation, the pH value of samples was standardized to pH 8.0 and samples were assayed for lipolytic activity by the pNPP standard method, at 25 °C and pH 8.0. Filled square mG1, open square sG1

Table 3 Stability of lipolytic activities of mG1 and sG1 in the presence of detergents and salts

Compound	Relative activity, %			
	2 mM		5 mM	
	mG1	sG1	mG1	sG1
Control ^a	100	100	100	100
Ca ²⁺	13	4	63	20
Zn ²⁺	44	14	6	2
Mg ²⁺	50	16	56	18
Co ²⁺	63	20	63	20
EDTA	50	16	31	10
PMSF	50	16	38	12
Triton X-100	50	16	19	6
Tween 80	50	16	69	22

The experiments were done in triplicate and the percentage error in each set of reading was within 2 %

^a Without the addition of any compound

temperature. Calcium and magnesium ions are required for many lipases for activity. Inevitably, the lipolytic activities of mG1 and sG1 lipases have increased in the presence of higher concentration of ions such as Ca²⁺, Mg²⁺ and detergent Tween 80 (Table 3). Excess of EDTA may cause complete inhibition of microbial lipases. *Acinetobacter* lipases have been assigned to be irreversibly inactivated by EDTA, while *P.aeruginosa* lipases are

exceptional in being almost unaffected by EDTA [26]. The catalytic activities of mG1 and sG1 lipases were neither enhanced nor inhibited in the presence of 5 mM of CoCl₂, while a drastic decrease in activity occurred in the presence of 5 mM of EDTA, ZnCl₂, PMSF and Triton X-100. The inhibition was greater when the concentration of the ion was increased by a magnitude of 2.5. Zinc is believed to be involved in enhancing the thermostability of lipases. However, in our study, the activities of mG1 and sG1 lipases had dropped in the presence of zinc ion. It is known that zinc, iron, mercury, nickel, copper and cobalt may cause inhibition of some lipases. In summary, mG1 lipase showed better stability for the influence of metals and detergents than sG1 lipase.

The expressed constructions differ only by codon usage profiles and the primary structure of the resulting proteins is supposed to be identical. However, proteins have slightly different characteristics. It could be assumed that the post-translational modification for mG1 and sG1 proteins undergoes differently. Not only glycosylation, but also phosphorylation and host-specific amino acid modification levels could be the causes of this phenomenon [20].

In general, both mG1 and sG1 lipases expressed in *K.lactis* showed promising characteristics for application in biocatalytic systems.

Conclusions

The constituted lipase purification scheme using single-step purification enabled to reduce costs and consumption of reagents and energy, which are needed for purification procedures in large scale. Despite the fact that lipase in the wild strain of *Serratia* is secreted extracellularly, the intracellular accumulation of recombinant *Serratia* lipase protein into exploited *E. coli* host strains was confirmed. However, attempts to express bacterial lipase gene composed of native codons and to express de novo-synthesized lipase gene, which possesses codons adapted to *K. lactis* yeast, gave desirable results—only extracellular lipase activity was determined. The following studies for estimation of optimal cultivation conditions showed that *K. lactis* can be successfully grown in the medium containing whey—a cheap raw material. Both variants of lipases (mG1 and sG1) expressed in *K. lactis* showed promising characteristics for application in biocatalytic systems. Also, our study showed that de novo synthesis technology used for production of targeted biocatalyst directly from genome sequence data had undoubtedly a future: it can improve the expression level in the system of choice for production of sufficient amount of biocatalyst; also, it may save time required for screening and isolation of enzymes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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