ACTA ABP BIOCHIMICA POLONICA

Vol. 68, No 3/2021
411–417
https://doi.org/10.18388/abp.2020_5730

Regular paper

Cloning, expression in *Komagataella phaffii*, and biochemical characterization of recombinant sequence variants of *Pseudomonas* sp. S9 GDSL-esterase*

Monika Wicka-Grochocka, Hubert Cieśliński and Marta Wanarska✉

Department of Molecular Biotechnology and Microbiology, Faculty of Chemistry, Gdańsk University of Technology, Gdańsk, Poland

Two recombinant *Komagataella phaffii* (formerly *Pichia pastoris*) yeast strains for production of two sequential variants of EstS9 esterase from psychrotolerant bacterium *Pseudomonas* sp. S9, i.e. αEstS9N (a two-domain enzyme consisting of a catalytic domain and an autotransporter domain) and αEstS9Δ (a single-domain esterase) were constructed. However, only one of recombinant *K. phaffii* strains, namely *Komagataella phaffii* X-33/pPICZαestS9Δ, allowed to successfully produce and secrete recombinant αEstS9Δ enzyme outside of the host cell. The purified αEstS9Δ esterase was active towards short-chain *p*-nitrophenyl esters (C2–C8), with optimal activity for the acetate (C2) ester. The single-domain αEstS9Δ esterase exhibits the highest activity at 60°C and pH 9.5. In addition, the enzyme retains 90% of its activity after 3 hour incubation at 70–90°C. What should be also noted is that αEstS9Δ esterase produced in the *K. phaffii* expression system has a much higher specific activity (0.069 U/mg of protein) than the recombinant EstS9Δ esterase produced in an *E. coli* expression system (0.0025 U/mg of protein) (Wicka *et al.*, 2016, *Acta Biochim Pol* 63: 117–125. https://doi.org/10.18388/abp.2015_1074).

Key words: GDSL-esterase, autotransporter, single-domain esterase, *Pseudomonas* sp. S9, *Komagataella phaffii*

Received: 03 June, 2021; revised: 25 July, 2021; accepted: 26 July, 2021; available on-line: 25 August, 2021

✉e-mail: marta.wanarska@pg.edu.pl

***This paper is dedicated to Professor Wacław Tadeusz Szybalski on the 100th anniversary of his birth**

INTRODUCTION

Lipolytic enzymes, due to their unique properties, such as the chemo-, regio-, and enantioselectivity, and the ability to catalyze reactions both in the aqueous and non-aqueous media, have become one of the most important groups of biocatalysts. They are mainly used in the detergent industry as additives in the laundry detergents, as well as in the food, pharmaceutical and organic synthesis industries (Jaeger & Eggert, 2002).

Due to their substrate specificity, lipolytic enzymes can be divided into lipases and esterases. Esterases catalyze hydrolysis of smaller ester molecules than lipases, partially water-soluble triacylglycerols, having short chains of monocarboxylic acids with less than 10 carbon atoms (Jaeger & Eggert, 2002).

Bacterial lipolytic enzymes were divided into eight families. This division was created based on the analysis of their amino acid sequences and their biological functions (Hausmann & Jaeger, 2010). Lipolytic enzymes belonging to the II family, in contrast to the α/β-hydrolase fold lipolytic enzymes, contain the active-site serine residue in a conserved Gly-Asp-Ser-Leu (GDSL) motif instead of the usually occurring evolutionally conserved pentapeptide Gly-Xaa-Ser-Xaa-Gly. The family of GDSL hydrolases was established in 1995 (Upton & Buckley, 1995). Some GDSL hydrolases share an additional domain located at the C-terminus of the respective proteins. This domain consists of 12 β-sheets which form a β-barrel inserted into the bacterial outer membrane. The N-terminal part of these GDSL hydrolases responsible for enzymatic activity is exported through this β-barrel. Enzymes of this group, termed autotransporters, have been identified in several pathogenic bacteria and are responsible for virulence of these microorganisms (Hausmann & Jaeger, 2010).

The EstS9 enzyme from psychrotolerant bacteria *Pseudomonas* sp. S9 is comprised of two domains: an N-terminal catalytic domain and a C-terminal autotransporter domain. Therefore, in our previous study (Wicka *et al.*, 2016), we tested the suitability of an *E. coli* expression system (pBAD expression system) for the production of two sequential EstS9 esterase variants, i.e. EstS9N and EstS9Δ proteins. Recombinant EstS9N was comprised of an N-terminal catalytic domain and C-terminal autotransporter domain of EstS9 esterase fused with the His-tag domain, whereas recombinant EstS9Δ was comprised of an N-terminal catalytic domain of EstS9 esterase fused with the His-tag domain. However, despite the differences in molecular structures, amino acid sequences and size of these recombinant enzymes, both EstS9N and EstS9Δ were produced as insoluble inclusion bodies in the *E. coli* cells. Unfortunately, after purification of both recombinant enzymes under denaturing conditions and renaturation, only EstS9N esterase was stable during storage and revealed remarkably higher enzymatic activity than the one observed for the purified EstS9Δ esterase. Hence, in our previous work, despite low purification efficiency of the recombinant enzyme from inclusion bodies, we could only characterize the EstS9N esterase.

Therefore, in this study we decided to test an alternative expression system based on methylotrophic *Komagataella phaffii* (formerly *Pichia pastoris*) yeast as the host because of the confirmed ability of this yeast to secrete recombinant proteins into the culture medium (Ahmad *et al.*, 2014; Spohner *et al.*, 2015). We choose the strong, methanol inducible *AOX1* promoter for expression of *aestS9N* and *aestS9Δ* genes to enable efficient production and secretion of both sequential variants of *Pseudomonas* sp. S9 esterase. However, in this case only the αEstS9Δ recombinant enzyme, a single-domain variant of the EstS9 esterase, was obtained and characterized.

MATERIALS AND METHODS

Bioinformatics analyses

Bioinformatics analyses of amino acid sequences of αEstS9N and αEstS9Δ proteins were done with bioinformatics tools used in our previous study (Wierzbicka-Woś *et al.*, 2013), but the PSORT program was not used here. Topographic presentations of the Pfam domains for αEstS9N and αEstS9Δ enzymes were done with the MyDomains – Image Creator (http://prosite.expasy.org/mydomains/). Dot blot results were analyzed using the Quantity One Software v. 4.5 (Bio-Rad, USA).

Construction of *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ strains

The first PCR product, called αestS9N, was obtained using the following primers: ForwardαEstAutoXhoI ATGCCTCGAGAAAAGA**GCGCCTAATCCTTACACCCATTTCGTC** and ReverseEstAutoXbaI GAGCTCTAGA**TAGAAGTCCAGCGCAACGCCTACATTAA**. The second DNA fragment, called αestS9Δ, was PCR amplified using the ForwardαEstAutoXhoI primer (as above) and ReverseEstXbaI primer CATGTCTAGA**AGGCGTTGCCCTGCTTCGGTC**. The XhoI restriction site was incorporated into the sequence of the forward primer and XbaI sites were incorporated into the sequences of both reverse primers. The recognition sites for the above mentioned restriction endonucleases are underlined in the sequences of presented primers and were designed to facilitate cloning. Sequences complementary to the template, i.e. genomic DNA of *Pseudomonas* sp. S9 (Wicka *et al.*, 2016), are given in bold. The thermal cycling conditions were the same in both PCRs performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA): initial denaturation at 98°C for 30 s, next 30 cycles with denaturation at 98°C for 10 s, annealing at 63°C for 30 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.

The αestS9N and the αestS9Δ PCR products were purified using the Extractme DNA Clean-Up kit (Blirt S.A., Poland), then digested with XhoI and XbaI restriction endonucleases (Thermo Fisher Scientific, USA), and purified by ethanol precipitation. Each purified DNA fragment was separately ligated to pPICZα A expression vector (EasySelect™ *Pichia* Expression Kit; Invitrogen, USA), previously digested with the same endonucleases. Then, the ligation products were used to transform chemically competent *E. coli* TOP10 cells (Invitrogen, USA). After transformation, *E. coli* TOP10 cells were plated on LB low salt agar (0.5% yeast extract, 1.0% peptone, 1.5% agar, 0.5% NaCl) containing Zeocin (Invitrogen, USA) at 25 µg/mL, and incubated at 37°C for 24 h. Then, all plates were analyzed for the presence of recombinant *E. coli* TOP10/pPICZαestS9N and *E. coli* TOP10/pPICZαestS9Δ colonies. The recombinant bacterial strains were grown in LB low salt medium containing Zeocin and pPICZαestS9N and pPICZαestS9Δ plasmids were then isolated with the ExtractMe Plasmid DNA Kit (Blirt S.A., Poland). Thus obtained plasmid constructs were validated by restriction analysis and DNA sequencing (Genomed, Poland).

Next, the recombinant plasmids pPICZαestS9N and pPICZαestS9Δ were linearized with PmeI restriction endonuclease (New England Biolabs, USA) and used to transform competent *K. phaffii* X-33 cells by electroporation. The *K. phaffii* X-33 strain was prepared for electroporation according to the pPICZα A, B, and C *Pichia* expression vectors for selection on Zeocin™ and purification of secreted, recombinant proteins user manual (Invitrogen, USA). Then, 40 µL of yeast cells were mixed with 5 µg of linearized plasmid DNA, incubated on ice for 5 min and transferred to an ice-cold 0.2 cm electroporation cuvette. The expotential decay pulse (1.5 kV, 5 ms) was performed using the Gene Pulser Xcell™ Electroporation System (Bio-Rad, USA). Immediately after the pulse, 1 mL of ice-cold 1 M sorbitol was added to the cuvette. The cuvette contents were transferred to a 15 mL tube and incubated at 30°C for 2 h. Subsequently, 1 ml of the YPD medium (1.0% yeast extract, 2.0% peptone, 2% D-glucose) was added and the tube was incubated for another 3 h at 30°C with shaking (180 rpm). Transformants were then plated on YPDS agar (1.0% yeast extract, 2.0% peptone, 2.0% D-glucose, 2.0% agar and 1 mol/L of D-sorbitol) containing 100 µg/mL Zeocin (Invitrogen, USA), and incubated at 30°C for 5 days.

Next, the recombinant *K. phaffii* cells from all colonies were transferred onto YPD agar plates (1.0% yeast extract, 2.0% peptone, 2.0% D-glucose, 2% agar) containing 100, 250, 500 or 1000 µg/mL Zeocin (Invitrogen, USA). The plates were incubated at 30°C for the next 5 days until colonies were formed. Transformants from YPD-agar plates with the highest concentration of Zeocin (1000 µg/mL) were cultivated in the YPD medium supplemented with Zeocin (100 µg/mL) at 30°C for 72 h with shaking at 180 rpm. Afterwards, the genomic DNA from recombinant yeast strains *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ was isolated by the ExtractMe DNA Yeast Kit (Blirt S.A., Poland), and assessed by PCR with ForwardαEstAutoXhoI and ReverseEstAutoXbaI or ForwardαEstAutoXhoI and ReverseEstXbaI primers, respectively.

Small-scale expression of *αestS9N* and *αestS9Δ* genes in *K. phaffii* X-33 cells

The *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ strains were grown in 25 mL of the BMGY medium (10 g/L of yeast extract, 20 g/L of peptone, 13.4 g/L of yeast nitrogen base, 4×10^{-4} g/L of biotin, 10 mL/L of glycerol and 0.1 mol/L of potassium phosphate buffer, pH 6.0) in 250 mL flasks at 30°C for 18 h with shaking (180 rpm). After centrifugation of the yeast cultures, the cell pellets were resuspended in 50 mL of the BMMY medium (BMMY, in contrast to the BMGY medium, contains 5 mL/L of methanol instead of glycerol) in 500 mL flasks and cultivated for 96 h at 25°C with shaking at 180 rpm. The cultures were supplemented with methanol every 24 h to its final concentration of 0.5% to induce expression of the *αestS9N* and *αestS9⊿* genes in *K. phaffii* X-33 cells.

Finally, the post-culture media were separated from the yeast cells by centrifugation at 3500×*g* for 5 min. The cell pellets were resuspended in 20 mM Tris-HCl buffer pH 7.5 and cells were disrupt using glass beads (212-300 µm, Sigma). The cell lysates were then centrifuged at 3500×*g* for 5 min to obtain cell-free extracts. The presence of recombinant proteins in post-culture media, cell lysates and cell-free extracts was tested by a dot blot method using HRP conjugated anti-His antibodies (Sigma).

Production and purification of the αEstS9Δ esterase

The *K. phaffii* X-33/pPICZαestS9Δ strain was grown in 150 mL of the BMGY medium, in a 1 L flask, at 30°C for 18 h with agitation (180 rpm). After centrifugation of the yeast culture, the cell pellet was resuspended in 300 mL of the BMMY medium in a 2 L flask and incubated at 25°C for 96 h with agitation (180 rpm). The yeast culture was supplemented with methanol every 24 h to the final concentration of 0.5% to induce expression of the *αestS9Δ* gene in the *K. phaffii* X-33 cells.

Next, the post-culture fluids from six yeast cultures were pooled and concentrated using a cross-flow ultrafiltration cassette with a molecular weight limit of 30 kDa (VIVA FLOW 50R, Sartorius Stedim Biotech GmbH, Germany), followed by exchange of the culture medium with a B5 buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9). The concentrated sample was applied onto an Ni-NTA column pre-equilibrated with the B5 buffer. Then, the Ni-NTA column was washed with an imidazole gradient from 5 to 60 mM in the B5 buffer. Finally, the elution step was carried out with an E500 buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9) at a flow rate of 0.5 mL/min.

The obtained protein sample was desalted using a VIVA FLOW 50R ultrafiltration cassette with a molecular weight limit of 30 kDa and 20 mM Tris-HCl buffer pH 7.5.

Molecular weight of the αEstS9Δ protein was estimated by SDS-PAGE (Bollag & Edelstein, 1991). Protein concentration was determined spectrophotometrically by the Bradford method (Bollag & Edelstein, 1991), using the commercially available Quick Start Bradford Protein Assay (Bio-Rad, USA).

Effects of temperature and pH on αEstS9Δ esterase activity and stability

The effect of temperature on the αEstS9Δ esterase activity was assayed by incubating the reaction mixtures at temperatures ranging from 5 to 85°C (in 5°C increments) and pH 7.5 (20 mM Tris-HCl buffer). Each reaction mixture contained *p*-nitrophenyl butyrate at a final concentration of 3.6 mM as the substrate. The enzymatic reactions were stopped after 40 min with isopropanol, and the absorbance of all analyzed mixtures was measured at 405 nm.

The optimum pH was determined by assaying the esterolytic activity of the αEstS9Δ enzyme in a 10 mM Britton-Robinson buffer, with pH values ranging from 2.0 to 12.0. The esterolytic activity in each analyzed reaction mixture was quantitated at 35°C with *p*-nitrophenyl butyrate (3.6 mM) as the substrate. The enzymatic reactions in all analyzed mixtures were stopped after 40 min with isopropanol, and the absorbance was measured at 405 nm.

For the thermal stability assays, the purified enzyme was pre-incubated at temperatures ranging from 40 to 90°C (in 10°C increments) in the absence of the substrate in reaction mixtures. After incubation for different times (20, 40, 80, 180 and 300 min), the esterolytic activity of αEstS9Δ against *p*-nitrophenyl butyrate (3.6 mM) was measured by assaying the residual activity of the enzyme at pH 9.0 and 35°C for 40 min.

For the pH stability assays, the reaction mixtures containing the purified enzyme were incubated at 35°C and pH ranging from 6.0 to 11.0. After incubation for 20, 40 and 60 min, small samples of the mixtures were withdrawn, and the residual enzymatic activities were measured with *p*-nitrophenyl butyrate in 20 mM Tris-HCl buffer pH 9.0 at 35°C. The enzymatic reactions were stopped after 40 min with isopropanol.

Effects of selected metal ions and reagents on the enzymatic activity of αEstS9Δ

The effects on the αEstS9Δ esterase activity of dithiothreitol (DTT), oxidized glutathione, reduced glutathione, β-mercaptoethanol, ethylenediaminetetraacetic acid disodium salt (EDTA), and salts of selected metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}) at final concentrations of 5 mM, were assayed for 40 min at 35°C in 20 mM Tris-HCl buffer pH 9.0, with 3.6 mM *p*-nitrophenyl butyrate as the substrate.

Substrate specificity of αEstS9Δ esterase

The substrate specificity of the purified αEstS9Δ enzyme was determined at 60°C and pH 9.0 using 3.6 mM *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caprylate, and *p*-nitrophenyl decanoate in acetonitrile and *p*-nitrophenyl palmitate, and *p*-nitrophenyl stearate in *n*-hexane as substrates. The enzymatic reactions were stopped after 40 min with isopropanol, and the absorbance of reaction mixtures was measured at 405 nm.

One unit of αEstS9Δ esterase activity (U) was defined as the enzyme activity required for release of 1 µmol of *p*-nitrophenol from *p*-nitrophenyl acetate per minute, at 60°C and pH 9.0.

RESULTS AND DISCUSSION

In this study, we decided to produce in the *Komagataella phaffii* yeast and characterize two recombinant sequence variants of the EstS9 esterase from a psychrotolerant bacterium *Pseudomonas* sp. S9. The first one, called αEstS9N, consists of an N-terminal catalytic domain and a C-terminal AB-domain of native EstS9 esterase. The second protein, called αEstS9Δ, is characterized by the lack of the C-terminal AB-domain of the EstS9 esterase, and its amino acid sequence was designed based on analysis of the InterProScan results. In both recombinant proteins the putative signal peptide of the EstS9 esterase was replaced by the signal peptide of *Saccharomyces cerevisiae* α-mating factor (α-MF) enabling secretion of recombinant esterases into the culture medium. Also, a six-histidine tag (His-tag) was added to the C-terminus of both proteins to facilitate detection and purification (Fig. 1). Since the signal peptide should be removed by the Kex2 endopeptidase in the *K. phaffii* Golgi apparatus, the mature esterases secreted into the medium should have the same amino acid sequences as the EstS9N and EstS9Δ proteins produced in *E. coli* (Wicka *et al.*, 2016).

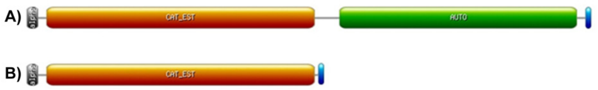

Figure 1. Topographic presentation of the Pfam domains for αEstS9N (A) and αEstS9Δ (B)
Alpha, signal peptide of *Saccharomyces cerevisiae* α-mating factor (grey domain); CAT_EST, catalytic domain (orange domain); AUTO, autotransporter domain (green domain); His-Tag (blue domain).

Construction of the recombinant *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ strains

In order to produce two sequence variants of the EstS9 esterase from *Pseudomonas* sp. S9 in *K. phaffii*, two integrating plasmids named pPICZαestS9N and pPICZαestS9Δ were constructed and used to transforme the yeast cells. Strong, methanol-inducible promoter of the alcohol oxidase 1 gene (P_{AOX1}) of the pPICZα A plasmid was used for expression of the *estS9N* and *estS9Δ* genes in *K. phaffii* X-33 cells. This plasmid also contains sequences encoding the α-MF signal peptide and the His-tag. The use of tightly regulated promoters, such as the *AOX1* promoter, holds advantages for overexpression of proteins. By uncoupling the growth from the production phase, biomass is accumulated prior to protein expression. Therefore, cells are not stressed by the accumulation of recombinant protein during growth phase, and even the production of proteins that are toxic is possible (Ahmad *et al.*, 2014). Furthermore, this system has already been used successfully for the production of lipolytic enzymes in *K. phaffii* (Minning *et al.*, 1998; Choi *et al.*, 2004; Jiang *et al.*, 2007; Yu *et al.*, 2007; Wang *et al.*, 2008; Ferrer *et al.*, 2009; Yu *et al.*, 2009; Eom *et al.*, 2013; Huang *et al.*, 2013; Sriyapai *et al.*, 2015).

Because the heterologous gene expression level in *K. phaffii* is dependent on the copy number of the recombinant plasmid integrated into the genome, and a higher copy number confers higher resistance to Zeocin, the transformants were screened on YPD-agar plates containing 100–1000 µg/mL Zeocin. About 60% of all *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ transformants were highly resistant to Zeocin (they were able to grow on the culture medium with Zeocin at final concentration of 1000 µg/mL). The PCR assays revealed that all yeast transformants highly resistant to Zeocin contained the *aestS9N* or *aestS9Δ* sequences as a result of integration of an appropriate recombinant plasmid into the *K. phaffii* X-33 genomic DNA.

Expression of genes encoding the αEstS9Δ and αEstS9N proteins

Small-scale expression of the *aestS9N* and *aestS9Δ* genes in the *K. phaffii* X-33/pPICZαestS9N and *K. phaffii* X-33/pPICZαestS9Δ cells was carried out in 50 mL of buffered methanol complex medium (BMMY) at 25 °C. Then, the post-culture fluids, cell lysates, as well as cell-free extracts (supernatants collected after centrifugation of cell lysates) were tested using the dot blot method. Dot blot results indicated presence of the αEstS9Δ protein in the post-culture fluids, cell lysates and cell-free extracts of *K. phaffii* X-33/pPICZαestS9Δ strains no. 2, 3 and 4 (Fig. 2A). Analysis of dot blot results using Bio-Rad's Quantity One Software showed that more than 50% of the single-domain esterase was secreted outside the *K. phaffii* cells. It was about 58% for recombinant strain no. 2, ~57% for strain no. 4 and ~55% for strain no. 3. In contrast, all of the αEstS9N protein was present in the cell lysate of *K. phaffii* X-33/pPICZαestS9N strain no. 6A (Fig. 2B), and it was not detected in the cell-free extract and post-culture fluid. Based on these results and analysis of the literature data (Rutherford & Mourez, 2006; Wilhelm *et al.*, 2011), it can be assumed that the αEstS9N protein is not transported outside the cell. Presence of the autotransporter domain can cause αEstS9N binding to cell membranes. Thus, in further research we decided to focus on production, purification and characterization of the αEstS9Δ esterase.

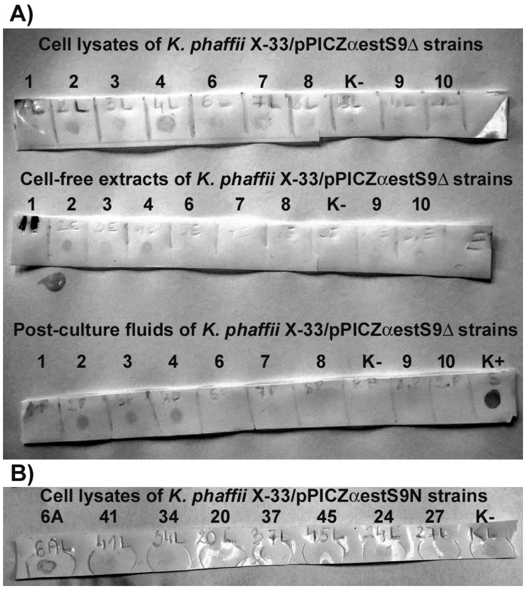

Figure 2. Dot blot assays of cell lysates, cell-free extracts and post-culture fluids of *K. phaffii* X-33/pPICZαestS9Δ recombinant strains (A) and cell lysates of *K. phaffii* X-33/pPICZαestS9N transformants (B)

Dot blot tests were performed using HRP conjugated anti-His antibodies. 10 µL samples were applied onto the membrane. Negative controls were samples of cell lysate, cell-free extract and post-culture fluid of *K. phaffii* X-33. Positive control was the protein with a His-tag.

Production and purification of the αEstS9Δ esterase

The fed batch strategy was applied to production and secretion of the αEstS9Δ esterase by recombinant *K. phaffii* X-33/pPICZαestS9Δ strain no. 2. In the first stage, yeast biomass was multiplied in the medium containing glycerol as a sole carbon source, and then the cells were transferred to the medium containing methanol for induction of the *aestS9Δ* gene expression. The cultivation was carried out for 4 days, and methanol was added every 24 h to maintain the induction. The αEstS9Δ esterase was then purified from the post-culture fluid by immobilized metal affinity chromatography (IMAC). SDS-PAGE analysis of the purified αEstS9Δ protein showed two bands at ~45 and ~35 kDa (Fig. 3), which are close to the predicted molecular mass of the deduced αEstS9Δ protein with or without the α-MF signal peptide, i.e. 42 and 33 kDa respectively. This may indicate incomplete removal of the signal peptide from

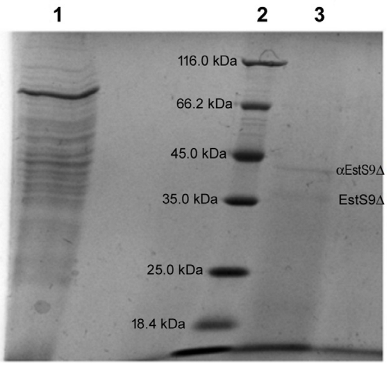

Figure 3. SDS-PAGE protein profiles:

(1) Post-culture fluid of *K. phaffii* X33/pPICZαestS9Δ, (2) Unstained Protein Molecular Weight Marker 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa (Thermo Fisher Scientific, USA), (3) Purified αEstS9Δ esterase

Table 1. Relative activity of the αEstS9Δ esterase against various chromogenic substrates

Substrate	No. of carbon atoms in the alkyl chain	Relative activity [%]
p-nitrophenyl acetate	2	100.0 ± 2.1
p-nitrophenyl butyrate	4	79.0 ± 3.3
p-nitrophenyl caprylate	8	37.7 ± 0.5
p-nitrophenyl caproate	10	4.8 ± 1.2
p-nitrophenyl palmitate	16	< 0.01
p-nitrophenyl stearate	18	< 0.01

recombinant protein. There are probably two forms of recombinant esterase in the resulting protein preparation. In summary, the production and purification resulted in 1.155 mg of αEstS9Δ esterase with a specific activity of 0.069 U/mg of protein per 1.8 liters of *K. phaffii* X-33/pPICZαestS9Δ yeast culture. For comparison, the EstS9Δ esterase produced in the *E. coli* expression system as inclusion bodies, purified under denaturing conditions and renatured, had a specific activity of 0.0025 U/mg of protein (Wicka *et al.*, 2016).

Substrate specificity, physicochemical characterization and determination of kinetic parameters of the αEstS9Δ esterase

p-Nitrophenyl esters of different alkyl chain lengths were used to determine substrate specificity of the purified αEstS9Δ esterase. As shown in Table 1, the αEstS9Δ enzyme is highly active towards short chain fatty acids (C2–C8), and has the maximum activity against the *p*-nitrophenyl acetate ester. Importantly, comparative analysis of results of different *p*-nitrophenyl esters hydrolysis by EstS9N (Wicka *et al.*, 2016) and αEstS9Δ enzymes revealed distinct differences in the substrate specificity for both compared proteins. In contrast to αEstS9Δ, the EstS9N enzyme showed the highest activity against *p*-nitrophenyl butyrate.

The effect of temperature on activity of the αEstS9Δ esterase was determined at temperatures ranging from 5 to 85°C. As shown in Fig. 4, the maximum esterolytic activity was recorded at 60°C. For comparison, the recombinant EstS9N esterase produced in the *E. coli* expression system was most active at 35°C and showed very little activity at 60°C (Wicka *et al.*, 2016). The αEstS9Δ esterase retained about 60% of its maximal activity at 35°C.

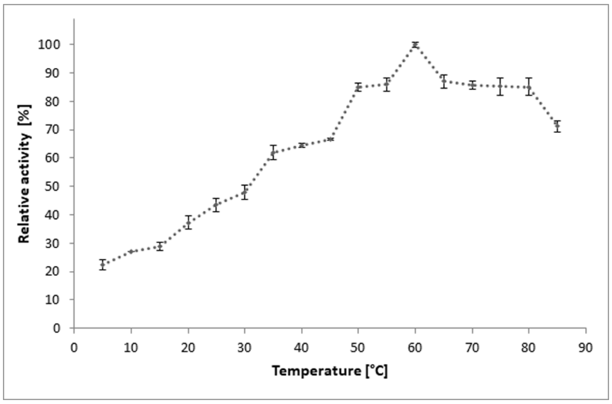

Figure 4. The effect of temperature on the recombinant αEstS9Δ esterase activity
The effect of temperature on the enzyme activity was assayed by incubating the αEstS9Δ protein at a temperature ranging from 5 to 85°C with 3.6 mM *p*-nitrophenyl butyrate, in the Tris-HCl buffer pH 7.5, for 40 min.

Since αEstS9Δ, a single-domain variant of the EstS9 esterase, is highly active in the temperature range of 50 to 80°C, it can be compared to enzymes active at high temperatures, for example the EstEP16 enzyme which also exhibits the highest activity at 60°C (Zhu *et al.*, 2013) or the GDSL esterase from thermophilic bacterium *Fervidobacterium nodosum* Rt17-B1, which is active in the temperature range of 30–80°C, with a maximum activity at 75°C (Yu *et al.*, 2010).

Moreover, 90% of the αEstS9Δ esterase activity was retained after 3 h of incubation over a temperature range of 70 to 90°C. Thus, the thermal stability of the αEstS9Δ enzyme is significantly higher than that of the EstS9N protein, which was gradually inactivated by heat treatment at temperatures above 70°C (Wicka *et al.*, 2016). The recombinant αEstS9Δ esterase can be successfully compared to enzymes from thermophilic microorganisms, for example the GDSL esterase from *Fervidobacterium nodosum* Rt17-B1, which loses half of its activity after 80 minute incubation at 80°C (Yu *et al.*, 2010), or the thermostable esterase EstEP16 which retains 80% of its activity after 6 h of incubation at 90°C (Zhu *et al.*, 2013). The increase in the thermostability of αEstS9Δ protein correlates with the shift in the optimal enzyme activity temperature.

The effect of pH on the esterolytic activity of the αEstS9Δ protein was determined over a pH range of 2.0 to 12.0. As shown in Fig. 5, the enzyme is active over a pH range of 7.5 to 10.0 (>50% of maximum activity), and prefers alkaline conditions with maximum activity at pH 9.5. Furthermore, the results from the pH stability assays showed that approximately 100% of the enzyme activity was retained after 1 h of incubation at the pH range of 9.0–11.0 (Fig. 6). The previously described EstS9N esterase (Wicka *et al.*, 2016) was also active and stable under alkaline conditions, especially at pH 9.0. The EstEP16 enzyme (Zhu *et al.*, 2013) and esterase from *Fervidobacterium nodosum* Rt17-B1 (Yu *et al.*, 2010) show maximum activity at pH 8.0 and 8.5, respectively.

As shown in Fig. 7, the hydrolytic activity of αEstS9Δ esterase against *p*-nitrophenyl butyrate as a substrate was clearly inhibited by Ni^{2+} and activated by Mg^{2+} ions. We also observed that the addition of ethylenedi-

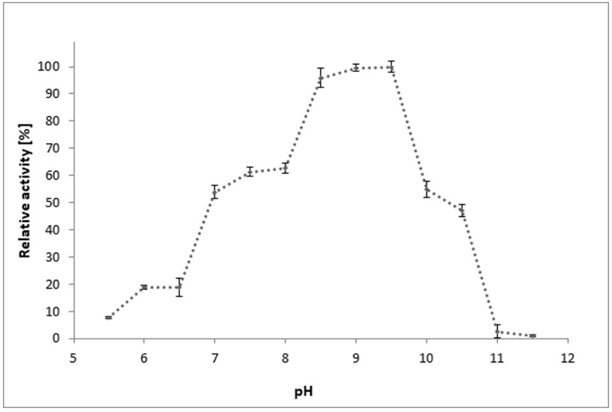

Figure 5. The effect of pH on the recombinant αEstS9Δ esterase activity
The enzymatic activity was assayed at the pH values ranging from 2.0 to 12.0, at 35°C, with *p*-nitrophenyl butyrate as the substrate.

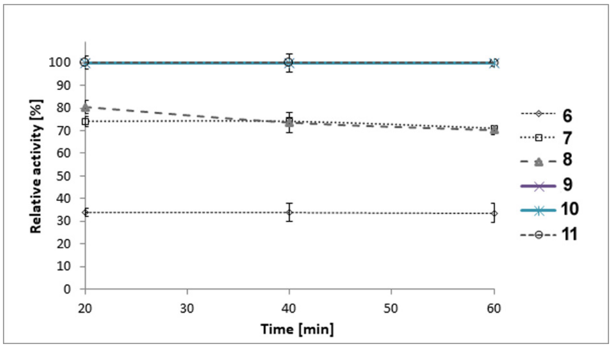

Figure 6. The effect of pH on the recombinant αEstS9Δ esterase stability
The pH-stability profile was determined by incubation of the enzyme for 20, 40 and 60 min at various pH, and then the residual activity of the esterase was assayed at 35°C with *p*-nitrophenyl butyrate as the substrate.

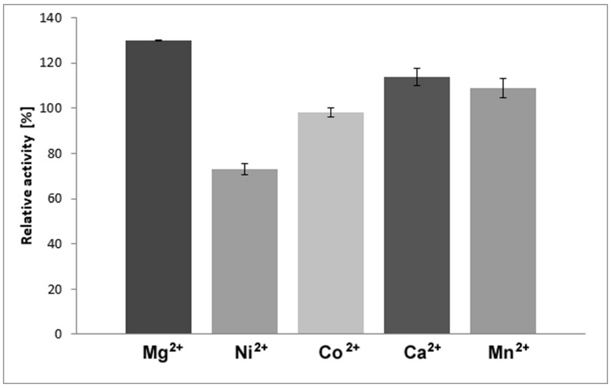

Figure 7. The effect of various metal ions on the recombinant αEstS9Δ esterase activity
The enzyme was incubated for 60 min at 35°C with 5 mM metal ions, and then the residual activity of the esterase was assayed with 3.6 mM *p*-nitrophenyl butyrate in the Tris-HCl buffer pH 9.0, at 35°C for 40 min.

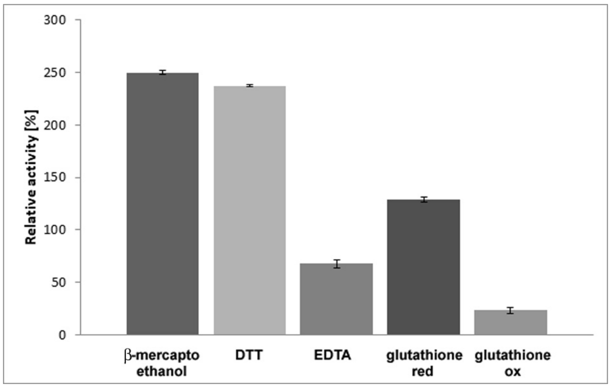

Figure 8. The effect of selected reagents on the recombinant αEstS9Δ esterase activity
The enzyme was incubated for 60 min at 35°C with 5 mM of β-mercaptoethanol, dithiothreitol (DTT), ethylenediaminetetraacetic acid sodium salt (EDTA), glutathione in the reduced and oxidized forms, and then the residual activity of the esterase was assayed with 3.6 mM *p*-nitrophenyl butyrate in the Tris-HCl buffer pH 9.0, at 35°C for 40 min.

aminetetraacetic acid sodium salt (EDTA) and oxidized glutathione to the enzyme markedly decreased its enzymatic activity (Fig. 8), whereas β-mercaptoethanol and dithiothreitol (DTT) were strong activators of the assayed enzyme. The EstS9N esterase was also inhibited by Ni^{2+} ions and oxidized glutathione, and activated by DTT (Wicka *et al.*, 2016).

Studies of the kinetics of *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate hydrolysis catalyzed by the αEstS9Δ esterase revealed that the enzyme had lower affinity for *p*-nitrophenyl butyrate at 60°C (optimal temperature of action) than at 35°C. It also exhibited low affinity for *p*-nitrophenyl acetate at 60°C. Moreover, as shown in Table 2, the catalytic efficiency towards *p*-nitrophenyl butyrate (k_{cat}/K_m value) at 60°C was similar to the k_{cat}/K_m value for *p*-nitrophenyl acetate at 60°C, and it was about two times lower than the one recorded at 35°C. These results show that the single-domain esterase still has the properties of a cold-adapted enzyme, i.e. higher affinity for the substrate and higher catalytic efficiency at lower temperature. Unfortunately, values of kinetic parameters (k_{cat} and k_{cat}/K_m) of αEstS9Δ, the single-domain esterase produced in *K. phaffii*, were much lower than those of EstS9N (a two-domain variant of the EstS9 esterase produced in *E. coli*). The catalytic efficiency of the αEstS9Δ protein against *p*-nitophenyl butyrate at 35°C was only 0.2% of the value recorded for the EstS9N enzyme (Wicka *et al.*, 2016). On this basis, it can be assumed that removal of the autotransporter domain of the EstS9 esterase had a negative effect on the enzyme activity.

CONCLUSION

The *E. coli* bacterium is the host of choice for expression of heterologous genes. However, some proteins do not fold properly in *E. coli*, leading to the formation of insoluble inclusion bodies. The choice of a different expression host, e.g. *K. phaffii* yeast, may allow production of the recombinant protein in a soluble form. Such a protein is more active and stable than its counterpart produced in *E. coli* cells in an insoluble form and refolded *in vitro*, as demonstrated for the single-domain variant of the GDSL-esterase of *Pseudomonas* sp. S9 (EstS9Δ). On the other hand, in the case of two-domain proteins, such as the EstS9 esterase, removal of the non-catalytic domain (autotransporter domain) may result in significant changes in the properties of the catalytic do-

Table 2. Kinetic parameters for *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate hydrolysis catalyzed by the αEstS9Δ esterase
The enzyme was incubated with the appropriate substrate at a final concentration ranging from 0.72 to 5.4 mM, in Tris-HCl buffer pH 9.0, at 35 or 60°C, for different time periods. After appropriate incubation, the reactions were stopped by the addition of isopropanol, and the absorbance was measured at 405 nm.

Substrate	Temperature [°C]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
p-nitrophenyl butyrate	35	0.727	0.034	0.047
p-nitrophenyl butyrate	60	6.151	0.145	0.024
p-nitrophenyl acetate	60	7.358	0.203	0.028

main and have a negative effect on the enzyme activity. Therefore, in further research we plan to produce the EstS9 esterase in its natural host *Pseudomonas* sp. S9 to determine the structure of the enzyme, its location in the cell and biochemical properties. First of all, we plan to determine if the two-domain protein is anchored in the outer membrane of *Pseudomonas* sp. S9, as in the case of the EstA esterase from *Pseudomonas aeruginosa* PAO1 (Wilhelm *et al.*, 1999). At the same time, we plan to produce wild-type EstS9 esterase in *E. coli* cells using the pZErO-S9 Lib1/NotI plasmid, containing a 3 kb genomic DNA fragment of *Pseudomonas* sp. S9 (Wicka *et al.*, 2016), and characterize the recombinant enzyme. In addition, we plan to clone the full-length *estS9* gene into the pBAD/Myc-His A expression vector, under the control of arabinose-inducible *araBAD* promoter, to produce the enzyme as a protein anchored in the outer membrane of *E. coli* and characterize the whole-cell biocatalyst. Such a biocatalyst is an attractive alternative for industry, as the enzyme bound to the cell surface (immobilized) is usually more stable than its free form and does not require multi-stage purification. Moreover, the whole-cell biocatalyst can be easily separated from the post-reaction mixture and used repeatedly, which reduces the cost of the process. On the other hand, the autotransporter domain of EstS9 esterase itself can be also used to transport and display other enzymes on the *E. coli* surface in order to obtain new biocatalysts with a biotechnological potential.

REFERENCES

Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol* **98**: 5301–5317. https://doi.org/10.1007/s00253-014-5732-5

Bollag DM, Edelstein SJ (1991) *Protein methods*. Wiley-Liss, New York

Choi YJ, Miguez CB, Lee BH (2004) Characterization and heterologous gene expression of a novel esterase from *Lactobacillus casei* CL96. *Appl Environ Microbiol* **70**: 3213–3221. https://doi.org/10.1128/AEM.70.6.3213-3221.2004

Eom GT, Lee SH, Song BK, Chung KW, Kim YW, Song JK (2013) High-level extracellular production and characterization of *Candida antarctica* lipase B in *Pichia pastoris*. *J Biosci Bioeng* **116**: 165–170. https://doi.org/10.1016/j.jbiosc.2013.02.016

Ferrer P, Alarcón M, Ramón R, Benaiges MD, Valero F (2009) Recombinant *Candida rugosa* LIP2 expression in *Pichia pastoris* under the control of the *AOX1* promoter. *Biochem Eng J* **46**: 271–277. https://doi.org/10.1016/j.bej.2009.05.018

Hausmann S, Jaeger KE (2010) Lipolytic enzymes from bacteria. In *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis KN ed, pp 1099–1126. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-77587-4_77

Huang J, Yang Z, Guan F, Zhang S, Cui D, Guan G, Li Y (2013) A novel mono- and diacylglycerol lipase highly expressed in *Pichia pastoris* and its application for food emulsifier preparation. *Process Biochem* **48**: 1899–1904. https://doi.org/10.1016/j.procbio.2013.08.021

Jaeger KE, Eggert T (2002) Lipases for biotechnology. *Curr Opin Biotechnol* **13**: 390–397

Jiang ZB, Song HT, Gupta N, Ma LX, Wu ZB (2007) Cell surface display of functionally active lipases from *Yarrowia lipolytica* in *Pichia pastoris*. *Protein Expres Purif* **56**: 35–39. https://doi.org/10.1016/j.pep.2007.07.003

Minning S, Schmidt-Dannert C, Schmid RD (1998) Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. *J Biotechnol* **66**: 147–156

Rutherford N, Mourez M (2006) Surface display of proteins by Gram-negative bacterial autotransporters. *Microb Cell Fact* **5**: 22. https://doi.org/10.1186/1475-2859-5-22

Spohner SC, Müller H, Quitmann H, Czermak P (2015) Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. *J Biotechnol* **202**: 118–134. https://doi.org/10.1016/j.jbiotec.2015.01.027

Sriyapai P, Kawai F, Siripoke S, Chansiri K, Sriyapai T (2015) Cloning, expression and characterization of a thermostable esterase HydS14 from *Actinomadura* sp. strain S14 in *Pichia pastoris*. *Int J Mol Sci* **16**: 13579–13594. https://doi.org/10.3390/ijms160613579

Upton C, Buckley JT (1995) A new family of lipolytic enzymes? *Trends Biochem Sci* **20**: 178–179

Wang CH, Guo RF, Yu HW, Jia YM (2008) Cloning and sequence analysis of a novel cold-adapted lipase gene from strain lip35 (*Pseudomonas* sp.). *Agric Sci China* **7**: 1216–1221. https://doi.org/10.1016/S1671-2927(08)60167-4

Wicka M, Wanarska M, Krajewska E, Pawlak-Szukalska A, Kur J, Cieśliński H (2016) Cloning, expression, and biochemical characterization of a cold-active GDSL-esterase of a *Pseudomonas* sp. S9 isolated from Spitsbergen island soil. *Acta Biochim Pol* **63**: 117–125. https://doi.org/10.18388/abp.2015_1074

Wierzbicka-Woś A, Bartasun P, Cieśliński H, Kur J (2013) Cloning and characterization of a novel cold-active glycoside hydrolase family 1 enzyme with beta-glucosidase, beta-fucosidase and beta-galactosidase activities. *BMC Biotechnol* **13**: 22. https://doi.org/10.1186/1472-6750-13-22

Wilhelm S, Tommassen J, Jaeger KE (1999) A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. *J Bacteriol* **181**: 6977–6986

Wilhelm S, Rosenau F, Kolmar H, Jaeger KE (2011) Autotransporters with GDSL passenger domains: molecular physiology and biotechnological applications. *Chembiochem* **12**: 1476–1485. https://doi.org/10.1002/cbic.201100013

Yu M, Lange S, Richter S, Tan T, Schmid RD (2007) High-level expression of extracellular lipase Lip2 from *Yarrowia lipolytica* in *Pichia pastoris* and its purification and characterization. *Protein Expres Purif* **53**: 255–263. https://doi.org/10.1016/j.pep.2006.10.018

Yu XW, Wang LL, Xu Y (2009) *Rhizopus chinensis* lipase: gene cloning, expression in *Pichia pastoris* and properties. *J Mol Catal B Enzym* **57**: 304–311. https://doi.org/10.1016/j.molcatb.2008.10.002

Yu S, Yu S, Han W, Wang H, Zheng B, Feng Y (2010) A novel thermophilic lipase from *Fervidobacterium nodosum* Rt17B1 representing a new subfamily of bacterial lipases. *J Mol Catal B Enzym* **66**: 81–89. https://doi.org/10.1016/j.molcatb.2010.03.007

Zhu Y, Li J, Cai H, Ni H, Xiao A, Hou L (2013) Characterization of a new and thermostable esterase from a metagenomic library. *Microbiol Res* **168**: 589–597. https://doi.org/10.1016/j.micres.2013.04.004