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

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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 also be noted is that αEstS9Δ esterase produced in the K. phaffii expression system has a much higher specificity (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

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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 methylo trophic 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 αestS9N and αestS9Δ genes to enable efficient production and secretion of both sequential variants of Pseudomonas sp. S9 esterase. However, in this case only the αEstS9Δ

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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 zestS9N, was obtained using the following primers: ForwardEstAutoXhoI ATGCGCTCGAGAAAAGAGGCCCTAATCCT-TACACCCATTTCGTC and ReverseEstAutoXbaI GAGGCTTGAAGTTGCCAGCGACGCCTACTTATAA- CATTA. The second DNA fragment, called zestS9Δ, was PCR amplified using the ForwardEstAutoXhoI primer (as above) and ReverseEstAutoXbaI primer CATGTCCTAGATAAGTCACGCGACGCCTACTTATAA-CATTAT. 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 zestS9N and the zestS9Δ 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 exponential 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 YPD 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, 2.0% 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 ForwardEstAutoXhoI and ReverseEstAutoXbaI or ForwardEstAutoXhoI and ReverseEstAutoXbaI primers, respectively.

Small-scale expression of aeztS9N and aeztS9Δ 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, 4×10⁻³ 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 BMYM 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 aeztS9N and aeztS9Δ 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 disrupted 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

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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 effects 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 pH values 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 (Mg²⁺, Ca²⁺, Mn²⁺, Ni²⁺, Co²⁺) 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 Est9 esterase from Pseudomonas sp. S9 in K. phaffii, two integrating plasmids named pPICZαestS9N and pPICZαestS9Δ were constructed and used to transform the yeast cells. Strong, methanol-inducible promoter of the alcohol oxidase 1 gene (P_{AOX1}) of the pPICZαA 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 over-expression 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 estS9N or estS9Δ 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 αestS9N and αestS9Δ 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 zEst9Δ 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 56% for recombinant strain no. 2, ~57% for strain no. 4 and ~55% for strain no. 3. In contrast, all of the zEst9N protein was present in the cell lysate of K. phaffii X-33/pPICZαestS9N strain no. 6Δ (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 zEst9N protein is not transported outside the cell. Presence of the autotransporter domain can cause zEst9N binding to cell membranes. Thus, in further research we decided to focus on production, purification and characterization of the αEstS9Δ esterase.

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 glucose as a sole carbon source, and then the cells were transferred to the medium containing methanol for induction of the αestS9Δ 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

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.
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 as inclusion bodies, purified under denaturing conditions and renatured, had a specific activity of 0.0025 U/mg of protein (Wicka et al., 2016).

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.

Substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No. of carbon atoms in the alkyl chain</th>
<th>Relative activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl acetate</td>
<td>2</td>
<td>100.0 ± 2.1</td>
</tr>
<tr>
<td>p-nitrophenyl butyrate</td>
<td>4</td>
<td>79.0 ± 3.3</td>
</tr>
<tr>
<td>p-nitrophenyl caprylate</td>
<td>8</td>
<td>37.7 ± 0.5</td>
</tr>
<tr>
<td>p-nitrophenyl caproate</td>
<td>10</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>p-nitrophenyl palmitate</td>
<td>16</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>p-nitrophenyl stearate</td>
<td>18</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

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

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.

Since αEstS9Δ, a single-domain variant of the Est9 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).

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

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.

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

Studies of the kinetics of \( \text{p-nitrophenyl butyrate} \) and \( \text{p-nitrophenyl acetate} \) hydrolysis catalyzed by the \( \alpha \text{EstS9}\Delta \) esterase revealed that the enzyme had lower affinity for \( \text{p-nitrophenyl butyrate} \) at 60°C (optimal temperature of action) than at 35°C. It also exhibited low affinity for \( \text{p-nitrophenyl acetate} \) at 60°C. Moreover, as shown in Table 2, the catalytic efficiency towards \( \text{p-nitrophenyl butyrate} \) (\( k_{\text{cat}}/K_m \) value) at 60°C was similar to the \( k_{\text{cat}}/K_m \) value for \( \text{p-nitrophenyl acetate} \) 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 (\( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \)) of \( \alpha \text{EstS9}\Delta \), the single-domain esterase produced in \( K. \text{phaffii} \), were much lower than those of EstS9N (a two-domain variant of the EstS9 esterase produced in \( E. \text{coli} \)). The catalytic efficiency of the \( \alpha \text{EstS9}\Delta \) protein against \( \text{p-nitrophenyl butyrate} \) 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 \( E. \text{coli} \) bacterium is the host of choice for expression of heterologous genes. However, some proteins do not fold properly in \( E. \text{coli} \), leading to the formation of insoluble inclusion bodies. The choice of a different expression host, e.g., \( K. \text{phaffii} \) 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 \( E. \text{coli} \) cells in an insoluble form and refolded \textit{in vitro}, as demonstrated for the single-domain variant of the GDSL-esterase of \( Pseudomonas \) sp. S9 (EstS9\( \Delta \)). 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 \( \text{p-nitrophenyl butyrate} \) and \( \text{p-nitrophenyl acetate} \) hydrolysis catalyzed by the \( \alpha \text{EstS9}\Delta \) esterase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temperature [°C]</th>
<th>( K_m ) [mM]</th>
<th>( k_{\text{cat}} ) [s(^{-1})]</th>
<th>( k_{\text{cat}}/K_m ) [s(^{-1}) mM(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{p-nitrophenyl butyrate} )</td>
<td>35</td>
<td>0.727</td>
<td>0.034</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.151</td>
<td>0.145</td>
<td>0.024</td>
</tr>
<tr>
<td>( \text{p-nitrophenyl acetate} )</td>
<td>60</td>
<td>7.358</td>
<td>0.203</td>
<td>0.028</td>
</tr>
</tbody>
</table>

### Figure 6. The effect of pH on the recombinant \( \alpha \text{EstS9}\Delta \) 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 \( \text{p-nitrophenyl butyrate} \) as the substrate.

### Figure 7. The effect of various metal ions on the recombinant \( \alpha \text{EstS9}\Delta \) 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 \( \text{p-nitrophenyl butyrate} \) in the Tris-HCl buffer pH 9.0, at 35°C for 40 min.

### Figure 8. The effect of selected reagents on the recombinant \( \alpha \text{EstS9}\Delta \) esterase activity

The enzyme was incubated for 60 min at 35°C with 5 mM of \( \beta \)-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 \( \text{p-nitrophenyl butyrate} \) in the Tris-HCl buffer pH 9.0, at 35°C for 40 min.
main and have a negative effect on the enzyme activity. Therefore, in further research we plan to produce the EssS9 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 PA01 (Wilmhelm et al., 1999). At the same time, we plan to produce wild-type EstS9 esterase in E. coli cells using the pZErO-S9 Lb1/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 araBAD promoter, to produce the enzyme with beta-glucosidase, beta-fucosidase and beta-galactosidase activity bound to the cell surface (immobilized) and used repeatedly, which reduces the cost of the process. On the other hand, the autotransporter domain of EstS9 esterase itself can also be used to transport and display other enzymes on the E. coli surface in order to obtain new biocatalysts with a biotechnological potential.

REFERENCES


