



# Production of Lysostaphin by Nonproprietary Method Utilizing a Promoter from Toxin–Antitoxin System

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## Abstract

Lysostaphin is a staphylolytic protein of growing interest from biotechnological and pharmaceutical industry due to its potential use in preventing and combating staphylococcal infections. Here, we describe an optimized method for production of lysostaphin in an inductionless system utilizing constitutive promoter from staphylococcal toxin–antitoxin system PemIK–Sa1. We investigated the influence of ribosome-binding site sequence, *Escherichia coli* producer strain and growth media on yield and kinetics of recombinant protein production. Lysostaphin was purified in its native active form using one-step cation-exchange chromatography. The system provides a method for cost-efficient and scalable protein production, and can be applied to produce other biotechnologically significant proteins.

**Keywords** Lysostaphin · Protein expression · Ion exchange chromatography · Staphylococcus · Recombinant protein · Toxin–antitoxin system

## Introduction

Many different expression systems have been developed to date, but the production of recombinant proteins in *Escherichia coli* remains the most widely used [1]. *E. coli* offer high growth rate and inexpensive substrates which allow for scalable and cost-efficient production. Additionally, broad prevalence of the system results in availability of numerous host strains and variety of expression vectors.

Most expression systems rely on inducible promoters enabling control over the onset of protein production. Biomass is usually first produced and only then the culture is switched

to production. Apart from clear advantages, the inducible systems, including the most widely utilized *lac* promoter [2], its derivatives, *tac/trc* promoter [3, 4] and *ara* promoter [5], require expensive inducers, a considerable burden in large scale production. Other inducible promoters respond to temperature [6], pH [7] or depletion of particular substrates [8, 9], but provide less stringent control and have not been widely used.

Tightly controlled strong promoters have been conventionally utilized [10], although often result in inactive, insoluble protein, deposited in inclusion bodies [11, 12]. Additionally, high level of mRNA can lead to ribosome destruction [13] and metabolic burden associated with overexpression [14] may lead to cell death. An alternative approach uses constitutive promoters. Continuous production from weak promoters allows gradual accumulation and often more efficient folding. Another advantage is in cost reduction by eliminating the inducers.

Lysostaphin is a bacteriolytic metalloprotease originating from *Staphylococcus simulans* biovar *staphylolyticus*. It degrades the cell wall of multiple species of staphylococci by hydrolysis of pentaglycine crosslinks within peptidoglycan [15, 16]. The gene encoding lysostaphin (*end*) is located on pACK1 plasmid together with *epr* gene which provides host resistance. The resistance is ensured by increased number of serine residues in the cross bridges [17, 18]. Native

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lysostaphin is composed of mature polypeptide of 246 amino acid residues, propeptide of 211 residues and signal peptide of 36 residues [19]. The signal peptide is removed upon secretion and the propeptide is processed outside the cell by an extracellular cysteine protease [20, 21]. The mature lysostaphin is a protein of 27 kDa and isoelectric point of approximately 9.5 [22].

Lysostaphin had been shown effective in treatment of staphylococcal infections [23, 24] and reduction of staphylococcal carriage [25, 26], but has not been translated to clinics. After a period of relative stagnation, medical lysostaphin is being rediscovered against multidrug-resistant MRSA (*Meticillin-resistant Staphylococcus aureus*) and VRSA (*Vancomycin-resistant Staphylococcus aureus*) [27, 28], as well as notoriously recalcitrant biofilms [29, 30]. Latest concepts incorporate lysostaphin into medically relevant materials, which include coating of orthopedic implants with polymer matrix containing lysostaphin and manufacturing gels or wound-dressing materials for the treatment of topical or wound infections [31–35]. Lysostaphin-containing materials were proven useful in both preventing and eradicating *S. aureus* infections linked to orthopedic implants in murine model. Furthermore, a biopolymer impregnated with lysostaphin had the antistaphylococcal activity comparable to commercially available antimicrobial wound dressings [36]. The growing interest in lysostaphin as an antistaphylococcal agent calls for a cost-effective and scalable method of production.

Aside potential clinical use, lysostaphin is an indispensable tool in research and diagnostics. Lysostaphin treatment remains the most effective method for lysing staphylococcal cells. This is because other common methods including alkaline lysis, sonication and homogenization are ineffective for staphylococci [37]. Lysostaphin is thus essential for extraction of nucleic acids and intracellular proteins. The enzyme is currently available from commercial sources, although relatively expensive. As a result, laboratories with sufficient experience may benefit from a non-complicated and reliable expression system to produce the enzyme on their own.

Several methods of lysostaphin expression and purification have been described to date. The enzyme was purified from *Staphylococcus simulans* conditioned media [38–42], however, with poor yield. Additionally, such produced enzyme may contain contaminating allergens and pyrogens. Alternatively, production in heterologous hosts has been reported including *E. coli* [43–50], *Bacillus subtilis* and *Lactobacillus casei* [51], *Pichia pastoris* [52] and even in mammalian cells [53]. The most promising system in terms of efficient large scale production was developed in *E. coli* and based on an inducible promoter. Prior proposed systems required multi-step purification and often relied on immobilized metal ions chromatography, which is known to compromise lysostaphin activity due to leakage of metal

ions, which are lysostaphin inhibitors [46]. No system based on a constitutive promoter and providing single-step purification has been proposed to date.

Toxin–antitoxin (TA) systems are widely spread among bacteria and contribute to maintenance of mobile genetic elements, mediate phage infection defense, stress adaptation [54] and other functions. While investigating the staphylococcal *pemIK-Sal* toxin–antitoxin system [55] we observed continuous expression of GFP (green fluorescent protein) under *pemIK-Sal* promoter increasing with culture density. This attractive characteristics prompted us to test the utility of the promoter in circumventing the need of inducible expression for efficient recombinant protein production.

In this study, we provide an efficient expression system for recombinant lysostaphin in *E. coli*. The system utilizes a constitutive, non-inducible promoter from *pemIK-Sal* toxin–antitoxin system. Furthermore, the system is constructed using an easily available pUC18 backbone suitable for cost-effective commercial production of recombinant proteins. Furthermore, we introduce a one-step ion exchange chromatography purification resulting in highly active and pure preparation. Altogether, our system allows rapid, efficient, scalable and cost-effective production of recombinant lysostaphin, which may easily be adapted for use in pharmaceutical and biotechnological applications.

## Materials and Methods

### Bacterial Strains and Culture Conditions

*E. coli* Top10 and DH5 $\alpha$  were used for cloning and plasmid propagation, respectively. Both above-mentioned strains and BL21(DE3) were used to test protein expression. The bacteria were cultivated in Luria–Bertani Broth (LB, Sigma) or Tryptic Soy Broth (TSB, Sigma). The media were supplemented with 100  $\mu$ g/ml ampicillin to assure plasmid maintenance. The cultures were cultivated at 37 °C with shaking.

### Construction of Expression Vectors

The promoter sequence of *pemIK-Sal* toxin–antitoxin system was cloned into pTZ57R/T plasmid using InsTA-clone™ PCR Cloning Kit (Thermo Scientific). The promoter sequence was amplified using pCH91 plasmid template [55] and RUN polymerase (A&A Biotechnology) with two different primer pairs. promF and promR1 were used to introduce an *E. coli*-derived RBS while promF and promR2 introduced staphylococcal RBS (Table 1). Gel-purified PCR products were ligated into pTZ57R/T plasmid resulting in pTZ57R/promEco and pTZ57R/promSau constructs.

GFP and the mature lysostaphin coding sequences were cloned into pUC18 vector. Additionally, lysostaphin with

**Table 1** Sequence of primers used in this study

Primer	Sequence (5'–3')
lizoHisPstI_Rev	<u>CGCCTGCAGTCAATGATGATGATGATGATGC</u>
lizoPstI_Rev	<u>CGCCTGCAGTCACTTTATAGTTCCCAAAAG</u>
lizoSacI_For	<u>GAAGAGCTCAGCTGCAACACATGAACATTC</u>
GFPSacI	<u>GGAGCTCAAAAGGAGAACTTTTCACTGG</u>
GFPPstI	<u>GCTGCAGTTATTTGTAAAGCTCATCCATGC</u>
promR1	<u>GTTCTGCAGAGCTCATCTGTATATCTCC</u> <u>TTTTGTTCATCATAACAAATG</u>
promR2	<u>GTTCTGCAGAGCTCATAAATAATCATCC</u> <u>TCCTTTTGTTCATCATAACAAATG</u>
promF	<u>GAGAATTCATTAAATTAACATAAATGGG</u>

Sequences recognized by restriction enzymes are underlined and RBS sequences are highlighted in bold

C-terminal His-tag was prepared. The sequences were amplified with following pairs of primers: GFPSacI and GFPPstI for GFP, and lizoSacI\_For and lizoPstI\_Rev or lizoSacI\_For and lizoHisPstI\_Rev for lysostaphin. Plasmids pALCP2G [55] and pBADLys [46] encoding GFP and lysostaphin, respectively, were used as templates. The PCR products and pUC18 plasmid were digested with *SacI* and *PstI* (Thermo Scientific) and ligated with T4 DNA ligase (Thermo Scientific). Resulting constructs were denoted as pUC18/GFP, pUC18/Lysostaphin and pUC18/Lysostaphin-His<sub>6</sub>.

Regulatory sequences were transferred from pTZ57R-based plasmids prepared before into pUC18/GFP, pUC18/Lysostaphin and pUC18/Lysostaphin-His<sub>6</sub>. All plasmids were digested with *EcoRI* and *SacI* and the regulatory sequences were ligated upstream the protein coding sequence. This resulted in a construct for expression of GFP and four constructs for expression of lysostaphin, namely pUC18/promEco/GFP, pUC18/promEco/Lysostaphin, pUC18/promEco/Lysostaphin-His<sub>6</sub>, pUC18/promSau/Lysostaphin and pUC18/promSau/Lysostaphin-His<sub>6</sub>. Best constructs for recombinant lysostaphin production (pUC18/promEco/Lysostaphin and pUC18/promSau/Lysostaphin) were deposited in Addgene (IDs 125765 and 125766, respectively).

### Lysostaphin Activity Assay

Lysostaphin activity was determined by monitoring the decrease in the optical density (OD) at 600 nm of suspension of susceptible *S. aureus* cells. An overnight culture of *S. aureus* RN4220 was diluted to an optical density of 1 in 0.1 M phosphate buffer, pH 7.5. The sample tested was diluted 10 times in L buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) in a 96-well plate. In each case 100 µl of the tested sample dilution was mixed with 100 µl of *S. aureus* cell suspension. The OD was followed with a microplate reader (PowerWaveXSelett, Bio-Tek Instruments)

for 30 min. The activity was expressed in arbitrary units calculated as follows. The blank-corrected  $\Delta OD_{600nm}$   $t_0 - t_{30}$  was set as a value of 100 and for each time point the respective  $\Delta OD_{600nm}$  the proportional value from the range 100 to 0 was assigned. Activity is expressed as 50 (corresponding to the half of initial OD<sub>600nm</sub> value) divided by the time (in minutes) when OD<sub>600nm</sub> reached the value.

### GFP Reporter Assay for Determination of Promoter Activity

Overnight cultures of *E. coli* Top10, DH5α and BL21(DE3) cells transformed with pUC18/promEco/GFP were diluted 1:100 in 20 ml of LB supplemented with 100 µg/ml ampicillin and cultivated at 37 °C with shaking. Samples of 0.5 ml were collected each hour. The cells were pelleted, resuspended in 0.5 ml of phosphate-buffered saline (pH 7.4) and sonicated (30 pulses, 500 ms, 80% amplitude) using UP50H ultrasonic processor (Hielscher). The fluorescence of the lysates was determined with PowerWave microplate reader (Biotek) at 480 nm (excitation) and 510 nm (emission).

### Optimization of Protein Production

To establish the best conditions for lysostaphin production, the kinetics of the recombinant protein expression from pUC18/promEco/Lysostaphin and pUC18/promEco/Lysostaphin-His<sub>6</sub> plasmids was examined. Bacterial host strain, the growth medium and the time of cultivation were subject of optimization. Lysostaphin production was monitored by determining the staphylolytic activity of tenfold diluted lysate as well as by SDS-PAGE of the whole lysate, and its soluble and insoluble fractions.

The overnight cultures were diluted 1:100 in 50 ml of tested medium supplemented with 100 µg/ml ampicillin. The bacteria were grown at 37 °C with shaking and first sample (2 ml) was taken 4 h after inoculation. Next samples were taken every hour for subsequent 7 h. The last sample was taken 24 h since the start of the culture. Samples were pelleted by centrifugation and the pellets were suspended in 200 µl of buffer L. 50 µl of every sample was set aside for total cell protein analysis. The remaining 150 µl were sonicated (5 series of 30 pulses, 800 ms, 80% power) to obtain clear lysates. The samples were clarified by centrifugation (10 min, 4 °C, 15,000×g). The supernatant was used to assay the protein content of the soluble fraction while the pellet included the insoluble fraction. The pellets were resuspended in 100 µl of buffer L. All samples were analyzed by SDS-PAGE [56].

## Production and Purification of Lysostaphin

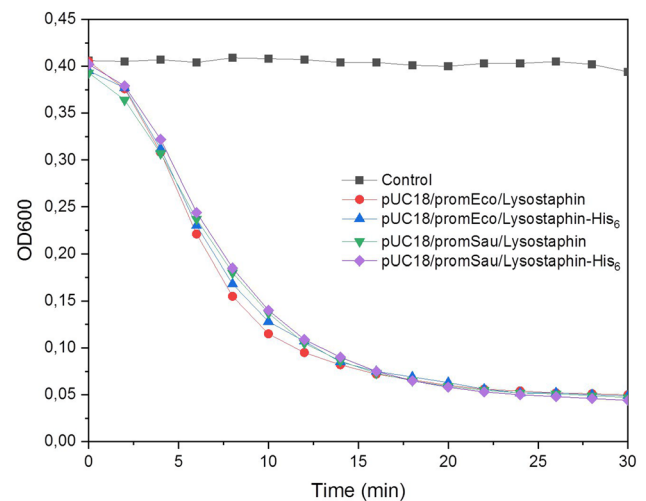
Overnight culture of *E. coli* DH5 $\alpha$  transformed with pUC18/promEco/Lysostaphin was diluted 1:100 in 200 ml of TSB supplemented with 100  $\mu$ g/ml ampicillin and cultured at 37  $^{\circ}$ C with shaking for 16 h. The cells were pelleted, resuspended in 10 ml of buffer A (50 mM sodium phosphate, pH 7.5) and sonicated on ice (6 pulses of 30 s, 1 min intervals, maximum power) with UD-11 ultrasonic disintegrator (Techpan). The lysate was clarified by centrifugation (20 min, 4  $^{\circ}$ C, 15,000 $\times$ g). The supernatant was applied (2 ml/min) onto 15 ml of cation-exchanger Source S30 resin (GE Healthcare Life Sciences) equilibrated with buffer A. The column was washed with 60 ml of buffer A. Elution was performed in linear gradient of buffer B (50 mM sodium phosphate, 1 M NaCl, pH 7.5) from 0 to 30% in a total volume of 150 ml. 2 ml fractions were collected. The fractions containing lysostaphin were pooled and concentrated 10 times using Amicon Ultra-15 Centrifugal Filter Units with MWCO of 3000 kDa (Merck). The preparation was dialyzed twice against buffer D (10 mM sodium acetate, pH 4.5) at 4  $^{\circ}$ C. The purified protein was aliquoted, frozen in liquid nitrogen and lyophilized (Christ LDC-1, Alpha 1–4 liophilizer). The protein concentration was determined by measurement of the absorbance at 280 nm (extinction coefficient: 2.43/cm/mg ml).

## Results

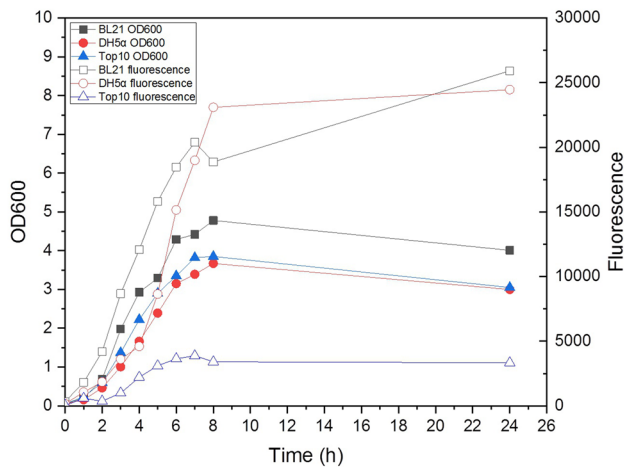
During functional investigation of staphylococcal PemIK-Sa1 toxin–antitoxin system we noticed that the *pemIK-Sa1* promoter is transcriptionally active in *E. coli*, despite of the fact that *E. coli* are phylogenetically distant from *S. aureus*. *E. coli* transformants carrying a shuttle plasmid encoding *pemIK-Sa1* driven GFP were green on agar plates. We took advantage of this observation to construct a vector for inductionless expression of recombinant proteins in *E. coli*. As a proof of concept, we first prepared a pUC18-based plasmid encoding a widely utilized GFP reporter. Another pUC18 based plasmids were prepared containing lysostaphin or lysostaphin appended with His-tag at the C terminus to demonstrate our idea using a protein of interest for biotechnology. *PemIK-Sa1* promoter was engineered into both plasmids in two variants. Since the original *pemIK-Sa1* TA promoter contains a non-canonical ribosome-binding site (AAGG GGGCTGATAGTAATG), our plasmid variants contained RBS optimized for *E. coli* and *S. aureus* (AAGGAGATA TCAGATG and AGGAGGATGATTATTTATG, respectively) [57]. Having the genetic constructs at hand, we first assessed our concept by evaluating recombinant lysostaphin production in *E. coli*, in a simple end-point assay. Cells were collected from overnight cultures, lysed by sonication and

lysostaphin activity in crude lysates was tested by monitoring a decrease in optical density of *S. aureus* cell suspension. All tested promoter and lysostaphin variants demonstrated significant staphylolytic activity. No differences in activity (around 8.5 A.U.) were observed regardless of RBS variant and/or the presence of C-terminal His-tag (Fig. 1).

In the next step, the effect of host strain on the efficiency of recombinant protein production and the production kinetics were evaluated. Here, we used the GFP-encoding plasmid with *pemIK-Sa1* promoter, which allowed us to track the transgene expression by quantifying the cell fluorescence. Moreover, since GFP is fluorescent only in its native conformation [58] we monitored only accumulation of properly folded protein rather than its accumulation in inclusion bodies. Three *E. coli* strains were tested: BL21(DE3), DH5 $\alpha$  and TOP10. The first strain is routinely used for production of recombinant proteins since it lacks Lon and OmpT protease encoding genes ensuring little or no recombinant protein degradation. The fact that the strain is additionally lysogenic for DE3 phage which allows T7 promoter-driven expression [59] was of no significance to this study. The two latter strains are generally used for DNA manipulations, but not in protein expression. This is because both are DNA recombinase deficient (*recA*<sup>-</sup>), which substantially reduces the risk of unspecific plasmid recombination, but not protease deficient. Recombinase deficiency is however of special interest when expressing toxic proteins in long-lasting biotechnological processes, and for that reason we included those strain in evaluation. Through the experiment, the optical density of tested *E. coli* cultures was monitored and samples were taken at different time points to evaluate



**Fig. 1** Recombinant lysostaphin expression in *E. coli*. Staphylolytic activity (shown as decrease in optical density of susceptible *S. aureus* suspension) of lysates prepared from *E. coli* DH5 $\alpha$  transformed with the plasmids prepared in this study and encoding lysostaphin under the control of the *pemIK-Sa1* promoter



**Fig. 2** Evaluation of recombinant protein expression in different *E. coli* strains. GFP was cloned under control of *pemIK-Sa1* TA promoter and expression was evaluated in indicated *E. coli* strains. Optical density (OD600 nm) and GFP fluorescence are shown

GFP fluorescence accumulation (Fig. 2). GFP production/fluorescence accumulation mirrored the growth curves of tested cultures, regardless of the strain. Continuous expression during the logarithmic phase of growth was observed which reached a plateau as the culture entered the stationary phase—no further protein accumulation was noted. Interestingly, the production yield differed substantially between tested strains. Top10 produced considerably less GFP (fluorescence) compared to two other strains. The fluorescence (GFP) level obtained in DH5 $\alpha$  and BL21(DE3) was comparable and roughly twice that observed in Top10. However, BL21(DE3) reached higher OD600 nm and thus the fluorescence level (GFP production) per biomass unit was slightly lower compared to DH5 $\alpha$ . As such, the latter strain was used for further optimization of lysostaphin production.

Composition of the growth medium is an important factor affecting the production yield of recombinant proteins [60]. Additionally, fusion tags may influence the expression efficiency, solubility and stability of the recombinant protein [61]. To optimize lysostaphin expression, we assessed the influence of the above factors on obtained yields. To this end, native and His-tagged lysostaphin were expressed in LB and rich TSB medium while monitoring the growth rate and transgene activity. Kinetics of lysostaphin production in LB was indifferent to the presence of C-terminal His-tag. The staphylolytic activity of lysates from both native and His-tagged lysostaphin expressing *E. coli* was roughly comparable at all time points tested (Table 2; Fig. 3). It increased gradually during the exponential growth phase indicating accumulation of the recombinant protein (Fig. 3). However, prolonged cultivation (24 h) resulted in decreased activity (Table 2; Fig. 3), likely because of secondary degradation. Both variants were produced only in the soluble form—no

**Table 2** Staphylolytic activity of lysates from *E. coli* DH5 $\alpha$  transformed with the plasmids encoding different variants of lysostaphin under the control of the *pemIK-Sa1* promoter cultured in LB and TSB medium

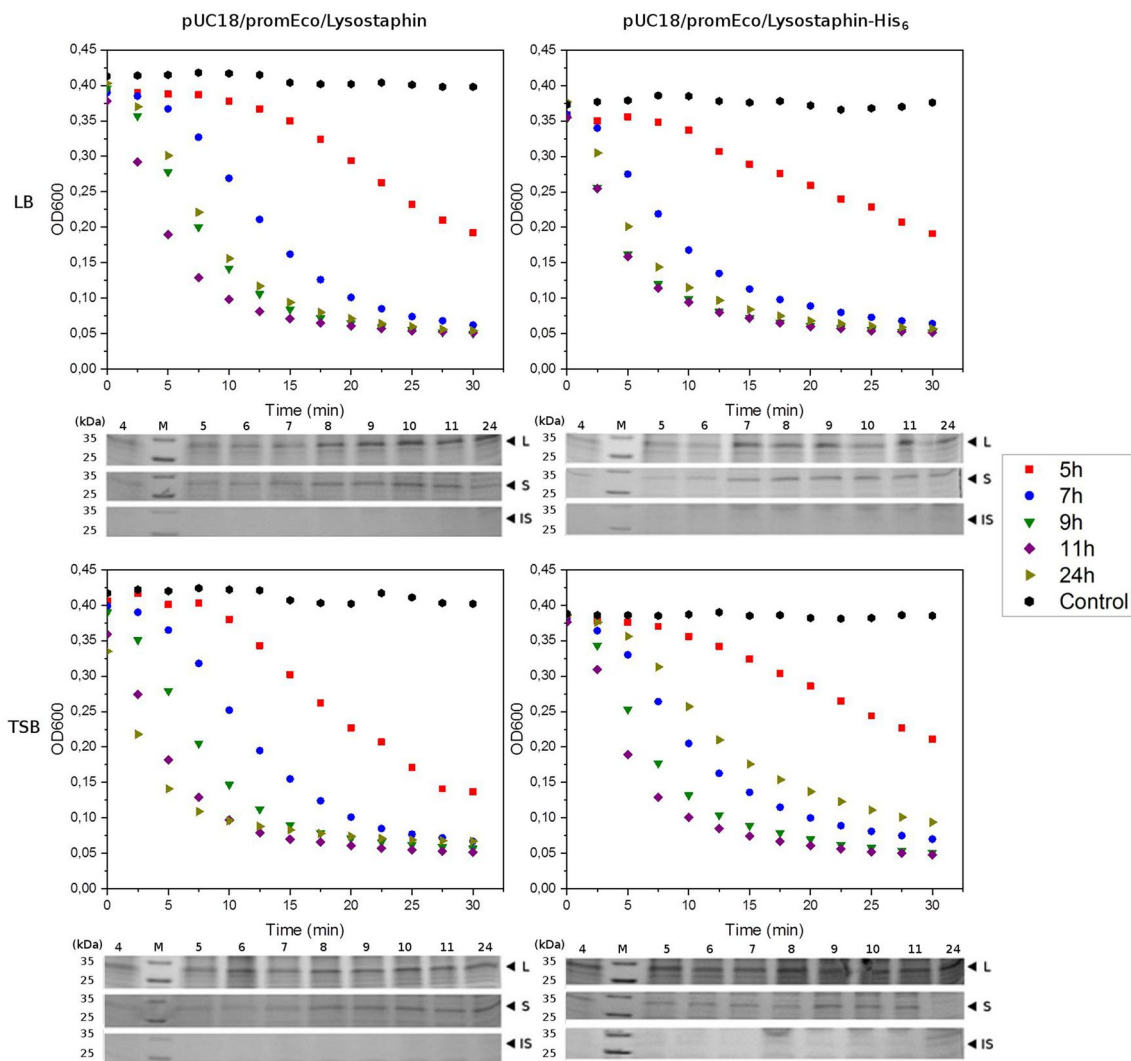
	Lysostaphin		Lysostaphin-His <sub>6</sub>	
Time post inoculation (h)	11	24	11	24
LB medium (A.U.)	14.8	8.0	18.1	11.0
TSB medium (A.U.)	17.7	33.8	13.6	4.9

bands corresponding to the respective proteins were detected in insoluble fraction during SDS-PAGE, even at prolonged incubation times (Fig. 3). The kinetics of recombinant lysostaphin production was largely different in TSB compared to LB. Time-dependent increase in staphylolytic activity of native lysostaphin was observed through the entire culture (up to 24 h), while the activity of His-tagged protein dropped substantially after prolonged cultivation (24 h) compared to that accumulated at 11 h. SDS-PAGE revealed decrease of the corresponding protein band in soluble fraction which suggests proteolytic degradation rather than conversion to inclusion bodies (Fig. 3). Interestingly, the activity of lysates containing lysostaphin of native sequence was substantially higher when the bacteria were grown in TSB compared to LB (Table 2). Therefore, we chose *E. coli* DH5 $\alpha$  transformed with pUC18/promEco/Lysostaphin cultivated in TSB up to stationary phase (16 h post inoculation) for preparative production of recombinant lysostaphin.

Protein purification in the absence of affinity tags is generally relatively challenging unless the protein of interest has unique features enabling application of dedicated isolation method. To recover the recombinant lysostaphin, we took advantage of its relatively high-isoelectric point (pI 9.5), which allowed advantageous fractionation using strong cation exchanger. Clarified *E. coli* lysate was passed over Source S30 at pH 7.5 retaining lysostaphin. Rather strikingly, this afforded a very efficient, single-step purification as the vast majority of endogenous proteins did not bind at these conditions. Virtually pure lysostaphin (>95% purity in SDS-PAGE) was recovered with low-sodium chloride in the loading buffer (Fig. 4). Lysostaphin-containing fractions were pooled, concentrated, dialyzed against storage buffer, and lyophilized. The procedure resulted in 20–25 mg of lysostaphin from 1 l of bacterial culture.

## Discussion

In this study, we took advantage of the features of the promoter derived from staphylococcal PemIK-Sa1 toxin–antitoxin system to design an induction-free platform for efficient production of recombinant proteins. The utility of the



**Fig. 3** Comparison of production efficacy of different variants of recombinant lysostaphin in different media. Native sequence and His-tagged lysostaphin were expressed in *E. coli* cultured in LB or TSB. Staphylolytic activity and protein levels were monitored. Samples were taken every hour till the 11th hour (exponential growth)

and at 24th hour (stationary phase)—selected time points are shown for clarity. Panels beneath the graphs show SDS-PAGE of the whole lysate (L) and the soluble (S) and the insoluble (IS) fractions. Bands corresponding to lysostaphin are marked with arrowheads

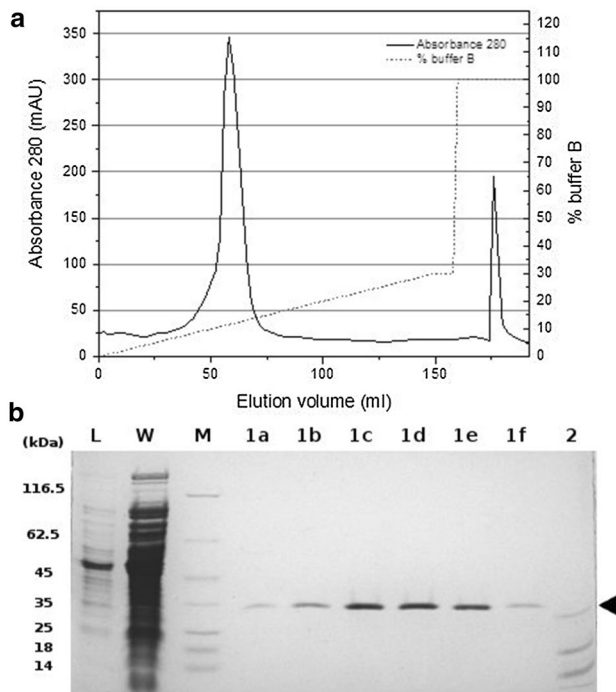
platform was exemplified by expression of lysostaphin, an important antistaphylococcal agent attracting a growing interest of pharmaceutical and biotechnology industry [34, 35, 62].

RBS sequences allow to fine-tune protein expression. Often the expression of genes clustered in operons is varied by differing the RBS sequences. In TA systems for example, the antitoxin is usually produced in higher amounts than the downstream-encoded toxin [63]. To bypass the expected low expression from non-canonical RBS, we optimized its original sequence to that characteristic for *E. coli* and *S. aureus*. Strikingly, we did not observe any difference in activity or kinetics of lysostaphin production from these two distinct RBS sequences when evaluated in *E. coli*. This indicates that

in our particular example, the limiting feature is the strength of the promoter, but not the RBS.

The kinetics of protein production driven by the tested *pemIK-Sa1* constitutive promoter matched that described previously for other staphylococcal toxin–antitoxin systems belonging to the same class, namely *mazEF* and *savRS* [64, 65]. The promoter was active during logarithmic growth phase. In the late stationary phase, degradation of recombinant protein was observed and for this reason the optimal time of harvest coincided with the peak of optical density at the early stationary phase.

Production of recombinant proteins is among biotechnological processes resulting in high value products. Factors influencing profitability include efficiency and the cost of an



**Fig. 4** Purification of native lysostaphin on Source S30 ion exchanger. **a** Purification chromatogram. The first absorbance peak corresponds to lysostaphin. **b** SDS-PAGE of samples obtained in (a): *L* whole lysate, *W* flow through (proteins which did not bind), *M* molecular weight standard, *1a–1f* eluate fractions corresponding to the first absorbance peak in (a). A band corresponding to lysostaphin is marked by an arrowhead, *2* eluate fraction corresponding to the second absorbance peak

expression platform and downstream processing—such as purification [66]. We provided the procedure based on generally accessible pUC18-based vectors and *E. coli* strains to produce mature lysostaphin in one-step purification. pUC18 is a high copy number plasmid which compensates the use of a relatively weak promoter.

A method of lysostaphin purification has been previously described based on immobilized metal ion affinity chromatography [46–48]. Histidine tagging has not affected the activity, but our results show that it may significantly affect protein stability. What is more, lysostaphin is a  $Zn^{2+}$ -dependent metalloprotease and trace  $Ni^{2+}$  or  $Co^{2+}$  ions released from the resin during purification result in significant decrease in enzymatic activity by substituting the catalytic  $Zn^{2+}$  ion [46].  $Zn^{2+}$  affinity chromatography was proposed as an alternative [67, 68], but a two-step procedure including additional ion-exchange chromatography was necessary to obtain relevant purity. Not only the two-step procedure, but even the relatively high concentrations of imidazole necessary for elution substantially increase the costs of high-scale industrial purification. Moreover, it was recently reported that excess exogenous  $Zn^{2+}$  inhibits lysostaphin [69]. Even the more, some applications require

the recombinant protein in its native form (i.e. without expression and purification tags) while precise tag removal is often impossible or requires additional reagents (i.e. proteases) and steps (i.e. tag removal, protease removal) which substantially increase the production costs and contribute additional impurities to the final preparations [46]. Here, we described a single-step, cation-exchange chromatography-based procedure yielding pure preparations of native sequence lysostaphin. Our approach overcomes the limitations of prior techniques proposed for lysostaphin purification. In the optimized conditions of pH 7.5, only a few proteins other than lysostaphin bind to the resin and none of those endogenous *E. coli* proteins co-eluted with the protein of interest. This phenomenon is explained by a bimodal distribution of isoelectric points (pI) in bacterial proteome [70]. Nearly two-thirds of *E. coli* proteins have theoretical pI below 7.5 and thus exhibit negative or neutral charge at the pH used for purification excluding their binding to a cation exchanger. The majority of remaining *E. coli* proteins with higher theoretical isoelectric points are classified among membrane proteins, and as such are absent in the clarified lysate. Such distribution of isoelectric points among *E. coli* proteins opens the possibility to apply cation exchange in one-step purification of not only lysostaphin, but also other recombinant proteins characterized by high values of pI, using procedures described in this study.

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**Author Contributions** BW designed the study. AM and AJ performed the study. AM, AJ, GD and BW analyzed the results. AM, GD and BW wrote the manuscript. All authors revised the manuscript and agreed to be accountable for all aspects of the presented work.

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