Al-Kufa University Journal for Biology / VOL.9 / NO.3 / Year: 2017

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Expression of Genetically Engineered BL21(DE3) with the Staphylokinase Gene from *Staphylococcus aureus*

Hêro Farhad Salah [1,2*], Adel Kamal Khider[1] and Sekaran Muniandy[2]

[1]Department of Biology, College of Education, University of Salahaddin, Erbil, Kurdistan Region, Iraq

[2]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Corresponding author: Hêro Farhad Salah

Adress: Department of Biology, College of Education, University of Salahaddin, Erbil, Kurdistan Region, Iraq

[*]**E-mail:** hero.salah@su.edu.krd

Abstract

The current study aimed to obtain staphylokinase (Sak) protein as a fibrinolytic agent from *Escherichia coli* as a nonpathogenic bacterium after transforming it with a cloned vector. From 630 clinical samples, 98 isolates of *S. aureus* were obtained and 81 (82.65%) were Sak producers. PCR amplification of the *sak* gene identified a single DNA band with the expected length of 408 bp in three *S. aureus* isolates. *E. coli* DH5α was successfully transformed with the *sak* gene. PCR amplification showed that the recombinant gene comprised 408 bp, which was the same size as the native gene amplified from *S. aureus*. Bacterial proteins of genetically engineered BL21(DE3) with cloned pET-28a-*sak* were separated according to their molecular weight and recombinant Sak appeared as a band with an estimated molecular weight ca 15.5 KDa. A non-induced sample with cloned vector was included as control. The fibrinolytic activity of recombinant Sak was higher than that of the native Sak. We found that the gene expression level and Sak production rate in the genetically engineered BL21(DE3) were similar to those of the native *S. aureus*.

Key words: Cloning, fibrinolytic property, isopropyl β-D-1-thiogalactopyranoside, protein expression, staphylokinase

Introduction

Cardiovascular diseases are a major cause of death and disability, where they are attributable to thrombolytic complications [1]. Thrombosis results in the formation of a solid mass of blood within the circulatory system, which can lead to changes in blood vessel walls, blood flow, and coagulability [2]. Every year, millions of deaths are reported due to thrombosis in the USA and Europe [3]. At present, thrombolytic enzyme therapies are becoming more widespread in medicine [4] and various fibrinolytic enzymes with helpful thrombolytic actions have been derived from microbial sources, including *Streptococcus pyogenes*, *Aeromonas hydrophila*, *Serratia* E15, *Bacillus natto*, *Bacillus amyloliquefaciens*, *Staphylococcus aureus*, and actinomycetes, as well as fungi such as *Fusarium* spp., *Mucor* spp., and *Armillaria mellea* [5]. The thrombolytic agents in current use for acute myocardial infarction include streptokinase, recombinant tissue-type plasminogen activator (rt-PA or alteplase), rt-PA derivatives such as reteplase and TNK-rtPA, anisoylated plasminogen-streptokinase activator complex (APSAC or anistreplase), 2-chain urokinase-type PA (tcu-PA or urokinase), recombinant single-chain u-PA (scu-PA, pro-u-PA, or prourokinase), and recombinant staphylokinase (Sak) and its derivatives [6].

Sak is produced during the late exponential growth phase by lysogenic strains of *Staphylococcus aureus* as well as many other species belonging to this genus, and is a promising blood clot dissolving agent [7]. Sak (163 amino acids (aa)) has comparatively better thrombosis specificity than t-PA, but producing it from native *S. aureus* possess a considerable risk during protein production because it is pathogenic [8]. Recombinant Sak (r-Sak) is more effective than streptokinase for the dissolution of platelet-rich arterial thrombi [9-11], where it has greater fibrinolytic and lower fibrinogenolytic effects [12].

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

The aim of study was to isolate and screen for the production of Sak in *S. aureus* isolates obtained from clinical sources, before expressing and producing Sak protein as a fibrinolytic agent in *Escherichia coli* as a non-pathogenic bacterium after transforming it with the cloned vector.

Material and Methods

Isolation and Identification of Bacterial Isolates

Six hundred and thirty samples were collected from hospitals in the Erbil-Kurdistan Region of Iraq between October 2013 and February 2014. Most of the isolates were isolated using swabs taken from the nasal passage of patients. At the Central Laboratory in Iraq, the isolates were identified using conventional methods based on culture, morphological Gram staining, and biochemical screening. Finally, 98 confirmed *S. aureus* isolates had been obtained and some of these isolates had been selected randomly for *sak* gene detection.

Other Bacterial Strains and Vector

The bacterial reference strains *S. aureus* ATCC 43300, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 12228 were obtained from Medya Diagnostic Centre in Erbil city and used as positive and negative controls for *sak* gene. *E. coli* DH5α (*sup*E44 Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end* A1 *gyr*A96 *thi*-1 *rel*A1) and BL21(DE3) (F^- *omp*T *hsdS*B (rB^- mB^-) *dcm gal* (DE3)) were obtained from Solarbio, and the pET-28a(+) vector (Novagen) was used for gene cloning and protein expression.

Detection of Sak

A heated plasma agar assay was used to detect the thrombolytic activity of the strains. First, 15 mL of nutrient agar was prepared and autoclaved. Second, 5 mL of heated human plasma was added (the plasma had been heated at 56 °C for 20 min) and mixed with the nutrient agar. The nutrient agar medium mixed with heated human plasma was poured into Petri dishes. After solidification, the *S. aureus* isolates were inoculated and the plates were incubated at 37 °C for 24–48 h [13].

DNA Extraction

Bacterial cells were lysed for DNA extraction depending on the method followed by Unal *et al.* [14] with a slight modification, where 1–3 colonies of the *S. aureus* isolates were suspended in 5 mL of sterilized Luria-Bertani (LB) broth and incubated overnight with shaking at 37 °C. Then, 1 mL of the suspension was transferred into an Eppendorf tube and centrifuged at 10000 rpm for 30 s. The pellet was resuspended in 120 µL of lysis solution (60 µL of lysostaphin solution 1mg/mL and 60 µL of lysozyme solution 10 mg/mL) and incubated at 37 °C for 60 min with mixing by inversion every 20 min. An Accuprep® Genomic DNA Extraction Kit (Bioneer, Korea) was used for genomic DNA extraction according to the manufacturer's instructions. The extracted genomic DNA from the isolates was run at 0.8% agarose gel (GeneDireX, Taiwan) to ensure that the genome was extracted. The concentration and purity of the genomic DNA samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). The samples were kept at –20 °C until further use.

Amplification of the *sak* Gene

The genomic DNA of *S. aureus* was used as a template to amplify the mature *sak* (M-*sak*) gene 408 bp, where the signal peptide sequence was eliminated from the gene to obtain the mature form by predicting the cleavage site between aa positions 27 and 28 using the signal peptide prediction program, SignalP 4.0 [15]. The two oligonucleotides supplied by SinaClon BioScience, i.e., Sak F: 5′-CGCGGATCCTCAAGTTCATTCGAC-3′ and Sak R: 5′-CCCAAGCTTTTTCCTTTCTATAACAAC-3′, where the sequence of primers was described by [16], where *Bam*HI and *Hind*III restriction sites were introduced at the 5′ ends of the forward and reverse primers, respectively. PCR was performed using a total volume of 25 µL (in 0.2 mL sterilized PCR tubes), which comprised 12.5 µL of PCR master mix, 1.5 µL of each

primer, 1.85-3.7 µL of template DNA (the volume of the template depended on the concentration of DNA extracted from the isolate in 1 µL), and the volume had been completed with sterile de-ionized/distilled water. The tubes were spun to ensure mixing and to move the contents to the bottom of the tubes. A positive reference strain (*S. aureus* ATCC 43300) and two negative reference strains (*S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228) were used in addition to another negative control (with all the components except the template DNA, where distilled water had been added).

The PCR program in the thermo-cycler comprised an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of three steps PCR cycle, comprising denaturation at 95 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 1 min [17], followed by an additional extension cycle at 72 °C for 15 min. The PCR products were analyzed by electro-phoresis at 1.2% agarose gel for 110 min at 5.5 V/cm.

Cloning of *sak* into *E. coli* DH5α

The PCR product of isolate 56 was purified with an AccuPrep® PCR Purification Kit (Bioneer, Korea). Both the purified PCR product and expression vector pET-28a(+) were digested with *Bam*HI and *Hind*III restriction enzymes (Thermo Scientific), and then incubated at 37 °C for 16 h. The reaction was stopped by purification with the same kit.

The digested *sak* gene was ligated in the linearized vector pET-28a(+) using T4 Ligase and incubated at 22 °C for 10 min, before insertion into chemically competent *E. coli* DH5α as a host. Ligation of the inserted *sak* in the extracted recombinant vector was confirmed by colony PCR amplification with a slight modification (the r-vector was extracted from the transformed *E. coli* DH5α using an AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Korea) and the gene was then amplified).

Sak Expression

To detect the production of r-Sak and to confirm the success of the cloning process, the transformant BL21(DE3)/pET-28a/*sak* was cultivated on heated plasma agar. The transformant BL21(DE3)/pET-28a/*sak* was screened for Sak expression, where 5 mL of autoclaved LB broth containing 50 µg/mL kanamycin was mixed with the transformant BL21(DE3)/pET-28a/*sak*, and then incubated overnight at 37 °C with agitation at 250 rpm. Then, 50 mL of autoclaved LB broth containing 50 µg/mL kanamycin was inoculated with 500 µL of the overnight culture and incubated at 37°C with agitation at 250 rpm until the optical density reached 0.6–1.0 at 600 nm (3–4 h). Then, 500 µL of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction, where a sample without induction was used as the control. The cultures were incubated in the same conditions for 4 h. Both the induced and non-induced samples were centrifuged at 13000 rpm for 10 min at 4 °C, and the pellet was preserved at –20 °C for subsequent analysis. Sak protein expression was analyzed by 15% Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) against a standard BLUeye pre-stained protein marker (Gene DireX, Taiwan).

Results and Discussion

Recombinant technology has been important for the development of various therapies. In the microbial environment, pathogenic organisms such as *S. aureus* produce the Sak protein [11], which has significant effects on thrombolysis.

In the present study, 98 confirmed *S. aureus* isolates had been obtained. The activity of Sak protein was detected in all 98 isolates by inoculating the isolates on heated plasma agar, which is a specific and reliable method for screening the activity of Sak [16, 18]. Table (1) shows the Sak activity results for the isolates. It has been found that for some of these isolates, fibrinolysis was visible according to the formation of a clear zone around the streaked isolate on the surface of the agar after 48 h (Figure 1), whereas fibrinolysis was observed after 24 h in others, although the width of the zone of hydrolysis was greater on the next day (48 h).

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Więckowska-Szakiel *et al.* [19] reported that Sak protein is produced during the late exponential growth phase by lysogenic strains of *S. aureus*.

Table (1): Staphylokinase producing *S. aureus* from different sources

No. of Isolate	Source of Isolate	Hydrolyzing activity of *sak* *	% Prevalence
1, 17, 21, 22, 23, 35, 38, 49, 56, 59, 60, 61, 63, 84	Nasal Passage	+	15.30%
90	Wound		
2, 3, 4, 16, 18, 24, 26, 28, 29, 30, 32, 34, 36, 39, 40, 41, 42, 43, 44, 50, 57, 58, 62, 66, 67, 68, 69, 70, 71, 83, 87, 88	Nasal Passage	++	33.67%
94	Wound		
6, 9, 11, 14, 19, 25, 31, 37, 45, 46, 47, 48, 64, 65, 79, 81, 85, 89	Nasal Passage	+++	19.38%
91	Wound		
8, 10, 12, 13, 20, 27, 33, 51, 52, 73, 74, 75, 76, 77	Nasal Passage	++++	14.28%
5, 7, 15, 53, 54, 55, 72, 78, 80, 82, 86	Nasal Passage	-	17.34%
92	Wound		
93	Urine		
95, 96	Semen		
97	Pus		
98			
Total	98		100%

*: +, ++, +++ and ++++ refer to the size of the clear zone

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

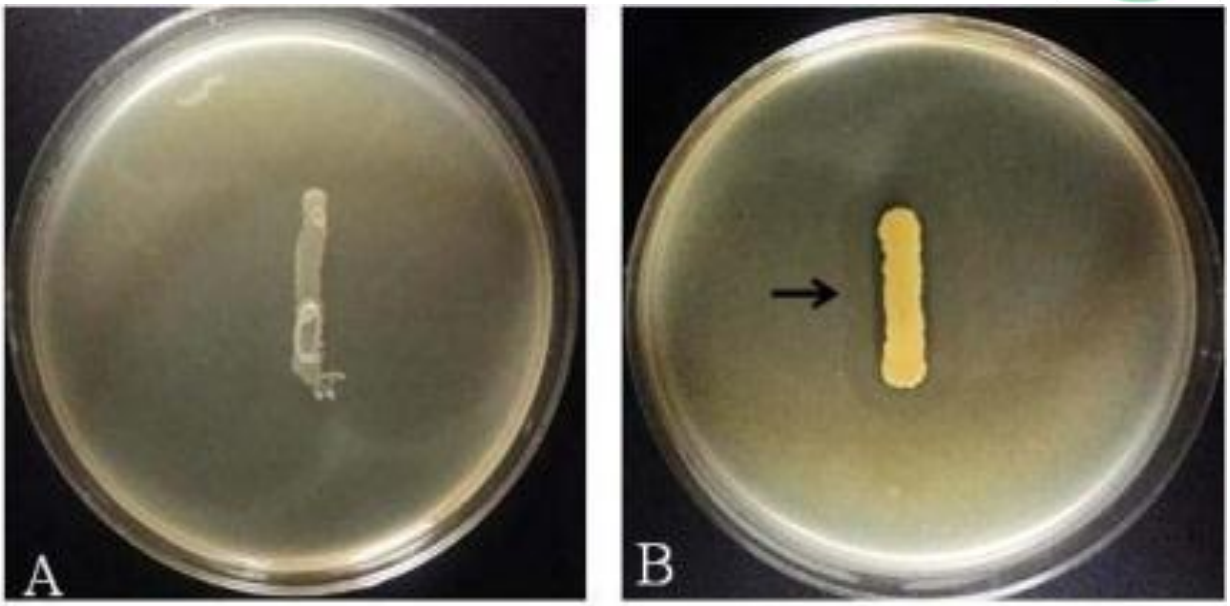

Figure 1: Analysis of M-Sak fibrinolytic activity on Heated Plasma Agar, A: Non-Sak producer isolate, B: Sak producer isolate, showing region of fibrinolysis (arrowed).

It has been found that 81 isolates (82.65%) of *S. aureus* ranged among weak (15.30%), moderate (33.67%), strong (19.38%), and very strong (14.28%) Sak producers (Table 1). These results are very similar to those reported by Naseer *et al.* [1] who found that 83.7% of *S. aureus* isolates were Sak producers. Many researchers have noted that Sak-producing *S. aureus* isolates differ in terms of the degree of fibrinolysis [11, 16, 20, 21].

Most of the isolates collected from nasal passage are Sak producers. The production of Sak is a feature that differentiates nasal isolates from staphylococci that invade internal organs [22]. In addition, Więckowska-Szakiel and his colleagues [19] postulated that nasal isolates produce Sak as an adaptive mechanism that facilitates *S. aureus* symbiosis with the host.

Sak forms an equimolar complex with human plasmin, which stimulates the activation of plasminogen. This complex is sensitive to rapid inhibition by alpha-2-antiplasmin unless it is bound to fibrin via the lysine-binding sites of plasmin. This mechanism explains the fibrin-specificity of Sak [23]. In contrast to streptokinase, Sak leads to the fibrin-specific activation of plasminogen and low immunogenicity [24]. Therefore, Sak could potentially be useful in the treatment of thrombosis and it may be a suitable thrombolytic agent for the treatment of patients with acute myocardial infarction or peripheral arterial occlusion [24].

In the present study, bacterial genomic DNA was extracted. Furthermore, the concentration and purity of the DNA extracted from the *S. aureus* isolates was measured, which showed that the concentration ranged between 11.7–31.2 ng/µL. This concentration range is comparable to that mentioned in the user guide for the kit used in this study. The purity of the genomic DNA ranged between 1.71–2.09 (based on the relative ratio of the absorbance at 260/280 nm).

The PCR product was a single DNA band with the expected length of 408 bp (Figure 2) and it was detected in *S. aureus* isolates 56, 66, and 88 (lanes 3, 4 and 6 respectively), as well as the reference strain *S. aureus* ATCC 43300 (lane 8) as a positive control. This band was not present in isolates 5, 74 (lanes 2 and 5 respectively), 92, and 94, the reference strain *S. aureus* ATCC 25923 (lane 7), and *S. epidermidis* ATCC 12228 (lane 9), which was used as a negative control. The absence of band in both isolates 5 and 92 supported our initial screening for Sak production (Table 1), whereas both isolates 74 and 94 were Sak producers but the gene was not amplified. This may due to mutations or deletion in the primer binding region [25].

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

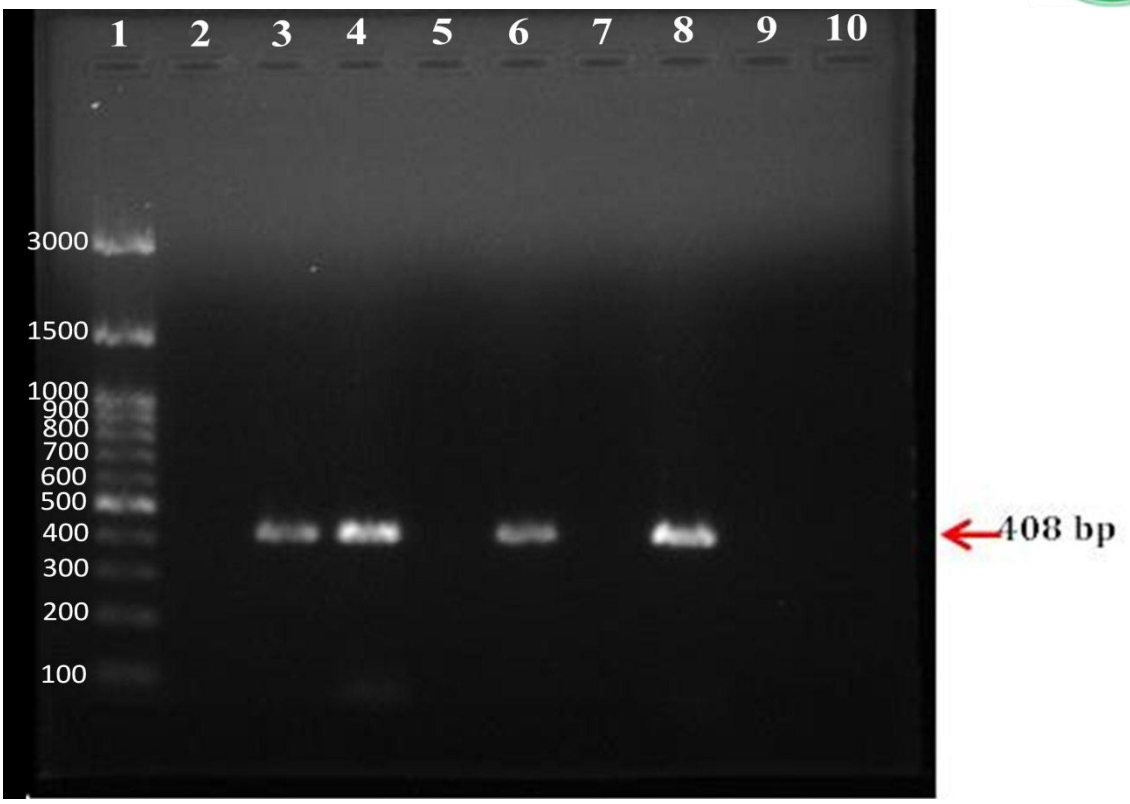

Figure 2: PCR amplified M-*sak* gene. Lane1: DNA ladder 100bp. Lanes 3, 4 and 6 represent isolates 56, 66 and 88 respectively, show *sak* gene. Lane 2 and 5 represent isolate 5 and 74 respectively show lacking *sak* gene, Lane 8: *S. aureus* ATCC 43300 is the positive control, Lane 7 and 9 represent *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 respectively, are negative control. Lane 10: Free tamplate control. Arrow indicate the size of PCR product approximately 408 bp.

The cloned vector pET-28a/*sak* was used to transform *E. coli* DH5α competent cells. The transformed cells were screened by spreading the transformation mixture onto the surface of LB agar containing kanamycin, X- Gal, and IPTG. The transformed *E. coli* DH5α competent cells produced white colonies, whereas the presence of blue colonies in the control indicated colonies that lacked the cloned vector.

Colony PCR was performed by amplifying the inserted gene into the vector by extracting the recombinant vector from each of the selected *E. coli* DH5α transformants, and then used as a template for amplification with *sak* F and *sak* R primers. Figure (3) shows the amplified recombinant gene measuring 408 bp obtained from 10 of 11 randomly selected recombinant colonies. The r-pET-28a/*sak* plasmid was extracted and the inserted gene was amplified by PCR and found to be the same size as the native gene amplified from *S. aureus*.

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

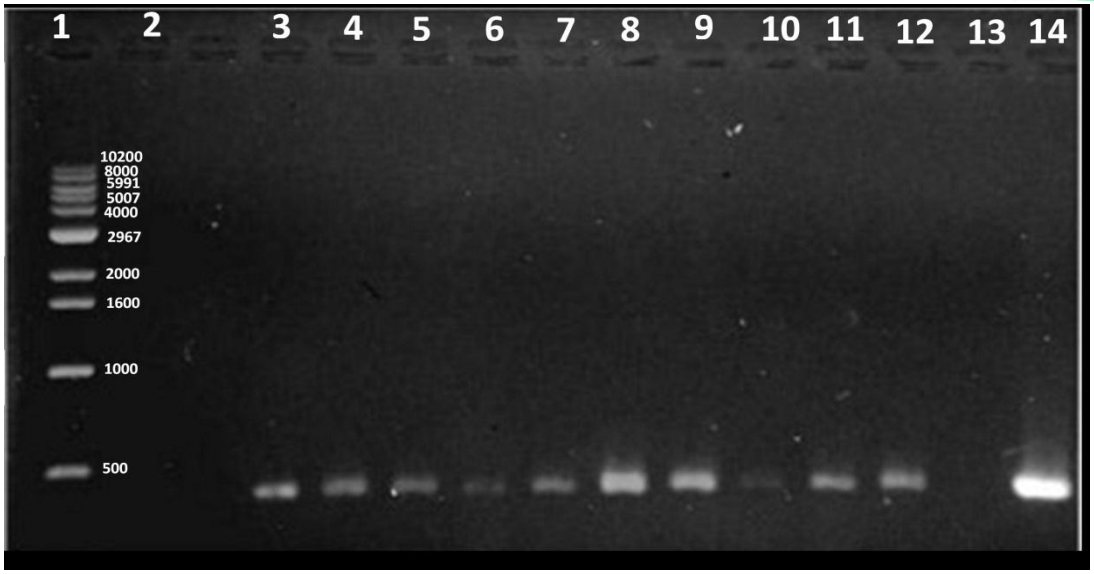

Figure 3: Gel of Colony PCR screening of 11 randomly chosen transformant colonies for colony PCR with paired of primers (Sak F and Sak R), Lane 1: DNA ladder 1Kb, Lane 2: negative control (without template), Lane 3: Native *sak* gene, Lanes 4-12 and 14 chosen colonies have the right fragment size except Lane 13 shows unsuccessing the transformation.

Sak (136 a.a.) has better clot specificity than t-PA, but its production from native *S. aureus* possess a great risk because it is pathogenic [20]. In addition, natural strains of *S. aureus* produce only negligible amounts of Sak [24]. Thus, we aimed to clone the *sak* gene to allow protein production in a non-pathogenic host, which would facilitate the production of cost-effective therapeutic protein for use in clinical practice.

To verify the expression of heterologous r-Sak activity by in-frame cloning and its translation, SDS-PAGE (15%) was performed using induced cell lysates from selected BL21(DE3) transformants. The bacterial proteins were separated according to their molecular weight and heterologous r-Sak was visible as a band among the other bacterial proteins with an estimated molecular weight of ca 15.5 KDa (lanes 2-5, Figure 4). In addition, a non-induced sample with pET-28a/*sak* was electrophoresed as a control (lane 6, Figure 4). Figure (4) shows that r-Sak was present in the samples with a molecular weight of ca 15.5 KDa (after considering the 6× Histidine tag with a molecular weight of 0.839 KDa [26]. Nguyen and Quyen [27] also reported that the molecular weight of Sak was ca 15.5 KDa.

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

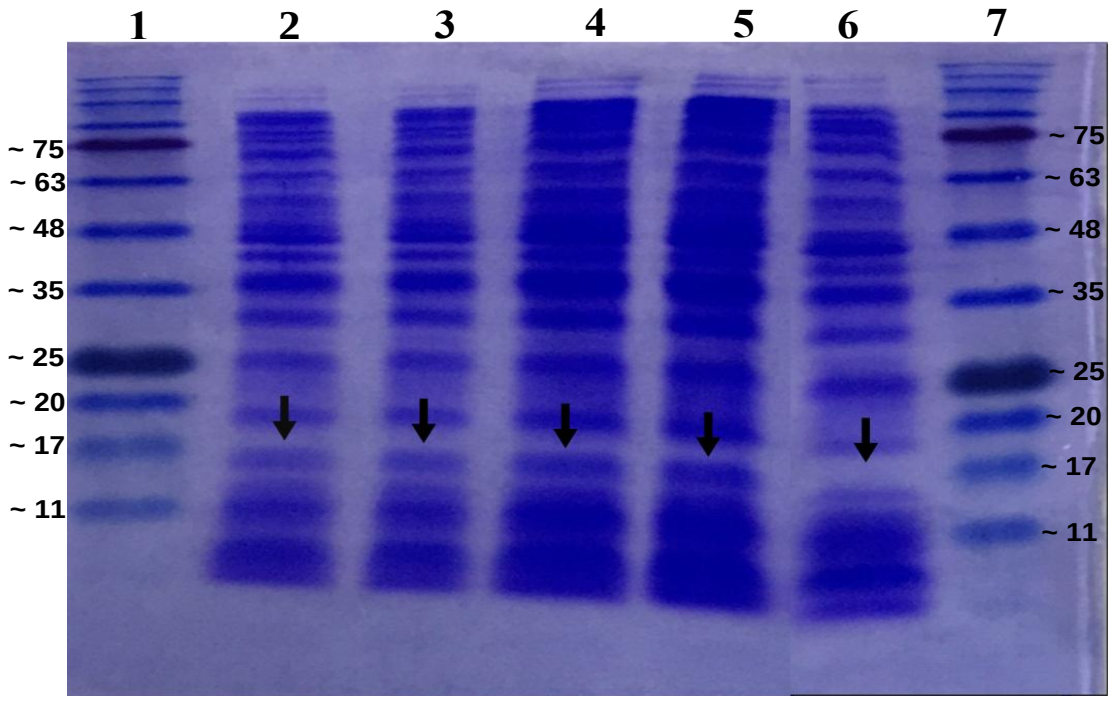

Figure 4: SDS-PAGE analysis of induced heterologous r-His-tagged-Sak. Lane 1 and 7: Protein ladder (KDa), Lanes 2-5 are induced BL21(DE3) cell lysates with IPTG and Lane 6 is non-induced BL21(DE3) cell lysates.

The expression of heterologous r-Sak based on its fibrinolytic property was investigated using heated plasma agar. A hydrolysis halo was visible around clone No. 5 of the streaked BL21(DE3) transformant after 24 h (Figure 5 B), whereas the size of halos be larger after 48 h in selected clones (Figure 5 C). The result showed that r-Sak is a good thrombolytic agent.

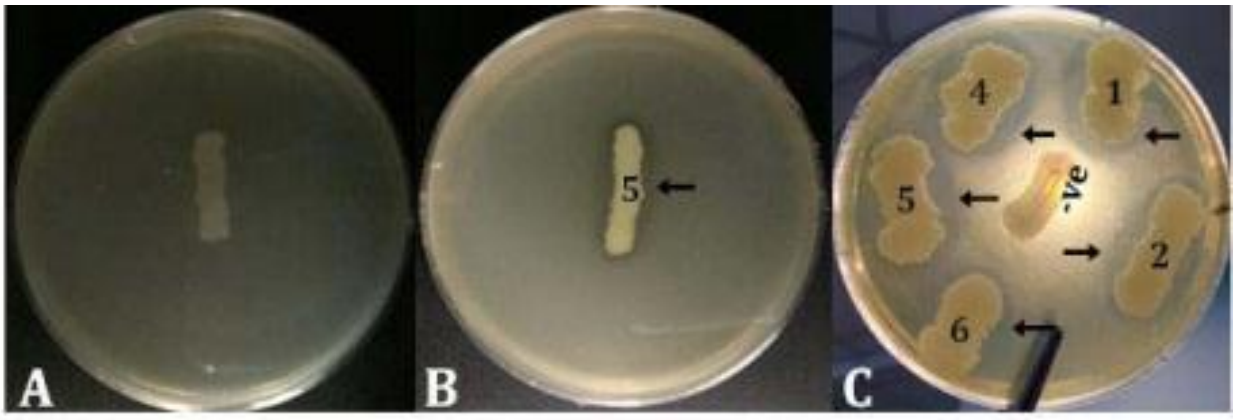

Figure 5: Analysis of r-Sak fibrinolytic activity on Heated Plasma Agar A: non-transformed DH5α is negative control, B: Transformed DH5α/ pET-28a/*sak* (arrowed) after 24 h, C: BL21(DE3)/pET-28a/*sak* (streaked colony No. 1, 2, 4, 5 and 6 of transformant BL21(DE3)/pET-28a/*sak* with fibrinolytic halo on Heated Plasma Agar after 48 h (arrowed), while streaked colony (-ve) of non-transformed BL21(DE3) is negative control.

Print ISSN: 2073-8854 & Online ISSN: 2311-6544

Conclusion

We conclude that most *S. aureus* are Sak producers and this protein is a strong fibrinolytic and thrombolytic agent. In this study, we successfully cloned the *sak* gene and the protein was expressed in transformed BL21(DE3) after induction with IPTG. The *sak* gene expression and Sak protein production levels in genetically engineered BL21(DE3) were similar to those in the native *S. aureus*. In addition, BL21(DE3) is a well-established host with a short culture time, which means that it can express large quantities of protein within a few hours. Furthermore, genetic manipulation is simple and only low-cost media are required.

Conflict of interest

All authors declare no conflict of interest.

References

1. Shagufta Naseer, B., Ravi, M. & Subhashchandra, M. G. (2014). Screening of staphylokinase producing *Staphylococcus aureus* from clinical samples. Int J Res Biol Sci, 4(2): 46-48.
2. Carton, J., Daly, R. & Ramani, P. (2007). *Clinical Pathology*, NewYork, Oxford University Press.
3. Vujcic, I. S., Sipetic, S. B., Dubljanin, E. S. & Vlajinac, H. D. (2013). Trends in mortality rates from coronary heart disease in belgrade (Serbia) during the period 1990–2010: A joinpoint regression analysis. BMC Cardiovasc Disord, 13(112): 1-8.
4. Kotb, E. (2012). *Fibrinolytic bacterial enzymes with thrombolytic activity*, Springer.
5. Mohanasrinivasan, V., Subathra Devi, C., Dhanmoni, K., Vaishnavi, B., Jemimah, N. S. & Kaustuvmani, P. (2015). Production and purification of staphylokinase from *Staphylococcus hominis* msd1isolated from kadi: A traditional indian fermented food. Int J PharmTech Res, 8(6): 265-272.
6. Laroche, Y., Heymans, S., Capaert, S., De Cock, F., Demarsin, E. & Collen, D. (2000). Recombinant staphylokinase variants with reduced antigenicity due to elimination of B-lymphocyte epitopes. Blood, 96(4): 1425-1432.
7. Kotra, S. R., Kumar, A., Rao, K. S. & Pulicherla, K. (2012). Statistical optimization of media components for enhanced production of the recombinant staphylokinase variant from salt inducible *E. coli* GJ1158. Int J Bio-Sci Bio-Technol, 4(4): 27-40.
8. Jasim, H. M., Dellol, R. A. & Hamzah, A. S. (2015). Cloning of staphylokinase gene produced from mutant *S.aureus* in *E.coli*. Int J Adv Res, 3(10): 276 - 285.
9. Collen, D., De Cock, F. & Stassen, J.-M. (1993). Comparative immunogenicity and thrombolytic properties toward arterial and venous thrombi of streptokinase and recombinant staphylokinase in baboons. Circulation, 87(3): 996-1006.
10. Li, C. J., Huang, J., Yang, Z. J. & Cao, K. J. (2007). Thrombolytic efficacy of native recombinant staphylokinase on femoral artery thrombus of rabbits. Acta Pharmacol Sin, 28(1): 58-65.
11. Reddy, Y. G. P., Prakash, R. & Anandakumar, S. (2014). Isolation, Cloning and expression of recombinant staphylokinase gene against thrombosis. Int J Pharm Pharm Sci, 6(4): 266-270.
12. Moussa, M., Ibrahim, M., El Ghazaly, M., Rohde, J., Gnoth, S., Anton, A., Kensy, F. & Mueller, F. (2012). Expression of recombinant staphylokinase in the methylotrophic yeast *Hansenula polymorpha*. BMC Biotechnol, 12(1): 96.
13. Devi, C. S., Sinha, D., Sharma, V. & Mohanasrinivasan, V. (2012). Screening for staphylokinase producing *Staphylococcus* spp. from different environmental samples. Asian J Pharm Clin Res, 5(4): 125-8.

14. Unal, S., Hoskins, J., Flokowitsch, J., Wu, C., Preston, D. & Skatrud, P. (1992). Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. J Clin Microbiol, 30(7): 1685-1691.
15. Petersen, T. N., Brunak, S., Von Heijne, G. & Nielsen, H. (2011). Signalp 4.0: Discriminating signal peptides from transmembrane regions. Nat Methods, 8(10): 785-786.
16. Pulicherla, K., Gadupudi, G., Rekha, V., Seetharam, K., Kumar, A. & Rao, K. (2011). Isolation, cloning and expression of mature staphylokinase from lysogenic *Staphylococcus aureus* collected from a local wound sample in a salt inducible *E. coli* expression host. Int J Adv Sci Technol, 30: 35-42.
17. Kotra, S. R., Peravali, J., Yanamadala, S., Kumar, A., Rao, K. S. S. & Pulicherla, K. (2013). Large scale production of soluble recombinant staphylokinase variant from cold shock expression system using iptg inducible *E. coli* BL21 (DE3). Int J Bio-Sci Bio-Tech, 5(4): 107-116.
18. Jasim, H. M., Dellol, R. A. & Hamzah, A. S. (2015). Effect of physical mutagenesis on the ability of *Staphylococcus aureus* in staphylokinase production. World J Pharm Res, 4(10): 202-212.
19. Więckowska-Szakiel, M., Sadowska, B. & RÓżalska, B. (2007). Staphylokinase production by clinical *Staphylococcus aureus* strains. Pol J Microbiol, 56(2): 97-102.
20. Jasim, H. M., Dellol, R. A. & Hamzah, A. S. (2015). Optimum conditions of staphylokinase production cloned in *E.coli* Jm109 (DE3). Int J Curr Microbiol Appl Sci, 4(12): 10-19.
21. Srinivasan, V. M., Devi, C. S., Saha, A., Mondal, M., Dhar, S., Suganthi, V. & Selvarajan, E. (2013). Screening for staphylokinase producing *Staphylococcus* spp. From bovine milk sample. Int J Pharm Pharm Sci, 5(2): 601-604.
22. Jin, T., Bokarewa, M., Mcintyre, L., Tarkowski, A., Corey, G. R., Reller, L. B. A. & Fowler Jr, V. G. (2003). Fatal outcome of bacteraemic patients caused by infection with staphylokinase-deficient *Staphylococcus aureus* strains. J Med Microbiol, 52(10): 919-923.
23. Peetermans, M., Vanassche, T., Liesenborghs, L., Claes, J., Velde, G. V., Kwiecinksi, J., Jin, T., De Geest, B., Hoylaerts, M. F. & Lijnen, R. H. (2014). Plasminogen activation by staphylokinase enhances local spreading of *S. aureus* in skin infections. BMC Microbiol, 14(310): 1-12.
24. Lee, S. J., Kim, D. M., Bae, K. H., Byun, S. M. & Chung, J. H. (2000). Enhancement of secretion and extracellular stability of Staphylokinase in *Bacillus subtilis* bywprA gene disruption. Appl Environ Microbiol, 66(2): 476-480.
25. Baum, C., Haslinger-Löffler, B., Westh, H., Boye, K., Peters, G., Neumann, C. & Kahl, B. C. (2009). Non-*spa*-typeable clinical *Staphylococcus aureus* strains are naturally occurring protein A mutants. J Clin Microbiol, 47(11): 3624-3629.
26. Nguyen, T. H. T. & Quyen, D. T. (2012a). High-level expression, purification and properties of a fully active even glycosylated staphylokinase variant SakϕC from *Staphylococcus aureus* QT08 in *Pichia pastoris*. Afr J Microbiol Res, 6(9): 2129-2136.
27. Nguyen, T. H. T. & Quyen, D. T. (2012b). Cloning, high-level expression, purification and characterization of a staphylokinase variant, sakøc, from *Staphylococcus aureus* QT08 in *Escherichia coli* BL21. Afr J Biotechnol, 11(22): 5995-6003.