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High expression of recombinant bacteria (Sak) and activity study's author

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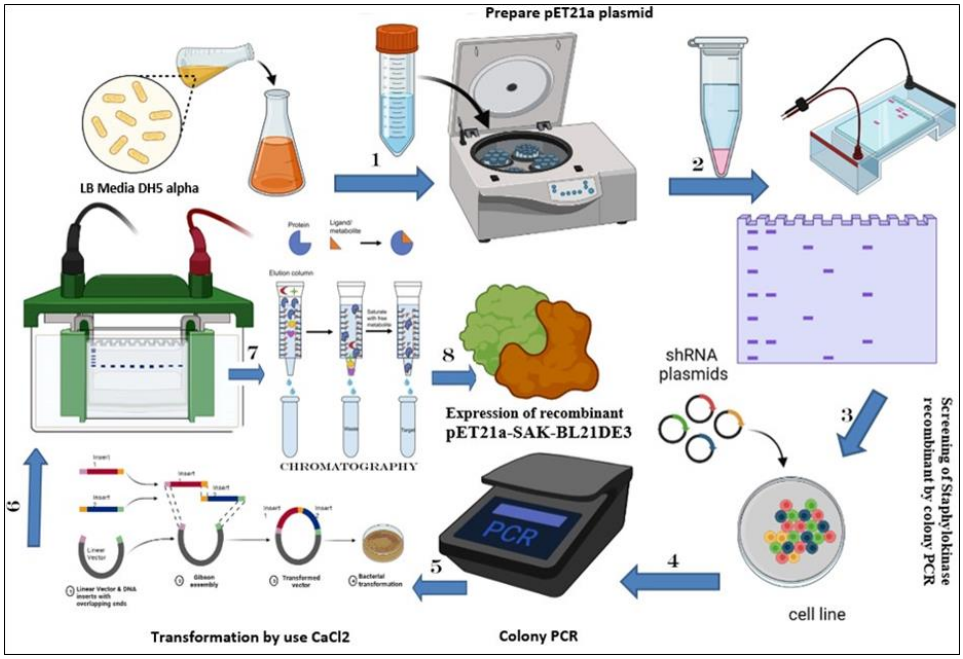
Abstract
Previous research and studies have shown the usefulness of using Staphylokinase as a thrombolytic treatments reported to have a therapeutic function and used as a potential alternative for the available clot dissolving drugs and some countries proved to be a potent alternative over the available clot dissolving drugs. The paper detailed the use of the *Escherichia coli* strain BL12DE3 for protein synthesis using the pET21a vector, and BL12DE3 activates the T7 polymerase, which encodes LacI, using the pET21a vector. It has been established that the highest level of protein expression may be obtained in the shortest amount of time. SDS-PAGE was used to obtain a more than 44% soluble protein, which was then purified using chromatography. The mature recombinant sak protein expressed in E.coli is also further extracted and analyzed by various methods to demonstrate thrombolytic activity.



Keywords: *E. coli* BL12DE3, pET21a, Recombination, DH5α- , expression, staphylokinase

Introduction
Staphylococcus aureus, discovered by Scottish physician Alexander Auguston, is the world's deadliest bacterium that threatens human life. Its presence in food is harmful not only to humans but also to animals (Louis, 1998). The biggest distinguishing feature of *Staphylococcus aureus* from other harmful bacteria is the diversity of its protein profiles, which allows it to contact host tissues and avoid the host's defense and immune systems [1] Damage to certain tissues and cells, as well as the breakdown of some infectious particles, leads to severe inflammation and significant damage, resulting in symptoms and disease [2]. *Staphylococcus aureus* is a therapeutic germ because it uses the proteins and enzymes it produces to treat some diseases, the most important of which are heart diseases and blood clots that affect blood vessels because they contain the SAK enzyme produced by a type of bacteria

known as (SAK) is the best treatment to remove the clot without additional negative consequences [3]. *Staphylococcus aureus* Kinase is an acronym for the strain from which this enzyme is formed. This protein, the enzyme found as a compound known as plasmin, has a molecular weight (15 kDa). Its composition as a proteolytic enzyme capable of activating and converting plasmin into plasminogen is critical in the breakdown and disintegration of infringement. Through plasminogen which has increased the importance of this enzyme and its active participation in the removal of blood coagulation (active clotting factor) and the production of drugs for heart diseases, coagulation disorders and atherosclerosis as well as the treatment of Blood clotting [4]. IPTG (Isopropyl ß-D-1-thiogalactopyranoside), is a molecular biology detector this molecular mimic of all lactose. IPTG considered a good metabolite for lactose [5]. This causes transcription of the lac operon which is then utilized to stimulate protein production in genes controlled by the lac operator [6]. The primary resources in the cell are transferred for the purpose of gene expression driven by T7 promoter and T7 polymerase combination. A few hours after induction, the intended product can account for more than half of total cell protein, in this potent system, expression levels may be adjusted (decreased) simply by changing the concentration of inducer [7]. This mechanism has the ability to keep target genes transcriptionally quiet in the unindicted condition [8]. The pET21a vector is a critical component of the pET system. The pET-21a vector has an N-terminal T7-Tag® sequence as well as an optional C-terminal His-Tag® motif for protein purification [9]. This vector also incorporates a selective ampicillin marker, which helps identify cells that possess this vector. The circular map shows many sites [10]. Figure 1 shows the pathway of gene induction in host cells.

Materials and Methods

1. Microbial Stains & Culture

The current investigation employed several bacterial strains to express Staphylokinase. Transduction and expression were carried out in *E. coli* strains DH5, BL21 (DE3), and the pET21a system, respectively .Except for *Escherichia coli* strain BL21 (DE3), all of the aforementioned bacterial strains were obtained from the Microbial Culture Kit at the Institute of Microbial Technology in Chandigarh, India. Genei Pvt. Ltd., Mumbai, India, provided the coli strain. Some samples were taken from patients carrying *Staphylococcus aureus* from Diwal Al-Batel Hospital, where these samples were cultured using nutrient broth and solid medium for multiplication of staphylococcal strains. "Place the isolated samples on blood agar medium (peptone: 7 g/L, beef extract: 5 g/L, NaCl: 7 g/L, blood: 7 mL/100 mL, agar: 1.5% and pH: 7.1) to distinguish Presence of a beta hemolytic organism. Selective medium, mannitol salt agar medium (beef extract: 1 g/l; peptone: 10 g/l; M sodium chloride: 75 g/l; mannitol: 10 g/l; phenol red: 0.025; agar: 1.6 pH: 7.1%, "used to screen for *Staphylococcus aureus* strains. In this study, blood agar medium (containing a base similar to nutrient agar) was added with 7% hop removed [11, 12].

2. Isolation and Screening of Staphylokinase

Most animals' skins are infested with *Staphylococcus aureus*. As a result, in the current investigation, samples were taken from pus-filled wounds and skin scrapings. The strains were carefully cultured on nutrient agar plates after being obtained using sterilized cotton swabs. Staphylokinase production in isolated samples was determined using a casein hydrolytic test and a heated plasma agar plate assay. 20 µl of bacterial samples were placed into casein and plasma agar wells and incubated overnight [13].

3. Cloning of Sak Gene from Isolated Sample

The genomic DNA was isolated from *Staphylococcus aureus* chosen on blood agar and mannitol salt agar media using the usual technique. In addition to basic lysozyme, lysostaphin is utilized in the staphylococcal cell wall lysis process. Using Gen Bank sequence information, gene specific forward and reverse primers were constructed, and PCR methods were used to isolate the sak gene using specialized custom oligos bought from Sigma Aldrich Chemicals Pvt. Ltd. The Isolated gene was used as a template to amplify mature sak with a set of primers, pET-21a expression vector is used for cloning and the sak gene was inserted in to the plasmid with the restriction sites of HindIII and NedlI [12].

4. Transformation in *E. coli* BL21 (DE3)

Transformation of a competent BL21DE3-expressing host cell with the chosen recombinant plasmid. It was prepared by preparing 20 ml LB broth and then adding BL21DE3 culture. Then it was placed in an incubator for 2 hours, then transferred to an Eppendorf, centrifuged at 5000 rpm for 8 minutes, then discarded, added 300 µl CaCl2 for 5 minutes, centrifuged at 5000 rpm for 5 minutes, discarded the suspension, added 600 µL of CaCl2, centrifuged at 4500 rpm for 10 minutes, discarded the suspension, added 700 µL LB broth, water bathed for 1 hour, centrifuged at 6000 rpm for 3 minutes, took out 900 µL of the suspension, mixed [13].

5. Expression of Recombinant SAK

Transform the expression plasmid into BL21 cells (DE3) Incubate overnight at 37 °C on antibiotic selection plates. Resuspend a single colony in 10 mL of antibiotic-laced liquid culture. 37 °C incubation Induce for 3 to 5 hours at 37 °C with IPTG (final concentration of 40 or 400 M`). Examine for expression using a Coomassie stained protein gel, a Western Blot, or an activity test. Examine expression in the entire cell extract (soluble + insoluble) as well as the soluble fraction solely. Repeat expression at a lower temperature (15 to 30 °C) or test expression in Lemo21 if a percentage of the target protein is insoluble (DE3) Inoculate 1 L of liquid medium (with antibiotic) with a newly developed colony or 10 ml of freshly generated culture for big scale 37 °C incubation [14].

Results and Discussion

1. Identification of Collected Sample

specimens taken from patients transferred to Diwal Patel Hospital were examined under a microscope and found to be Gram-positive as in (2), Samples in blood agar culture media were cultured and incubated for 48 hours at 37 °C, and it was noted that there were lytic areas on the agar plate cultured for *Staphylococcus aureus*, which was characterized by its yellow color as shown in the figure 2.

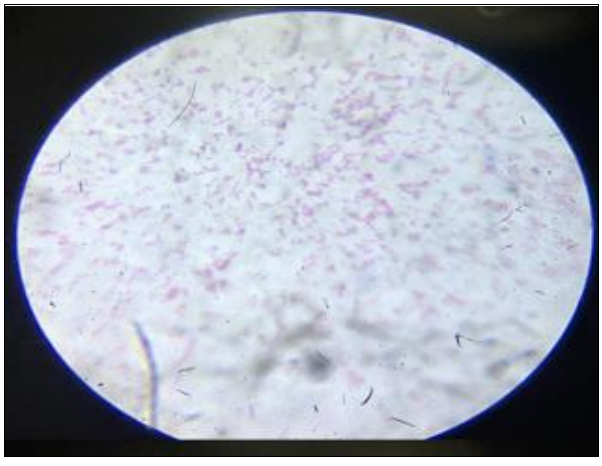


Fig 1: Test under a microscope of sample *S. aureus*

Fig 2: Clearance Zones by Hemolysis on Blood Agar Plate

2. Screening for Staphylokinase by use *Escherichia coli* D DH5α

The SAK product was digested with specific enzymes HindIII and NdeI, and the vector was digested with the same enzymes before ligating the pET21a and SAK amplicon from NdeI and HindIII using the ligase enzyme. As described in the Materials section, the reaction was set up at room temperature. Ligation was done at room temperature overnight. *Escherichia coli* DH5α strain as shown in the figure [4].

Fig 3: Screening for Staphylokinase by use *Escherichia coli* D DH5α

3. Transformation &expression protein (SAK-pET21a) in *E. coli* BL21 (DE3)

We transferred the SAK gene from the DH5α strain to the other strain (BL21DE3), as the previous strain could not express the protein. Cell harvesting after induction was performed at different time intervals (three hours) at 37 °C, respectively to find the optimal induction time. At the end cells were separated by centrifugation. Cell pellets were suspended in 1.5 mL cold lysis buffer (10 mM Tris-Cl pH 8.0), then we used a sonication apparatus for the purpose of extracting the protein from inside the cell since the sac is a protein found inside the cell and then examined on 15% SDS-PAGE gels to visualize it. Protein profile as in Figure 4.

4. Purification Recombinant SAK-pET21a Protein in *E. coli*

The expression plasmid was created by inserting the Sak gene into the expression vector pET32(a), resulting in 44% fusion protein expression. Following that, a quick and easy chromatographic approach for large-scale purification of therapeutic-grade r-Sak from *E. coli* was devised, which included Ni2+-affinity chromatography, ultrafiltration, and Q-Sepharose Fast Flow chromatography. According to SDS/PAGE and HPLC analyses, this approach resulted in the synthesis of very pure r-Sak (>99%) fig (6) .

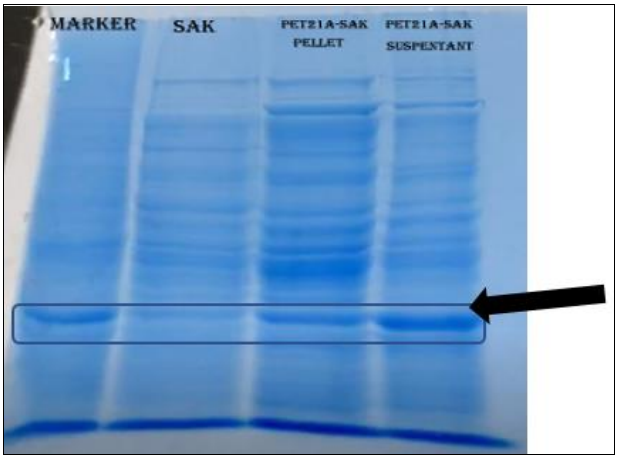


Fig 4: 12% SDS PAGE showing Expression protein (SAK-pET21a) in *E. coli* BL21 (DE3) Lane 1 marker, Lane 2 SAK, Lane 3 SAK-pET1a pellet, Lane 4 SAK-pET1a pellet supernatant

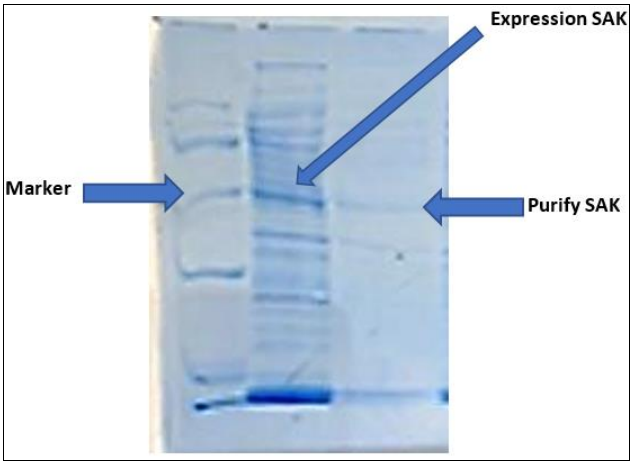


Fig 5: 11: 15% SDS PAGE Affinity Purification Recombinant Protein in *E. coli* (Purification of Proteins Using Polyhistidine Affinity Tags) chromatography

5. SAK Activity

The process of investigating the enzymatic activity of SAK by using the hot plasma agar plate method, where the results show the hydrolysis and proteolytic cleavage of plasminogen in the plasma and its conversion to plasmin by SAK protein Thus, the fibrinolysis that appears through a region devoid of inhibitory plates is in the presence of plasminogen activators that activate human plasminogen. The enzymatic activity of Staphylokinase was measured by the diameter of the area on the plasma agar plate fig (7).

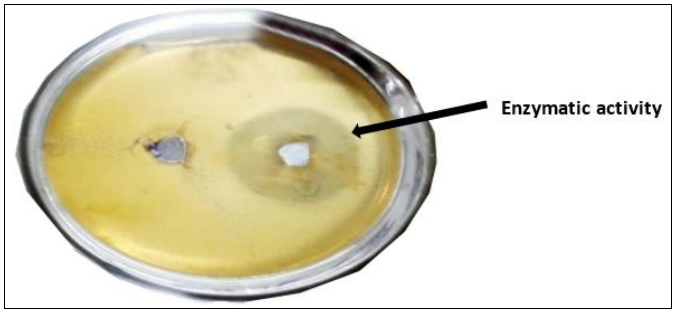


Fig 6: SAK Activity by Heated Plasma Agar Plate Assay

Conclusion

Thrombolytic treatment requires more clot-specific third-generation molecules to achieve optimum patency in a short period of time while minimising adverse effects such as bleeding risk and reclusions. SAK is one of the bacterial proteins with better clot specificity than t-PA, although manufacture from pathogenic *Staphylococcus aureus* poses a significant danger in protein manufacturing. Recombinant protein synthesis in a nonpathogenic host might be advantageous for producing therapeutic proteins at a low cost in clinical application. Staphylokinase gene was effectively extracted from a local wound isolate of lysogenic *Staphylococcus aureus* and converted into BL21 (DE3) with pET21a vector in the current work. This study clearly shown that *E. coli* BL21(DE3) with stable sakpET21a may be exploited for commercial purposes production of Staphylokinase.

When discussing the high expression of recombinant Staphylokinase and its activity, the key conclusions typically involve several aspects:

- ****Efficient Production**:** High expression levels of recombinant Staphylokinase indicate that the expression system (e.g., bacterial, yeast, or mammalian cells) used is highly efficient in producing the protein. This is often a sign that the genetic constructs, expression conditions, and purification protocols are well-optimized.
- ****Functional Activity**:** High levels of expression generally suggest that the recombinant Staphylokinase is correctly folded and active. Functional assays, such as fibrinolytic activity tests, are crucial to confirm that the protein is not only present in large quantities but also retains its biological activity.
- ****Purity and Stability**:** Achieving high expression can sometimes lead to challenges with protein purity and stability. It is important to assess whether the recombinant Staphylokinase is produced in a pure form and whether it maintains its stability over time, which can affect its effectiveness.
- ****Applications and Impact**:** High expression and activity levels enhance the potential applications of recombinant Staphylokinase in medical or industrial contexts. For instance, it could be used as a therapeutic agent for dissolving blood clots or in other biochemical processes where fibrinolytic activity is desired.
- ****Scalability**:** The successful high expression of recombinant Staphylokinase in small-scale experiments often needs to be translated into larger-scale production for practical applications. This requires addressing issues related to scaling up production, maintaining activity, and ensuring cost-effectiveness.

In summary, high expression of recombinant Staphylokinase coupled with confirmed activity indicates a successful production process, suggesting that the protein can be effectively utilized for its intended applications.

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