

## 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

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### **Abstract**

*Staphylokinase, a 163 amino acid bacterial protein from Staphylococcus aureus is 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. In the present study, sak gene is isolated from a lysogenically converted S.aureus and expressed in non-pathogenic E.coli expression host. The sak gene is cloned into pET-28a vector and expressed in an IPTG and salt induced E. coli strain (GJ1158). The mature recombinant sak protein expressed in E.coli is also further extracted and analyzed by various methods to demonstrate thrombolytic activity.*

**Keywords:** *Staphylokinase, Salt induction system, lysogenically converted S.aureus, thrombosis, thrombolysis.*

## 1. Introduction

Thrombolytic disorders have emerged to be one of the main causes of human mortality worldwide [1, 2]. A blood clot (thrombus) developed in the circulatory system can cause vascular blockade leading to life threatening consequences. A healthy homeostatic system suppresses the development of such blood clots in normal circulation, however reacts extensively during vascular injury to prevent blood loss [3]. The failure of the system to produce the bodily clot lysins such as tissue plasminogen activator (t-PA) and Urokinase leads to stroke, pulmonary embolism, deep vein thrombosis and acute myocardial pathologies. The clinical intervention to cure these disorders is carried out by the external administration of thrombolytic agents [4]. Staphylokinase (SAK), a 163 amino acid profibrinolytic agent sourced from *Staphylococcus* sp. is a potential alternative plasminogen activator [5, 9, 10, 11, 12, 13]. The structure and mechanism of action of SAK are now gaining medical significance and better understood. Staphylokinase forms 1:1 stoichiometric complex with plasmin, which activates plasminogen to plasmin [16, 17] and there on help in clot lysis by its proteolytic action [18] on fibrin, a major constituent of the thrombus formed [9, 14, 19]. The recombinant Staphylokinase has been produced in bacteria such as *Escherichia coli* [6, 7, 11, 12, 15] and shown to induce fibrin specific clot lysis in human plasma milieu *in vitro* unlike other thrombolytic agents which act on free fibrinogen in the circulating blood [10, 14]. Attempts have been made to compare the fibrinolytic properties of Staphylokinase and Streptokinase using animal models of venous thrombosis and other methods [8, 11, 14]. The clinical administration of staphylokinase however, induces neutralizing antibody formation in a majority of patients due to the heterologous nature of bacterial protein.

Staphylokinase, could be relatively inexpensive when compared to that of other thrombolytic agents and scaled up into large amounts for industrial production [20]. Apparently, the only limitation with this thrombolytic agent is its bacterial origin that could raise undesired immune responses [21]. Keeping the scope for the industrial scale development of sak as a thrombolytic agent, present work is under taken to isolate the mature staphylokinase gene from a local isolate and to clone and express from a salt inducible *E.coli* expression host.

## 2. Materials and Methods

### 2.1 Microbial Stains

Various bacterial strains were used in the present study for expression of staphylokinase. *E. coli* strains of DH5 $\alpha$  (MTCC No.1652), BL21 (DE3) (MTCC No.1679), and GJ1158 were used as maintenance and expression hosts respectively. All the above mentioned bacterial strains except *E. coli* strain GJ1158, were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. *E. coli* strain GJ1158 was procured from Genei Pvt. Ltd., Bangalore, India.

### 2.2 Culture Conditions

Nutrient broth and solid medium were used for propagation of *Staphylococcal* strains. The isolated samples were streaked on blood agar medium (Peptone: 5g/L; Beef Extract: 3g/L; NaCl: 5 g/L; Blood: 5ml/100ml; Agar: 1.5% and pH: 7.0) to find the  $\beta$ -hemolytic organism. The selective medium, Mannitol salt agar medium (Beef extract: 1g/L; Peptone: 10g/L; NaCl: 75g/L; Mannitol: 10 g/L; Phenol red: 0.025; Agar: 1.5% and p<sup>H</sup>: 7.0) served for the screening of *Staphylococcus aureus* strains. In the present study, blood agar medium (contains a base similar to nutrient agar) was added with 5% defibrinated

sheep red blood cells and used for discriminating the collected microbes. LB medium was used for culturing *E. coli* strains.

### 2.3 Isolation and Screening of Staphylokinase Producing Microbial Source

The *Staphylococcus aureus* are commonly found on the skin of most mammals [22]. Therefore, in the present study the samples were collected from pus laden wounds and skin scrapings. The strains were collected by using sterile cotton swabs and carefully grown on nutrient agar plates.

The isolated samples were analyzed for Staphylokinase production by casein hydrolytic assay and heated plasma agar plate assay. 25  $\mu$ l of bacterial samples were loaded on the wells made in Casein and plasma agar plates and incubated for overnight.

### 2.4 Identification and Cloning of Sak Gene from Isolated Sample

The *Staphylococcus aureus* selected on blood agar and mannitol salt agar medium was used for genomic DNA isolation as per the standard protocol. In addition to basic lysozyme, lysostaphin is also used in the protocol for lysing the staphylococcal cell walls. With the sequence information from GenBank data base (gi 21283614), gene specific forward and reverse primers were designed and PCR methodologies were applied for isolation of *sak* gene with specific custom oligos purchased from Sigma Aldrich Chemicals Pvt. Ltd. The Isolated gene was used as a template to amplify only the 420 bp mature *sak* with a set of primers (24 nt forward primer: CGCGGATCCTCAAGTTCATTCGAC and 27 nt reverse primer: CCCAAGCTTTTCTTTCTATAACAAC). pET-28a expression vector is used for cloning and the *sak* gene was inserted in to the plasmid with the restriction sites of *Bam*HI and *Eco* RI.

### 2.5 Expression and Activity Analysis of Recombinant SAK

Recombinant proteins were expressed in a NaCl induced *E.coli* expression host GJ1158 and IPTG inducible BL 21 (DE3). Both the bacterial strains GJ1158 and BL 21 (DE3) were grown in M9 medium. The GJ1158 and BL 21 (DE3) were induced with 100mM NaCl and 1mM IPTG respectively for 4 hrs when the cells reached to the required cell density (0.8 to 1.0 OD). The protein expression was analyzed on 15% SDS-PAGE.

Recombinant SAK is analyzed for its thrombolytic activity by casein as well as heated plasma agar plate assays. Protein activity is also analyzed by an *in-vitro* tube test described by Swetha Prasad et al. [23].

## 3. Results and Discussion

### 3.1 Identification of Collected Sample

The colony morphology was examined and they were identified as cocci and gram staining results revealed that the bacterial species to be a gram positive. All the collected microbial sources were streaked on blood agar medium and yellow colored bacterial colonies were observed after 48 h of incubation at 37<sup>0</sup>C (Figure 1). The hemolytic zones were observed on the blood agar plates.

*Staphylococcus aureus* is distinguished from other staphylococcal species using differential growth media like mannitol salt agar medium with the phenol red pH indicator (Figure 2). The results are in consonance with Schoenbaum *et al.* [24], which reported that characteristic golden yellow color colonies can be used in the identification of pathogenic *Staphylococcus aureus* from a mixture of various Staphylococcal species.

Although *Staphylococcus aureus* have the hemolytic activity but Lack (9) suggested that the hemolytic capability of *Staphylococcus aureus* is not always a specific selective medium for identifying *Staphylococcus aureus*.

### 3.2 Screening for Staphylokinase Activity

The heated plasma agar plate assay is reported to be the more specific and accurate method for determining the Staphylokinase activity. The observation revealed that when SAK producing strains were inoculated overnight at 37°C onto the heated plasma agar plate, there is a formation of clear fibrinolytic halos. After confirmation of the presence of *sak* gene in the isolated *Staphylococcus aureus*, the further experiments were carried out only with these Sak +ve *Staphylococcus aureus* strains.

To further explore and confirm the thrombolytic activity of Staphylokinase, casein hydrolytic assay (one of the specific tests for detecting the thrombolytic activity of Staphylokinase) was carried out. The zones of clearance were clearly observed on the plates after an overnight incubation. The diameter of the halo around the well was measured to check the functional activity of SAK proteins. Among all the sources, maximum zone of clearance of 3 cm was observed for *Staphylococcus aureus* strains from the wound pus. This test involves cleavage of casein analogous to fibrin in the clot atmosphere when activated by plasma milieu.

### 3.3 Isolation of Gene Coding for SAK

The full length 489 bp staphylokinase gene was extracted from the genomic DNA of screened *S.aureus*. The results of the PCR methodologies were analyzed and confirmed the 489 bp amplified *sak* gene product on 2% agarose gel by running against a 100bp DNA ladder (Figure 3). Further this gene is used as template to amplify the *Msak* with a different set of primers in PCR and amplicon is confirmed on 2% agarose (Figure 4).

### 3.4 Generation of Recombinant DNA

The recombinant DNA was generated successfully by ligating the vector pET-28a with passenger DNA *Msak* gene by T4 DNA ligase. The vector DNA and the insert (*Msak*) were ligated as per the protocol described earlier and the ligation reaction was confirmed by PCR with vector specific primers. The desired product size of 780 bp DNA was identified against a 100 bp DNA ladder (Figure 5). The rDNA was confirmed by PCR and transformed into *E.coli* DH5 $\alpha$  by heat shock method.

### 3.5 Expression and Activity Analysis

For the expression studies, the *E. coli* expression host GJ1158 and BL21 (DE3) were used for NaCl and IPTG induction respectively. This expression system was chosen owing to the simplicity of Staphylokinase that it will not require any glycosylation machinery as it is a non glycosylated protein with simple structure. The rDNA *Msak*pET-28a was extracted from the DH5 $\alpha$  and was successfully transformed into GJ1158 and BL21 (DE3) bacterial cells. Cells containing the *sak* gene were grown up to 0.8-1 O.D and induced with appropriate inducer (NaCl for GJ1158 and IPTG for BL 21 (DE3)), for 4 h at 37°C. The induced protein expression profiles were resolved on 15% SDS-PAGE. The Staphylokinase was visualized on the gel at the position corresponding to the reference or standard protein. 100mM NaCl induction in GJ1158 was identified as the optimal inducer concentrations. The time of induction was also optimized for both the cultures and the induction time of 4 hours was identified as the optimal time required for the inducer for the maximal production of recombinant staphylokinase. As mentioned earlier all protein expressions were analyzed by running on 15% SDS-PAGE and a very

clear 28.6 KDa protein band was identified against a high molecular weight protein ladder (Figure 6). The observed results in the present investigation were coincided with the similar expression patterns in *E. coli* as an extra cellular protein, the difference being only the cytoplasmic nature of the protein in the present system. Similar attempts were made to express SAK in a protease deficient *Bacillus subtilis* strain [25].

The activity of the Staphylokinase which was expressed in *E. coli* GJ1158 and BL 21 (DE3) bacterial host system was determined by heated plasma agar plate assay and by an *in vitro* tube test. The heated plasma agar plate test showed a very good clearance zones after overnight incubation through induction however prolonged incubation for 48 h increased not only the clearance zone for induced samples but also exhibited clearance zone in un-induced samples due to leaky expression of SAK by *E. coli* (Figure 7).

The clot lytic activity of newly developed thrombolytic drugs was done by adopting the *in vitro* model developed by Sweta Prasad et al. [23]. The tube test has shown nearly 30% clot degradation within two hours of clot incubated with cell lysates.

#### 4. Conclusion

Thrombolytic therapy is demanding more clot specific third generation molecules to get maximum patency in a short time with fewer side effects like minimal bleeding risk and reocclusion. SAK is one of the bacterial proteins having relatively good clot specificity than t-PA, but production from native *Staphylococcus aureus* poses a great risk in the protein production as it is pathogenic. Recombinant protein production in the non pathogenic host would be useful for cost effective therapeutic protein production in the clinical practice.

In the present study Staphylokinase gene was successfully isolated from a local wound isolate of lysogenic *Staphylococcus aureus* and transformed into BL21 (DE3) and GJ1158 with pET-28a vector. This investigation certainly has shown that *E.coli* GJ1158 with stable *sak*pET28a could be used for economic production of Staphylokinase.

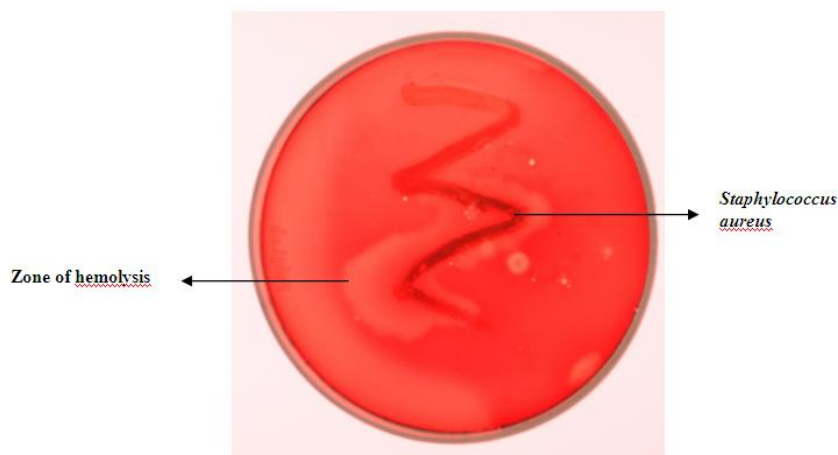
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**Figure 1. Zones of Clearance by Hemolysis on Blood Agar Plate**

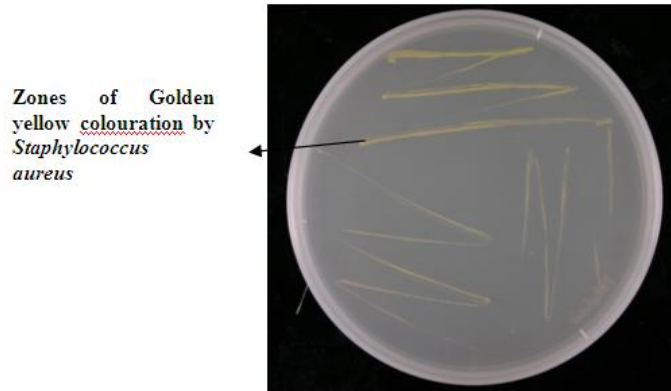


Figure 2. Zone of Clearance by Hemolysis on Blood Agar Plate

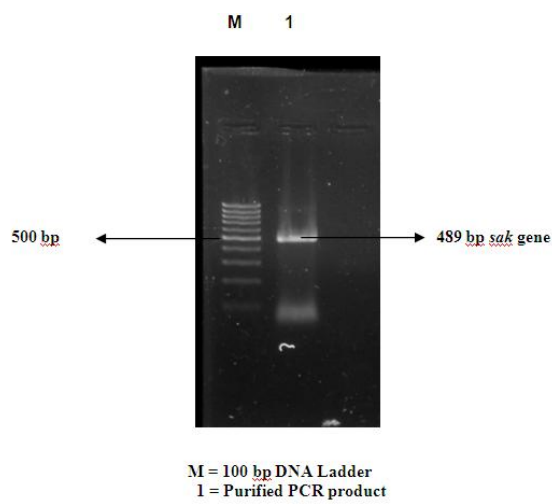


Figure 3. Purified *Sak* Gene

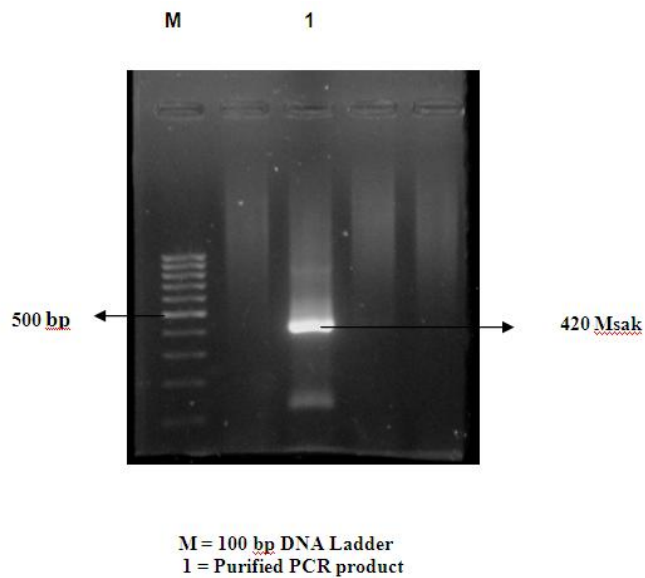
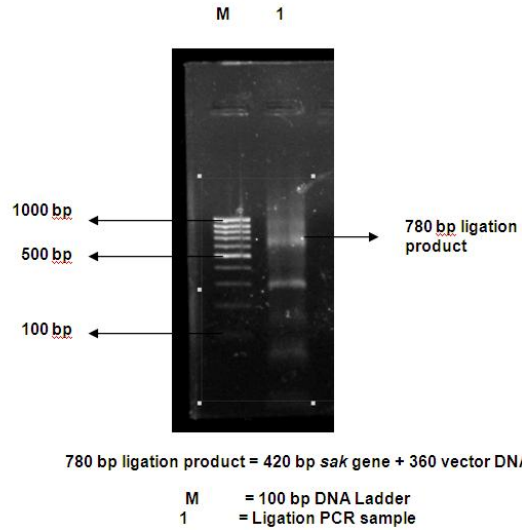
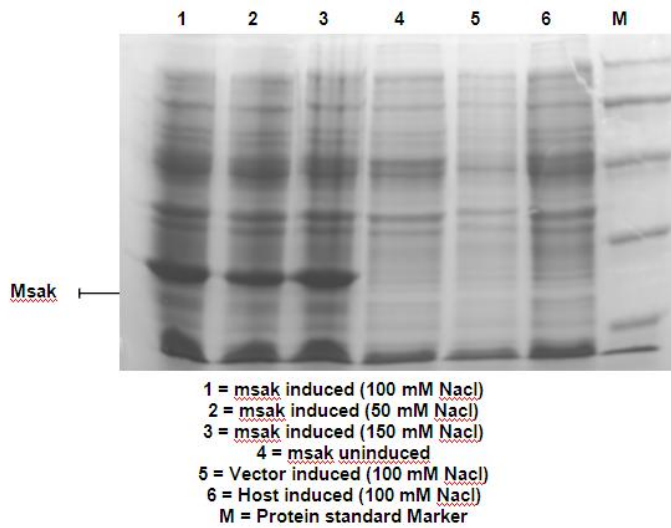


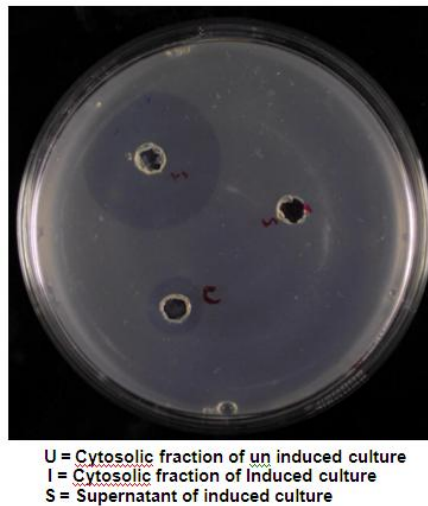
Figure 4. Purified *Msak* (420 bp)



**Figure 5. Ligation Confirmation by PCR with Vector Specific Primers**



**Figure 6. SDS Analysis of Mature Staphylokinase (Msak)**



**Figure 7. Analysis of SAK Activity by Heated Plasma Agar Plate Assay**