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# Cloning of mutant staphylokinase by novel cloning vector M13 and PET32a from mutant *Staphylococcus aureus* and role in medical applications

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**Abstract**

Study design of cases are Cross-sectional study design that descriptive study design and in analytical case-control study design by internal comparison of 500 isolates collected from different provenance of human infections including 280(56%) from tonsils , 100(20%) nose, 40(8%) tumors, 17(3.4%) urine, 27(5.4%) skin and 36(7.2%) blood, the isolation and identification achieved by using $Vitek_2$-GP and Genotypic detection of PCR to confirmative identification of *S. aureus* isolated from tumors. Fibrinolytic enzyme (Staphylokinase) assay done on plasma agar plate and Casein agar plate to determine fibrinolysis, caseinolysis on medium,

The first step of cloning occurs by using restriction enzyme BamH1 and EcoRI for cutting DNA M13 Novel cloning vector and plasmid vectors PET32a cloning vector is to grant sticky ends from DNA of mutant *S. aureus*. The second step of cloning inclusive ligated by ligate digested fragment of DNA mutant *S. aureus* (*sak* gene) with plasmid cloning vector with sticky ends that compatible with ends of *sak* gene. The third step of cloning including transform recombinant vector (recombinant PET32a and M13) into *E. coli*(DH5$\alpha$), the results showed positive transformants of *E. coli*(DH5$\alpha$) was selected on plasma agar medium containing 50 mg/ml of ampicillin as a selectable marker for 20 isolates, the result exhibit 14 isolates able to grown in presence of ampicillin and producing stapylokinase enzyme. Staphylokinase is recombinant drug therapy for hydrolyzing thrombosis. Purification of recombinant staphylokinase from recombinant *E. coli*(DH5$\alpha$) by utilizing Ammonium sulfate, Dialysis, Gel filtration and Ion Exchange chromatography of 14 transformants *E. coli* (DH5$\alpha$) express staphylokinase from mutant *S. aureus*.

Study fibrinolysis achieved by using thrombolytic enzyme *in vitro* within tubes containing blood clot (thrombi) to determine enzyme activity *in vitro* to hydrolyze thrombosis, results showed dissolve thrombosis after adding 40,50,100µl from recombinant staphylokinase (thrombolytic enzyme) through seconds.

**Key words:** Cloning, PET32a , Cloning vector, Recombinant drug therapy

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## Introduction

*Staphylococcus aureus* is a major bacterial human pathogen that causes a wide variety of clinical manifestations causing skin infection, tissue infection, deep abscess, wound infections, sepsis, endocarditis, septic arthritis and osteomyelitis [1,2]. Staphylokinase known as thrombolytic enzyme and proteolytic enzyme that act to dissolve fibrin in order to help the bacteria in spreading (invasiveness) and causing damage to tissue by interact with plasminogen that convert into plasmin (proteolytic enzyme) which hydrolyses fibrin clots and inhibiting phagocytosis [3].

Study designs are the set of methods and execution used to stack and analyze data particular in a specific seek question. One of the headmost procedures in arranging a research study is the predilection of study design. There are two types of study design in global inclusive study designs observational and interventional, in the observational study designs out of the descriptive design is the simple, in order that permit the investigator to study with depict the allocation of one or more vary, wanting regard to any causal or other proposition [4].

Cloning vector is DNA molecules carry foreign DNA into a host cell and replicates bacterial cells or yeast cells which produces numerous copies of one self with foreign DNA. The cloned genes in the vectors are not expecting to express at transcription or translation level, the vectors are utilized for generating genomic libraries for provided that the probes or utilizing in genetic engineering experiment. Cloning vectors are small, autonomously replicating DNA molecules with diverse size and shape [5].

Plasmid vector of PET32a applied as a standard cloning vector carry T7 RNA polymerase promoters flanking a unique multiple cloning site, that inclusive restriction sites for diverse endonucleases [6].

The filamentous bacteriophage M13 features as a tool in several lines of investigation, The genome of the filamentous bacteriophage M13 comprises a single-stranded circular DNA molecule, designated the (+) strand, which is converted into a double-stranded replicative form (RF) on infection of *Escherichia coli*. [7].

## Methodology

### Study design

Descriptive (Cross-sectional study designs), Analytic (Case-Control).

### Study Setting

Baghdad hospitals in 2018/ 2019.

**Study population:**

Descriptive (Cross-sectional study designs), Analytical (case-control study design) was done on 500 isolates of *S. aureus* were collected from different clinical sources of human are 280 tonsils swab, 100 nose swab, 40 tumors (swab), 17 urine samples, 27skin swab (Acne) and 36 Blood sample from hospital / Baghdad, Iraq, the study was conducted during the period from **17/11/**2018 to **17/11/**2019

**Data collection tools**

Clinical examination from different infections Include; laboratory tests for antibiotics, blood hemolysis Staphylokinase production, chemical mutagenesis and screening procedures records for chemical mutagenesis, Gene Cloning with best Cloning Vector, Screening procedures for characterization of fibrinolytic enzyme in different conditions.

**Genotypic detection of S. *aureus* by Polymerase Chain Reaction (PCR)**

Direct PCR done amplification of 16SrRNA genes (30s Subunit of ribosomal of *S. aureus*) (Kai *et al*., 2018) showed in table 3 and amplification of *sak* gene from mutant *S. aureus* with primers listed in table 1.

**Table 1: Oligonucleotide primers used for the amplification of *16srRNA* geneand *sak* gene**

| Specific primer | Primers of | Length | Product Size (bp) | Refrences |
|---|---|---|---|---|
| ***16sRNA*gene for identification of *S.aureu*** | | | | |
| **Forward primer** | 5′TCAAAGGAATTGACGGGGGC -3′ | **20** | **479** | [8] |
| **Reverse primer** | 5′AGGCCCGGGAACGTATTCAC-3′ | **20** | | |
| ***sak* gene encoded for thrombolytic enzyme** | | | | |
| **Forward primer** | 5’ AGAGATTGATTGTGAAAGAAGT GTT 3’ | 25 | **492** | [9,10,11] |
| **Reverse primer** | 3’CGAAGTACCTGCCTAAAAAAG GAT 5’ | 24 | | |

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Forward and reverse primers of *Sak* gene used to amplify specific gene of mutant *S. aureus* that supplied from Alpha DNA Company in lyophilized form were dissolved in free nuclease distilled water to give final concentration of 100 picomole /µl , then 10picomole /µl of primer (10µl of primer stock solution added to 90µl of free nuclease distilled water), mixed and stored at -20°C until use.

The amplification of *sak* gene of mutant *S. aureus* prepared by taking 12.5 µl of TaqGreen master mix, 5 µl of DNA template and 4.5 µl of nuclease free water showed in table 2.

**Table 2: Conditions for amplification of *sak* gene of mutant *S. aureus***

| Initial denaturation | No. of cycles | Denaturation | Annealing | Extension | Final extension |
|---|---|---|---|---|---|
| 95 °C for 5 min. | 35 | 94 °C for 1min. | 52 °C for 1min. | 72 °C for 1min. | 72 °C for 10 min. |

The mixture was incubated at 37°C for over night , subsequently taken 5µl in order to analyzed on 1% agarose gel electrophoresis.

**Table 3: Components of mixtures digestion with M13 and PET32a Cloning vector.**

| Component | Volume (µl) |
|---|---|
| **Sterile distilled water** | 25.6 |
| **Restriction enzyme 10x buffer** | 4 |
| **Acetylated BSA** | 0.4 |
| **Plasmid DNA (50ng/µl) PET32a and M13(cloning vector)** | 6 |
| **Restriction enzyme *Bam*HI 10 U/µl** | 2 |
| **Restriction enzyme EcoRI 10 U/µl** | 2 |
| **Total volume** | 40 |

The mixture was incubated at 37°C for 24 hr., subsequently taken 5µl in order to analyze on 1% agarose gel.

**Table 4: Component of mixture ligation of PET32a with gene insert (*sak* gene).**

| Component | Volume (µl) |
|---|---|
| T4 Ligase 10x buffer | 1 |
| T4 DNA Ligase 3 U/µl | 0.5 |
| M13 and Plasmid DNA(50ng/µl) PET32a | 1 |
| Gene Insert (50ng/µl) *Sak* gene | 3 |
| Sterile distilled water | 4.5 |
| Total volume | 10 |

The ligation mixture was incubated at a room temperature for 5 hr., then stopped the reaction by using heat at 70°C for 15 min. to inactivation of ligation reaction cool the mixture at room temperature , after that used for transformation in competent cell *E. coli* (DH5***α***).

**Bacterial transformation**

Bacterial Competent cells of *E. coli* (DH5***α***) were kept in deep freezing at -80 ºC, taken 100 µl aliquots of bacterial suspension with transferred into Eppendorf tubes with size 1.5 ml from 10 µl (50 ng/µl) of DNA ligation mixture added to the bacterial competent cells (*E. coli* (DH5***α***) ), then mixed gently with incubated in ice bath for 30 min. after that competent bacterial cells exposition to heat at 42 ºC for 45 sec without shaking, then kept in ice bath for 2 min., 400 µl of MHB (without antibiotics) was added to each reaction with shaked at 37 ºC for 1 h. , then 200 µl of transformed bacterial suspension were incubated on selective medium (plasma agar medium) containing 50µg/ml of ampicillin, 100µg/ml of IPTG ,kept at 37 ºC overnight to determine the expression of fibrinolytic enzyme (staphylokinase ) within transformed *E. coli* (DH5***α***)[12].

**Results and discussions**

**Isolation and Identification of *S. aureus***

In this descriptive study and analytical study design of 500 isolates of *S. aureus* were collected from different hospitals in Baghdad. The results of isolation from different clinical sources from patients are 280(56%) tonsils, 100(20%) nose, 40(8%) tumors, 17(3.4%) urine, 27(5.4%) skin (Acne) and 36(7.2%) Blood showed in figure (1). The results of isolation from different clinical sources of human were identified by Vitek2-GP were 280 (56%) tonsils , 100 (20%) nose, 40 (8%) tumors, 17 (3.4%) urine, 27 (5.4%) skin(Acne) and 36 (7.2%) Blood as shown in figure (1).

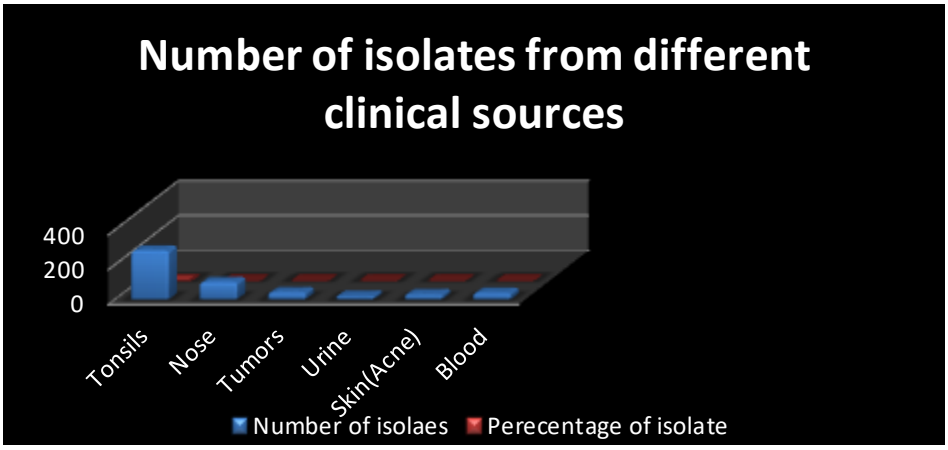


**Figure (1): Prevalence of *S. aureus* from different clinical sources with percentage of isolates.**

**Genotypic detection for mutant *sak* gene from mutant VSSA and MSSA S. *aureus* by PCR**

Genomic DNA for 100 mutant VSSA and MSSA *S. aureus* extracted by DNA extract kit, amplified by using Go Taq master mix for amplification *sak* gene , purity the genomic DNA measured by the ratio of absorbance at 260nm and 280nm (260 /280 ratio) 1.8 pure DNA. PCR technique used for amplification *Sak* gene from mutant VSSA and MSSA *S. aureus* with annealing temperatures in 52 °C , denaturation DNA in 95°C and extension in 72°C by using specific design primers, forward primer and reverse primer explain in figure(2).

Agarose gel electrophoresis achieved with products of size in PCR in order that determine presence *Sak* gene (492bp) compared with DNA Ladder Marker in size (1500bp). These results showed in figure (2) for mutant VSSA and MSSA *S. aureus* possess *sak* gene encoded for staphylokinase.

Results the genotypic analysis of sak gene showed presence of *sak* gene in all mutant VSSA and MSSA *S. aureus* after treated by Hydroxylamine (HA),Acridine Orange (AO) and Ethylemethansulfonate(EMS) chemical mutagenesis compared with control before chemical mutagenesis accomplished in agarose gel electrophoresis with product size of *sak* gene (492bp) compared with DNA Ladder (1500bp) as shown in figure(2).

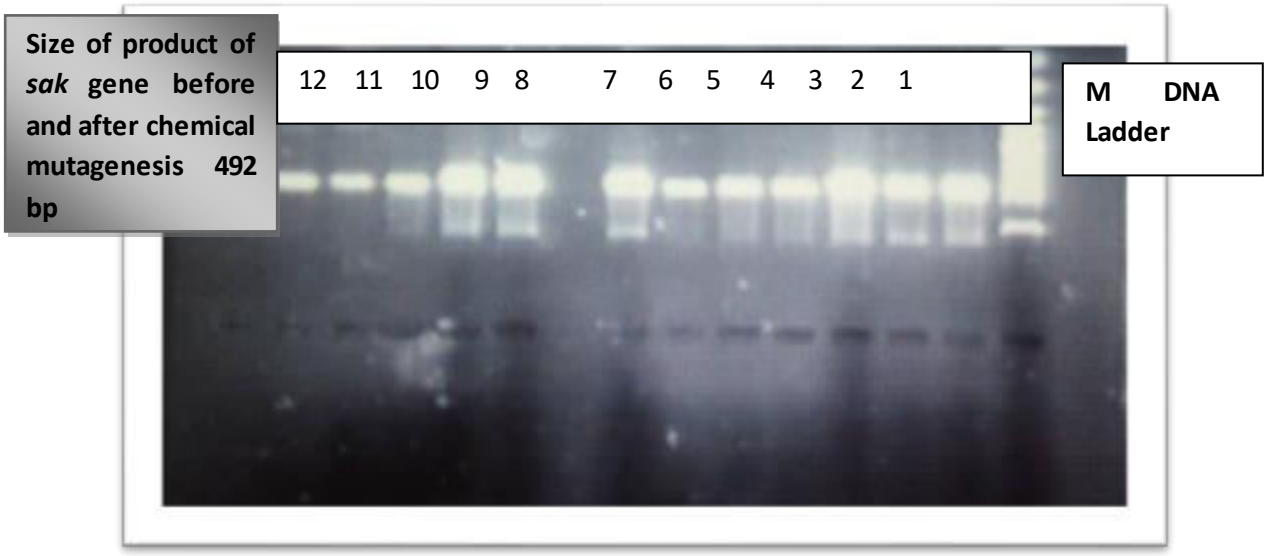


**Figure (2) : Gel electrophoresis for detection amplified mutant *sak* gene with 492bp on agarose gel (1% ), 50V for 1 hr., Lane (1 – 12) represents mutant VSSA and MSSA *S.aureus* , (M) DNA Ladder (1500 bp).**

**Cloning of *sak* gene (Genetic engineering) from mutant VSSA and MSSA *S. aureus* in competent cell E. *coli* (DH5α)**

Cloning technology has conclusive impact in therapy evolution. Staphylokinase has great decisive role in thrombotic disturbance and utilized as a drug versus thrombosis. This study was achieved for cloning *sak* gene in non-pathogenic organism as *E.coli*(DH5α) utilized for recombinant drug synthesis [13].

**Restriction of *sak* gene**

Restriction of *sak* gene from mutant VSSA and MSSA *S.aureus* achieved in order to cloning *Sak* gene in competent *E.coli*(DH5α), design primers that utilized hold two restriction sites for *Bam*HI (in forward primer) and *EcoRI*(in reverse primer) .

Results digestion of *sak* gene with *Bam*HI and *EcoRI* create fragment of *sak* gene with sticky ends.

**Restriction of novel cloning vector M13 and plasmid PET32a cloning vector**

Restriction of PET32a cloning vector was achieved by exposition with *Bam*HI and *EcoR*I in order to grant linear molecule of cloning vector with two sticky ends of *sak* gene fragment.

Processing of M13 and PET32a cloning vector with *Bam*HI and *EcoR*I extirpate the fragment of DNA within multiple cloning site (MCS).

**Ligation of digested M13 andPET32a cloning vector with digested *sak* gene by T4 DNA Ligase**

Restriction fragment of *sak* gene exposition with *Bam*HI and *EcoR*I ligated with M13 and PET32acloning vector position with two enzymes incubated at room temperature for 5 hr., subsequently ligation reaction finished by heat inactivation at 70 °C for 15 min., then cooled at room temperature, finally utilized for transformation trial.

Transformation of *E. coli* (DH5$\alpha$) with recombinant vector M13 and PET32a cloning vector with *sak* gene was achieved. Results in figure (3) shown grown recombinant *E. coli* with successful cloning process on Nutrient agar after added 50mg/ml ampicillin as a selectable marker for 14 isolates with white colony.

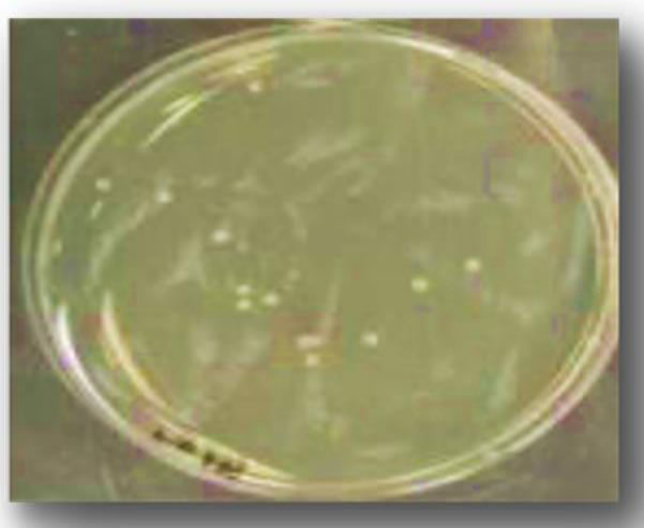

**Figure (3): recombinant *E. coli* with successful cloning on Nutrient agar added ampicillin.**

The treatment of bacterial disease by bacteriophage is known as phage therapy. Early work focused on the treatment of humans [14] although modern studies have demonstrated bacteriophage antimicrobial action in animals, aquaculture [15], food products, as well as humans [16].

A phage display construct is generated by the insertion of DNA encoding for a foreign (heterologous) protein into the bacteriophage genome. This DNA is added as a transcriptional fusion to one of the coat protein genes meaning that progeny bacteriophage subsequently express the heterologous protein as a surface fusion to the corresponding coat protein [17].

M13 bacteriophage allowed the display in technique of heterologous proteins on all five coat proteins, although by far the most commonly used are coat proteins pVIII and pIII (Russel*et al*., 2004). A fusion to these coat proteins is not consequence-free, however: peptides longer than six residues fused to every pVIII heavily decreases phage viability while substantial fusions to pIII can reduce infectivity. Nevertheless the pIII coat protein can generally tolerate much larger phage display fusions, up to 100 residues [19].

**Transformation of *E. coli* (DH5α) with recombinant M13 and PET32a Cloning vector**

Transformation of *E. coli* (DH5α) with recombinant vector M13 and PET32a cloning vector with *sak* gene was achieved, subsequently aliquot of 200 µl of transformed cells competent cells with cloning vector carrying *sak* gene was cultivated on selective medium (plasma agar medium containing 50µg/ml of ampicillin incubated at 37 ºC overnight to reveal the expression of Sak (thrombolytic enzyme) in positive transformants of *E. coli* (DH5α) hold recombinant M13 and PET32a cloning vector.

Extraction of recombinant vector (PET32a and M13 cloning vector with *sak* gene from efficacious transformant *E. coli* (DH5α) achieved by utilizing plasmid extraction kit. Results in figure (4) elucidate the recombinant vector with molecular size of 900bp. This result assured the successful transformation into *E. coli* (DH5α) with overproduction of staphylokinase.

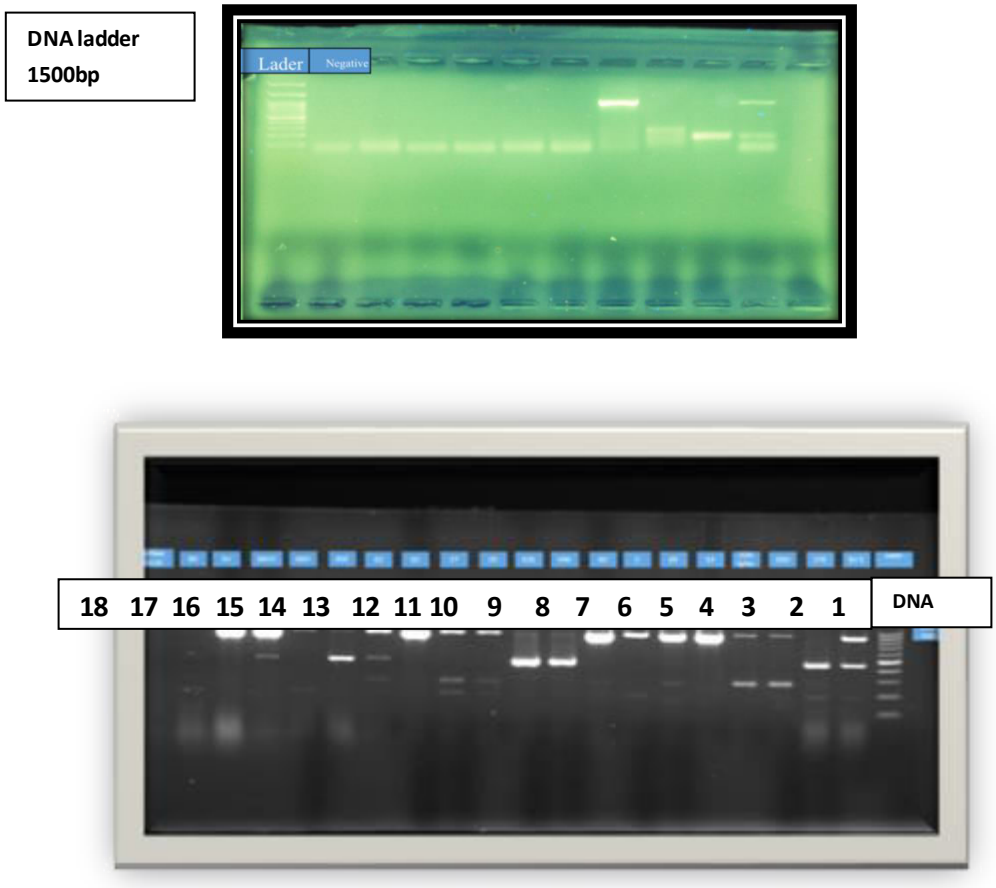


**Figure (4): Gel electrophoresis for detection of amplified recombinant M13 and PET32a (mutant *sak* gene with cloning vector M13 and PET32a ), size of product of mutant *sak* gene 492bp, size of product of Cloning vector and PET32a are 500; size of product of recombinant M13 and PET32a cloning vector are 900bp on agarose gel (1% ) , 50V for 1 hr. , (L) DNA Ladder (1500 bp).**

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A previous study by Hamza, (2015) amplification of *Sak* gene by PCR technique was carried out for (A15-M1, A15-M4, A15 , A31, A34, A43) mutant *S. aureus* when exposure to UV .light.

The treatment of bacterial disease by bacteriophage is known as phage therapy. Early work focused on the treatment of humans [20], although modern studies have demonstrated bacteriophage antimicrobial action in animals, aquaculture [21], food products [22] as well as humans [23]. Almost all trials utilize naturally occurring lytic bacteriophage for treatment, although success with genetically modified variants has been demonstrated [24,25].

A second important characteristic distinguishing phage therapy from antibiotics use is the specificity that bacteriophage exhibit for a target bacteria. This specificity is quite unlike the broad spectrum action of antibiotics. Therefore, phage therapy often uses so-called bacteriophage cocktails, where more than one bacteriophage type is applied concurrently [26].

A phage display construct is generated by the insertion of DNA encoding for a foreign (heterologous) protein into the bacteriophage genome. This DNA is added as a transcriptional fusion to one of the coat protein genes, meaning that progeny bacteriophage subsequently expresses the heterologous protein as a surface fusion to the corresponding coat protein [27].

Gene delivery utilizing bacteriophage Developed using the technique of phage display, bacteriophage (both filamentous and lytic) now show applicability in the field of gene delivery [28,29,30]. Phage display enables the expression of specific surface molecules on bacteriophage that cause binding to mammalian receptors [31]. The scientific advance lies in the additional insertion of mammalian promoter and reporter genes into the bacteriophage genome for transcription within the mammalian cell: successful internalization and expression of reporter genes GFP and luciferase [32] have been demonstrated. In a related approach not necessarily requiring phage display, the use of bacteriophage as vehicles for DNA vaccine delivery has 34 been reported [33,34]. Here, bacteriophage uptake is by antigen-presenting cells. Once engulfed and the protein coat broken down, the DNA is freed and the vaccine expression cassette is transcribed, resulting in vaccine protein expression.

Clinical and food-related investigations of bacteriophage technology can be broadly categorised into the major areas of antibacterial action, vaccination and gene therapies. Further niche applications of bacteriophage include use as groundwater tracers, as bio-defense agents and as bacterial diagnostic tools [35]. In recent years interest in bacteriophage as antibacterial agents has been somewhat revived as the limitations of antibiotic therapy have become apparent. With a thorough understanding of 35 bacteriophage biology, many of the issues described as afflicting early studies in the 20th century can now be avoided. Modern, controlled studies have successfully demonstrated microbial control by bacteriophage in a variety of settings. Advanced examples are the commercial control of *Listeria monocytogenes* on food by bacteriophage [22] and their use as antibacterial "pesticides" on commercial tomato and pepper crops [36]. Clinical trials of bacteriophage-based therapies for human use include treatment of chronic *Pseudomonas aeruginosa* infections of the ear [37]and against *Staphylococcus aureus* infections [38].

A further alternative method to control microbial infection has been to apply bacteriophage derived lytic enzymes rather than whole bacteriophage. These have been shown effective in vivo against *Streptococcus pneumonia* infections of mice [39].

Bacteriophage has been demonstrated to have a dual ability to act as tools for vaccine delivery. Firstly, using phage display, they can display vaccine antigens as fusions to their coat proteins, which elicit the desired immune response [40] Secondly, the bacteriophage particle can be used as a gene delivery vehicle to deliver a DNA vaccine expression cassette to eukaryotic cells [34].

Overproduction of staphylococcal fibrinolysin in transformants *E .coli*(DH5***α***) was revealed on plasma agar medium in accordance with formulation of zones of fibrinolysis nearly each clone, the degree of expression was revealed via measuring diameters of fibrinolysis on the medium zones. Results exhibit 14transformants successfully gene cloning of *sak* gene with cloning vector M13 and PET32a cloning vector expressed for Staphylokinase.

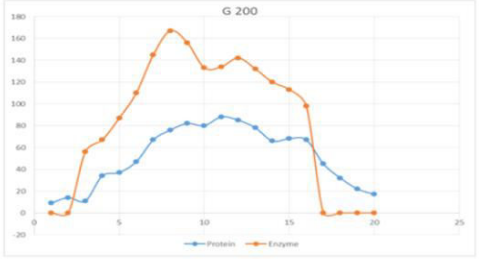
**3-10 Purification of recombinant fibrinolytic enzyme (protein engineering) by Ion Exchange chromatography and Gel filtration chromatography**

Ion Exchange chromatography can be serve as one of the utmost efficient path for protein decisiveness and purification, this mechanism is headmost, fast, quite modest and so efficient [41].

Ammonium sulphate precipitation, concentration of recombinant staphylokinase is one of the utmost excessively utilized technicality in recombinant staphylokinase purification: this method based on precipitation via ammonium sulfate salts (Salting out), its rather utilized as an costly way for condense a protein extract [42].

Results indicated in table 5 and figure(5) showed there is one protein peak which appeared in the washing step, all these protein peaks were detected by measuring the absorbance at 280nm of each eluted fraction.

| Fraction | Protein | Enzyme |
|---|---|---|
| 1 | 9 | 0 |
| 2 | 14 | 0 |
| 3 | 11 | 56 |
| 4 | 34 | 67 |
| 5 | 37 | 87 |
| 6 | 47 | 110 |
| 7 | 67 | 145 |
| 8 | 76 | 167 |
| 9 | 82 | 156 |
| 10 | 80 | 133 |
| 11 | 88 | 134 |
| 12 | 85 | 142 |
| 13 | 78 | 132 |
| 14 | 66 | 120 |
| 15 | 68 | 113 |
| 16 | 67 | 98 |
| 17 | 45 | 0 |
| 18 | 32 | 0 |
| 19 | 22 | 0 |
| 20 | 17 | 0 |




**Figure(5):peak of Recombinant Staphylokinase produced from transformants*E.coli*(DH5α)**

**Table 5: Specific Activity of Recombinant Staphylokinase produced from transformants *E.coli*(DH5α) compared with Staphylokinase produced from S.aures before cloning, absorbance at 280 nm with protein concentration at 595 nm.**

| No.Steps | Steps purification of Recombinant Staphylokinase produced from Transformants Clone *E.coli*(DH5$\alpha$) | Specific activity Before Cloning | Specific activity After Cloning By both M13 and PET32a |
|---|---|---|---|
| | **Ammonium Sulfate Precipitation** | 11.3- 15.6 U/mg | 40.2-70.3 U/mg |
| | **Dialysis** | 9.5-13.2 U/mg | 37.4-65.6 U/mg |
| | **Ion Exchange Chromatography** | 8.0-11.4 U/mg | 30.2- 62.3 U/mg |
| | **Gel Filtration Chromatography** | 8.4-10.3 U/mg | 30.5- 60.6 U/mg |

Ion exchange chromatography can be seve as one of the utmost efficacious way for protein separation and purification.This technicality is fast,quite simple and so effective, furthermore, the method depend reversible binding of a protein to reverse charge of ion exchange.DEAE-Cellulose exchanger was utilized to purify streptokinase from *Bacillus lichniformis*[41].

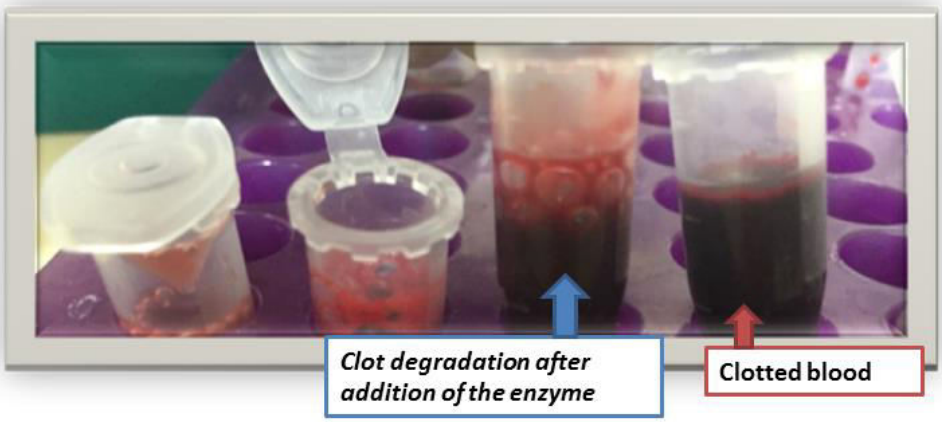
**Fibrinolytic activity (thrombolytic activity) of recombinant Staphylokinase by lysis fibrin clot *In vitro* (within tubes)**

Recovery of blood for dissolving by recombinant Staphylokinase, clot lysis activity of the purified enzyme was established according to Holmstrom method [43,44].

Determination of Staphylokinase Activity Thrombolytic activity of the filtered SAK enzyme was specified according to the modulate Holmstrom procedure. This is one of the utmost significant methods to exam the thrombolytic activity of an enzyme. In this procedure both purified SAK enzyme and ammonium sulphate precipitated specimen were utilized. 1 ml of human blood were possessed in

Appendorff tubes and pliable to clot. After the blood clotted perfectly, enzyme was added at a concentration.

Results all purified enzyme in concentration 10, 20,30,40,50,100 µl has Thrombolytic activity as shown in figure(6).




**Figure(6): Thrombolytic activity of recombinant thrombolytic enzyme*in vitro*.**

While for **ammonium sulphate** precipitated only 40,50,100 µl has Thrombolytic activity.

**Conclusions**

1-Gene expression of thrombolytic enzyme within genetically engineered *E.coli* was higher compared with non-mutant *S.aureus* before chemical mutagenesis.

2-Purification of fibrinolytic enzyme(Staphylokinase) from genetically engineered *E.coli* by using Ion Exchange Chromatography because used PET32a and M13 (Cloning vector) because current technique used to purification staphylokinase.

3-Production recombinant Staphylokinase from genetically engineered *E. coli* (DH5$\alpha$) increased highly compared before mutagenesis.

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