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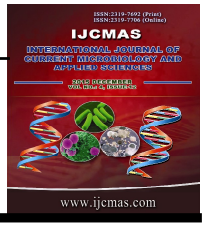

Original Research Article

Optimum Conditions of Staphylokinase Production Cloned in *E.coli* Jm109 (De3)

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Keywords

Staphylo-kinase Production, *E.coli* JM 109 (DE3), Growth factors

A B S T R A C T

Cloning of staphylokinase gene carried out in *E.coli* JM 109 (DE3). recombinant pSP72 was used to transform competent *E.coli* JM 109 (DE3) as a new and safe host for different genes and different organisms. Then positive transformants of *E.coli* (containing recombinant pSP72) was selected on plasma agar medium containing 50 µg/ml of ampicillin as a selectable marker. The degree of staphylokinase gene expression in the new host (*E.coli*) was asymbtotic to the degree of expression of the same gene in the original host (*S.aureus*). Optimum conditions for staphylokinase gene expression in genetically engineered *E.coli* was studied under two growth factors. Results of optimization showed that the degree of gene expression in new host was increased when transformant *E.coli* JM 109 (DE3) was grown in Luria – Bertani agar containing IPTG (as inducer for gene expression) in a concentration of 100 µg / ml and incubated at 37 °C for 24 hours. Effect of incubation temperature on staphylokinase activity produced by genetically engineered *E.coli* JM 109 (DE3) transformant was studied in range of temperature. Results showed that the optimum temperature for staphylokinase activity was 37°C, at this temperature the diameter of zone of hydrolysis was 30mm, while the enzyme activity was decreased above and under this temperature.

Introduction

Thrombolytic therapy is needing more clot specific third generation molecules to get maximum patency in a short time with fewer side effects like minimal bleeding risk and re occlusion. SAK (420 bp) is one of the bacterial proteins having relatively good clot specificity than t-PA, but production from native *S. aureus* poses a great risk in the protein production as it is pathogenic. Cloning of SAK gene to investigation protein production in the non pathogenic host would be useful for cost effective therapeutic protein production in the clinical practice (Pulicherla *et al*., 2011).

There are very important substances acts as inducer substance for Lac operon of *E.coli* like Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG binds to the lac repressor and altering its conformation causing lac

repressor and reducing its affinity to the lac operator and initiate protein synthesis (Wink, 2006). Effect of incubation temperature on staphylokinase activity produced by genetically engineered *E.coli* BL21 affected with changing temperature (Thi and Dinh, 2012).

Materials and Methods

Isolation of *S. aureus* from Clinical Samples

In order to isolate *S. aureus*, a total of 200 clinical samples were collected from hospitals in Baghdad governorate during the period between November and December 2013. After identification by use selective media and biochemical tests according to (Harlly and Prescott, 2002) and confirmed by using VITECK-2 we got 54 isolates.

Assay of Staphylokinase Production

Assay of staphylokinase production on plasma agar plate was carried out according to Pulicherla *et al*. (2011).

Genomic DNA Extraction

Genomic DNA of *S.aureus* was extracted according to boiling method described by klingenberg *et al*.(2004).

Amplification of Staphylokinase (*Sak*) Gene

Amplification conditions summarized in table (1) by use :

Forward primer: 5'- CGCGGATCCTCAA GTTCATTCGAC-3'

Reverse primer: 5'- GAATCTAGACCCA AGCTTTTTCCTTTCTATAACAAC-3'
The conditions of polymerase chain reactions indicated in (table 1) were optimized at different annealing temperatures between 50 an 60 ˚C by using gradient thermo cycler.

PCR products were analyzed on agarose gel (1%) using horizontal electrophoresis unit, gel was immersed in 0.5X TBE buffer, then samples were loaded into the wells of the gel. Electrophoresis was carried out for one - two hours at 50V.

After electrophoresis gel was stained with 10µl of ethedium bromide stock solution. DNA bands were visualized by using U.V transilluminator at 365 nm. in presence of 1500bp DNA ladders marker (Maniatis *et al*., 1982).

Sequencing of Staphylokinase Gene

PCR products from the amplification of staphylokinase gene were sent to NICEM/USA ABI3730XL Applied BIOSYSTEMS to determine the complete nucleuotide sequence of the gene.

Cloning of Staphylokinase Gene

Cloning of staphylokinase gene steps carried out according manufacturer company (Promega,2015), transformation of Sak gene in *E.coli* JM 109(DE3) by use pSP72 cloning vector was carried out according to (Sambrook, and Russell,2001)

Optimum Conditions for Staphylokinase Production

Optimum conditions for recombinant staphylokinase production by transformats of *E.coli* JM109 (DE3) were carried out according to Thi and Dinh (2012). After each experiment, production of stataphylokinase was assayed.

Effect of Temperature on Staphylokinase activity

Effect of temperature on staphylokinase activity for recombinant staphylokinase production by transformats of *E.coli* JM109 (DE3) were carried out according to Thi and Dinh (2012).

Results and Discussion

Isolation and Identification of *S.aureus*

In this study, and in order to isolate *S. aureus*, a total of 200 clinical samples were collected from Baghdad hospitals includes urine samples (100 samples) and another 100 samples collected from skin infections. All sample were then cultured on blood agar and manitol agar,then incubated at 37 ºC for 24 hours. Identification isolates carried out by biochemical test and confirmed by VITECK-2 tests. Results showed there are 35 isolates obtained from samples collected from skin infections (35%),while only 19 isolates were obtained from samples collected from urinary tract infections(19%). A study by Al-Marjani *et al*.(2015) identify 60 isolates of *S.aureus* from wound infections.

In other study carried by Nandita and Stanley (2014), they collected 101 samples from urine infections and they identify 23 isolates of *S.aureus* from total samples in a percentage of 22.7 %. In locally study by Al -Marjani and Hadi(2013), 86 Staphylococcus isolates from urine, blood and swaps from different hospitals in Baghdad, 64.1% of were identified as methicillin resistant *Staphylococcus spp.*

Staphylokinase Production

In this study, ability of staphylokinase production by local isolates of *S.aureus* was examined by using well diffusion method. Results showed that all of the 54 isolates were able to produce staphylokinase on plasma agar medium according to formation of zone of hydrolysis around each well containing culture filtrate of each bacterial isolate. Results also showed that these local isolates differ in their abilities in staphylokinase production due to differences in size of zone of hydrolysis around each well. Results showed that the zones of hydrolysis were ranged between 25 mm and 36 mm while mutant *S.aureus* was 38mm. Hence the most efficient isolate in staphylokinase production was mutant *S.aureus* A15-M1 isolated from skin infections because the size of zone of hydrolysis around the well containing its culture filtrate was 38 mm.

These results are in consonance with other studied achieved by Pulicherla *et al*. (2011) and Yerasi *et al*.(2014), Shagufta *et al*. (2014), who detect staphylokinase production by *S.aureus* isolated also from skin infections and obtaining clear zones of hydrolysis on plasma agar medium after an overnight incubation at 37 ºC. Genomic DNA of the (54) isolates *S.aureus* were extracted by boiling method and considerd as a templet DNA for amplification of staphylokinase gene. Concentration of genomic DNA was 50 µg /µl. From this DNA concentration, aliqoutes of DNA were taken and used for amplification of *Sak* gene. Amplification of *Sak* gene by PCR technique was carried out for all *S.aureus* isolates and mutatnt (*S.aureus* A15-M1). Forward primer contain site restriction for *BamHI*, while reverse primer contain site restriction of *XbaI*. Reaction products of gradient PCR were analyzed on (1%) agarose gel to identify the amplified Sak gene in presence of 1500 bp DNA ladder marker. Results of amplification shown in figure (1) represents that there is a DNA

fragment of about 400 bp obtained after electrophoresis on agarose gel.

Nucleotides Sequencing of *Sak* Gene

The nucleotide sequence alignment between *Sak* gene of locally isolate *S.aureus* A15-M1 (Query) and standard strain (Sbjct) was carried out to ensure that there is no restriction site for *BamHI* and *XbaI* in *Sak* gene of standard strain and the size of PCR product (*Sak* gene) was 411bp. This study consonance with Thi and Dinh (2012) and *Pulicherla et al*.(2013) in which *Sak* gene was isolated from *S.aureus* and amplified by PCR and analyzed on agarose gel in presence of 100bp DNA ladder marker wich the size of PCR product (*Sak* gene) was 411bp.

Cloning of *Sak* Gene in *E.coli* JM 109 (DE3)

Cloning technology has crucial impact in therapy development. Staphylokinase has major crucial role in thrombotic disorders and used as a drug against thrombosis (Yerasi *et al*., 2014). So that, this study was carried out for cloning *Sak* gene in non-pathogenic organism well known as *E.coli* JM 109 (DE3) used for recombinant drug synthesis.

Restriction Digestion of *Sak* Gene

In order to cloning *Sak* gene from *S.aureus* in *E.coli JM 109 (DE3)*, two specific primers were used first used for amplification of *S.aureus Sak* gene. These primers were designed to be consist of two restriction sites for *BamHI* (in the forward primer) and for *XbaI* (in the reverse primer). Hence the complete nucleotide sequence of *Sak* gene indicated in figure (2) doesn't have restriction sites for these two restriction enzymes to ensure that treatment of staphylokinase structural gene with *BamHI* and *XbaI* doesn't destroy the nucleotide sequence of the gene. Results of restriction digestion of staphylokinase with *BamHI* and *XbaI* generates a restriction fragment of *Sak* gene with stick ends then purification of digested *Sak* gene was achieved with concentration 50 ng/µl and kept in -20 °C until use for further experiment.

Restriction Digestion of pSP72 Cloning Vector

Restriction digestion of pSP72 cloning vector was carried out by treatment with *BamHI* and *XbaI* to obtain linear molecule of the cloning vector with two sticky ends compatible with Sak gene fragment treated previously with the same restriction enzymes. Results illustrated in figure (2) showed the linearized DNA molecule of the cloning vector after electrophoresis on agarose gel (1%) in presence of 10kb DNA ladder marker. This vector of 2462 bp by carrying AP^r as a selectable marker was purified for the cloning staphylokinase gene.

Treatment of pSP72 cloning vector with *BamHI* and *XbaI* excise the fragment of DNA between these two enzymes within the multiple cloning site (MCS) in genetic restriction map of pSP72 cloning vector.

Cloning of Staphylokinase Gene in pSP72

Restriction fragment of staphylokinase gene treated with *BamHI* and *XbaI* was ligated with pSP72 treated with the same two enzymes, and inoculated at room temperature for five hours, then ligation reaction was terminated by heat inactivation at 70 °C for 15 minutes and cooled at room temperature, then used for transformation experiment.

Expression of *Sak* gene Cloned into *E.coli* JM 109 (DE3) Transformants

Transformation of *E.coli* JM 109 (DE3) with recombinant vector pSP72 (cloning vector with *Sak* gene insert) was carried out according to Sambrook and Russell (2001), aliquot of 200 µl of transformed cell suspension (DNA ligation mixture and competent cells) was plated on selective medium (plasma agar medium) containing 50µg/ml of ampicillin and 100µg/ml of IPTG, the plates were kept at 37 ºC overnight to detect the expression of staphylokinase gene in transformed *E.coli* JM109(DE3) and positive transformants of *E.coli* JM 109 (DE3) containing recombinant pSP72 were selected. IPTG was caused induction of *sak* gene expression which its binds to the lac repressor and altering its conformation causing lac repressor and reducing its affinity to the lac operator and initiate protein synthesis. Then gene expression of transformants was detected on plasma agar medium according to the formation of zones of hydrolysis around each clone. For conformation of the gene expression, well- difussion agar (specific method) was used and the degree of expression was detecting by measuring the diameters of zones of hydrolysis on plasma agar medium. Results showed that five transformants out of 83 (6%) were succefully expressed its own *Sak* gene according to the formation of zone of hydrolysis on plasma agar medium between 21-30mm as shown in figure (3) and genotype shown in.

Sak gene isolated from clinical sources was cloned into non-pathogenic *Escherichia coli* (DH5α) and it was produced recombinant staphylokinase (r-SAK) protein used for thrombolysis (Yerasi *et al*., 2014). A study achieved by Seetha *et al*.(2012) *Sak* gene from *S.aureus* was cloned in *E.coli* GJ1158 and gene expression in *E.coli* transformants was detected by formation of zone of hydrolysis on plasma agar medium.

Optimum Conditions for Gene Expression of *Sak* gene in *E.coli* JM 109 (DE3) Transformnts

Several factors affecting Sak gene expression was examined. These factors include the effect of IPTG as an inducer for gene expression and effect of growth medium. Expression of *Sak* gene under the effect of both factors was studied by detection the hydrolysis zones on plasma agar medium according to the well diffusion method.

Effect of IPTG on Staphylokinase Production

Effect of IPTG in induction of gene expression of *Sak* gene in transformants was examined by using different concentrations of IPTG (0, 25, 50, 75, 100 µg/ml) . Results illustrated in figure (5) showed that maximum gene expression was obtained when IPTG was added to the culture medium in a concentration of 100 µg/ml. At this concentration of inducer the diameter of hydrolysis on plasma agar medium was reached 30mm after incubation at 37 ºC for 24 hours for the most efficient transformant of *E.coli* JM 109 (DE3).

This result was consonance with those obtained by Pulicherla *et al*.(2011) when they used IPTG for induction of *Sak* gene expression in *E. coli strain (GJ1158)* and detect the expression on plasma agar medium. On the other hand, Yerasi *et al*. (2014) was used IPTG in induction of staphylokinase production from *E.coli DH5α* on plasma agar.

Effect of Growth Medium on Staphylokinase Production

Effect of growth medium on *Sak* gene expression in genetically engineered *E.coli JM109(DE3)* was studied. These growth media (Luria agar medium, brain – heart infusion agar medium and nutrient agar medium) were supplemented with 20% human plasma to detect thrombolytic activity of staphylokinase. Diameters of zones of hydrolysis on these media were used as indicator for gene expression and staphylokinase production. Results illustrated in figure (6) showed that Luria agar medium was the optimum for induction of gene expression because the diameter of zone of hydrolysis around well in this medium was 30 mm in comparison with diameters around wells in other media.

Effect of Temperature on Staphylokinase activity

Effect of incubation temperature on staphylokinase activity produced by genetically engineered *E.coli* JM 109 (DE3) transformant was studied in range of temperature (30, 32, 34, 36, 37, 39, 40°C). Results illustrated in figure (7) showed that the optimum temperature for staphylokinase activity was 37 °C, at this temperature the diameter of zone of hydrolysis was 30mm, while the enzyme activity was decreased above and under this temperature due to the effect of these temperatures on enzyme tertiary structure and leads to denature the enzyme or decrease the activation energy for transforming the substrate to product.

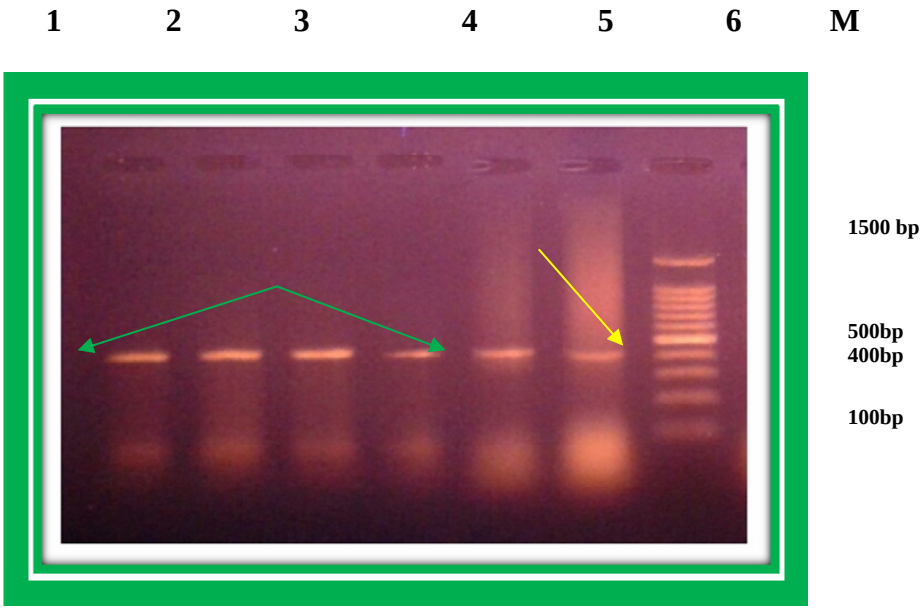
These results were consonance with these obtained by Thi and Dinh (2012) who mentioned that the optimum temperature for staphylokinase production by *E. coli* BL21 was 37 °C after incubation for 24 hours.

In conclusion, Gene expression and staphylokinase production in genetically engineered *E.coli* was in similar level of the gene from source microorganism (*S.aureus*). Optimum conditions for staphylokinase gene expression in genetically engineered *E.coli* includes the addition of IPTG as inducer to Luria medium in concentration of 100μg/ml. Incubation at 37 °C for 24 hours was best temperature of staphylokinase activity.

Table.1 Conditions for Amplification of Staphylokinase Gene

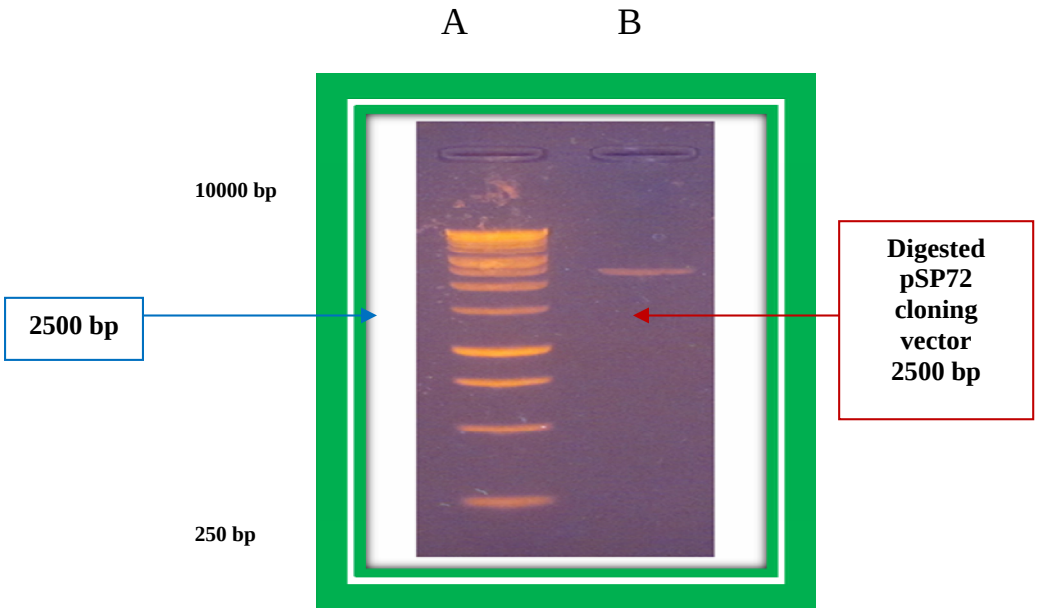
Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
95 ˚C for 5 min.	35	94 ˚C for 1min.	51 ˚C for 1mins.	72 ˚C for 1 min.	72 ˚C for 10 min.
			52 ˚C for1min.	72 ˚C for 1min.	72 ˚C for 10 min.
			53 ˚C for 1 min.	72 ˚C for 1 min	72 ˚C for 10 min.

Figure.1 Gel Electrophoresis for Amplified Staphylokinase Gene on Agarose Gel (1%), 50V for 1 Hour



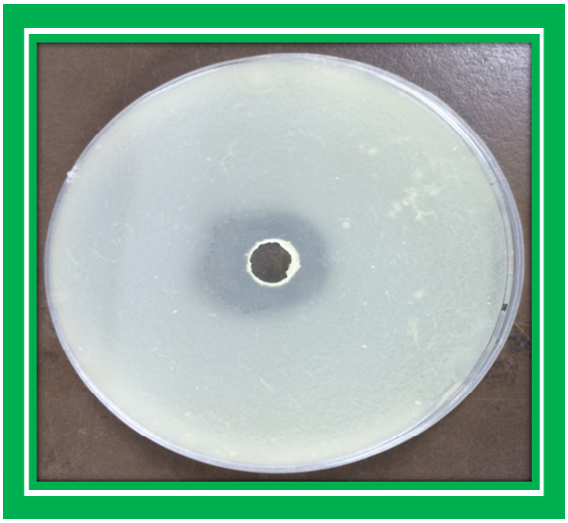
Lane (1 – 6) : *S.aureus* isolates (A15-M1 , A15-M4 , A15 , A31, A34 , A43)
(M) : DNA ladder (1500 bp)

Figure.2 Gel Electrophoresis on Agarose Gel (1%) for pSP72 Cloning Vector Digested with *BamHI and XbaI*



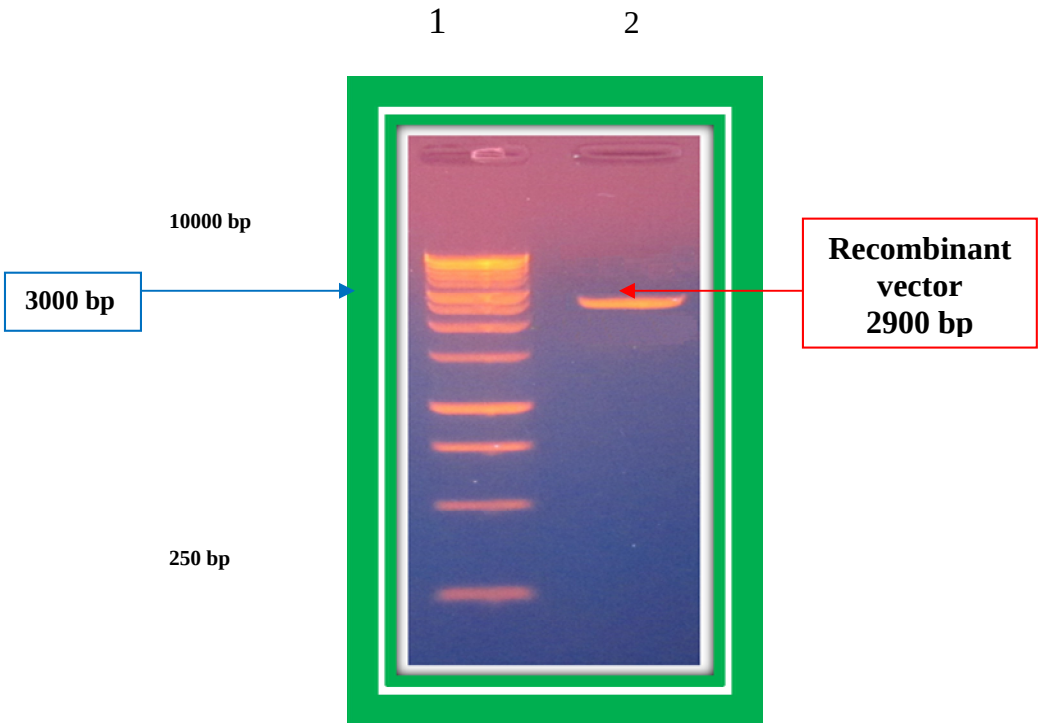
Lane (A) : DNA ladder marker (10000 bp) .
Lane (B) : Digested pSP72 cloning vector .

Figure.3 Expression of *Sak* Gene Cloned into *E.coli* JM 109 (DE3)



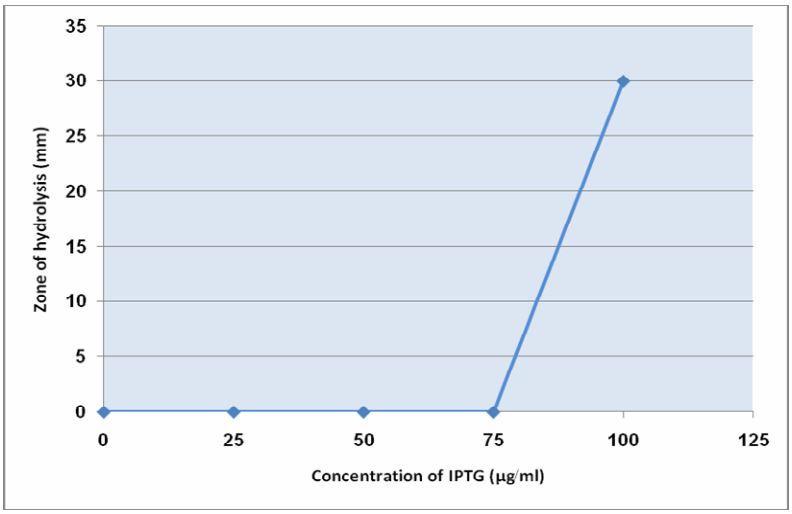
Transformants on plasma agar on the bases of zone of hydrolysis after incubation at 37°C for 24hours

Figure.4 Gel Electrophoresis for Recombinant pSP72 on Agarose gel (1%) after Extraction after Extraction from Transformant *E.coli* JM 109 (DE3)



Lane (1) : DNA ladder marker (10000 bp) .
Lane (2) : Recombinant vector (pSP72 cloning vector with *Sak* gene)

Figure.5. Effect of IPTG on Staphylokinase Gene Expression in



E.coliJM109(DE3) transformant on plasma agar medium after incubation at 37 ^{o}C for 24 hours

Figure.6 Effect of Growth Medium Type on *Sak* Gene Expression in Genetically Engineered *E.coli* JM109 (DE3) after Incubation at 37 °C for 24 Hours

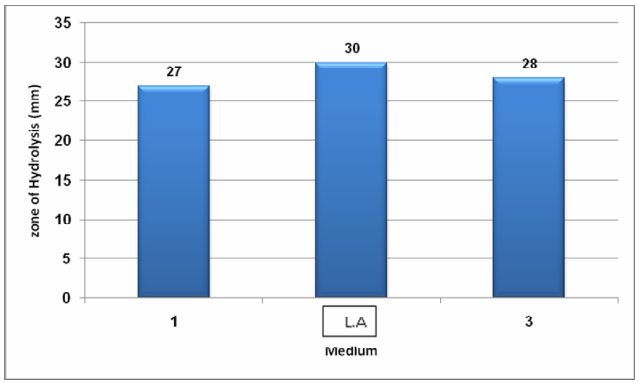
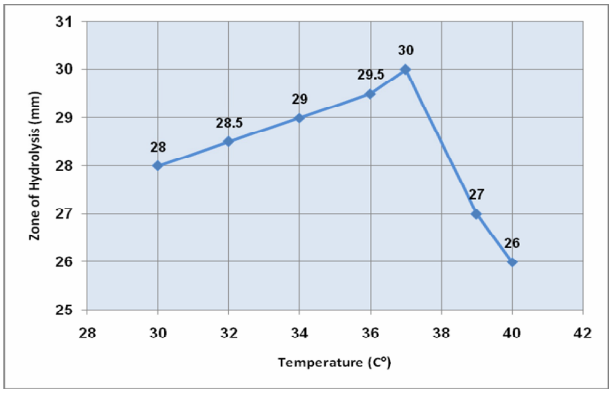


Figure.7 Effect of Temperature on Staphylokinase Activity in *E.coliJM109(DE3)*



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