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Research Article

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Over Expression of Recombinant Staphylokinase and Reduction of Inclusion Bodies Using IPTG as Inducer in E. coli BL21 DE3

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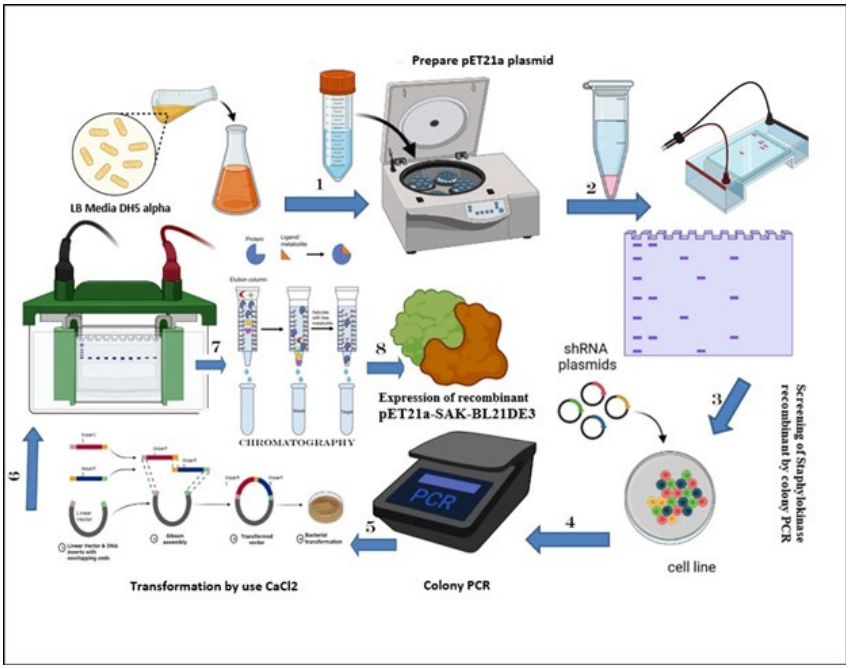
Abstract

A previous research and studies have established the significance of describing Staphylokinase as a thrombolytic treatment, particularly in patients with cardiovascular disease, stroke, and other life-threatening disorders. Staphylokinase plays a significant part in the coagulation process through formation plasmin-Staphylokinase complex on the surface of the clot which activates plasminogen is generated by some strains of Staphylococcus aureus. The publication described the use of the E. coli strain BL12DE3 to produce the protein via the pET21a transporter is predicated on the use of a chemical stimulator (IPTG) which plays an important role in regulating protein recombinant by stimulating protein synthesis. Strain BL12DE3 activates T7 polymerase, encoding LacI via the pET21a transporter. It has been demonstrated that the IPTG inducer achieves the maximum level of protein expression in the shortest amount of time. A Staphylokinase was generated as a soluble protein in excess of 45 % by SDS-PAGE and subsequently purified by chromatography.

Keywords: *E. coli* BL12DE3, pET21a, Recombination, IPTG, Expression, Staphylokinase

Graphical Abstract



Introduction

Staphylococcus aureus which is identified by Scottish surgeon Alexander Auguston is the most dangerous human life-threatening bacteria on the planet, its presence that spreads through food is not affects humans only but also animals [1]. The most important characteristic of Staphylococcus aureus from the rest of the pathogenic bacteria is the multiplicity of its protein profiles that help it attach to host tissues and escape from the host's defense and immune systems [2]. Damage to some tissues, cells and the disintegration of some infectious particles cause severe inflammation and significant damage resulting in symptoms and disease [3].

S. aureus can be considered 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 it contains the SAK enzyme that is generated by a kind of bacteria known as (SAK) is the best treatment to remove the clot with no additional negative consequences [4]. Staphylokinase is an abbreviation for the strain from which this enzyme produced Kinase from *Staphylococcus aureus*. This protein, the enzyme that takes the form of a complex called as plasmin has a molecular weight (15.5-16.5 kDa). Its structure of a proteolytic enzyme that is able to activate and convert plasmin to plasminogen plays an important role in the fragmentation and fragmentation of infringe. Through plasminogen, which increased the importance of this enzyme and its active role in the removal of blood clotting (active clotting factor) and the manufacture of drugs for heart diseases, thrombotic diseases and atherosclerosis, as well as in the treatment of blood [5].

IPTG (Isopropyl ß-D-1-thiogalactopyranoside), is a molecular biology detector this molecular mimic of all lactose. IPTG considered a good metabolite for lactose [6]. This causes transcription of the lac operon which is then utilized to stimulate protein production in genes controlled by the lac operator (Gomes et al., 2020). 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. This mechanism has the ability to keep target genes transcriptionally quiet in the unindicted condition [7]. One of the most essential vectors in the PET system is the pET21a vector. The pET-21a vector has an N-terminal T7-Tag® sequence as well as an optional C-terminal His-Tag® sequence that may be utilized to purify recombinant proteins [8]. This vector also includes a selective ampicillin marker, which aids in the identification of cells containing this vector. The circular map depicts distinct locations [9]. The induction of gene in host cell mechanism was described in below Fig. 1.

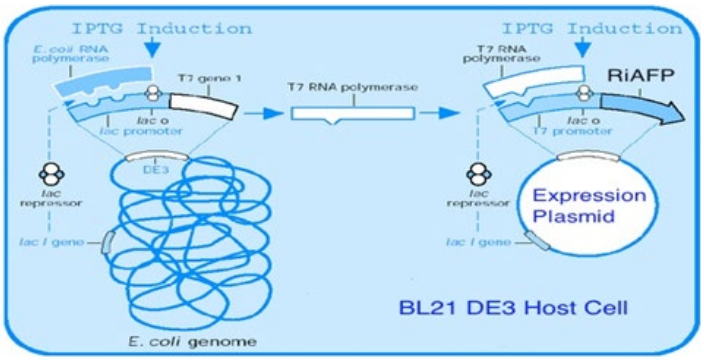


Figure 1: Overall IPTG Induction in the Host Cell

Materials and Method

Bacterial Genetic Isolation (Preparation pET21a Plasmid)

Plasmids are circular extrachromosomal DNA fragments found in bacteria and yeast. The expression plasmid pET21a was obtained from an E. coli cell DH5 harboring the plasmid using an alkaline lysis technique in this experiment. DH5α containing 5 ml of pET21a5 plasmid was cultured overnight, and the following day, the bacterial culture was centrifuged at 8000 rpm for 2 min at room temperature, discarded suspended, 600µL of TE buffer was added, separated and discarded, Add 10µL of suspended lysozyme and place the incubator in a water bath at 37°C for 30 min, sonicate the samples for 10 /20 sec pulses, then add 50µL 1% SDS, add 10µL of proteinase after that incubation in a water bath at 56°C For 45 min, add P:C:I the same volume of sample mixed well and then centrifuged at 10,000 rpm. For 10 min, carefully transfer to a fresh tube, add 10µl of Ranse and incubate in a water bath at 37°C for 30 min, add C: I in an equal volume of sample mixture centrifuged at 10,000 rpm. For 10 minutes, transfer fresh tube only Layer Aqueous, add 2 x vol. Sample chilled ethanol with 15µl NaCl reverse mixture by hand 3 times by centrifugation at 10,000 rpm. -10 minutes, add 500µl of 70% ethanol by centrifugation at 10,000 rpm. For 10 minutes, discard the suspended dry spin: at 56°C at 20 minutes, add 50µL distilled water and store [10].

Purification and Extraction pET21a plasmid

The above reagents were added as mentioned in the table in an autoclaved Eppendorf, tube with the sequence of plasmid, water, buffer, and finally enzyme. The tubes were incubated at 37°C for 1 hr. After 1 hour the reaction mixture was pooled in one tube, so that the volumes in 120µl, the tube was incubated at 37ºC for another hour (Tsai et al., 2017). During incubation period the preparatory gel was cast. The sample was loaded in 6X gel loading dye and the gel was run. The UV Trans illuminator was cleaned, the gel was places on the Trans illuminator and the DNA band was cut to utilize a perfect cutting edge. The excised DNA of digested pET21a vector was transferred in a fresh Eppendorf tube. The gel piece was weighed and to it 3 volumes of buffer QG was added. 1 volume of isopropanol was added to the above blend. The spin section was spun on 2 ml tube and the above blend was

applied to the column. The section with accumulation tube was spun at 10000rpm for 2mins. (For more specimen, step 3 and 4 was repeated utilizing same column). The flow through discarded. 750µl of PE buffer added to column. The segment was spun with the accumulation tube at 10000rpm for 1min. Dry spin: The segment with accumulation tube was spun again at 10000rpm for 1min. Elution: The segment was placed on fresh Eppendorf tube and 60µl of DNase free water was added. It was allowed to remain for 2min. The flow through was saved. This is purified digested vector is to be used for cloning [11]

Isolation Staphylokinase

Synthetic DNA was manufactured by Integrated DNA Technologies (IDT) in California, USA. Strains, Vectors and Chemicals, Reproduction was carried out using Escherichia coli BL21DE3, which is the expression host, the bacteria were cultured in Luria Bertani agar medium, the ratios were 1.25 g yeast and tryptophan isolates from Sodium chloride with the antibiotic represented by penicillin at a rate of 100µg. A 10 ml of Luria Bertani broth were taken from Staph*ylococcus Aris*. After that, the SAK gene was isolated by special isolation methods, and then tested in a 1% electrophoresis device at 150 volts, 50 A for 15 minutes [12].

Development of SAK recombinants using digested pET21a vector and SAK amplicon

The NdeI and *Hind III* Enzymes digested pET-SAK vector amplicon were ligated using ligase enzyme. The reaction was set up at room temperature as mentioned in Material section. The ligation was performed overnight at room temperature, Competent *E. coli* DH5 cells were transformed with ligated product and plated on LB agar + Amp plates. The plates were incubated at 37°C overnight, and the recombinants produced on the plates are shown in the figure below. Transformed cells were plated on LB agar + Amp plates. The plates were incubated at 37°C overnight, and the recombinants produced on the plates are presented in the figures below [13].

Screening of Staphylokinase recombinant by colony PCR

To screen the Staphylokinase-containing recombinants, colony PCR was performed with the SAK gene specific primers PCD8 and PCD10, as described in the technique. The PCR cycle conditions are also followed exactly as indicated in the methods section. The PCR result was placed onto a 1% agarose gel for a rapid clone confirmation testing. The third colony PCR in the above PCR figure revealed a very strong intensity and the correct size band, indicating the SAK gene insertion into the pET21a vector (Table 1) [14].

Table 1: Restriction Digestion of pET21a

Requirements	*NdeI (µl)*	*Hind III*
Vector (pET21a)	40	40
Buffer	Buffer-O 6	Buffer-*EcoRI* 6
Enzyme	2	2
Water	12	12
Total	60	60

Transformation of *E. coli* BL21 (DE3)

Transformation of the selected recombinant plasmid into a competent BL21DE3-expressing host cell. It was made by preparing 20 ml of LB broth and then introducing BL21DE3. cultures, and placing them in the incubator for 2 hours, then transferred to Eppendorf and put in centrifuge 5000rpm 8 min ice after that discard supinated and add 300µL CaCl2 for 5 min, centrifuge 5000rpm at 5 min discard suspensions and add 600µL CaCl2, cetrifuge4500rpm at 10 min, discarded suspensions and add 1ml CaCl2 mix properly and put in ice for 30min, heat shock 42°C for 2m and transfer in ice for 5 min. add 700µL from LB broth and keep in water bath 1 hour, centrifuge 6000rpm for 3min, removed 900µL suspensions & mix 100µL and plated [15].

Results and Discussion

Isolation, purification and extraction pET21a plasmid

The pET21a plasmid (the vector) was isolated from cultured Escherichia coli DH5α carrying the plasmid by alkaline lysis method. Using a protocol for the plasmid isolation kit in the Methodology section as shown in Figure 2, the intrinsic restriction enzymes cleave the double-stranded DNA at specific loci known as the restriction recognition sequence. PET21a is doubly digested with NdeI and HindiIII in this experiment, and the restriction recognition sites for these enzymes are presented below. NdeI: CA/TATG/Hindi III: AAGCTT Then we purified the PET21a plasmid by special enzymes for the purpose of obtaining the required plasmid and using it in the process of gene insertion for the purpose of cloning and expression of the recombinant proteins. Positron emission tomography is commonly used. Target genes are cloned into PET plasmids under strong control of T7 phage transcription and translation. The T7 RNA polymerase gene is inserted into the modified E. coli host (BL21DE3) in front of the LacUV5 promoter. When T7 RNA polymerase is fully catalyzed by IPTG [16]. We extract the purified plasmid from generation and according to the methods mentioned in the mode of action as shown in Figure (2). Recombinant production became one of the fundamentals of the industrial revolution, which began in various fields of the pharmaceutical industry and knowledge of living organisms. It has been essential in most programs for the discovery of modern drugs and small molecules over thirty years [17].

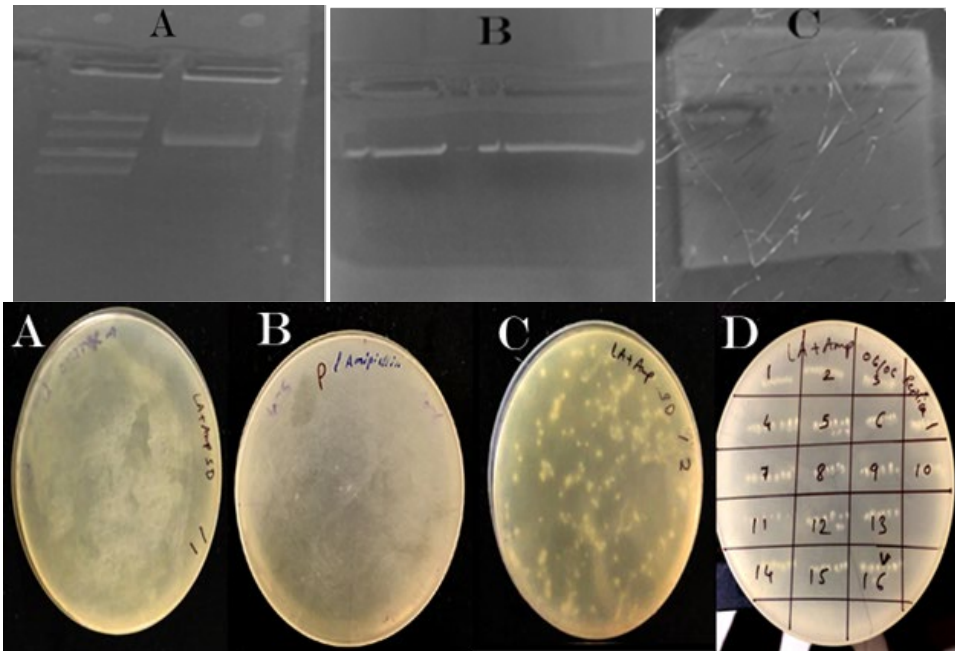


Figure 3: Isolation pET21a plasmid and loading 5 µl from S1, S2in 1% agarose gel showing: lane 1 and 2

To screen the Staphylokinase-containing recombinants, colony PCR was performed with the SAK gene specific primers PCD8 and PCD10, as described in the technique (Fig 3). The PCR result was placed onto a 1% agarose gel for a rapid clone confirmation testing (Fig 4 and 5). The third colony PCR in the above PCR figure revealed a very strong intensity and the correct size band, indicating the SAK gene insertion into the pET21a vector [18].

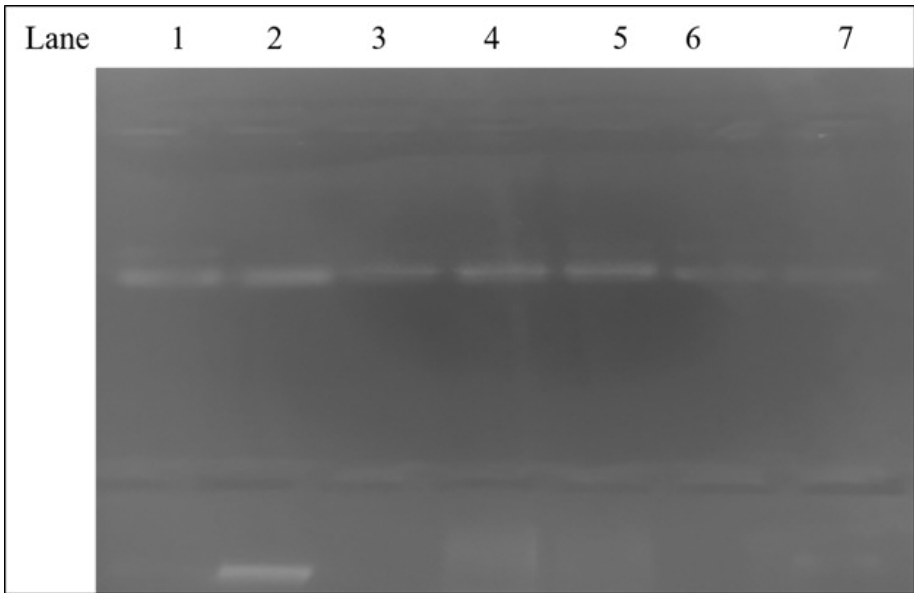


Figure 4: Colony PCR Loading sequence
1) Negative Control:10µL
2) Colony V-10µL
3) Colony No:1 -10µL
4) Colony No:2-10µL
5) Colony No:3-10µL
6) Colony No:4-10µL
7) Colony No.5:10µL.

Forward Primer: for cloning SAK gene with N Terminal His tag in pET vector 5' CCG CAT ATG CAT CAT CAT CAT CAT CAT TCA AGT TCA TTC 3' Reverse Primer: for cloning SAK in PET 21a vector with STOP codon 5' CCG CCG GAA TTC AAG CTT TTA TTT CTT TTC TAT AAC 3'.

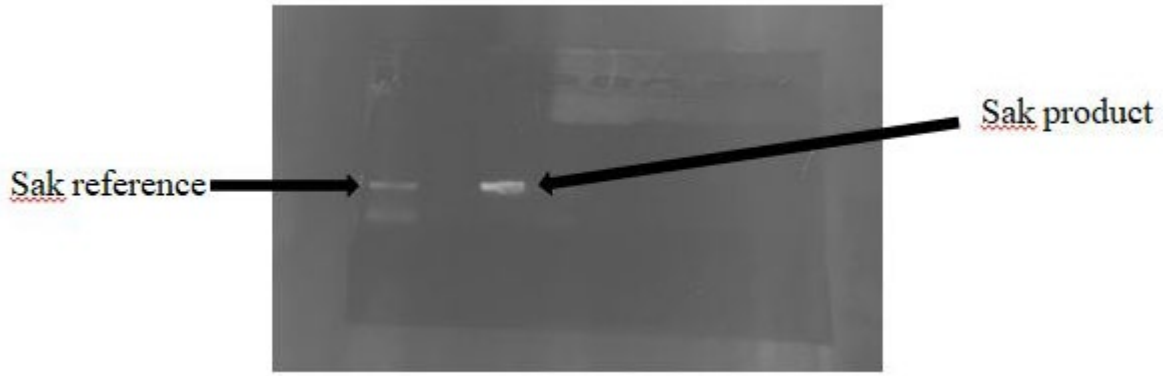


Figure 5: Transformation of expression host with recombinant plasmid (pET-SAK) and in gel electrophoreses

IPTG Induction of BL21DE3

E. coli strain BL21DE3 was transfected with the recombinant pET21a-SAK plasmid. Transformed cells were selected on LB medium plates supplemented with ampicillin. The experiment was performed as above, in order to improve the time of induction, cultures were stimulated by adding IPTG inducer at different concentrations (0, 1, 2, 3, mM) concentration and the growth rate was measured at different times starting with (1.5, 3, 4.5, 6) hours at 32 °C respectively as mention in Table 2 [19]. The study found that the optimum concentration was (1mM) and 2-6 hours at the conclusion of the induction duration (Fig 6). Centrifugation at 10,000 rpm for 15 minutes was used to extract the cells. To minimize warming of the samples, cell pellets were suspended in 1.5 ml cold lysis buffer (10 mM Tris Cl pH 8.0) and disrupted by glass bead lysis in an ice bath. Following lysis, the soluble fractions were centrifuged and put onto SDS-PAGE gels to observe the induced protein profile as described in the section [20].

Table 2: OD600 and concentration of protein expression in E. coli strain BL21 (DE3) after IPTG induction at various concentrations

Concentration IPTG	1.5 hours	3 hours	4.5 hours	6 hours
0.0 mM	0.3	0.43	0.49	0.78
0.5mM	0.59	0.78	0.85	1
1mM	0.7	0.92	1.25	1.49
2mM	0..54	0.73	1.19	1.35
3mM	0.5	0.75	1.24	1.36

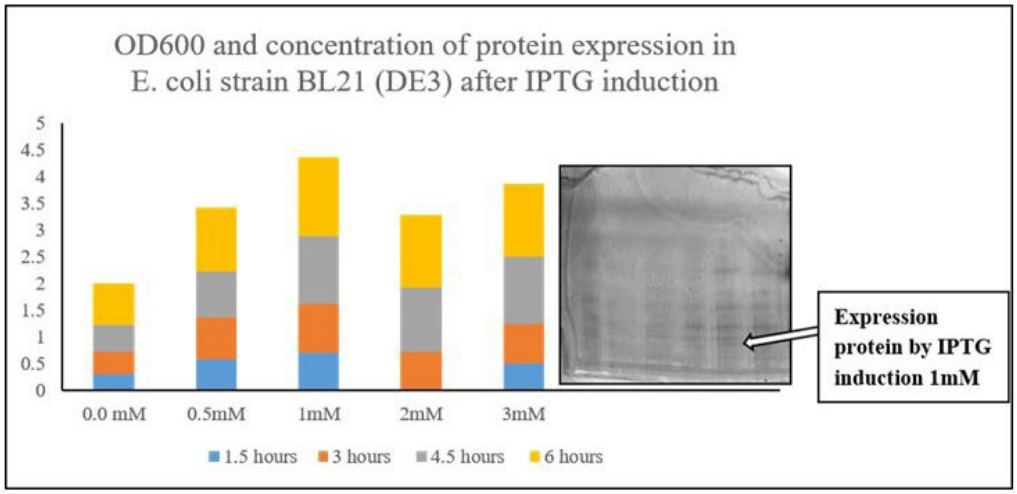


Figure 6: OD600 and concentration of protein expression in E. coli BL21 (DE3) with viability of cells not induced or pre-stimulated with IPTG that is determined by using different concentrations at different times to know the extent of growth intensity at 32 °C

SDS PAGE analysis of recombinant PET21a-SAK protein expression stained with coomassie staining

We used 15% SDS-PAGE to establish protein parameters. BL21DE3 expression culture hosts recombinant pET-SAK-plasmid in LB + Amp media for three hours before being stimulated with 1 mM IPTG for three hours. At the conclusion of induction, cells were extracted and sorted into soluble and insoluble fractions, which were then put onto 15% lysis and 5% stacking. Coomassie dye was used to stain the SDS-PAGE gel (Fig 7). They were granule fractions as well as supernatant. Loaded with 20 µL 40 µL, respectively. Together with a 10 µL medium run marker.

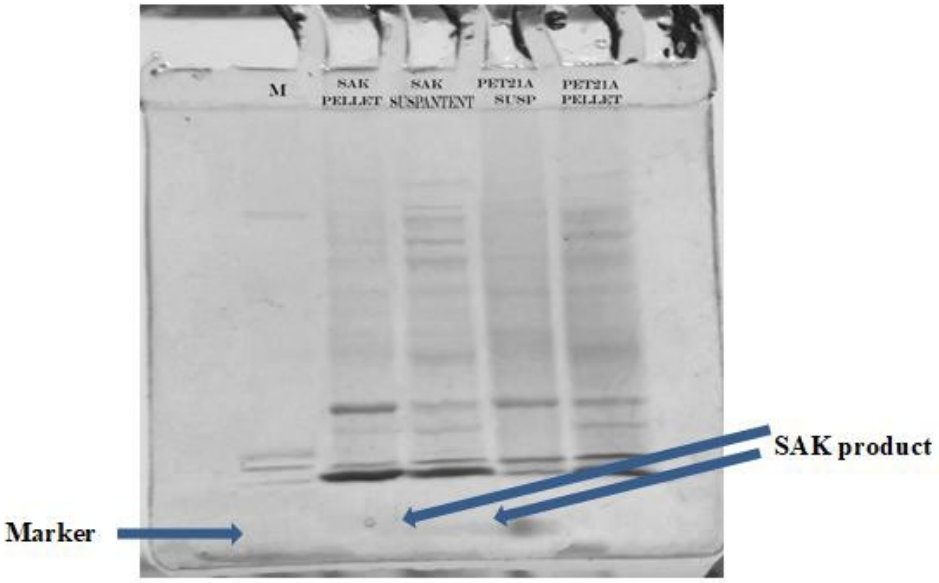


Figure 7: 12% SDS PAGE showing Expression protein in E. coli, when induced BL21DE3by IPTG Post induction cell harvesting was done as various time 3 hours at 32°C, respectively to find optimum induction time. Lane 1 marker, Lane 2 SAK pellet, Lane 3 SAK supernatant, Lane 4 pET21a supernatant, Lane 5 pET21a pellet

Recombinant Protein Affinity Purification in BL21D (DE3)

An E. coli recombinant protein (recombinant SAK protein) was purified using affinity chromatography with an imidazole ring and a histidine-tag and then analyses in a 12 % SDS PAGE. Recombinant Protein Affinity Purification in BL21D (DE3) was described in fig 8. While similar result was find by [21].

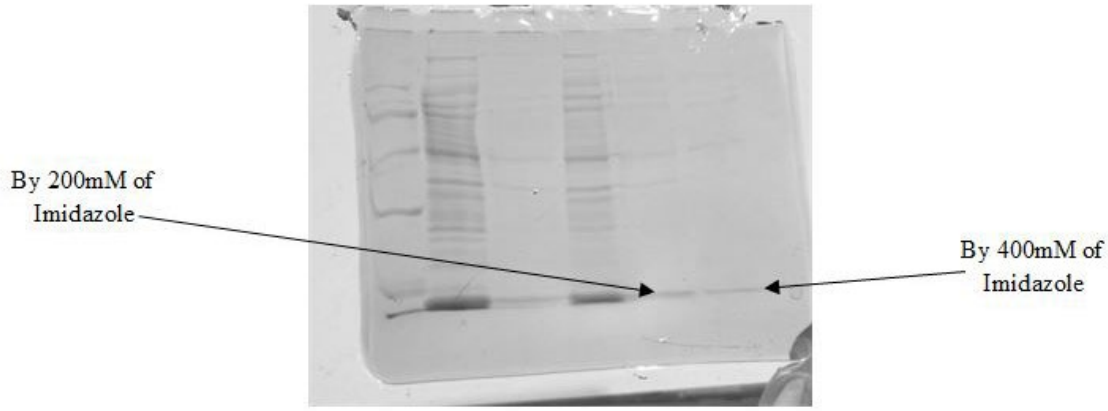


Figure 8: 15% SDS PAGE Affinity Purification Recombinant Protein in E. coli (Purification of Proteins Using Polyhistidine Affinity Tags) chromatography; lane 1 use marker; lane 2 original protein; lane 3 flow through; lane 4 wash purification protein; lane 5 elution by 200mM of Imidazole concentration; lane 6 elution by 400mM of Imidazole concentration

Conclusion

PET is one of the best systems used in the process of expression of recombinant proteins especially with Escherichia coli strain BL21DE3. Because of its many advantages that convinced us to use it especially with target gene expression, we used IPTG inducer for the purpose of increasing cell mass by selecting a specific concentration which in turn it suppresses protein production and reduces inclusion bodies formation depending on protein expression by IPTG induction of a chromosomal integration strand containing T7 RNA polymerase from the lacUV5 promoter. The clonality of target genes is very low in strains that lack a source of T7 RNA polymerase upon induction. High-potency polymerase primarily competes for transcription by the host RNA polymerase. This phenomenon together with highly efficient translation that achieves expression levels at which the target protein may constitute the majority of the cellular protein. After only a few hours, the rate of gene expression of Staphylokinase is affected by differences in IPTG concentration. The optimum concentration of IPTG to stimulate gene expression was (1mM). When the concentration of IPTG increases, it causes risks that have a negative effect on the amount and solubility of protein. This helps in the formation of agglomeration (included bodies) of the amount of protein in the insoluble part due to the increased rate of gene expression [22].

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Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no conflict of interest.

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