MÆDICA - a Journal of Clinical Medicine

ORIGINAL PAPERS

Optimizing of Nutrients for High Level Expression of Recombinant Streptokinase Using pET32a Expression System

Shima MAHMOUDI[a,b]; Hamid ABTAHI[c,d]; Abbas BAHADOR[b]; Ghasem MOSAYEBI[b]; Ali Hatef SALMANIAN[e]; Mostafa TEYMURI[a]

[a]Pediatrics Infectious Diseases Research Center, Tehran University of Medical Sciences, Tehran, Iran

[b]Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

[c]Research center of Molecular Medicine, Arak University of Medical Sciences, Arak, Iran

[d]Department of Microbiology and Immunology, School of Medicine, Arak University of Medical Sciences, Arak, Iran

[e]National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

ABSTRACT

Streptokinase (SK) is being increasingly used in the treatment of acute myocardial infarction and ischemic stroke. The feeding control method using substrate balance equations is vital to maintain the preferred specific growth rate for the high-level expression of recombinant proteins. In this study, initial experiments on chemical and temperature inducible expression systems were carried out to identify appropriate expression conditions to improve production of recombinant Streptokinase. Streptokinase gene of group C Streptococci *was cloned into prokaryotic expression vector pET32a. Gene expression was optimized by changing levels of glucose, tryptone, and MgSO4 in the media and temperature-inducible expression system and recombinant protein was confirmed by western blot analysis with anti streptokinase sera of immunized rats. Among the various expression systems used, the quantity of recombinant streptokinase produced in the medium containing 2.4% glucose was more than two-fold compared to the medium containing 0.2% glucose. In addition, temperature induction system (37°C) was found to result in higher productivities compared to room temperature. In conclusion we have demonstrated that significant improvement in the streptokinase yield can be obtained by modifying the media and feeding of substrate. These results indicate that efficient process control strategy is important for the mass production of streptokinase.*

Keywords: recombinant streptokinase, optimizing of nutrients, pET32a

Address for correspondence:
H. Abtahi, Department of Microbiology and Immunology, School of Medicine, Arak Univercity of Medical Sciences, Arak, Iran.
Tel: +98- 0861- 4173504
E-mail: mahmoudi18033@gmail.com

Article received on the 29th March 2012. Article accepted on the 22nd August 2012.

INTRODUCTION

Streptokinase (SK) is being increasingly used in the treatment of acute myocardial infarction and ischemic stroke (1). The increased prevalence of thrombo-embolic disorders have led to a remarkable rise in the demand for clot-dissolver drugs preferably at low price especially in developing countries and to meet this, it is crucial to develop a production process with high yield (mg protein/L). The low SK production yields from natural host and its pathogenicity are the main reasons for the investigation of recombinant DNA technology for production of this important protein (2).

The streptokinase gene (*skc*) has been expressed in several gram positive and gram negative bacteria, However, there are some disadvantages of producing recombinant proteins in some of them like heavily glycosylated protein that secreted into the culture medium by *Pichia pastoris* and low cell density obtained by *Lactococcus lactis* due to the growth inhibition caused by acidification of the medium by lactic acid (3,4).

Escherichia coli is the first-line system for producing recombinant proteins, because of its favorable behavior, readily available host-vector systems, ease in cultivation and high growth rate (5). Fed-batch cultures for production of recombinant protein by *E. coli* have been generally used (6). All proteins do not accumulate maximal levels in *E. coli* and the production of the target proteins requires medium optimization. The feeding method to maintain the preferred specific growth rate is simple and agreeable to feed control using substrate balance equations (7). The aim of our study was to optimize nutrients for achieving high expression levels of streptokinase. ❑

MATERIAL AND METHODS

Bacterial strains and plasmids

Streptococcus dysgalactiae subsp. *equisimilis* ATCC 12388 (Culti-Loops^{R} of Remel Inc.) was used as templates to amplify the target DNA fragments by polymerase chain reaction (PCR). pET32a (Novagene, USA) was used as expression vector. *E. coli* DH5α (Stratagene, USA) (f-gyr A96 Nalr, recA1 relA1 Thi-1 hsdR17 r-k m+k) was used as the primary host for the construction of plasmid and *E. coli*, BL21 (DE3) pLysS (f -ompt hsdB, rB^{-} mB^{-}, dcm gal, DE3, pLYsS cmr) was used to express the recombinant protein.

Cloning of streptokinase gene

Streptococcus dysgalactiae subsp. *equisimilis* of the group C (ATCC 12388) was cultivated in LB broth (overnight, at 37°C). Bacterial genome was extracted by CTAB-NaCl method and its concentration was measured by spectrophotometer (OD 260 and 280 nm) (8). All DNA manipulations were performed under standard conditions as described by Sambrook et al. (8). Oligonucleotides were designed with suitable restriction enzyme sites. The following oligonucleotides were used for gene amplification by PCR technique, forward: 5′- TCG GAT CCA TTG CTG GAC CTG AGT G -3′, reverse: 5′-GAC TCG AGG TTA TTT GTC GTT-3′ These primers were designed to introduce a BamHI site at the 5′-end of forward primers and an XhoI site at the 5′-end of reverse primers. The PCR product was purified from the Agarose gel by high pure PCR product purification kit (Roche, Germany) according to manufacturer recommendation. We used InsTAclone™ PCR Cloning Kit (Fermentas) for direct one-step cloning of PCR-purified DNA fragments. The target product was gel-purified and then ligated into pTZ57R/T at the molar ratio 3:1 (fragment:vector). The ligation mixture incubated overnight at 4°C and subsequent ligation mix was used for transforming *E. coli* DH5α competent cells. The recombinant clones were confirmed by PCR for *skc* gene, by restriction digestion and DNA sequencing.

Finally, the *skc* fragments containing the *Bam*HI and *Xho*I sites separated and purified by 1% agarose gel electrophoresis. The purified products were then ligated into the pET32a expression which had been precut with the same enzymes. The ligated products were transformed into competent *E. coli BL21* (DE3) plysS cells and the ampicillin-positive clones identified by PCR and restriction enzyme digestion. Finally, the identified recombinant plasmid pET32a-*skc* was further sequenced on both strands by Macrogen (Seoul, Korea).

Media and culture conditions

Cultivation conditions

Although changing one variable parameter at a time while keeping the others constant was

used for experimental optimization in the classical method, it is not practical to carry out experiments with every possible factorial combination of the test variables because of the large number of experiments required (9). Besides this, it is time-consuming process especially when a large number of parameters are taken into account (10). In our study the four critical independent variables, glucose, tryptone, $MgSO_4$, and temperature were chosen to optimize the SK production by *E. coli* (Table 1).

Induction conditions

The cultures were induced at the mid-exponential phase 0.6 OD (600 nm) in the shake flask experiments. Expression of the SK protein was then induced by the addition of isopropyl-thio-P-D-galactoside (IPTG) to a final concentration of 1 mM and incubation was continued for a further 4 hour.

Purification of SK

The expressed protein was purified using Ni-NTA column according to Manufacture instruction (Qiagene). Starting from the insoluble fraction a first step of solubilization was needed. In this case urea was used. After solubilization samples were charged in an affinity column that allowed the fusion protein to bind to the matrix charged with nickel carrying a histidine tag. SDS–PAGE electrophoresis gel was carried out to see whether the eluted protein was found. The purified protein was dialyzed twice against PBS (pH 7.2) at 4°C overnight and quantity of purified SK Protein was analyzed by Bradford methods (8).

Production of antibody and western blotting

For producing of antibody 100 µl (50µg SK) of commercial streptokinase (Behring, Canada) with the same volume of Freund's complete adjuvant (Sigma, St. Louis, Ma) was injected into 5 rats by intramuscular injection, after 10 days the rats were boosted with 50 µg SK prepared in Freund's incomplete adjuvant. Blood was collected 7 days after the second injection.

The blood of control rats was also collected. The antibody was used for western blotting.

For Western blot analyses, 0.5 µg of purified SK protein was used per well. After SDS-PAGE, bacterial proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF Membrane, Roche) membrane using transfer buffer containing 25 mM Tris (pH= 8.3), 192 mM glycine and 20% methanol at 90 volts for 1.5 hours at 4°C. The blotting membrane was blocked with 2.5% (w/v) BSA in TBST buffer (0.5 M NaCl, 0.02 M Tris pH 8.5, 0.05% Tween20™) for one hour at room temperature. For detection of streptokinase, the membranes were immunoblotted using diluted rat sera. Normal serum from rat was used as controls. After reactions with the primary antibody, the blots were washed three times with TBST, the antigen-antibody complexes were detected by using peroxidase conjugated goat anti-rat Ig (G, A, M) The blots were then washed three times with TBST and reactions were developed by diamino Benzidine Solution (DAB; Sigma, USA). ❑

RESULTS

Cloning of SK

The *skc* gene (GenBank: AP010935.1) was isolated by PCR from *Streptococcus dysgalactiae* subsp. *equisimilis* group C. This gene was cloned in pET32a plasmid yielding pET32a-*skc* for expression in *E. coli BL21* (DE3). Recombinant plasmid was screened by PCR then double digested by *Bam*HI and *Xho*I. The digestion

Content of medium	Medium1	Medium2	Medium3	Medium4
Yeast extract (g)	0.37	0.37	0.37	0.37
Tryptone (g)	1.2	0.6	0.6	0.6
Glucose (g)	0.05	0.6	0.05	0.05
NaCl (g)	0.25	0.25	0.25	0.25
KCl (g)	0.025	0.025	0.025	0.025
MgCl2(g)	0.013	0.013	0.013	0.013
CaCl2 (g)	0.013	0.013	0.013	0.013
Nutrient broth (g)	0.019	0.019	0.019	0.019
$MgSO_4$ (g)	-	-	-	0.25
Total volume	25 ml	25 ml	25 ml	25 ml

TABLE 1. Quantity of additives in Different media using for expression of streptokinase.

product was visualized on 1% agarose gel electrophoresis. We demonstrated that recombinant plasmid contained the target gene by PCR, restriction enzyme digestion and DNA sequencing. The sequencing result showed that the correct sequences of *skc* gene were inserted into the vector.

Analysis of *skc* expression

We reported the cloning of the skc gene of *Streptococcus dysgalactiae* subsp. *Equisimilis* under T7 promoter based vector (pET-32a) with optimization of its expression in *E. coli*. The expression of SK was observed in case of induced lysate using different expression conditions (Figure 1).

Comparison of temperature induction

Induction at higher incubation temperature of 37°C led to higher expression of SK in *E. coli* BL21 (DE3) compared to 25°C. Due to the higher productivity obtained during the increase in temperature (37°C), this condition was used in all induction experiments.

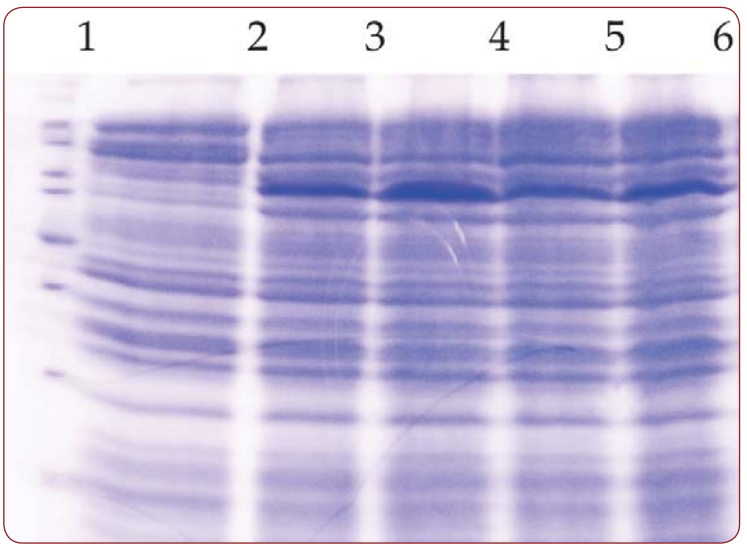

FIGURE 1. Expression of streptokinase in synthetic media. Lanes 1 marker; lane 2, uninduced cells carrying expression vector; lane 3, induced cells with 1 mM IPTG (medium1), lane 4, induced cells with 1 mM IPTG (medium2), lane 5, induced cells with 1 mM IPTG (medium3), lane 6, induced cells with 1 mM IPTG (medium4).

Sample	Protein (µg/ml)
SK elute sample medium1	480
SK elute sample medium2	800
SK elute sample medium3	360
SK elute sample medium4	580

TABLE 2. Yield of Streptokinase obtained from pET32a-*skc* in different media after purification.

Effect of glucose on SK production

As was evident from Table 2, addition of 2.4% glucose (0.6g/25 ml) affecting the productivity of the recombinant protein. It could be observed that, the quantity of SK produced in this condition was more than two-fold higher (800 µg/ml) compared to the medium containing 0.2% glucose (0.05 g/25 ml).

Effect of tryptone on SK production

It was observed that the quantity of recombinant SK produced in medium with two-fold higher tryptone supplement was higher (480 µg/ml) compared to the quantity of the protein observed in the medium containing 0.6 g/25 ml tryptone (360 µg/ml).

Effect of Inorganic compound ($MgSO_4$) on SK production

The higher level of SK concentration (580 µg/ml) was found in presence of $MgSO_4$ (0.25g/25 ml) compared to the condition with the same medium without $MgSO_4$ (360 µg/ml).

Purification of SK and Western blotting analysis

Recombinant protein was purified using immobilized metal affinity chromatography, SDS-PAGE analysis of eluted streptokinase showed a single band (Figure 2) and the quantity of SK during these conditions was shown in Table 2. All purified recombinant protein was reacted with rat sera (only western blot result of one purified streptokinase was shown in Figure 3). ❑

DISCUSSION

These works demonstrate that the wide disparity found in recombinant product due to variation in nutrient levels or cultural condition. The use of chemically defined media like glucose as carbon sources and yeast extracts or peptones as nitrogen sources in producing recombinant proteins is a common practice (11-13). In the present work, the chemical and temperature inducible expression systems for the production of recombinant streptokinase were compared and high level production of protein was obtained in the presence of 2.4% glucose.

Until now, SK has been expressed in several gram positive and gram negative bacteria. Expression of SK was 24.5 mg/L by *Schizosaccha-*

romyces pombe (14), 0.17 mg/ml by *Streptomyces lividans* (4), 45 mg/L and 50 mg/L by E.coli JM109 (15,16), 100mg/L by *E. coli* K-12 W3110 (17), 250 mg/L by *E. coli* Bl21 (DE3) (18) and 140 mg/L by *E. coli* GJ1158 (19) as host. The productivity of streptokinase obtained in several hosts was lower than the productivity obtained from our study using optimized medium with 2.4% glucose.

In this study we have demonstrated that significant improvement in the SK yield can be obtained by modifying the media and feeding of substrate. Our investigations have shown that presence of 2.4% glucose has the most significant effect on the productivity of the recombinant streptokinase. Although High glucose causes Crabtree effect and leads to accumulation of acetate which is inhibitory to cell growth [20], L8-UV5 promoter of lambda DE3 prophage encoding T7 RNA polymerase in pET expression hosts, is less sensitive to the glucose effect by the net effect of the three-point mutations. Two point mutations in the -10 region, decrease dependence of promoter on CAP/cAMP stimulation for full activation and the third-point mutation which located in the CAP/cAMP binding site decreases the affinity for CAP/Camp. This allows strong IPTG induction of T7 RNA polymerase expression even in the presence of glucose (21).

In order to enhance SK yield in fed-batch process, the concentration of organic nitrogen sources like tryptone in feed medium was increased. It has been observed that the presence of nitrogen sources in the medium provides nutrients and precursors for synthesis of the building blocks of cells and helps to decrease the inhibitory effect of acetic acid (22-24). In addition, Nitrogen sources are known to result in increased plasmid stability and cell mass (25,26).

Also addition of inorganic compound like Mg2+ result in increased SK yield. This compound at specific concentrations might cause better coupling of supply and demand for the amino acids by regulation of the pathways (27). In the present study we used *E. coli* BL21 (DE3) plysS that is deficient in the known cytoplasmic protease gene products, such as Lon, OmpT, DegP or HtpR (28) These strains prevent the escape of expression and contribute to an over expression of the recombinant.

The difference in the molecular weight of the SK expressed using the pET32a–*skc* construction is due to the SK is fused to thioredoxin increasing the molecular weight from 7900 to 28000. This vector encodes 109aa trxA (Trx-tag) domain immediately upstream from His-Tag and S-Tag sequences that allow easy detection and purification of target proteins (29). With SK fused to thioredoxin it has been shown that purification has been successful. Compared to other methods, these purification protocols had a great advantage and sufficient amount of protein was obtained in a few steps.

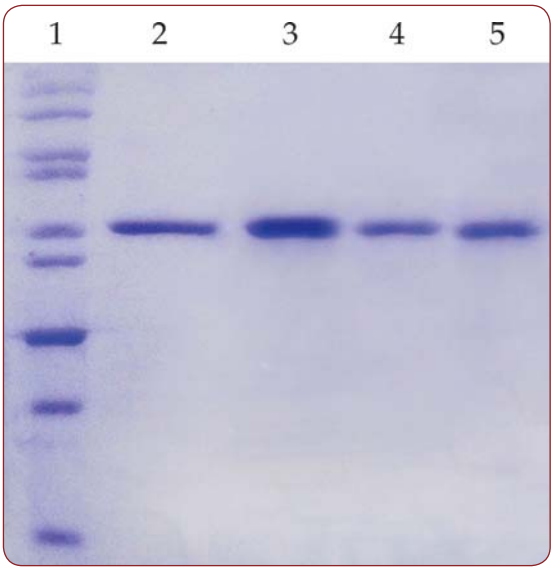

FIGURE 2. GNi-NTA purification of recombinant streptokinase produced in *E. coli* BL21(DE3)plysS; Proteins were resolved by SDS-PAGE on 15% of polyacrylamide gel and stained with Coomassie brillant blue. Lanes 1, marker; lane 2, extract protein from medium 1; lane 3, extract protein from medium 2; lane 4, extract protein from medium 3; lane 5, extract protein from medium 4 after Ni-NTA affinity chromatography.

Expression at lower temperatures is expected to lower the translation rate, which might slow down demands on the translocon and also might provide the target proteins with an increased chance of folding correctly.

Regardless of the complexity of recombinant protein production, nutrient optimization could result in higher productivity. On the other hand, local biotechnological production of streptokinase especially in developing countries would considerably contribute to reducing the costs of this vital thrombolytic drug (30). ❑

CONCLUSION

Nowadays, growing number of protein-based drugs significantly accelerates the

development of efficient production methods. In this study, variation of parameters had a critical effect on the productivity of recombinant streptokinase. Therefore, careful optimization of parameters could allow design of experimental strategies which would be very useful in enhancing the productivity of recombinant proteins. Although our results are specific to a recombinant streptokinase and the *E. coli* expression system, we believe that optimized expression system has several advantages and could be in principle extended to other recombinant proteins and expression systems.

References

1. **Banerjee A, Chisti Y, Banerjee UC –** Streptokinase-a clinically useful thrombolytic agent. *Biotechnol Adv.* 2004; 22:287-307
2. **Goyal D, Sahni G, Sahoo DK –** Enhanced production of recombinant streptokinase in Escherichia coli using fed-batch culture. *Bioresour Technol.* 2009; 100:4468-74
3. **Sriraman K, Jayaraman G –** Enhancement of recombinant streptokinase production in Lactococcus lactis by suppression of acid tolerance response. *Appl Microbiol Biotechnol.* 2006; 72:1202-9
4. **Pimienta E, Ayala JC, Rodríguez C, et al. –** Recombinant production of Streptococcus equisimilis streptokinase by Streptomyces lividans. *Microb Cell Fact.* 2007; 5:20
5. **Zhao K, Liu M, Burgess RR –** The global transcriptional response of Escherichia coli to induced sigma 32 protein involves sigma 32 regulon activation followed by inactivation and degradation of sigma 32 in vivo. *J Biol Chem.* 2005; 280:17758-68
6. **Clementschitsch F, Jürgen K, Florentina P, et al. –** Sensor combination and chemometric modelling for improved process monitoring in recombinant E. coli fed-batch cultivations. *J Biotechnol.* 2005; 120:183-96
7. **De Anda R, Lara AR, Hernández V, et al. –** Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of Escherichia coli for recombinant protein production without impairment of growth rate. *Metab Eng.* 2006; 8:281-90
8. **Sambrook J, Fritsch EF, Maniatis T –** Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, New York, 2001
9. **Bursali N, Ertunc S, Akay B –** Process improvement approach to the saponification reaction by using statistical experimental design. *Chemical Engineering and Processing: Process Intensification* 2006; 45:980-989
10. **Vellanki RN, Potumarthi R, Mangamoori LN –** Constitutive expression and optimization of nutrients for streptokinase production by Pichia pastoris using statistical methods. *Appl Biochem Biotechnol.* 2009; 158:25-40
11. **Lim HK, Jung KH –** Improvement of heterologous protein productivity by controlling postinduction specific growth rate in recombinant Escherichia coli under control of the PL promoter. *Biotechnol Prog.* 1998; 14:548-53
12. **Cserjan-Puschmann M, Kramer W, Duerrschmid E, et al. –** Metabolic approaches for the optimisation of recombinant fermentation processes. *Appl Microbiol Biotechnol.* 1999; 53:43-50
13. **Zhang J, Greasham R –** Chemically defined media for commercial fermentations. *Appl Microbiol Biotechnol* 1999: 51;407-421
14. **Kumar R, Singh J –** Expression and secretion of a prokaryotic protein streptokinase without glycosylation and degradation in Schizosaccharomyces pombe. *Yeast* 2004; 21:1343-1358
15. **Kim DM, Lee SJ, Yoon SK, et al. –** Specificity role of the streptokinase C-terminal domain in plasminogen activation. *Biochem Biophys Res Commun* 2002; 290:585-8
16. **Lee SH, Jeong ST, Kim IC, et al. –** Identification of the functional importance of valine-19 residue in streptokinase by N-terminal deletion and site directed mutagenesis. *Biochem Mol Biol Int* 1997b; 41:199-207
17. **Estrada MP, Hernandez L, Perez A, et al. –** High-level expression of streptokinase in Escherichia coli. *Biotechnology* 1992; 10:1138-1142
18. **Ramalingam S, Gautam P, Mukherjee KJ, et al. –** Effects of postinduction feed strategies on secretary production of streptokinase in Escherichia coli. *Biochem. Eng. J.* 2007; 33:34-41
19. **Pal Y, Gupta JC, Mukherjee KJ –** Optimizing recombinant protein expression in the T7 system under the control of the proUp promotor. *Biotechnol. Lett.* 2001; 23:41-46
20. **Panda AK –** Bioprocessing of therapeutic proteins from the inclusion bodies of Escherichia coli. *Adv. Biochem. Eng. Biotechnol* 2003; 85:43-93
21. **Novy R, Morris B –** Use of glucose to control basal expression in the pET system innovations BioTechniques 2001; 12:1-3
22. **Shiloach J, Fass R –** Growing E. coli to high cell density–A historical perspective on method development. *Biotechnology Advance* 2005; 23:345-57
23. **Tripathi NK, Babu JP, Shrivastva A, et al. –** Production, and characterization of recombinant dengue virus type 4 envelope domain III protein. *Journal of Biotechnology* 2008; 134:278-86
24. **Tripathi NK, Shrivastva A, Pattnaik P, et al. –** Production, purification and characterization of recombinant dengue multiepitope protein. *Biotechnol. Appl. Biochem.* 2007; 46:105-13
25. **Matsui T, Sato H, Sato S, et al. –** Effect of nutritional conditions on plasmid stability and production of tryptophan synthase by a recombinant Escherichia coli. *Agric. Biol. Chem* 1990; 54:619-624
26. **Li X, Robbins Jr, Taylor KB –** The production of recombinant beta-galactosidase in Escherichia coli in yeast extract enriched medium. *J. Ind. Microbiol* 1990; 5:85-94
27. **Calik P, Bilir E, Ozçelik IS, et al. –** Inorganic compounds have dual effect on recombinant protein production: influence of anions and cations on serine alkaline protease production. *J Appl Microbiol.* 2004; 96:194-200
28. **Sugimura K, HigashiJ N –** A novel outer-membrane-associated protease in Escherichia coli. *Bacteriol.* 1988; 170:3650-3654
29. **McCormick M, Mierendorf R –** S-Tag a multipurpose fusion peptide for recombinant proteins innovations 1994; 1:4-6
30. **Al Sohaimy S, Aleem E, Hafez EE, et al. –** Expression of recombinant Streptokinase from local Egyptian Streptococcus sp. SalMarEg. *African Journal of Biotechnology* 2011; 10:9001-9011