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High Cell Density Cultivation for the Production of Industrially Important Engineered Bi-functional Recombinant Staphylokinase Variant from Salt Inducible *Escherichia coli* GJ1158

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Abstract

Most of the bacterial and other simple non glycosylated recombinant proteins were conventionally produced from IPTG inducible Escherichia coli BL21(DE3). Considering the factors like cost and toxic nature of IPTG, as an alternative, salt inducible Escherichia coli GJ1158 was used in this study for the over production of staphylokinase variant (sak – hirulog) using fed batch fermentation, cost effectively. Optimization of physico chemical factors viz., dissolved oxygen (DO), carbon, nitrogen and phosphate sources on bacterial growth for the production of recombinant sak – hirulog using batch and fed batch fermentation was studied. In batch culture, increased DO at more than 30 % did not influence the enhanced expression of sak – hirulog, but significant improvement was observed in fed batch cultivation. Supplementation of production medium with different nutrient sources like dextrose, yeast extract and dipotassium hydrogen phosphate (K_2HPO_4) enhanced the sak – hirulog expression in fed batch fermentation process even without disturbing the cell growth by providing 50 % DO. Approximately 1178 mg/L of specific protein was obtained using cost effective modified glucose yeast exgtract (GYE) media devoid of sodium chloride. This study also reports the highest concentration of recombinant protein from salt inducible expression host till to date, which manages to satisfy the production of bifunctional staphaphylokinase variant using economically feasible bacterial expression host Escherichia coli GJ1158.

Keywords: *Staphylokinase hirulog, Escherichia coli BL21(DE3) and Escherichia coli GJ1158, Fed batch fermentation, dissolved oxygen, modified GYE media*

1. Introduction

Thrombosis is one of the important causes of cardiovascular and cerebrovascular diseases (CVD) worldwide [1]. To treat the patients alleged from thrombosis, different clinical and surgical methods like unfractionated heparin (UFH), low molecular weight heparin (LMWH), balloon angioplasty and bypass surgery are in use by medical practitioners. But all these are cost affective and have low success rate. Hence lot of research was focused on thrombolytics especially bacterial derived thrombolytics, alternative to the existing clinical and surgical methods. Recombinant thrombolytics are administered to save the patients alleged from cardiovascular diseases like acute

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myocardial infarction and stroke [2]. Even though these drugs are known from a decade, cost of the treatment and availability are the major drawbacks to become ideal molecule. Therefore it is critical time to hunt for an economical clot buster with enhanced yield without compromising the activity. Different types of drugs are available in market viz., streptokinase, tissue plasminogen activator and urokinase. But major drawbacks like re-occlusion and poor production attracted the researchers for alternative. Among all, staphylokinase (sak) has the highest fibrinolytic activity without fibrinogen depletion when compared to other thrombolytics like streptokinase (SK), urokinase (UK) and tissue Plasminogen Activator (t-PA) [3]. The clot specificity of sak was shown to be more capable than other thrombolytics for the dissolution of platelet-enriched and retracted blood clots [4, 5]. Sak is not a proteolytic enzyme, but forms 1:1 stoichiometric complex with plasmin(ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix [6]. Generally the native expression of sak from *Staphylococcus aureus* is very low and *Staphylococcus aureus* is a potent pathogen, for this reasons r-DNA technology is explored to produce the protein in large quantity with the use of cost effective media. IPTG inducible *Escherichia coli* BL21(DE3) is one of the most extensively employed expression system for recombinant protein, inspite of disadvantages like cost and toxicity associated with production. To overcome many of such hindrances experiments with salt inducible expression host like *Escherichia coli* GJ1158 is used for the heterologous protein expression where the protein yield somewhat reduced, but activity of the recombinant protein is not compromised [7]. The recombinant protein expression slightly enhanced even if the cultivation switched from shake flask to reactor level. Therefore some influential parameters need to be optimized for cell proliferation and protein expression. In particular, nutritional factors like carbon, nitrogen and phosphate sources along with dissolved oxygen percentage are studied.

The effect of oxygen transfer rate (OTR) and dissolved oxygen (DO) are the two aspects for the cultivation process [8]. The dissolved oxygen in the range of 30 – 50 % yields the optimal expression level of different recombinant proteins during fed-batch cultivation [9-11]. But in many *E. coli* fermentation studies the expression levels were found to be elevated with the addition of different nutritional factors.

The recombinant strain (*E. coli* GJ1158 pRSET-A mature sak – hirulog) constructed in our lab is used in the present study. The study was designed to check the influence of dissolved oxygen, nutritional factors like dextrose, yeast extract and K_2HPO_4 on the over production of sak variant using salt inducible expression host *E. coli* GJ1158.

2. Materials and Methods

2.1. Strains and Plasmid

Expression host *E. coli* GJ1158 was procured from Genei, Bangalore, India, was used for the production of bi-functional recombinant protein. The cloned gene was under the regulation of strong T7 promotor and lac operator in pRSET-A vector with the ampicillin resistance gene as selectable marker in the plasmid containing bacterial clones. The r-DNA was maintained in the host *Escherichia coli* DH5α [12]. The recombinant plasmid was isolated and transformed into GJ1158 for further studies.

2.2. Medium Preparation

The seed culture was prepared using LB broth (1% tryptone and 0.5% yeast extract, pH 6.8 ± 0.2). Fermentation trails were carried out using modified M9 medium (MM9ON) (K_2HPO_4 – 6g/L, KH_2PO_4 – 3 g/L, NH_4Cl – 1 g/L, Yeast extract – 5 g/L, Dextrose – 5g/L, 1M $MgSO_4$ – 2 mL, Trace Metal Mix – 1 mL $(Al_2 (S0_4)_3$. $7H_2O$ – 10 mg/L, $CuSO_4$. H_2O – 2 mg/L, H_3BO_4 – 1 mg/L, $MnCl_3$. $4H_2O$ – 20 mg/L, $NiCl_2$. $6H_2O$ – 1

mg/L, Na_2MoO_4. $2H_2O$ – 50 mg/L, $ZnSO_4$. $7H_2O$ – 50 mg/L, $FeSO_4$ – 50 mg/L) and modified GYE medium (MGYE) (Dextrose – 11 g/L, $(NH_4)_2$ SO_4 – 3.6 g/L, $MgSO_4$. $7H_2O$ – 7.2 g/L, yeast extract – 20 g/L, K_2HPO_4 – 10 g/L, $FeSO_4$. $7H_2O$ – 80 mg/L, $CaCl_2$. $2H_2O$ – 80 mg/L, $MnSO_4$. H_2O – 20 mg/L, $AlCl_3$ – 11.04 mg/L, $CoCl_2$. $6H_2O$ – 8 mg/L, $ZnSO_4$. $7H_2O$ – 4 mg/L, Na_2MoO_4. $2H_2O$ – 4 mg/L, $CuCl_2$. $2H_2O$ – 2 mg/L, H_3BO_3 – 1 mg/L). Aliquots are transferred into bottles and sterilized for 20 min at 121^0C. Sodium chloride was excluded while working with GJ1158.

2.3. Inoculum Preparation

2 % glycerol stock was added to LBON plate (ampicillin concentration 100 μgml^{-1}) and incubated at 37 ^{0}C for 16–24 hrs. A loopful of single colony was transferred to LBON medium with appropriate concentration of antibiotic and incubated on rotatory shaker at 37 ^{0}C for 4-6 hrs at 150 rpm. After the incubation time, 4-5 % of the pre-inoculum was transferred to MM9ON and MGYE medium and incubated at 37 ^{0}C for 4-6 hrs.

2.4. Shake Flask Cultivation

3% inoculum was transferred to 50 ml working volume of LBON, MM9ON and MGYE broth with appropriate antibiotic and incubated on a rotary shaker at 37 ^{0}C and 150 rpm. When the cell density (OD_{600}) reaches to 2.5 in all the flasks, induce the culture with final concentration of 150 mM sterile NaCl. After induction, the cultures were again incubated on a rotary shaker at 37 ^{0}C and 150 rpm for 4 hrs.

2.5. Fermentation

Fermentation trials were conducted using 10 L laboratory bioreactor with a working volume of 6 L. Sterile 5N NH_4OH and 3N OPA (Ortho phosphoric acid) were used as pH controlling agents. For batch and fed batch cultivations, different stocks of DMH, K_2HPO_4 and trace metal mix were prepared and autoclaved separately. After autoclaving of remaining components or before addition of inoculum to the fermentor vessel, stocks were be added in appropriate amounts. Therefore three feed bottles containing dextrose, yeast extract and K_2HPO_4 were added under aseptic conditions. Sterile polyethylene glycol was used to prevent foaming.

2.5.1. Batch Fermentation: Induction was carried out when the cell density (OD_{600}) of the batch culture reaches 9.0 (at 7-8 hrs). After this procedure the culture was harvested for 4 hrs. The dissolved oxygen percentage was maintained at 30 (before and after induction) by cascading agitation rate (500–750 rpm). The constant aeration rate was maintained 1.25 ± 0.25 vvm.

2.5.2. Fed Batch Fermentation: After complete consumption of nutritional factors the batch cultivations were switched to fed batch cultures. Four hours of post induction was carried after inducing with 150 mM NaCl when the OD_{600} reaches 70 (~ 10 – 12 hours). 30 % DO was maintained as the set point by cascading impeller speed. Oxygen enrichment was recorded as percentage of pure oxygen added to the total air/gas (taken as 100 %) supplied for aeration [13]. After three hours of inoculation, a pulse of dextrose monohydrate was given at the rate of 3 gm/L/hr for first three hours. This was enhanced to 6 gm/L/hr for the next six hours. Likewise, a pulse of yeast extract at the rate of 5 gm/L/hr for first three hours and 10 gm/L/hr for next six hours and also a pulse of K_2HPO_4 at the rate of 3 gm/L/hr for first three hours and 6 gm/L/hr for next six hours was provided. While giving the pulse the wet weight of the biomass was determined at the regular intervals of time and when the cell density reached to ~ 78 gm/L, appropriate concentration of sterile inducer is added (150 mM NaCl) and rate of pulse of three

components gradually reduced to 60 % for first three hours, 40 % for the next three hours, 20 % for the next three hours and at the 0 feed rate at the last three hours of post induction.

2.6. Analytical Methods

Dextrose levels in the medium is determined by 3,5-dinitrosalicylic acid (DNS) method [14]. One optical density (OD_{600}) unit was found to be equivalent to 0.5 gm dry cell weight (DCW). After induction, cell pellets were dissolved in phosphate buffered saline (PBS) followed by the addition of sample solubilizing buffer (0.0625 M Tris - HCl (pH 6.8), 5 % βME, 2 % SDS, 10 % glycerol, 0.01 % bromophenol blue) and boiled at 100 ^{0}C for 10 min. Samples were run on 12 % SDS-PAGE with reference to the low range protein marker. To investigate both the soluble and insoluble fractions, cells were lysed by sonication [15]. After sonication, the samples were loaded on 12 % SDS-PAGE [16].

2.7. Fibrinolytic and Thrombolytic Activity

Fibrinolytic activity was measured on a fibrin plate consisting of 1 NIH U/mL of thrombin, 1 % (w/v) agarose, 15 nM of human plasminogen and 0.25 mg/mL of bovine fibrinogen. 15 µL of sample were added to the wells on the plate and incubated at 37 ^{0}C for 8 – 12 hrs. Activity of recombinant protein was obtained from the standard curve. The diameter of digested fibrin on the plate was considered as the measurement of fibrinolytic activity of the enzyme. The international units (I.U.) of the enzyme activity of the protein was measured by the mean diameter of the zone of clearance around the well according to the standard curve plotted using the other thrombolytic protein streptokinase as a reference.

For thrombolytic activity, recombinant protein is analyzed by *in-vitro* tube test described by Swetha Prasad [17]. Blood is collected and citrated immediately with the addition of 2 % sodium citrate solution [18]. Two ml of this citrated blood is added to pre weighed microfuge tube and centrifuged at 3000 rpm for three minutes. After centrifugation the tube is incubated for one hour at 37 ^{0}C. After formation of clot, serum was decanted carefully. 50 µL of recombinant protein (C), 50 µL of standard streptokinase (M) and 50 µL of sterile water is added to the tubes containing clots respectively. Standard streptokinase serves as a positive control and sterile water serves as negative control. Three tubes were incubated at 37 ^{0}C for 90 minutes. Weight of the clot (W) is taken by substracting the preweight (W_1) from the weight of clot containing tube (W_2). The results are shown in Figure – 5. Approximately 50 % of clot lysis is observed.

Weight of the clot (W) = weight of clot containing tube (W_2) – pre weighed tube (W_1).

2.8. Purification of Sak - Hirulog

The Sak – hirulog was purified by IMAC (immobilized-metal affinity chromatography) for 6X His – tagged protein described according to manufacturer instruction using Ni – NTA spin column (Qiagen, Germany). The intracellular recombinant protein expression in *E. coli* resulted in formation of soluble proteins (SP) and purified under standard conditions.

The cell pellet was suspended in lysis buffer (7 M urea, 100 mM NaH_2PO_4, 100 mM Tris-HCl, pH 8.0 and benzonase nuclease (3 U/ml)) and cell suspension was allowed for sonication at 50 W intermittently in 10 sec on/off cycle for 10 min. The colour change in cell suspension results the complete lysis of cells. Centrifuge the sample at maximum speed for 15 min at 4 ^{0}C. The supernatant containing his-tagged protein was collected and transferred to a Ni-NTA spin column, previously pre-equilibrated by centrifuging at 3000 rpm for 5 min with lysis buffer. The protein sample is incubated on spin column for 5 min and centrifuged for 10 min at 2000 rpm. The column was washed with wash buffer (8M urea, 100 mM NaH_2PO_4, 100mM Tris-HCl, pH 6.3) for two times at 2000 rpm for 2 min.

Finally, the protein was collected in an appropriate volume of elution buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-HCl, pH 4.5) at 3000 rpm for 5 min and stored at in freezing conditions. The concentration of the protein was determined according to Lowry's method [19] with bovine serum albumin (BSA) as a standard.

3. Results and Discussion

3.1. Shake Flask Cultivation

Growth of recombinant salt inducible *Escherichia coli* GJ1158 strain is evaluated in shake flasks and sak variant expression levels in LBON, modified M9ON media and modified GYE medium (later used for batch and fed batch cultivations) were compared. The protein expression was analysed using 12 % SDS–PAGE of the three samples from LBON, modified M9ON media and modified GYE medium were shown in the following figure – 1. In LBON broth, the recombinant protein specific yield was 83 mg/L. Likewise, in modified M9 media, the recombinant protein specific yield was 67 mg/ L and in modified GYE media, the specific yield was 78 mg/ L respectively. The recombinant protein was expressed in soluble fractions. The recombinant staphylokinase variant production in bacterial expression systems is higher compared to the other production from other *E. coli* strains (50 mg/L) [20] but slightly low in comparison to *Bacillus* (140 mg/L) [21].

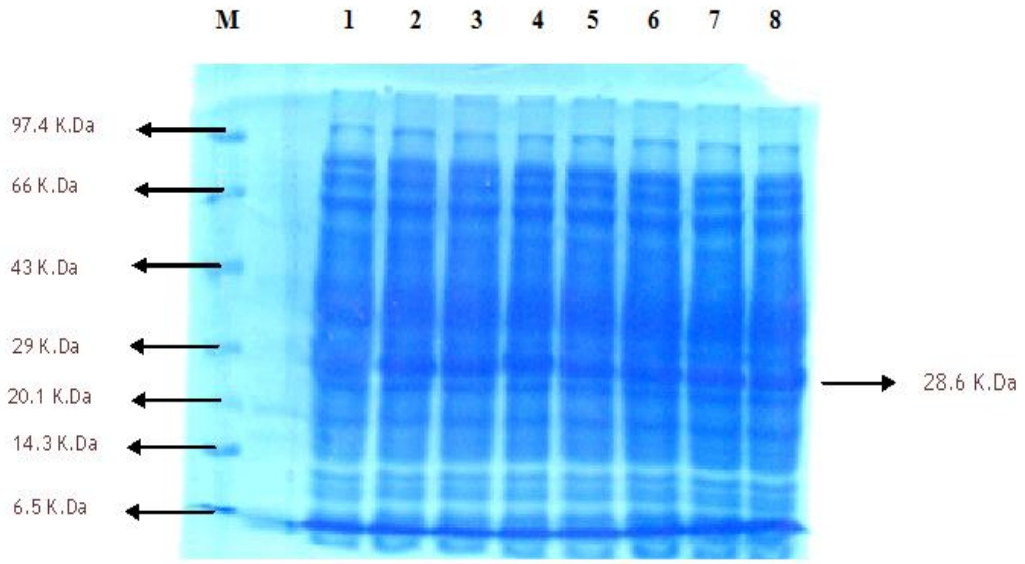

Figure 1. SDS-PAGE of Staphylokinase Hirulog Variant

M: Aprotinin (6.5 K.Da) and protein molecular weight marker; Lane 1: Uninduced Msak – hirulog pET28a+ GJ1158; Lane 2: Induced Msak – hirulog pET28a+ in LB from shake flask, Lane 3: Induced Msak – hirulog pET28a+ in modified GYE from shake flask, Lane 4: Induced Msak – hirulog pET28a+ in modified GYE from batch cultivation, Lane 5: Induced Msak – hirulog pET28a+ in modified GYE from fed batch cultivation (D0 30%), Lane 6: Induced Msak – hirulog pET28a+ in modified GYE from fed batch cultivation (D0 50%) and dextrose as pulse, Lane 7: Induced Msak – hirulog pET28a+ in modified GYE from fed batch cultivation (D0 50%) with dextrose and yeast extract as pulse, Lane 8: Induced Msak – hirulog pET28a+ in modified GYE from fed batch cultivation (D0 50%) with dextrose, yeast extract and K_2HPO_4 as pulse.

3.2. Batch Cultivation

Based on the results of shake flask studies, recombinant protein expression was slightly higher in LBON broth when compared to modified M9ON medium and modified GYE medium. Batch cultivation, performed using synthetic medium was carried out in a 10 L bioreactor with a working volume of 6 L and 30 % of dissolved oxygen is maintained throughout the experiment. Approximately, similar yield is obtained in modified GYE media and it is more preferred to be run in bioreactor because of its low cost and good specific yield. At a final OD_{600} (~12) in modified GYE medium (Figure – 2), induced with 150 mM NaCl and after 3 – 4 hrs of post induction process the recombinant specific yield was 247 mg/L. This is comparatively high when modified M9 media was used (216 mg/L), but slightly less when compared to the production from LB media (278 mg/L). Even enhanced DO beyond 30 % did not influence the production of recombinant protein in batch fermentation.

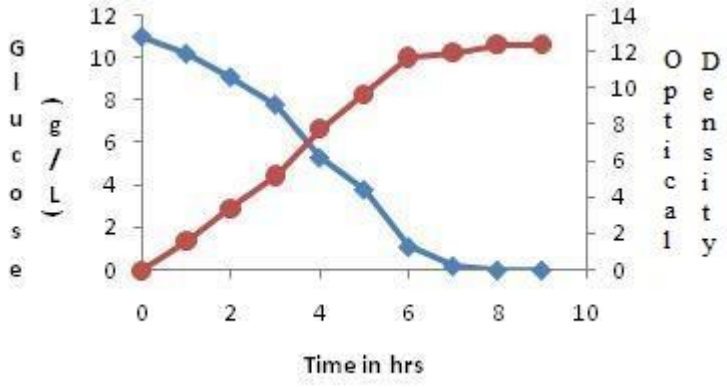

Figure 2. Batch Fermentation

Figure shows cell growth (●) and dextrose utilization (■)

3.3. Fed batch Cultivation

Fed batch cultivations were carried out by the consequent addition of nutrients like dextrose, K_2HPO_4 and yeast extract to build up high density of recombinant *E. coli* cells. Dissolved oxygen ranging from 30, 40, 50 and 60 % was supplied to the medium. The fed-batch cultivation run using 50% dissolved oxygen as set point under nutrients supply before and after induction process gave maximum yield. Fed-batch experiment with K_2HPO_4 for 3 gm/L/hr for first three hours then 6 gm/L/hr for next six hours, yeast extract feeding rate is 5 gm/L/hr for first three hours then 10 gm/L/hr for next six hours and dextrose feeding rate is 3gm/L/hr for first three hours and 6 gm/L/hr for next six hours resulting in a maximum cell density (OD_{600}) of 78 within 12 hrs (Figure – 3). Induce the culture with 150 mM NaCl at OD_{600} 78. After 12 hrs of post induction, cell density of 81 (~ 89 g/L) was obtained. After specific yield quantification, induction at OD_{600} 78 was noticed as the best OD for induction. The produced recombinant protein was found to have good fibrinolytic activity on plasma agar plate (Figure – 4). Approximately 1178 mg/L of protein was achieved using 30 % of dissolved oxygen. It was two fold increases in the production when compared with the batch cultivation of sak – hirulog.

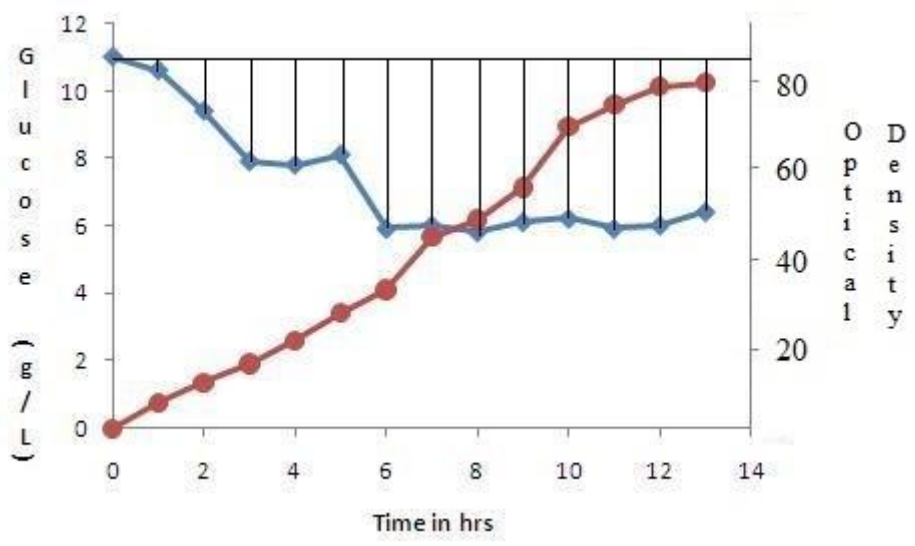

Figure 3. Fed Batch Fermentation Supplied with Dextrose Monohydrate at 50% DO

Dextrose utilization (■) and optical density at OD_{600} nm (●). Vertical lines indicating the addition of dextrose at each and every hour interval and finally maintained 11 gm/L throughout the experiment for getting maximum OD upto before induction process.

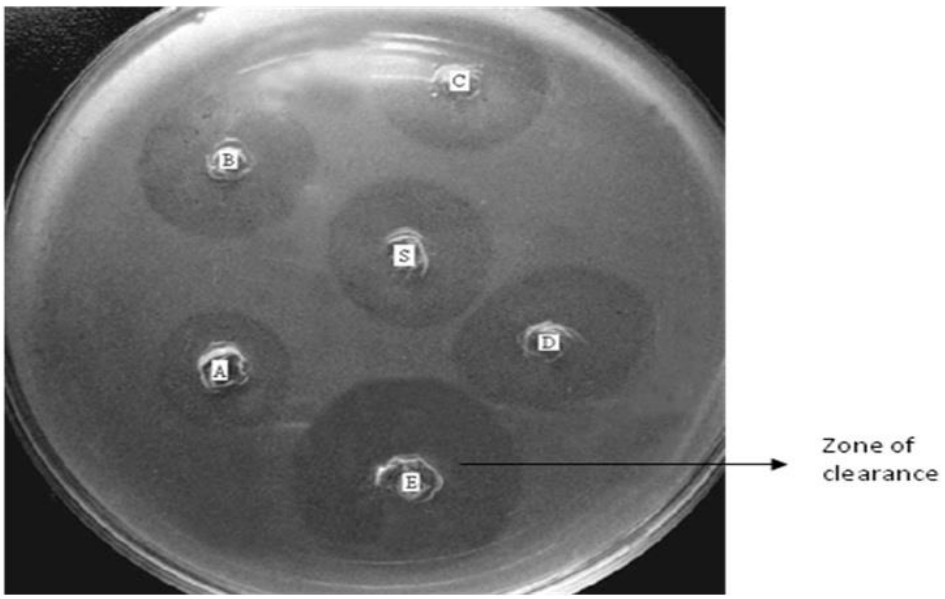

Figure 4. Fibrinolytic Activity of Staphylokinase-Hirulog

S = 30 micro liters of standard streptokinase; A = 20 micro liters of standard streptokinase; B = 30 micro liters of soluble fraction of sak from shake flask fermentation; C = 30 micro liters of soluble fraction of sak – hirulog from batch fermentation at 30% DO; D = 30 micro litres of insoluble fractions of sak – hirulog from fed batch fermentation at 30% DO; E = 30 micro liters of purified soluble fractions of sak – hirulog from fed batch fermentation at 50% DO.

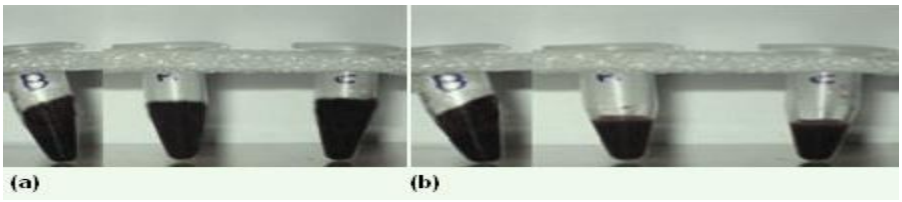

Figure 5. In Vitro Tube Test for Thrombolytic Activity of Streptokinase and Staphylokinase Hirulog

(a) Before treated with recombinant protein. (b) After treating with recombinant protein. B – Blank (clot treated with saline water), M – streptokinase (clot treated with streptokinase), C – chimera (staphylokinase - hirulog) (clot treated with staphylokinase – hirulog).

3.4. Effect of Dextrose

In order to improve the cell density to get maximum yield of recombinant protein, concentration of dextrose in medium after inoculation is maintained as 11 gm/L throughout the experiment before induction process by providing the pulse 3 gm/L/hr for first 3 hours to 6 gm/L/hr for next 6 hours. Each and every time while giving dextrose as pulse, OD was recorded. There was no significant difference between the OD at 12^{th} and 13^{th} hour. At 50 % dissolved oxygen, dextrose alone as pulse gave 869 mg/L of recombinant protein, this is one and half folds greater than the protein achieved at 30 % DO in fed batch.

3.5. Effect of Dissolved Oxygen

Different levels of dissolved oxygen were studied in batch fermentation process. 30 % DO in batch cultivation resulted in a good expression level. Whereas, in the fed batch fermentation, the threshold levels of dissolved oxygen was found to be in the range of 50 %. The value of DO remained at 30 % in batch cultivation, while in fed-batch it was found to be 50 %. 20 % dissolved oxygen is enough to produce the recombinant proteins in fed batch cultivation [22, 23]. The threshold level in order to maintain the DO, the next fed-batch cultivation is carried out at 50 % DO and although the cell growth slightly increased when compared to 30 % DO and residual dextrose profiles reduced similar to 30 % DO fed batch fermentation. The number of cells during post-induction phase did not increase. The final enhancement in cell proliferation is observed while giving the pulse to be in direct agreement with increase in the DO and hence, concentration of recombinant protein, is estimated to be 1178 mg/L, where the combination of dextrose, yeast extract and K_2HPO_4. So, 50 % of DO is always effective in fed batch cultivation and increase of DO to 60 % did not influence the improvement in expression of sak variant in other experiment trails. In some studies 10 % DO is enough to produce the protein [24]. But, we achieved highest quantity of protein at 50 % DO in fed batch. The low and high levels of DO are not unusual and supposed to happen due to changes in growth kinetics [25, 26], aeration or agitation rates and increase in viscosity [27]. Based on the results, the decrease in level of DO in cultivation medium after induction, below a threshold levels, can affect the cell proliferation and expression of recombinant protein.

Very inconsiderable cell proliferation is observed during the initial 4-5 hrs of post induction time by raising the DO from 50 % to 60 % and the protein expression levels are not enhanced. During the bioreactor cultivations of recombinant *E. coli*, the maintenance of DO above a certain threshold level and oscillations in DO should also be taken in consideration. For this reason the DO is critical for both growth and it provides the energy for the proliferation of bacteria and expression of the recombinant protein [28]. The initial post-induction time is crucial for the expression of recombinant protein under the strong

promoter T7 as product formation ceased after the 4 – 5 hours of induction period [13, 29].

3.5. Effect of Yeast Extract

To increase the expression of sak variant in fed-batch cultivation, the effect of different nitrogen sources were investigated and yeast extract is identified as the optimal nitrogen source for bacterial growth as it resulted in increased recombinant protein expression [30, 31] and cell mass [32]. The final cell density (OD_{600}) in fed batch cultivation is 40 which resulted in the highest expression from recombinant *E. coli* by providing the 5 gm/L/hr for first three hours and 10 gm/L/hr for next 6 hours to achieve the maximum cell density. The continuous medium supplementation [33-35] resulted in the highest specific protein concentration of 1098 mg/L when compared to batch fermentation 278 mg/L from LB broth. But on combination with dextrose the production yield is 1098 mg/L, where the maximum yield was achieved compared to the dextrose alone as pulse at 50 % fed batch fermentation.

3.6. Effect of Disodium Hydrogen Phosphate

The phosphorus compounds serve as major nutrients in cell growth and protein expression. A pulse of K_2HPO_4 is given in the rate of 3 gm/L/hr for first three hours. Then pulse has been enhanced to 6 gm/L/hr for next six hours. While giving the pulse the dry weight of the biomass was determined at the regular intervals of time and when the cell density reached to maximum biomass, appropriate concentration of inducer is added (150 mM NaCl) and after induction, the rate of pulse will gradually reduced to 60 % for first three hours, 40 % for the next three hours, 20 % for the next three hours and at the 0 feed rate at the last three hours of the induction. The protein production slightly increased (1178 mg/L) along with addition of other nutrients like dextrose and yeast extract at 50 % DO in fed batch fermentation [Table-1].

Table 1. Recombinant Staphylokinase Hirulog at Different Stages of Production

Cultivation type	Medium used	OD_{600} [1]	Specific protein (mg/L)[1]
Shake flask cultivation	LB	2.6	83
Shake flask cultivation	MM9	2.1	67
Shake flask cultivation	MGYE	2.4	78
Batch cultivation	LB	14	278
Batch cultivation	MM9	9.8	216
Batch cultivation	MGYE	12.9	247
Fed batch cultivation at 30 % DO	MGYE	70	549
Fed batch cultivation at 50 % DO	MGYE + Dextrose as pulse	72	869
Fed batch cultivation at 50 % DO	MGYE + Dextrose + Yeast extract as pulse	74	1098
Fed batch cultivation at 50 % DO	MGYE + Dextrose + Yeast extract + K_2HPO_4 as pulse	78	1178

[1] values are ± means of two experimental trails

The feeding rate of K_2HPO_4 improves the cell biomass and the protein production. Even after increasing the feeding rate more than 6 gm/L/hr after 9th hour does not show any influence on cell proliferation and protein production indicates that maximum feeding rate is 3 gm/L/hr for first three hours and 6 gm/L/hr for next six hours. The essentially unaffected protein yield of sak - hirulog with scale up from flask level to fed-batch reactor has a practical involvement in improved protein production.

4. Conclusion

The third generation molecule staphylokinase and its variant will have demand in future because of its maximum patency with fewer side effects like minimal bleeding risk and reocclusion. Sak is a prokaryotic protein with good clot specificity when compared to t-PA. The optimization of cultivation parameters leads to the enhanced production of therapeutic protein, staphylokinase variant is the best of our knowledge from salt inducible system till to date. At flask and batch cultivation level the highest quantity of protein is achieved using LB medium, very similar results were observed from modified GYE medium. This study mainly concentrated on influence of dissolved oxygen and other nutritional parameters on growth and protein production. Dissolved oxygen (50 %) is the influencing parameter for the production of recombinant protein with the combination of different nutritional factors. Finally 4.76 folds of protein production is observed using the batch fermentor via through modified GYE medium and 2.14 folds increase is observed in comparison to fed batch run using the same medium with 30 % dissolved oxygen.

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