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A Novel Induction Strategy Based on Temperature and pH Optimization to Improve the Yield of Recombinant Streptokinase in *Escherichia coli*

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Abstract

Introduction:

Streptokinase (SK) is used for thrombolytic therapy of acute myocardial infarction. Recombinant fermentation is the most common and cost-effective procedure in the production of SK**.** Nutrients composition, as well as such fermentation variables as temperature and pH, can affect the production level of protein-based drugs expressed in *E. coli*. In the present study, optimized recombinant streptokinase (r-SK) production in *E. coli* in the form of inclusion body (IB), the effect of induction temperature, and extracellular pH were evaluated.

Methods and Results:

To maximize the expression level of r-SK, three different induction temperatures were used to obtain the optimal temperature for r-SK production. The effect of extracellular pH on the level of r-SK production was also studied and the optimal pH was determined. To this purpose, eight fermentation processes were applied and followed by: Cell disruption, Isolation of IBs, and determination of target protein (SDS-PAGE, Western-blot). The biological activity of r-SK obtained from optimal conditions was determined in comparison with reference standard, using gel clot method.

The highest amount of expression level and IB formation were performed by combining two temperatures: 42˚C for the first two hours of induction, followed by 39˚C at pH 6. The expression level of r-SK under these conditions demonstrated an 11.6% increase in comparison with the control.

Conclusion:

Choosing appropriate cultivation conditions can increase IB formation and utilise its advantages to produce higher quantities of r-SK with appropriate biological activity.

Keywords: *Escherichia coli, Fermentation, recombinant streptokinase, Inclusion body*

1. Introduction

The *Escherichia coli (E.coli)* is widely used as an expression host for production of recombinant proteins, some of which are used for therapeutic purpose [1,2]. In fact, some of the most important protein-based drugs, including insulin, human growth hormone, interferons, interleukins, and (SK) have

been successfully produced in *E. coli* [3-6]. Recombinant protein expression using *E. coli* can result in the formation of insoluble aggregates known as (IB) [7]. Provided refolding, with high degree of recovery of the active form of the protein, can be achieved, expression as IBs is useful for obtaining a large amount of the target protein [8]. Since IBs mainly consist of the protein of interest and are easily isolated by centrifugation, their formation has often been exploited to simplify the purification process [9]. On the other hand attempts have been made to obtain the protein of interest in the soluble form by adjusting media composition, growth temperature, inducer concentration, promoter strength, and plasmid copy number [10,11].

However, recent studies have suggested that properly folded proteins may be formed as natural components of IBs [12,13], indirectly compromising the paradigm of recombinant protein solubility as equivalent to protein conformational quality [14]. In any case, maximizing the yield of a recombinant protein expressed in *E. coli* can be achieved by increasing the formation of IBs. Furthermore, it is worth noting that in the case of proteolytic-sensitive proteins, like SK, which do not contain any disulfide bonds, expression as IBs can be the method of choice. So, optimization of fermentation variables can effectively increase the yields of (r-SK) in the form of IBs and, consequently , the overall yield of production process.

In this study, the effects of the induction temperature, as well as the extracellular pH during induction phase, on productivity of temperature-induced r-SK were investigated using a one-factor-at-a-time strategy. One-factor-at-a-time, in which all parameters are kept constant except one, is the most popular technique for improving fermentation processes [15].

2. Materials and Methods

2.1. Strain and plasmid:

E. coli K-12 (Invitrogen) was used as the host microorganism for SK production in all fermentation experiments. An Ampicillin (Sigma-Aldrich, Germany) resistance plasmid under the control of the λ_{PL} promoter was used for temperature-inducible expression of r-SK.

2.2. Cultivation:

2.2.1. Shake flask:

E. coli cells were cultured in 10 mL of Luria-Bertani medium containing 50µg/ mL of ampicillin and shaken at 35˚C for 10 hrs (Kuhner ISF-1-W, Switzerland). When the OD_{600} reached 0.5-2, the seed culture was used to inoculate a flask containing 200 mL of LB medium shaken at in the same conditions as above.

2.2.2. Fermentor culture:

The culture obtained was used as inoculum for a 13L fermentor (Infors, Switzerland) containing 3L medium, prepared essentially as described previously (16,17). The fermentor medium was composed of Glycerol (120mL), $(NH_4)_2HPO_4$ (4g), KH_2PO_4 (13.2g), $MgSO_4.7H_2O$ (1.2g), Ampicillin (5mg), Citric Acid (1.7g), EDTA (0.0141g), and trace elements ($CuSO_4.5H_2O$, KI, $ZnCl_2$, $CoCl_2$, $MnSO_4.H_2O$). Fermentation was continued until OD_{600} reached 4-6.

This was followed by the fed batch stage, which was carried out using glycerol as the carbon source. The culture conditions were as follows: air flow= 0.5-1.0 (liters of air/ liter of medium / min), Stirrer speed= 300 to 700 rpm. The temperature was held at 35˚C during the growth phase and then raised to initiate the expression of SK. The dissolved oxygen concentration was maintained at 30% of air saturation by adjusting the stirrer speed. The pH was adjusted by addition of aqueous ammonia solution (25%w/v). The control of temperature, pH, foam, and dissolved oxygen was carried out by the digital control unit of the fermentor. The foam was suppressed, when necessary, by the addition of anti-foam reagent B (Sigma-Aldrich, USA). The culture was then induced at mid-exponential phase (around OD_{600} of 8-10) by an increase in temperature. The production of SK was studied at four fermentation runs, three times each with different induction temperatures, namely 39 ˚C, 42˚C, 44˚C, and a combination of 42˚C and 39˚C.

In these experiments the extracellular pH was kept constant at 7.0 ± 0.2. To find the optimal pH, three fermentation runs were carried out using the optimal induction temperature at different pHs including 5, 6, and 8. Sampling was carried out every hour during the induction phase to analyze the expression level of the desired protein using SDS-PAGE and Lowry protein assay. The comparative expression level between optimized and control conditions were estimated per 100 mg of IB and defined as a normalization procedure (r-SK/total proteins ratio×100).

To check the biological activity of the produced r-SK, an extra fermentation run was performed under optimal conditions, followed by solubilization and refolding of the SK obtained.

2.3. Cultivation control

The induction temperature of 40˚C and pH of 7.0 maintained for four hours were considered as the control condition, which is routinely exploited utilised for production of recombinant protein in the form of IB in *E. coli* [16, 17]. The average of the results obtained from all the experiments were compared with the mean results of three cultivation control runs.

3.4. Cell fractionation

The cells were harvested by centrifugation (Sigma 8K10, Germany) at 4000 g for 30 min at 4˚C. The cell pellet obtained was re-suspended in Tris.HCl buffer (20 mM, pH 8) and brought to OD_{600} of around 0.5-1.Then, the cells were disrupted in 3 cycles using a high-pressure homogenizer (GEA, NiroSoavi, Italy) at 1800 bar. The final suspension was centrifuged (Sigma 8K10, Germany) for 30 min at 4000 g to collect the IBs. These were washed three times in Tris.HCl buffer (20mM, pH 8). The weight of the IBs was measured and recorded.

2.5. Solubilization

Solubilization was performed on the IBs obtained by addition of 6M guanidine hydrochloride and 2mL/liter of β-mercaptoethanol.

2.6. Refolding by rapid dilution:

The solubilized SK was refolded in 20 mM Tris-HCl pH 7.5 with sucrose by diafiltration (Proflux M12, Milipore, USA) in three cycles.

2.7. Analytical Methods

Cell growth during cultivation was monitored by observation of the optical density at a wavelength of 600 nm (Lambda 25, PerkinElmer, USA). Sampling was carried out every hour during growth phase and post-induction.

Following cell disruption, to determine the total soluble and insoluble proteins, the samples were centrifuged at 15000 g for five minutes. Then, the soluble protein content in the supernatant was determined with the Lowry method [18] using 1 mg/mL of bovine serum albumin (Thermo scientific, USA) as standard. The insoluble protein quantification was performed by densitometeric analysis (TotalLab, Nonlinear dynamics, USA) of proteins on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean® 3 cell, Bio-Rad, USA) and calculation of the ratio of the target protein expression level to the amount of total protein. SDS-PAGE was carried out according to the method of Laemeli on 15% resolving gel [19]. The gels were stained with Coomassie Brilliant Blue R250.

Identification of r-SK was carried out using Western blotting; the samples were run on 15% SDS-PAGE gel and electroblotted (Trans-Blot®, Bio-Rad, USA) to Hybond-P PVDF membrane (GE healthcare, USA) according to the method of Towbin [20]. Rabbit anti-streptokinase (American diagnostic Inc., USA) and anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma–Aldrich, USA) were applied as primary and secondary antibodies respectively. The biological activity of SK was measured using fibrin clot lysis according to the method of Marsh and Gaffney [21]. The 3rd international standard of streptokinase 00/464 (WHO/NIBSC-UK EN63QG) containing 1030 IU per vial was used as reference standard.

3. Results

3.1. Effect of induction temperature:

The results of the effect of induction temperature on the level of IB formation, as well as the expression level of SK, are summarized in Figure1. Induction and production at 42˚C resulted in a significant increase in IBs formation during the first two hours post induction. After that a reduction in the concentration of IBs was observed. On the other hand, IBs formation at 39˚C showed a significant increase during the second two hours of induction in comparison with other induction temperature.

Furthermore, the highest cell density was observed at 4 hours post induction at 39˚C, which was statistically significant. In terms of expression level, induction at 42˚C during the first two hours, and 39˚C for the second two hours was significantly higher among others. Therefore, a fermentation run was performed by combining these two temperatures: 42˚C for the first two hours of induction, followed by 39˚C. The expression level of SK under these conditions was found to be 58.3% compared to 44% at 39˚C and 46.7% at 42˚C (Figure 1).

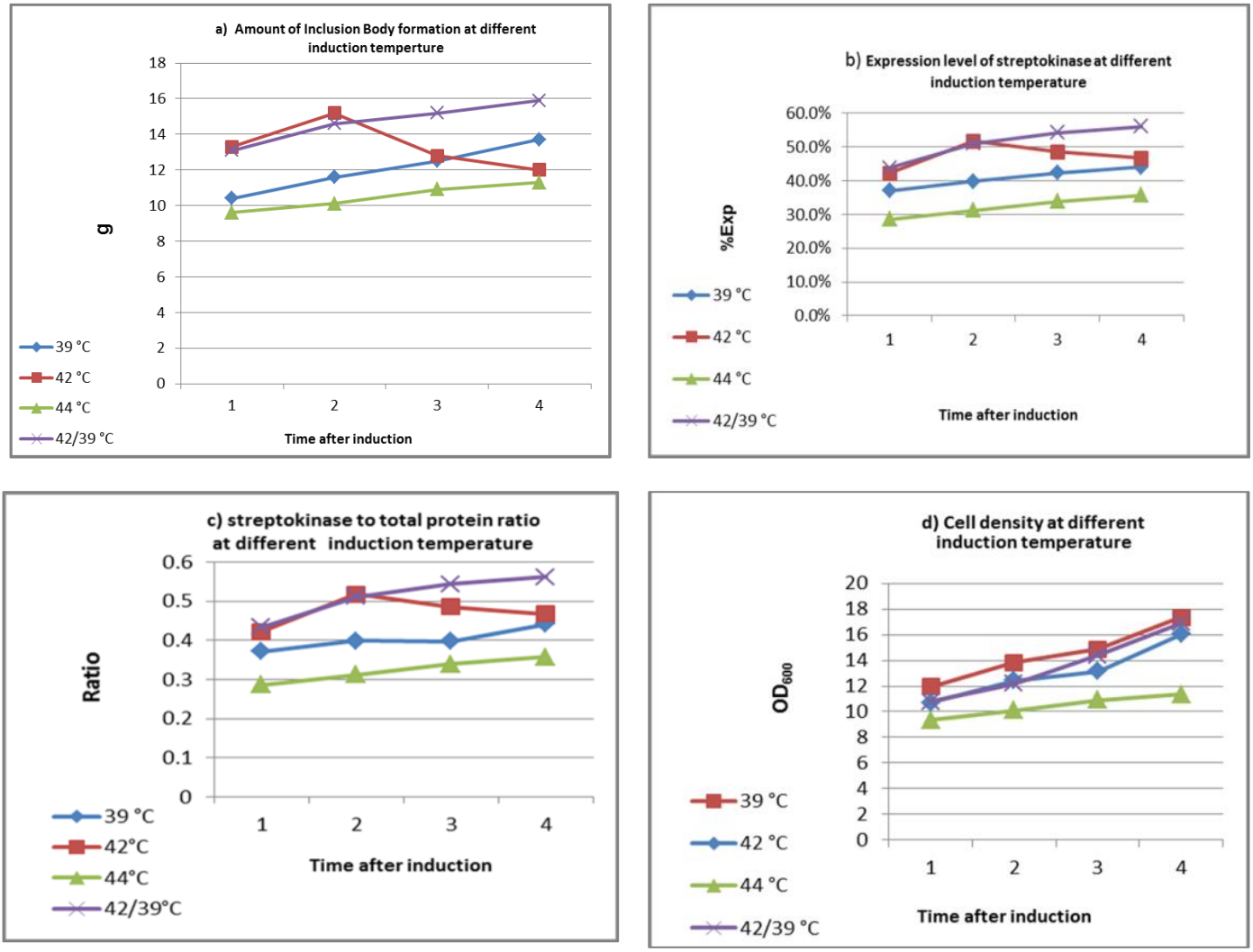


Figure 1. Effect of different induction temperature on (a). IB formation (b) Expression level of streptokinase. (c) Streptokinase to total protein ratio. (d) Cell density.

3.2. Effect of pH

In the first set of experiments, the optimal temperature for obtaining the highest amount of IBs and target protein expression was found to be a combination of 42˚C and 39˚C.

Since reduction of pH has been found to lead to an increase in IB formation [22], the second set of investigative experiments focused on the determination of the optimal pH. So, fermentation runs were carried out at different pHs including 5, 6, and 8; whilst the pH 7 was used as the control condition ANOVA. At pH 5, the amount of total cell protein, cell mass, IB, and SK declined with time. In fact, the results were comparable with the results of the temperature induction at 44˚C. At pH 6, the maximum amount of IB and SK was observed. The expression level of SK at pH 7 (cultivation control) was similar to pH 6, but total cell protein and the ratio of target protein to total cell protein were lower. So, the optimal pH for production of SK was found to be 6 (Figure 2).

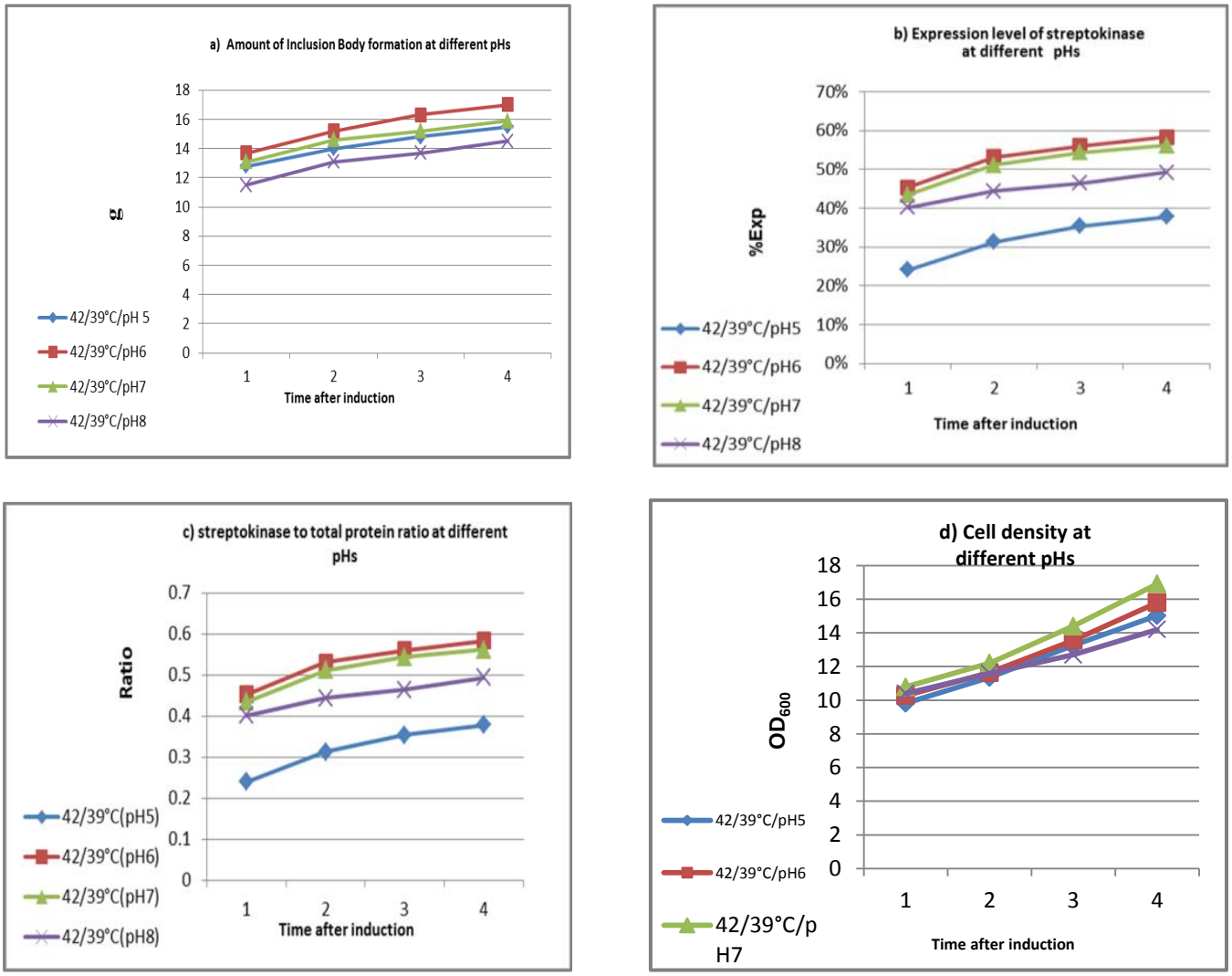
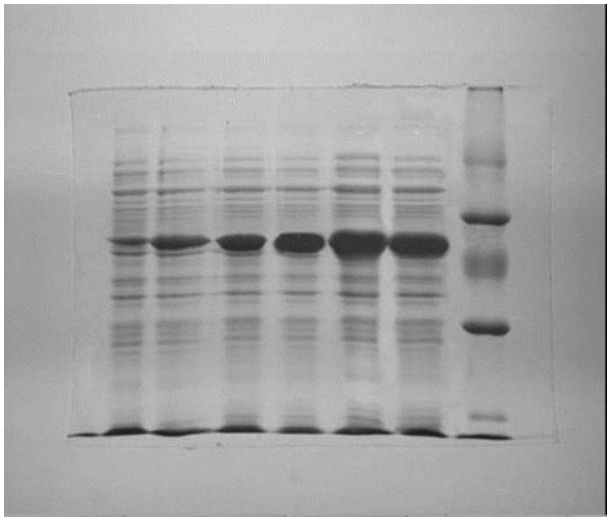


Figure 2. Effect of different pHs on (a) IB formation. (b) Expression level of streptokinase. (c) Streptokinase to total protein ratio and (D) Call density

3.3. Analysis of recombinant streptokinase

SDS-PAGE and densitometric analysis of r-SK under optimal conditions and control is shown in Figure 3. Under optimal conditions, expression level of SK was found to be 58.3%. The result of identification test for the expressed SK is shown in Figure 4. Furthermore, the specific activity of SK obtained under optimal

conditions was found to be 91000 IU per mg of protein in comparison with reference standard. This amount was approximately 98% of SK produced under cultivation control.



Process Step	Lane	Expression level	Total Protein (Lowry)	Total r-SK	OD600
Time zero	1	–	–	–	–
1 hour after induction	2	45.3%	(1612 µg/mL)	(730.2 µg/mL)	10.28
2 hours after induction	3	53.1%	(1725 µg/mL)	(916.0 µg/mL)	11.64
3 hours after induction	4	56.0%	(1829 µg/mL)	(1024.2 µg/mL)	13.56
4 hours after induction	5	58.3%	(1922 µg/mL)	(1120.5 µg/ mL)	15.80
5 hours after induction	6	51.7%	(1945 µg/mL)	(1005.6 µg /mL)	16.38

Figure 3. SDS-PAGE analysis of r-SK under optimal conditions at 5 hours post induction

4. Discussion and Conclusion

Over-expression of heterologous genes in *E. coli* leads to accumulation of proteins as IBs [1]. The main concern about IBs formation is the problems sometimes encountered during the *in-vitro* refolding process, which needs to be adapted for each individual protein [23,24]. Also sometimes refolding from IBs is the method of choice [10]. In fact, SK being a single-chain polypeptide which does not contain cystine and cysteine residues as well as conjugated carbohydrate and lipid [25], can easily adopt an active conformation following *in-vitro* refolding. The results obtained from biological activity of SK support this view. Furthermore, from the molecular point of view, IBs are considered to be formed by unspecific hydrophobic interactions between disorderly deposited polypeptides, and are observed as 'molecular dust-balls' in productive cells [13]. So, isolation of the desired protein with high purity is more convenient due to density differences [26]. Hence, increasing the amount of r-SK via choosing appropriate cultivation conditions can effectively maximize the volumetric yield of the production process in *E. coli*. Also, temperature-inducible expression systems have been used for

production of several recombinant proteins in *E. coli* due to the ease of induction [27].

In this study, in order to produce r-SK with high productivity and biological activity, the effects of induction temperature and extracellular pH were investigated using one-at-a-time strategies. The results obtained indicated that induction at 39ºC leads to higher cell density. On the other hand, an induction temperature of 42ºC led to the highest amount of IB at two hours post induction. So, starting the induction at 42ºC, continuing it for 2 hours and then continuing the process at 39ºC was carried out. These conditions yielded the highest total amount of IBs and rSK. This finding can be attributed to the function of molecular chaperons which were originally identified by their increased abundance due to heat shock [28]. DnaK, GroEL and GroES are the major chaperons involved in protein folding and solubilized production of recombinant proteins in *E. coli*. The level of heat shock proteins can be influenced by the degree of heat shock. The steady-state level of some heat shock proteins differs about two-fold between 37 and 42ºC, but transiently, after a heat shock the level of chaperones is higher [29]. Hence, a lower temperature increase results in lower response. Since the highest amount of SK production takes place during the first hour after induction, at 42ºC, the expression level of misfolded SK might be too high and the level of chaperons might not be adequate enough to interact with all of the misfolded proteins. This results in higher accumulation of SK in the form of IB during the first hour after induction at 42ºC. Therefore, continuous production of IBs at 39ºC, compared with the transient production at 42ºC, is likely to be the result of difference in the level of chaperons. Accordingly, continuing the induction at 39ºC (Figure1) accelerates IB formation due to decrease in the level of heat shock proteins [29].

The other parameter studied was the pH of the culture medium. When the pH of the medium is reduced below a certain value, the cells will encounter difficulties in maintaining the intracellular pH at a constant level. Studies have shown that decreasing the external pH from 7.0 to 5.0 changes the intracellular pH of 7.7 in *E.coli* by 1 unit [30]. On the other hand, changes in intracellular pH affect the folding process of proteins. So, in this study, based on the assumption that decreasing the pH might enhance IB formation, fermentation runs were carried out at pHs 5, 6, and 8 to obtain optimal condition (Figure2). At pH 6, the highest amounts of IB and rSK were obtained. However, further reduction of the pH to 5 was accompanied by a reduction in cell mass and IB because of reduction in cell proliferation.

Under the optimal conditions determined, expression level of SK was found to be 58.3%, which shows an 11.6% increase in comparison with the control.

This study shows the importance of careful examination of the cultivation conditions for production of recombinant proteins such as SK. In other words, by choosing appropriate cultivation conditions we can increase IB formation and use its advantages to produce higher quantities of r-SK with appropriate biological activity. However, it must be borne in mind that more than one temperature may need to be used during induction to control the effect of heat shock proteins in IB formation. Therefore, careful study of the effect of different induction temperatures on different parameters of the process must be undertaken to determine the overall optimal conditions.

List of abbreviations

r-SK: recombinant streptokinase

IB: Inclusion body

E. coli: Escherichia coli

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