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REGULAR ARTICLE

RECOMBINANT PROTEIN PRODUCTION OF ABUNDANT LARVAL TRANSCRIPT (ALT-2) IN *ESCHERICHIA COLI*

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ABSTRACT

Lymphatic filariasis is a major tropical disease caused by mosquito born nematodes *Brugia malayi and Wuchereria bancrofti*. Vaccine against filariasis must generate immunity to infective mosquito derived L3 stage. Two highly expressed genes designated abundant larval transcript-1 and -2 (alt-1 and alt-2). ALT-1 and ALT-2 represent closely related protein (79%) it. Now, expression of this alt gene in *E. coli* BL21plysS for the production of vaccine is major challenge as no vaccine is available against this disease. Work was carried out to express this protein at laboratory scale bioreactor. At first optimization of different parameter like suitability of media, inducer concentration, induction time was done for getting maximum amount of recombinant protein. In shake flask studies, after induction (max cell density and max specific growth rate stage) good expression of ALT-2 protein was found. However, at laboratory scale production done in bioreactor, expression level drastically decreased. Plasmid stability analysis was done in reactor and was found to be cause for decreased productivity. The stability was improved by increasing antibiotic concentration in the medium and also by pulsing antibiotic during induction. This led to better plasmid stability and increased expression levels in reactor similar to expression levels in shake flask studies.

Keywords: Lymphatic filariasis, IPTG, plasmid stability, abundant larval transcript, shake flask

INTRODUCTION

Lymphatic filariasis (LF) is a mosquito-transmitted disease caused by parasitic nematodes *Brugia* and *Wuchereria*. More than 120 million people living in 83 endemic countries of the tropics and subtropics were affected from this disease. Over 1.3 billion people (one-fifth of the world's population) are at the risk of infection through their exposure to LF's mosquito borne infective larvae **(WHO, 2006)**. Two highly expressed genes designated abundant larval transcript-1 and -2 (alt-1 and alt-2). ALT-1 and ALT-2 represent closely related protein (79%) it ALT-2 is the most abundantly expressed protein in the infective stage larvae (L3) of the filarial life cycle **(Joseph *et al.,* 1998)**. The Global Programme to eliminate Lymphatic Filariasis (GPELF) has been initiated by World Health Organization (WHO) in 1997 with two major goals, which is to disrupt transmission of the parasite and morbidity control by providing care for those who suffer the overpowering clinical manifestations of the disease **(Addiss and Brady, 2007)**. *Escherichia coli* is commonly used to produce recombinant proteins because it can be grown to high densities on inexpensive media and its genetics has been well understood. These attributes led to the use of *E. coli* as the most popular host cell in bio fermentation. Although filarial parasite is a eukaryote, and we had chosen a prokaryotic expression system (*E. coli*) for production of ALT-2 recombinant protein based on two reasons. First, an *E. coli* system is used because the proteins expressed do not have post translational modifications. Second, an *E. coli* expression system allows production of large amounts of recombinant proteins **(Kathleen *et al.,* 1996)**. However, synthesis of recombinant protein places substantial metabolic burden on the producing cells. In order to prevent this burden from compromising the growth, by using transcription regulators. Common regulators used in *E. coli* include the lac, trp, ara BAD and tac promoters **(Makrides, 1996).** The lac promoter used in this experiment is controlled by the use of isopropyl-ß-Dthiogalactoside (IPTG) **(Kweon *et al.,* 2001).** Recombinant protein production is normally induced in the late exponential growth phase. However, induction in the late exponential phase or early stationary phase reduces the culture viability and can lead to production of proteases that can break down the desired recombinant protein. On the other hand, early induction can unnecessarily slow the doubling time of bacterial cells **(Chisti, 1998, Corchero *et al.,* 2001**). Protein expression levels of a culture depend on cultivation

conditions, such as culture temperature, medium composition, induction time, inducer concentration and inducer type, which can be optimized for over expression of a soluble protein **(Hettwer *et al*., 2002; Kopetzki *et al*., 1989).**

Hence it is important to optimize the type of medium, IPTG concentration and induction time, to achieve high cell density culture towards the aim of producing a high yield of the recombinant protein. Generally the development of fermentation strategies is paying attention on improving the cultivation techniques, manipulating the physiology of the bacteria and the level of recombinant protein expression. As a result, batch and fed batch fermentation techniques have been developed **(Shiloach and Fass, 2005).**

Here in this experiment, we first optimized the different parameter for the production of larger amount of abundant larval transcript-2 recombinant protein then after production was done at reactor scale. This is because of its potential use in vaccines.

MATERIAL AND METHODS

Host and vectors pRSETB-alt-2 construct coding for the His-tagged ALT-2 protein was previously constructed in our laboratory. It was expressed in *E. coli* BL21(DE3)pLysS (Novagen, Darmstadt, Germany).

Reagents and media

Reagents and chemicals were purchased from Merck (Darmstadt, Germany) and BioRad (Hercules, USA). Inoculum was grown in Luria-Bertani medium (LB) that contained the following: peptone (10 $g.L^{-1}$), yeast extract (5 $g.L^{-1}$), NaCl (5 $g.L^{-1}$) and the pH was adjusted to 7.2.

Competent cell preparation

There are two main methods are available for transformation of competent bacterial cells, the calcium chloride and the electroporation method **(Dargert *et al*., 1979; Okamoto *et al*., 1997; Topcu, 2000).** Here we have chosen the calcium chloride method.

A single colony was inoculated from fresh plate of *E.coli* BL21 (DE3) plysS in 15 ml of LB medium. One ml of this culture was inoculated in 100 ml of LB flask. Flask was incubated on orbit shaker at 37˚C at 200 rpm for 2 hrs. When the OD reached 0.6 on 600 nm, the culture

flask was chilled on ice immediately. The culture was centrifuged at 4˚C at 3500 rpm for 5 minutes. The supernatant was decanted and the tube was kept in an inverted position for 1 min to allow the last trace of media to drain away. The pellet was suspended in 100 mM of $CaCl_2$ (one fourth volume of culture), kept in ice for 30 minute. The cells were recovered by centrifugation at 3500 rpm for 5 min at 4˚C. The fluid was decanted from the tube and the pellet was re suspended in 100 mM $CaCl_2$ and stored for 30 minute in ice. Again we recovered the cell by centrifugation at same speed. Discarded the liquid and suspend the cell in 2 ml 0.1M $CaCl_2$.

Preparation of competent cells for storage as glycerol stocks

Transfer 1.6 ml of the competent cell suspension to sterile cryo-storage tubes, and add 0.4 ml of sterile 100% glycerol to give a final concentration of 20% glycerol, and then mix together. The glycerol stocks are placed at -4ºC, -20ºC and -70ºC separately for later use.

Transformation in bacterial cell

Bacterial transformation, Plasmid transformation and antibiotic selection

Calcium chloride treatment of bacterial cells produces competent cells that will take up DNA following a heat shock step. DNA molecules, *i.e.* plasmids, which are introduced by this method, will then be replicated in the bacterial host cells. To aid the bacterial cells' recovery, the cells are incubated briefly with non-selective growth medium following the heat shock treatment. However, due to the low percentage of bacterial cells that have been transformed with the plasmid and the potential for the plasmid not to propagate itself in all daughter cells, it is necessary to select for bacterial cells that contain the plasmid. This is commonly performed using antibiotic selection.

Bacterial transformation protocol

We took 200 µL of competent cell in tube and add 2µL of plasmid DNA, kept in ice for 30 minutes. For negative control we took competent cell alone without adding plasmid. After 30 min cells were transferred in to 40˚C water for 90 sec. (Heat shock). After 90 seconds the tube was rapidly transferred to an ice-bath for 1 min. 800 µL of LB medium was

added to tube and incubated at 37˚C. This step is to allow the bacteria to recover and to express the antibiotic resistant marker encoded by the plasmid. After 1 hr. incubation plate 100 µL of cell in LB with agar ampicillin plate. A negative control was also prepared accordingly. The plates were incubated at 37˚C overnight (12 to 16 hrs.). After 16 hr of overnight incubation, the colonies were screened and the results compared. The transformed colonies were incubated in 3 mL of LB ampicillin medium.

Initial inoculum concentration

A single colony of Alt-2 was inoculated in 10 mL of LB broth followed by sub-culturing into 100 mL modified LB (supplemented with 100 $\mu g.mL^{-1}$ ampicillin) and incubated overnight (± 16 h) at 37 °C at 180 rpm in an incubator shaker. In order to optimize the initial inoculum, different concentrations of initial inoculum were varied at 1% v/v, 3% v/v, 7% v/v and 10% v/v of the recombinant bacteria in shake flask culture. Optimization of culture medium. Media trials were performed to evaluate the *ALT-2* recombinant antigen production on several media that are commonly used for culturing *E. coli*. Triplicate cultures were performed in four different medium namely Luria-Bertani broth (LB), Terrific broth (TB), Super broth (SB), and M9 minimal medium **(Atlas, 1997)**. Culture working volume was set at 10% of total volume, where the optimized inoculum was added to 100 mL of each of the different medium in 1 L Erlenmeyer flask incubated at 180 rpm at 37 °C. Samples were taken at regular time intervals. Cultures were induced with 1.0 mM IPTG once the cell density reached (OD600)0.62. The amounts of total cell protein recombinant protein and biomass were measured.

Optimization of inducer concentration

To assess the effect of various concentrations of IPTG on the induction of the recombinant protein synthesis, the optimized inoculum was introduced in 1 L shake flasks, each containing 200 mL of the optimized medium supplemented with 100 $\mu g.mL^{-1}$ ampicillin. Once the cell density reached (OD600) 0.62, triplicate cultures were induced with 0.1, 0.2, 0.4, 0.6, 1.0, 2.0, and 3.0 mM concentrations of IPTG. All cultures were incubated at 180 rpm and 37 °C. The amounts of total cell protein, recombinant protein and biomass were measured.

Optimization of induction time

To examine the effect of induction time on the production of ALT-2, the optimized inoculum was added to 1 L shake flasks containing 200 mL of the optimized medium supplemented with 100 µg/mL ampicillin. Triplicate cultures were induced at initial ODs of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 using 1.0 mM IPTG, at 180 rpm and 37 °C. The amounts of total cell protein, recombinant protein and biomass were measured.

Shake flask experiment

E.coli BL21(DE3)pLysS host was transformed chemically with the pRSETB-alt-2 vector construct. LB media used for culturing *E. coli*. Culture working volume was set at 18% of total volume, where the optimized inoculum was added to 200 mL of LB medium in 1 L Erlenmeyer flask incubated at 180 rpm at 37 °C. Samples were taken at regular time intervals. Cultures were induced with 1.0 mM IPTG once the cell density reached (OD600) 0.62. Expression of protein was checked by running SDS-PAGE (Fig 1). The amounts of total recombinant protein were measured.

Batch Culture

Batch fermentation using DO-stat: batch culture was carried out in a 2L lab-scale bioreactor (Bioengineering, Switzerland). The initial culture volume was 1.2L of LB medium. A 100 mL sample of culture at an OD (600 nm) of 0.62 was used as seed inoculum. Agitation speed was set to 500 rpm(constant throughout batch culture) with an aeration rate of 2 l/min. LB media was used. Culture pH was controlled at 7.2 by the addition of 1N sodium hydroxide. Temperature was maintained at 37^{o}C and 0.02% poly (propylene glycol) was used as antifoaming agent.The culture was induced with 1 mM IPTG. The culture was allowed to grow for 3 h after induction. Biomass was monitored as optical density at 600 nm. DO concentration, pH and temperature were monitored by using online monitoring probes. SDS-PAGE analysis was done to check the expression.

Protein Recovery

Biomass was monitored by measuring optical density at 600nm using a spectrophotometer (Thermo Spectronic, USA). The dry cell weight (DCW) was estimated from a calibration curve that interrelated experimentally measured dry weight to spectrophotometric measurement of optical density. Total cell protein was estimated at various concentrations of bovine serum albumin (Sigma, USA) by BioRad Protein Assay (BioRad, USA), using protein standards. This is comprising Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples and was carried out as described previously **(Laemmli, 1970)**. The gels were stained with Coomassie Brilliant blue R-250 (BioRad, USA). Low molecular weight marker proteins were used as standards (BioRad Precision Plus Protein Standards Unstained, BioRad, USA).

RESULTS AND DISCUSSION

The growth and protein production characteristics of the cell cultured on various media have been studied. LB produced the highest cell protein among them(Table 1).
The only chemically defined medium used was minimal medium (M9) which yielded poor growth, this may be due to the low glucose level in the medium. Chemically defined media are generally known to produce slower growth and lower protein titres than complex media **(Zanette *et al.*, 1998).** In the present study, the volumetric titre of recombinant antigen declined as the concentration of IPTG increased over the 1.0 mM threshold.This was obvious because the presence of excessive IPTG reduced the final biomass as a consequence of the growth inhibition. Thus the results shown that expression of total protein using 0.5 mM (2.0 $mg.L^{-1}$), 0.8 mM (2.3 $mg.L^{-1}$) and 1.0 mM (2.4 $mg.L^{-1}$) IPTG. Thus, 1.0 mM IPTG was found to be the optimum inducer concentration and was used in all subsequent experiments.

Optimization of induction time

The effect of induction time on the production of ALT-2 recombinant protein is based on biomass production and it was determined by using cultures induced with the optimal concentration of 1.0 mM IPTG. Results shows that the culture density at OD600 1.0 was found to be optimal induction time since it gave the highest volumetric titre of recombinant protein.

Shake flask experiment

From shake flask experiment, it was noted that the protein expression in induced pellet is more better as compared to uninduced pellet and supernatant (Fig 1).

Batch Culture

In this experiment we have seen that in long run of batch culture plasmid is no longer stable and did not produce any expression (Table 2). Hence plasmid analysis experiment was carried out.

Plasmid stability analysis

Appropriate dilution of each sample of cells(uninduced and induced) were plated as in two LB agar plates-one containing ampicillin at a conc.of 100 ug.mL^{-1} (X) and 200 ug.mL^{-1} (2X) and other without antibiotics. The cells were incubated at 30^{o}C.The no. of colonies grown in both plates were counted and the stablity of plasmids were expressed in terms of percentage of plasmid bearing cells using the following formula (Table 3).

$$\text{Percentage of plasmid bearing cells} = \frac{\text{No.of colonies in LB ampicillin containing plate}}{\text{No.of colonies in LB media containing plate}} \times 100$$

From the above data we took colonies from 10^{-1} and 10^{-2} dilution of 1st hour sample after induction and patched it into separate LB medium containing ampicillin.For the preparation of inoculum in 100 ml LB media, pre inoculum was done from these cells. In reactor initially pulsed 2X conc. of ampicillin and then added 1X concentration of ampicillin just before induction and at last pulsed 1X concentration of ampicillin one hr after induction. After 3 hr, harvested and centrifuged the sample. We got the pellet and run on SDS-PAGE to check the expression (Fig. 2) and recombinant protein and biomass were measured (Table 4). Plasmid stability experiment shows that antibiotic concentration plays very important role in maintaining plasmid stability. Since plasmid is no longer stable during long run of batch culture due to high level of metabolic burden on *Ecoli.* In this experiment we have seen that different concentration of antibiotic at different interval of time helps in maintaining plasmid

stable.The expression of protein was seen in SDS-PAGE analysis (Fig 2) and concentration of protein were measured.

After getting good results from these experiments, batch culture reactor was run for 3hr after induction with three uniduced and three induced sample in different hour interval (Fig 3). It was seen that every lane showing expression but in lane7 the expression level was very good and protein concentration was calculated and found to be highest among all lanes (Table 4).During batch fermentation specific growth rate plays very important role in expression of protein. High specific growth rate may cause acetate build up which would be deleterious to both cell growth and recombinant protein expression**(Jensen and Carlsen, 1990; Sakamoto *et al*., 1994; Kwon *et al*., 1996)**. Other parameter include agitation rate (rpm) which regulate the dissolve oxygen concentration in the bioreactor and also provided us an on-line estimate of the growth rate. Stress situations has important impact on recombinant protein production and can be avoided and should be circumvented if the desired quality and quantity of recombinant protein is impaired (**Sorensen and Mortensen., 2005**). If target protein is highly expressed, stress response like heat shock, amino acid depletion or starvation may be induced in maintainces of plasmid **(Hoffmann and Rinas, 2004)**. A recent approach which reduces the acetate formation and increase the production of recombinant protein would be involving altering the genetic profile of the organism itself **(Mark *et al* ., 2006)**. Temperature also plays an important role in the solubility as well as productivity of the expressed protein (**Michel and Doriano ., 2002).**

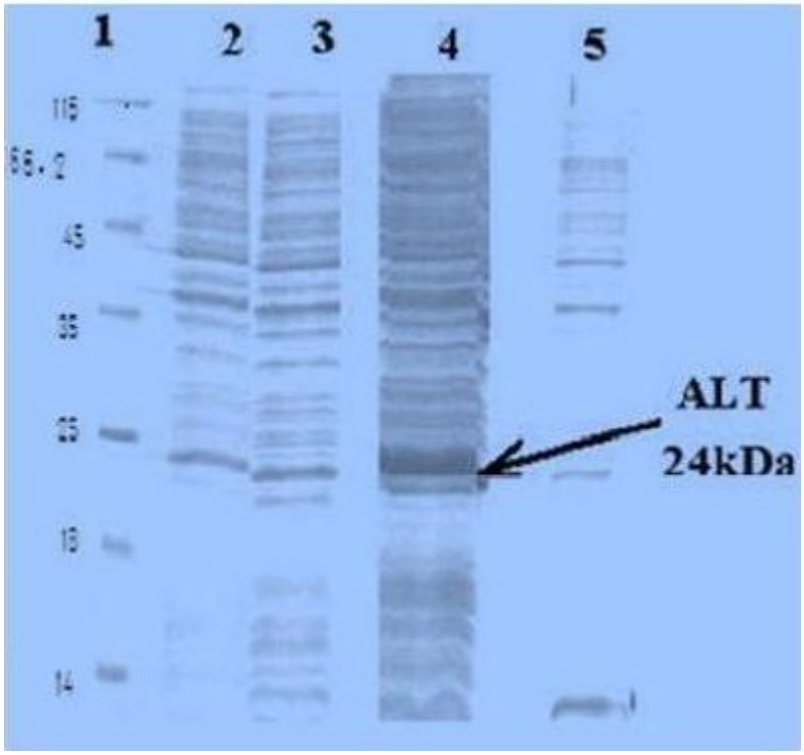

Figure 1 SDS-PAGE (12%) analysis of BL21(DE3) plysS

Experiment was done in flask with 200 ml LB media. Lane1: standard protein marker (kDa), Lane2: uninduced supernatant, Lane3: uninduced pellet, Lane 4: induced supernatant, Lane5: induced pellet.

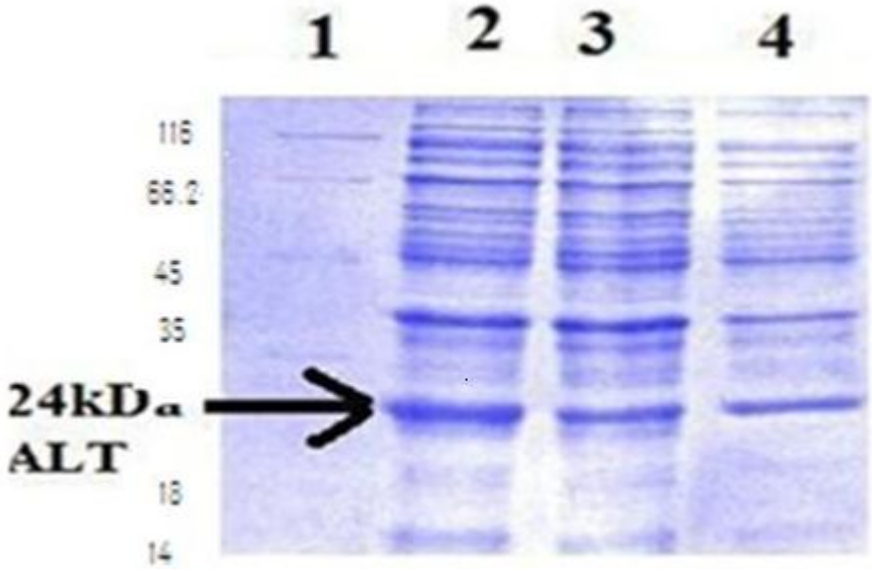

Figure 2 SDS-PAGE analysis of *E.coli* BL21(DE3) plysS

Experiment was done in shake flask in 100 ml of LB media. Lane1:Standard protein marker, Lane2: 2X concentration of ampicillin at 1st hour of sample after induction, Lane3: 3X concentration of ampicillin at1st hour sample after induction, Lane4: X concentration of ampicillin, 1st hour sample before induction (where X conc.= 100 ug.mL^{-1}).

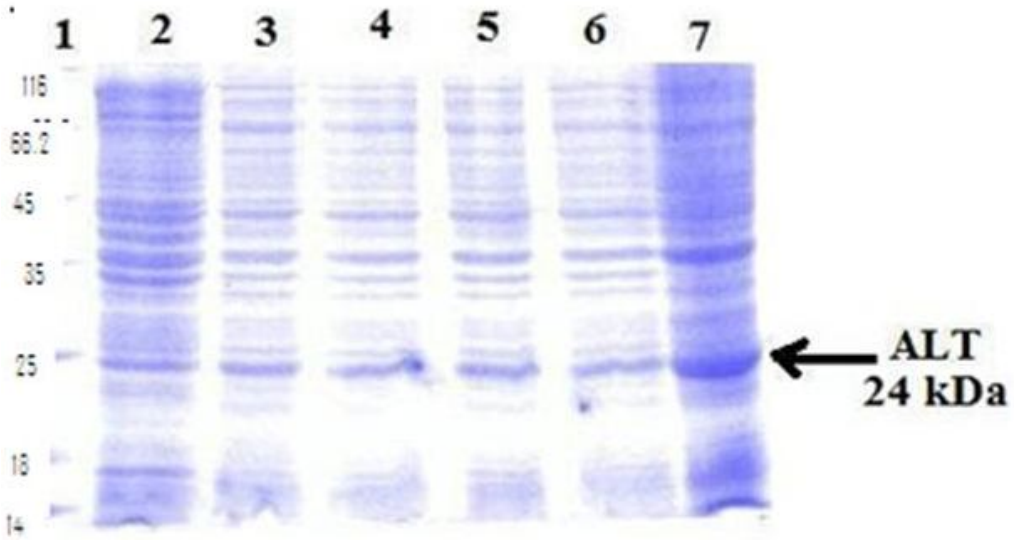

Figure 3 SDS-PAGE (12%) analysis of BL21(DE3) plysS of batch culture in fermenter

Lane1:Std protein marker (kDa), Lane2: uninduced sample, Lane 3:uninduced sample, Lane4: uninduced sample, Lane5: induced sample, Lane6: induced sample, Lane7: induced sample.

Table 1 Production characteristics of *ALT-2* expressing Total Cell Protein (TCP) cultured in various media (n=2)

Post inoculation time(h)	LB		TB		SB		M9 minimum media	
	TCP(mg/L)	**DCW(g/L)**	**TCP(mg/L)**	**DCW(g/L)**	**TCP(mg/L)**	**DCW(g/L)**	**TCP(mg/L)**	**DCW(g/L)**
3	103.54 ±3.26	3.25 ±0.12	93.25 ± 3.95	1.33 ±0.06	64.08 ±2.79	1.84 ±0.08	51.69 ±2.29	0.77 ±0.03
4	97.29 ±4.16	6.20 ±0.24	98.07 ±3.18	1.54 ±0.06	76.44 ±3.80	2.79 ±0.11	69.22 ± 2.40	0.93 ±0.04
5	103.54 ±3.26	3.25 ±0.11	98.23 ± 1.34	1.53 ±0.05	88.34 ±2.08	1.44 ±0.03	61.09 ±3.18	0.98 ±0.06
6	102.79 ±2.21	3.99 ±0.06	93.25 ± 3.95	1.33 ±0.06	60.09 ±3.17	0.98 ± ±0.05	66.97 ±3.35	2.11 ±0.11

TCP- Total cell protein (mg/L), DCW- Dry Cell Weight (g/L)

(n=Number of times experiment repeat)

Table 2 Profile of Batch fermentation with induction (n=2)

Time	OD at@600nm	Temperature(^{0}c)	DO
00	0.03	37	100
1	0.08	37	95
2	0.19	37	87.4
3	0.27	37	85.45
4	0.37	37	78.40
5	0.49	37	86.34
6	0.57	37	76.46
7	0.602	37	84.50
8	0.775	37	74.88
9	0.880	37	78.43
10	0.940	37	75.85
11	1.08	37	76.56
12	1.18	37	78.90
13	1.24	37	77.85

OD-optical density, DO-Dissolve Oxygen

Table 3 Plasmid stability analysis at different dilution (n=2)

Before induction,1st hr sample		
S.N	No of times dilution	% plasmid bearing cell
1	10^{-1} dilution	83
2	10^{-2} dilution	81
3	10^{-3} dilution	80
After induction,1st hr sample		
4	10^{-1} dilution	76
5	10^{-2} dilution	72.8
6	10^{-3} dilution	67.7
After induction,2nd hr sample		
7	10^{-1} dilution	59
8	10^{-2} dilution	59
9	10^{-3} dilution	46
After induction,3rd hr sample		
10	10^{-1} dilution	36
11	10^{-2} dilution	26
12	10^{-3} dilution	25

Table 4 Concentration of protein in different sets of experiment (n=2)

S.N.	Type of experiment	TCP(mg/L)	DCW(g/L)
1	Shake Flask (induced pellet sample)	98.09± 3.18	1.53 ±0.06
2	Shake flask after plasmid stability analysis (3X conc.)	103.34m±1.42	3.26 ±0.05
3	Batch culture induced sample of 7hr	102.78 ±2.21	3.08 ±0.06

X concentration-100 ug.mL^{-1}

CONCLUSION

Expression of ALT in *E.coli* Bl21plysS at flask level was found very good as compared to large scale production. This is because of at shake flask expression was very good because all the controlling parameter like PH, dissolve oxygen, plasmid stability etc was maintained but at large scale it is difficult to do. Since plasmid in *E.coli* is no longer stable in running the reactor for longer duration. Hence pulsation of antibiotic of different concentration at different interval of time have overcome this problem and plasmid become stable and expression of ALT was recorded very good. When target protein is highly expressed, maintenance of plasmid induces stress reponse **(Hoffmann and Rinas, 2004)**. Plasmid copy number play very important role in plasmid maintenance may often induced by stress **(Bailey, 1993)**. In this experiment good expression was recorded in soluble form and hence maximum amount of protein was found in soluble form.

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