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Research Article

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Production improvement of recombinant epidermal axolotls in *Escherichia coli* batch culture

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ABSTRACT

Lipoxygenase enzymes in eukaryotes play a significant role in the healing process. Based on recent studies, epidermal lipoxygenase enzyme extracted from amphibious is more effective in the healing process in comparison with human lipoxygenase. By the potential future significant effect in wound healing and tissue engineering, recombinant lipoxygenase axolotls (LOXe) production in E. coli was mentioned in this research. Clinical trial studies require sufficient amount of recombinant protein to generate. High level recombinant membrane protein LOXe over expresion in E. coli can be achieved by optimization of fermentation parameters such as induction conditions and culture medium. In this study, E. coli BL21 DE3 [pET21a-LOXe] (constructed by this research group) was used for enhanced r-LOX production. Production improvement was done in two stages: first evaluation of induction temperature, inducer concentration and medium culture effects on recombinant protein production in three levels by L9 array Taguchi experiment design approach, second optimization of carbon source amount and induction time in three levels by full factorial experiment deign in bioreactor, in turn. Experiments were done two times and responses (the amount of production of r-LOX) evaluated with design expert software. The obtained first step optimized conditions were induction temperature of 28 ^{o}C, inducer concentration 0.05 mg/l and TB medium culture. Optimized recombinant protein production and final dry cell weight under optimized conditions (glucose concentration of 10 g/l and induction time of OD_{600}=5) were 18.8 and 3.54 g/l, respectively. The obtained values are of the highest amounts cell density and recombinant membrane proteins that has been reported up to now. The obtained high cell density cultivation in batch mode may be due to individual properties of LOXe and its effect on host cell growth rate.

Keywords: lipoxygenase, *E. coli*, membrane Protein, Optimization, Overexpression.

INTRODUCTION

Lipoxygenases (LOXs, EC 1.13.11.12) are a family of non-heme iron containing dioxygenases that catalyze the regio- and stereo-specific oxidation of polyunsaturated lipids containing pentadiene systems and produce conjugated hydroperoxide products. LOXs are found in plants, animals, fungi and at least two bacterial species [1][2]. Enzyme Structure lipoxygenase proteins have a single polypeptide chain with a molecular mass of 75–80 kDa in animals and

94–104 kDa in plants. The proteins have a N-terminal β-barrel domain and a larger catalytic domain containing a single atom of non-heme iron [1] [3]. In eukaryotes, this enzyme converts arachidonic acid to a variety of inflammatory mediators. This inflammatory mediator plays an important role in the healing process. Recent studies have shown that the epidermal lipoxygenase enzyme extracted from an amphibious (Ambystoma mexicanum) is more effective in the healing process in comparison with human lipoxygenase. This membrane protein has a molecular weight of 71.6 KDa and composed of 623 amino acid residues with N-teminal PLAT domain and C-terminal lipoxygenase activity domain. [4] [5].

E. coli is one of the most a widespread prokaryotic host for recombinant protein expression. *E. coli* can grow fast on simple and inexpensive culture media. It has well known molecular genetics and cellular physiology, and its implementation is easy [6] [7] [8]. Intracellular over expression of recombinant protein in *E. coli* result in insoluble protein aggregates formation as inclusion bodies. Recombinant protein expression as IB protects product from protease and simplify recombinant protein purification [9] [7]. In the case of intracellular expression of recombinant protein, productivity enhanced with the increasing of final cell density and the specific yield of recombinant protein (i.e. the amount of product formed per time unit) [10]. Therefore, optimization of these culture conditions results in increase of productivity. Although the heterologous proteins production in *E. coli* expression system have been established well but optimal scheme can vary for each target protein. In the most cases optimized conditions for recombinant protein production is different from those for cell growth. Culture conditions, induction conditions and culture media have a dramatic effect on protein expression yield [11]. Expression of recombinant protein impose metabolic burden on host cell which can decrease final cell concentration that result in lower productivity[12] [13]. Therefore, Growth rate of recombinant bacteria correlates inversely with the recombinant protein production rates. The conditions that result in decrease the rate of protein expression, such as induction at low temperature and low inducer concentration, decrease metabolic burden on host cell and increase obtained biomass concentration, too. Therefore, induction at low temperature and low inducer concentration may result in increase of target protein production [14] [15]. This metabolic burden results in starvation of some nutrients include amino acids, therefore using rich complex media can decrease nutrient starvation and improve recombinant protein productivity [16].

Like any protein characterization, the first step is production of a large amount of purified protein, but the recombinant production of r-LOX protein in bacterial expression system has not yet been reported. Therefore, the aim of this study is improvement of r-LOX production in shake flask and bioreactor by introducing optimized induction conditions which leads to decrease of metabolic burden on host cell and consequently overexpression of r-LOX in *E. coli*. Eventually, effect of overexpression of *loxe* gene on *E. coli* cell growth kinetics was evaluated as probable growth factor.

EXPERIMENTAL SECTION

Host and cell culture

E. coli BL21 (DE3) (Novagen, Inc.), which contained pET21a inducible expression vector, and carried the *loxe* synthetic gene, that constructed and confirmed by this research group, was used as host cell. Transformed cells using the calcium chloride procedure were cultured on Luria- Bertani agar (LB) contains 10g Yeast extract, 5 g Pepton and 10 g NaCl per liter. All media supplemented with 100 µg/l ampicillin.

In order to cultivation of the recombinant *E. coli*, it was removed from –70 °C freezer and grown at 37°C for 24 h on LB agar plates. One colony from LB plate was transferred into culture media (according to each experiment) and incubated at 37 °C and 200 rpm on a rotary shaker incubator as seed culture. The batch culture was started by inoculation of seed culture into broth in shake flask or bioreactor. After obtaining the appropriate OD_{600} (according to the each experiment), the gene expression was induced by IPTG. LB, TB (24 g Yeast extract, 12 g Pepton, 4 g glycerol, 12.54 g KH_2PO_4 and 2.31 g K_2HPO_4 per liter) and 32YT (8 g pepton, 32 Yeast extract and 5 g NaCl per liter) medium were used to evaluation of medium type effect on r-LOX production in shake flask.

A batch culture bench scale bioreactor was initially ongoing by adding 100 ml of seed culture into 3.5 l bioreactor with working volume of 1.5 l containing optimized media (achieved in shake flask) comprising 0.1% antifoam and 100 µg/l ampicilin. pH was sustained at seven by the addition of 1M HCl or 1M NaOH solution. Dissolved oxygen was controlled at 30-40% (v/v) of air saturation by adjusting both inlet air and agitation rate. Foam was controlled by the addition of silicon-anti-foaming reagent. Cell culture was induced by the addition of IPTG then temperature diminished to optimized induction temperature acquired in shake flask section.

Analytical procedure

Cell growth was monitored by measuring culture turbidity at 600 nm and dry cell weight [9]. The expression of r-LOX was analyzed by Coomassie brilliant blue-stained SDS-PAGE with 12.5% polyacrylamide and quantified by a gel densitometer. Total soluble protein was analyzed by Bradford's method [17].

Optimization of induction condition and culture media type

At first, for expression enlargement of r-LOX in *E. coil* BL21 (DE3), the effects of induction conditions and medium type were optimized in 250 ml shake flasks. Effect of inducer concentration (in three levels of 0.05, 0.10 and 0.15 mM of IPTG), temperature of induction (24, 28 and 32 ^{o}C) and medium type (LB, TB and 32YT) were investigated by Taguchi method L_9 array (table 1). Design expert software was used for analysis the responses. It should be mentioned that any trial Taguchi design was duplicated and the presented results are the average between 2 replications.

Afterward, optimized glucose concentration and time of induction (OD_{600}) were determined in 3.5 l bioreactor with working volume of 1.5 l. It must be mentioned that obtained optimized condition in shake flask was applied in this section. Effect of these parameters at three levels (glucose concentration: 5, 10 and 20 g/l; induction time (OD_{600}) 1, 5 and 10) were investigated with full factorial method.

Liopxygenase assay

Assay were carried out as reported previously with some modifications [18]. Enzyme activity was determined spectrophotometrically by monitoring the increase in the absorbance at 234nm (Perkin Elmer 552a UV/VIS Spectrophotometer, Germany) due to the transformation of linoleic acid to hydroperoxydienes. Assays were performed in 1ml quartz curvet in a total assay volume of 1000 µl. 5 µl of Crude Recombinant LOXe in 945 µl of assay buffer (0.1 M potassium phosphate buffer, pH 7.4.) were added to cuvet. The enzymatic reaction was initiated by adding 50 µl of linoleic acid solution (2 mM stock solution) to a final concentration of 100 µM and was terminated after 5 min at room temperature.

RESULT AND DISCUSSION

Recombinant membrane protein production in *E. coli* as host cell has been thoroughly investigated in recent years but it must be mentioned that optimum condition may be different for each protein and expression system (vector and host cell). There are several parameters like induction temperature, inducer concentration, induction time and culture media which have dramatic effect on recombinant protein yield.

Production enhancement in shake flask: r-LOXe expression confirmation by SDS-PAGE gel electrophoresis in *E. coli* was indicated in figure 1. Results of r-LOXe optimization in shake flask were presented in table 1. Statistical analysis of Taguchi design of experiments was shown in table 2. Recombinant protein production and final dry cell weight in optimized conditions were 1.84 g/l and 6.68 g DCW/l, respectively. Figure 2 shows the main effect plots of temperature after induction, inducer concentration and media type on r-LOXe production.

It was found (Figure 2a) that TB medium is more suitable for overexpression of r-LOXe in *E. coli*. Overexpression of recombinant protein impose metabolic burden on host cells which lead to decrease of cell growth rate. TB medium with high amount of pepton and yeast extract compensate amino acid shortage during recombinant protein overexpression period and result in decrease of metabolic burden on host cell. Furthermore, TB medium contains phosphate buffer which maintains the pH of the medium at constant level in shake flask. Therefore, TB medium with higher ratio of yeast extract to peptone is more suitable for overexpression of r-LOXe in *E. coli*. In the other hands, lower content of pepton in TB medium in comparison with 32YT leads to increase growth rate and the maintenance of cells in stationary phase. The pH adjusting of batch culture of recombinant *E. coli* in flask is impossible, therefore phosphate buffer has important role in control of pH culture. For these reasons, TB is the best media for r-LOXe overexpression in shake flask.

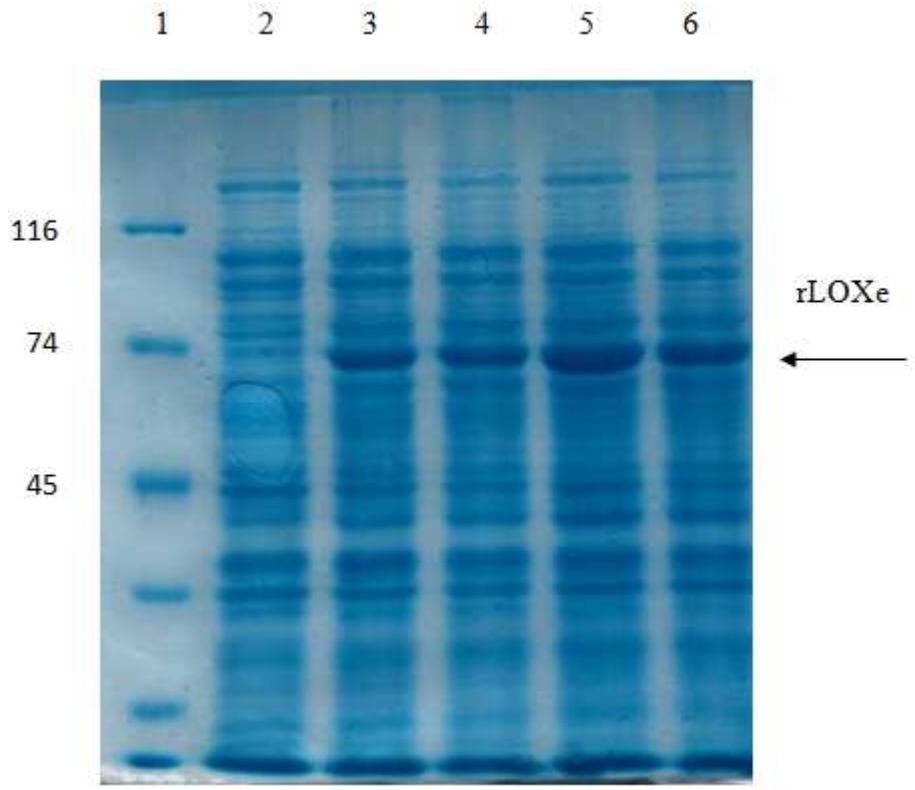
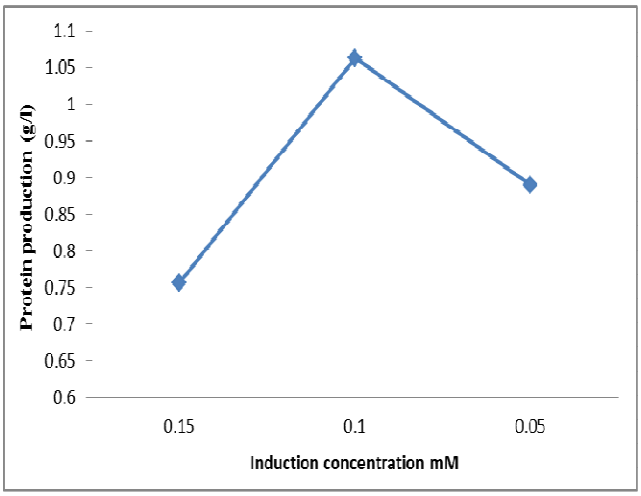
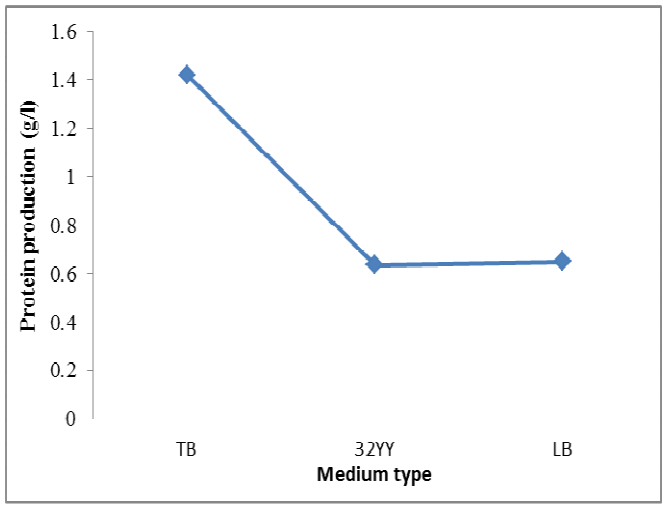


Figure 1: SDS PAGE analysis of lipoxygenase expression in recombinant *E. coli*. Lane 1 and 2 show Molecular weight marker and sample before induction, lane 3 to 6 show r-LOXe expression 1 to 4 hours after induction, respectively

Based on the results presented in table 1, optimized temperature and inducer concentration were 28°C and 0.05 mM, respectively. High rate of recombinant protein production impose more metabolic burden on host cells and decrease specific growth rate of bacteria. The combination of lower temperature during induction and low inducer concentration lead to decrease of recombinant protein production rate and metabolic burden on host cell. IPTG is high cost inducer with some toxic effects on host cell therefore; using lower concentration of IPTG has economic effect on large scale recombinant protein production process and increase process productivity. Results indicate that lowest amount of IPTG was suitable for efficient expression of r-LOXe and had a lower toxic effect on host cells. For this reason high expression of r-LOXe and higher cell concentration were obtained by low amount of IPTG.



A



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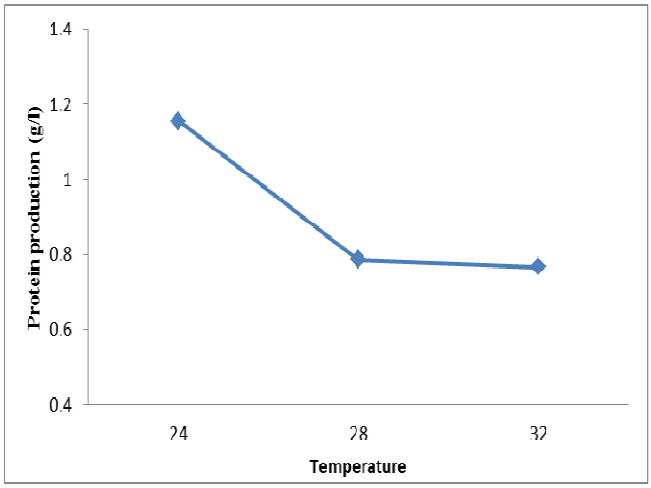

Figure 1. The main effect plots of each parameter in Taguchi design, (A) inducer concentration (B) temperature after induction and (C) medium type at different levels

Table 1. Obtained results from Taguchi design (L_9 array) of batch culture optimization of r-LOXe at three levels

Experiment number	Temperature after Induction (°C)	Inducer concentration (mM)	Media type	Dry cell weight (g)	r-LOXe expression (%)	Total r-LOXe production (g/l)
1	24	0.05	LB	4.82	41٪	1.2
2	24	0.10	TB	5.05	37٪	1.18
3	24	0.15	32YT	1.77	31%	0.33
4	28	0.05	TB	6.64	44٪	1.84
5	28	0.1	32YT	4.01	43٪	0.84
6	28	0.15	LB	2.37	36٪	0.46
7	32	0.05	32YT	4.87	42٪	.97
8	32	0.1	LB	2.93	37٪	0.59
9	32	0.15	TB	3.94	46%	1.06

Table 2: Statistical analysis of r-LOXe production optimization by L_9 array Taguchi design in three levels

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.64	6	0.27	21.25	0.0456	significant
A-A	*0.067*	*2*	*0.034*	*2.61*	*0.2772*	
B-B	*0.87*	*2*	*0.43*	*33.62*	*0.0289*	
C-C	*0.71*	*2*	*0.36*	*27.52*	*0.0351*	
Residual	0.026	2	0.013			
Cor Total	1.67	8				

In the other hand metabolic burden on host cell will increase with an increase in temperature during induction and expression rate. It must be mentioned that decrease induction temperature result in decrease in metabolic burden on host cells but it decrease cell growth rate, too. For this reason, induction in lower temperature effect on r-LOXe production was less significant than those for other parameters. Therefore, applying optimized conditions and using TB medium increase recombinant protein overexpression and final cell concentration of bacteria.

Production improvement in the bench scale bioreactor: In the next step, recombinant protein overexpression optimization in bioreactor was investigated under obtained optimized condition and suitable media in shake flask. As overexpression of recombinant protein result in decrease of final cell concentration, it's very important obtaining optimized induction time and higher cell concentration can be obtained by induction in appropriate cell concentration. Therefore, cell culture induction in higher cell density results in higher final cell concentration and increase of recombinant protein productivity. For this reason, glucose concentration and induction time were optimized in 3.5 l bioreactor. Result of r-LOXe production optimization in bench scale bioreactor illustrated in table

3 and statistical analysis (Table 4) show that optimum induction time was OD_{600}=5. As mentioned, overexpression of heterologous protein impose metabolic burden on host cells and reduce cell growth rate and result in lower final cell density and protein productivity. Therefore, optimization of induction time or induction in higher cell density results in increase of final cell density and productivity. However, accumulation of byproduct in higher induction time (OD_{600}=10) is more sever and can inhibit recombinant protein overexpression. In the other hand, increasing glucose concentration as carbon source leads to increasing final cell concentration. Results show that effect of induction time is more significant than glucose concentration. Glucose concentration of 20 g/l has inhibitory effect on host cells and induces production of byproduct acetate. In the other hand, accumulation of acetate on medium reduce cell growth rate and reduce recombinant protein productivity. Optimized recombinant protein production and final cell density was 4.11 g/l and 19.7 g DCW/l, respectively. Statistical analysis indicates that induction time effect is more severe than those for glucose concentration.

Table 3: Results of r-LOXe production optimization in bench scale bioreactor by full factorial design in three levels

Experiment number	Glucose concentration (g/l)	Induction Time (OD600)	Dry cell weight (g)	r-LOXe expression (%)	Total r-LOXe production (g/l)
5	5	1	12.94	40	2.498
4	5	5	17.38	37	3.21
6	5	10	16.3	33	2.69
7	10	1	14.39	35	2.52
8	10	5	19.7	44	4.11
9	10	10	15.80	38	3.00
2	20	1	12	30	1.80
3	20	5	16.8	40	3.36
1	20	10	14.86	36	2.65

Table 4: Statistical analysis of r-LOXe production optimization by full factorial design in three levels

Term	df	Sum of Squares	Mean Square	F Value	Prob > F
Intercept					
A-A	2	0.40	0.20	148.84	< 0.0001
B-B	2	7.05	3.53	2601.90	< 0.0001
AB	4	1.29	0.32	237.88	< 0.0001
Lack Of Fit	0	0.000			
Pure Error	9	0.012	1.356E-003		
Residuals	9	0.012	1.356E-003		

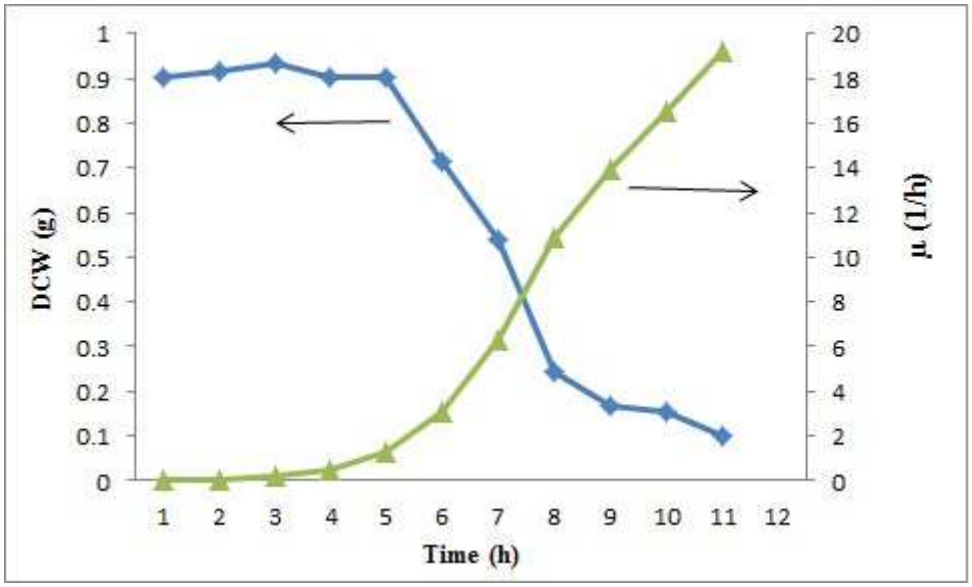

Figure 3: Growth kinetics of recombinant *E. coli* BL21 DE3 [pET 21a-*LOXe*]. Specific growth rate (◇), Dry cell weight (△

CONCLUSION

The optimum induction conditions are the temperature after induction of 25(°C), inducer concentration of 0.05 mM, and TB medium was the best media for overexpression of r-LOX in *E. coli in* shake flask and optimum glucose concentration and induction time obtained in bioreactor were 10 g/l and OD_{600}=5, respectively. The improvement of recombinant production indicates that reducing recombinant protein expression rate and using enriched TB medium can compensate metabolic burden on host cells and increasing product yield. In the other hand, using higher induction time result in induction in higher final cell concentration and increase of recombinant protein production yield. Therefore, using *E. coli* as host and improved induction conditions lead to optimized overexpression of r-LOXe.

REFERENCES

[1] AR Brash, *J. Biol. Chem.*, **1999**, 274(34), 23679–23682.
[2] H Porta; M Rocha-Sosa, *Plant Physiol.*, **2000**, 130(1), 15–21.
[3] SA Gillmor; A Villaseñor; R Fletterick; E Sigal; MF Browner, *Nat. Struct. Mol. Biol.*, **1997**, 4(12), 1003–1009.
[4] B Menger; PM Vogt; C Radtke; JW Kuhbier; J Killat; K Reimers, *Plast. Reconstr. Surg.*, **2010**, 126(2), 726.
[5] B Menger; PM Vogt; C Allmeling; C Radtke; JW Kuhbier; K Reimers, *Ann. Surg.*, **2011**, 253(2), 410–418.
[6] E Seyedi; M Rahaie; MR Mofid; L Haddad; MM Namvaran; J Fallah; S Jafari; V Babaeipour, *Res. Pharm. Sci.*, **2014**, 9(6), 453–461.
[7] V Babaeipour; S Khanchezar; MR. Mofid; MP Haji Abbas; *Iran. Biomed. J.*, **2015**, 19(2), 102,.
[8] J Shiloach; R Fass, *Biotechnol. Adv.*, **2005**, 23(5), 345–357.
[9] AK Panda; RH Khan; KBC Appa Rao; SM Totey, *J. Biotechnol.*, **1999**, 75(2), 161–172,.
[10] R Khalilzadeh; SA Shojaosadati; A Bahrami; N Maghsoudi, *Biotechnol. Lett.*, **2003**, . 25(23), 1989–1992.
[11] FA Kahaki; V Babaeipour; HR Memari; MR Mofid, *Appl. Biochem. Biotechnol.*, **2014**, 174(4), 1558–1571.
[12] MS Wong; S Wu; TB Causey; GN Bennett; KY San, *Metab. Eng.*, **2008**, 10(2), 97–108.
[13] F Faraji; MR Mofid; V Babaeipour; A Divsalar; SA Dehaghani, *Int J Env. Sci Dev*, **2010**, 1, 15–19.
[14] HP Sørensen; KK Mortensen; *J. Biotechnol.*, **2005**, 115(2), 113–128.
[15] P Balbás; A Lorence, Recombinant Gene Expression: Challenges and Solutions, 2nd ed, Humana Press, New Jersey, **2004**, pp. 15–51.
[16] NK Tripathi, *Def. Sci. J.*, vol. 59, no. 2, pp. 137–146, 2009.
[17] F Baneyx, *Curr. Opin. Biotechnol.*, vol. 10, no. 5, pp. 411–421, **1999**.
[18] X Lu; J Zhang; S Liu; D Zhang; Z Xu; JW Jianghua Li; G Du, J. Chen, *Appl. Microbiol. Biotechnol.* (**2013**) 97, 5793–5800.