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ORIGINAL ARTICLE

Sequential optimization of production of a thermostable and organic solvent tolerant lipase by recombinant *Escherichia coli*

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Abstract Several medium formulations were screened for the production of a thermostable and organic solvent tolerant lipase by a recombinant *Escherichia coli* BL21. The highest lipase production (28.9±4.1 IU/mL) was obtained in Luria Bertani medium with the addition of 1% (w/v) glucose. The medium formulation and fermentation conditions were then subjected to sequential optimization. Using a Plackett-Burman design, glucose, NaCl, temperature and induction time were found to be the most significant variables affecting lipase production, and these were then optimized using response surface methodology (RSM). The large value of R^2 (0.979) showed that the quadratic model used for the prediction is highly significant. The optimum levels of these four significant variables (glucose, NaCl, temperature and induction time) as predicted by RSM were 32.4 g/L, 5 g/L, 31.7°C and 2.1 h, respectively. The amount of lipase activity (50.2±4.5 IU/mL) produced under these optimal conditions fitted well to the value (48.9 IU/mL) predicted by RSM. Production of lipase in optimized fermentation was about 2.5-fold higher than in non-optimized fermentation.

Keywords Thermostable lipase · Solvent tolerant lipase · *Escherichia coli* · Fermentation · Optimization · Response surface methodology

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Introduction

Lipases (triacylglycerile hydrolases; EC 3.1.1.3) are industrially important enzymes due to their regio-, stereo-, chemoselective reactions and kinetic resolution of racemates. Conventionally, lipases are used in the detergent industry, as flavour enhancers in the food industry, as fat remover in the meat industry, and as pitch remover in the paper and pulp industry (Hasan et al. 2006). Recently, these enzymes have been used for biodiesel production (Adamczak et al. 2009), enantioselective deacetylation (Kumar and Gupta 2008), cyclic resolution of racemic ibuprofen (Liu et al. 2009), production of medium-chain triacylglycerols (Low et al. 2007) and the preparation of diacylglycerol-enriched palm olein (Wang et al. 2009). Thermostability, organic solvent tolerance and stability over a wide range of pH are the most desired characteristics of industrial lipases.

Industrially, lipases are produced in large quantities from microbial fermentation. Lipase-producing strains are isolated from various sources such as oil mill effluent, hot springs and fatty waste (Eltaweel et al. 2005; Rahman et al. 2007). Various microorganisms, such as *Staphylococcus caseolyticus* (Volpato et al. 2008), *Burkholderia multivorans* (Dandavate et al. 2009), *Aureobasidium pullulans* (Liu et al. 2008) and *Candida cylindracea* (Brozzoli et al. 2009), have been identified as lipase producers. Natural

isolates usually produce lipase in small quantities, while thermostable lipases are normally produced by thermophilic microorganisms, which are difficult to cultivate.

To overcome the problems related to natural isolates, lipase-producing genes are cloned in mesophiles for over-expression. *Escherichia coli* is the expression system most commonly used for recombinant proteins. This recombinant bacterium can be cultivated using a simple medium formulation at room temperature. *Escherichia coli* can accumulate recombinant proteins up to 80% of its dry weight and survives in a variety of environmental conditions (Demain and Vaishnav 2009). *Escherichia coli* has been used widely as host for lipase gene expression (Nthangeni et al. 2001; Oginoa et al. 2008; Gao et al. 2009). However, development of lipase production by recombinant *E. coli* is focused more toward cloning, expression and characterization of the enzymes. Studies on engineering problems related to the fermentation process for production of lipase by the recombinant strain are lacking. Optimization of medium formulations and culture conditions play an important role in the development of fermentation process for improvement of production in terms of final enzyme activity, yield, and overall productivity to ensure the process is economically viable.

The conventional method of optimization by changing one parameter at a time is time-consuming and inefficient. Statistical methods like response surface methodology (RSM) and artificial neural networks (ANN) are used commonly nowadays for the optimization of processes (Singh et al. 2009; Wolski et al. 2008). Using such optimization methods, the number of experiments can be reduced significantly compared to the number required by conventional methods. In addition, interactions between process variables can also be estimated using statistical methods. RSM has been used successfully for the optimization of lipase production by natural isolates (Khare et al. 2008; Liu et al. 2006; Rajendran et al. 2008; Ebrahimpour et al. 2008). To our knowledge, there are no reports on the optimization of lipase production by RSM using recombinant *E. coli*. However, RSM has been applied to the optimization of processes for production of various other biotechnology products by recombinant *E. coli* (Maldonado et al. 2007; Zhang et al. 2009; Farliahati et al. 2010; Azaman et al. 2010).

A natural producer of thermostable and organic solvent tolerant lipase (Lip 42) has been isolated from palm oil mill effluent and identified as *Bacillus* sp. 42 (Eltaweel et al. 2005). The lipase Lip 42 has a molecular weight of about 43 kDa, with optimal activity at pH 8 and 70°C. This lipase is stable in many polar organic solvents like DMSO, DMF, acetone, methanol, heptanol and octanol, which shows its potential for use as a biocatalyst in biodiesel production. Since lipase production by *Bacillus* sp. 42 was very low (< 1 IU/mL), the Lip 42 gene isolated from this wild strain was cloned in *E. coli* BL21 for over-expression (Hamid et al. 2009). The objective of the present study was to optimize the medium formulation and culture conditions for improvement of lipase Lip 42 production by recombinant *E. coli* using RSM. Preliminary screening of lipase production was carried out in four different medium formulations available from the literature. The medium that gave the highest lipase activity was then subjected to statistical optimization.

Materials and Methods

Microorganism

The recombinant bacterium, *E. coli* strain BL21 (DE3) pLysS harbouring the plasmid pET51b-Lip 42, was used in this study. The details of the insertion of lipase (Lip 42) gene in plasmid pET51b and the development of recombinant *E. coli* are described in detail by Hamid et al. (2009). The recombinant *E. coli* BL21 culture was maintained in glycerol and preserved at −80°C.

Inoculum preparation and fermentation

The stock culture was activated in LB broth and streaked on an LB agar plate, which was then incubated for 24 h at 37°C. Inoculum was prepared by transferring a single colony from the LB agar plate into 50 mL sterile LB broth in a 250 mL bottle and incubated at 37°C for 12–16 h. All fermentations were conducted in 250 mL screw cap bottles containing 50 mL medium. Different sizes of inoculum were used for the different experiments. In the screening experiments, the inoculum size was varied from 1% to 9% (v/v), while for optimization experiments the inoculum size was fixed at 4% (v/v). The bottles were seeded with the required size of inoculum and incubated at different temperatures (ranging from 25°C to 49°C) in a rotary shaker agitated at 200 rpm. The cultures were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) after 1–5 h of inoculation and the fermentation was then extended according to the requirements of each experiment (8 h and 25 h for screening experiments, and 12 h for optimization experiments). The variations in medium composition and fermentation conditions are presented in Tables 1 and 2. A mixture of antibiotics (50 μg/mL ampicillin and 35 μg/mL chloramphenicol) was added to all media for inoculum preparation as well as for fermentations to avoid contamination.

Table 1 Plackett-Burman (PB) experimental design for the initial screening of fermentation variables. *IPTG* Isopropyl β-D-thiogalactoside

Experiment number	Tryptone (g/L) (X_1)	Yeast extract (g/L) (X_2)	Glucose (g/L) (X_3)	NaCl (g/L) (X_4)	Inoculum size (%) (X_5)	Time (h) (X_6)	Temperature (°C) (X_7)	IPTG (mM/mL) (X_8)	Induction time (h) (X_9)	Lipase activity (IU/mL)[a]	Cell concentration (mg/mL)[a]
1	25	1	90	1	1	8	25	2.5	5	26.9±2.8	2.3±0.5
2	25	9	10	9	1	8	25	0.5	5	0.6±1.9	1.2±0.7
3	5	9	90	1	9	8	25	0.5	1	33.2±2.6	3.5±0.5
4	25	1	90	9	1	25	25	0.5	1	24.0±3.6	2.4±0.7
5	25	9	10	9	9	8	49	0.5	1	1.0±2.5	1.3±0.3
6	25	9	90	1	9	25	25	2.5	1	31.9±4.1	3.1±0.6
7	5	9	90	9	1	25	49	0.5	5	0.2±2.6	1.0±0.7
8	5	1	90	9	9	8	49	2.5	1	11.0±2.5	2.2±0.6
9	5	1	10	9	9	25	25	2.5	5	4.4±1.4	2.1±0.6
10	25	1	10	1	9	25	49	0.5	5	0.9±3.7	1.8±0.3
11	5	9	10	1	1	25	49	2.5	1	6.4±1.4	1.5±0.8
12	5	1	10	1	1	8	49	2.5	5	0.8±3.2	1.4±0.4
Estimates	0.0277	-0.2619	0.14665	-1.2322	0.1276	-0.0562	-0.5287	-0.11205	-2.1805	–	–
t values	0.3574	-1.3349	7.5688	-6.9091	0.7155	-0.7269	-8.2645	-0.1349	-5.2484	–	–
P values	0.7550	0.3135	0.0170*	0.0203*	0.5486	0.5428	0.0143*	0.0905	0.0344*	–	–

*Columns with significant variables and their calculated values

[a] ± Standard deviation of triplicate data

Table 2 Box-Wilson 2^4 factorial central composite design for the optimization of lipase fermentation by recombinant *Escherichia coli*

Experiment number	Glucose (g/L)(X_3)	NaCl (g/L) (X_4)	Temperature (°C) (X_7)	Induction time (h) (X_9)	Observed lipase activity (IU/mL)[a]	Predicted lipase activity (IU/mL)	Cell mass (mg/mL)[a]	E/x (IU/g/mL)
1	70	7	43	4	4.21±0.05	7.17	2.6±0.6	1.615
2	70	7	43	2	12.97±1.4	14.38	2.8±0.5	4.666
3	70	7	31	4	18.93±2.2	18.21	3.6±0.3	5.187
4	70	7	31	2	29.65±3.5	30.04	3.6±0.8	8.238
5	70	3	43	4	6.04±0.1	8.02	2.5±0.3	2.367
6	70	3	43	2	15.72±1.9	16.99	2.3±0.4	6.720
7	70	3	31	4	22.03±3.6	20.62	3.0±0.5	7.245
8	70	3	31	2	35.35±3.8	34.23	3.6±0.7	9.657
9	30	7	43	4	0.021±0.4	1.93	0.05±0.6	0.42
10	30	7	43	2	12.93±1.4	14.66	2.3±0.2	5.722
11	30	7	31	4	22.65±3.6	21.70	3.3±0.5	6.781
12	30	7	31	2	40.24±4.4	39.06	3.7±0.3	10.706
13	30	3	43	4	0.04±0.06	-0.02	0.8±0.7	0.053
14	30	3	43	2	12.96±2.7	14.48	1.9±0.6	6.547
15	30	3	31	4	21.93±2.3	21.32	2.3±0.2	9.332
16	30	3	31	2	43.07±3.9	40.45	4.0±0.3	10.741
17	90	5	37	3	23.38±2.6	21.57	3.0±0.7	7.591
18	10	5	37	3	21.84±1.8	22.55	1.7±0.3	8.120
19	50	9	37	3	26.92±3.8	24.73	2.1±0.8	9.579
20	50	1	37	3	25.87±2.6	26.96	3.0±0.5	8.374
21	50	5	49	3	0.05±0.3	-5.74	0.4±0.2	0.140
22	50	5	25	3	26.59±2.6	31.27	2.6±0.6	10.227
23	50	5	37	5	2.96±0.4	1.98	2.0±0.6	1.451
24	50	5	37	1	28.43±3.6	28.31	2.8±1.5	10.084
25	50	5	37	3	34.2±2.4	35.33	3.4±0.5	10.006
26	50	5	37	3	35.78±3.7	35.33	3.4 ±0.6	10.494
27	50	5	37	3	34.95±2.3	35.33	3.2±0.4	10.624
28	50	5	37	3	35.25±3.5	35.33	3.4±0.3	10.338
29	50	5	37	3	34.96±2.7	35.33	3.4±0.7	10.223
30	50	5	37	3	35.5±2.6	35.33	3.5±0.5	10.038
31	50	5	37	3	36.01±3.5	35.33	3.6±0.2	9.975
32	50	5	37	3	35.89±3.7	35.33	3.4±0.4	10.587

[a] ± Standard deviation of triplicate data

Experimental design

Four different medium formulations were tested initially for lipase production by recombinant *E. coli*. The composition of each medium was as follows (in g/L):

M1: Yeast extract, 25.4; tryptone, 10.9; $Na_2HPO_4 \cdot 12 H_2O$, 9.2; KH_2PO_4, 3.3; NH_4Cl, 1.1; NaCl, 0.54; $MgSO_4 \cdot 7H_2O$, 0.53; $CaCl_2 \cdot 2H_2O$, 0.16; glucose, 6.5; thiamine, 0.00109; pH 7

M2: LB broth, 20; glucose, 10; pH 7

M3: LB broth, 20; glycerol; 10; pH 7

M4: LB broth, 20; pH 7

Optimization of fermentation variables for lipase production was carried out using the statistical approach, where Plackett-Burman (PB) design was used for screening of significant variables. The selected variables were then subjected to optimization using a Box-Wilson 2^4 factorial central composite design. The PB design was used to screen out nine variables—tryptone (X_1), yeast extract (X_2), glucose (X_3), NaCl (X_4), inoculum size (X_5), fermentation time (X_6), temperature (X_7), IPTG concentration (X_8) and induction time (X_9)—in 12 experiments. The design used for the experiments is presented in Table 1, where each variable was set at its high and low levels. The high level of the variables was set far enough from the low level to allow

the significance of levels to be seen. Each variable was tested an equal number of times at its high and low levels. This equal allocation provided a fair and efficient estimate of linear effect.

RSM was used for sorting out the optimum levels of significant variables. A Box-Wilson (BW) 2^4 full factorial central composite design (CCD) with 32 experiments was used for this purpose. Five levels of variation were used for each variable (Table 2). According to the design (2^4=16, factorial CCD), 16 experiments were carried out for factorial points, 8 for axial points (α=2) and 8 replications for central points. In these experiments, non significant variables from the PB design were set at their center points. A second order model (Eq. 1) was used to calculate the predicted response.

$$Y = \beta_o + \sum \beta_{ii} + X \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y represents a response variable, β_o is the coefficient of interception, β_i is the coefficient of linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect.

Analytical procedure

Culture samples withdrawn from fermentations were centrifuged at 12,000 *g* and 4°C for 10 min. The cell pellets were resuspended in 20 mM phosphate buffer at pH 7 and then lysed by sonication for 2 min on ice. The supernatant was used for lipase analysis after removal of cell debris by centrifugation. Lipase activity was determined according to the method as suggested by Hamid et al. (2009), which was a modification of a method proposed by Kwon and Rhee (1986). In this method, 1 mL diluted sample was added to 2.5 ml olive oil emulsion with phosphate buffer in a 1:1 ratio. The mixture was added to 20 μL 20 mM $CaCl_2$ and then agitated at 200 rpm and 60°C for 30 min. The reaction was terminated by the addition of 1 mL 6 *N* HCl. Isooctane (5 mL) was added to the solution to extract the liberated fatty acids by the action of lipase. The upper layer (4 mL) was mixed with 1 mL pyridine cupric acetate reagent and vortexed. The absorbance of the upper layer was recorded at 715 nm and compared with a free fatty acid standard curve. One unit of lipase is defined as 1 μM free fatty acids released per minute. Cell concentration was determined by drying the cell pellet at 80°C for at least 24 h, until constant weight was achieved.

Statistical analysis

The statistical analysis of data and plots was performed using STATISTICA software version 7. For PB design, variable estimates and their *t* and *P* values were determined by multiple regressions to see the significance of variables. For RSM, response surface regression analysis was conducted. *F* and *t* tests were employed to determine the significance of model parameters. The coefficient of correlation determination (R^2) was calculated to evaluate the performance of the regression equation. The optimum levels of the selected variables were obtained from the desirability charts.

Results

Screening of different media for lipase production from *E. coli* BL21

Among the four medium formulations tested for lipase production by *E. coli* BL21, medium M2 gave the highest lipase activity (28.9±4.1 IU/mL), which also related to the highest cell concentration obtained during the fermentation (Fig. 1). A slight reduction in growth and lipase activity (25.4±4.5 IU/mL) was observed in media M1and M3. Medium M1 contains lower glucose and higher nitrogen source (yeast extract and tryptone) concentrations than medium M2. On the other hand, medium M3 contains glycerol as a primary carbon source. From this screening result, it can be concluded that carbon source is an important medium component for lipase production by the recombinant *E. coli*. Glucose seems to be preferred over glycerol as a carbon source for growth and lipase production, as medium with glucose (M2) gave better results than medium with glycerol (M3). Medium M2 was selected for further optimization as it gave the highest growth of *E. coli* and highest lipase production.

Evaluation of significant variables using PB design

From the results of the PB design, the highest (33.2 IU/mL) and the lowest (0.2 IU/mL) lipase activity were obtained in

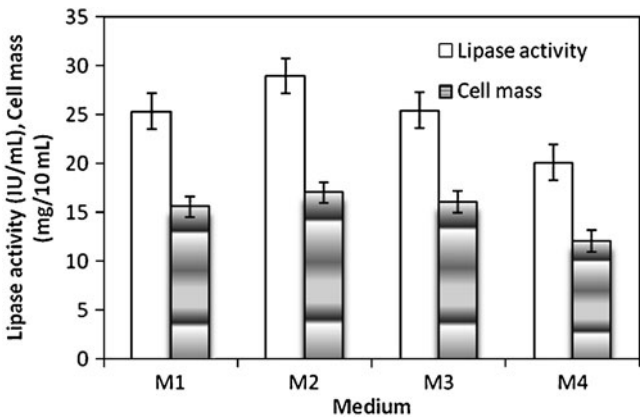

Fig. 1 Lipase activity in different production media

experiments 3 and 6, respectively. The highest and lowest lipase activities also corresponded to the highest (3.5 g/L) and the lowest (1.0 g/L) cell concentration attained during fermentation. The coefficient estimates, *t* and *P* values obtained after regression analysis of the PB design are presented in Table 1. Variables X_3 (glucose), X_4 (NaCl), X_7 (temperature) and X_9 (induction time) were significant for lipase production as their *P* values were lower than 0.05. The variable with the largest coefficient value—either positive or negative—is the most significant variable. This fact is used to rank the significant variables. The ranking order for the significant variables was X_7 (temperature), X_3 (glucose), X_4 (NaCl) and X_9 (induction time). The positive and negative signs indicate that the effect is positive or negative. Positive effect indicates that variable and response are directly proportional and, conversely, a negative effect shows that the variable and response are inversely proportional. Results from this study also show that X_3 (glucose) has a positive effect and the other three significant variables have a negative effect on lipase production. These significant variables [X_3 (glucose), X_4 (NaCl), X_7 (temperature) and X_9 (induction time)] were then selected for further optimization using RSM.

Optimization using response surface methodology

A total of 32 experiments with variable settings and their observed responses are given in Table 2. The factorial experiments (16) were used to estimate the main effects and two factor interactions, whereas axial point experiments (8) were used to estimate quadratic effects and central point experiments (8) were used to observe the process variability. The explanatory model obtained from the data is shown in Eq. 2;

$$\begin{aligned} Y = & -110.933 + 0.024X_3 + 4.657X_4 + 8.401X_7 \\ & + 11.996X_9 - 0.008{X_3}^2 - 0.593{X_4}^2 \\ & - 0.157{X_7}^2 - 5.046{X_9}^2 - 0.017X_3{*}X_4 \\ & + 0.018X_3{*}X_7 + 0.033X3{*}X_9 + 0.069X_4{*}X_7 \\ & + 0.221X_4{*}X_9 + 0.193X_7{*}X_9 \end{aligned} \quad (2)$$

Where *Y* is lipase activity.

Statistical analysis of the quadratic regression demonstrated that the above equation is a highly significant model (Table 3), as can be observed from Fisher's *F* test, which gave a calculated *F* value of 16.4 with a very small *P* value (0.0008). The model's accuracy of fit can also be checked by determining the coefficient of correlation (R^2=0.97). It is obvious from the value of R^2 that only 3% of the total variation was not explained by the model. The value of adjusted R^2 (0.96) also supported the significance of the model. Main effects of temperature (X_7) and induction time (X_9) are significant according to their *P* values (Table 3). Quadratic effects of all four variables glucose (X_3), NaCl (X_4), temperature (X_7) and induction time (X_9) are significant. The interaction effects of glucose and temperature (X_3*X_7) and glucose and induction time (X_3* X_9) are significant. The interaction effects can be represented by a three-dimensional (3D) surface plot (Fig. 2).

The effect of the four significant variables can also be seen from desirability charts (not shown). Lipase activity increased with increasing glucose concentration (X_3) up to 32 g/L and decreased drastically at very high X_3 (>45 g/L). Lipase activity decreased equally at concentrations lower and higher than the maximum at 5 g/L NaCl (X_4). Lipase activity increased with increasing temperature (X_7) up to 31.7°C and decreased above this value. Lipase activity also increased with induction time (X_9) up to 2.1 h and decreased with further increase in induction time (X_9).

The ability of cells to express lipase as measured by the E/x (lipase activity/cell concentration) value ranged from 0.14 to 10.74 IU/g cell in different medium formulations and culture conditions. Maximum lipase producing conditions (experiment number 16) also favoured the highest cell mass and the highest E/x value. A reduced E/x value was observed for fermentation performed at higher temperatures (experiments 1, 2, 5, 6, 9, 10, 13, 14 and 21), which produced only about 2 mg/mL cell mass. This observation suggests that the ability of cells to express lipase enzymes may vary with changes in medium composition and cultivation conditions.

The levels of the four tested variables before optimization were 10 g/L, 5 g/L, 37°C and 1.7 h for X_3 (glucose), X_4 (NaCl), X_7 (temperature) and X_9 (induction time), respectively. X_4 (NaCl) level predicted by RSM was the same as before optimization. Significant changes were observed only in the levels of X_3 (glucose) and X_7 (temperature) after optimization.

Verification experiments

The optimum levels for the tested variables as predicted by RSM were 32.4 g/L, 5 g/L, 31.7°C and 2.1 h for X_3 (glucose), X_4 (NaCl), X_7 (temperature) and X_9 (induction time), respectively. The highest predicted response on these levels was 48.9 IU/mL. Experiments were conducted in triplicate on these optimum levels to determine the observed response. The experimental data fitted well to the optimal predicted response, with a few small differences (Table 4). In the optimized fermentation, the experimental value of final lipase activity was 50.2±4.5 IU/mL. This corresponds to an improvement in lipase production of about 2.5-fold as compared to that obtained in non-optimized fermentation using a basal medium (M4, LB

Table 3 Analysis of variance for optimization of lipase production by recombinant *E. coli* using Box-Wilson design

Variable	Analysis of variance				Parameter estimates		
	SS	Degrees of freedom	MS	F	Estimates	*t* values	*P* values
Intercept	103.5437	1	103.5437	16.4015	-110.933	-4.0499	0.000832*
X_3	0.0560	1	0.0560	0.0089	0.024	0.0942	0.926090
X_3 *2	325.1179	1	325.1179	51.4991	-0.008	-7.1763	0.000002*
X_4	20.5663	1	20.5663	3.2577	4.657	1.8049	0.088831
X_4*2	166.1063	1	166.1063	26.3114	-0.593	-5.1295	0.000084*
X_7	388.8201	1	388.8201	61.5896	8.401	7.8479	0.000000*
X_7*2	939.7712	1	939.7712	148.8609	-0.157	-12.2009	0.000000*
X_9	32.5716	1	32.5716	5.1594	11.996	2.2714	0.036401*
X_9*2	752.0353	1	752.0353	119.1233	-5.046	-10.9144	0.0000008*
X_3*X_4	7.8338	1	7.8338	1.2409	-0.017	-1.1140	0.280802
X_3*X_7	76.2758	1	76.2758	12.0822	0.018	3.4759	0.002891*
X_3*X_9	2.4380	1	2.4380	0.3862	0.033	0.6214	0.542560
X_4*X_7	30.4991	1	30.4991	4.8311	0.069	2.1980	0.042092*
X_4*X_9	3.1237	1	3.1237	0.4948	0.221	0.7034	0.491317
X_7*X_9	21.4360	1	21.4360	3.3955	0.193	1.8427	0.082885
Error	107.3224	17	6.3131				

*Rows with significant variables and their calculated values

broth), which gave a final lipase activity of only 20.1± 0.8 IU/mL. Cell mass increased from 1.7 g/L to 4 g/L after optimization. It was also observed that the highest cell mass was obtained in the same experiment that gave the highest lipase activity.

Discussion

LB medium is a minimal medium normally used for recombinant protein expression in *E. coli* at laboratory scale fermentation. For industrial use, this medium needs to be modified to ensure that its components and composition are suitable to support the growth of host cells and also the expression of the target proteins or enzymes. Results from this study have demonstrated that LB medium should be supplemented with a carbon source such as glucose or glycerol to enhance growth of *E. coli* and lipase production. The highest cell concentration obtained to date in batch fermentation of *E. coli* with LB with optimal culture conditions was only 1 g/L (Shiloach and Fass 2005). In our study, with optimal addition of glucose in LB medium, a final cell concentration of up to 4 g/L was attained.

Results from this study also indicate that the optimum levels of temperature (X_7) and glucose (X_3) as suggested by RSM are significantly different from their initial values. A wide range of temperature (30–37°C) has been used for the cultivation of *E. coli*, depending on the strain and the protein to be expressed. In this study, the optimum temperature for growth of *E. coli* BL21 and lipase production was 31.7°C. A temperature used widely for the cultivation of *E. coli* Bl21 is 30°C (Kogure et al. 2007; Phue et al. 2008). The quality of soluble and insoluble recombinant proteins and the quantity of soluble recombinant proteins was increased in *E. coli* fermentation at temperatures lower than 37°C (Vera et al. 2007). At low temperature, the rate of transformation and transcription was reduced and the protein has more time to reach its final folded configuration during the fermentation. A temperature-dependent phenomenon has also been reported for the expression of other proteins (Kane and Hartley 1988; Seeger et al. 1995; Hoffmann et al. 2002). Therefore, it can be predicted that the solubility of recombinant protein is favoured by growth at lower temperature. This phenomenon can be used as a possible explanation for the higher lipase production at 31.7°C compared to at 37°C.

The optimal glucose concentration (32.4 g/L) observed in this study for growth of *E. coli* BL21 and lipase production was higher than those reported in the literature, which range from 1 to 10 g/L (Sharma et al. 2007; Gao et al. 2009; Zhang et al. 2009). The *E. coli* B strain used in this study is not as sensitive as other *E. coli* strains, such as *E. coli* K, to high glucose concentrations in the medium. During cultivation of *E. coli* B strain in higher glucose concentrations (e.g., 40 g/L), very low acetate was accumulated in the culture (Shiloach and Rinas 2009). Under normal growth conditions, *E. coli* B strain has activates various pathways of central carbon

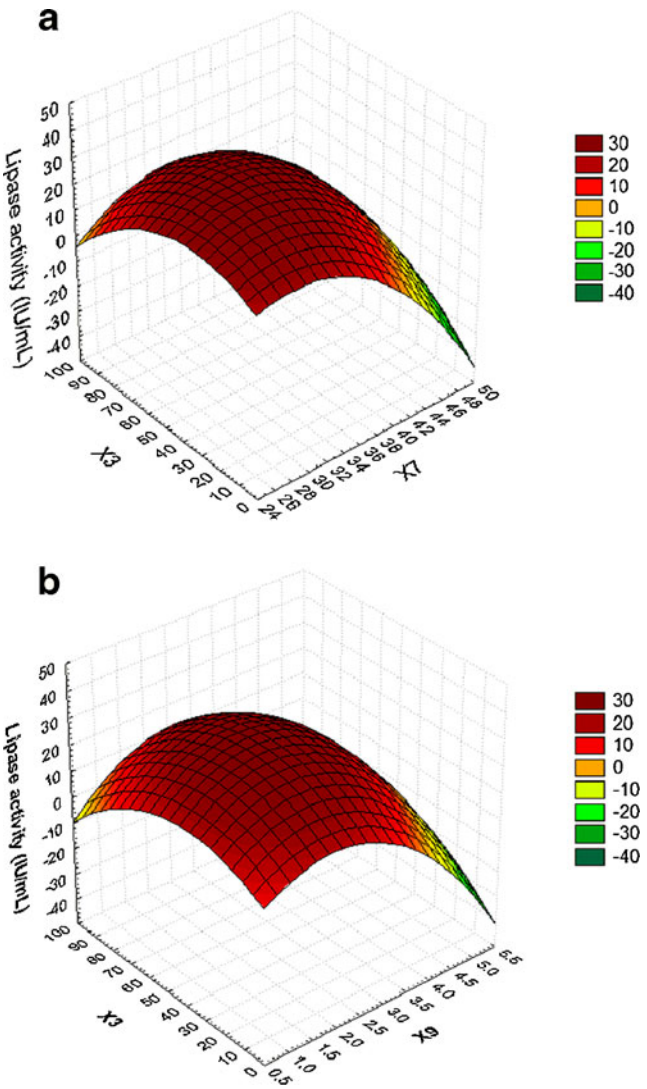

Fig. 2a,b Three-dimensional (3D) surface plots. **a** Interaction of glucose (X_3) and temperature (X_7). **b** Interaction of glucose (X_3) and induction time, keeping all other parameters constant

metabolism, such as glyoxylate shunt and pentose phosphate shunt, and thus growth does not depend on the glucose concentration in the culture. On the other hand, high glucose concentrations in cultures of *E. coli* K strain trigger high accumulation of acetate, in which growth is significantly inhibited (Shiloach and Rinas 2009; Phue et al. 2005).

The expression of recombinant proteins by *E. coli* B strain is also less affected by high concentrations of glucose in the culture as compared to other *E. coli* strains even when using an IPTG induction strategy via the lac operon. Pre-induction growth and acetate accumulation patterns of two *E. coli* strains (BL21 and K12) used widely for the production of various recombinant proteins have been compared (Shiloach et al. 1996). The yield of recombinant exotoxin A obtained in batch culture of *E. coli* BL21 (lambdaDE3) with high initial glucose concentration (40 g/L) was similar to that obtained in fed batch cultures where glucose was controlled at low levels (2 g/L). Acetate accumulated in batch culture of *E. coli* BL21 to only 2 g/L and was reduced to less than 1 g/L in fed batch culture. In contrast, the productivity of a recombinant-fusion protein by *E. coli* K12 (JM109) was greatly reduced in batch culture with high initial glucose as compared to fed batch technique, as higher acetate accumulated in batch (10 g/L) compared to fed batch (5 g/L) culture. *E. coli* BL21 was less sensitive to variation in glucose concentrations as compared to *E. coli* K12. As observed in this study the expression of Lip 42 in *E. coli* BL21 was increased up to 32 g/L glucose but decreased drastically at higher concentrations. This result indicated that the lac operon was not catabolically repressed at the levels of glucose at which the *E. coli* BL21 strain did not exhibit the overflow metabolism.

Substantial increases in lipase production, ranging from five- to six-fold, after optimization using RSM, have been reported by several researchers (Liu et al. 2006; Khare et al. 2008; Pan et al. 2008). However, in most cases, the fermentation employed natural isolates for lipase production. Improvement of protein production by recombinant *E. coli* through medium optimization using RSM has been reported by several researchers. For example, a five-fold increase in the production of human interferon beta for expression of a synthetic gene in *E. coli* was achieved after optimization using RSM (Maldonado et al. 2007). Results from this study clearly indicate that lipase production was associated with growth of *E. coli* B21, but the ability of cells to express the lipase varied with medium composition and culture conditions. This means that the optimization of lipase production by recombinant *E. coli* cannot simply be focused on the

Table 4 Predicted optimum values of significant variables for lipase production by the recombinant *E. coli* using response surface methodology (RSM)

Variable	Range studied	RSM predicted optimum values	Lipase activity (IU/mL)	
			Observed	Predicted by RSM
X_3	10-90	32.4	50.261±4.576	48.902
X_4	1-9	5		
X_7	25-49	31.7		
X_9	1-5	2.1		

maximization of cell production, commonly known as high density cell cultivation technique, as practised for the production of many recombinant proteins by *E. coli* (Shiloach and Fass 2005; Nikerel et al. 2006).

Conclusion

The production of intracellular lipase by recombinant *E. coli* was influenced greatly by the medium composition and culture conditions. The variables having the most significant effect, as evaluated by PB design, were cultivation temperature and induction time as well as glucose and NaCl concentrations. The optimized medium composition based on LB medium obtained using RSM was 32.40 g/L glucose and 5 g/L NaCl with a cultivation temperature of 31.7°C and induction time of 2.1 h. Lipase production (50.2 IU/mL) using this optimized medium and culture conditions was about 2.5 fold higher than production in non-optimal fermentation (20.1 IU/mL).

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