

Optimization of immobilization conditions by conventional and statistical strategies for alkaline lipase production by *Pseudomonas aeruginosa* mutant cells: scale-up at bench-scale bioreactor level

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Abstract: Suitability of 3 matrices, agarose, sodium alginate, and polyacrylamide, for immobilization of mutant cells of *Pseudomonas aeruginosa* MTCC 10,055 was investigated. Of these, agarose was proven to be the best as exhibiting maximum enzyme production (4363.4 U/mL), followed by polyacrylamide gel (2172.3 U/mL). Alginate beads were the poorest. The one-variable-at-a-time approach suggested agarose at 2.0%, immobilized bead at 4.0 g blocks/50 mL, and initial cell loading of 0.8 g in the matrix as optimum conditions for maximum lipase production (5982.3 U/mL) after 24 h of incubation. However, response surface methodology studies determined the optimum values of these variables as 1.96%, 4.06 g blocks/50 mL, and 0.81 g of cells in the matrix for maximum lipase production (6354.23 U/mL) within 22.54 h of incubation. The agarose blocks were reusable for 7 cycles without any significant loss in lipase yield. Bench-scale bioreactor level optimization resulted in further enhancement in lipase yield (6815.3 U/mL) at 0.6 vvm aeration and 100 rpm agitation within only 20 h of incubation. Presumably, this is the first attempt for lipase production by immobilized cells of *P. aeruginosa* at the bioreactor level. The agarose-immobilized mutant cells showed potential candidature for alkaline lipase production at the industrial level.

Key words: Alkaline lipase, bench-scale bioreactor, immobilized cells, response surface methodology

1. Introduction

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are hydrolytic enzymes, which catalyze the hydrolysis of the ester linkage of long-chain acylglycerols at the oil–water interface. They are an important group of biotechnologically relevant enzymes because of their activity in both aqueous and nonaqueous media. They have diverse applications in a wide variety of industries such as detergent, oleochemical, organic synthesis, dairy, fat and oil modification, and pharmaceutical (1).

Microbial products are usually produced either by free or immobilized cells. Recently, immobilized biocatalysts such as enzymes, microorganisms, organelles, plants, and animal cells have added new dimensions to the application potential of the rapidly advancing frontier of biotechnology because these biocatalysts display better operational stability (2,3) and higher efficiency of catalysis, and they are reusable (4). Whole-cell immobilization appears to be an interesting alternative to conventional free-cell fermentation because of easier downstream processing without loss of biomass, increased productivity, and operational stability or reduction of the fermentation time in enzyme

production (5–7). In addition, whole-cell immobilization has been a better choice over enzyme immobilization (8,9). Immobilized cells are mainly used for economic benefits of continuous production or repeated use in continuous as well as in batch mode. Because of this, during the last few years immobilized living microbial cells have been applied to the fermentative production of enzymes (10–12).

For the development of an industrially viable microbial fermentation system, the results of flask-level experiments must be reproduced at the bioreactor level. A drastic change in the response was observed when operation conditions obtained by flask-level experiments were implemented at bioreactor level. Thus, various operation parameters, such as agitation and aeration, pH, dissolved oxygen, and inoculum levels require extensive investigation at bioreactor level (13). For aerobic fermentation, oxygen supply is an imperative variable that is controlled by aeration and agitation. The agitation and aeration rates not only affect the productivity of the microbial process, but also affect the overall energy requirement of the production process. Therefore, it is necessary to establish an optimum combination of airflow and agitation for maximum yield (14).

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Keeping the above in mind, the conventional one-variable-at-a-time and statistical approach employing response surface methodology (RSM) was used to assess the immobilization parameters of agarose concentration, inoculum size, and cell concentration on lipase production addition to select a suitable support material for immobilization of *Pseudomonas aeruginosa* whole cells. Moreover, studies have been done to perform reuse experiments with the immobilized blocks and storage stability of the blocks. For scale-up strategy, we have found a balanced level of agitation and aeration rates for lipase production by *P. aeruginosa* immobilized cells in a bench-scale fermentor.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The mutant strain under investigation was developed in our laboratory by chemical mutagenesis of *P. aeruginosa* MTCC 10,055 isolated from oil-contaminated soil (15). It was maintained on nutrient agar with 1% tributyrin and stored at 4 °C.

2.2. Inoculum preparation

A loopful of *P. aeruginosa* mutant strain from the 24-h-old slants were inoculated into 50 mL of inoculum medium containing (in g/L) $(\text{NH}_4)_2\text{SO}_4$ 1.0; KH_2PO_4 0.6; MgSO_4 0.2; yeast extract, 0.1; and olive oil, 20 (pH 9.0), and inoculated media were incubated at 37 °C with shaking (120 rpm) for 24 h.

2.3. Preparation of immobilized cells

2.3.1. Entrapment in sodium alginate

Exponentially growing bacterial cells were harvested from culture broth by centrifugation at $12000 \times g$ for 10 min. The supernatant was removed and the pellet (0.2 g) was washed twice with sterile distilled water and mixed with sterilized 20 mL of 4% (w/v) sodium alginate prepared in distilled water. The slurry was extruded through a syringe into 0.2 M calcium chloride solution with constant stirring. The beads were allowed to cure in 0.2 M calcium chloride solution for 24 h at 4 °C followed by washing with sterile distilled water 3 to 4 times, and they were preserved in Tris-HCl buffer (pH 8.0) at 4 °C until use.

2.3.2. Entrapment in agarose

The harvested cells (0.2 g) from culture broth were suspended in 20 mL of sterilized agarose solution cooled to 40–45 °C. The agarose–cell suspension was poured onto a sterile petri plate aseptically. After solidification, agarose blocks were cut into cubes of equal size (approximately 4 mm³), added to sterile 50 mM phosphate buffer (pH 8.0), and kept at 4 °C until use.

2.3.3. Entrapment in polyacrylamide

A cell suspension was prepared by adding 0.2 g of cells to 10 mL of chilled sterile distilled water. To another

10 mL of 0.2 M sterile phosphate buffer (pH 8.0), the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium persulfate, and TEMED. The cell suspension and the above phosphate buffer mixture were mixed well and poured into sterile petri plates. After polymerization, the acrylamide gel was cut into cubes of equal size (approximately 4 mm³), transferred to 50 mM phosphate buffer (pH 8.0), and kept in the refrigerator for 1 h for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile buffer (Tris-HCl, 50 mM, pH 8.0) at 4 °C until use. All the immobilization steps were carried out under aseptic conditions.

2.4. Screening of different matrices for immobilization of mutant cells for lipase production

Immobilized cells prepared by the above methods were transferred to 250-mL Erlenmeyer flasks containing 50 mL of production medium, obtained after the optimization using RSM (Box–Behnken method) containing (in g/L): starch, 15; castor oil, 1.77; yeast extract, 0.2; $(\text{NH}_4)_2\text{SO}_4$ 1.0; KH_2PO_4 0.6; MgSO_4 0.4; Triton-X-100, 0.93, and gum arabic, 5.0; with pH 8.1 (16). The flasks were incubated on a rotary shaker (120 rpm) at 34.1 °C for 48 h. For the free cell culture, 50 mL of production medium was inoculated with bacterial cells equivalent to those used for immobilization.

2.5. Optimization of immobilization conditions for alkaline lipase production

In the conventional one-variable-at-a-time approach, various immobilization parameters were optimized by maintaining all factors at a constant level, except the variable under study. To determine the optimum concentration of agarose, various concentrations of 1%–5% (w/v) were used to prepare the blocks. Thereafter, the inoculum size (weight of immobilized blocks/50 mL production medium) was optimized by varying the weight from 2 to 10 g. For the effect of different cell concentrations in the matrix, different amounts of mutant *P. aeruginosa* cells (0.2–1.0 g wet weight in matrix) were investigated by incorporating in 2% (w/v) agarose solution (20 mL). These blocks were employed for lipase production and the fermentation was conducted for 24 h under optimized fermentation conditions.

2.6. Optimization of immobilization conditions using response surface methodology

A response surface methodology, using a Box–Behnken design (17), was adopted to appraise the interactions and to identify the optimal level of the factors of agarose concentration (A), inoculum size (B), cell concentration (C), and incubation time (D) affecting the lipase production by *P. aeruginosa* mutant under shake-flask fermentation.

The factors at 3 different levels (–1, 0, +1), with minimum and maximum range of values and the treatment schedule for the model, are given in Tables 1 and 2, respectively. Five replicates (runs) at the center of the

Table 1. Experimental range and levels of the independent variables used in RSM in terms of actual and coded units.

Variables	Coded units		
	-1	0	+1
Agarose concentration (%)	1.0	2.0	3.0
Inoculum size (g)	3.0	4.0	5.0
Cell concentration (g)	0.6	0.8	1.0
Incubation time (h)	20	24	28

design were used for the estimation of pure error and sum of squares. The experiments were randomized to maximize the effects of unknown variability due to irrelevant factors in the observed responses.

2.7. Statistical analysis

A second-order polynomial equation, fitted to the data by multiple regression procedure, resulted in an empirical model:

$$Y = \beta_0 + \sum \beta_n X_n + \sum \beta_{nn} X_n^2 + \sum \beta_{nm} X_n X_m \quad (1)$$

For analysis of design based on 4 factors, the following model equation was used:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \varepsilon \quad (2)$$

Table 2. Box-Behnken design matrix for RSM studies of 4 independent variables for alkaline lipase production.

Std. order	A: Agarose concentration (%)	B: Inoculum size (g)	C: Cell concentration (g)	D: Incubation time (h)	Observed response (U/mL)	Predicted response (U/mL)
1	1.00	3.00	0.80	24.00	3809.70	3780.20
2	3.00	3.00	0.80	24.00	3405.60	3423.60
3	1.00	5.00	0.80	24.00	4089.40	4039.62
4	3.00	5.00	0.80	24.00	3942.80	3940.52
5	2.00	4.00	0.60	20.00	5202.10	5183.25
6	2.00	4.00	1.00	20.00	5323.40	5349.94
7	2.00	4.00	0.60	28.00	4806.30	4747.99
8	2.00	4.00	1.00	28.00	4951.10	4938.17
9	1.00	4.00	0.80	20.00	4667.40	4688.83
10	3.00	4.00	0.80	20.00	4518.40	4551.68
11	1.00	4.00	0.80	28.00	4391.80	4356.02
12	3.00	4.00	0.80	28.00	4061.40	4037.47
13	2.00	3.00	0.60	24.00	3829.30	3841.60
14	2.00	5.00	0.60	24.00	4865.30	4864.62
15	2.00	3.00	1.00	24.00	4656.70	4654.88
16	2.00	5.00	1.00	24.00	4423.00	4408.20
17	1.00	4.00	0.60	24.00	4232.20	4294.65
18	3.00	4.00	0.60	24.00	3913.60	3960.70
19	1.00	4.00	1.00	24.00	4291.80	4322.98
20	3.00	4.00	1.00	24.00	4273.40	4245.23
21	2.00	3.00	0.80	20.00	4977.60	4929.76
22	2.00	5.00	0.80	20.00	4820.20	4805.63
23	2.00	3.00	0.80	28.00	3945.10	3993.95
24	2.00	5.00	0.80	28.00	4812.30	4894.41
25	2.00	4.00	0.80	24.00	5980.5	5954.42
26	2.00	4.00	0.80	24.00	5930.3	5954.42
27	2.00	4.00	0.80	24.00	5980.5	5954.42
28	2.00	4.00	0.80	24.00	5940.3	5954.42
29	2.00	4.00	0.80	24.00	5940.5	5954.42

Here, Y is the predicted response for alkaline lipase produced; β_0 is the value of the fitted response at the center point of the design; β_1 , β_2 , β_3 , and β_4 are the linear coefficients; β_{11} , β_{22} , β_{33} , and β_{44} are the quadratic coefficients; β_{12} , β_{13} , β_{14} , β_{23} , β_{24} , and β_{34} are the interaction coefficients; and ε is the random error. The software package Design-Expert 8.0.5.2 (Stat-Ease Inc., Minneapolis, MN, USA) was used to obtain the coefficients of Eq. (2) based on the data provided in Table 2. The responses under different combinations as defined by the design (Table 2) were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters.

2.8. Production of alkaline lipase by repeated-batch fermentation

One of the advantages of using immobilized biocatalysts is their repeated and continuous use. Therefore, the reusability of *P. aeruginosa* cells immobilized in agarose gel was examined. After attaining the maximum production of alkaline lipase, the spent medium was replaced with fresh production medium (50 mL), and the process was repeated for several cycles until the blocks started disintegrating or lipase production was decreased. The storage stability of immobilized cells was also investigated up to the period of 6 months. After each month, the beads were washed and used for lipase production.

2.9. Optimization of different airflow and agitation rates at bench-scale bioreactor level

Further studies were performed in a stirred tank bioreactor (Yorko, New Delhi, India) of 7-L capacity. The lipase production was carried out in 5 L of the same production medium used in the flask-level studies. Three levels of airflow rates, 0.3, 0.6, and 0.9 vvm, were studied, and at each airflow rate, 3 different agitation rates of 50, 100, and 150 were also tested. The cultivation was carried out for 36 h and samples (5.0 mL) were drawn periodically at 4-h intervals. The cell-free culture broth was used for the estimation of lipase production.

2.10 Analytical methods

Lipase activity was determined spectrophotometrically by following the method of Winkler and Stuckman (18) with slight modifications. The substrate solution containing 10 mL of isopropanol with 30 mg of *p*-nitrophenyl palmitate was mixed with 90 mL of Tris-HCl buffer (50 mM, pH 9.0), containing 0.4% Triton-X 100 and 100 mg of gum arabic. Freshly prepared substrate solution (2.4 mL) was incubated at 37 °C with 25 μ L of suitably diluted cell-free supernatant for 15 min. After incubation, absorbance was measured at 410 nm by using a spectrophotometer (UV-1601, Shimadzu) against a control with heat-inactivated enzyme. One unit of enzyme is defined as the amount of enzyme liberating 1 μ g of *p*-nitrophenol $\text{mL}^{-1} \text{min}^{-1}$ under the assay conditions.

3. Results and discussion

3.1. Screening of different matrices used for immobilization of *P. aeruginosa*

Immobilized microorganisms improve the productivity of bioreactors and provide several advantages over free cells in industrial fermentation and environmental and agricultural applications (19). Figure 1 represents the comparative lipase production by different matrices along with cell leakage. The agarose (without glutaraldehyde as binding agent)-entrapped cells started the enzyme production after 6 h and gradually approached maximum titer (4363.4 U/mL) within 24 h of incubation, which was 32% higher than free cells with the cell leakage of 0.43 mg/mL (dry cell weight). The free cell system started lipase production after 12 h of incubation, and the maximum enzyme yield was 3305.6 U/mL within 30 h of incubation. The cells entrapped in agarose, containing glutaraldehyde as binding agent, showed the least enzyme production (1972.2 U/mL). The negative effect of glutaraldehyde on lipase production can probably be attributed to its biocidal activity. Glutaraldehyde readily alkylates sulfhydryl, hydroxyl, carboxyl, and amino groups of nucleic acids and proteins (20,21). In the case of microorganisms, the amine groups present on the outer cell walls and cell membranes of bacteria and other microorganisms are cross-linked and this is thought to be responsible for their efficacy against gram-negative bacteria, fungi, and viruses (22,23). Our findings are in concurrence with those of Abdel-Fattah et al. (24), who also reported that the use of glutaraldehyde as a binding agent exerted an adverse effect on levansucrase production by *Bacillus circulans* cells entrapped in agar. However, contrasting reports on the use of glutaraldehyde as a binding agent are also available; for example, Krastanov (25) found it the best for immobilization of yeast cells on wool. Glutaraldehyde probably had no effect on the yeast strain used by him.

The sodium alginate-entrapped cells showed peak activity (3669.2 U/mL) after 30 h of incubation, which was 11% more compared to the free cell system with maximum cell leakage (1.1 mg/mL, dry cell mass). The immobilized beads prepared with sodium alginate have an inherent problem of dissolving in phosphate solution. The low stability of sodium alginate beads is attributed to the presence of potassium phosphate in the production medium that tends to dissolve the beads (26).

Polyacrylamide-entrapped cells exhibited 2172.3 U/mL of enzyme production after 36 h of incubation, which was less than that of agarose and sodium alginate. The low yields with polyacrylamide beads may be due to the poor viability of cells (27). Therefore, agarose was considered to be the best matrix for the production of alkaline lipase.

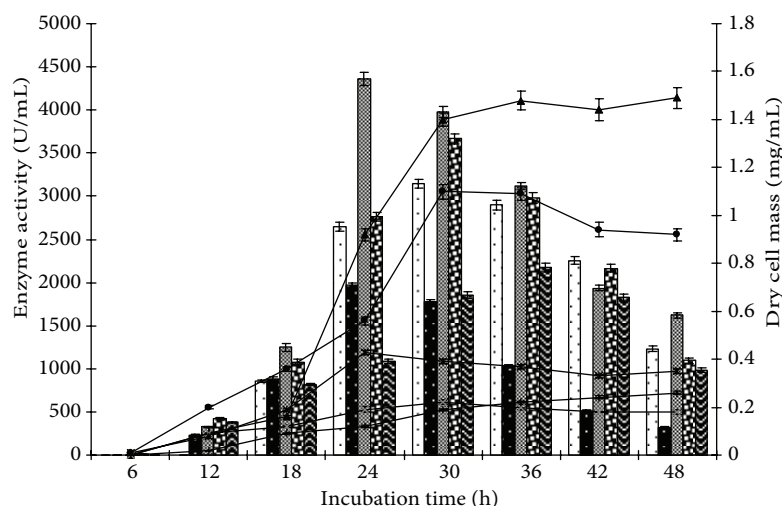


Figure 1. Comparative analysis of free and immobilized cells of *P. aeruginosa* entrapped in different carriers for alkaline lipase production. The fermentation medium was inoculated with free cells and immobilized cells entrapped in different matrices. The flasks were incubated at 34.1 °C at 120 rpm for 48 h. Lipase production (U/mL) and cell leakage (in the form of dry cell mass) were observed at the interval of every 6 h. Fermentation medium was inoculated with different matrices: (□) – lipase production by free cells, (■) – lipase production by cells entrapped in agarose (with glutaraldehyde), (▨) – lipase production by cells entrapped in agarose (without glutaraldehyde), (▩) – lipase production by cells entrapped in sodium alginate, (▧) – lipase production by cells entrapped in polyacrylamide for 48 h. (—▲—) – Dry cell weight of free cells, (—□—) – cell leakage by agarose (with glutaraldehyde), (—*—) – cell leakage by agarose (without glutaraldehyde), (—●—) – cell leakage by sodium alginate, (—+—) – cell leakage by polyacrylamide. Bars presented are mean values ± standard deviations of triplicates of 3 independent experiments.

3.2. Optimization of immobilization conditions for lipase production

3.2.1. Effect of agarose concentration

Figure 2 depicts the effect of the agarose concentration for immobilization on alkaline lipase production and cell leakage. The highest lipase production (4359.6 U/mL) was obtained with blocks prepared by using 2% (w/v) agarose, in comparison to the other concentrations used. In a similar study, Joseph et al. (27) reported maximum

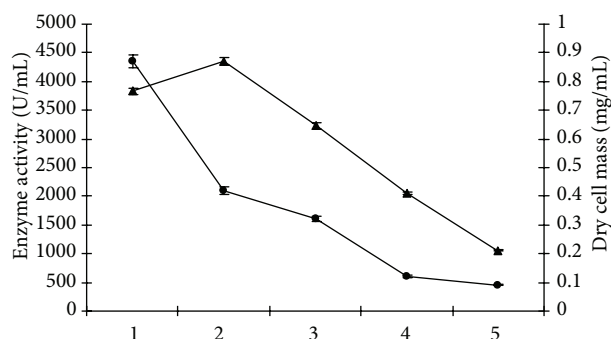


Figure 2. Effect of agarose concentration (%) on alkaline lipase production by immobilized cells. The flasks containing blocks prepared in agarose (2.0%) were taken as the control. Inoculated flasks were incubated at 34.1 °C for 24 h. (—▲—) – lipase production (U/mL), (—●—) – cell leakage (in the form of dry cell mass). Bars presented are mean values ± standard deviations of triplicates of 3 independent experiments.

lipase production when cells were immobilized in 3% agar. The increase in agarose concentration (above 2.0%, v/v) resulted in a drop in the enzyme yield. This could be ascribed to the reduction in the diffusion efficiency of the nutrients and oxygen into the gel matrix (27), or to limitation of lipase release out of it due to its high rigidity. However, use of 1.0% (w/v) agarose concentration for block preparation resulted in very soft blocks, which were easily broken due to their low mechanical strength, resulting in leakage of a significant amount of cells from the blocks and hence incomplete immobilization.

3.2.2. Effect of inoculum size

Figure 3 shows that 4 g of immobilized cells (blocks) in 50 mL of medium was the most suitable inoculum level exhibiting maximum lipase yield of 4378.8 U/mL. The increase in weight of blocks in the fermentation medium led to decrease in alkaline lipase productivity. This could be attributed to the fact that when the number of blocks increased, the nutrient/block ratio decreased, in turn declining the lipase production. A maximum production of lipase was reported by Zakaria et al. (28) by 1.5 g of alginate-immobilized bacteria in 50 mL of culture broth. Ahmed (29) obtained maximum invertase production with 200 alginate beads/flask.

3.2.3. Effect of cell concentration

Evidently, the lipase productivity by immobilized cells was dependent on the initial cell concentration in the agarose

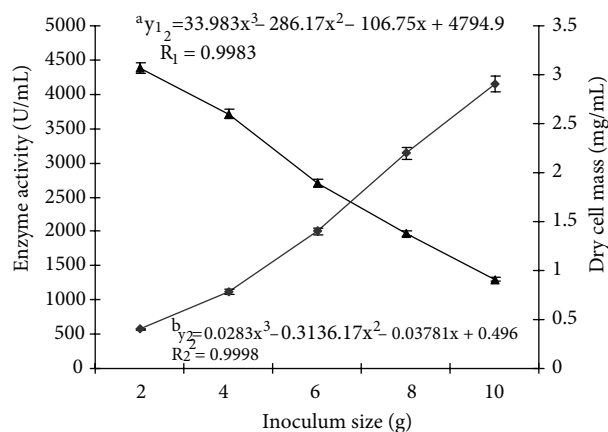


Figure 3. Effect of inoculum size on alkaline lipase production by immobilized cells. The control flasks contained the same inoculum level (2 g blocks). Test flasks contained different inoculum levels. Inoculated flasks were incubated at 34.1 °C for 24 h. (▲) – lipase production (U/mL), (◆) – cell leakage (in the form of dry cell mass). Bars presented are mean values \pm standard deviations of triplicates of 3 independent experiments. ^a: denotes the line equation for lipase production, ^b: denotes the line equation for cell leakage.

matrix (Figure 4). In this study, an increase in initial cell concentration resulted in gradual augmentation in lipase yield, and maximum production (5982.3 U/mL) was achieved at 0.8 g of wet cell weight in the matrix. With the increase in cell concentration, there was dearth of nutrient supply (30) and that may be the reason why lipase production did not improve beyond 0.8 g of cell weight. Our findings are supported by those of Jouenne et al. (31), who reported that low levels of entrapped cells led to rapid enzyme biosynthesis, while high levels caused diffusion limitation of nutrients. However, Cheetham et al. (32) reported that at very high cell concentrations, the beads were actually less active because the porous structure of the beads was lost.

3.3. Statistical optimization of immobilization conditions by response surface methodology

The lipase production by *P. aeruginosa* immobilized cells was assessed using an experimental design technique. This study showed that the independent parameters evaluated had significant impact on alkaline lipase production.

3.4. Determination of significant variables by Box-Behnken design

RSM using Box-Behnken design was used to assess the interactive effects of 4 variables: agarose concentration, inoculum size, cell concentration, and incubation time. Table 1 shows the maximum and minimum levels of variables chosen for trials in the Box-Behnken design. For RSM, 29 experimental runs with 5 center points were designed using 4 factors at 3 levels. Table 2 represents the

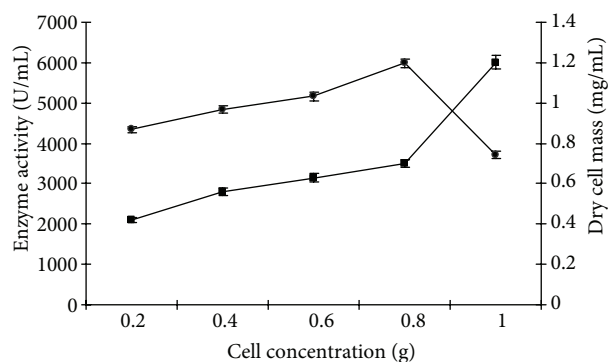


Figure 4. Effect of cell concentration on alkaline lipase production. Inoculated flasks with different cell concentrations (g) entrapped in 2.0% agarose were incubated at 34.1 °C for 24 h. (▲) – lipase production (U/mL), (◆) – cell leakage (in the form of dry cell mass). Bars presented are mean values \pm standard deviations of triplicates of 3 independent experiments.

effects of test variables on lipase production and the observed response, along with the predicted response obtained from the regression equation for each run. Observations (Table 2) clearly explain that there was considerable variation in the lipase production depending on the test variables. Thus, by optimizing the immobilization parameters using RSM, the yield of alkaline lipase increased from 4359.6 U/mL to 5954.42 U/mL. The center point in the design was carried out 5 times to estimate the error.

Multiple regression analysis on the experimental data resulted in the following coefficients for alkaline lipase production (Y), obtained after ANOVA: agarose concentration (A), inoculum size (B), cell concentration (C), and incubation time (D).

Alkaline lipase activity (Y U/mL) = $5954.42 - 113.92 \times A + 194.08 \times B + 89.22 \times C - 211.76 \times D + 64.37 \times AB + 75.05 \times AC - 45.35 \times AD - 317.42 \times BC + 256.15 \times BD + 5.88 \times CD - 1202.94 \times A^2 - 955.50 \times B^2 - 556.60 \times C^2 - 342.98 \times D^2$.

The analysis of variance for the quadratic regression model demonstrates the aptness of the model for alkaline lipase production. Several indicators, such as model significance (F value), determination coefficient (R^2 value), predicted coefficient of determination ($R^2_{\text{predicted}}$), adjusted coefficient of determination (R^2_{adjusted}), and coefficient of variation (CV), were used to judge the adequacy of the model.

The high computed F value (479.71) for the present model suggested the significance of the model (Table 3). There is only a 0.01% chance that a large “model F value” could occur due to noise. In general, calculated F values should be several times more than the tabulated value, if the model was a good prediction of experimental results and estimated factors effects were real (33). A high F value

Table 3. Analysis of variance (ANOVA) for fitted second-order polynomial model as per Box–Behnken design.

Source	Sum of squares	df	Mean square	F value	P value Prob > F	Significance
Model	1.523E+007	14	1.523E+007	479.71	<0.0001	Significant
Agarose concentration (A)	1.557E+005	1	1.557E+005	68.70	<0.0001	
Inoculum size (B)	4.520E+005	1	4.520E+005	199.39	<0.0001	
Cell concentration (C)	95515.36	1	95515.36	42.13	<0.0001	
Incubation time (D)	5.381E+005	1	5.381E+005	237.36	<0.0001	
AB	16576.56	1	16576.56	7.31	0.0171	
AC	22530.01	1	22530.01	9.94	0.0071	
AD	8226.49	1	8226.49	3.63	0.0775	
BC	4.030E+005	1	4.030E+005	177.78	<0.0001	
BD	2.625E+005	1	2.625E+005	115.77	<0.0001	
CD	138.06	1	138.06	0.061	0.8087	
A ²	9.386E+006	1	9.386E+006	4140.41	<0.0001	
B ²	5.922E+006	1	5.922E+006	2612.27	<0.0001	
C ²	2.010E+006	1	2.010E+006	886.42	<0.0001	
D ²	7.631E+005	1	7.631E+005	336.60	<0.0001	
Residual	31737.91	14	2266.99			Not significant
Lack of fit	29402.66	10	2940.27	5.04	0.0665	
Pure error	2335.25	4	583.81			
Corr. total	1.526E+007	28				

and a very low probability ($P > F = 0.0001$) indicated that the present model was a good prediction of experimental results. R^2 or the determination coefficient is the proportion of variation in the response attributed to the model rather than to random error (34). The R^2 value always lies between 0 and 1 and for a good fit of model, R^2 should be at least 0.80 (35). Similarly, Doddapaneni et al. (36) suggested that the closer the value of R^2 is to 1.0, the stronger the model and the better its prediction efficiency of the responses will be. The R^2 (0.9979) for this model implied that 99.79% of the sample variation for lipase activity was attributed to the independent variables, and only about 0.21% of the total variation was not explained by the model. Its value being closer to 1.0 suggested that the model represents a better correlation between experimental and predicted values.

The “lack of fit F value” of 5.04 demonstrated that the lack of fit is not significant relative to the pure error. There was only 6.65% chance that the lack of fit F value could occur due to noise. Nonsignificant lack of fit is good as we want the model to fit (Table 3). Adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable. An adequate precision of 69.105 indicates a low signal-to-noise ratio. The CV is the ratio of the standard error of the estimate to the mean value of the observed response and is expressed as a percentage. A model can be considered practically reproducible if the

CV is not greater than 10% (35). Here, a relatively lower value of the coefficient of variation ($CV = 1.02\%$) indicated the precision and reliability of the conducted experiments (Table 4). On the basis of results obtained from ANOVA, it can be concluded that the model is highly significant and sufficient to represent the actual relationship between the response, and the significant variables can be used successfully to navigate the design space.

In our study, 13 models terms were found to be significant. The variables having prevalent effects on lipase production were the linear terms of A ($P < 0.0001$), B ($P < 0.0001$), C ($P < 0.0001$), and D ($P < 0.0001$) and the

Table 4. Analysis of variance (ANOVA) for response-surface quadratic fitted model.

Parameter	Value
Standard deviation	47.61
Mean	4689.03
CV (%)	1.02
PRESS	1.730E+005
R^2	0.9979
Adjusted R^2	0.9958
Predicted R^2	0.9887
Adequate precision	73.908

quadratic terms of A^2 ($P < 0.0001$), B^2 ($P < 0.0001$), C^2 ($P < 0.0001$), and D^2 ($P < 0.0001$), followed by interaction effects of BC ($P < 0.0001$) and BD ($P < 0.0001$). A value of P of less than 0.05 indicated the significance of the model terms. The effect order of the linear terms on the yield of alkaline lipase were as follows: incubation time ($F = 237.36$), inoculum size ($F = 199.39$), and agarose concentration ($F = 68.70$).

The second-order model can be plotted as a 3-dimensional surface representing the response (lipase production) as a function of 2 factors at a time while maintaining the other 2 factors at fixed levels (center point) to understand both the main and the interaction effects of these 2 factors. The 3-dimensional response surfaces obtained after analysis showed different shapes, which indicated variation in the combined effect of independent variables on lipase production. The observation of interaction of agarose concentration and inoculum size (Figure 5a) indicated that lipase production (4089.40 U/mL) was maximum at lower concentrations of agarose and a higher concentration of inoculum size; however, lipase production was decreased at a higher concentration of agarose (3.0) and at a central point of inoculum size (3.0).

The effect of agarose concentration and cell concentration is shown in Figure 5b. Maximum lipase was produced at a higher cell concentration (1 g) and at a lower agarose concentration (1%) in the design space. However, lower cell concentration (0.6 g) and higher agarose concentration (3.0%) had a negative effect on lipase production.

Figure 5c depicts the production of lipase with respect to agarose concentration versus incubation time. Maximum lipase production with these variables was observed at 1.0% agarose concentration after 20 h of incubation. However, further elevation in agarose concentration (3%) and incubation time (28 h) decreased the lipase production.

Figure 5d represents the response for the interaction of inoculum size with cell concentration. According to the plot, the optimal value (4865.30 U/mL) lay towards the higher concentration of inoculum size (5 g) at a lower cell concentration (0.6 g). However, decrease in inoculum size (3 g) decreased the lipase production at the same cell concentration (0.6 g).

Figure 5e illustrates the interactive effect of inoculum size with incubation time. Maximum lipase production with these variables was observed at lower inoculum size (3 g) after 20 h of incubation. However, increase in incubation time (28 h) reduced the lipase production at the same inoculum size (3.0 g).

Figure 5f represents the response for the interaction of cell concentration with incubation time. The observation of interactions of cell concentration with incubation time indicated that lipase production was maximum with

increase in cell concentration (0.6–1.0 g) after 20 h of incubation. However, at a lower cell concentration (0.6 g) after 28 h of incubation, the lipase production was minimum.

Analysis of response-surface curves and contour plots indicated optimum levels of the variables, necessary to achieve better results. The results obtained as well as those predicted by the Box–Behnken design showed that the combination of center points, i.e. agarose concentration at 2.0%, inoculum size at 4.0 (g), and cell concentration at 0.8 g, and incubation time of 24 h would favor maximum lipase production (5954.42 U/mL).

With an aim to test the desirability of the model, optimum values of the variables were determined as agarose concentration, 1.96%; inoculum size, 4.06 g; cell concentration, 0.81 g; and incubation time, 22.54 h, yielding a maximum lipase production of 5994.56 U/mL.

3.5. Comparison of observed and predicted response and validation of the model

A high degree of similarity was observed between the predicted response (5994.56 U/mL) by the empirical model and the experimental values (6354.23 U/mL) in the range of the operating variables, which reflected the applicability of RSM to optimize the process of cell immobilization.

The suitability of the model was validated at shake-flask level by additional independent experiments under the optimal conditions as predicted by the appropriate equation. Table 5 shows the predicted and observed responses of the validation experiments. Under the optimum conditions obtained by point prediction, the immobilized cells were able to produce 6354.23 U/mL of enzyme production, which was 106% of the predicted value (Table 5). The results showed that the actual values were closer to the predicted values, supporting the data and the model as valid. However, other tested combinations did not reveal improvement in the response. Thus, by optimizing the immobilization parameters using RSM, the yield of alkaline lipase increased from 5994.56 U/mL to 6354.23 U/mL.

3.6. Production of alkaline lipase by repeated-batch fermentation: reusability test of immobilized beads

One of the advantages of using immobilized cells is the possibility of their reuse in repeated processes. However, reuse requires high operational stability. The immobilized cells obtained under the optimal conditions were repeatedly used for a number of successive cycles and their activity was retained for about 11 cycles (Figure 6). The lipase production by immobilized cells remained in the range of 5900–6000 U/mL throughout the 7 cycles. Further replacement of the production medium after every 22.54 h was accompanied by a minor decrease in lipase activity and it reached 5041.7 U/mL at the end of the ninth cycle, which was still higher than that obtained by free cells

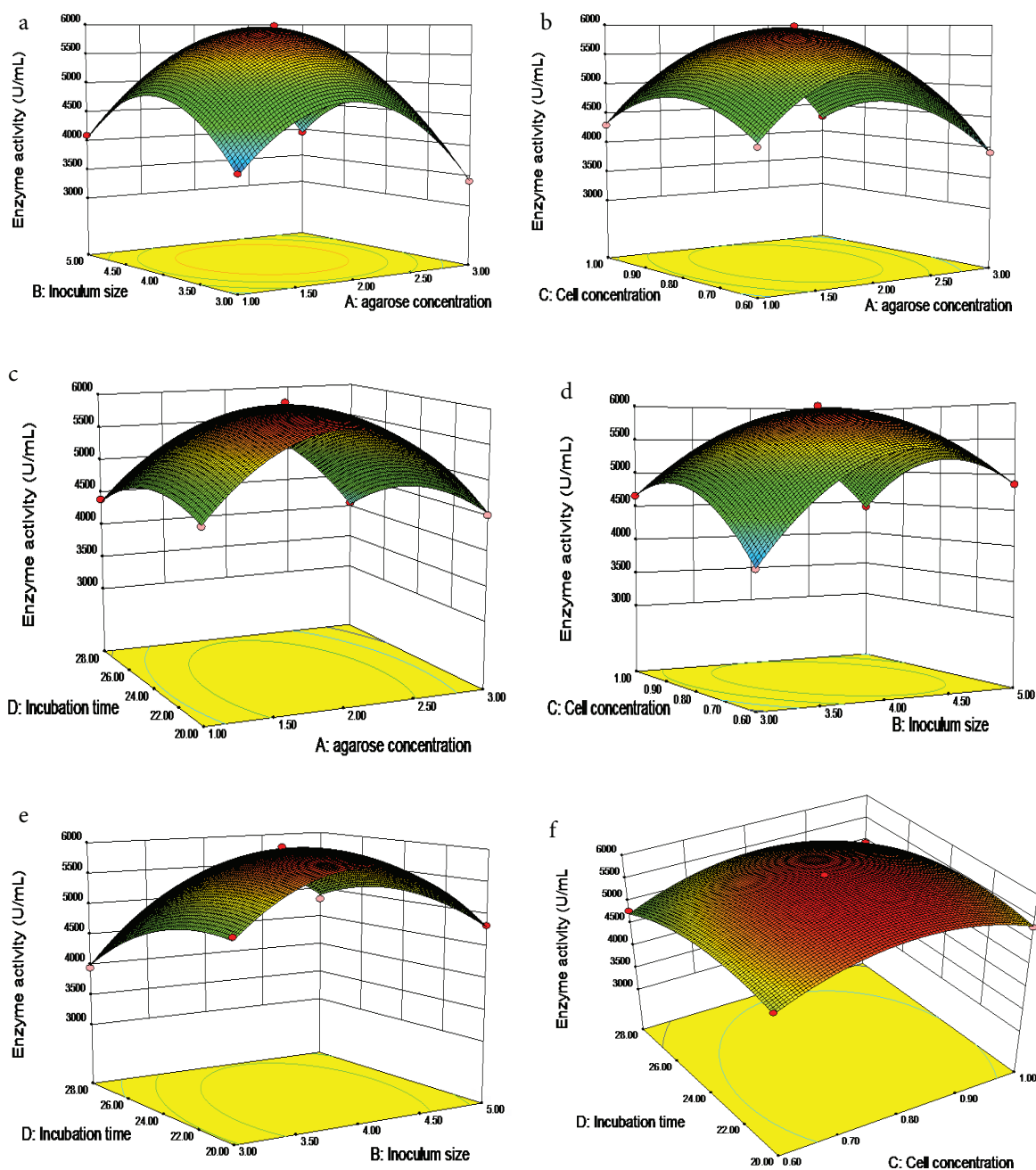


Figure 5. Response surface plots for alkaline lipase production by immobilized cells of *P. aeruginosa*. Each subfigure illustrates the interaction of 2 independent variables while others were kept at their respective center points. a) Agarose concentration and inoculum size, b) agarose concentration and cell concentration, c) agarose concentration and incubation time, d) inoculum size and cell concentration, e) inoculum size and incubation time, f) cell concentration and incubation time.

(3305.6 U/mL). This high activity after 9 cycles is another valuable feature of this mutant strain of *P. aeruginosa*, which could be imperative from an economic point of view at industrial-level production. These results were in agreement with those of Yang et al. (37), who reported 9

repeated batches with polyurethane-entrapped cells by *Rhizopus arrhizus* for lipase production. Rao et al. (38) and Anwar et al. (39) reported 11 and 4 repeated batch cycles, respectively, for protease production with alginate-entrapped cells.

Table 5. Validation of the response surface model.

S. no.	A: Agarose concentration (%)	B: Inoculum size (g)	C: Cell concentration (g)	D: Incubation time (h)	Observed response (U/mL)	Predicted response (U/mL)
1*	1.96	4.06	0.81	22.54	6354.23	5994.56
2	2.00	4.05	0.81	23.0	5845.62	5992.24
3	2.50	3.50	0.80	24.0	5148.94	5244.71
4	1.80	4.00	1.00	24.0	5512.71	5446.69
5	2.00	4.50	0.75	23.0	5678.43	5795.02
6	1.90	4.25	0.90	22.0	5793.96	5787.38
7	2.25	4.75	0.80	22.0	5398.87	5400.66
8	2.00	4.00	0.85	20.0	5767.32	5809.24

*Optimum values predicted by the design.

The economics of the immobilization process depends on the lifespan of the biocatalyst immobilized (40). In this study, the immobilized cells were stored at 4 °C and lipase production was found to be constant even after 6 months of storage (data not shown).

3.7. Optimization of airflow and agitation rates for lipase production by *P. aeruginosa* immobilized cells at bench-scale bioreactor level

Experiments were conducted to analyze the effect of airflow and agitation rates on lipase production in a lab-scale bioreactor to find the optimum combination of airflow and agitation levels. Three different airflow rates (0.3, 0.6, and 0.9 vvm) were investigated and at each airflow rate, 3 agitation rates (50, 100, and 150 rpm) were also tested. Figure 7 portrays the lipase production in the stirred tank bioreactor under each experimental condition. It is evident from the figures that each batch of the fermentation has

resulted in a different level of lipase production, indicating variable effects of airflow and agitation rates on lipase yield. The agitation rate of 100 rpm along with an airflow rate of 0.6 vvm resulted in maximum lipase production (6815.3 U/mL) within only 20 h of fermentation (Figure 7b). This experimental setup not only resulted in maximum enzyme yield but also reduced incubation time by about 3 h, as the enzyme production was optimum at 22.54 h under flask-level optimized conditions using RSM. However, at the same level of agitation (100 rpm), increased airflow rate of 0.9 vvm resulted in only 5926.1 U/mL of lipase production after 24 h of incubation (Figure 7c). The lower enzyme activity at the higher aeration rate may be due to the oxygen toxicity. Although suitable oxygen availability is vital for bacterial growth and enzyme production under aerobic fermentation, a higher aeration rate may adversely affect the enzyme production. Foam formation also affects the enzyme yield by causing denaturation of produced proteins at higher aeration rates. Similarly, at 0.3 vvm aeration and 100 rpm agitation, enzyme production was only 5526.3 U/mL after 24 h of incubation (Figure 7a). Less enzyme yield at lower airflow rate could be due to insufficient supply of oxygen to the cells.

With regard to the lipase yield at 150 rpm, it was observed that at every airflow rate the lipase production was decreased and agarose beads were damaged. The breakage of beads caused release of immobilized cells into the medium, which resembled the free-cell fermentation system; as a consequence, less enzyme yield was obtained. In another study on lipase production by *Rhizopus delemar* in batch-baffled stirred reactor, Açikel et al. (41) found 200 rpm as the optimum for maximum lipase yield.

Under aerobic fermentation, aeration is an indispensable factor for microbial growth and production of certain metabolites of scientific and industrial interest (42). The solubility of oxygen decreases with the increase in temperature and concentration of solutes in the fermenting medium. The oxygen supply to the microorganisms must

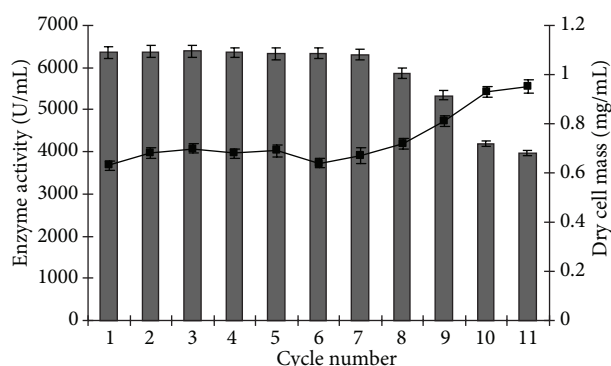


Figure 6. Repeated-batch production of alkaline lipase by mutant *P. aeruginosa* cells immobilized in agarose. Immobilized cells in agarose beads were repeatedly used for lipase production under fully optimized conditions and were transferred to fresh production medium at every 24 h for up to 11 cycles. (■) – lipase production (U/mL), (—■—) – cell leakage (in the form of dry cell mass). Bars presented are mean values \pm standard deviations of triplicates of 3 independent experiments.

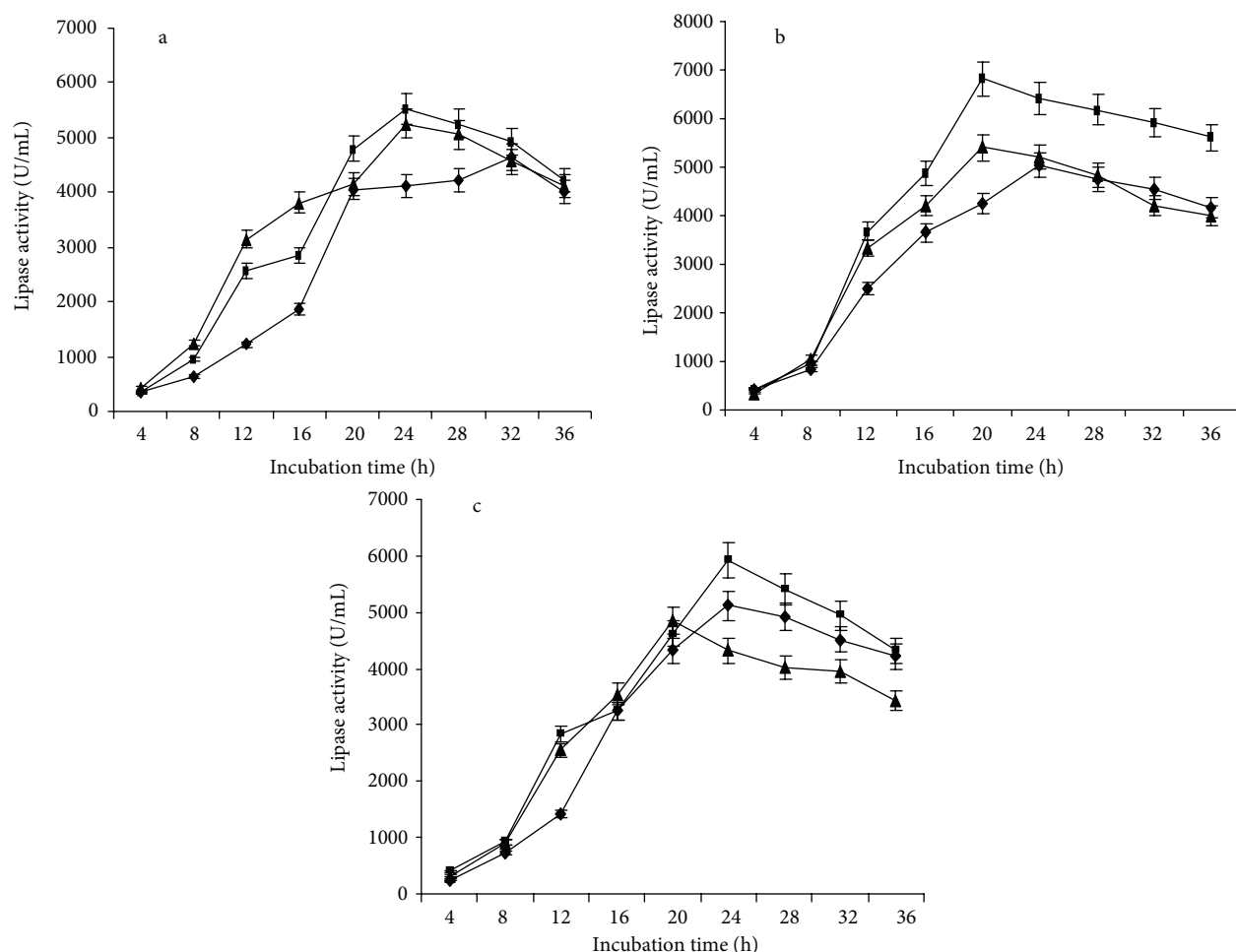


Figure 7. Production of alkaline lipase by immobilized cells in stirred tank bioreactor under different airflow and agitation rates. a) (—◆—) – 0.3 vvm and 50 rpm, (—■—) – 0.3 vvm and 100 rpm, (—▲—) – 0.3 vvm and 150 rpm; b) (—◆—) – 0.6 vvm and 50 rpm, (—■—) – 0.6 vvm and 100 rpm, (—▲—) – 0.6 vvm and 150 rpm; c) (—◆—) – 0.9 vvm and 50 rpm, (—■—) – 0.9 vvm and 100 rpm, (—▲—) – 0.9 vvm and 150 rpm. Bars presented are mean values ± standard deviations of triplicates of 3 independent experiments.

be regulated accordingly for successful execution of aerobic fermentation processes. Optimization of the aeration rate at a fixed agitation speed of 100 rpm indicated that oxygen supply to immobilized cells was a crucial parameter for production of lipase. Our finding showed that optimization of aeration speed at a constant agitation rate was necessary to get an appropriate balance of agitation and aeration. Increased agitation/aeration leads to mechanical damage of bacterial cells, while at lower agitation/aeration rates, the oxygen supply gets reduced, and both conditions affect the enzyme yield adversely.

3.8. Conclusion

Based on the above results, it can be concluded that agarose matrix was better than alginate and polyacrylamide for cell immobilization with the aim of substantial alkaline lipase production. Agarose has various desirable features over other gels. For example, it does not require polymerization

like polyacrylamide does, and it does not require any cations, since they are not utilized in its gelation. The presence of cations used for formation of other types of gels (alginate) influences cell protein and cell metabolic function and therefore is undesirable. Optimization of immobilization process by one-variable-at-a-time and RSM approaches resulted in lipase production of 6354.3 U/mL within 22.54 h of fermentation time. The cells entrapped in the agarose matrix could be reused effectively 7 times without any significant loss in enzyme yield for each constitutive use. Scale-up at bench-scale bioreactor level and optimization of aeration and agitation rates further enhanced the lipase production by about 7%, with maximum yield of 6815.3 U/mL within 20 h. This is presumably the first report on lipase production by immobilized *P. aeruginosa* cells at the bioreactor level. The significantly high level of lipase production by these agarose-entrapped cells warrants

their candidature for alkaline lipase production at a pilot scale for industrial applications.

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