OPTIMIZATION OF LIPASE IMMOBILIZATION

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Abstract *Pseudomonas aeruginosa* BBRC-10036 was used for lipase production. The organism secreted the enzyme extracellulary. In order to purify the enzyme, precipitation was carried out first, and then this lipase was purified by Ion Exchange Chromatography leading to 2.3-fold purification and 11.47% recovery. Lipase from *P.aeruginosa* was entrapped into Ca-alginate gel beads and effect of independent variables such as alginate concentration (%w/v), $CaCl_2$ concentration (M) and enzyme load (%v/v) on immobilization yield and activity of immobilized enzyme were investigated. Media optimization for immobilization of lipase was carried out by Response Surface Methodology. The optimum conditions were: sodium alginate concentration 2.5% (w/v), calcium chloride concentration 2.5 (M) and enzyme load 50% (v/v). Under these conditions, the highest immobilization yield and the optimum activity of immobilized enzyme obtained were 93.65% and 2.64 unit/g (IME), respectively.

Keywords *Pseudomonas aeruginosa*; Lipase; Production; Purification; Immobilization; Calcium Alginate.

چکیده در این پروژه، سودوموناس آئروژینوزا BBRC-10036 برای تولید لیپاز مورد استفاده قرار گرفت. این سویه‌ی میکروبی لیپاز خارج سلولی را تولید می‌نماید. روش‌های متفاوتی جهت خالص سازی وجود دارد که در این تحقیق ابتدا از روش راسب سازی استفاده شد تا شرایط بهینه‌ی ترسیب مشخص شود و سپس با استفاده از کروماتوگرافی تبادلگر یونی، خالص سازی انجام شد. درصد بازیافت آنزیم پس از استفاده از این ستون تبادل‌گر یونی، ۱۱/٤۷٪ به دست آمد و آنزیم به میزان ۲/۳ برابر خالص شد. سپس لیپاز به دست آمده از سودوموناس آئروژینوزا در دانه‌های آلژینات کلسیم تثبیت شد و تأثیر پارامترهای مستقل مانند غلظت آلژینات سدیم، کلرید کلسیم و آنزیم بر روی درصد تثبیت و میزان فعالیت آنزیم تثبیت شده، بررسی شد. بهینه‌سازی شرایط برای تثبیت لیپاز توسط روش پاسخ سطح انجام شد. با توجه به نتایج، شرایط بهینه‌ی تثبیت در ۲/۵ درصد وزنی آلژینات سدیم ، کلرید کلسیم ۲/۵ مولار و ۵۰ درصد حجمی آنزیم، اتفاق افتاد. که تحت شرایط مذکور، مقدار بهینه برای درصد تثبیت ۹۳/٦۵٪ و برای میزان فعالیت باقیمانده ۲/٦٤ واحد بر گرم آنزیم تثبیت-شده به دست آمد.

1. INTRODUCTION

Lipases constitute one of the most important groups of industrial enzymes. This is due to their unique ability to hydrolyze fatty acid ester bonds in aqueous environments and synthesize them in non-aqueous medium. These find diverse applications in fats and oil hydrolysis, food industry, detergent industry, peptide synthesis and pharmaceutical industries [1]. The lipases used are usually of fungal or bacterial origin; *Pseudomonas* being the most important bacterial genus[3,4]. Amongst 3 recognized groups of *Pseudomonas* lipases, subgroup 1 with its prototype, the 29 kDa *P.aeruginosa* lipases, attracts industrial attention since the enzyme has broad substrate specificity, accepting triglycerides substrates with fatty acyl chain length varying from C_6-C_{18} stereo selectively for the *sn*-1 position of the substrate trioctanoin [2]. Lipases are further categorized based on their preference for acyl group positions in triglycerides viz.1, 3-specific, 2-specific, and nonspecific or random. It is evident that *P. aeruginosa* lipase cleaved not only the 1,3- positioned ester bonds but also the 2-positioned ester bond leading to the formation of a mixture of products. This indicates that *P. aeruginosa* lipase possesses random specificity. This kind of random positional

specificity by *P. aeruginosa* lipase has been reported in the literature [1]. *P. aeruginosa* strain produces extracellular enzyme so the reason why this strain was selected relates to extracellular enzyme production. Many other strains produce intracellular enzyme.

Immobilization of enzymes can offer several advantages including its reuse, ease in application of both batch and continuous systems, possibility of better control reactions, ease in removal from the reaction medium and improved stability [3, 4]. Entrapment, one of the immobilization techniques, can be defined as physical restriction of enzyme within a polycationic polymer by the addition of multi-valent counter-ions is a simple and common method of enzyme entrapment. Alginates are one of the most frequently used polymers due to their mild gelling properties ease of formulation, biocompatibility, and acceptability as food additive and as oral drug delivery systems and non-toxicity [5]. The optimization of the immobilization procedures are usually based on one-variable-at-a-time approach, which facilitate the interpretation of the results , but interactions between variables are not taken into consideration. Consequently, a false minimum or maximum may be attained, leading to the use of certain conditions in which the combination of the variables is not that which provides the best analytical response. Experimental design methods have been recently applied extensively to optimize the immobilization procedures. One of the most used multivariate tools is two-level factorial design (full or fractional). It is used to check the preliminary significance of the variables in the system under study. In this approach, the main effects of the variables and their interactions are estimated. This is one of the greatest advantages of multivariate optimization compared to univariate optimization. Another advantage is that the numbers of experiments are considerably reduced particularly in the case with many factors. Additionally, optimum values of factors can be determined with higher degree of reliability using central composite design (CCD) [6].

In this study, *Pseudomonas aeruginosa BBRC-10036* was used for lipase production. The organism secreted the enzyme extracellulary. These lipases are widely used in industry, especially for the production of chiral chemicals that serve as basic building blocks in the synthesis of pharmaceuticals, pesticides and insecticides [7, 8]. After production, this enzyme must be separated from culture and then the enzyme must be purified for use in analysis and industry.

In this study, lipase from *P.aeruginosa* was entrapped by drop-wise addition of an aqueous solution of sodium alginate and the biocatalyst to a hardening solution of a Ca^{2+} salt. The cation acts as a cross-linking agent towards the alginate biopolymer and the droplets precipitate as beads with the biocatalysts entrapped within the network. Then, the effect of independent variables such as alginate concentration (%w/v), $CaCl_2$ concentration (M) and enzyme load (%v/v) on immobilization yield and activity of immobilized enzyme were investigated [6, 9]. Media optimization for immobilization of lipase was carried out by Response Surface Methodology which is a collection of a polynomial equation to the experimental data, describes the behavior of a data set with the objective of making statistical previsions [1, 10].

2. MATERIALS AND METHODS

Sodium alginate, $CaCl_2$ anhydrous, nutrient agar, $(NH_4)_2HPO_4$, K_2HPO_4, HCl, $MgSO_4$. $7H_2O$, acetone, methanol, n-propanol were purchased from Merck. Polyvinyl alcohol was purchased from Nakalai Chemical. All the other chemicals used were of analytical grade.

2.1. Organism *P. aeruginosa* BBRC No.10036, procured from Institute of Microbial Technology, Russia.

2.2. Lipase Production from *P.aeruginosa* *Pseudomonas aeruginosa* was maintained at 4℃ on nutrient agar. Inoculum was prepared by transferring loopful of this stock culture to the nutrient medium containing (%): soya flour, 3; $MgSO_4.7H_2O$, 0.21; K_2HPO_4, 0.5; $(NH_4)_2HPO_4$, 3; pH 7.1. The cultivation was performed at 30℃ with shaking at 150 rpm for 20 h.

One hundred milliliters of optimized media in 500 ml Erlenmeyer flask was seeded with 5 ml of inoculums. The incubation was carried out at 150 rpm in shaker maintained at 30℃. After 48 h, cells were harvested by centrifugation at 6000 rpm at 6 ℃ for 15-20 min and enzyme activity was

assayed in the supernatant.

2.3. Lipase Activity Assay Lipase activity was estimated with olive oil emulsion following the procedure of Ota and Yamada [11]. One unit of activity is defined as the amount of enzyme, which release 1μmole fatty acid per min per ml under specified assay conditions.

$$\text{Lipase activity}= \frac{N\times V\times 1000}{t}$$

where $V=V_2-V_1$ is the volume of NaOH used against control flasks, V_2 the volume of NaOH used against experimental flasks, N the normality of NaOH and t is the reaction time [12].

2.4. Protein Determination Protein analysis of the different supernatants was determined by spectrophotometer at 540 nm. The protein different concentrations were derived from Bovine Serum Albumin standard curve [13, 14].

2.5. Purification of Lipase All purification steps were performed at -20°C.

2.5.1. Organic Solvent Precipitation Cold organic solvent (acetone, ethanol, methanol, n-propanol) was slowly added with stirring to the supernatant until its concentration reached 20% (v/v). The precipitate formed was settled for 1 h at -20°C, then it was moved by centrifugation at 3000 rpm for 10 min. Then, the supernatant additional organic solvent was added to bring the concentration to 40% (v/v). The mixture was centrifuged to remove the precipitate, while the resulting supernatant was further subjected to ethanol precipitation in a sequential manner (50, 60, 80 v/v). The lipase activity and protein content were determined.

2.5.2. Anion Exchange Chromatography The enzyme solution was loaded onto the column of Q Sepharose High Performance preequilibrated with 30 mM Tris-HCl buffer (pH 8.5). The proteins bound to the anion exchanger were eluted by increasing the concentration of NaCl in 30 mM Tris-HCl buffer. Eluted fractions were assayed for both lipase activity and proteins.

2.6. Lipase Immobilization into Calcium Alginate Gel Beads Four milliliters of lipase solution was mixed with 16 ml of sodium alginate solution (1-2.5%, w/v) and then the mixed solution was dripped into 20 ml of $CaCl_2$ solution. Calcium alginate beads formed by cross-linking. After 20 min of hardening time, Ca-alg beads were separated from $CaCl_2$ solution by vacuum filtration and washed on the filter two times with buffer (pH 7.1) [3,15].

2.7. Immobilized Lipase Activity Assay Immobilized lipase activity was also determined by the method proposed by Ota and Yamada [10]. The activity of immobilized enzyme [unit/gr(IME)] is calculated using the following formula:

$$U=\frac{N\times V\times 1000}{t\times m}$$

where m is the mass of immobilized enzyme.

2.8. Protein Assay The amount of protein content before and after immobilization was determined by the method of Lowry using BSA as the standard. The percent of immobilized protein was calculated from the relationship [16]:

% immobilization = (total amount of protein in supernatant before immobilization – total amount of protein after immobilization) / total amount of protein in supernatant before immobilization × 100.

3. RESULTS AND DISCUSSION

3.1. Partial Purification of Lipase Enzyme Different precipitation agents were used for the partial purification of lipase, namely acetone, ethanol, methanol, n- propanol. Organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media.

By using acetone for precipitation, maximum recovery (22.13) and purification factor (2.2) were observed at 50% (Table 1) and Ethanol precipitation data revealed that 60% was the best for total activity, specific activity and yield (Fig. 1).

3.2. Ion Exchange Chromatography The purification of lipase was carried out by a single-

step purification method using Anion Exchange Chromatography leading to 2.33-fold purification and 11.47% recovery (Table 3). The pattern of Anion Exchange Chromatography is shown in Fig. 2. Amongst the observed protein peaks, Peak (No. 43) was the best data that we used it for further uses.

TABLE 1. Partial purification of lipase using acetone

Purification step	Volume (ml)	Lipase activity (U ml^{-1})	Yield (U)	Protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification factor	Recovery %
Culture filtrate	25	11.7	293.7	0.454	25.8	1	100
%40	10	3.6	35.8	0.081	44.2	1.7	12.19
%50	10	6.5	65	0.112	58.1	2.2	22.13
%60	10	5.5	55	0.122	45.1	1.7	18.72
%80	10	2.8	28.3	0.084	33.7	1.3	9.63

TABLE 2. Partial purification of lipase using 4 organic solvent at 50%(v/v)

Purification step	Volume (ml)	Lipase activity (U ml^{-1})	Yield (U)	Protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification factor	Recovery %
Culture filtrate	25	11.7	293.7	0.454	25.8	1	100
%50 Acetone	10	6.5	65	0.112	58	2.2	22.13
%50 Methanol	10	3.3	33.3	0.102	32.6	1.2	11.45
%50 Ethanol	10	3.2	32.5	0.078	41.7	1.6	11.06
%50 n-propanol	10	2.7	26.7	0.071	37.6	1.4	9.09

TABLE 3. Partial purification of lipase using Q sepharose high performance anion exchange

Purification step	Volume (ml)	Lipase activity (U ml^{-1})	Yield (U)	Protein (mg ml^{-1})	Specific activity (U mg^{-1})	Purification factor	Recovery %
Culture filtrate	25	11.7	293.7	0.454	25.8	1	100
50 % Acetone	15	7.8	117	0.122	63.9	2.1	35.32
Anion exchange chromatography	8	4.8	38	0.065	73.1	2.3	11.47

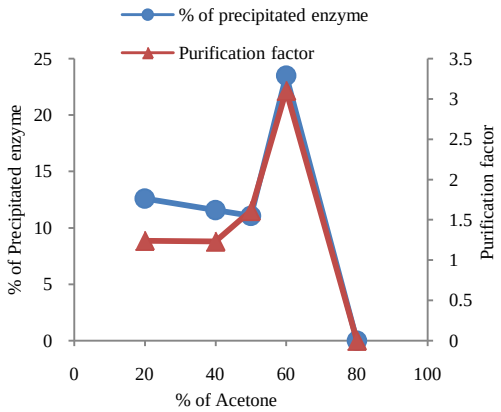

Figure 1. Partial purification of lipase using ethanol

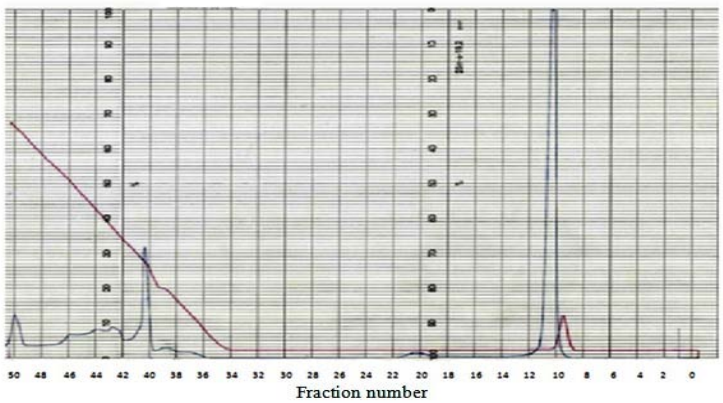

Figure 2.The chromatogram of lipase from *P.aeruginosa*

3.3. Optimization of the Immobilization Conditions In this study, the central composite design was used [17, 18]. It allows the determination of both linear and quadratic models. In general, a CCD for k factors, coded as (x_1,. . ., x_k), consists of three parts: a factorial (or cubic) design containing a total of $n_{fact} = 2^k$ points with coordinates $x_i = -1$ or $x_i = +1$, for $i = 1,. . . , k$; an axial (or star) part, formed by $n_{ax} = 2k$ points with all their coordinates null, except for the one that is set equal to a certain value α (or $-\alpha$) which usually ranges from 1 to $\sqrt{k}$. Finally, a total of n_c runs are performed at the center point of the experimental region, where, of course, $x_1 = x_2 = ... = x_k = 0$.

In this study, a rotatable CCD with $\alpha = 1.683$ was used for optimization of the effective parameters on lipase immobilization. Based on the preliminary studies, 3 factors including alginate concentration (%w/v), $CaCl_2$ concentration (M) and enzyme load (%v/v) were considered. Tables 4 and 5 show the main factors and levels and the design layout, respectively.

TABLE 4. Factors and their levels for central composite design (CCD)

Factor	Units	Low level (-1)	Medium level (0)	High level (+1)
Na-alg concentration	% (w/v)	1	1.75	2.5
$CaCl_2$ concentration	M	1	1.75	2.5
Enzyme concentration	% (v/v)	30	40	50

3.3.1. Effect of Factors on Immobilization Yield ANOVA table was used to verify the quality of model fit to the data, so we can choose the suitable response surface model to represent the data.

The central idea of ANOVA is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses. A quadratic response surface model based on a higher F- and R-value and lower lack of fit (LOF) to fit the experimental data was selected (Table 6). Table 7 shows the ANOVA table of the quadratic response surface model.

TABLE 5. Design layout and the responses for central composite design (CCD)

	Na-alg concentration W/V %	$CaCl_2$ concentration M	Enzyme concentration V/V %	Response 1 mobilization%	Response 2 Activity of immobilized enzyme Unit/g (IME)
1	1	1	30	87.98	0.58
2	2.5	1	30	91.48	1.84
3	1	2.5	30	88.94	1.57
4	2.5	2.5	30	92.89	2.53
5	1	1	50	88.79	1.05
6	2.5	1	50	90.54	2.3
7	1	2.5	50	90.14	1.68
8	2.5	2.5	50	94.46	3.01
9	1.75	1.75	40	91.1	1.67
10	1.75	1.75	40	92.34	1.48
11	1.75	1.75	40	90.78	1.63
12	1.75	1.75	40	91.32	1.52
13	0.49	1.75	40	84.8	0.51
14	3.01	1.75	40	89.32	2.07
15	1.75	0.49	40	87.64	0.75
16	1.75	3.01	40	90.55	1.78
17	1.75	1.75	23.18	91.4	0.59
18	1.75	1.75	56.82	94	1.69
19	1.75	1.75	40	90.2	1.58
20	1.75	1.75	40	89.39	1.23

The F-value of 23.40 implies that the model is significant. Value of "Prob>F" less than 0.05 for a factor indicates that its effect is significant. Non-significant lack of fit shows the model is significant. On the other hand, the absolute error is very low.

For the graphical interpretation of the interactions, the use of three-dimensional plots of the model is highly recommended.

This is useful to visualize the relationship between the responses and the experimental levels of each factor. The response model is mapped against two experimental factors while two other factors are held constant at their central levels.

Figs. 3, 4 and 5 are the response surfaces showing the effect of factors on the immobilization yield. It allows us to generate predicted responses for any set of factors. Fig. 3 clearly shows the positive effects of parameters on the response, interaction between Na-alg and $CaCl_2$ concentration and presence of curvature in the model. It can be seen that when Na-alg concentration (A) or $CaCl_2$ concentration (B) was increased, the peak area of target peaks increased as well, and because of the interaction between A and B, no linear relation existed between the response and factors. It can be seen that the optimum value are far from the center of the design. Enzyme concentration had little effect on immobilization yield (Figs. 4 and 5).

Fitting the data to various models (linear, two factorial, quadratic and cubic) and their subsequent ANOVA showed that linear model was able to explain the effects of factors on activity of immobilized enzyme.

Table 8 shows the analysis of variance of the linear response surface model. The computed model F-value of 63.73 implies that the model is significant and there is only 0.01% chance that a "model F-value" could occur that is due to noise effect. The lack of fit is not significant relative to the pure error. With very small "model P- value" (< 0.0001) and large "lack of fit P- value" (0.2973) from the analysis of ANOVA and a suitable coefficient of determination (R^2=0.9272), the linear model was significant to represent the actual relationship between the response and the significant variables.

The optimum level of each variable and the effect of factors on activity of immobilized enzyme as a function of two variables were studied by plotting three dimensional response surface curves (by keeping the other variable at its central level).

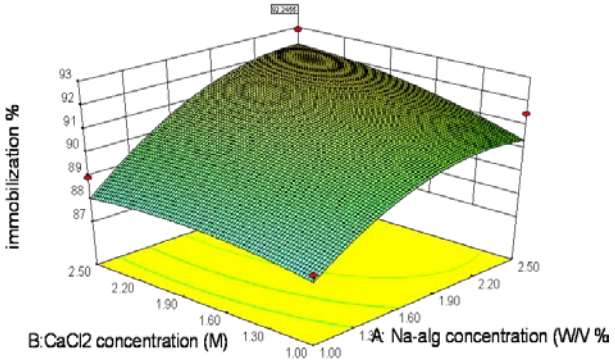

Figure 3. Three-dimensional (3D) response surface for Na-alg concentration (A) -$CaCl_2$ concentration (B)

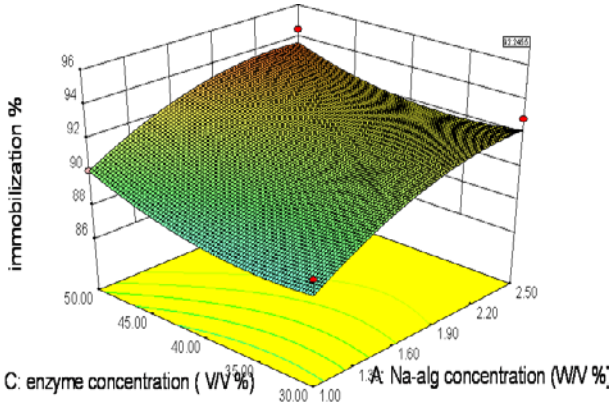

Figure 4. Three-dimensional (3D) response surface for Na-alg concentration (A) - enzyme concentration (C)

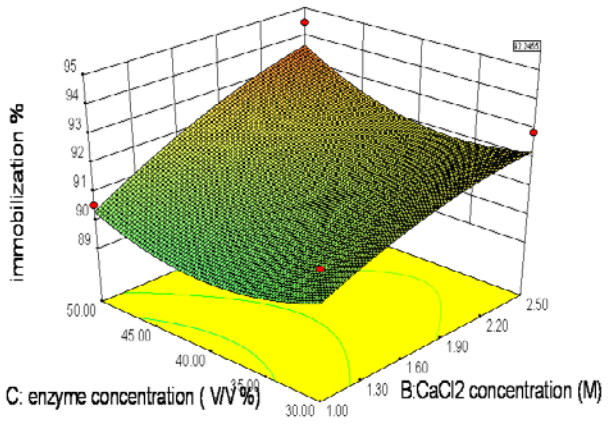

Figure 5. Three-dimensional (3D) response surface for CaCl2 concentration (B) - enzyme concentration (C)

TABLE 6. Sequential Model Sum of Squares

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	1.635E+005	1	1.635E+005			
Block vs Mean	7.31	1	7.31			
Linear vs Block	47.77	3	15.92	5.99	0.0068	
2FI vs Linear	2.43	3	0.81	0.26	0.8531	
Quadratic vs 2FI	33.84	3	11.28	28.27	< 0.0001	Suggested
Cubic vs Quadratic	1.64	4	0.41	1.05	0.4679	Aliased
Residual	1.96	5	0.39			
Total	1.635E+005	20	8177.45			

TABLE 7. Analysis of variance (ANOVA) table of the quadratic response surface model

Source	Sum of squares	DF	Mean square	F Value	Prob>F	
Model	84.05	9	9.34	23.40	<0.0001	Significant
A-Na-alg concentration	32.67	1	32.67	81.86	<0.0001	
B-$CaCl_2$ concentration	11.50	1	11.50	28.83	0.0005	
C-enzyme concentration	3.60	1	3.60	9.02	0.0149	
AB	1.14	1	1.14	2.86	0.0231	
AC	0.24	1	0.24	0.6	0.0523	
BC	1.05	1	1.05	2.63	0.0339	
A^2	17.05	1	17.05	42.72	0.0001	
B^2	1.96	1	1.96	4.90	0.0541	
C^2	11.82	1	11.82	29.61	0.0004	
Residual	3.59	9	0.4			
Lack of fit	1.90	5	0.38	0.9	0.5567	not significant
Pure Error	1.69	4	0.42			
Core Total	94.95	19				

TABLE 8. Analysis of variance (ANOVA) table of the linear response surface model

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F	
Model	6.52	3	2.17	63.73	<0.0001	
A-Na-alg concentration	4.04	1	4.04	118.31	<0.0001	
B-$CaCl_2$ concentration	1.65	1	1.65	48.48	<0.0001	
C-enzyme concentration	0.83	1	0.83	24.38	0.0002	
Residual	0.51	15	0.034			
Lack of fit	0.43	11	0.039	1.82	0.2973	Not significant
Pure Error	0.085	4	0.021			
Core Total	8.06	19				

TABLE 9. Optimum response and the corresponding levels

Factor	Name	Level	Low Level	High Level	Std.Dev.	Coding	
A	Na-alg conc.	2.50	1.00	2.50	0.000	Actual	
B	$CaCl_2$ conc	2.50	1.00	2.50	0.000	Actual	
C	Enzyme conc	50.00	30.00	50.00	0.000	Actual	
Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
immobilization	93.6525	0.52	92.48	94.82	0.82	91.81	95.50
activity	2.64498	0.096	2.44	2.85	0.21	2.20	3.09

Figs. 6, 7 and 8 reveal that Na-alg and $CaCl_2$ concentration had significant effects on activity of immobilized enzyme and Na-alg concentration is one of the most important parameters for immobilization of lipase.

3.3.3. Response prediction at the optimum The optimal conditions for lipase immobilization were predicted as presented in Table 9. The main objective of the optimization is to determine the optimum value of effective factors of immobilization from the model obtained using experimental data. Optimization was carried out using the Design-Expert 8.0, trial version, software.

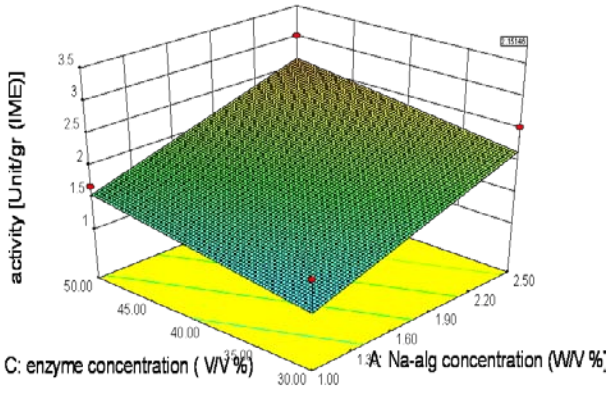

Figure 6. Three-dimensional (3D) response surface for Na-alg concentration (A) -$CaCl_2$ concentration (B)

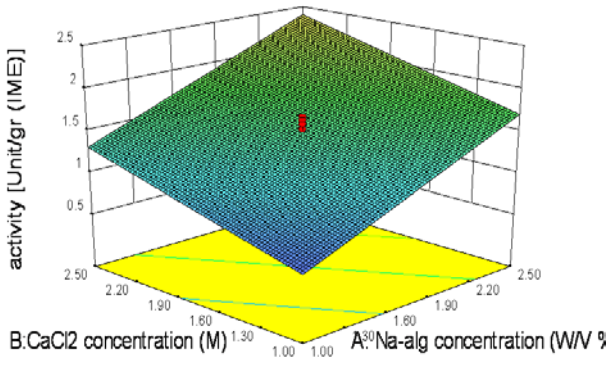

Figure 7. Three-dimensional (3D) response surface for Na-alg concentration (A) -enzyme concentration (C)

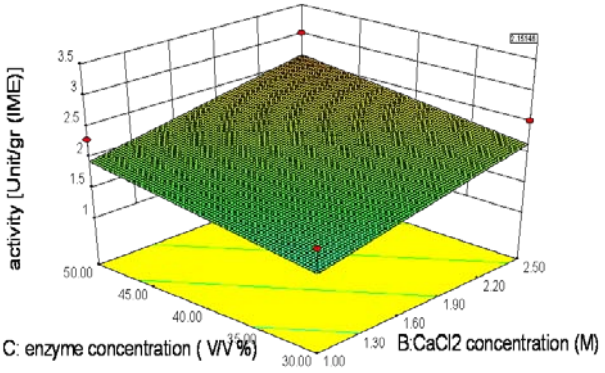

Figure 8. Three-dimensional (3D) response surface for CaCl2 concentration (B) - enzyme concentration (C)4.

4. CONCLUSION

The main aim of the present study was optimization of lipase immobilization. Effects of lipase entrapment conditions on immobilization yield and activity of immobilized enzyme were investigated. Media optimization for immobilization of lipase was carried out by Response Surface Methodology.

This optimization technique includes the interactive effects among the variables studied and decrease the number of experiments necessary to conduct the research, which leads to decrease of time and expenses as well as decrease in the consumption of reagents and materials.

The optimum conditions were as follows: sodium alginate concentration 2.5% (w/v), calcium chloride concentration 2.5 (M) and enzyme load 50% (v/v). Under these conditions, the highest immobilization yield and the optimum activity of immobilized enzyme, were 93.65% and 2.64 unit/g (IME), respectively. Results showed that Na-alg and $CaCl_2$ concentration had significant effects on responses and Na- alg concentration is one of the most important parameters for lipase entrapment. Enzyme concentration had little effect on responses.

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