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ORIGINAL PAPER

N. Saisubramanian · N. G. Edwinoliver
N. Nandakumar · N. R. Kamini · R. Puvanakrishnan

Efficacy of lipase from *Aspergillus niger* as an additive in detergent formulations: a statistical approach

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Abstract The efficacy of lipase from *Aspergillus niger* MTCC 2594 as an additive in laundry detergent formulations was assessed using response surface methodology (RSM). A five-level four-factorial central composite design was chosen to explain the washing protocol with four critical factors, viz. detergent concentration, lipase concentration, buffer pH and washing temperature. The model suggested that all the factors chosen had a significant impact on oil removal and the optimal conditions for the removal of olive oil from cotton fabric were 1.0% detergent, 75 U of lipase, buffer pH of 9.5 and washing temperature of 25°C. Under optimal conditions, the removal of olive oil from cotton fabric was 33 and 17.1% at 25 and 49°C, respectively, in the presence of lipase over treatment with detergent alone. Hence, lipase from *A. niger* could be effectively used as an additive in detergent formulation for the removal of triglyceride soil both in cold and warm wash conditions.

Keywords Additive · *Aspergillus niger* · Detergent · Lipase · Oil removal

Introduction

Detergent enzymes account for about 32% of the total world-wide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology [20]. The use of enzyme-based detergents is preferred over the conventional synthetic ones due to their better cleaning properties, lowering of washing temperatures and reduction in pollution [13]. Lipases improve the washing capacity of detergents as well as removal of fatty food stains and sebum from fabrics, which are difficult to remove under normal washing conditions [1, 9]. In recent years, research on lipases, especially of microbial origin, has increased due to their great commercial potential [22]. However, their major application in terms of quantity is as an additive for laundry detergents [13].

Response surface methodology (RSM) is a collection of mathematical and statistical techniques widely used to optimize different biotechnological processes [15, 23]. Factorial design of a limited set of variables is advantageous compared to the conventional method, which handles single parameter per trial and as an approach; this frequently fails to consider the effect of possible interactions between factors [10]. Reports are available on RSM for the production of lipase from various microbes including *A. niger* [3, 8]. Similarly, application of lipase using RSM has been reported for esterification [7], transesterification [18], interesterification [5] and biodiesel production [21]. Though there have been reports on application of lipases as an additive in detergent formulations using conventional methods [6,12], there is no report on the use of lipase in detergent formulations and optimization of washing conditions using RSM. In this paper, an attempt was made to use central composite rotatable design (CCRD), a tool of RSM, for optimizing the conditions for removal of oil from the soiled fabric using lipase from *Aspergillus niger* MTCC 2594 as an additive in laundry detergents.

N. Saisubramanian · N. G. Edwinoliver · N. Nandakumar
N. R. Kamini · R. Puvanakrishnan (✉)
Department of Biotechnology,
Central Leather Research Institute,
600 020, Adyar, Chennai, India
E-mail: puvanakrishnan@yahoo.com
Tel.: +91-44-24430273
Fax: +91-44-24911589

Materials and methods

Enzyme

Lipase used in this study was obtained from *A. niger* MTCC 2594, a laboratory isolate and it was maintained on Czapek Dox agar slants at 4°C. Lipase production was carried out in the optimized production medium using submerged culture fermentation by growing the fungus for 48 h at 30°C [11] and the crude enzyme was recovered by filtration and used in the present study. Lipase activity was assayed by the method of Yamada et al. [25]. One unit of activity is defined as the amount of enzyme releasing 1 μmol of free fatty acid in 1 min at 37°C and pH 7.0. The crude enzyme with an activity of 35 U/ml was used.

Detergents

Detergents used were selected from the laboratory or commercially available products viz. anionic detergent—sodium dodecyl sulphate (SDS), nonionic detergent—Tween 80 and commercial detergents such as Ariel, Tide (Procter and Gamble Home Products Ltd.), Henko (Henkel Spic India Ltd.), Surf Excel and Surf Ultra (Hindustan Lever Ltd.). All other reagents used were of analytical grade.

Effect of detergents on lipase activity and stability

To investigate the activity of lipase in detergents, different detergents were added to the reaction mixture at a concentration of 7 mg ml^{-1} and assayed under standard assay conditions and expressed as percent relative activity. To determine the stability, an aliquot of enzyme sample was incubated with equal volume of detergent solution (7 mg ml^{-1} of respective detergent) in 0.1 M sodium phosphate buffer pH 7.0 for 1 h at 30°C. The residual activity (%) of each sample was determined and compared with the control without detergent.

Preparation of soiled fabric and washing solution

The cotton fabric (5×10 cm^2) was defatted in boiling chloroform for 4 h and soiled by spotting with 0.5 ml of olive oil in benzene (100 mg ml^{-1} concentration) twice with a micropipette.

The washing solutions were prepared as shown in Table 1. Solution BDL contained buffer and the detergent solution, pre-incubated at 37°C for 10 min to which lipase solution (100 U) was then added. The volume of the final solution was adjusted to 100 ml by adding distilled water. Ten pieces of the soiled fabric were put into the flask containing the washing solutions.

Table 1 Composition of the washing solutions

Constituents	Volume (ml)			
	B	BL	BD	BDL
0.1 M Tris buffer (pH 8.5)	40	40	40	40
Detergent solution (1.0%)	–	–	50	50
Lipase solution (100 U)	–	10	–	10
Distilled water	60	50	10	–

B buffer, *BL* buffer + lipase, *BD* buffer + detergent, *BDL* buffer + detergent + lipase

Washing procedure

The soiled fabrics were washed for 20 min at 37°C with shaking at 100 rev min^{-1} using B/BL/BD/BDL. At the end of 20 min, the fabrics were removed and rinsed thrice with 100 ml of water, each for a period of 2 min and then air-dried.

Determination of olive oil

Olive oil was extracted from the fabrics using petroleum ether (B.P. 40–60°C) for 6 h in a soxhlet extractor. The petroleum ether was completely evaporated and the weight of olive oil was determined. The removal of olive oil was calculated by the following equation based on the weight of olive oil before and after washing.

$$\%\,\text{Oil removal} = \frac{W_b - W_a}{W_b} \times 100$$

where, W_b was the weight of olive oil before washing and W_a was the weight of olive oil after washing.

Factorial design

A five-level four-factorial CCRD was employed in this study with an α value of ±1.414 for four critical factors, viz. detergent concentration (*A*), lipase concentration (*B*), washing temperature (*C*) and buffer pH (*D*). The range and levels of variables investigated in this study were chosen based on preliminary experimentation. The relationship between the variation of the response, Yc (% oil removal) and the variation of factors *A*, *B*, *C* and *D*, was represented by a second-order mathematical model using the following equation:

$$\begin{aligned}\text{Yc} = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 \\ & \text{(Intercept and main effects)} \\ & + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 \\ & \text{(Interactions)} \\ & + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\ & + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \\ & \text{(Quadratic effects)}\end{aligned}$$

where, Yc was the response calculated by the model and X_1, X_2, X_3 and X_4 were the coded variables corresponding to factors *A*, *B*, *C* and *D*, respectively. Coding was required, since the factors were expressed in different units and ß$_0$ represented the regression coefficient at the centre. ß$_1$, ß$_{11}$ and ß$_{12}$ were coefficients estimated by the model, which represented the linear quadratic and interactive effects of X_1, X_2, X_3 and X_4 factors on the response respectively. The treatment combinations of CCRD were allocated in three blocks and each block had ten runs. The first two blocks each had eight factorial points and two centre points. The last block had eight axial points and two centre points. Thus, in total, the experimental setup consisted of 30 trials and the value of the dependent response was the mean of two replications. The increase in percent removal of olive oil between BD and BDL was determined and the results were analysed by multiple regression analysis to calculate the equation coefficients. Based on second-order equation, three-dimensional response surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones. They were drawn imposing constant values (i.e. the centre points of the interval taken into consideration) to two of the independent variables of the CCRD. Statistical analysis of the model was performed using the 'Design Expert' software package (Version 6.0.11, Stat-Ease Inc., Minneapolis, USA).

Results and discussion

Lipases are important enzymes for the detergent industry in terms of degreasing potential, to remove oil and fat stains from fabrics. An ideal detergent enzyme should be thermo-tolerant at low- and high-temperature and also stable at alkaline pH. Accordingly, lipase from *A. niger* MTCC 2594 showed broad pH stability between pH 4.0 and 10.0 and temperature stability between 4 and 60°C. Hence an attempt was made to use this fungal lipase as an additive in detergent formulations.

Effect of detergents on lipase activity and stability

Lipase from *A. niger* was tested for its activity in presence of various detergents. The enzyme showed increased stability in presence of SDS, Tween 80 and all commercial detergents (Fig. 1) and similar results were reported for lipases from *Aspergillus* sp. and *Rhizopus* sp. [1]. Among various detergents, Tide, showed a maximum of 19% increase in activity, while SDS exhibited an increase of approximately 6% in activity over control. On the other hand, SDS inhibited the lipase activity of *Aspergillus carneus* [19] while it had positive effect on the *Humicola lanuginosa* lipase [17].

Effect of lipase on the removal of olive oil from cotton fabric

The enzyme exhibited a wide pH stability of 4.0–10.0 [12] and hence, a buffer pH of 8.5 (0.1 M Tris buffer) was selected as one of the constituents in the washing solution for the removal of olive oil from cotton fabric (Table 1). Olive oil was selected in this study because it is the standard substrate for lipase assay. Moreover, this enzyme effectively hydrolysed different vegetable oils including olive oil, coconut oil and sunflower oil (data not shown).

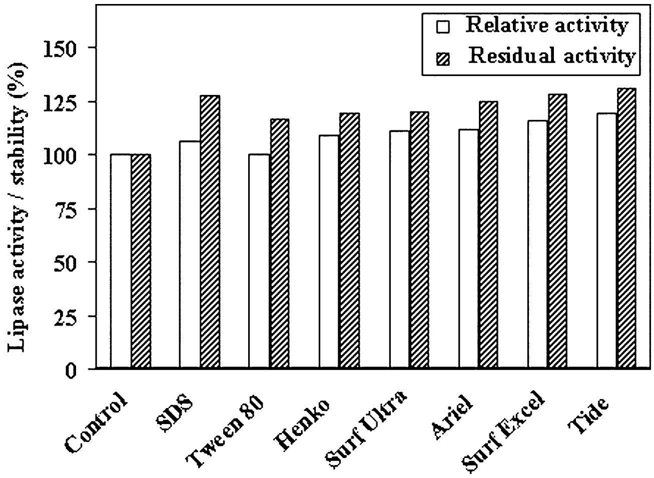

Fig. 1 Effect of different detergents on lipase activity and stability. For the control, enzyme was incubated with buffer devoid of detergents

Various detergents were first evaluated for their efficiency in removal of olive oil from cotton fabric with and without lipase. The percentage of oil removed from the fabric was higher (7–12%) in the presence of lipase with all detergents (Table 2). Similar results were reported for lipases from *Candida cylindracea* [6], *A. niger* [12] and *Ralstonia pickettii* [9].

Among the detergents, Tween 80 and Tide were more effective in removing the triglycerides from the fabric and the removal of olive oil with the addition of lipase was 9 and 11.9% higher respectively than without lipase (Table 2). However, only 10% of the oil was removed when washing was carried out in buffer solution and addition of lipase to the buffer solution increased the percent oil removal to 22.5% under the same conditions. Since the percent oil removal was high with Tide, a popular commercial detergent, it was selected for further optimization studies using RSM.

Table 2 Effect of lipase on removal of olive oil from cotton fabric with various detergents

Detergents	Percent oil removed	
	BD	BDL
SDS	58.0	65.0
Tween 80	62.0	71.0
Ariel	54.0	64.0
Henko	53.0	61.0
Surf Excel	57.0	68.0
Surf Ultra	55.0	62.0
Tide	60.3	72.2

BD buffer + detergent, *BDL* buffer + detergent + lipase

Factorial design

Based on the results of our earlier report [12], experiments were planned to obtain a quadratic model using four independent variables viz. detergent concentration (*A*), lipase concentration (*B*), pH of the buffer (*C*) and washing temperature (*D*) with the centre points obtained from preliminary studies (Table 3). The design matrix for CCRD and the results on increase in percentage oil removal using *A. niger* lipase were shown in Table 4.

The results of CCRD indicated that the percent increase in oil removal was from 3 to 33% when compared to BD wash alone. The varied nature of results indicated that the interactions among the factors played a more significant role than the effect of individual factors alone. This is exactly the significance of RSM since interactions between the factors can only be discerned through statistical design approach and not by conventional one factor at a time method [4].

The adequacy of the model and fitness were evaluated by ANOVA and regression coefficients. The ANOVA for the quadratic model was highly significant with an *F* value of 19.06 as shown by Fisher's *F* test, along with a very low probability value ($P_{\text{model}} > F = 0.0001$), which was significant at 95% confidence interval. At the same time, relatively lower value of coefficient of variation (CV = 16.36 %) indicated a better precision and reliability of the experiments carried out. The determination coefficient (R^2) of the model was 0.9181 indicating that 91.8% of variability in the response could be accounted by the model (Table 5) and it showed that the model was suitable to represent the real relationship among the selected factors. The insignificant lack of fit test also indicated that the model was suitable to navigate the design space. The final predictive equation was as follows:

$$\begin{aligned}\text{Percentage oil removal} = {} & 6.68 + 2.18(A) - 1.26(B) \\ & - 1.27(C) + 1.40(D) + 1.94(B^2) \\ & + 3.34(C^2) + 4.99(D^2) \\ & - 1.31(A)(B) - 3.34(A)(C) \\ & - 1.62(C)(D) \end{aligned} \tag{1}$$

From the model, it was clear that all the linear coefficients, three quadratic coefficients and three cross product coefficients were highly significant ($P < 0.05$, Table 6). Among the four factors, lipase concentration, washing temperature and buffer pH showed significant quadratic effects as shown by their respective probability values (Table 6) while detergent concentration contributed mainly to interaction effects.

A significant quadratic regression, insignificant lack of fit and a small total variation (8.2%), which was not explained by the model, suggested that the model accurately represented the data in the experimental region. This also indicated that the second order terms were sufficient and higher order terms were not essential [16]. The adequacy of the model was examined using additional independent experiments that were not employed in model generation. It was observed that the predicted values for percent oil removal were in good agreement with RSM plots.

The cumulative effect of detergent concentration and enzyme concentration on removal of olive oil from cotton fabric at 37°C and pH 8.5 was depicted in the response surface plot of Fig. 2. The percent oil removal was optimum, when the washing solution contained an enzyme concentration of 75 U and a detergent concentration of 1.0%. At higher detergent concentration, a decrease in lipase concentration from 125 to 75 U led to an increase in percent oil removal from 8 to 13%, whereas at lower detergent concentration, reduction in lipase concentration did not have a significant impact on percent oil removal. Changes in detergent concentration from 0.6 to 1.0% increased the percent oil removal significantly irrespective of the lipase concentration employed. An ideal detergent–enzyme should be effective at very low concentrations. Accordingly, our results also demonstrated that lipase from *A. niger* was effective in removing the olive oil from the fabric using low concentrations of enzyme. Similar results were observed with *C. cylindracea* lipase as reported by Fujii et al. [6]. However, an enzyme concentration of 100 U of lipase from *R. pickettii* was used for the removal of olive oil from soiled fabric [9].

Figure 3 illustrated the interactive effect of detergent concentration and temperature on removal of olive oil from soiled fabric using a lipase concentration of 100 U and a buffer pH of 8.5. The percent oil removal increased with increase in detergent concentration from 0.6 to 1.0% and decrease in temperature from 49 to

Table 3 Range of variables for the central composite design

Variable	Coded symbol	Coded levels				
		-α	−1	0	1.0	α
Detergent concentration (%, w/v)	X_1	0.52	0.6	0.8	1.0	1.08
Lipase (U)	X_2	64.65	75	100	125	135.35
Temperature (°C)	X_3	20.03	25	37	49	53.97
pH	X_4	7.09[a]	7.5[a]	8.5[b]	9.5[c]	9.91[c]

[a]0.1 M Phosphate buffer
[b]0.1 M Tris buffer
[c]0.1 M Glycine–NaOH buffer

Table 4 Composition of the various runs of the central composite design and the percentage oil removed using *A. niger* lipase

Run	Detergent (*A*) [%]	Lipase (*B*) [U]	Temperature (*C*) [°C]	pH (*D*)	Percent increase in oil removal	
					Observed	Predicted
1	0.6	75	25	9.5	16.4	17.5
2	1.0	75	25	7.5	25.0	25.1
3	0.6	125	25	7.5	12.0	11.6
4	1.0	125	25	9.5	27.0	26.0
5	0.6	75	49	7.5	18.0	18.8
6	1.0	75	49	9.5	17.1	18.7
7	0.6	125	49	9.5	20.0	18.5
8	1.0	125	49	7.5	15.2	14.0
9	0.8	100	37	8.5	9.0	8.5
10	0.8	100	37	8.5	8.0	8.5
11	0.6	75	25	9.5	12.0	9.3
12	1.0	75	25	9.5	33.0	29.0
13	0.6	125	25	9.5	15.7	15.4
14	1.0	125	25	7.5	16.1	17.8
15	0.6	75	49	9.5	15.9	16.2
16	1.0	75	49	7.5	16.0	17.0
17	0.6	125	49	7.5	13.8	16.8
18	1.0	125	49	7.5	11.0	11.4
19	0.8	100	37	8.5	6.5	6.3
20	0.8	100	37	8.5	6.0	6.3
21	0.52	100	37	8.5	3.0	2.0
22	1.08	100	37	8.5	8.0	8.2
23	0.8	64.7	37	8.5	9.8	10.7
24	0.8	135.4	37	8.5	8.0	7.2
25	0.8	100	20	8.5	10.0	13.5
26	0.8	100	54	8.5	13.4	10.0
27	0.8	100	37	7.09	15.0	13.1
28	0.8	100	37	9.91	15.0	17.0
29	0.8	100	37	8.5	4.0	5.1
30	0.8	100	37	8.5	6.0	5.1

Table 5 ANOVA for quadratic model for percent oil removal

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	$P > F$
Block	302.19	2	151.10		
Model	933.92	10	93.39	19.06	< 0.0001[a]
A	95.36	1	95.36	19.47	0.0004
B	31.62	1	31.62	6.45	0.0211
C	32.24	1	32.24	6.58	0.0201
D	39.20	1	39.20	8.00	0.0116
B^2	34.43	1	34.43	7.03	0.0168
C^2	102.03	1	102.03	20.82	0.0003
D^2	227.71	1	227.71	46.49	< 0.0001
AB	27.56	1	27.56	5.63	0.0298
AC	178.22	1	178.22	36.38	< 0.0001
CD	42.25	1	42.25	8.63	0.0092
Residual	83.28	17	4.90		
Lack of fit	80.65	14	5.76	6.58	0.0733 [b]
Pure error	2.63	3	0.88		
Total	1319.39	29			

$R^2 = 0.9181$; CV = 16.36%
[a]Significant
[b]Not significant

25°C. The variation in temperature caused a significant impact on percent oil removal at 1% detergent concentration and similarly, the change in detergent concentration had a prominent impact at 25°C. The optimal oil removal was obtained using 1.0% detergent at 25°C.

The cumulative effect of pH and temperature on removal of olive oil from cotton fabric at 0.8% detergent concentration and 100 U of lipase was shown in Fig. 4. The percent oil removal was optimum, when the washing was performed at pH 9.5 and 25°C. Decrease in

Table 6 Coefficients of quadratic model for percent oil removal

Coefficient	Value	Standard error	t Value	$P(P>\|t\|)$
β_o (Intercept)	6.68	0.82	8.14	
$\beta_1(A)$	2.18	0.49	4.44	$<$ 0.0001
$\beta_2(B)$	−1.25	0.49	−2.55	0.0004
$\beta_3(C)$	−1.26	0.49	−2.57	0.0211
$\beta_4(D)$	1.40	0.49	2.85	0.0201
$\beta_{22}(B^2)$	1.94	0.73	2.65	0.0116
$\beta_{33}(C^2)$	3.34	0.73	4.57	0.0168
$\beta_{44}(D^2)$	4.99	0.73	6.83	0.0003
$\beta_{12}(AB)$	−1.31	0.55	−2.38	$<$0.0001
$\beta_{13}(AC)$	−3.34	0.55	−6.07	0.0298
$\beta_{34}(CD)$	−1.62	0.55	−2.94	$<$0.0001

the variation of pH from a low level to a high level indicating that the quadratic term for buffer pH played an important role on oil removal. This was confirmed by the corresponding high "t" value as indicated in Table 6. The maximal increase in oil removal (33%) observed under the optimized conditions (Table 4) was not depicted in response surface plots. This could be due to the fact that response surface plots were drawn by imposing constant values (i.e. the central points of the interval taken into consideration) to two of the independent variables of the factorial design.

The design illustrated that the best conditions for the removal of olive oil from cotton fabric was 1.0%

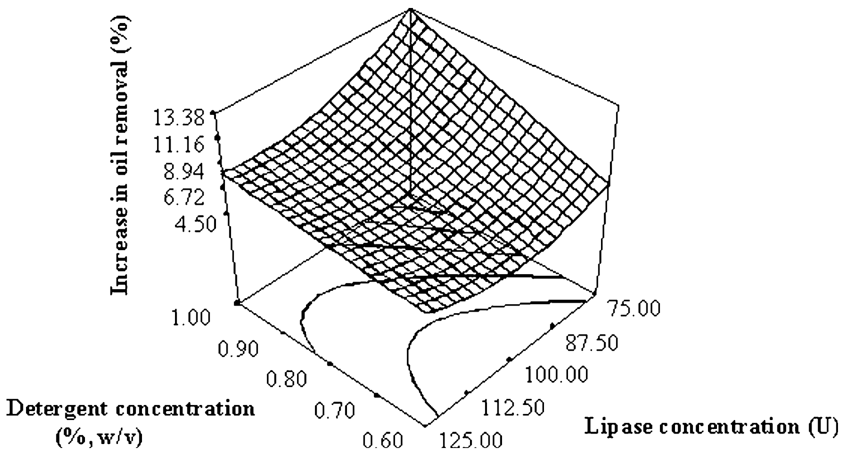

Fig. 2 Response surface plot showing the effect of detergent concentration and lipase concentration and their mutual effect on the removal of olive oil from cotton fabric using *A. niger* lipase

temperature from 49°C to 25°C caused a significant increase in percent oil removal at a buffer pH of 9.5. At lower pH, the variation in temperature did not cause much impact on the response. The oil removal was observed to be the lowest at the centre points of pH and temperature. A significant curvature was produced by detergent, 75 U of lipase, a buffer pH of 9.5 and washing temperature of 25°C. To validate the model, when washing was performed under these optimized conditions, the results showed an increase of 31 ± 2% increase in oil removal when compared to the wash by detergent alone. The predicted value for percent oil removal under

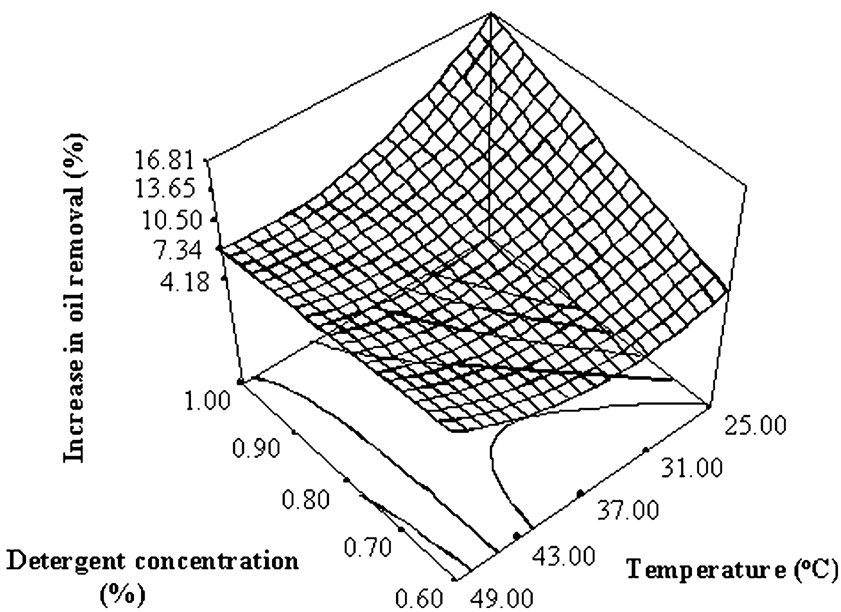

Fig. 3 Response surface plot showing the effect of detergent concentration and temperature and their mutual effect on the removal of olive oil from cotton fabric using *A. niger* lipase

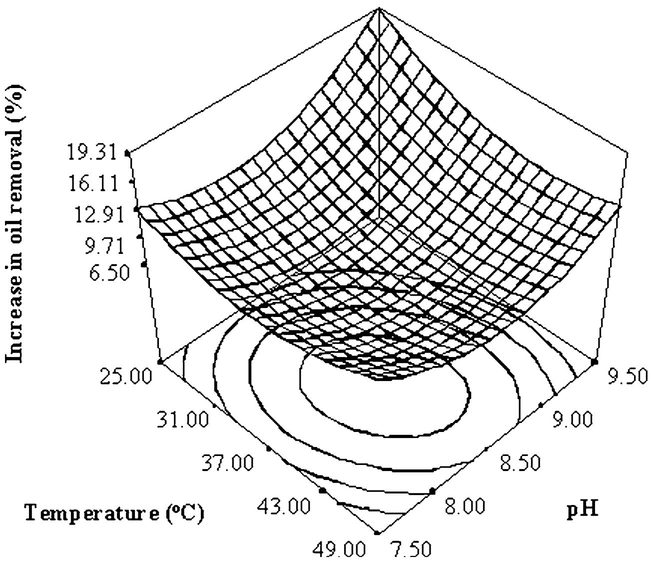

Fig. 4 Response surface plot showing the effect of temperature and pH and their mutual effect on the removal of olive oil from cotton fabric using *A. niger* lipase

these optimized conditions was 29.04%. The present model could be considered suitable, since the predicted value was in reasonable agreement with the observed value.

The importance of choosing enzymes as additives in laundry detergents results not only from their high efficacy, but also from reasons of environmental protection because phosphates, which are used in synthetic washing agents, are known to pollute waste-waters [24]. Another factor was that use of enzymes in detergent formulations allowed laundering at lower temperatures, thus reducing energy expenditure. Lipase from *A. niger* MTCC 2594 was an ideal candidate for use in laundry detergent formulations, since it possessed most of the desired properties. The enzyme showed considerable stability in presence of 1.0% hydrogen peroxide for a period of 45 min (data not shown). By the use of statistical design, the enzyme concentration required for maximum oil removal was reduced by 25% with *A. niger* lipase, while 100 U of lipase was used for *R. pickettii* lipase using conventional method [9]. Hence, the lipase from *A. niger* could be effectively used as an additive in laundry detergents in both cold wash and warm wash conditions.

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