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Statistical optimization of Factors influencing the activity and the kinetics of partially purified Lipase produced from *Aspergillus japonicus* (MTCC 1975) cultured in protein enriched medium

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Abstract: Extracellular lipase produced by *Aspergillus japonicus* was tested for its activity on sesame, groundnut and sunflower oil substrates. The enzyme showed significant activity in the pH range of 6-8 and a temperature range of 30^0C-40^0C but with sunflower oil, the activity is optimum at 50^0C. This is indicative of the substrate playing a role in the optimization of physico-chemical characteristics affecting the enzyme activity. Of the eleven metal ions tested, only Mg^{2+} (2mM) enhanced the enzyme activity while others, inhibited. EDTA significantly ($P < 0.05$) enhanced the enzyme activity suggesting that metal ions do not in general affect the lipase isolated from *A. japonicus.* Organic solvents and acids tested showed significant ($P < 0.05$) enhancement of the lipase activity at higher concentrations, presumably because of their influence on the interfacial area. K_m and V_{max} values of the partially purified lipase determined from Lineweaver Burk and Eadie-Hofstee plots were 63.09 (mM/L), 5.33 (mM/L/min) and 71.76 (mM/L), 5.25 (mM/L/min) respectively.
Key Words: Lipase, *A. japonicus*, pH, Temperature, Metal ions, Organic solvents, Enzyme kinetics

Introduction

A significant increase in the number of lipases (triacylglycerolhydrolases – EC 3.1.1.3) is encompassed during the recent past due to the success in genetic engineering studies and the ever increasing demand for this class of enzymes owing to their applications in food, pharma, oleochemical and other industries. Lipases have certain industrially essential characteristics like wide range of stability in different physico-chemical conditions, broad substrate range, independence from cofactors for their activity, high stereoselectivity etc. There are several industrial processes established[1] based on lipase catalysis and one such industrial success is exemplified by the large scale process operated by BASF (Ludwigshafen, Germany) for the kinetic resolution of amines[2]. There is the need to develop and adopt novel biocatalytic process development strategies including screening and selection of suitable biocatalyst for the intended process from various biological processes. The rationale for this continuous search is being that, what is good for one particular reaction/conversion, may not be so for another and thus becomes industrially inapplicable. When a biocatalyst is found to be suitable, it should be evaluated for its activity, stability and availability and if these parameters are satisfactory, then the biocatalyst should be characterized, purified and its large scale economical production should be explored for industrial use. In case, one or a few desired characteristics are missing, then biocatalyst improvement could be effected through physico-chemical methods and/or rDNA technology[3].

The present study is aimed at investigating the activity of lipase, produced from *A. japonicus* using olive oil substrate, on sesame, groundnut and sunflower oils and find out if the physico-chemical conditions influencing the enzyme activity vary in comparison to our earlier studies[4] with olive oil. Also, the pH and the temperature stability, the effect of metal ions and organic solvents and the kinetic parameters have been investigated. A search of the lipase engineering database (University of Stuttgart, Germany) did not show the listing of *A. japonicus* lipase and hence efforts are to be made to elucidate the sequence-structure-function relationship of this fungal lipase and pave way for developing novel products by green manufacturing processes.

Materials and Methods

I. Organism, Maintenance and Production Media

Fungal strain *A. japonicus* (MTCC 1975) was procured from IMTECH, Chandigarh, India. The fungal strain was stored and maintained in nutrient agar slants at 4^0C. The culture medium contained malt extract – 20g/L, distilled water – 1L and the pH was adjusted to 7 before sterilization. First time, the procured lyophilized powder was put in 50 ml of the culture medium and incubated for a period of 24 hours. This served as master culture and glycerol stocks were prepared for maintenance of the strain at -20 ^{0}C. Production medium was prepared with soybean flour 5g/L, Wheat mill bran 10g/L, Olive oil substrate 1% and whey 1% added to the culture medium containing malt extract and pH adjusted to 7. Initially, master culture was prepared with the culture medium and then 5 ml of master culture was inoculated into 45 ml of production medium and incubated at 30 ^{0}C for 72 hours in an orbital incubator shaker at 120 rpm.

Ii. Crude Enzyme Preparation

The culture was filtered through Whatman no. 1 filter paper to remove the fungal mycelia and the filtrate was centrifuged at 10,000 rpm at 4 ^{0}C for 15 minutes. The supernatant was stored at -20 ^{0}C till further use.

Iii. Determination of Lipase Activity

Lipase enzyme activity was determined by titrimetric method in an emulsifier free system. The reactions were carried out in 100 ml conical flasks kept in water bath adjusted to the desired temperature and shaking at 120 rpm. The reaction mixture consisted of 2 ml of 0.1M potassium phosphate buffer, 1ml of oil substrate (Sesame/Groundnut/Sunflower) and 1 ml of culture supernatant incubated for 30 minutes at 40 ^{0}C. The enzyme action was terminated by the addition of 5 ml of 96% ethanol followed by titration with 0.05N KOH solution and phenolphthalein indicator. When the reaction mixture turned to pink colour, titration was stopped and the volume of titrant run down was noted. The enzyme activity was calculated using the formula

$$\text{Lipase activity (U/ml)} = \frac{\text{Vol.of titrant rundown} \times \text{Normality of titrant} \times 1000 \times 2 \times \text{Dilution factor}}{\text{Vol.of culture supernatant /Enzyme}}$$

Iv. Experimental Design and Statistical Analysis

For studies on enzyme activity, pH, Temperature and oil substrate were taken as design variables. The three variables, in each case, were varied over 3 levels (-1, 0 and +1) and culture conditions were optimized by full factorial design (27 runs) under response surface methodology (RSM). Three level full factorial design of response surface methodology was used to optimize the chosen design variables for the optimum activity of the enzyme lipase produced by the organism *A. japonicus*. Experiments with three initial pH values of 6, 7 & 8, initial temperatures 30^0C, 40^0C & 50^0C and initial substrate concentrations of 1%, 2% & 3% were employed simultaneously as per the design (Table 1). Experiments (27 runs) were conducted to describe the affect of all the three variables on Lipase activity. The coded values of the process parameters are determined by the equation

$$x_i = \frac{X_i - X_0}{\Delta X} \qquad \text{(Eqn. 1)}$$

Where x_i is the coded value of the ith variable, X_i is the uncoded value of the ith test variable and X_0 is the uncoded value of the ith test variable at the center point. The range and levels of the coded variables are given in Table 1. The behavior of the experimental design is explained by the following second order polynomial

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \text{(Eqn. 2)}$$

Design expert 8.0.1.7 (Stat-Ease Inc., Minneapolis, MN, USA) software trial version was used for statistical analysis of the experimental results (Tables 2 to 7). The experimental design regression equations (3, 4 & 5) were solved using fmincon function of matlab2008 and with the optimum values obtained, surface plots were drawn.

V. Effect of Ph on the Activity and Stability of the Lipase

To determine the effect of pH on the lipase activity, the reaction mixtures were made with the crude enzyme (1 ml), olive oil substrate (1 ml), phosphate buffer (2ml) at a pH range of 4 to 11 and incubated at 40^0 C in a water bath for 30 min. Then the reaction was terminated with 5 ml of 96% ethanol and the activity was determined. The pH stability of the enzyme extract was determined by preincubating the crude enzyme (1 ml) with the phosphate buffer (2 ml) at different levels of pH ranging from 4 to 11 at 37±2 ^{0}C. The residual lipase activity was determined by further incubating the treated enzyme with the olive oil substrate (1 ml) at 40 ^{0}C for 30 min. followed by titration with KOH in presence of phenolphthalein indicator. A comparison is made with the control kept under optimum conditions of pH 7.5 and temperature 40 ^{0}C[5,6]. Relative activity % was calculated by the formula

Relative activity % = $\frac{\text{Enzyme Activity } (\frac{U}{ml}) \text{ shown by the test sample}}{\text{Highest enzyme activity } (\frac{U}{ml}) \text{ shown in the test series}} \times 100.$ Residual activity % was calculated using the formula

Residual activity % = $\frac{\text{Activity } (\frac{U}{ml}) \text{ of the treated enzyme solution}}{\text{Activity } (\frac{U}{ml}) \text{ of the untreated enzyme solution}} \times 100.$

Vi. Effect of Temperature on the Activity and the Stability of the Enzyme

1 ml of the crude enzyme was incubated at varying temperatures (20 ^{0}C – 80 ^{0}C) along with 2 ml of phosphate buffer with pH 7.5 and 1 ml of olive oil as substrate. The enzyme activity was measured after incubation of the reaction mixture for 30 min and the relative activity % calculated. For determining the residual activity, first seven sets of test tubes (in triplicates) were taken and 1 ml of the crude enzyme preparation was taken into each of the test tube sets and incubated at temperatures ranging from 20 ^{0}C to 80 ^{0}C (in increments of 10 ^{0}C) for 1 hour. Then the enzyme samples were quickly cooled down to room temperature and the corresponding reaction mixtures containing the treated crude enzyme (1 ml), olive oil substrate (1 ml) and phosphate buffer (pH 7.5; 2 ml) were incubated at 40 ^{0}C for 30 minutes to determine the lipase activity[5,6].

Vii. Effect of Metal Ions And Chemical Reagents on the Lipase Activity

Role of metals in enzyme structure and function is highly significant. While certain metal ions enhance the enzyme activity, others may function as inhibitors. In the present study, eleven metal ions were chosen and their effect on the lipase activity was studied by incubating the enzyme preparation with the metal ion at a concentration of 2mM in 1:1 ratio at 30^{0}C for 1 hour. Then the respective reaction mixtures were prepared by adding phosphate buffer (pH 7.5; 2 ml) and olive oil substrate (1 ml) and then incubated at 40^{0}C for 30 minutes to determine the enzyme activity. Similar tests with chemical reagents including mineral acids, organic solvents and certain salts were carried out, relative activity % were calculated and presented in Tables 8 & 9.

Viii. Ammonium Sulphate Precipitation and Dialysis

The cell free culture supernatant was precipitated with ammonium sulphate (HiMedia) upto 70% W/V saturation and the precipitated proteins were pelleted by high speed centrifugation at 12000 rpm for 30 minutes at 4^{0}C. The pellet was then dissolved in 50mM phosphate buffer (pH 7.5) and was subjected to dialysis. The dialysis membrane purchased from HiMedia with a MCO 12-14 kDa was pretreated using standard protocol, loaded with the sample and dialysed for 12 hrs in 50mM phosphate buffer, replacing the buffer once in every 3 hours.

Ix. Determination of Kinetics of the Lipase Enzyme

The method is based on the hydrolysis of tributyrin by lipase, and titrating the butyric acids produced with 0.05N KOH in distilled water (NOVO industrials, Analytical methods handout, 1995). The alkali consumption was registered as a function of time at pH 7.5. Emulsifying reagent was first prepared by dissolving 8.95g NaCl and 0.2g KH_2PO_4 in 200 ml deionized water with 270 ml glycerol and 3g gum Arabic was added under vigorous stirring using a magnetic stirrer. After complete dissolution, the mixture was transferred to a 500 ml measuring flask and deionized water was added to make up the volume to 500 ml. Then the substrate emulsion was prepared by mixing 50 ml of emulsifying reagent with 250 ml of tributyrin water mixture having different concentrations of tributyrin so as to get final ester bond concentrations [S] of the values 6.8, 9.11, 13.7, 20.5, 27.3, 68.4, 102.6,136.8 and 171 mol m^{-3} [7].

For determining the reaction rate, 20 ml of the substrate emulsion was taken into a conical flask and the pH was adjusted to 7. 2 ml of the enzyme having 50 U/ml of the enzyme activity was added to the substrate mixture to initiate the reaction. 2 ml of the sample was withdrawn at an interval of 2 minutes, reaction terminated with the addition of 5 ml of 96% ethanol, titration was done with 0.05N KOH and the volume of alkali run down was noted. The procedure was continued till the completion of 20 minutes from the start of the reaction. The rate of alkali addition was used to determine the initial reaction rate by the following formula.

$$\text{Rate of reaction, } v\left(\frac{mol}{m^3\,min}\right) = \frac{Slope\left(\frac{ml}{min}\right) \times Normality_{KOH}\left(\frac{mol}{L}\right) \times 10^{-3}\left(\frac{L}{ml}\right)}{Volume\ of\ the\ sample\ (m^3)}$$

From the graph plotted between the initial rate of the reaction and the tributyrin substrate concentration, rates of the reactions for chosen substrate concentrations were calculated and the kinetic parameters V_{max} and K_m were calculated from Lineweaver Burk and Eadie-Hofstee plots.

Results

I. Effect of Physico-Chemical Factors Affecting Lipase Activity in Different Oil Substrates

Multiple regression analysis of the data for the effect of factors on lipase activity has yielded the following regression equations:

For Sesame oil,

$$Y = +102.27 - 39.02X_1 + 13.48X_2 - 18.88X_3 - 10.73X_1X_2 + 9.80X_1X_3 + 0.55X_2X_3 - 8.06X_1^2 - 20.36X_2^2 + 1.68X_3^2$$

(Eqn. 3)

For Ground nut oil,

$$Y = +75.09 - 35.01X_1 - 6.51X_2 - 10.93X_3 - 2.51X_1X_2 + 3.51X_1X_3 - 0.66X_2X_3 + 5.69X_1^2 - 6.77X_2^2 - 2.63X_3^2$$

(Eqn. 4)

For Sunflower oil,

$$Y = +99.34 - 41.29X_1 - 0.44X_2 - 12.67X_3 - 7.62X_1X_2 - 0.31X_1X_3 - 2.64X_2X_3 - 24.44X_1^2 + 3.96X_2^2 - 2.72X_3^2$$

(Eqn. 5)

where 'Y' is the enzyme activity expressed in U/ml, X_1 is the initial pH, X_2 is the temperature and X_3 is the initial substrate concentration. Solving the regression equations using fmincon function of matlab2008, the optimum values for the factors affecting the enzyme activity were determined.

Optimum pH for the lipase activity tested on the three oils – sesame, groundnut and sunflower is 6 and the substrate concentration is 1%. But the optimum temperatures were different, with 35.8 ^{0}C for sesame, 37.5 ^{0}C for groundnut and 50 ^{0}C for sunflower oil. From the model F-values, the suggested models are inferred as quadratic for sesame oil (Tables 2 & 3; Fig. 1-3) while for groundnut (Tables 4 & 5; Fig. 4-6) and sunflower oils (Tables 6 & 7; Fig. 7-9), the model is linear. For sesame oil, the significant model terms are pH, temperature, substrate concentration, interactive effects of pH and temperature, pH and substrate and square

term of the temperature. R^2 value of 0.9311 is obtained and the predicted R^2 of 0.8131 is in reasonable agreement with adjusted R^2 of 0.8947. For groundnut oil, the significant model terms are pH and substrate concentration; R^2 is 0.8653 and the predicted R^2 of 0.6683 is in reasonable agreement with the adjusted R^2 of 0.7940. In the case of sunflower, pH alone appears to be a significant factor with an R^2 value of 0.7710. The predicted R^2 of 0.4499 is in reasonable agreement with adjusted R^2 of 0.6498. In all the three cases, adequate precision measuring the signal to noise ratio was adequate enough suggesting that the respective models could be used to navigate the design space.

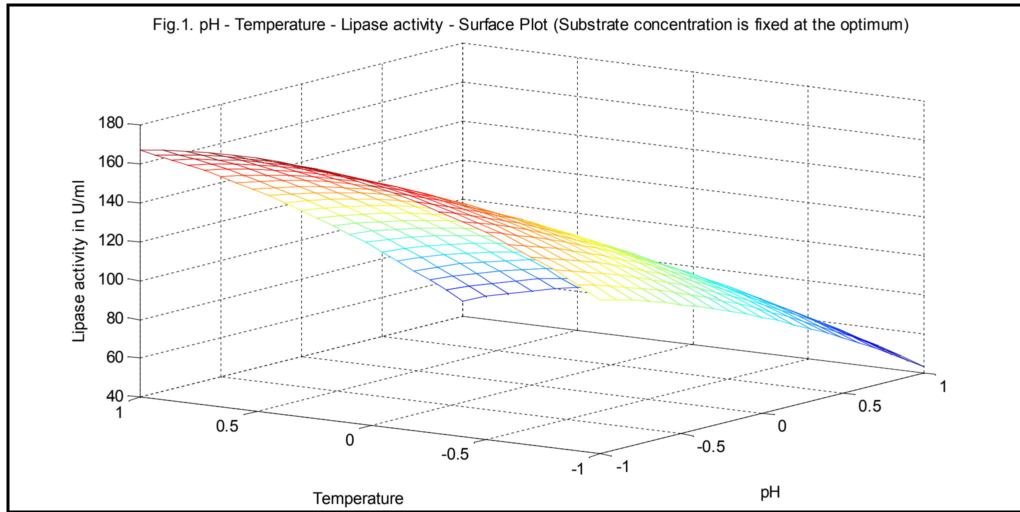


Fig.1. pH - Temperature - Lipase activity - Surface Plot (Substrate concentration is fixed at the optimum)

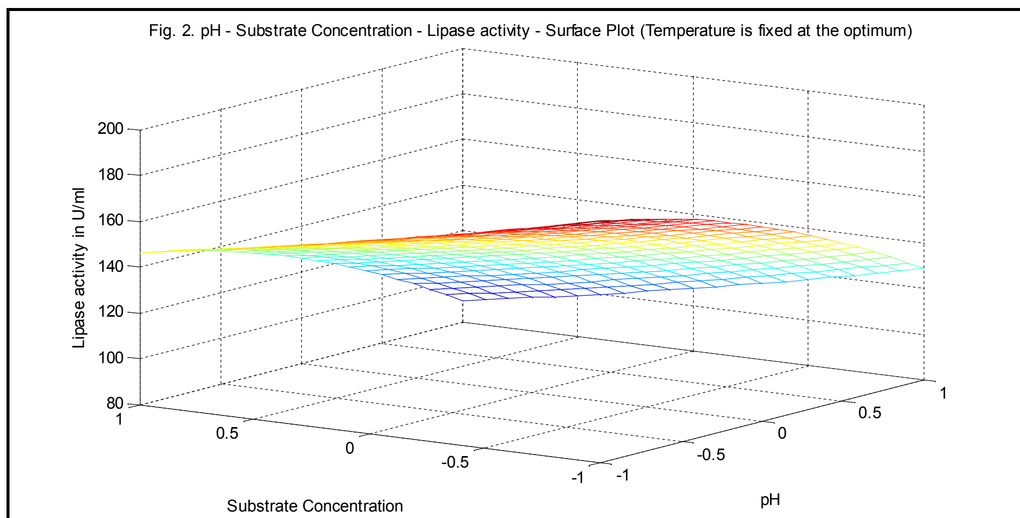


Fig. 2. pH - Substrate Concentration - Lipase activity - Surface Plot (Temperature is fixed at the optimum)

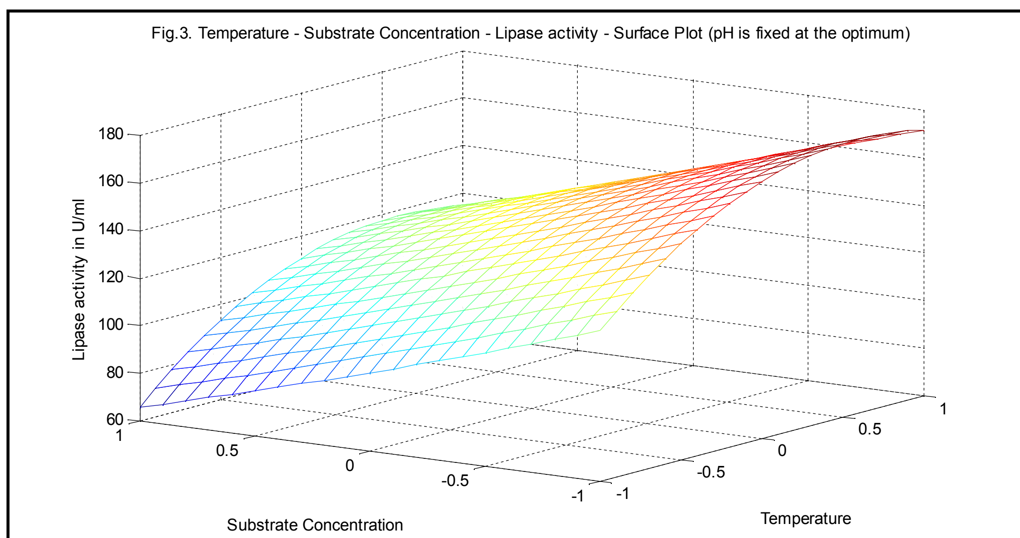


Fig.3. Temperature - Substrate Concentration - Lipase activity - Surface Plot (pH is fixed at the optimum)

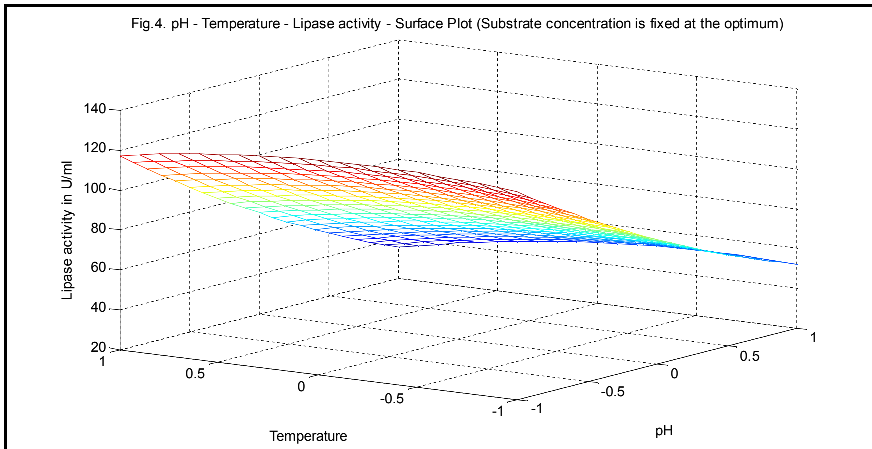


Fig.4. pH - Temperature - Lipase activity - Surface Plot (Substrate concentration is fixed at the optimum)

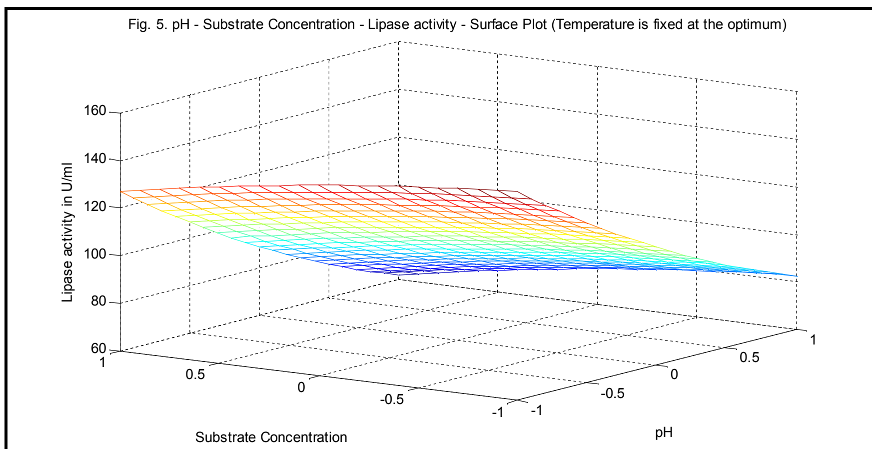


Fig. 5. pH - Substrate Concentration - Lipase activity - Surface Plot (Temperature is fixed at the optimum)

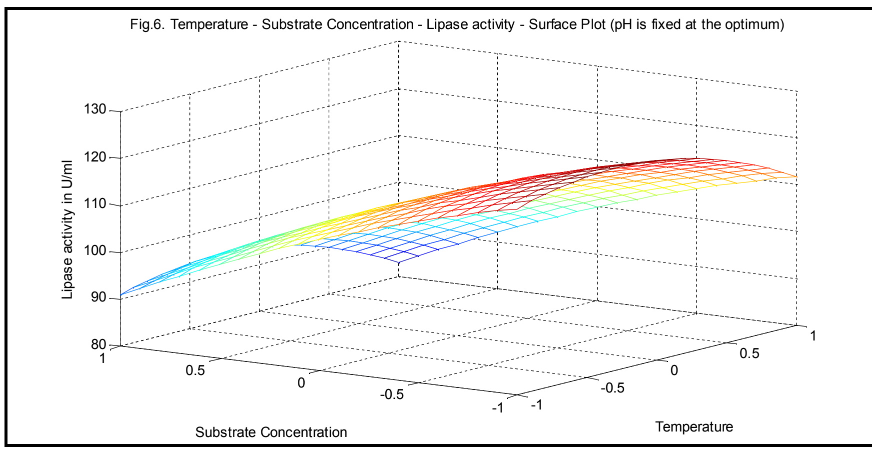


Fig.6. Temperature - Substrate Concentration - Lipase activity - Surface Plot (pH is fixed at the optimum)

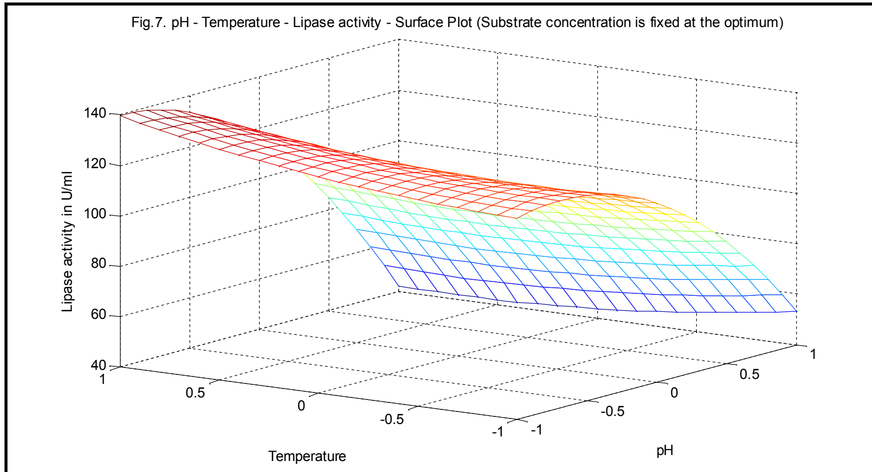
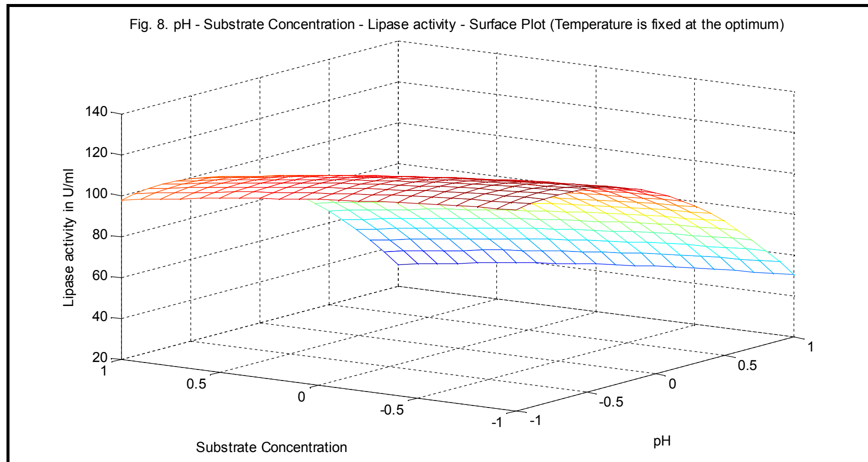
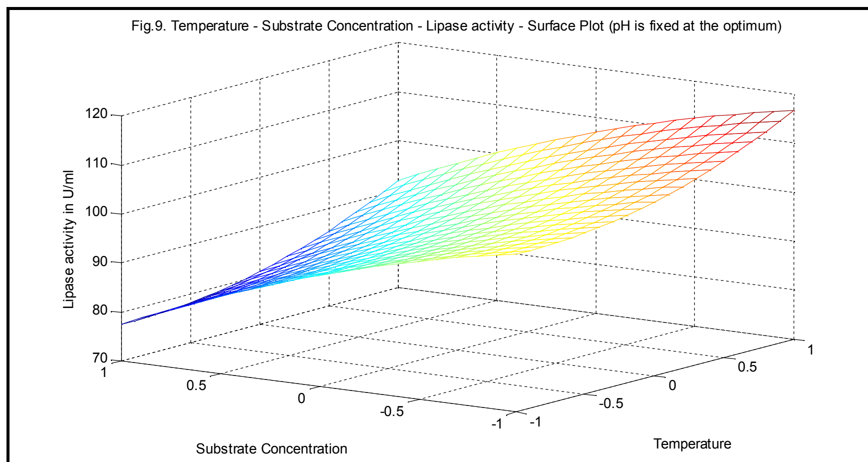


Fig.7. pH - Temperature - Lipase activity - Surface Plot (Substrate concentration is fixed at the optimum)





Ii. Effect of pH and Temperature on Lipase Stability in the Presence of Olive Oil Substrate

A steady increase in the activity of the enzyme was noticed with the increase in pH from 4 to 8 when the activity was the highest and with further increase in alkalinity up to pH 11, there was a steep decline (Fig. 10). From the residual activity %, it is evident that the lipase from *A. japonicus* has highest stability at pH 7 & 8 and the stability drastically reduced with an increase in pH to 9 (Fig. 11). Thus the ideal pH for this enzyme appears to be in the range of 6-8. Temperature in the range of 30^0C to 40^0C is optimal for the enzyme activity (Fig. 12) and the enzyme is quite stable in the temperature range of 20^0C to 40^0C but with further increase in temperature, the stability of the enzyme decreased significantly (Fig. 13). For all these tests, olive oil was used as the substrate.

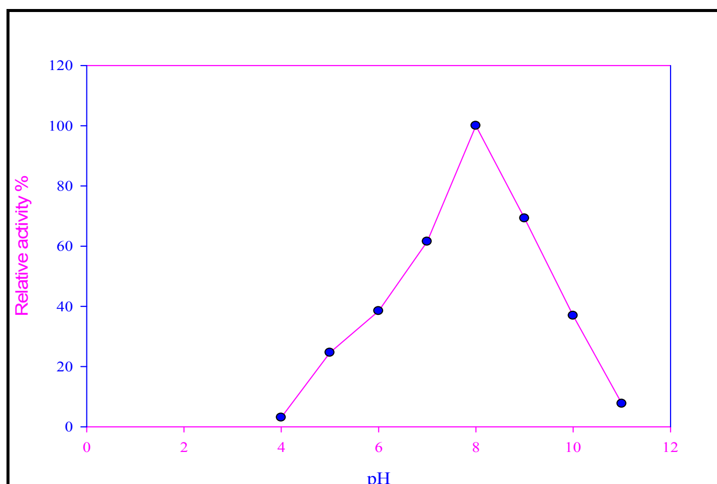


Fig. 10 Effect of pH on the lipase activity from the fungus *A. japonicus*

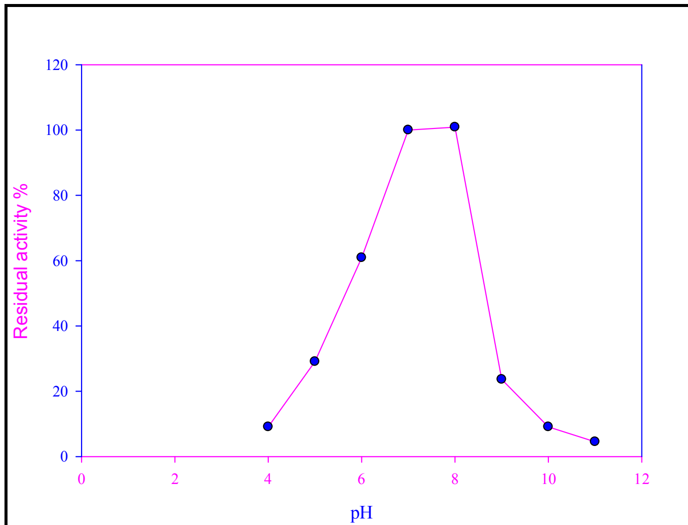


Fig. 11 pH and residual activity percentage of the lipase enzyme from *A. japonicus*

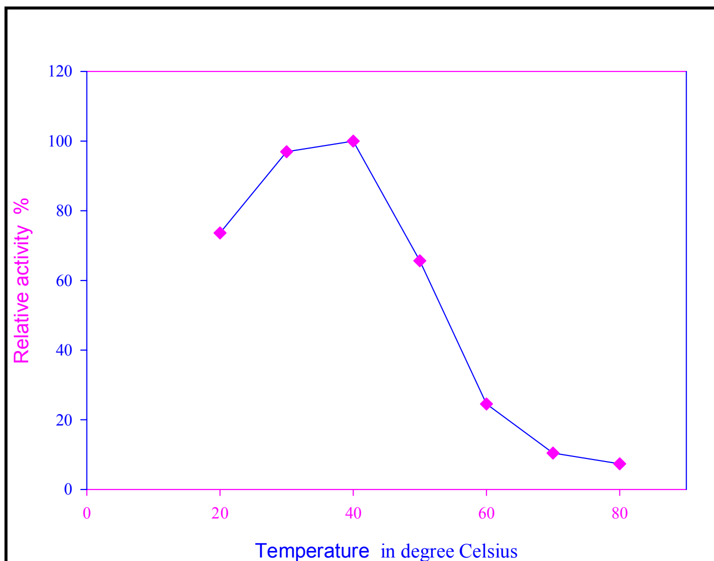


Fig. 12 Effect of Temperature on the activity of the lipase from *A. japonicus*

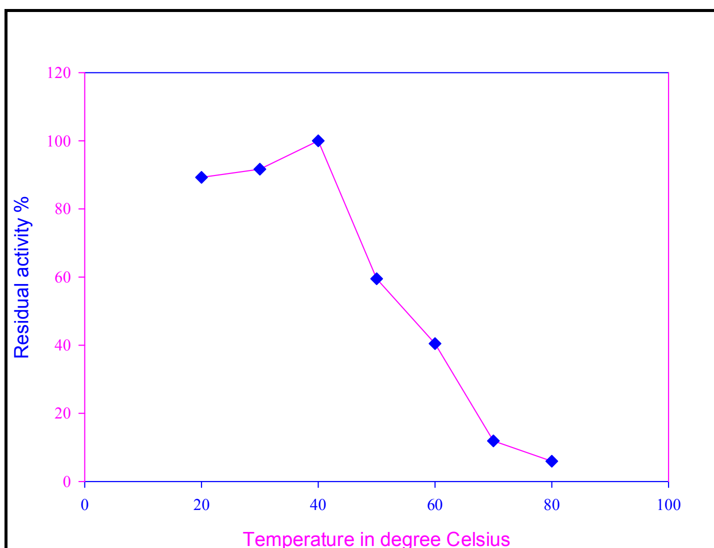


Fig. 13 Effect of Temperature on the residual activity of the lipase from *A. japonicus*

Iii. Effect of Metal Ions, Chemical Reagents on Lipase Activity

Among the eleven metal ions tested for their influence on lipase activity, Mg^{2+} alone enhanced the enzyme activity while all others had a depressive effect, Pb^{2+} to inhibit the highest. The results indicated that the lipase activity was inhibited by chloroform, sodium azide and tween. The enzyme activity increased with the increase in the concentrations of ethanol. In the case of HCl and Phenol, the enzyme activity was very low in !% while was significantly higher in 5% and 10% concentrations (Tables 8 & 9). Reasonably good activity was obtained with triton.

Iv. Kinetics of the Lipase

Experiments were conducted to determine the initial rate of enzymatic hydrolysis of tributyrin in micro-emulsion system using the lipase at different substrate concentrations. Michaelis - Menten kinetic parameters were determined by Lineweaver Burk and Eadie-Hofstee methods. According to Lineweaver Burk method, V_{max} was determined to be 5.33 mM/L/min and the K_m 63.09 mM/L; From the Eadie-Hofstee method, the kinetic parameters V_{max} and K_m were 5.25 mM/L/min and 71.76 mM/L respectively. The plots were drawn using Originpro software (Fig. 14, 15).

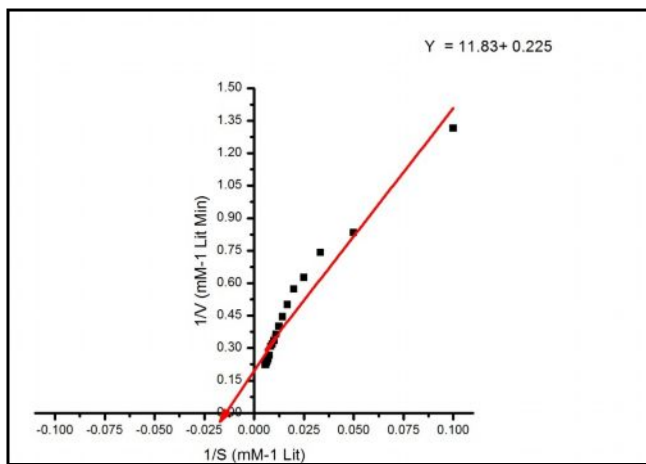


Fig. 14 Lineweaver Burk plot

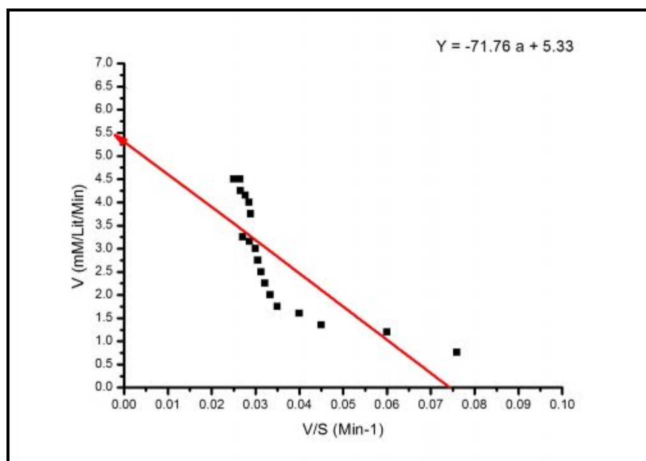


Fig. 15 Eadie-Hofstee Plot

Table 1. Experimental ranges and levels of the independent variables for the lipase activity.

Variables	Symbol	Range and Levels		
		-1	0	+1
pH	X_1	6	7	8
Temperature, ^{0}C	X_2	30	40	50
Oil substrate (%)	X_3	1	2	3

The actual values of the variables are pH: 6, 7 & 8; Temperature: 30^0C, 40^0C & 50^0C; Oil substrate: 1%, 2% & 3% for the three vegetable oils tested viz. Sesame, Groundnut and Sunflower.

Table 2. Effect of pH, Temperature and Substrate on the lipase activity using sesame oil

Run No.	Coded Values			Enzyme activity U/ml		Residual	% Error
	X_1	X_2	X_3	Actual	Predicted		
1	0	1	0	100.0	95.40	4.6	4.6
2	1	1	0	44.0	37.59	6.41	14.57
3	1	0	0	48.0	55.20	-7.2	15.0
4	-1	1	1	105.6	110.65	-5.05	4.78
5	-1	-1	0	90.0	88.67	1.33	1.48
6	1	0	1	30.4	47.80	-17.4	57.2
7	-1	0	1	112.0	106.24	5.76	5.14
8	0	0	-1	125.0	122.83	2.17	1.74
9	0	0	0	112.0	102.27	9.73	8.69
10	-1	1	0	130.0	137.10	-7.1	5.46
11	-1	0	-1	197.5	163.60	33.9	17.16
12	0	0	1	76.8	85.07	-8.27	10.76
13	-1	-1	-1	107.5	119.58	-12.08	11.24
14	1	-1	1	32.0	24.15	7.85	24.53
15	-1	1	-1	152.5	166.90	-14.4	9.44
16	-1	-1	1	64.0	61.12	2.88	4.5
17	1	0	-1	52.5	65.95	-13.45	25.62
18	0	1	1	86.4	78.75	7.65	8.85
19	1	-1	-1	60.0	43.40	16.6	27.66
20	0	-1	1	51.2	50.69	0.51	0.99
21	1	1	-1	45.0	47.79	-2.79	6.2
22	0	-1	-1	75.0	89.55	-14.55	19.4
23	1	1	1	36.8	30.74	6.06	16.47
24	0	-1	0	62.0	68.44	-6.44	10.39
25	0	1	-1	120.0	115.4	4.6	3.83
26	1	-1	0	36.0	32.10	3.9	10.83
27	-1	0	0	128.0	133.24	-5.24	4.09

Table 3. Regression analysis (RSM) for the lipase activity using Sesame oil

Model term	Coefficient estimate	Standard Error	F- Value	P-Value
Intercept	102.27	6.93		
X_1	-39.02	3.21	148.17	<0.0001
X_2	13.48	3.21	17.68	0.0006
X_3	-18.88	3.21	34.68	<0.0001
X_1X_2	-10.73	3.93	7.47	0.0141
X_1X_3	9.80	3.93	6.23	0.0231
X_2X_3	0.55	3.93	0.020	0.8902
X_1^2	-8.06	5.55	2.10	0.1650
X_2^2	-20.36	5.55	13.44	0.0019
X_3^2	1.68	5.55	0.091	0.7662

Table 4. Effect of pH, Temperature and Substrate on the lipase activity using groundnut oil

Run No.	Coded Values			Enzyme activity U/ml		Residual	% Error
	X_1	X_2	X_3	Actual	Predicted		
1	0	1	0	48.0	61.80	-13.8	28.75
2	1	1	0	38.0	29.98	8.02	21.10
3	1	0	0	32.0	45.77	-13.77	43.03
4	-1	1	1	83.2	87.29	-4.09	4.91
5	-1	-1	0	100.0	113.02	-13.02	13.02
6	1	0	1	30.0	35.72	-5.72	19.06
7	-1	0	1	112.0	98.73	13.27	11.85
8	0	0	-1	110.0	83.39	26.61	24.19
9	0	0	0	64.0	75.09	-11.09	-17.33
10	-1	1	0	102.0	105.01	-3.01	2.95
11	-1	0	-1	125.0	127.6	-2.6	2.08
12	0	0	1	50.68	61.53	-10.85	21.41
13	-1	-1	-1	108.5	124.17	-15.67	14.44
14	1	-1	1	30.0	38.62	-8.62	28.7
15	-1	1	-1	125.0	117.48	7.52	6.02
16	-1	-1	1	95.0	96.62	-1.62	1.70
17	1	0	-1	35.5	50.57	-15.07	42.45
18	0	1	1	41.6	47.59	-5.99	14.39
19	1	-1	-1	45.0	52.15	-7.15	15.88
20	0	-1	1	68.0	61.93	6.07	8.93
21	1	1	-1	42.5	35.44	7.06	16.61
22	0	-1	-1	95.0	82.47	12.53	13.19
23	1	1	1	36.8	19.27	17.53	47.63
24	0	-1	0	84.6	74.82	9.78	11.56
25	0	1	-1	57.5	70.77	-13.27	23.07
26	1	-1	0	65.7	48.01	17.69	26.92
27	-1	0	0	135.0	115.79	19.21	14.23

Table 5. Regression analysis (RSM) for the lipase activity using groundnut oil

Model term	Coefficient estimate	Standard Error	F- Value	P-Value
Intercept	75.09	7.81		
X_1	-35.01	3.62	93.71	<0.0001
X_2	-6.51	3.62	3.24	0.0897
X_3	-10.93	3.62	9.14	0.0077
X_1X_2	-2.51	4.43	0.32	0.5790
X_1X_3	3.51	4.43	0.63	0.4396
X_2X_3	-0.66	4.43	0.022	0.8831
X_1^2	5.69	6.26	0.83	0.3761
X_2^2	-6.77	6.26	1.17	0.2946
X_3^2	-2.63	6.26	0.18	0.6802

Table 6. Effect of pH, Temperature and Substrate on the lipase activity using sunflower oil

Run No.	Coded Values			Enzyme activity U/ml		Residual	% Error
	X_1	X_2	X_3	Actual	Predicted		
1	0	1	0	100.0	102.86	-2.86	2.86
2	1	1	0	24.0	29.51	-5.51	22.9
3	1	0	0	30.0	33.60	-3.6	12.0
4	-1	1	1	96.0	109.61	13.61	14.18
5	-1	-1	0	124.0	112.98	11.02	8.89
6	1	0	1	20.8	17.91	2.89	13.89
7	-1	0	1	157.5	101.11	56.39	35.8
8	0	0	-1	130.0	109.29	20.71	15.93
9	0	0	0	96.0	99.34	-3.34	3.48
10	-1	1	0	122.0	127.33	-5.33	4.37
11	-1	0	-1	110.0	125.83	-15.83	14.39
12	0	0	1	78.4	83.95	-5.55	7.08
13	-1	-1	-1	130.0	119.99	10.01	7.7
14	1	-1	1	17.6	32.57	-14.97	85.01
15	-1	1	-1	142.5	139.61	2.89	2.03
16	-1	-1	1	92.8	100.54	-7.74	8.34
17	1	0	-1	30.0	43.86	13.86	46.2
18	0	1	1	81.6	84.84	-3.24	3.97
19	1	-1	-1	35.0	53.25	-18.25	52.14
20	0	-1	1	68.8	91.0	-22.2	32.26
21	1	1	-1	32.5	42.41	-9.91	30.49
22	0	-1	-1	105.75	111.06	-5.31	5.02
23	1	1	1	19.2	11.17	8.03	41.82
24	0	-1	0	96.0	103.75	-7.75	8.07
25	0	1	-1	145.0	115.45	29.55	20.38
26	1	-1	0	100.8	45.63	55.17	54.73
27	-1	0	0	78.4	116.19	-37.79	48.20

Table 7. Regression analysis (RSM) for lipase activity using Sunflower oil

Model term	Coefficient estimate	Standard Error	F- Value	P-Value
Intercept	99.34	13.14		
X_1	-41.29	6.08	46.12	<0.0001
X_2	-0.44	6.08	5.276E-003	0.9429
X_3	-12.67	6.08	4.34	0.0526
X_1X_2	-7.62	7.45	1.05	0.3207
X_1X_3	-0.31	7.45	1.714E-003	0.9675
X_2X_3	-2.64	7.45	0.13	0.7276
X_1^2	-24.44	10.53	5.39	0.0330
X_2^2	3.96	10.53	0.14	0.7113
X_3^2	-2.72	10.53	0.067	0.7993

Table 8. Effect of metal ions on the lipase activity expressed as relative activity % in comparison with the control with an activity of 95.625 U/ml.

S. No.	Metal ion (2 mM)	Relative activity %
1	Cu^{2+}	94.12
2	Zn^{2+}	54.38
3	Ca^{2+}	54.38
4	Cd^{2+}	60.65
5	Cr^{3+}	58.56
6	As^{3+}	57.52
7	Pb^{2+}	52.28
8	Mn^{2+}	71.1
9	Ni^{2+}	69.02
10	Co^{2+}	70.06
11	Mg^{2+}	140.13

Table 9. Effect of different chemical reagents on the lipase activity expressed as relative activity %.

S. No.	Chemical reagent	Relative activity %
1.	Control	91.5
2.	1% HCl	36.06
3.	5% HCl	172.68
4.	10% HCl	327.87
5.	1% Chloroform	24.59
6.	5% Chloroform	24.59
7.	10% Chloroform	46.99
8.	1% Phenol	75.41
9.	5% Phenol	113.11
10.	10 % Phenol	154.09
11.	1% Ethanol	184.69
12.	5% Ethanol	213.11
13.	10% Ethanol	239.89
14.	EDTA (mM)	269.4
15.	NaZ (2mM)	30.6
16.	Tween	59.02
17.	Triton	129.51

Discussion

Fungal lipases are more attractive than those of bacteria because of their extracellular nature and the consequent reduction in the cost of their production[8]. The occurrence of inducible and extracellular lipases in fungi grown on vegetable oils is well known in lipid biotechnology. A lipase that is stable at acidic conditions and normal temperatures is generally rare and it has been found that the fungal organism *Rhizopus sps.* produces an inducible extracellular and acidophilic lipase that could be exploited for commercial purpose[9]. Physico-chemical characteristics like pH, temperature, cofactors etc. are the major factors that strongly influence the production and the activity of the microbial enzymes and in general the micro-organisms are very sensitive to changes in temperature and pH.

Purified lipase from *A. japonicus* of 43 kDa mol. wt. was shown to have an optimum pH of 7.5 and the temperature was 40^0C. Further metal ions Mn^{2+} and Co^{+2} were reported to have an inhibitory effect on the enzyme[6]. The optimum pH and temperature for the crude lipase activity isolated from various strains of *Aspergillus sps.* were 40^0C and 6.5 respectively[10]. Extracellular lipase from *Pseudomonas sps.* was reported to work optimally at 50^0C, pH 8 and 15% olive oil as substrate[11]. *A. japonicus* isolated from the paper nest of *Ropalidia marginata*[6] produced an extracellular lipase with maximum enzyme activity of 120 U/ml working at an optimum pH of 7.5 and the temperature 40^0C.

Lipase isolated from the yeast, *Cryptococcus albidus* showed highest activity at pH 6.5 and beyond that the activity dropped. But in *Candida rugosa*[12] and *Pichia lynferdii*[13], maximum activity was reported to be pH 7. The crude enzyme from *C. albidus* showed maximum activity and stability at 50^0C and retained 57% of its activity at 60^0C indicating thermotolerant nature of the enzyme[14]. Further Mukesh et al (2011) showed that the activity of the lipase increased on addition of metal ions like Ba^{+2}, Ca^{+2}, Mg^{+2} and NH_4^+ while others like Mn^{2+}, Cu^{2+}, Fe^{2+} etc. inhibited the activity. In C. albidus, the chemical agent EDTA inhibited the enzyme activity while in our study, it increased the enzyme activity. This enhanced activity in the presence of EDTA in our study perhaps suggests that metal ions except Mg^{2+} are inhibitory for the enzyme lipase and hence their removal by the chelating agent EDTA would have a positive effect on enzyme activity. Lipase produced from *Trichoderma harzianum* had an optimum pH and Temperature of 8.5 and 40^0C. Further, K_m and V_{max} values of the crude enzyme for P-nitrophenylbutyrate substrate hydrolysis were found to be 7.15mM and 7.067mM/min respectively[5]. The lipase activity was found to be maximum at a pH of 5 and a temperature of 40^0C in the case of newly isolated marine bacterium *Bacillus sonorensis*[15]. Broadly, the envisaged experimental results of our study suggest a pH range of 6-8 and a temperature range of 30^0C-40^0C for the best activity of the lipase from *A. japonicus*.

A careful survey of the literature suggests that different metal ions affect the lipase activity differently and it is difficult to draw general conclusions as to a particular metal ion invariantly as a promoter and some other as inhibitor. But it is apparent that metal ions do affect the enzyme structure and function. Lipase from *A. japonicus* produced using pig fat production medium[6] was shown to be induced by Ca^{2+} followed by Hg^{2+}, Mg^{2+} and Ba^{2+} while was inhibited by Mn^{2+} and Co^{2+}. The results of this study are in reasonable agreement with the earlier reports and also, there are subtle differences which are to be considered when the enzyme is put to industrial scale applications.

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