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The effect of olive cake types on lipase production by isolated *Rhizopus sp.* and process statistical optimization

ABSTRACT

The aim of this work was to study the production of extracellular lipase by solid-state fermentation with different olive cakes varieties including Mary, Shenghe and Yellow from isolated fungi using agro-industries waste such as rice straw, rice barn and wheat straw. The highest yields of enzyme were obtained in solid-state fermentation using rice straw as solid substrate in combination with 40% Mary olive cakes as inducer. The initial screening by using Plackett-Burman's design demonstrated that among the tested factors, lactose and ammonium sulfate of the medium significantly ($p < 0.05$) enhanced the lipase production. Further optimization of lipase production by isolated fungi in solid-state fermentation by applying response surface methodology was achieved, which revealed these as follows: 0.42 (% w/v) for lactose and 0.09 (% w/v) for ammonium sulfate. Also the enzyme kinetics parameters, biochemical properties, thermodynamic of thermal deactivation and deactivation rate constant of enzyme were determined.

Key words: lipase, olive cake, solid-state fermentation, rice straw, design of experiment

Introduction

Lipases are enzymes belong to the group of the hydrolases, whose main biological function is to catalyze the hydrolysis of insoluble triacylglycerols to free fatty acids, mono- and diacylglycerols and glycerol. Besides its natural function, lipases can catalyze esterification, interesterification and transesterification reactions in non-aqueous media (Houde et al., 2004; Rigo et al., 2010).

Fungi are widely recognized as the best lipase sources and are used preferably for industrial applications. Use of waste biomaterials for biotechnological products, especially enzymes, have been noticed in the recent years. Solid substrate fermentation (SSF) has built up credibility in recent years for the production of microbial products including enzymes through inexpensive media and it is an appropriate process for developing countries (Anvari & Khayati, 2011; Singhania et al., 2009).

Guilan province is the largest rice producer in Iran. Rice cultivation area in Guilan is over 230000 ha with an average farm yield of 6.3 tons/ha and an approximate straw production of 3.5 tons/ha (Nguyen & Tran, 2009). Also it is the largest different olive varieties producer for example Mary, Feishami, Yellow and Shenghe. There are 450 ha olive cultivation area and 6000 tons product yield per year. So, significant amount of olive cakes are obtained followed by large olive oils capacity productions (4480 tons/year). Potentially combination with agrochemical wastes is a cheap and valuable source as growth media for different biotechnological processes including microbial enzymes production.

The objective of the study was optimization of lipase production under SSF conditions by different varieties of olive cakes using isolated *Rhizopus sp*. as a microorganism. To propose an efficient lipase extraction method, the fermented solids and their biochemical properties were studied. Also the enzyme kinetics parameters, thermodynamic of thermal deactivation and deactivation rate constant of enzyme were determined.

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Materials and Methods

Nomenclature

Y	predicted response (lipase activity) (U/g)
E_t	enzyme activity at time t (U/g)
E_0	initial enzyme activity (U/g)
ΔH^*	Enthalpy energy (kJ/ mol)
ΔS^*	Entropy energy (J /mol K)
ΔG^*	Gibbs free energy (kJ /mol)
E_d	activation energy (kJ /mol)
t	time of treatments (h)
K_d	first-order deactivation rate constant
$t_{1/2}$	half-life of an enzyme (h)
R	gas constant (8.314 J/K mol)
T	absolute temperature (K)
κ	Boltzmann's constant (1.38×10^{-23} J/K),
h	Planck's constant (6.626×10^{-34} Js)
β_0	Coefficients of equation (1)

Microorganisms

Fungal strain used in this study was isolated from oily food waste in Guilan - Iran. The fungus was identified as *Rhizopus microspores*. The cultures were maintained in potato dextrose agar (PDA) at 30°C with monthly sub culturing.

Inoculum and fermentation conditions

Rice straw, rice bran and wheat straw were used as solid substrates for solid-state fermentation (SSF). Varieties of three different olive cakes including Mary, Shenghe and Yellow were tested as inducers to produce lipase. Solid- state fermentation was performed using a combination of agro–residual and oil cake as the solid substrate. Ten grams of mixed substrates taken in 500 ml Erlenmeyer flasks was moistened with Czepek-dox media ($NaNO_3$ - 2.5 g/l, KH_2PO_4 - 1.0 g/l, $MgSO_4\,7H_2O$ - 0.5 g/l, KCl - 0.5 g/l) to reach a final moisture content of 70% (w/v). After sterilization (autoclaved at 121°C at 15 psi pressure for 20 min), an inoculum size of 10^6 spores/g substrate was used for each flask under aseptic conditions. The contents of each flask were mixed thoroughly with inoculating needle for uniform distribution of fungal spores in the medium. The flasks were incubated at 30 ± 1°C. After incubation period of fermentation, extraction of the enzyme was carried out according to Kamini et al. (1998) and the supernatant was used for analytical assays.

Enzyme assay

Lipase assay was done spectrophotometrically using *p*-nitrophenyl palmitate (*p*NPP) (Sigma) as the substrate. The assay mixture contained 1 ml of 16.5 mM solution of *p*-nitrophenyl palmitate in 2-propanol along with Tris-HCl buffer, pH 8 (supplemented with 0.1% arabic gum and 0.4% Triton X-100) in a ratio of 1:9. The enzyme solution (0.025 ml) was added to it and incubated in water bath at 37°C for 20 min. *p*-nitrophenol was liberated from *p*-nitrophenyl palmitate by lipase mediated hydrolysis imparting a yellow color to the reaction mixture. After incubation, 2 ml of distilled water was added and the absorbance was measured at 410 nm (Kordel et al., 1991). Absorbance of control was also recorded. One unit (U) of lipase activity was defined as the amount of enzyme that liberates one micromole of *p*-nitrophenol per min under the assay conditions.

Optimization procedure and experimental design

Plackett–Burman's factorial design was employed for screening the most significant factors effecting the lipase production by strain under study. Plackett–Burman design serves as a valuable tool for initial screening of effects of various factors in a small number of experiments for reliable short-listing of relevant factors, indicating how each factor affects the production process for further optimization (Ruchi et al., 2008). The effect of addition of various carbon sources (glucose, lactose and sucrose), nitrogen sources (ammonium sulfate, di-ammonium hydrogen phosphate, yeast extract and urea), and incubation time was studied for optimal lipase production (Table 1). Based on Plackett–Burman's factorial design, each factor was examined at two levels: - 1 for low level and + 1 for high level.

Sequential, a central composite design (CCD) with five coded levels (- 1.41, -1, 0, +1 and +1.41) was used to describe the nature of the response surface in the optimum region. According to this design, the total number of treatment combinations was $2^k + 2k + n_0$, where k is the number of independent variables and n_0 is the number of repetitions of experiments at the centre point (Song et al., 2007). The experimental results of the CCD were fit with a second-order polynomial equation by a multiple regression technique:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i\neq j}^{k} \beta_{ij} x_i x_j \qquad (1)$$

where Y is the predicted response (lipase activity), β_0 is the offset term, β_i is the i-th linear coefficient, β_{ii} is the i-th quadratic coefficient and β_{ij} is the ij-th interaction coefficient. The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear,

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quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the *p*-value at a probability of 0.05. The regression coefficients were then used to make statistical calculations to generate response surface from the regression models. The analysis of data and the optimizing process were generated using Minitab statistical software version 15.

Table 1. *Plackett–Burman experimental design for screening of significant variables affecting lipase production*.

Run	Glucose (%w/v)	Lactose (%w/v)	Sucrose (%w/v)	$(NH_4)_2SO_4$ (%w/v)	$(NH_4)_2HPO_4$ (%w/v)	Yeast extract (%w/v)	Urea (%w/v)	Incubation time (day)	Lipase activity (U/g)
1	1.0	0.2	1.0	0.1	0.1	0.1	0.5	7	329.36
2	1.0	1.0	0.2	0.5	0.1	0.1	0.1	7	330.94
3	0.2	1.0	1.0	0.1	0.5	0.1	0.1	5	463.69
4	1.0	0.2	1.0	0.5	0.1	0.5	0.1	5	241.22
5	1.0	1.0	0.2	0.5	0.5	0.1	0.5	5	466.06
6	1.0	1.0	1.0	0.1	0.5	0.5	0.1	7	465.07
7	0.2	1.0	1.0	0.5	0.1	0.5	0.5	5	336.37
8	0.2	0.2	1.0	0.5	0.5	0.1	0.5	7	259.48
9	0.2	0.2	0.2	0.5	0.5	0.5	0.1	7	313.67
10	1.0	0.2	0.2	0.1	0.5	0.5	0.5	5	358.87
11	0.2	1.0	0.2	0.1	0.1	0.5	0.5	7	494.19
12	0.2	0.2	0.2	0.1	0.1	0.1	0.1	5	406.74

Biochemical characterization of the enzyme

Effect of pH and temperature on lipase activity and stability

In order to find the optimum pH, lipase activity was tested at different pH (from 6 to 10) in a suitable buffer by incubating enzyme-substrate under standard assay conditions. The optimal temperature enzyme activity was determined by incubating the reaction mixture at 30, 40, 50 and 60°C.

To investigate the effect of pH stability, enzyme solutions were incubated at various pHs (4–10.6) at 30°C for 60 min and the residual activity was measured against *p*-NPP. Thermo-stability (at pH=8.0) of enzyme was measured by incubating the lipase alone at different temperatures (40–80°C) for 60 min followed by standard assays.

Effect of metal ions and organic solvents on the enzyme activity

The extracted enzymes were incubated with various metal ions (10 mM) and organic solvents (50%, v/v) at 30°C under shaking at 150 rpm for 60 min. At the end of the incubation period, residual lipase activity was measured against *p*-NPP under standard assay conditions (see above). Control experiments were performed without any compound.

Estimation of enzyme kinetics parameters

The kinetic parameters, K_m and V_{max} were determined by measuring the lipase activity reaction rates at different *p*-NPP concentrations ranging from 0.05 to 0.5 (% w/v) with a fixed amount of the enzyme at 30°C for 1 h. The K_m and V_{max} values were obtained by analysis of data according to Lineweaver–Burk equation, allowing the catalytic efficiency i.e., the ratio V_{max} /K_m, to be determined (Maisuria et al., 2010).

Estimation of deactivation rate constant and thermodynamic parameters for thermal deactivation

As described by Ortega et al. (2004) thermal deactivation of enzymes can often be explained by a first-order kinetic model. According to this model an enzyme activity decreases log-linearly as a function of time as described in the following equation:

$$Ln\left({}^{E_t}/_{E_0}\right) = -K_d t \qquad (2)$$

where E_t is the enzyme activity at time t (h), E_0 is initial enzyme activity, t is time of treatments, and K_d is the first-order deactivation rate constant.

The K_d values were calculated from a plot of time (t)

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versus Ln(E_t/E_0) at a particular temperature. The half-life of an enzyme is defined as the time required by the enzyme to lose half of its initial activity, which is calculated as:

$$t_{1/2} = {Ln2}/{K_d} \qquad (3)$$

The energies and entropies of deactivation can be estimated by making use of absolute reaction rates (Narayana Naidu & Panda, 2003). The temperature dependency of deactivation rate constant can be expressed as:

$$K_d = {\kappa T}/{h}\, exp({\Delta S^*}/{R}) exp\left(-\frac{\Delta H^*}{RT}\right) \qquad (4)$$

or

$$Ln\left({K_d}/{T}\right) = Ln\left({\kappa}/{h}\right) + {\Delta S^*}/{R} - \left({\Delta H^*}/{R}\right){1}/{T} \qquad (5)$$

ΔH^* and ΔS^* values were estimated from the slope and intercept of a 1/T versus ln(K_d/T) plot, respectively. So that,

$$\Delta H^* = -(slope)R \qquad (6)$$

and

$$\Delta S^* = R\left[intercept - Ln\left({\kappa}/{h}\right)\right] \qquad (7)$$

where κ is the Boltzmann's constant (1.38×10^{-23} J/K), R the gas constant (8.314 J/K mol) and h the Planck's constant (6.626×10^{-34} Js).

Free energy change were calculated by using the following relationship

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad (8)$$

where T is the absolute temperature (K).

Energy of deactivation was estimated using the Arrhenius equation:

$$K_d = A exp\left({E_d}/{RT}\right) \qquad (9)$$

so that,

$$LnK_d = -\frac{E_d}{RT} + Ln\, A \qquad (10)$$

Energy (E_d) involved in this deactivation process was calculated from the slope of a linear plot of 1/T versus (Ln K_d), $E_d = -(slope)\, R$.

Results and Discussion

Effect of solid substrate and inducers to produce lipase

There are various important factors that produce immense impact on success of a particular technology hence, needed to be considered for the development of any bioprocesses and so is the SSF. It includes selection of microorganism, different substrates and optimum process parameters, which has been a challenge for solid state fermentation (Singhania et al., 2009). Selection of substrate depends upon several factors mainly related with cost and availability and thus may involve the screening of several agro-industrial residues.

The results obtained for SSF production of lipase with rice straw, wheat straw and rice bran combination with three types of olive cakes (in 20 and 40%) as inducer are given in the Tables 2 and 3. As the results show, the maximum enzyme activity for rice straw and rice bran were found with 20% Mary and Yellow olive cakes, respectively. Sun and Xu (2008) also reported that the olive oil was the best lipase production inducer between four different tested inducers. Despite of olive cake types in compare to wheat straw and rice bran the enzyme activity was higher in medium containing rice straw (Table 2).

Table 2. *The enzyme activity (U/g) using different solid substrate with 20% different olive cakes.*

Solid substrate	Olive cakes		
	Shengeh	**Mary**	**Yellow**
Wheat straw	160.68	151.68	135.12
Rice bran	94.52	78.28	101.16
Rice straw	226.56	286.44	256.44

It may be related for increasing fungus growth rate on the rice straw. The reported enzyme activity with our isolated strain was high when compared to enzyme activity *Penicillium chrysogenum* in similar culture medium containing wheat bran and Czapek-dox solution (Kumar et al., 2011).

Table 3. *The enzyme activity (U/g) using different solid substrate with 40% different olive cakes.*

Solid substrate	Olive cakes		
	Shengeh	**Mary**	**Yellow**
Wheat straw	196.28	205.23	179.14
Rice bran	83.72	77.24	91.16
Rice straw	304.28	350.8	310.56

In comparison with three different solid substrates, the rice straw was also the best one with 1.40 and 4.54 fold greater enzyme activities with 40% Mary olive cakes (Table 3). There was 22.4% enzyme production increasing rate with 40% compare to 20% Mary olive cakes. Study on the effect

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of different type's oil cakes on lipase production showed that all of tested oil cakes supported enzyme production however, with 40% Mary type maximum yields of lipase activity was obtained, so it was chosen for further experiments.

Evaluation of factors effecting lipase production using design of experimental

The experimental lipase activity is given in Table 1. The Plackett–Burman experimental design proved to be a valuable tool for the rapid evaluation of the effects of the various medium components. Since this design is a preliminary optimization technique, which tests only two levels of each factor, it cannot provide the optimal quantity of each factor required for the optimum enzyme production. This technique, however, provides indications of how each factor tends to effect microbial growth and enzyme production (Mukherjee & Rai, 2011).

Estimated effects and coefficients for lipase activity are shown in Table 4. The magnitude of the effects indicates the level of the significance of the variable on lipase production. When the sign of the concentration effect of the tested variable was positive, the influence of the variable upon the lipase activity was greater at a high concentration, and when it was negative, the influence of the variable was greater at a low concentration (Gangadharan et al., 2008).

Table 4. *Estimated effects and coefficients for lipase activity according to the Plackett-Burman experimental design.*

Term	Effect	Coefficient	*p*- value
Constant		372.14	0
Glucose (% w/v)	-13.77	-6.88	0.668
Lactose (% w/v)	107.83	53.91	0.034
Sucrose (% w/v)	-45.88	-22.94	0.212
$(NH_4)_2SO_4$ (% w/v)	-95.03	-47.52	0.047
$(NH_4)_2HPO_4$ (% w/v)	31.34	15.67	0.36
Yeast extract (% w/v)	-7.81	-3.91	0.805
Urea (% w/v)	3.83	1.92	0.903
Incubation time (day)	-13.37	-6.69	0.677

The results showed that the effects of lactose and ammonium sulfate concentrations had confidence levels >95% and were considered to influence the lipase activity significantly (Table 4). A similar observation has been reported using *Penicillium restrictum* in SSF (Palma et al., 2000). Also, Lima et al. (2003) found that lipase production in *Penicillium aurantiogriseum* was stimulated using ammonium sulfate.

The main effects of each parameter are presented in Figure 1, which serve as a measure to view individual components' contributions on the production. This was estimated based on the difference between the averages of measurements made at the high level (+1) and at the low level (−1) of each factor. It can be seen that sucrose and ammonium sulfate were the factors that highly affect the lipase production at negative levels, followed by glucose, incubation time and yeast extract. It is not surprising that glucose appeared non-contributory to the production, because reports on lipase production linked its presence with catabolite repression (Salihua et al., 2011).

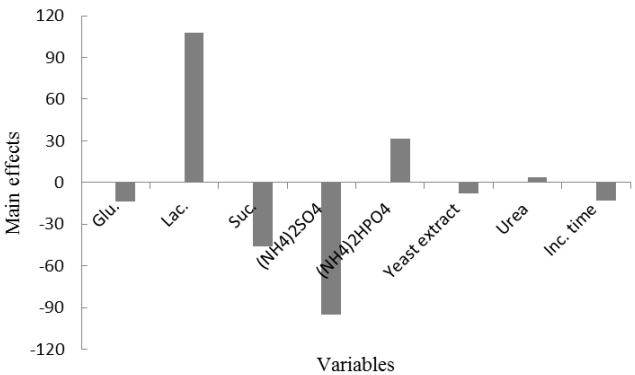

Figure. 1. *Main effects of the medium constituents for lipase production based on the Plackett–Burman experimental results.*

At positive levels, the medium components affecting the lipase production could be ranked as lactose > di-ammonium hydrogen phosphate > urea.

Since Plackett–Burman design on its own does not determine the exact quantity of components to be used in further experiments, but rather provides information about each factor. On the basis of calculated *p*-values (Table 4), lactose and ammonium sulfate were chosen for further optimization by RSM. Incubation time (day) was kept at low level.

The CCD was conducted in the vicinity of the optimum to locate the true optimum concentrations of lactose (X_2) and ammonium sulfate (X_4) for lipase production. The levels of the variables for the CCD experiments were selected according to the results of the previous experiments. The design matrix and the corresponding experimental data are given in Table 5.

The experimental results of the CCD were fit with a second-order polynomial equation:

$$Y = 428.6+618.9X_2–862.X_2^2–1652.X_4^2+1174.8\,X_2\,X_4 \qquad (2)$$

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Table 5. *Design and responses of the central composite design (CCD).*

Run	Lactose (%w/v)	$(NH_4)_2SO_4$ (%w/v)	Lipase activity. (U/g)
1	0.20	0.10	557.39
2	1.00	0.10	312.67
3	0.20	0.50	71.93
4	1.00	0.50	203.16
5	0.03	0.30	262.38
6	1.17	0.30	200.06
7	0.60	0.02	427.93
8	0.60	0.58	321.84
9	0.60	0.30	583.98
10	0.60	0.30	464.04
11	0.60	0.30	463.40
12	0.60	0.30	462.92
13	0.60	0.30	518.98

The plot of experimental values of lipase activity versus those calculated from Eq. (2) indicated a good fit, as presented in Figure 2. Coefficient of determination (R^2) is defined to be the ratio of the explained variation to the total variation and is a measurement of the degree of fitness (Nath & Chattopadhyay, 2007). The closer of R^2 value to unity, the better the empirical models fits the actual data (Sin et al., 2006). On the other hand, the smaller of R^2 value the less relevance the dependent variables in the model have in explaining the behavior of variations (Lee et al., 2006). By analysis of variance, the R^2 value of this model was determined to be 0.875. Therefore, the developed model could adequately represent the real relationship among the parameters chosen.

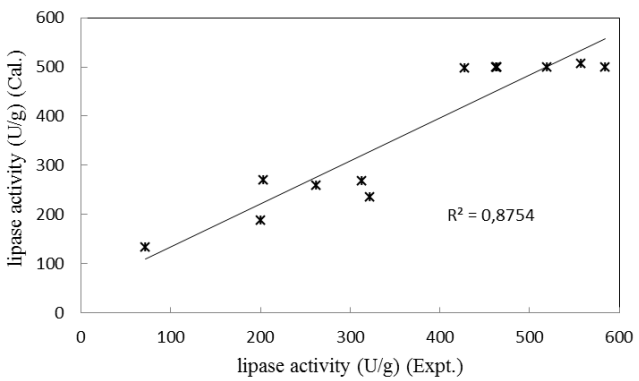

Figure 2. *Correlation of calculated with experimental lipase activity.*

The results of analysis of variance (ANOVA) are shown in Table 6. The *p*-value of the model was 0.005 (Table 6).

Table 6. *Analysis of variance (ANOVA) of the regression parameters for the response surface model.*

Source	DF [a]	SS [b]	MS [c]	*F*-value	*P*-value
Model	5	258479	51696	9.83	0.005
Residual	7	36800	5257		
Lack of fit	3	25388	8463	2.97	0.16
Pure error	4	11411	2853		
Total	12	295279			

Legend: [a] degree of freedom, [b] sum of squares, [c] mean square

Meanwhile, the lack of fit value of the model was 0.160 which was not significant. These two values confirmed that the model fitness was good. The lack of fit, which measures the fitness of the model, was found to be non-significant ($p > 0.05$), indicating that the number of experiments were sufficient for determining the effect of independent variables on percentage fat yield (Montgomery, 2001).

The response surface plot described by the model *Y* is represented in Figure 3. The results show that the optimal concentration for the two components obtained from the maximum point of the model was 0.42 (% w/v) for lactose and 0.09 (% w/v) for ammonium sulfate.

Lipase extraction from the solid fermented medium

Extraction of the enzyme from SSF was carried out distilled water, NaCl (0.8%), $(NH_4)_2SO_4$ (0.5%) buffer phosphate (pH=7) and Tween 80 (1%). The recovery of the enzyme was optimal with Tween 80 (1%). The lipase activity was as high as 936.67 U/g (Table 7). This could be due to the use of surfactant, Tween 80, which might increase the permeability of cells resulting in higher recovery of enzyme from SSF. Also, Edwinoliver et al. (2010) found that surfactants are the best compounds for enzyme extraction.

Table 7. *Lipase extraction from solid fermented medium using different solutions.*

Extraction solutions	Lipase activity (U/g)
Control (Distilled water)	548.61
Sodium chloride (0.8%)	664.43
Ammonium sulphate (0.5%)	653.36
Phosphate buffer (0.05 M, pH 7.0)	571.87
Tween 80 (1.0%)	936.67
NaCl (0.8%) + Tween 80 (1.0%)	876.61

Conversely, ammonium sulfate (0.5%) and sodium chloride (0.8%) prevented the enzyme release; this could be due to the increased hydrophobic interactions between lipase

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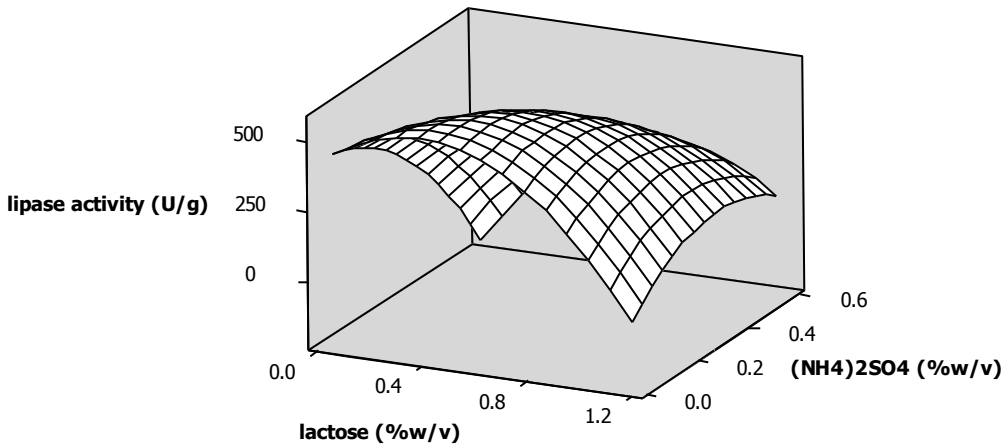

Figure 3. *Response surface plot for the effects of lactose and ammonium sulfate concentration on the lipase activity (Y).*

and solid support, thereby preventing the enzyme release as reported by Rodriguez et al. (2006). Buffer phosphate (pH=7) did not aid lipase recovery. However, supplementation of sodium chloride (0.8%) with Tween 80 helped the recovery of enzyme from SSF (Table 7).

Biochemical characterization on hydrolytic activity

Effect of pH and temperature on lipase hydrolytic activity and stability

The optimum activity of lipase was observed between pH 8.0 and 9.0 using *p*-NPP as the substrate. In compare with pH=7, there was a 25% increasing rate on the lipase activity. But the hydrolytic activity decreased sharply when the enzyme-substrate solution incubated at acidic pHs (below 7.0), since over 75% of the original activity was lost (Figure 4). In the pH range from 7.0 to 10.0, the enzyme was more stable when were incubated for 1 h (Figure 4). This enzyme is active and stable in alkaline media and it probably has a potential for different industrial applications such as leather manufacture, detergent formulation, pulp and paper manufacture, and dairy industry.

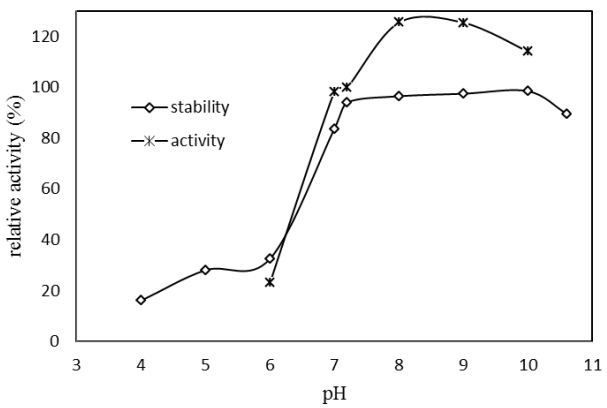

Figure 4. *Effect of pH on the stability and activity of the lipase produced by Rhizopus sp.*

The optimum temperature for the lipase activity was observed at 40°C with substantial activity between 30 and 50°C (Figure 5). At 50 and 60°C the activity decreased to 97% and 85%, respectively.

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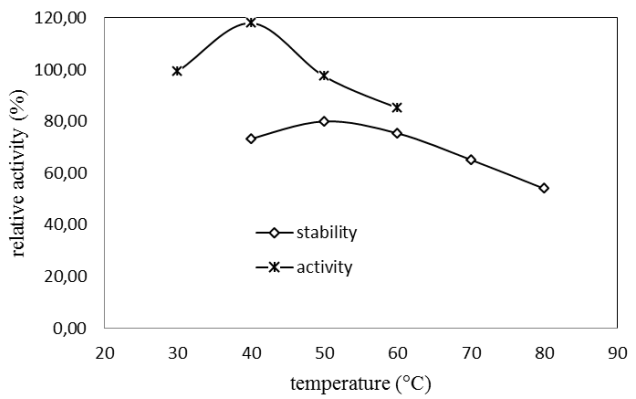

Figure 5. *Effect of temperature on the stability and activity of the lipase produced by Rhizopus sp.*

Thermostability was examined by measuring the residual activity after 1h of incubation at 40–80°C at pH=8. It was stable at 40-60ºC, since it retained greater than 75% of the initial activity after 1 h incubation (Figure 5). At 70 and 80°C the stability decreased to 65% and 53%, respectively. Our results showed that enzyme should be thermostable.

Some works presented in the literature report that the optimum temperature and pH for lipase activity is approximately 40°C and pH 8, respectively (Sun & Xu, 2009; Sun et al., 2009).

Effect of organic solvent on the lipase stability

In addition to activity, the stability of lipases in organic solvents is an important parameter for industrial applications. Log $P_{o/w}$ value is generally used to correlate solvent polarity with enzyme activity and stability in non-aqueous phases (Hernandez-Rodriguez et al., 2009). Generally, the less hydrophobic solvent (related to lower log $P_{o/w}$ values) has higher affinity to water and the more likely to strip the essential water from the enzyme molecules (Sun & Xu, 2009).

In order to determine lipase stability, each organic solvent was incubated for 1 h in different organic solvents at 30°C; then the residual hydrolytic activity was measured with *p*-NPP as the substrate. The effects of various organic solvents on the stability of the lipase are shown in Table 8. Organic solvents such as 2-ethyl-1-hexanol and oleyl alcohol enhanced the activity of enzyme by 23.88%, and 8.01%, respectively, when compared to the control. 1,3-butanediol, 2,3-butanediol and 1-propanol were destabilizing enzyme activity, resulting in separately 63.35%, 56.40% and 56.31% of activity loss. Lower relative stability was also observed in 2-methyl-1-propanol. In summary, the stability of our lipase in organic solvents follow the log $P_{o/w}$ trends and there was clear correlation between the stability of enzyme and the log $P_{o/w}$ values (exception for oleyl alcohol) (Table 8).

Table 8. *Effect of organic solvents on the lipase activity.*

Organic solvents	log $P_{o/w}$	Residual activity (%)
2,3-butanediol	-0.36	43.6
1,3-butanediol	0.74	36.65
1-propanol	0.33	43.69
2-methyl-1-propanol	0.76	78.82
1-Hexanol	1.85	98.64
2-ethyl-1-hexanol	2.71	123.88
Oleyl alcohol	7.5	108.01

The obtained results suggest that the activity values are influenced by several phenomena, including not only inhibition and activation, but also denaturation.

Effects of metal ions on lipase hydrolytic activity

The effect of various ions at a concentration of 10 mM on lipase hydrolytic activity was also assessed (Figure 6). Inhibitory effects were observed in the presence of Zn^{2+}, Co^{2+} and Fe^{3+} after 1 h-incubation with 35– 50% for enzyme. On the other hand, the enzyme was positively activated by 39% in the presence of K^{+} in comparison to the activity in the absence of this ion.

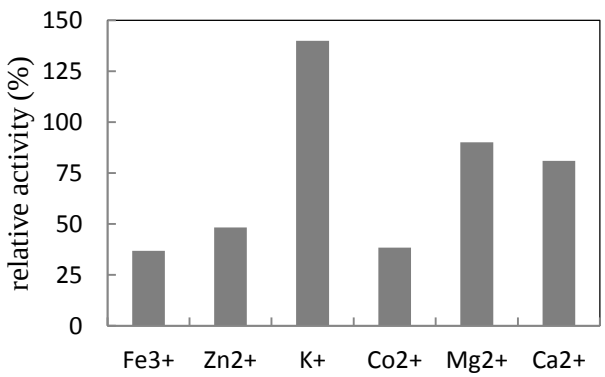

Figure 6. *Effect of various ions on the hydrolytic activity of the lipase produced by Rhizopus sp.*

Many enzymes have been found to require certain metals to sustain or enhance activity and/or stability (Salameh & Wiegel, 2007). Metal enzymes and metal-activated enzymes both belong to this group. Metal enzymes contain tightly bound metal ion cofactors, mostly commonly transition ions

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such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+} and Zn^{2+}. In contrast, metal-activated enzymes loosely bind metal ion from solution, usually the alkali metal ions Na^{+} and K^{+} (Rahman et al., 2005). The lipase produced by *Rhizopus sp.*, in this study, was strongly inhibited by Fe^{3+}, $Zn^{2+}$and Co^{2+} but stimulated by alkaline metal ions, K^{+} ,after 60 min of incubation, thus, this enzyme is suggested to be a metal-activated enzyme.

Michaelis–Menten kinetic parameters

Lipase activity showed a typical Michaelis–Menten profile with *p*-NPP as substrate; the curves were linear with correlation coefficient (R^2) of 0.998. The values of K_m and V_{max} were 1.94 g/l and 614.06 U/g for substrate. The values of K_m indicate that the produced enzyme had a relatively high affinity for *p*-NPP. The catalytic efficiency (V_{max} / K_m) of lipase was found to be higher for *p*-NPP (316.52). The catalytic efficiency value provides a practical model for selecting the most efficient enzyme for a commercial application process using a fixed initial substrate concentration (Ortega et al., 2004).

Thermodynamic parameters for substrate hydrolysis

The deactivation rate is proportional to the active enzyme concentration, and k_d (deactivation rate constant) is the proportional constant. The deactivation process is modeled as first-order kinetics and the deactivation rate constant was evaluated (Figure 7).

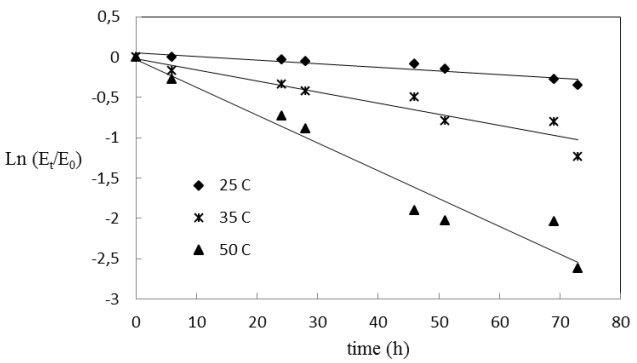

Figure 7. *Thermal inactivation kinetic of lipase at different temperatures.*

It is also observed that at higher temperatures, the deactivation occurs slowly (Figure 8). Daniel (1996) observed that protein degradative reactions at high temperature occur only slowly in conformationally intact proteins, implying that the structural conformation of the protein dictates upper temperature limit for enzyme activity.

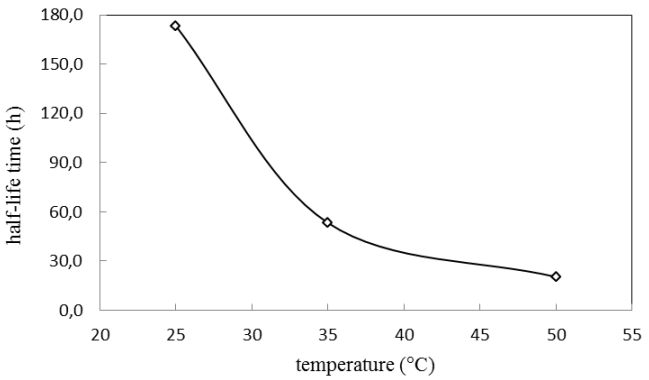

Figure 8. *Effect of temperature on half-life time during deactivation of lipase.*

In order to understand the behavior of lipase and the complex process of enzyme deactivation thermal inactivation thermodynamic parameters (ΔG^*, ΔH^*, ΔS^*, and E_d) were determined. The change in enthalpy (ΔH^*) and entropy (ΔS^*) were estimated based on the transition state theory. The ΔS^* values were obtained negative for system used in this study (Table 9), which is unique property of biocatalyst processes. The solvent and structural effects are two important factors which influence the numeric values of ΔH^* and ΔS^*. The negative values of ΔS^* may be due to aggregation of the partially unfolded enzyme molecules. The Gibbs free energy (ΔG^*) measures the combination of changes in heat and entropy that occurs during the spontaneity of a reaction. The resistance of enzyme to thermal denaturation is due to the 'intrinsic' contribution of polypeptide chain i.e. hydrophobic interactions, hydrogen bonding and ionic stabilization (Maisuria & Nerurkar, 2012).

The temperature dependency of first-order deactivation rate constant was studied by Arrhenius equation [Eq. (8)]. The activation energy E_d was estimated and it is shown in Table 9.

Table 9. *Estimated thermodynamic parameters during the thermal deactivation of enzyme* [a].

ΔH^* (kJ.mol^{-1})	ΔS^* (J.mol^{-1}.K^{-1})	E_d (kJ.mol^{-1})	ΔG^* (kJ.mol^{-1})[b]
64.96	-71.91	67.54	86.40-88.19

Legend: [a] R^2 for plot of ln(k_d/T) versus 1/T is 0.975; R^2 for plot of ln(k_d) versus 1/T is 0.977. [b] The temperature range was 25–50°C.

Conclusion

During the recent years, efforts have been directed to explore the means to reduce the enzyme production costs

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through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for enzyme production (Singhania et al., 2009). Our finding showed that combination of rice straw and Mary olive cakes could be used as suitable and low-cost substrate for lipase production. The statistical optimization of enzyme production showed that lactose has a significant effect on lipase production so whey as could be a suitable candidate for the lipase production as low cost dairy waste. Lipase extraction from the fermented solid was also studied. Efficient recovery of the enzyme was achieved using Tween 80 at 1.0% (w/v). The pH and temperature optimal activity for lipase were pH 8-9 and 40°C, respectively. Lipase produced by isolated fungus was characterized with respect to its biochemical properties including the thermodynamic parameters of substrates hydrolysis and thermal behavior. Our studies suggested that this enzyme can be considered as potential candidate for various applications like leather manufacture, detergent formulation, food and textile industries.

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