African Journal of Biotechnology Vol. 10(81), pp. 18704-18708, 16 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2741
ISSN 1684–5315 ©2011 Academic Journals

Full Length Research Paper

Evaluation of nutritional components by Plackett-Burman design for *Penicillium citrinum* lipase production using palm oil mill effluent

Aliyu Salihu[1,2], Md. Zahangir Alam[1]*, M. Ismail AbdulKarim[1] and Hamzah, M. Salleh[1]

[1]Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), 50728 Kuala Lumpur, Gombak, Malaysia.
[2]Department of Biochemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Accepted 7 November, 2011

A number of medium components influencing lipase production by *Penicillium citrinum* (ATCC 42799) were studied using palm oil mill effluent (POME) as the basal medium. The medium components (peptone, yeast extract, malt extract, NH_4Cl, $NaNO_3$, KH_2PO_4, $CaCl_2$, $MgSO_4$, olive oil and Tween-80) were analyzed in twelve experimental trials using Plackett–Burman (PB) design. The most significant components affecting lipase production were found to be Tween-80, peptone, yeast extract, malt extract and $NaNO_3$ at $p < 0.05$. The results indicate the efficiency of using PB design for screening processes. However, optimal concentration of the significant components can be determined by further statistical analysis.

Key words: Lipase, *Penicillium citrinum,* Plackett–Burman design, palm oil mill effluent.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that are unique in catalyzing the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface, and have the ability of reversing the reaction in non-aqueous media (Saxena et al., 2003). Microbial lipases are currently receiving more attention than lipases from plants and animals because of their diversity in catalytic activity, high yield and low cost production, as well as relative ease of genetic manipulation. Moreover, micro-bial lipases are also stable in organic solvents, requiring no cofactor and possess broad substrate specificity (Gupta et al., 2004; Sharma et al., 2001). The recent interest in the production of lipases is associated with their applications as additives in food (flavor modification), fine chemicals (synthesis of esters), detergent (hydrolysis of fats), waste water treatment (decomposition and removal of oily substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather processing (removal of lipids from animal skins) and biomedical assays (blood triglycerides) (Burkert et al., 2004; Elibol and Ozer, 2002; Kamini et al., 2000). Additionally, lipases have an important application in the field of bioenergy, especially in biodiesel production, which is an expanding sector, as a result of the worldwide rising demand on the use of renewable energy (Colla et al., 2010). Thus, a research is on to ensure the reduction of lipase production costs, which is the major problem that often restricts its use. Carbon source was estimated to account for about half of the final production cost (Miranda et al., 1999). The use of inexpensive substrates can make the process economically viable.

In Malaysia, large amounts of palm oil mill effluent (POME) produced from palm oil mills could be used as a basal medium for lipase production. This effluent has been characterized as a brownish colloidal suspension with 95 to 96% water, 0.6 to 0.7% oil, 2 to 4% suspended solids, high organic and inorganic nutrients, and carbohydrates ranging from hemicellulose to simple sugars (Foo

*Corresponding author. E-mail: zahangir@iium.edu.my or zahangir@yahoo.com. Tel: +603-61964571. Fax: +603-61964442.

Abbreviations: PB, Plackett–Burman; **POME,** palm oil mill effluent.

and Hameed, 2010; Habib et al., 1997). Therefore, designing an appropriate fermentation medium using a productive microbial strain is of crucial importance to improve the efficiency and productivity of lipase fermentation process because medium composition can significantly affect the product concentration, yield, volumetric production and the ease and cost of downstream product separation (Kennedy and Krouse, 1999).

The present investigation was aimed at evaluating the effects of medium components on *Penicillium citrinum* lipase production using palm oil mill effluent as a basal medium through Plackett-Burman design as an initial screening step in formulating a suitable medium for maximizing the lipase production.

MATERIALS AND METHODS

Sample collection and reagents

POME was collected from West Oil Mill of Sime Darby Sdn Bhd. Carey Island Malaysia in clean containers and immediately brought to the laboratory and stored at 4°C. *p*-nitrophenyl palmitate (*p*-NPP) for lipase assay was purchased from Sigma (St. Louis, USA). All other reagents used were of analytical grade.

Microorganism and inoculum preparation

Penicillium citrinum (ATCC 42799) was obtained from American Type Culture Collection (ATCC). The stock culture was maintained on potato dextrose agar (PDA) plates and subcultured monthly. Inoculum preparation was done according to Alam et al. (2004). Seven-day PDA plate of *P. citrinum* (ATCC 42799) was washed with 25 ml of sterile distilled water using a bent glass rod followed by filtration through Whatman No.1 filter paper to remove the mycelia from the spore suspension. The filtrate (spore suspension) was then used as inoculum after measuring the spore concentration (10^7 spore/ml) using a hemocytometer.

Selection of important media components by Plackett–Burman design

Plackett–Burman (PB) design was used to screen the important medium constituents that influence the lipase production. The POME sample having 1.0% (w/v) of total suspended solids (TSS) was prepared by addition of distilled water into the original sample. This was used as a basal medium where the eleven medium constituents screened were dissolved. The constituents studied include glucose as carbon source; peptone, yeast extract, malt extract, NH_4Cl and $NaNO_3$ as nitrogen sources; KH_2PO_4, $CaCl_2$ and $MgSO_4$ as inorganic mineral sources, olive oil and Tween-80 as inducers.

Design Expert 6.0.8 (Start Ease Inc., Minneapolis, USA) was used to generate a set of 12 experimental runs based on PB design, each variable was examined at two levels: low level (-1) and high level (+1). Table 1 presents the design of the variables under investigation as well as the response obtained in the experimental design. All experiments were carried out in triplicate and the averages of lipase activity were taken as response. The PB design was based on linear equation model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

Where, Y is the response (lipase activity); β_0 is the model intercept; β_i is the linear coefficient and X_i is the level of the independent variable. This model does not describe the interaction among the factors, as such, it is only used to evaluate and select the important factors that influence the response.

Fermentation medium preparation and lipase production

Lipase production medium was prepared using POME (1% total suspended solid, TSS) as the basal medium, containing different concentrations of nutrients that were tested according to the statistical design of experiments. The initial pH was adjusted to pH 6.0 using 1 M NaOH and then sterilized at 121°C and 15 psi for 15 min. Two percent (2% v/v) of the prepared inoculum (*P. citrinum*) was added each to 50 ml medium in 150 ml Erlenmeyer flasks according to the design. The flasks were incubated for 7 days at 28 ± 2°C under orbital shaking at 150 rpm. After the incubation, the culture broth was centrifuged at 10,000 x*g* for 10 min (4°C) and the cell-free supernatant was used as a source of extracellular lipase.

Assay for lipase activity-colorimetric method

Lipase activity was assayed quantitatively as described by Gopinath et al. (2005) using *p*-nitrophenyl palmitate (*p*NPP) as the substrate. First, 10 ml of isopropanol containing 30 mg *p*NPP was mixed with 90 ml of 0.05 M sodium phosphate buffer (pH 8.0) containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total amount of 2.4 ml of freshly prepared substrate solution was pre-warmed at 37°C and mixed with 0.1 ml enzyme solution. After 15 min incubation at 37°C, the absorbance at 410 nm was measured against an enzyme-free control. One enzyme unit was defined as 1 µmol *p*-nitrophenol enzymatically released from the substrate in milliliter per minute (ml/min). All the enzyme assays were carried out in triplicate and the average values were calculated.

RESULTS AND DISCUSSION

Plackett-Burman design is a well-established and widely used statistical design for the screening and selection of medium components in shake flask cultures. PB design offers a good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which saves time and maintains convincing information on each component (Abdel-Fattah et al., 2005). A total of eleven medium components were analyzed with regard to their effects on lipase production using a PB design. For each run, the experimental together with the predicted lipase activity from the regression equation (II) for the combinations are shown in Table 1. Based on the design matrix selected for the screening of significant variables, the highest lipase activity realized was 0.651 U/ml at run 11 and the lowest amount was observed in run 6 (0.004 U/ml), where all the components were at their low concentration levels.

Thus, of the eleven components screened during PB design, all except glucose affected the response at a positive level (Figure 1). Glucose was the only one that affected the lipase production at a negative level. This is in agreement with several findings where glucose was found to have inhibitory effects on lipase production.

Table 1. Plackett-Burman experimental design for evaluation of 11 components with the actual and coded values for lipase production by *P. citrinum* (ATCC 42799), showing the experimental and predicted response.

Run	Parameter*											Lipase activity (U/ml)	
	G% (w/v)	O% (v/v)	T % (v/v)	P % (w/v)	YE % (w/v)	ME % (w/v)	NH_4Cl % (w/v)	$NaNO_3$ % (w/v)	KH_2PO_4 % (w/v)	$CaCl_2$ % (w/v)	$MgSO_4$ % (w/v)	Experimental	Predicted
1	0.5(+1)	0.1(–1)	0.5(+1)	0.5(+1)	0.0(–1)	0.4(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.01(+1)	0.2(+1)	0.451	0.450
2	0.0(–1)	0.5(+1)	0.5(+1)	0.0(–1)	0.5(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.2(+1)	0.01(+1)	0.2(+1)	0.474	0.470
3	0.0(–1)	0.1(–1)	0.0(–1)	0.5(+1)	0.5(+1)	0.4(+1)	0.0(–1)	0.2(+1)	0.2(+1)	0.0(–1)	0.2(+1)	0.618	0.620
4	0.0(–1)	0.5(+1)	0.5(+1)	0.5(+1)	0.0(–1)	0.4(+1)	0.3(+1)	0.0(–1)	0.2(+1)	0.0(–1)	0.0(–1)	0.568	0.570
5	0.5(+1)	0.5(+1)	0.0(–1)	0.5(+1)	0.5(+1)	0.0(–1)	0.3(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.2(+1)	0.392	0.400
6	0.0(–1)	0.1(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.0(–1)	0.004	0.007
7	0.0(–1)	0.5(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.4(+1)	0.3(+1)	0.2(+1)	0.0(–1)	0.01(+1)	0.2(+1)	0.348	0.340
8	0.5(+1)	0.1(–1)	0.0(–1)	0.0(–1)	0.5(+1)	0.4(+1)	0.3(+1)	0.0(–1)	0.2(+1)	0.01(+1)	0.0(–1)	0.334	0.330
9	0.5(+1)	0.5(+1)	0.0(–1)	0.5(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.2(+1)	0.2(+1)	0.01(+1)	0.0(–1)	0.361	0.360
10	0.0(–1)	0.1(–1)	0.5(+1)	0.5(+1)	0.5(+1)	0.0(–1)	0.3(+1)	0.2(+1)	0.0(–1)	0.01(+1)	0.0(–1)	0.649	0.650
11	0.5(+1)	0.5(+1)	0.5(+1)	0.0(–1)	0.5(+1)	0.4(+1)	0.0(–1)	0.2(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.651	0.650
12	0.5(+1)	0.1(–1)	0.5(+1)	0.0(–1)	0.0(–1)	0.0(–1)	0.3(+1)	0.2(+1)	0.2(+1)	0.0(–1)	0.2(+1)	0.348	0.350

*G - glucose, O - olive oil, T - Tween-80, P - peptone, YE - yeast extract, ME - malt extract, (–1) indicates the low level and (+1) indicates the high level.

Brozzoli et al. (2009) reported a significant decrease in lipase production by *Candida cylindracea* NRRL Y-17506 from 6.4 ± 0.6 to 0.62 ± 0.14 U/ml in the presence of glucose at 5 g/L.

Based on this, Ferrer et al. (2001) indicated that microbial synthesis of lipases as a function of their regulation mechanisms can be grouped into two classes, one of which is constitutively expressed and the other is induced by free fatty acids. Thus, synthesis of inducible lipases is inhibited at a transcriptional level by the presence of glucose, while oleic acid appears to hinder the synthesis of the constitutive ones.

The first order model equation developed by PB design showed the dependence of *P. citrinum* lipase production on the medium constituents:

Y (Lipase activity U/ml) = +0.43 - 0.010A + 0.033B + 0.090C + 0.073D + 0.087E + 0.062F + 6.574E - 003G + 0.063H + 0.017J + 5.278E - 003L (II)

where, A, B, C, D, E, F, G, H, J and L represent the concentrations of glucose, olive oil, Tween-80, peptone, yeast extract, malt extract, ammonium chloride, sodium nitrate, dihydrogen potassium phosphate and magnesium sulphate, respectively. Statistical analysis of the responses is represented in Table 2. The model F value of 324.06 implies that the model is significant. The values of Prob < 0.05 indicate that model terms are significant. A *p*-value of less than 0.05 for the five variables viz., peptone, malt extract, yeast extract, Tween-80 and $NaNO_3$ indicates that they are significant. Also, olive oil was found to be at the margin as its *p*-value was found to be 0.0597. The other inorganic mineral sources (KH_2PO_4 and $MgSO_4$) used in this study were not significant (Table 2), despite the positive effects shown in Figure 1. Although, inorganic minerals are required by microorganisms in small amounts during lipase production, POME on its own was reported to contain essential amounts of amino acids, inorganic nutrients (sodium, potassium, calcium, magnesium, manganese, and iron), nitrogenous constituents, free organic acids and carbohydrates ranging from hemicellulose to simple sugars (Foo and Hameed, 2010; Habib et al., 1997), as such addition of minerals into this medium is not required. In addition, the predicted R^2 was found to be 0.9556, which is in reasonable agreement with the R^2 of 0.9997 and adjusted R^2 of 0.9966. This revealed that there is good agreement between the experimental and the theoretical values predicted by the model and almost all the variation could be accounted for by the model equation. Overall, the percentage contribution of the significant variables indicated

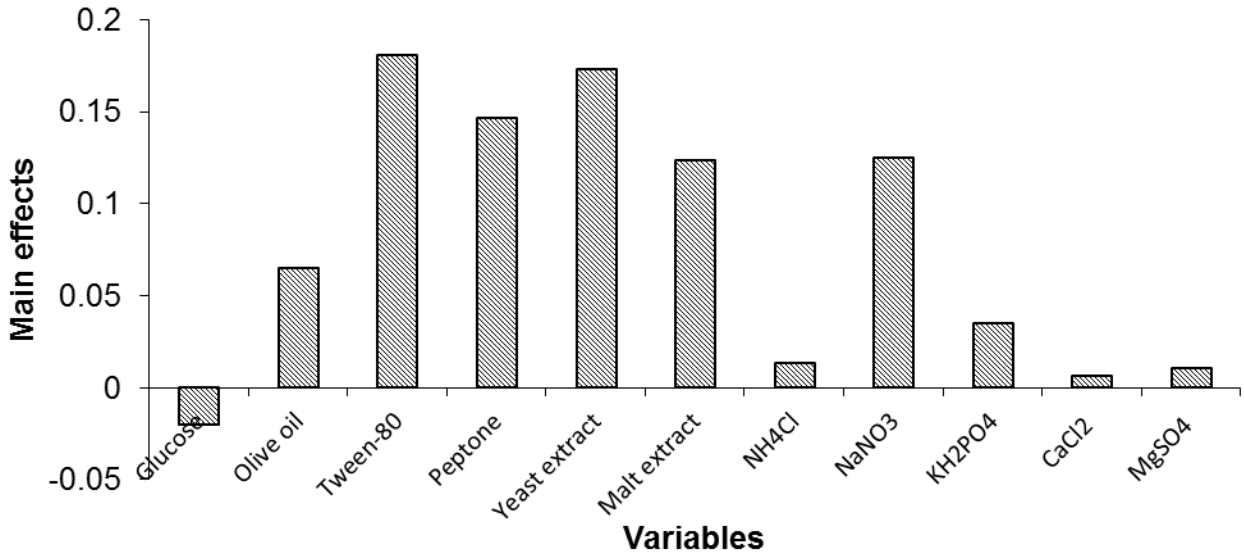

Figure 1. Main effects of the medium constituents on *P. citrinum* lipase production by the Plackett–Burman experimental results.

Table 2. Statistical analysis (ANOVA) for evaluating the significance of variables.

Source	Sum of squares	F-value	*p*-value[a]	
Model	0.363155	324.0614	0.0432	Significant
A (Glucose)	0.001268	11.31062	0.1840	
B (Olive oil)	0.012675	113.1079	0.0597	
C (Tween-80)	0.097802	872.7342	0.0215*	
D (Peptone)	0.064371	574.4168	0.0265*	
E (Yeast extract)	0.089942	802.5951	0.0225*	
F (Malt extract)	0.045771	408.4383	0.0315*	
G (NH_4Cl)	0.000519	4.628432	0.2770	
H ($NaNO_3$)	0.046876	418.2946	0.0311*	
J (KH_2PO_4)	0.003598	32.105	0.1112	
L($MgSO_4$)	0.000334	2.983216	0.3341	

R^2 = 0.9997; adjusted R^2 = 0.9966; predicted R^2 = 0.9556, adequate precision = 63.804, cummulative variance = 2.44

[a]significant at probability > F < 0.05. **p* < 0.05 was considered to be significant.

that 27% was for Tween-80, 25% for yeast extract, 18% for peptone, 13% for malt extract and $NaNO_3$ each, 3% for olive oil and the remaining 1% for KH_2PO_4 (Figure 2).

It is not surprising that Tween-80 appeared to be the most contributing variable during lipase production by *P. citrinum* in POME based medium with the highest percent contribution and *p*-value of 0.0215; this is because it can serve as a carbon source and an inducer. The oleic acid present gives it the ability to act as an inducer, especially for extracellular lipases. The lipase production of *Rhizopus chinensis* was improved by oleic acid related surfactants, Span-80 and Tween-80 (Wang et al., 2008). Maliszewska and Mastalerz (1992) showed the stimulatory effects of lipase production by *P. citrinum* where the maximum stimulation (5.5-fold increase of lipase production) was observed at 0.7% Tween-80 with no inhibitory effect.

The type of nitrogen source influences the production levels of lipases, organic nitrogen sources are favoured by *Aspergillus wentii*, *Mucor racemosus* and *R. nigricans* (Ghosh et al., 1996), while inorganic nitrogen sources proved effective for *C. cylindracea* (Brozzoli et al., 2009) and *P. citrinum* (Miranda et al., 1999) in olive mill waste water and groundnut oil refinery residue, respectively. However, the work of Pimentel et al. (1997) showed that organic nitrogen sources led to higher lipase production of 2850 U/L by *P. citrinum* as compared to 1585 U/L when ammonium sulfate was used. This indicated that variation in nitrogen sources could lead to 55% reduction in *P. citrinum* lipase production. This is in agreement with

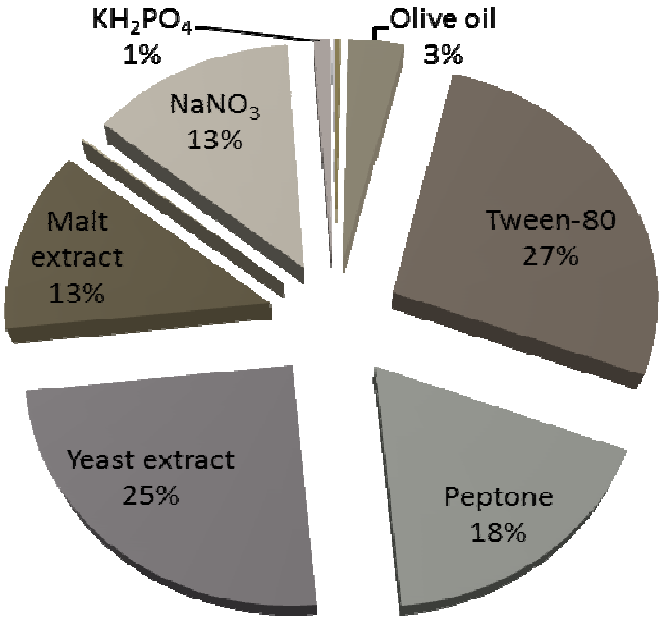

Figure 2. Pie chart representing the percentage contribution of the nutrient components.

the findings of this work, since the organic nitrogen sources (peptone, yeast extract and malt extract) were found to contribute significantly to the lipase production.

Conclusion

This design allows reliable short listing of a small number of parameters for further optimization and allows one to obtain unbiased estimates of linear effects of all the factors with maximum accuracy for a given number of observations, the accuracy being the same for all effects. The results also showed the use of cheap and available agro-residue (POME) as a basal medium for lipase production.

Thus, the present study identified the effect of various constituents on the enzyme yield and the production was found to be significantly influenced by nutritional components viz., Tween-80, peptone, yeast extract, malt extract and $NaNO_3$.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Biotechnology Engineering for providing the laboratory facilities and to West Oil Mill, Sime Darby Plantation for the experimental samples.

REFERENCES

Abdel-Fattah YR, Saeed HM, Gohar YM, El-Baz MA (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochem. 40: 1707-1714.

Alam MZ, Fakhru'l-Razi A, Molla AH (2004). Evaluation of fungal potentiality for bioconversion of domestic wastewater sludge. J Environ Sci. 16:132-137.

Brozzoli V, Crognale S, Sampedro I, Federici F, D'Annibale A, Petruccioli M (2009). Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor. Bioresour. Technol. 100: 3395-3402.

Burkert JFM, Maugeri F, Rodrigues MI (2004). Optimization of extracellular lipase production by *Geotrichum sp.* using factorial design. Bioresour. Technol. 91: 77-84.

Colla LM, Rizzardi J, Pinto MH, Reinehr CO, Bertolin TE, Vieira Costa JA (2010). Simultaneous production of lipases and biosurfactants by submerged and solid-state bioprocesses. Bioresour. Technol. 101: 8308-8314.

Elibol M, Ozer D (2002). Response surface analysis of lipase production by freely suspended *Rhizopus arrhizus*. Process Biochem. 38: 367-372.

Ferrer P, Montesinos JL, Valero F, Sola C (2001). Production of Native and Recombinant Lipases by *Candida rugosa*. Appl. Biochem. Biotechnol. 95: 221-256.

Foo KY, Hameed BH (2010). Insight into the applications of palm oil mill effluent: A renewable utilization of the industrial agricultural waste. Renew. Sustain. Energy. Rev. 14: 1445-1452.

Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson S (1996). Microbial lipases: production and applications. Sci. Prog. 79(2): 119-157.

Gopinath SCB, Anbu P, Hilda A (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Mycosci. 46: 119-126.

Gupta R, Gupta N, Rathi P (2004). Bacterial lipases: an overview of production, purification and biochemical properties. Appl. Microbiol. Biotechnol. 64: 763-781.

Habib MAB, Yusoff FM, Phang SM, Ang KJ, Mohamed S (1997). Nutritional values of chironomid larvae grown in palm oil mill effluent and algal culture. Aquaculture 158: 95-105.

Kamini NR, Fujii T, Kurosu T, Iefuji H (2000). Production, Purification and characterization of an extracellular lipase from yeast, *Cryptococcus* sp. S-2. Process Biochem. 36: 317-324.

Kennedy M, Krouse D (1999). Strategies for improving fermentation medium performance: a review. J. Ind. Microbiol. Biotechnol. 23: 456-475.

Maliszewska I, Mastalerz P (1992). Production and some properties of lipase from *Penicillium citrinum*. Enzyme Microb. Technol. 14: 190-193.

Miranda OA, Salgueiro AA, Pimentel MCB, LimaFilho JL, Melo EHM, Dur'an N (1999). Lipase production by Brazillian strain of *Penicillium citrinum* using an industrial residue. Bioresour. Technol. 69: 145-147.

Pimentel MCB, Melo EHM, Filho JLL, Ledingham WM, Durán N (1997). Lipase from a Brazilian Strain *Penicillium citrinum* Cultured in a Simple and Inexpensive Medium: Heat-Denaturation, Kinetics, and pH Stability. Appl. Biochem. Biotechnol. 66(2): 185-188.

Saxena RK, Sheoran A, Giri B, Davidson WS (2003). Purification strategies for microbial lipases. J. Microbiol. Methods 52: 1-18.

Sharma R, Chisti Y, Banerjee UC (2001). Production, purification, characterization, and applications of lipases. Biotechnol. Adv. 19: 627-662.

Wang D, Xu Y, Shan T (2008). Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media. Biochem. Eng. J. 41: 30-17.