Statistical screening of medium components affecting lipase production using Palm oil mill effluent by *Penicillium citrinum*

Aliyu Salihu, Md. Zahangir Alam, Mohamed Ismail Abdulkarim and Hamzah Mohd Salleh
Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, 50728, International Islamic University Malaysia.
Phone: +603-61964571; Fax: +603-61964442. Email: zahangir@iium.edu.my

Abstract— **A number of medium components influencing lipase production by *Penicillium citrinum* (ATCC 42799) were studied using palm oil mill effluent 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 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 indicated the efficiency of using PB design for screening processes. However, optimal concentration of the significant components can be determined by further statistical analysis.**

Keywords: 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, with the ability of reversing the reaction in non-aqueous media [1]. 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, microbial lipases are also stable in organic solvents, requiring no any cofactors and possess broad substrate specificity [2, 3].
The recent interest in the production of lipases is associated with its 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) [4, 5, 6]. 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 [7]. Thus, a search is on to ensure the reduction of lipase production costs, which is the major problem that often restricts its use. Since carbon source was estimated to account for about half of the final production cost [8]. 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 95–96% water, 0.6–0.7% oil, 2–4% suspended solids, high organic and inorganic nutrients, and carbohydrates ranging from hemicellulose to simple sugars [9, 10]. 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 [11].
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 by 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

Palm oil mill effluent (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. [12]. 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 the 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:

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

Where, Y is the response (lipase activity), β_o is the model intercept, β_i is the linear coefficient, and Xi 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 1M NaOH and then sterilized at 121°C and 15psi 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°C under orbital shaking at 150 rpm. After the incubation, the culture broth was centrifuged at 10,000 x g for 10 min at 4°C in 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. [13] using *p*-nitrophenyl palmitate (*p*NPP) as the substrate. First, 10ml isopropanol containing 30mg *p*NPP was mixed with 90ml 0.05M sodium phosphate buffer (pH 8.0) containing 207mg sodium deoxycholate and 100mg gum arabic. A total amount of 2.4ml freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1ml enzyme solution. After 15min incubation at 37°C, absorbance at 410nm was measured against an enzyme-free control. One enzyme unit was defined as 1µmol *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 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 [14]. A total of eleven medium components were analyzed with regard to their effects on lipase production using a PB design. The design matrix selected for the screening of significant variables and the corresponding responses are shown in Table 1. The lipase activity realized showed a wide variation from 0.004U/ml to 0.651 U/ml.
Thus, of the eleven components screened during PB design, all except glucose affected the response at a positive level (Fig. 1). Glucose was the only one affecting 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. Brozzoli, et al. [15] reported a significant decrease in lipase production by *Candida cylindracea* NRRL Y-17506 from 6.4 ± 0.6 U/ml to 0.62 ± 0.14 U/ml in the presence of glucose at 5g/L. Based on this, Ferrer, et al. [16] indicated that microbial synthesis of lipases as a function of their regulation mechanisms can be grouped in 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.
Statistical analysis of the responses were performed which 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 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.

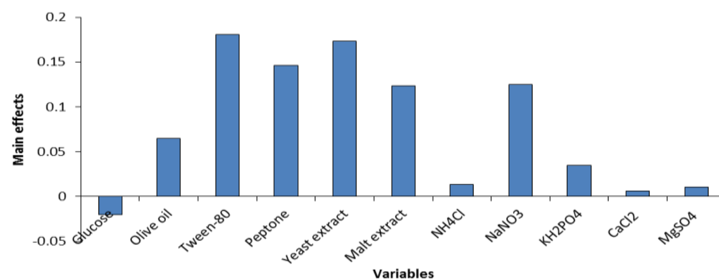

Fig. 1: Main effects of the medium constituents on *P. citrinum* lipase production by the Plackett–Burman experimental results

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) and the design response

Run	G %(w/v)	O %(w/v)	T %(w/v)	P %(w/v)	YE %(w/v)	ME %(w/v)	NH_4Cl %(w/v)	$NaNO_3$ %(w/v)	KH_2PO_4 % (w/v)	$CaCl_2$ % (v/v)	$MgSO_4$ %(v/v)	Lipase activity (U/ml)
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
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
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
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
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
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
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
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
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
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
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
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

G, glucose., O, Olive oil., T, Tween-80., P, peptone., YE; Yeast extract., ME, Malt extract. The (–1) indicates the low level, (+1) indicates the high level

All the inorganic mineral sources (KH_2PO_4, $CaCl_2$ and $MgSO_4$) used in this study were found to be not significant (Table 2), despite the positive effects shown in Fig. 1. Although inorganic minerals are required by microorganisms in small amounts during lipase production, but 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 [9, 10]; as such addition of minerals into this medium is not required.

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

Source	Sum of Squares	df[a]	Mean Square	F-value	*p*-value[b]	
Model	0.363155	10	0.036315	324.0614	0.0432	significant
A (Glucose)	0.001268	1	0.001268	11.31062	0.1840	
B (Olive oil)	0.012675	1	0.012675	113.1079	0.0597	
C (Tween-80)	0.097802	1	0.097802	872.7342	0.0215*	
D (Peptone)	0.064371	1	0.064371	574.4168	0.0265*	
E (Yeast extract)	0.089942	1	0.089942	802.5951	0.0225*	
F (Malt extract)	0.045771	1	0.045771	408.4383	0.0315*	
G (NH_4Cl)	0.000519	1	0.000519	4.628432	0.2770	
H ($NaNO_3$)	0.046876	1	0.046876	418.2946	0.0311*	
J (KH_2PO_4)	0.003598	1	0.003598	32.105	0.1112	
L($MgSO_4$)	0.000334	1	0.000334	2.983216	0.3341	

R^2 = 0.9997; Adjusted R^2 = 0.9966; Predicted R^2 = 0.9556, Adequate precision = 63.804
[a] degree of freedom
[b] Significant at Prob > F < 0.05
* $p < 0.05$ were considered to be significant.

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. Overall, the percentage contribution of the significant variables indicated that 27% goes to tween-80, 25% to yeast extract, 18% to peptone, 13% to malt extract and $NaNO_3$ each and 3% to olive oil. It is not surprising that tween-80 appeared to be the most contributing variable in POME based medium with the highest percent contribution and *p*-value of 0.0215; this is because it can serve as both 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 [17]. Maliszewska and Mastalerz [18] 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 any 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* [19], while inorganic nitrogen sources proved effective for *C. cylindracea* [15] and *P. citrinum* [8] in olive mill waste water and groundnut oil refinery residue respectively.
However, the work of Pimentel, et al. [20] showed that organic nitrogen sources led to higher lipase production of 2850 U/L by *P. citrinum* 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 the findings of this work, since all the nitrogen sources (peptone, yeast extract, malt extract and $NaNO_3$) except NH_4Cl were found to contribute significantly to the lipase production.

CONCLUSION

This design allows reliable short listing of a small number of ingredients 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 [21]. 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 $NaNO_3$.

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