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Statistical Screening and Optimization of Factors that Influence Lipase Production from Palm Kernel Cake using *Candida cylindracea* in Solid-state Bioconversion

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Although lipases are important class of industrial enzymes but the high cost of their production limits their applications. In this study, an abundant raw material in Malaysia, palm kernel cake (PKC), was investigated as a substrate ina fermentation process to produce lipase using solid-state bioconversion (SSB) with minimum requirements of machinery and external nutrients. This requires an optimization process in order to achieve maximum production with the minimum requirements. Optimization for the parameters that influence lipase production from *Candida cylindracea* was conducted using statistical experimental designs including Plackett-Burman (PB) design, one-factor- at-a-time (OFAT) and response surface methodology (RSM). Optimization enhanced lipase production over 4-fold compared to the screening stage, in PKC media supplemented with 1.5% (w/w) yeast extract, 2.0% (v/w) Tween-80, 0.5% (v/w) olive oil and 7.0% (v/w) inoculum at pH 7.0 and 30°C within 72 hrs fermentation. Maximum activity of 400±2 U/g dry PKC was achieved. The analysis of variance (ANOVA) indicated that the model was significant ($p<0.05$) with coefficient of determination (R^2) 0.9893 which was very close to the adjusted R^2 (0.9816) pointing the reliability of the model.

Key words: Enzymes (Lipase); bioprocess design; palm kernel cake; Bioconversion; optimization; yeast (*Candida cylindracea*).

Lipases (EC.3.1.1.3) are one of the most important industrial enzymes. Due to their applications, they are gaining much interest in research, especially lipases from microbial sources since they are considered the best choice for commercial production [1]. Despite the potential applications of lipases, the high production costs still limit their use [2]. However, to overcome this challenge, the use of different microorganisms, low-cost nutrients and available substrates can assist in obtaining the best combination to produce lipases with high value as well as reasonable costs in industrial scale. For instance, the use of agro-industrial residues as substrates to produce the enzyme using solid-state bioconversion could reduce the cost significantly and add value to low-cost material [3].

During the last few years, researches are being engaged towards management of waste and turning waste to wealth. One of the proposed solutions is to reuse the wastes [4] to obtain other products such as enzymes and organic acids [5-6] through fermentation process in order to meet the demand of the current market. Malaysia is one of the largest producers of palm oil in the world with plantation area of 11% of the land along with a yearly production of 13 million tons of crude palm

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oil [4]. The processing of palm oil production includes many stages which results in several forms of wastes. Generally, all wastes resulting from the palm industry are referred as palm oil mill wastes that include palm kernel cake (PKC). PKC is one of the solid wastes obtained after extraction of the oil from palm kernel seeds[7]. In 2009, Malaysia produced 17.56 million tons of palm oil along with 2.31 million tons of palm kernel cake[4] and it reached 2.4 million tons in 2012[8]. PKC contains 50% carbohydrates and 15.0- 20.0 % proteins. Analysis also showed that PKC has high oil content. PKC mineral concentrations were reported as follows: Mg (0.27%), P (0.79%), Fe (4.05 mg/kg), Cu (28.5 mg/kg), Zn (77.0 mg/kg) and Mn (225.0 mg/kg) [9-10].

Solid-state bioconversion (SSB) or solid-state fermentation (SSF) is defined as the fermentation process occurring in the absence or near-absence of free water [11]. Agro-industrial residues such as oil cakes (e.g. coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake, etc) [12], are the most often and commonly used substrates for SSB processes. During the growth on such substrates hydrolytic exo-enzymes are synthesized by the microorganisms and excreted outside the cells, which makes accessing the products more feasible [13]. However, to date only few studies employed SSB for lipase production from different substrates, whereas many applied submerged fermentation for this purpose. Some of the substrates that have been used for lipase production are olive oil cake, cassava, sweet potato, pineapple and carrot waste and wheat bran[12, 14], rice bran, groundnut oil cake, sesame oil cake, coconut oil cake, and wheat bran [15-16]. Using SSF, few studies reported lipase production from different solid wastes such as wheat bran [17], Rice bran [18], and wheat rawa[19]. Among the reported data, optimization of media and process conditions has not received much attention. Falony et al [17]studied the effect of different nitrogen sources on lipase production. Several carbon and nitrogen sources were screened for lipase production by Imandi et al [8]where maximum lipase activity achieved was 18.58 U/gds. Single factor optimization was reported for process parameters such as incubation period and initial pH and their effect of lipase production from *Aspergillus niger* by Adinarayana et al[19]. Experimental design was employed to study the relation between olive oil concentration, glucose concentration and humidity ratio and their influence on lipase production which resulted in activity of 121 U/gds[20].

However, not much works have been conducted on lipases, especially employing SSB to ferment solid agro-industrial wastes and particularly, PKC using *Candida cylindracea*. Furthermore, SSB seemed to be more attractive economically because the cost was reported to be much lower than the market price [21]. Hence, more investigation is required and more challenges need to be overcome to achieve high value as well as lower-cost product. PKC is a rich source for nutrients; therefore the availability of abundant quantities of this substrate requires a considerable utilization. The use of PKC as a low-cost renewable mass source in industrial bioprocesses can reduce the production cost of lipase as well as reduce the impact of PKC on the environment [22]. Important factors that influence lipase production by SSB are particle size, moisture content, inoculum concentration, temperature, pH, media composition including carbon and nitrogen sources, inducers and inorganic minerals [8, 11, 14, 17]. This work aimed to explore the potential of using PKC in optimizing a fermentation medium and to determine the optimal process conditions for microbial lipase production, using *Candida cylindracea* by employing SSB. This study in expected to provide a contribution for scaling-up the process for industrial applications.

MATERIALS AND METHODS

Raw Materials

The main substrate in this study was palm kernel cake (PKC) was collected in clean autoclaved bags, from West Oil Mill, Sime Darby Sdn. Bhd. (Supercritical Fluid Extraction Unit) in Carey Island, Banting, Selangor, Malaysia.A sample of PKC was grinded to 1.0 mm downgraded. The weight of the sample was measured and dried at 60°C for 72 hrs to reduce the moisture content to approximately 3.0% [8]. After drying, the weight was measured again to identify the moisture content.

All chemicals and consumables used is this research were of analytical grade and commercially purchased from Oxoid Ltd (UK), Merck Sdn Bhd. (Germany), Fisher Scientific (UK), R & M Chemicals (UK), MHM Global (Germany),

BumiPharmaSdn Bhd. and Nano-life Quest (Malaysia).

Sub-Culturing and Inoculum Preparation

The microbial strain, *Candida cylindracea*(ATCC 14830) used in this study was purchased from the American Type Culture Collection, USA. *C. cylindracea* and grown on (PDA) plates at 28°C for four days and sub-cultured every two weeks. Each culture plate was washed with 10.0 ml sterile distilled water and the suspension was used to prepare the inoculum. 1.0 ml of suspension was cultivated in a medium containing 0.7% (v/v) Tween-80, 0.2% (v/v) olive oil and 0.5% (w/v) peptone at 28ºC in an orbital shaker (MaxQ-4000, UK) at 150 rpm for 48 hrs[23].

Fermentation Process

Experiments were carried out in 250 ml Erlenmeyer flask where 6.0 g of PKC was moisturized using sterilized distilled water and supplements to a total weight of 20 grams. The medium components consisted of the solid substrate supplemented with nutrients with concentrations as described in the experimental design later. pH was adjusted using 1.0 M of NaOH. The flasks were autoclaved at 121°C for 15 min (Hirayama, Japan), cooled down to room temperature and inoculated with the inoculum solution. Flasks were incubated at 30°C(Incucell, Germany) and samples were taken every 24 hrs for analysis.

Analytical Approaches

Determination of solid substrate pH

To measure the pH of PKC, 10 g of the dried solid substance was dissolved in 100 ml deionised water and agitated for 5 minutes. The mixture then was filtered through Whatman No.1 filter paper. The pH for the filtrate was measured using a pH meter[19].

Lipase extraction

The crude enzyme from the fermented media was recovered using a simple extraction method. To each flask, the fermented material was mixed with 50 ml sterile distilled water and agitated for 2 hrs at room temperature (30±2°C) on a rotary shaker (SK-600, UK) at 150 rpm to facilitate the enzyme extraction. The suspension was then filtered through Whatman No.1 filter paper and the resulting clear filtrate was used for lipase assay [8, 17]. To get the cell-free supernatant, the filtrate was centrifuged at 10,000 rpm (Eppendorf 5804, UK) for 10 min at 4°C [23].

Lipase assay

Analysis of lipase was carried out according to the method described by Gopinath et al [24] using *p*-nitrophenylpalmitate (*p*NPP) as the substrate. Substrate solution was prepared by mixing 10.0 ml of isopropanol containing 30.0 mg of *p*NPP, with 90 ml of 0.05 M phosphate buffer (pH 8.0) containing 207.0 mg sodium deoxycholate and 100 mg gum Arabic. Twenty micro-litre of the enzyme [23] (diluted to appropriate concentration with deionised water) was added to 2.4 ml of freshly prepared substrate solution and incubated for 15 min at 37°C. The optical density was measured at 410 nm (Infinite M200, TECAN) against an enzyme free control. One unit of lipase activity was defined as the enzyme amount that releases 1µmol of *p*-nitrophenol per minute under assay conditions. Enzyme activity was expressed as Unit/ ml of the extract solution and units/gram of the initial dry substrate (U/gds). Assays were carried out in triplicates and the average values were calculated. Standard curve of *p*-nitrophenol was prepared using different concentrations[24].

Statistical Optimization of Fermentation Conditions

Optimization for lipase production was conducted in three stages: Plackett-Burman design (PB), one-factor-at-a-time (OFAT) and response surface methodology (RSM). In this study, Design Expert v.6.0.8 was used for designing the experiments for screening, optimization and validation of the model. IBM SPSS Statistics v.19 was used to analyse the contribution and the significance of the parameters which affected lipase production.

Plackett-Burman design for screening of variables

Plackett-Burman design (PB) has been widely used for the screening and selection of medium components in flask cultures. PB design assists in giving a screening procedure that calculate the significance of large number of factors mathematically. The insignificant factors can be removed from the design to get controllable set of experiments. Each factor was examined at two levels: low level (-1) and high level (+1) [25]. Based on previous studies [8, 17, 23], eleven components have been selected for screening which are media components: glucose, sodium nitrate, magnesium

sulphate, peptone, yeast extract, Tween-80 and olive oil, and for process conditions: pH, temperature, inoculum concentration and moisture content. The range of each parameter was selected according to many studies that showed the effect of those parameters on the production of lipase: organic and inorganic nitrogen sources, carbon source, minerals, inducers, pH, temperature, inoculum size and moisture content [8, 17, 23]. In this study, the eleven components which have been selected generated a set of 12 experimental designs as stated in Table 1. All experiments were conducted in triplicates and the average was recorded as lipase activity. The main effect of each factor was calculated as the difference between the average of the measurements at the high level and the average of measurements at the low level as follows:

Main Effect (E) = Total responds at high level – Total responds at low level/ Number of trials (1)

One Factor at-a-Time (OFAT) Analysis

PB design was followed by classical one-factor at-a-time (OFAT) method to evaluate the optimum levels of the factors that contributed to lipase production. The factors that were investigated were pH, temperature, inoculum size, yeast extract, Tween-80 and olive oil concentrations. From PB design results, factors that contributed at negative level or had relatively small values for the main effect were eliminated from the media. Following that, OFAT experiments were conducted to find out the minimum requirement needed for the production of lipase while factors with clear effect were fixed as stated in Table 2. For pH, a range of 5.0 to 11.0 was tested. Production of lipase was also studied at 25, 30 and 35ºC. Yeast extract concentrations ranged from 0.3 to 2.4% (w/w), Tween-80: 0.25 to 4.0% (v/w), olive oil: 0.1- 2.0% (v/w) and inoculum size: 1.0- 12.0% (v/w).

Response Surface Methodology (RSM)

Face centred central composite design (FCCCD) under RSM was employed using Design Expert software (version 6.0.8, Stat-Ease Inc., Minneapolis, USA) to optimize the two significant factors: yeast extract and inoculum concentration. After obtaining OFAT results, the data was subjected to One-Way ANOVA Post-hoc Tukey's test ($p \leq 0.01$). Factors that gave maximum activity at certain points and showed a clear optimum level were selected for FCCCD while other components were fixed at the minimum concentrations. A set of 13 experimental runs with five replicate centre points (Run 3, 6, 9, 10 and 12) was generated as stated in Table 3. Variables were studied using three different levels: low (-1), (0.0) and high (+1). The remaining factors concentrations were fixed based on the results of one-factor-at-a-time study.

Data analysis and validation of the model

The statistical model was validated using values within the optimal range. Ten solutions of the optimization equation were suggested while three within the range were selected. Experiments were conducted in triplicates for each and compared with the predicted values generated by the software. To evaluate the model, Analysis of Variance (ANOVA) was used along with 2D contour plot and 3D response surface curve. Main effect calculation was used to analyse PB design results in Microsoft Office Excel 2007. IBM SPSS Statistics v.19 was used to analyse the contribution and the significance of the parameters which affect lipase production in OFAT experiments employing One-Way ANOVA Post-Hoc Tukey's test ($p \leq 0.01$).

RESULTS AND DISCUSSION

Plackett-Burman Design for Screening Of Variables

The use of PB design is an effective tool for screening factors that influence a particular process. In the present work, eleven factors were examined in 12 experimental runs with two levels for each factor and the response (lipase activity) was expressed as U/g dry PKC (Table 1). The main effects of each factor that influence the production of extracellular lipase are presented in Figure1.

The results showed that $MgSO_4$, olive oil and $NaNO_3$ highly affected the production of lipase at negative levels, followed by glucose. The low level for all the above compounds was zero except for olive oil which seems to enhance the production at low concentration (0.2% v/w). Glucose was reported to significantly decrease lipase production while using olive oil as the carbon source showed an increase in lipase activity by five folds when glucose was removed from the media. It has been also reported that glucose and glycerol act as repressors for lipase production[26], while organic nitrogen sources and long chain fatty

Table 1. Plackett-Burman experimental design for evaluation of 11 factors with high and low levels for lipase production by *C .cylindracea* and the responses expressed as lipase activity

Factors* Run	G %(w/w)	YE %(w/w)	N %(w/w)	P %(w/w)	Tw %(v/w)	O %(v/w)	M % (w/w)	pH	T °C	In % (v/w)	MC %	Activity U/ml	Activity U/g PKC
1	0.06(-1)	0.0(-1)	0.12(+1)	0.3(+1)	0.5(+1)	0.2(-1)	0.06(+1)	8.0(+1)	25(-1)	10.0(+1)	55.0(-1)	6.12	34.02
2	0.06(+1)	0.3(+1)	0.12(+1)	0.0(-1)	0.5(+1)	1.0(+1)	0.00(-1)	8.0(+1)	25(-1)	2.00(-1)	55.0(-1)	5.70	31.66
3	0.00(-1)	0.0(-1)	0.12(-1)	0.0(-1)	0.0(-1)	0.2(-1)	0.00(-1)	4.0(-1)	25(-1)	2.00(-1)	55.0(-1)	5.39	29.90
4	0.06(+1)	0.0(-1)	0.12(+1)	0.3(+1)	0.0(-1)	1.0(+1)	0.00(-1)	4.0(-1)	25(-1)	10.0(+1)	75.0(+1)	5.49	54.86
5	0.06(+1)	0.0(-1)	0.12(+1)	0.0(-1)	0.0(-1)	0.2(-1)	0.06(+1)	8.0(+1)	35(+1)	2.00(-1)	75.0(+1)	6.46	64.57
6	0.00(-1)	0.0(-1)	0.00(-1)	0.3(+1)	0.5(+1)	1.0(+1)	0.00(-1)	8.0(+1)	35(+1)	2.00(-1)	75.0(+1)	7.43	74.27
7	0.06(+1)	0.0(-1)	0.00(-1)	0.0(-1)	0.5(+1)	1.0(+1)	0.06(+1)	4.0(-1)	35(+1)	10.0(+1)	55.0(-1)	6.58	36.54
8	0.00(-1)	0.3(+1)	0.12(+1)	0.3(+1)	0.0(-1)	1.0(+1)	0.06(+1)	4.0(-1)	35(+1)	2.00(-1)	55.0(-1)	5.67	31.50
9	0.06(+1)	0.3(+1)	0.00(-1)	0.3(+1)	0.0(-1)	0.2(-1)	0.00(-1)	8.0(+1)	35(+1)	10.0(+1)	55.0(-1)	7.33	40.70
10	0.00(-1)	0.3(+1)	0.12(+1)	0.0(-1)	0.5(+1)	0.2(-1)	0.00(-1)	4.0(-1)	35(+1)	10.0(+1)	75.0(+1)	9.41	94.13
11	0.06(+1)	0.3(+1)	0.00(-1)	0.3(+1)	0.5(+1)	0.2(-1)	0.06(+1)	4.0(-1)	25(-1)	2.00(-1)	75.0(+1)	5.70	57.00
12	0.00(-1)	0.3(+1)	0.00(-1)	0.0(-1)	0.0(-1)	1.0(+1)	0.06(+1)	8.0(+1)	25(-1)	10.0(+1)	75.0(+1)	6.02	60.24

*G- glucose, YE- yeast extract, N- $NaNO_3$, P-peptone, Tw- Tween-80, O-olive oil, M-$MgSO_4$, pH- initial pH, T- temperature,In-inoculum concentration, MC- moisture content. (-1) indicates the low level and (+1) indicates the high level

acids were found to be the most common enhancers[27].Since PKC contains many minerals and most of them seem to activate the enzyme except Cu^{2+} and Fe^{3+} [9-10, 23, 28], only $MgSO_4$ was tested and it showed negative effect according to the main effects results. The negative effect of $MgSO_4$ showed that additional amount of minerals from external sources was not needed because they were required in small quantities and excessive amounts could have a negative effect on lipase production. This is also comparable to the findings of Iwai et al [29] which presented that at low concentrations, magnesium ions slightly increased the activity of lipase. Another study reported that magnesium ions could bind to the fatty acids and stimulate the reaction with lipase which leads to the complete reaction [28].

At positive level, factors that influenced the production could be ranked as: temperature > inoculum concentration > Tween-80 > moisture

Table 2. One-factor-at-a-time (OFAT) tested factors showing the range studied and the fixed parameters during the experiments

Factor	Range tested	Fixed parameters
Yeast extract % (w/w)	0.3 – 2.4	Tween-80: 0.5% (v/w), olive oil: 0.2% (v/w), moisture content: 70%, temperature: 30˚C, pH 6.0-7.0 and inoculum 10.0% (v/w).
Olive oil % (w/w)	0.1 – 2.0	Tween-80: 0.5% (v/w), yeast extract: 0.3% (w/w), moisture content: 70%, temperature: 30°C, pH 6.0-7.0 and inoculum 10.0% (v/w).
Tween-80 % (w/w)	0.3- 4.0	Yeast extract: 0.3% (w/w), olive oil: 0.2% (v/w), moisture content: 70%, temperature: 30˚C, pH 6.0-7.0 and inoculum 10.0% (v/w).
Inoculum % (w/w)	1.0-	12.0 Yeast extract: 0.3% (w/w), olive oil: 0.2% (v/w), Tween-80: 0.5% (v/w), moisture content: 70%, temperature: 30˚C and pH 6.0-7.0.
Initial pH	3.0, 4.0, 5.0, 6.0. 7.0, 8.0, 9.0, 10 and 11.0	Yeast extract: 0.3% (w/w), olive oil: 0.2% (v/w), Tween-80: 0.5% (v/w), moisture content: 70%, temperature: 30˚C and inoculum 10.0% (v/w
Fermentation temperature°C	25, 30 and 35	Yeast extract: 0.3% (w/w), olive oil: 0.2% (v/w), Tween-80: 0.5% (v/w), moisture content: 70%, pH: 6.0-7.0 and inoculum 10.0% (v/w).

Table 3. Experimental design using FCCCD of two parameters with actual and coded value, centre points, actual and predicted lipase activity

Run order	A: Yeast extract concentration (% w/v)	B: Inoculum concentration (% w/v)	Lipase Activity U/g dry PKC		
			Actual	Predicted	Error%
1	0.6 (-1)	7.00 (0)	256.33	267.2915	4.100
2	0.6 (-1)	10.0 (0)	286.77	279.0259	-2.775
3	1.5 (0)	7.00 (0)	394.26	394.2521	-0.002
4	0.6 (-1)	4.00 (-1)	241.00	237.7826	-1.353
5	2.4 (+1)	10.0 (+1)	372.03	369.4126	-0.785
6	1.5 (0)	7.00 (0)	400.22	394.2521	-1.514
7	2.4 (+1)	4.00 (-1)	375.00	376.9093	0.507
8	1.5 (0)	4.00 (-1)	375.62	376.9282	0.347
9	1.5 (0)	7.00 (0)	402.33	394.2521	-2.489
10	1.5 (0)	7.00 (0)	393.00	394.2521	0.3176
11	2.4 (+1)	7.00 (0)	381.34	382.0482	0.1854
12	1.5 (0)	7.00 (0)	393.12	394.2521	0.2872
13	1.5 (0)	10.0 (+1)	383.44	393.8015	2.6311

content > yeast extract> pH > peptone. Lipase production based on the experimental design showed a fluctuated activity which ranged from 29 U/gds to 94.13 U/gds. The highest lipase activity was obtained in Run 10 in the presence of organic and inorganic nitrogen sources, olive oil, Tween-80 and in the absence of glucose and $MgSO_4$. The lowest activity was observed in Run 3 where the medium was supplemented with only olive oil. The most significant factors that resulted in a maximum lipase activity were Tween-80, yeast extract and olive oil while the effective process conditions were the temperature and inoculum concentration.

PB design did not provide a complete idea about the exact effect of each parameter. Thus, based on main effects calculations, the factors that contributed the most in lipase production were further investigated using one-factor-at-a-time (OFAT) method. After OFAT, the optimal level for each factor was determined to be used in the optimization step using RSM.

Table 4. Analysis of variance for response surface quadratic model for lipase production (ANOVA)

Source	Sum of squares	DF	Mean Square	*F*- value	*p*-value	
Model	38194.35	5.00	7638.87	129.08	< 0.0001	significant
A*	4838.35	1.00	19753.64	328.47	< 0.0001	
B*	54.77	1.0	427.06	7.08	0.03681	
A^2	13372.28	1.0	13372.28	222.47	< 0.0001	
B^2	218.14	1.0	218.14	3.55	0.0963	
AB	593.90	1.0	593.90	9.87	0.0158	
Residual	414.26	7.0	59.			
Lack of Fit	337.78	3.0	112.59	5.89	0.0598	not significant
Pure Error	76.48	4.0	19.12			
Total	38608.62	12.0				

*A= yeast extract concentration, B= inoculum concentration, R^2= 0.9893, Adjusted R^2 = 0.9816, C.V= 2.15, Predicted R^2= 0.9252, Adequate precision= 29.939.

Table 5. Validation of FCCCD experimental model with the predicted lipase activity for three suggested solutions from the software

Experiment	Yeast extract concentration (%w/w)	Inoculum concentration (%v/w)	Activity U/g dry PKC	Predicted Activity	Error %
1	1.79	8.32%	406.79	405.77	-0.25
2	1.82	8.99%	401.42	404.69	+0.81
3	1.69	7.06%	398.37	403.64	+1.31

One-Factor-at-a-Time (OFAT) Experiments and Analysis

The aim of this study is to determine the effects of the parameters that influenced lipase activity and the optimal levels to be used in the optimization process. The parameters included nitrogen source, carbon source, inducer, inoculum, pH and temperature. There is no record on optimal conditions for lipase production from PKC using *C. cylindracea* in SSF. Furthermore, by employing PB design, we could only eliminate the factors that affected negatively at their low levels (0). Other factors were required to be tested further to choose the three levels for the optimization process.

Effect of nitrogen sources

Organic nitrogen sources are favoured by organisms that produce lipase[1]. Peptone and yeast extract are organic nitrogen sources and both showed positive main effects in PB experiments while $NaNO_3$ showed negative effect. Moreover, based on PB experiments, yeast extract was more contributing than peptone and thus only yeast extract was chosen for OFAT as well as further optimization study. The effect of yeast extract is illustrated in Figure 2A.

Based on the results, the maximum lipase activity (234.5 U/g dry PKC) was obtained by

0.9%to 1.8% (w/w) concentrations of yeast extract, where the mean differences of the used concentrations were statistically significant by using One-Way ANOVA Post Hoc Tukey's test ($p \leq 0.01$). Thus, this concentrations range has been selected for the optimization studies. Further increase of yeast extract beyond 1.8% (w/w) in the medium resulted in a slight decrease in lipase activity.

The results of the current study are in agreement with the reported data which showed that high concentration of nitrogen sources in the medium effectively improve lipase production. According to Kumar and Kanwar[30], yeast extract and urea gave higher lipase activity with almost six times compared to other nitrogen sources in SSF. Reduction in lipase production was noticed when yeast extract concentration increased when producing extracellular lipase from *Bacillus sp*[31]. Among the organic nitrogen sources, yeast extract gave the highest lipase production [32].

Effect of olive oil

Generally, lipase production is enhanced by lipids such as fats, oils, fatty acids and other lipids. Most microbial lipases are specific toward long chain fatty acids available in olive oil, sesame oil and corn oil. Oils and fats are required only in small amounts while excessive amounts result in biphasic system formation and thus prevent oxygen transfer and nutrient absorption by the microorganism from the substrate or might be cytotoxic [30]. As presented in Figure 2B, maximum lipase activity (244.25 U/gds) was achieved in a media supplemented with 1.0% (v/w) olive oil while further increase to more than 1.0% (v/w) of the total weight, showed slight decrease in the activity. Using One-Way ANOVA Post-Hoc Tukey's test ($p \leq 0.01$), there was no significant differences between the olive oil concentrations (0.5 – 2.0% v/w) while the mean difference between 0.1, 0.2 and the group of (0.5- 2.0%) was significant. Thus, the minimum concentration 0.5% (v/w), among the group was chosen to be fixed during the optimization process. Tamilarasan and Kumar[32]showed that maximum lipase activity was obtained using 1.0 - 1.5% (v/v) of olive oil by *Bacillus sphaericus* MTCC 7542 in the production medium. Fadilo Glu and Erkmen[33] showed that both lipase activity and biomass were the highest in the presence of olive oil. Moreover, olive oil was found to increase the production of extracellular lipase in *C. cylindracea* NRRL Y-17506 [34].

Effect of Tween-80

Tween-80 is one of the surfactants used in lipase production. The function of surfactants is to improve the permeability of the cell membrane

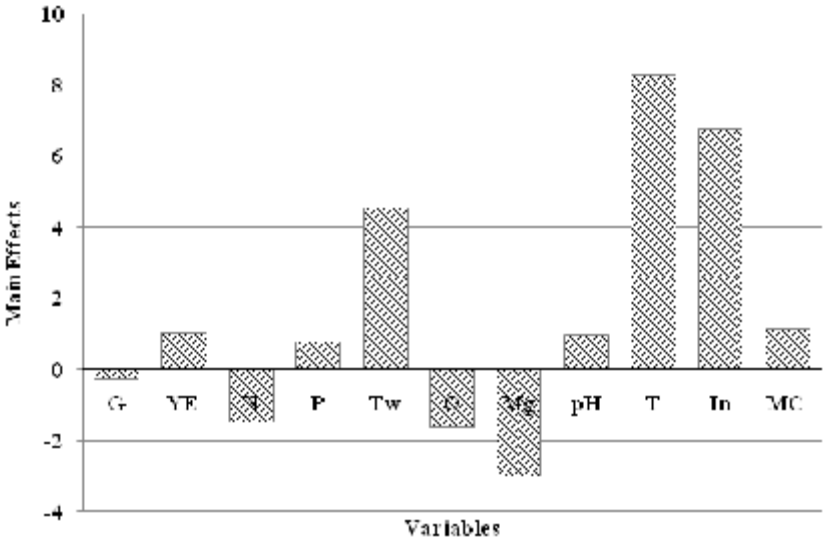

Fig. 1. Main effects of the medium components and process conditions for lipase production using Plackett-Burman results: G-glucose (0.0- 0.06 %w/w), YE- yeast extract: (0.0- 0.3 %w/w), N- $NaNO_3$: (0.0- 0.12 % w/w), P- peptone: (0.0- 0.3 %w/w), Tw- Tween-80: (0.0- 0.5% v/w), O-olive oil: (0.0- 0.5% v/w), Mg- $MgSO_4$: (0.0- 0.06%w/w),pH: initial (4.0- 8.0), T- temperature: (25- 35°C), In- inoculum concentration: (2.0- 10.0% v/w), MC-moisture content (55-75%)

which facilitates the transmission of several molecules across the cell membrane and increases lipase secretion or assists the contact between the enzyme and the substrate[28].

Increasing the concentration of Tween-80 from 0.30 to 2.0% (v/w) in the media enhanced the production of lipase by *C. cylindracea*(Figure 2C). Further increase did not affect the production significantly. The maximum lipase activity (250 U/gds) was achieved by using 2.0 – 4.0% (v/w) Tween-80 in the medium. One-Way ANOVA Post Hoc Tukey's test, ($p \leq 0.01$) showed no statistical

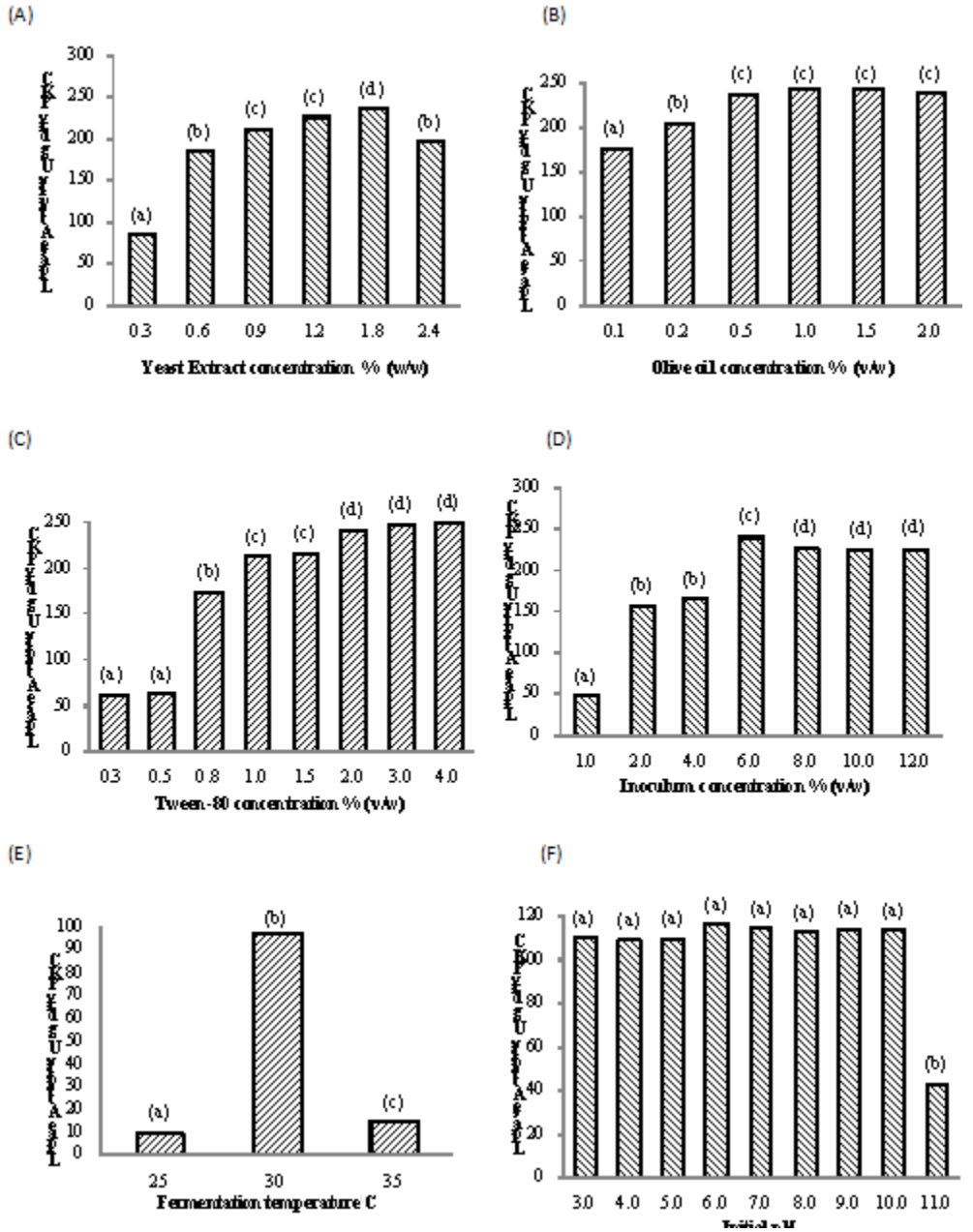

Fig. 2. Effect of different (A) concentrations of yeast extract (B) concentrations of olive oil (C) concentrations of Tween-80 (D) inoculum concentrations (E) initial pH (F) fermentation temperatures, on lipase activity by *C. cylindracea*. (Culture conditions are presented in Table 1). Mean values with the same letter are not significantly different from each other using One -Way ANOVA Post-Hoc Tukey's test ($p \leq 0.01$)

significant difference between concentrations 2.0 to 4.0% (v/w). Although there was a slight increase in the lipase activity, 2.0% (v/w) of Tween- 80 was chosen to be fixed during the optimization process. It was reported that increasing Tween-80 concentration in the media resulted in decrease in activity due to fatty acids accumulation which inhibited the synthesis of lipase [35]. Another study indicated that 1.0% (w/w) resulted in increasing of lipase activity in solid state fermentation[30]. In the case of *C. rugosa*, the microorganisms could not grow in the presence of Tweens alone while the growth was improved if oleic acid was available which proved that Tweens are used as inducers but not as carbon sources for growth in the fermentation medium. Moreover, it was suggested that addition of Tweens to the media in the first six hours might turn on the expression of some *lip* genes and thus leads to the stimulation of the lipase production. It has been stated that Tween-80 could enhance the secretion of the enzyme in the fermentation media [36].

Effect of inoculum concentration

Inoculum concentration is one of the most important factors which influence the fermentation process and thus affects the enzyme production. Lower inoculum concentration may result in inadequate biomass which in turn decreases the production of the desired products, whereas excessive concentration of inoculum may generate too much biomass which leads to poor production [8]. Therefore, the effect of inoculum level on lipase production was studied in more detail. The range of concentrations tested was: 1.0 – 12.0% (v/w) as illustrated in Figure 2D. An average count of 2.0×10^7 cells/ ml was used. Based on the results, maximum activity (240.42 U/gds) was obtained with 6.0% (v/w) (1.2 ml) of the inoculum when other parameters were fixed at PB design levels (Table 1). The mean difference between and within the tested concentrations were significant. The comparison between 2.0 and 4.0% (v/w) was not significant using One-Way ANOVA Post Hoc Tukey's test ($p \leq 0.01$). Similar observation was also noticed fro concentrations 8.0 to 12.0% (v/w). The most significant concentration in the current study was 6.0%, and thus the range 4.0 to 10.0% (v/w) were chosen for further investigation during optimization process due to its importance for the production of lipase.

Concentration of 3×10^7 spores/g of dry substrate was used for maximum productivity of lipase by*R. homothallicus*[37]. Increasing the inoculum concentration was predicted to reduce the lag phase of the fungal growth leading to shorter time of maximum lipase production. Low productivity of the enzyme with high inoculum concentration might be referred to biomass accumulation which reduced the enzyme synthesis because of the limited nutrients[8].

Effect of pH

The pH of the fermentation media is one of the most important factors that influence the microbial growth and metabolic activities of the cells. Changes of pH can influence the stability of the enzyme during the fermentation [38]. Moreover, changes in pH can affect the metabolic activities due to the release of organic acids (such as acetic,

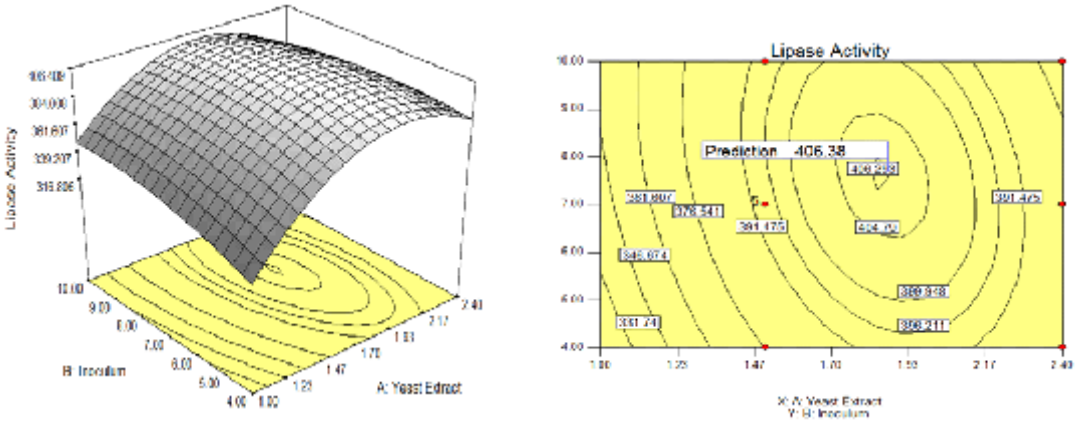

Fig. 3. (A) 3D response surface curve of the interaction effects of yeast extract and inoculum concentrations on lipase activity by *C. cylindracea*; (B) Contour plot of the interaction between yeast extract concentration % (w/w) and inoculum concentration % (v/w)

citric and lactic acid) in the media which results in a decrease in pH. The pH profile during the growth depends on the type of microorganisms and the nature of the substrate itself which might have buffering effect due to the presence of lingo-cellulosic materials [39].

Initial pH of the fermentation medium was varied between 4.0 and 11.0 as shown in Figure 2E. Lipase production increased at pH between 6.0 and 10.0 and dropped dramatically when the initial pH was ≥ 11.0. The maximum activity was achieved at pH 6.0 although the production was also high for all pH ranges except 11.0. Meanwhile, one-Way ANOVA Post Hoc Tukey's test ($p \leq 0.01$) showed no statistical significant differences in lipase activities in the range of pH 4.0 and pH 10.0, although there was a slight increase in the activity at pH 6.0. Thus the pH of the culture medium was selected to be fixed for the following steps between 6.0 and 7.0.

According to Tamilarasan and Kumar [32], the nature of yeast and fungal lipases is alkaline .It has also been reported that lipase activity decreased at pH > 9.0 and < 5.0. It has been also observed that *C. cylindracea* and *Y. lipolytica* could grow and produce lipase at pH between 6.0 and 8.0[40]. For subsequent studies, it was stated that pH 6.2 has been used for lipase production by *C. cylindracea*. This particular pH would enable the substrate to be taken by the cells, promote their growth and induce the enzyme production [34].

Effect of temperature

Temperature ranges from 28.0 to 45.0ºC were reported to be suitable for lipase production [41]. Normally, high temperature results in deactivation of metabolic pathways enzymes while low temperature may prevents the nutrients flow across cell membrane which leads to high demand for maintenance energy for lipase production [42].

Maximum lipase activity was achieved at 30ºC (Figure 2F) while the activity dropped almost nine-folds at both 25 and 35ºC which indicate the sensitivity of the growth and enzyme production to temperature changes. These results agreed with the reported data showed that maximum lipase production was supported at 30ºC [43]. One-Way ANOVA Post Hoc Tukey's test, ($p \leq 0.01$) showed significant difference between the temperature ranges in terms of lipase production. In addition, comparing the results to the ones obtained from PB design, the temperature had a positive contribution to lipase production with large mean effect. It was clear that the maximum activity achieved at 30°C, thus the temperature was fixed during the fermentation. In contrast, maximum enzyme activity using *Rhizopusoligosporus*was obtained at 30ºC [42]. It was also documented that the production of the lipase by *C. rugosa* was the maximum at 32ºC while the production reduced when the temperature increased above 32ºC [15]. As reported in many studies, lipases from *Candida* species were produced at temperatures between 28 and 30ºC [8, 15, 23, 40].

The process conditions tested by OFAT showed that temperature was the most influencing factor hence production was 9 times at 30ºC than the production at both 25 and 35ºC. The factors that showed clear trends (temperature, pH, olive oil and Tween-80) were fixed during optimization at the lowest level to achieve a cost effective process. The remaining parameters (yeast extract and inoculum concentrations were subjected to FCCCD experimental design.

Optimization of Media Components and Process Conditions by Face-Centered Central Composite Design (FCCCD)

The most contributing parameters in OFAT were subjected to the optimization process in order to achieve high lipase production. Based on PB and OFAT experiments, FCCCD was employed to determine the optimal conditions of the most two significant factor (yeast extract and inoculum concentrations) while keeping the other parameters fixed at OFAT levels. For each run, predicted lipase activities from regression equation and the experimental results are presented in Table 3.

The results showed that the highest lipase production (400 U/gds) was observed at the centre points (runs 3, 6, 9, 10 and 12). The minimum activity was observed in run 4 (241 U/gds), where both factors were at their low concentrations. This actually showed that the FCCCD design improved the lipase production where the difference between the maximum and minimum responses (241 and 400 U/gds) was significant. A second order equation which described the relation between the two factors and the lipase activity was obtained from the software by analysing the experimental results. The equation obtained was as follow:

$$Lipase\ Activity = +272.10 - 162.08\ A - 5.10\ B - 193.28\ A^2 - 8.89\ B^2 - 20.31\ AB \quad (2)$$

Where the response is lipase activity, A and B are the concentrations of yeast extract and inoculum respectively.

Analysis of variance (ANOVA) was used to prove the efficacy of the model as presented in Table 4. The Model *F*-value of 129.08 and *p*-value of <0.0001 implied that the model was significant meaning that there was only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise. Values of "Prob>*F*" less than 0.05 indicated that the model terms are significant. In this case A, B, A^2 and AB were significant model terms. Values greater than 0.1000 indicated the model terms were not significant. The "Lack of Fit *F*-value" of 5.89 implied there was a 5.89% chance that a "Lack of Fit *F*- value" this large could occur due to noise. The insignificancy of lack of fit suggested that the experimental results obtained fit with the model. The coefficient values are listed below Table 4.

For further validation of the model, the goodness of fit was evaluated by the coefficients of determination (R^2). The high value of R^2 (0.9893) implied a high degree of correlation between the experimental and the predicted values by the model. The R^2 value of 0.9893 pointed that only about 1.07 % of the total variations could not be explained by the model. The adjusted R^2 of 0.9816 in this study was close to R^2 value which indicates the better prediction of the model. The purpose of calculating the adjusted R^2 was to correct the R^2 value for the sample size and the number of terms in the model [42]. The closer the value of R^2 to 1.0, the more the variation in the model could be explained. Adequate precision compared the range of the predicted values at the design points to the average prediction error. Ratios greater than 4.0 indicated adequate model discrimination. In this study, the value was 29.939, well greater than 4.0. Moreover, the low value of the coefficient of variation (CV) showed the reliability of the experiment.

The *p*- value is also used to check the strength of the interaction between the variables. If the *p*-value is very small (less than 0.05), it means that the factors (the term) in the model have a significant effect on the activity of the lipase (response). According to our results, only one interaction term (B^2) was not significant ($p > 0.05$) while all the remaining terms were significant ($p < 0.05$). The 3D response surface (Figure 3A) was plotted to describe the interaction between yeast extract and inoculum concentrations and the optimum concentrations required for maximum lipase production. The figure represented that the interaction resulted in a significant higher enzyme activity by *C. cylindracea*. The contour plot (Figure 3B) clearly showed the optimal predicted lipase activity at (x= 1.87 and y= 7.71). The maximum lipase activity was achieved at the centre points of the experimental design region. According to the graphs, increasing the yeast extract and the inoculum concentrations increased the lipase productivity where further increase resulted in reduction in the productivity. The elliptical shape produced from the results indicated the significance of the interactions between the variables. This nature of the plot pointed that the interaction between the two factors has a significant effect on lipase production [43]. Lipase activity was very low (241 U/gds) at the low levels of both yeast extract and the inoculum compared to the highest activity (400 U/gds) obtained at the centre points. Moreover, it was noticed that if one of the two factors decreased less than the centre point level, the production decreased.

Validation of The Model

To further prove the reliability of the model, validation has been conducted using values within the optimal range. Three sets of experiments were selected and the remaining parameters were fixed at the optimal conditions. Validation results are presented in Table 5. The predicted and the experimental results were in good agreement which prove the validity of the model and the existence of the optimal conditions.

From the optimization study the production of lipase using the optimum conditions obtained from OFAT followed by FCCCD increased by 4.23-fold and 1.67-fold compared to the lipase production obtained by PB (94.13 U/gds) and OFAT (250 U/gds), respectively. Moreover, fermentation time decreased to 72 hours instead of 6 days (144 hours) in OFAT experiments.

Improvement of lipase production by using RSM has been used in many studies in both liquid-state and solid-state fermentation, despite the fact that there were fewer reports on SSF optimization. Moreover, by optimizing the media

components, the fermentation period was also decreased to 72 hours which was the end of the exponential growth phase.

Yeast extract had positive effect on lipase production but excessive amounts could inhibit the activity. This might be due to the ability of yeast extract to enhance the secretion of proteases into the culture medium which results in deactivation of the lipase. Yeast extract was also noticed to be the best nitrogen source for the production of lipase. This ability might be explained by the presence of some trace factors in yeast extract that stimulate the production of lipase which might also lead to the decrease in fermentation time. Yeast extract stimulated the production of lipase while after that, some protease were also secreted in the medium which resulted in the reduction of the activity [44].

Inoculum concentration has also been discussed previously. Sufficient inoculum level is required for optimal production. Low inoculum concentration is insufficient to consume the nutrients while excessive inoculum concentration will result in accumulation of the biomass and affect the lipase production. A concentration of 20.0% (v/w) inoculum was suggested to be optimal by Imandi et al[8] in SSF by *Yarrowia lipolytica*. The percentage could vary according to the cell count in the inoculum preparation.

It was predicted that only small concentrations of additional nutrients were required for the production of lipase since PKC is a rich source of carbon and amino acids as well as several minerals.

A successful and significant improvement of lipase production was achieved by the end of this study. The results revealed that only yeast extract, Tween-80 and small amount of olive oil were associated with high productivity of lipase.

CONCLUSIONS

By optimizing the process conditions, the maximum lipase production (400±2 U/gds) was achieved within 72 hrs. The production was 4.23-fold higher compared to the results obtained by PB (94.13 U/gds). Although this study has been conducted in lab-scale, it developed a combination of media components and process conditions for lipase production using SSB with no requirement of complicated controlling system which reduced the production cost. The process considered to be cost effective compared to submerged fermentation. It is also contributing in agro-industrial waste utilization. However, scaling up the process will require more control due to the heat transfer problems in SSB which needs to be studied further.

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