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Optimization of lipase production by *Candida cylindracea* in palm oil mill effluent based medium using statistical experimental design

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ABSTRACT

Sequential optimization strategy based on statistical experimental design including one-factor-at-a-time (OFAT) method was used to enhance the production of lipase by *Candida cylindracea* ATCC 14830 using palm oil mill effluent as a basal medium in shake flask cultures. The two-level Plackett–Burman (PB) design was implemented to screen the medium components that significantly influence the production. Following the OFAT method, three significant components influencing lipase production were identified as peptone, Tween-80 and inoculum. The optimum values of these three components were determined by response surface methodology (RSM) based on the face-centered central composite design (FCCCD). The validity of the model developed was verified, and the optimum medium containing 0.45% (w/v) peptone, 0.65% (v/v) Tween-80 and 2.2% (v/v) inoculum led to a maximum lipase production of 20.26 U/ml, which was 5.19-fold higher than the unoptimized medium. The analysis of variance indicated that the established model was significant ($P < 0.05$). This study innovatively developed a fermentation medium utilizing renewable palm oil mill effluent (POME) to produce lipase at considerable level.

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are carboxylesterases that catalyze the hydrolysis of acylglycerides to glycerol and free fatty acids at the water–liquid interface. Besides this, they catalyze the reverse reactions such as esterification, interesterification and transesterification in non-aqueous conditions [1,2]. The unique characteristics of lipases such as substrate specificity, regio-specificity and chiral selectivity have attracted considerable attention from both physiological and biotechnological point of view [3].

Lipases have been found in many species of animals, plants, and microorganisms. The enzyme from microbial sources are currently receiving more attention because of their potential diverse applications in industries such as detergent, oleochemical, organic synthesis, dairy, fat and oil modification, tanning, pharmaceutical and sewage treatment [1,4]. By contrast, the high costs of production often restrict their use [5]. Based on this industrial challenge, the use of different microorganisms, supplements and substrates for lipase production can contribute in getting the best combinations to obtain lipases with high value, using substrate and operational conditions that facilitate the reduction of the production costs in industrial scale [6].

Candida cylindracea is a well-known industrial lipase producing yeast. It is non-ascosporic, often unicellular, non-pathogenic and recognized as GRAS (generally regarded as safe). Lipase produced by *C. cylindracea* has been one of the most widely used enzymes in research due to its high activity in hydrolytic reactions as well as synthetic chemistry [7]. Besides using a productive strain, optimized culture medium composition and fermentation conditions play a significant role in the improvement of lipase production [8]. Among the techniques used in optimizing the culture medium, experimental statistical techniques such as Plackett–Burman design (PB) and response surface methodology (RSM) have proved to be the most effective methods in optimizing the medium components for lipase production, eliminating the limitations of a single factor optimization process [4,9]. Although several optimized culture media using different carbon, nitrogen and inducer compositions have been extensively studied [10–12], there are only a few reports on culture medium optimization using agro-industrial residues in submerged fermentation for lipase production.

Considering the fact that a substantial part of industrial enzymes production cost is contributed by the cost of the fermentation medium, the present investigation was aimed at evaluating the effects of medium components on lipase production by formulating a suitable medium containing palm oil mill effluent (POME) as a low-cost renewable substrate alternative supplemented with

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minimal nutrients for lipase production by *C. cylindracea* (ATCC 14830). In Malaysia, palm oil extraction generates about 67 million tons of palm oil mill effluent (POME) annually [13]. Thus, POME can be sustainably used as a fermentation medium in the production of lipase. The growing interest in POME utilization as renewable substrate is mainly associated with its high organic contents [14].

In this study, factors that significantly affect the lipase production were screened by Plackett–Burman design; the one-factor-at-a-time approach to obtain the possible optimum levels of all the factors and the optimization process by response surface methodology, so that relationship between the factors and the response variable can be determined. Even though there are several literatures on lipase production using different substrates, the present work serves as the first report on the production and optimization of lipase using POME.

2. Materials and methods

2.1. Chemicals

p-Nitrophenyl palmitate (*p*-NPP) for lipase assay was purchased from Sigma (St. Louis, USA). All other chemicals used were of analytical grade and commercially available in Malaysia.

2.2. Sample collection

Palm oil mill effluent (POME) was collected from West oil mill of Sime Darby Plantation Sdn. Bhd., Carey Island, Malaysia in clean containers and immediately brought to the laboratory and stored at 4 °C.

2.3. Microorganism and preparation of inoculum

C. cylindracea ATCC 14830 was obtained from American Type culture collection. The strain was grown on potato dextrose agar plates at 28 °C for 4 days and subcultured every two weeks. It was then maintained and preserved at 4 °C. Four-day old PDA-plate culture of *C. cylindracea* ATCC 14830 was suspended in 10 ml of sterile distilled water and 1.0 ml of this suspension was used as the inoculum for pre-cultures. Incubations were carried out at 28 °C for 48 h at 150 rpm in 100 ml of seed medium containing Tween-80 (0.7%, v/v), olive oil (0.2%, v/v) and peptone (0.5%, w/v) to yield a microbial density of about 10^8 cells/ml.

2.4. 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 was added each to 50 ml medium in 150 ml Erlenmeyer flasks according to design of the matrix. The flasks were incubated for 6 days at 28 °C under orbital shaking at 150 rpm. The cell-free filtrate was used as a source of extracellular lipase.

2.5. Assay for lipase activity

Lipase activity assay was carried out according to the method described by Gupta et al. [15], where 1 ml of isopropanol containing 3 mg of *p*-nitrophenyl palmitate was mixed with 9 ml of 0.05 M Tris–HCl pH 8.0, containing 40 mg Triton X-100 and 10 mg gum arabic. The substrate solution mixture was stirred until all the constituents were dissolved completely. A total amount of 2.4 ml of freshly prepared substrate solution was dispensed into each test tubes. Thereafter, 0.1 ml of enzyme solution was added to initiate hydrolysis. After 15 min of incubation at 37 °C, the optical density at 410 nm was measured against an enzyme free control. One lipase unit (U) was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per milliliter per minute under the standard assay conditions. All the enzyme assays were carried out in triplicate and the average values were calculated.

Table 1
Medium components used in Plackett–Burman design showing the low and high levels of each variable.

Components[a]	Actual low level (%)	Actual high level (%)
Glucose	0.0	0.5
Peptone	0.0	0.5
Yeast extract	0.0	0.5
Malt extract	0.0	0.4
NH_4Cl	0.0	0.3
$NaNO_3$	0.0	0.2
$FeCl_3{\cdot}6H_2O$	0.0	0.005
$MgSO_4$	0.0	0.1
K_2HPO_4	0.0	0.2
Tween-80	0.0	0.5
Olive oil	0.2	1.0

[a] The components as well as their concentration levels were decided on the basis of literature reports on lipase production.

2.6. Medium optimization of lipase production by statistical approach

2.6.1. Identifying the significant variables using Plackett–Burman design

Plackett–Burman (PB) design is a valuable method for screening significant medium components that influence lipase production from a lot of variable components, so that insignificant ones can be eliminated to obtain a smaller, reliable and more manageable set of components. Based on PB design, each component was examined in two levels: '−1' for low level and '+1' for high level [16]. In this study, 11 components were screened using the statistical software package Design-Expert 6.0.8 (Stat Ease Inc., Minneapolis, USA), which generated a set of 12 experimental designs. All experiments were carried out in triplicate and the average of each was recorded as lipase activity. Table 1 shows the medium components under investigation as well as levels of each variable used in the experimental design, whereas Table 2 represents the design matrix. PB experimental design is based on the first order polynomial model:

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

where Y is the response (lipase activity U/ml), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. Thus, this model does not describe interaction among variables and only used to screen and evaluate the important variables that influence the response. The effect of individual component on lipase production was determined based on the main effect equation (E):

$$E = \frac{\text{Total response at high level} - \text{total response at low level}}{\text{Number of trials}} \quad (2)$$

2.6.2. One-factor-at-a-time analysis

Following PB design, the classical one-factor-at-a-time (OFAT) approach was employed to evaluate the possible optimum levels of the medium components that showed positive effects. This is necessary since POME (the basal medium used) on its own, has been reported to contain high concentrations of protein, carbohydrate, nitrogenous compounds, lipids and minerals [17]. The components investigated include peptone, yeast extract, Tween-80, olive oil, malt extract, $MgSO_4$ and inoculum. Thus, appropriate

Table 2
Plackett–Burman experimental design for evaluation of 11 components with the actual and coded values for lipase production by *C. cylindracea* (ATCC 14830) and the design response.

Run	G (%w/v)	P (%w/v)	YE (%w/v)	ME (%w/v)	NH_4Cl (%w/v)	$NaNO_3$ (%w/v)	$FeCl_3{\cdot}6H_2O$ (%w/v)	$MgSO_4$ (%w/v)	K_2HPO_4 (%w/v)	T (%v/v)	O (%v/v)	Lipase activity (U/ml)
1	0.5(+1)	0.0(−1)	0.5(+1)	0.0(−1)	0.0(−1)	0.0(−1)	0.005(+1)	0.1(+1)	0.2(+1)	0.0(−1)	1.0(+1)	0.209
2	0.0(−1)	0.5(+1)	0.5(+1)	0.4(+1)	0.0(−1)	0.2(+1)	0.005(+1)	0.0(−1)	0.2(+1)	0.0(−1)	0.2(−1)	1.209
3	0.0(−1)	0.5(+1)	0.0(−1)	0.0(−1)	0.0(−1)	0.2(+1)	0.005(+1)	0.1(+1)	0.0(−1)	0.5(+1)	1.0(+1)	3.888
4	0.0(−1)	0.0(−1)	0.0(−1)	0.4(+1)	0.3(+1)	0.2(+1)	0.0(−1)	0.1(+1)	0.2(+1)	0.0(−1)	1.0(+1)	0.004
5	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)	0.5(+1)	1.0(+1)	3.864
6	0.5(+1)	0.0(−1)	0.0(−1)	0.0(−1)	0.3(+1)	0.2(+1)	0.005(+1)	0.0(−1)	0.2(+1)	0.5(+1)	0.2(−1)	0.004
7	0.5(+1)	0.5(+1)	0.0(−1)	0.4(+1)	0.0(−1)	0.0(−1)	0.0(−1)	0.1(+1)	0.2(+1)	0.5(+1)	0.2(−1)	3.884
8	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)	0.0(−1)	0.2(−1)	0.600
9	0.0(−1)	0.0(−1)	0.5(+1)	0.4(+1)	0.3(+1)	0.0(−1)	0.005(+1)	0.1(+1)	0.0(−1)	0.5(+1)	0.2(−1)	2.664
10	0.5(+1)	0.5(+1)	0.5(+1)	0.0(−1)	0.3(+1)	0.2(+1)	0.0(−1)	0.1(+1)	0.0(−1)	0.0(−1)	0.2(−1)	2.671
11	0.5(+1)	0.5(+1)	0.0(−1)	0.4(+1)	0.3(+1)	0.0(−1)	0.005(+1)	0.0(−1)	0.0(−1)	0.0(−1)	1.0(+1)	0.556
12	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)	0.5(+1)	1.0(+1)	3.899

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

concentrations and relative availability of these components are critical in overall media design. Fixed inoculum concentration was used in PB design but its concentration is of importance especially when considering its interaction with other medium components. As such the range of 1.0–5.0% (v/v) inoculum was examined. For peptone, yeast extract and malt extract, the same concentration range was studied i.e. from 0.1 to 0.6% (w/v), while 0.1–1.0% (v/v) and 0.25–1.25% (v/v) were investigated for olive oil and Tween-80, respectively.

2.6.3. Response surface methodology (RSM)

RSM was used to optimize the screened variables for enhanced lipase production. This process involves three important steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model.

Face centered central composite design (FCCCD) under the response surface methodology (RSM) developed by the Design Expert software (Version 6.0.8, Stat-Ease Inc., Minneapolis, USA) was used to optimize the concentrations of the three significant factors namely peptone, Tween-80 and inoculum, to find a set of 20 experimental runs with six replicated center points. The independent variables were studied at three different levels, low (−1), medium (0) and high (+1). The experimental design used for the study is shown in Table 3. The remaining factors (olive oil and POME) were maintained at the fixed concentration throughout the analysis based on the result of one-factor-at-a-time study. Experiments were conducted in 150 ml Erlenmeyer flasks containing 50 ml media (pH 6.0) prepared according to the design. The incubation was carried out at 28 °C and 150 rpm. All the experiments were carried out in triplicate and the average of lipase production obtained was taken as the response (Y). The relationship between dependent and independent variables is explained by the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3)$$

where Y is the dependent variable (lipase production); X_1, X_2 and X_3 are independent variables (peptone, Tween-80 and inoculum concentrations); β_0 is an intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are the interaction coefficients; and β_{11}, β_{22} and β_{33} are the quadratic coefficients.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability values, coefficient of determination R^2 which measures the goodness of fit of regression model. The fitted polynomial equation was then expressed in the form of contour and surface plots in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

2.6.4. Validation of the experimental model

The statistical model was validated with respect to all the three variables within the design space. Experiments predicted by the point prediction feature of the Design Expert software were conducted in triplicates. Six combinations of media constituents were used to determine the lipase production and the results were compared with the predicted values.

3. Results and discussion

3.1. Screening of media constituents for lipase production using the Plackett–Burman design

The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its propensity and relevance. In the present study, the medium components necessary for enhanced lipase production were screened and selected using the Plackett–Burman (PB) design.

Table 3
Experimental design using FCCCD of three independent variables with their actual and coded values and six center points showing the experimental and predicted response.

Run order	Peptone (%w/v)	Tween-80 (%v/v)	Inoculum (%v/v)	Lipase activity (U/ml)	
				Experimental	Predicted
1	0.40 (0)	0.75 (0)	2.00 (0)	19.89	19.48
2	0.40 (0)	0.75 (0)	2.00 (0)	19.01	19.48
3	0.60 (+1)	1.00 (+1)	3.00 (+1)	15.26	15.07
4	0.40 (0)	0.75 (0)	2.00 (0)	19.80	19.48
5	0.60 (+1)	0.50 (−1)	3.00 (+1)	18.26	18.12
6	0.20 (−1)	0.50 (−1)	1.00 (−1)	14.53	14.66
7	0.40 (0)	0.50 (−1)	2.00 (0)	18.35	18.94
8	0.40 (0)	1.00 (+1)	2.00 (0)	18.58	18.21
9	0.20 (−1)	0.75 (0)	2.00 (0)	18.14	18.10
10	0.20 (−1)	0.50 (−1)	3.00 (+1)	16.75	16.39
11	0.40 (0)	0.75 (0)	2.00 (0)	19.90	19.48
12	0.20 (−1)	1.00 (+1)	3.00 (+1)	16.23	16.41
13	0.20 (−1)	1.00 (+1)	1.00 (−1)	16.17	16.25
14	0.60 (+1)	0.50 (−1)	1.00 (−1)	17.46	17.23
15	0.40 (0)	0.75 (0)	2.00 (0)	18.89	19.48
16	0.40 (0)	0.75 (0)	3.00 (+1)	17.97	18.47
17	0.40 (0)	0.75 (0)	2.00 (0)	19.80	19.48
18	0.40 (0)	0.75 (0)	1.00 (−1)	18.24	17.95
19	0.60 (+1)	1.00 (+1)	1.00 (−1)	15.45	15.75
20	0.60 (+1)	0.75 (0)	2.00 (0)	18.47	18.72

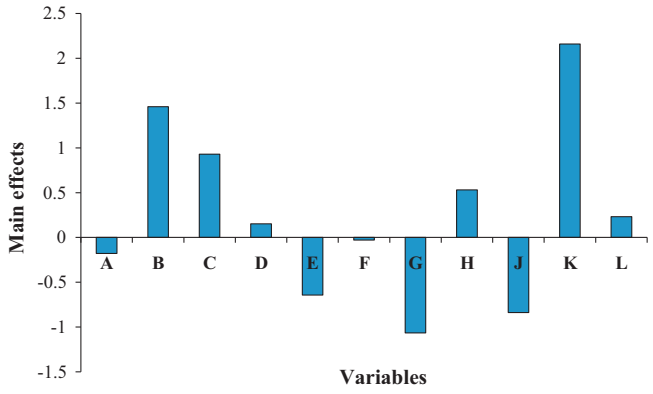

Fig. 1. Main effects of the medium constituents for lipase production based on the Plackett–Burman experimental results (A, glucose; B, peptone; C, yeast extract; D, malt extract; E, NH_4Cl; F, $NaNO_3$; G, $FeCl_3{\cdot}6H_2O$; H, $MgSO_4$; J, K_2HPO_4; K, Tween-80; L, olive oil).

A total of eleven medium components were screened by the Plackett–Burman design through twelve experimental runs with two levels each (Table 1) for lipase production. 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 [11]. The main effects of each medium component are presented in Fig. 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 component. It can be seen that $FeCl_3{\cdot}6H_2O$, K_2HPO_4, and NH_4Cl were the components that highly affect the lipase production at negative levels, followed by glucose and $NaNO_3$. It is not surprising that glucose appeared non contributory to the production, because reports on lipase production linked its presence with catabolite repression. Fickers et al. [18] reported that extracellular lipase production by *Yarrowia lipolytica* CBS6303 was found to be repressed by glucose and glycerol but increased in the presence of oleic acid. Habib et al. [17] reported the presence of several inorganic minerals in POME including Fe (11.08 ± 2.20 μg/g), P ($14{,}377.38 \pm 1206.88$ μg/g), Na (94.57 ± 6.45 μg/g), and K (8951.55 ± 256.45 μg/g). Thus, the negative effect of minerals in the PB experimental results suggested that exogenous source of minerals in POME based medium is not needed since they are required in smaller quantities, and their high concentration may affect the overall productivity.

At positive levels, the medium components affecting the lipase production could be ranked as Tween-80 > peptone > yeast extract > $MgSO_4$ > olive oil > malt extract. Based on the design, the lipase produced by *C. cylindracea* (ATCC 14830) ranges from 0.004 to 3.899 U/ml, which indicates a strong influence of medium components on enzyme production. The highest activity was found in run 12, where all the medium components present (peptone, yeast extract, NH_4Cl, K_2HPO_4, Tween-80 and olive oil) were at their highest levels, while runs 4 and 6 showed the lowest activity of 0.004 U/ml. This could be related to the absence of organic nitrogen sources despite the presence of inducers.

The result obtained in this study followed what was obtained using synthetic substrates by Rajendran et al. [19], where Plackett–Burman statistical design was used to evaluate the fermentation medium components. Among the twelve medium components screened in 16 experimental trials, glucose, olive oil, peptone, and $FeCl_3{\cdot}6H_2O$ were found to have more significant influence on lipase production by *Candida rugosa* with an activity of 3.8 U/ml. In case of *Rhizopus arrhizus*, lipase production was screened by Plackett–Burman experimental design. The most significant variables that led to a maximum lipase activity of 3.98 U/ml were found to be olive oil, peptone, KH_2PO_4, $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ [20]. 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. Based on these, the factors that positively contributed to lipase production i.e. peptone, yeast extract, malt extract, $MgSO_4$, Tween-80 and olive oil, were further screened using the one-factor-at-a-time experiments, so as to get the optimum levels of each that can be used for optimization using RSM.

3.2. One-factor-at-a-time (OFAT) to determine the optimum levels of parameters

The improvement of microbial lipase production is the purpose of several investigations. Each strain has its specific requirement in special conditions for maximizing the enzyme production. Various components in the media influence the production of extracellular lipase enzyme which includes inducers, carbon and nitrogen substrates and trace elements. Yeasts require a range of metals for optimal growth, metabolism and fermentation performance. The requirement for metal ions varies so widely with the different strains [21]. Magnesium ion is required by most microorganisms and plays some regulatory functions through increased adenosine triphosphate metabolism and nucleic acid synthesis [22]. However, POME on its own was found to contain high amount of magnesium up to 911.95 μg/g [17]. This concentration was found to be adequate for maximizing the lipase activity (data not shown). As such, further addition of magnesium into the medium was not considered, since the requirements of the microorganisms were fulfilled by the components of POME. It was found that magnesium ion up to a maximum concentration of 1.5 mM stimulated the production of extracellular thermostable lipase by *Aspergillus terreus* and further increase in this concentration resulted in a remarkable decrease in production [23].

Malt extract was used to augment the nitrogen sources during fermentation processes [24]. Muralidhar et al. [10] studied the effects of two different media for lipase production by *C. cylindracea* and found that olive oil, peptone, yeast extract and malt extract led to higher production. Even though malt extract appeared to be positively contributing based on PB results, its contribution with respect to lipase production in POME-based medium was found to be insignificant based on the OFAT results (data not shown). Since our target in this study is to use POME as a basal medium with minimal nutrient supplementation, then malt extract was excluded.

Lipases are mostly inducible enzymes and their inducers in form of oils, are necessary for the enzyme production [1]. The production of lipase by *Y. lipolytica* 681 was enhanced significantly ($P < 0.05$) by olive or corn oil when used as both carbon and inducer sources. Olive and corn oil were the best inducers and carbon sources for growth and production of lipases [25]. Olive oil was found to increase the yield of extracellular lipase in *C. cylindracea* NRRL Y-17506 [26]. Effect of different concentrations of olive oil was studied (Fig. 2), and the maximum lipase activity of 6.15 U/ml was found at 0.2% (v/v) olive oil. Addition of more than 0.2% (v/v) olive oil resulted in decrease in overall lipase activity. As such olive oil was fixed during optimization experiments.

Peptone and yeast extract are organic nitrogen sources and showed positive main effects. Organic nitrogen sources tend to be favored by lipase producing organisms. Both peptone and yeast extract were found to have positive effects on lipase production by *R. arrhizus* MTCC 2233, at confidence levels of 97.48% and 83.28% respectively, and peptone was found to be statistically significant [20]. However, Brozzoli et al. [26] obtained maximum extracellular lipase production of 10 U/ml in shake flask cultures using *C.*

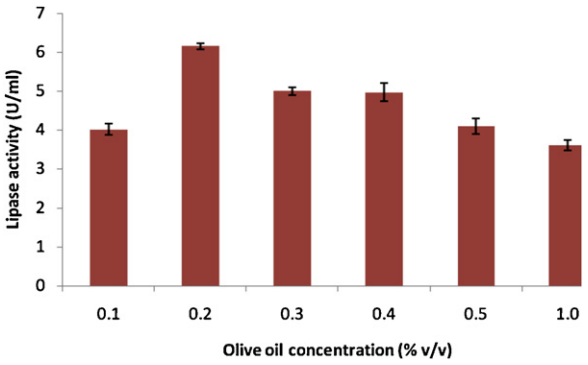

Fig. 2. Effects of different concentrations of olive oil (0.1–1.0%, v/v) on lipase activity by *C. cylindracea*.

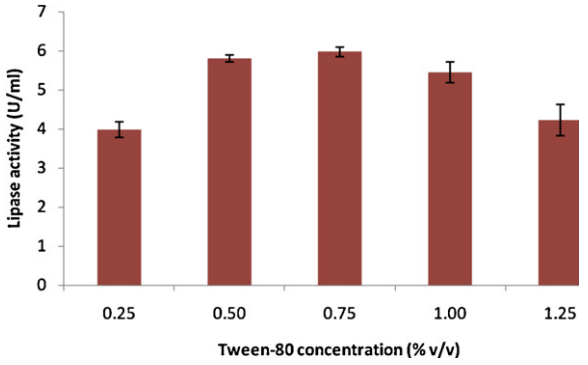

Fig. 4. Effects of different concentrations of Tween-80 (0.25–1.25%, v/v) on lipase activity by *C. cylindracea*.

cylindracea NRRL Y-17506 when olive mill wastewater was supplemented with ammonium chloride as an inorganic nitrogen source and olive oil. Apparently, this is in contrast with the findings of this work, as ammonium chloride screened by PB showed strong negative effect. Using OFAT experiments, peptone was found to be more contributing than yeast extract and thus only peptone was chosen for optimization. Fig. 3 shows the maximum activity of 5.92–5.96 U/ml with the different concentration of peptone at 0.4–0.5% (w/v). This was similar to the criteria used by Ruchi et al. [11] where both tryptone and peptone appeared to be positively contributing based on PB experiments but since they serve the same function, tryptone was favored.

In case of Tween-80, lipase production was enhanced by increasing the concentration of Tween-80 into the culture medium. A slight increase in lipase production was observed between 0.5 and 0.75% (v/v) Tween-80 but further increase in concentration led to a decrease in the production (Fig. 4). This agrees with the findings of Dalmau et al. [27], where Tween-80 was found to stimulate both lipase biosynthesis and its secretion in *C. rugosa*. In contrast, the addition of Tween-80 to *C. cylindracea* NRRL Y-17506 in olive mill wastewater supplemented with olive oil (3 g/L) resulted in an unexpected 50% reduction of the lipase activity [26]. Also, the yeast *Pseudozyma hubeiensis* HB85A lipase was strongly stimulated by 150.8% in the presence of Tween-80 [3]. Based on this, Tween-80, a nonionic surfactant with a molecular structure of polyoxyethylene sorbitan monooleate can act effectively as a carbon source in the lipase fermentation medium in some microorganisms, due to its ability to increase cell wall permeability and/or release cell bound enzymes [28].

Even though the inoculum concentration was fixed in Plackett–Burman design, but optimization of inoculum density is quite important, as high inoculum density can affect the enzyme production due to competition for available nutrients. In a similar way, low density can result in a mitigation of enzyme secretion, owing to a drop in cell numbers [29]. Different levels of inoculum with the concentrations of 10^8 cells/ml from 1.0 to 5.0% (v/v) were tested to determine the level of inoculum for the maximum production of lipase. The highest lipolytic activity of 6.2 U/ml was obtained at about 2% (v/v) inoculum levels when other parameters were fixed (Fig. 5). Several investigators have used different levels of inoculum for lipase production employing different microorganisms. Muralidhar et al. [10] used up to 10% (v/v) inoculum levels of *C. cylindracea* to obtain a maximum lipase production of 47.25 U/ml using olive oil, yeast extract, malt extract, peptone and Tween-80.

Since the aim of using OFAT in this study is the product promoting ability and the need to keep the number of factors as low as possible for optimization studies using FCCCD, only three factors, viz., peptone, Tween-80 and inoculum were identified as the most effective, while keeping the concentration of olive oil and POME constant.

3.3. Optimization of medium components by response surface methodology

Several fold increment in lipase production showed that statistically based experimental design is a valuable tool in optimizing the medium. This method offers a number of important advantages such as the factor effects, obtaining the optimum values and also developing a system model with considerably less experimental requirements [30]. Based on the PB and OFAT experiments, face centered central composite design under response surface methodology was used to determine the optimum conditions of the three significant factors (peptone, Tween-80 and inoculum). For each run, the experimental along with the predicted lipase activity obtained

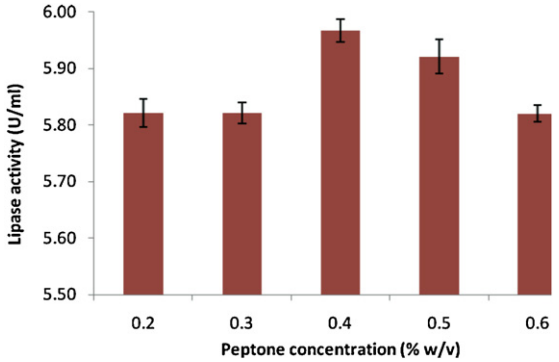

Fig. 3. Effects of different concentrations of peptone (0.1–0.6%, w/v) on lipase activity by *C. cylindracea*.

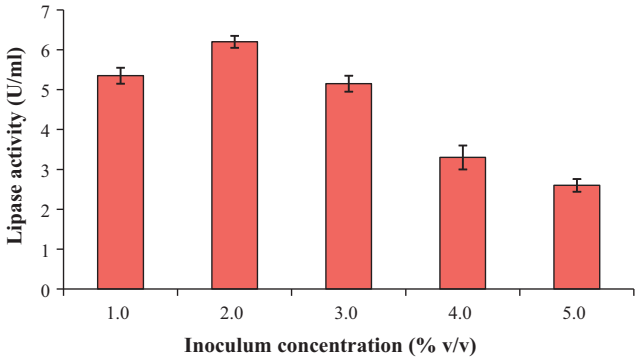

Fig. 5. Effects of different concentrations of inoculum (1.0–5.0%, v/v) on lipase activity by *C. cylindracea*.

Table 4
Analysis of variance of quadratic model for lipase production.

Source	Sum of squares	*F*-value	*p*-value
Model	47.97	22.34	<0.0001
Peptone, A	0.94	3.95	0.0749
Tween-80, B	1.34	5.62	0.0392
Inoculum, C	0.68	2.87	0.1212
A^2	3.15	13.20	0.0046
B^2	2.25	9.43	0.0118
C^2	4.43	18.56	0.0015
AB	4.70	19.72	0.0013
AC	0.35	1.47	0.2535
BC	1.24	5.19	0.0460
Lack of fit	1.30	1.19	0.4252

R^2 = 0.956, adjusted R^2 = 0.91, CV = 2.73, adequate precision = 13.943.

from the regression equation for the 20 combinations are shown in Table 3. The results demonstrated that the highest amount of lipase produced by *C. cylindracea* ATCC 14830 (18.89–19.90 U/ml) was observed in the runs representing the center points (runs 1, 2, 4, 11, 15 and 17) and the lowest amount was observed in run 6 (14.53 U/ml).

A second order regression equation showed the dependence of *C. cylindracea* lipase activity on the medium constituents. The parameters of the equation were obtained by multiple regression analysis of the experimental data. An empirical relationship between the response and the screened variables was expressed in terms of second-order polynomial equation:

$$Y\,(\text{Lipase activity, U/ml}) = +19.48 + 0.31A - 0.37B + 0.26C - 1.07A^2 - 0.90B^2 - 1.27C^2 - 0.77AB - 0.21AC - 0.39BC \quad (4)$$

where the lipase production is the response (Y) and A, B and C are the concentrations of peptone, Tween-80 and inoculum, respectively.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results are shown in Table 4. The model F value of 22.34 and p-value of <0.0001 imply that the model is significant, suggesting that there is only 0.01% chance that the model F value could occur due to noise. Model terms with Prob > F (less than 0.05) are considered significant, while those greater than 0.10 are insignificant [31]. The non significant lack of fit suggested that the obtained experimental responses sufficiently fit with the model.

The R^2 value closer to 1 denotes better correlation between the observed and predicted values. The higher values of R^2 (0.9526) and adjusted R^2 (0.9100) also indicated the efficacy of the model suggesting that 95.26% and 91% variation could be accounted for by the model equation respectively. Thus, for a good statistical model, the R^2 value should be in the range of 0–1.0, and the closer the value is to 1.0, the more fit the model is deemed to be [29]. Adequate precision measures the signal to noise ratio and a value >4 is considered appropriate for desirable models. The adequate precision value of 13.94 for lipase production indicates that the model can be used to navigate the design space. Also, the coefficient of variation (CV) indicates the degree of precision with which the treatments are compared, and the low value of CV showed the reliability of experiment. In this study, a relatively lower value of the coefficient of variation (CV = 2.73) suggested a good precision and reliability of the experiment.

The coefficient values of the regression equation are listed in Table 4. The p-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable; the smaller the p-values, the bigger the significance of the corresponding coefficient. The responses revealed that only one (B, Tween-80), out of the three linear coefficients, two interaction terms AB (peptone and Tween-80) and BC (Tween-80 and inoculum), and all the three quadratic coefficients were significant ($p < 0.05$) and had remarkable effects on the overall production.

The 3D response surface plot is the graphical representations of the regression equation used to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum lipase production by *C. cylindracea* in POME based medium. The 3D plots shown in Fig. 6 were based on the function of concentrations of two variables with the other variable being at its optimum level. Significance of the interactions between the corresponding variables is indicated by an elliptical or saddle nature of the contour plots [10]. Fig. 6a represents the interaction between peptone and Tween-80. Lower and higher levels of both the peptone and Tween-80 did not result in higher enzyme yields. The shape of the response surface curves showed a moderate interaction between these tested variables. While Fig. 6b shows the 3D plot corresponding to Tween-80 and inoculum concentration, this plot showed somewhat an elliptical contour suggesting that not only there were well defined optimum operating conditions but also the interaction effect between the two factors was significant. In case of peptone and inoculum (Fig. 6c), the response plot was elliptical depicting interaction between them with optimum production by *C. cylindracea*. Thus, it can be seen that the optimized combinations of the selected media components showed strong synergistic effects on lipase production. Media manipulation tends to be a better alternative for the overproduction of enzymes, since the secretion of metabolism products is an important component of survival strategies of some microorganisms occupying certain environments [32].

To investigate the applicability of the second-order model developed, some sets of experiments with three replications each were performed according to the media constituents presented in Table 5. Some of the experimental results were slightly higher than those predicted by the model. The slight deviation of the experimental results may be explained based on the experimental conditions, in which experiments were run in the border zone of the technological space considered in the FCCCD. From the optimization study, it is clearly observed that the production of lipase of 20.26 U/ml with optimum conditions obtained by the FCCCD was 5.19-fold and 3.27-fold higher than the production obtained by the PB design (3.89 U/ml) and the OFAT method (6.20 U/ml), respectively.

Several folds increase in lipase production has been reported in the literature using response surface methodology by different organisms. He and Tan [9] reported the culture medium optimization using RSM for lipase production by the strain *Candida* sp. 99-125, where 20% higher lipase production was obtained under optimized conditions. Burkert et al. [33] studied the effects of carbon source (soybean oil, olive oil, and glucose) and nitrogen source concentrations (corn steep liquor and NH_4NO_3) on lipase production by *Geotrichum* sp. using the methodology of response surface. The lipase activity reached 20 U/ml under optimized conditions at 30 °C. In case of pure cheese whey, the extracellular lipolytic activity after 120 h of fermentation by *C. rugosa* was 5.18 U/ml. A complete 2^4 factorial design was used to determine the effects of various co-substrates (brewery coproduct, yeast extract, malt extract, Tween-80, and olive oil) on the overall production. This resulted in activity of 15 U/ml which is about 2.8 fold higher than the activity obtained using only cheese whey as the substrate [34].

A 2^5 design matrix using response surface approach was developed to study the production of extracellular lipase by *C. cylindracea* with only the carbon sources being varied. The optimal yield of lipase obtained was 17.30 U/ml using glucose as the carbon source and 47.25 U/ml when olive oil was the main carbon source [10].

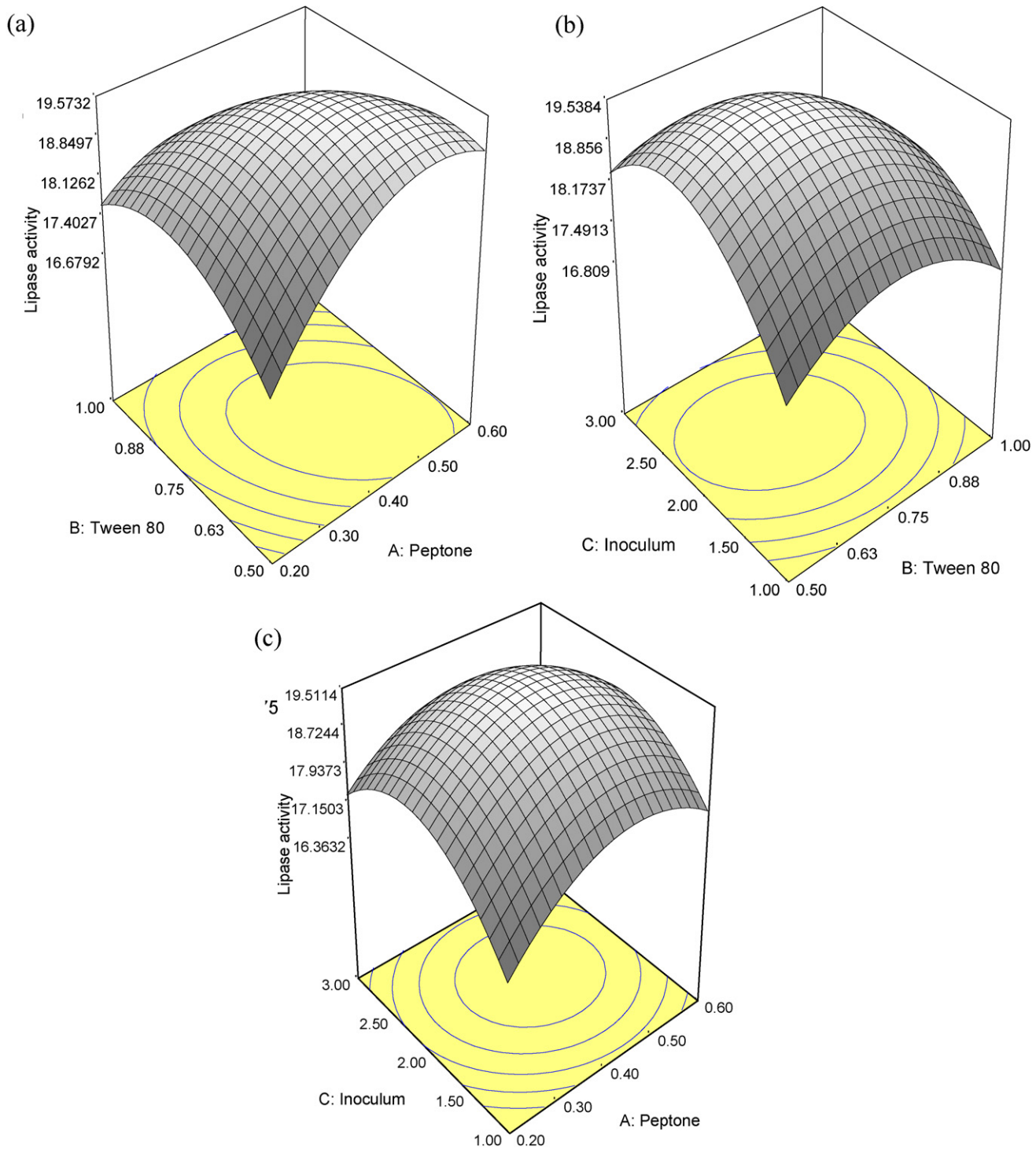

Fig. 6. The 3D response surface curves of the combined effects of peptone, Tween-80 and inoculums on lipase production by *C. cylindracea* ATCC 14830: (a) peptone and Tween-80 at fixed level of inoculum, (b) Tween-80 and inoculum at fixed level of peptone, and (c) peptone and inoculum at fixed level of Tween-80.

In this study, POME being a rich source of organic carbon, nitrogen as well as minerals was used, with other components for the production of lipase using *C. cylindracea* by means of PB design, OFAT method and FCCCD. Using PB design as the first screening step, different components (peptone, yeast extract, malt extract, $MgSO_4$, Tween-80 and olive oil) were found to contribute positively to the lipase production in POME-based medium. However, the classical OFAT methods revealed that peptone, Tween-80 and small amount of olive oil were associated with high lipase production. Since microbial fermentation processes are influenced significantly by inoculum concentration, the OFAT method used in this study indicated that inoculum concentration was essential. Based on this, optimization of three components (peptone, Tween-80 and inoculum) was carried out using FCCCD to develop a second-order regression model. A successful and significant improvement (5.19-fold) in the production of lipase was accomplished.

Table 5
Validation of the experimental model.

Experiment	Peptone (%w/v)	Tween-80 (%v/v)	Inoculum (%v/v)	Lipase activity (U/ml)	
				Experimental	Predicted
1	0.45	0.65	2.20	20.26 ± 0.02	19.59
2	0.40	0.70	2.00	19.48 ± 0.10	19.52
3	0.50	0.70	2.00	19.97 ± 0.05	19.48
4	0.50	0.60	2.50	19.37 ± 0.01	19.37
5	0.60	0.80	2.50	18.10 ± 0.02	18.26
6	0.45	0.70	2.00	20.07 ± 0.08	19.57

4. Conclusion

The observed lipase activity in this study seems promising for efficient production of microbial lipases using POME as a basal medium (agro-industrial waste) in submerged fermentation. The combined effects of PB design and OFAT method proved effective in finding the important medium components that have significant effect on lipase production in POME-based medium. Using face centered central composite design, the optimum components for high lipase activity were peptone 0.45% (w/v), Tween-80 0.65% (v/v) and inoculum 2.2% (v/v). The predicted lipase production was validated to be 20.26 U/ml with the optimum medium constituents. This indicated that optimization resulted in 5.19-fold increase in overall lipase production. Similarly, the utilization of inexpensive and available substrates like POME as an alternative to more commonly used and expensive medium would result in a considerable reduction in the production costs.

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