Optimization of thermostable organic solvent-tolerant lipase production by thermotolerant *Rhizopus* sp. using solid-state fermentation of palm kernel cake

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Abstract This study enhanced the production of thermostable organic solvent-tolerant (TS-OST) lipase by locally isolated thermotolerant *Rhizopus* sp. strain using solid-state fermentation (SSF) of palm kernel cake (PKC). The optimum conditions were achieved using a series of statistical approaches. The cultivation parameters, which include fermentation time, moisture content, temperature, pH, inoculum size, various carbon and nitrogen sources, as well as other supplements, were initially screened by the definitive screening design, and one-factor-at-a-time using PKC as the basal medium. Three significant factors (olive oil concentration, pH, and inoculum size) were further optimized using face-centred central composite design. The results indicated a successful and significant improvement of lipase activity by almost two-fold compared to the initial screening production. The findings showed that the optimal conditions were 2% (v/w) inoculum size, 2% (v/w) olive oil, 0.6% (w/w) peptone, 2% (v/w) ethanol, 70% moisture content at initial pH 10.0 and 45 °C within 72 h of fermentation. Process optimization resulted in maximum lipase activity of 58.63 U/gram dry solids (gds). The analysis of variance showed that the statistical model was

significant (p value <0.0001) and reliable with a high value of R^2 (0.98) and adjusted R^2 (0.96). This indicates a better correlation between the actual and predicted responses of lipase production. By considering this study, the low-cost PKC through SSF appears to be promising in the utilization of agro-industrial waste for TS-OST lipase production. This is because satisfactory enzyme activity could be attained that promises industrial applications.

Keywords Optimization · Palm kernel cake · *Rhizopus* sp. · Thermostable organic solvent-tolerant lipase · Solid-state fermentation

Introduction

Lipase, triacylglycerol acyl hydrolase (EC 3.1.1.3), is a lipolytic enzyme used as a biocatalyst for a wide range of industrial processes. Since the majority of the industrial processes operate at high temperatures and in the presence of organic solvent, there is demand for discovering enzymes that function optimally in hostile conditions without losing their activities. Enzymes produced from the isolation of thermophilic organisms are expected to have higher thermostability, resistance toward chemical denaturation and have the capability to catalyze biochemical reactions at a temperature higher than those of mesophiles. The isolation of thermophilic organisms has also shown organic solvent tolerance to the enzyme being secreted (Rahman et al. 2009), making them more attractive to industry. Thus, the industrial demand for thermostable organic solvent-tolerant (TS-OST) lipase has driven the search for novel thermophilic microorganisms from nature. Most of the previous studies on microbial lipase were explored via bacteria (Yele and Desai 2015; Mo et al.

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2016; Yang et al. 2016) and a limited number of thermophilic fungal strains producing TS-OST lipases have been reported (Romdhane et al. 2010). In fact, filamentous fungi are more favorable to be used as microorganisms in the SSF process than bacteria and yeast since they promote fungal growth similar to their natural habitat, which results in higher metabolic activities.

Since the production cost of the industrial enzyme is affected by the cost of the fermentation medium, the selection of effective medium formulations that yield high enzyme production is a significant consideration. Various low-cost nutrients from organic agricultural residues or wastes can be utilized in the fermentation process as a carbon and energy source for microorganisms to produce more economical lipase (Salihu et al. 2012). The palm kernel cake (PKC), a by-product of oil palm processing, is produced at low-costs and contains most of the macro- and micro-nutrients for the fermentation process of various bio-products including lipase. Malaysia, as one of the world's main producers of palm oil, has produced approximately 2.5 million tons of PKC annually (MPOC 2014). In this study, PKC was used as a basal medium that contains carbon, nitrogen, phosphorous, potassium and minerals, which needed to be optimized for the maximum production of lipase with other supplementary nutrients as well as the inducer and stimulant. The production cost of lipase can be reduced by avoiding other supplements in the basal medium of PKC, which render the PKC a low-cost substrate.

A new thermo solvent-tolerant, *Rhizopus* sp., was recently isolated from PKC with potential production of TS-OST lipase useful for various industrial applications (Riyadi et al. 2016). The present study seeks to optimize the production of lipase by the newly isolated fungi by formulating and analyzing the effects of various solid-state fermentation (SSF) culture mediums and process conditions on lipase production. A series of statistical experimental designs comprising definitive screening design (DSD), one-factor-at-a-time (OFAT) and face-centered composite design (FCCCD) were used to construct extensive experimental design for the selection of the most suitable condition for optimum lipase production. The fermentation conditions and culture medium being studied include fermentation time, initial moisture content, temperature, pH, inoculum size, various carbon (glucose, ethanol, methanol) and nitrogen sources (peptone, yeast extract, $(\text{NH}_4)_2\text{SO}_4$), as well as other supplements (olive oil, Tween 80, KH_2PO_4 , Na_2HPO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). This study is the first report on the utilization of PKC as the basal medium in the SSF process to produce TS-OST lipase from thermotolerant *Rhizopus* sp.

Materials and methods

Sample collection

PKC was used as the main sample in the study, which was collected from West Oil Mill of Sime Darby Sdn Bhd. Carey Island, Malaysia, in a clean container and stored at 4 °C.

Microorganism and inoculum preparation

Thermotolerant *Rhizopus* sp. was locally isolated from PKC in Malaysia, following the methods of Cooney and Emerson (1964). The seven-day grown culture was washed with approximately 25 ml of sterile distilled water using a bent glass rod. The suspended fungal cultures were then filtered using Whatman No. 1 filter paper to remove the mycelia from the spore suspension. The filtrate was transferred into a 150 ml Erlenmeyer flask and used as inoculum (Alam et al. 2004).

Lipase production in SSF

SSF was conducted according to the methods by Iluyemi et al. (2006) in a 250 ml Erlenmeyer flask containing the concentration of media components, inoculum and process parameters as adjusted based on the experimental design (Table 1). The flask was incubated at 45 °C. The crude enzyme was acquired by mixing the fermentation with 50 ml of sterile distilled water in flasks and the contents were agitated in a rotary shaker at 180 rpm for 1 h and 30 min at room temperature (32 °C) to initiate the enzyme extraction. The supernatant was then centrifuged at 4.0 °C and 8500 rcf for 15 min. The resulting clear filtrate was used for lipase assay (Ong et al. 2004).

Assay for lipase activity

Lipase activity was determined using para-nitrophenyl palmitate (*p*NPP) as the substrate, where 10 ml of isopropanol containing 30 mg of para-nitrophenyl palmitate was mixed with 90 ml of 0.05 M phosphate buffer at pH 8.0 consisting of 207 mg of sodium deoxycholate and 100 mg of gum arabic. Approximately 2.4 ml of the freshly prepared substrate solution was mixed with 0.02 ml of enzyme solution and incubated at 45 °C for 15 min. The enzyme absorbance was measured using spectroscopy at 410 nm against an enzyme-free control (Gopinath et al. 2005).

Table 1 Definitive screening design (DSD) for evaluation of 11 combination factors of medium compositions and process conditions for TS-OST lipase production and the response expressed as lipase activity

Run	A (%) v/w)	B	C (%)	D (%) v/w)	E (%) v/w)	F (%) w/w)	G (%) w/w)	H (%) w/w)	I (%) w/w)	J (%) w/w)	K (%) w/w)	Lipase activity (U/ gds)
1	5.5	6.5	65	1.15	1.05	0.5	0.65	0.35	0.15	0.35	0.05	14.00
2	1	6.5	50	2	0.1	0	0.3	0.6	0.3	0.6	0	9.27
3	10	3.0	80	2	2	0	0.3	0.1	0.3	0.1	0.05	10.31
4	1	3.0	80	1.15	0.1	1	0.3	0.1	0	0.6	0.1	10.45
5	1	10.0	80	2	0.1	1	0.65	0.1	0.3	0.1	0	13.79
6	1	3.0	50	0.3	2	1	1	0.1	0.3	0.35	0	8.73
7	10	10.0	80	2	0.1	0	0.3	0.6	0	0.35	0.1	17.25
8	1	3.0	80	0.3	0.1	0	1	0.6	0.3	0.1	0.1	16.77
9	10	3.0	50	0.3	2	0	0.65	0.6	0	0.6	0.1	4.68
10	10	3.0	65	2	0.1	1	1	0.6	0	0.1	0	7.58
11	10	10.0	50	0.3	0.1	1	0.3	0.35	0.3	0.1	0.1	7.51
12	10	10.0	50	1.15	2	0	1	0.6	0.3	0.1	0	5.38
13	5.5	10.0	80	2	2	1	1	0.6	0.3	0.6	0.1	8.57
14	10	3.0	80	0.3	1.05	1	0.3	0.6	0.3	0.6	0	6.72
15	10	3.0	50	2	0.1	0.5	1	0.1	0.3	0.6	0.1	6.24
16	1	3.0	50	2	2	1	0.3	0.6	0.15	0.1	0.1	4.60
17	10	6.5	80	0.3	2	1	1	0.1	0	0.1	0.1	9.01
18	10	10.0	80	0.3	0.1	0	1	0.1	0.15	0.6	0	8.29
19	5.5	3.0	50	0.3	0.1	0	0.3	0.1	0	0.1	0	25.87
20	1	10.0	50	0.3	0.1	1	1	0.6	0	0.6	0.05	30.91
21	10	10.0	50	2	2	1	0.3	0.1	0	0.6	0	21.04
22	1	10.0	80	0.3	2	0.5	0.3	0.6	0	0.1	0	26.97
23	1	3.0	80	2	2	0	1	0.35	0	0.6	0	21.10
24	1	10.0	65	0.3	2	0	0.3	0.1	0.3	0.6	0.1	29.95
25	1	10.0	50	2	1.05	0	1	0.1	0	0.1	0.1	23.09

A inoculum size, B pH, C moisture content, D Tween 80, E olive oil, F peptone, G yeast extract, H $(\text{NH}_4)_2\text{SO}_4$, I KH_2PO_4 , J Na_2HPO_4 , K $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

Statistical analysis for optimum lipase production

DSD method

DSD was used to screen the potential medium compositions and process conditions for lipase production. Eleven factors comprising the medium components and process conditions were selected for screening, with three levels of each factor. Lipase activity expressed in U/gds dry PKC was used as the response. A set of 25 experimental designs were generated (Table 1). All the experimental runs were conducted in triplicate and average values were taken for the response. DSD eliminated factors with insignificant effect from the subsequent medium preparation.

OFAT analysis

Following DSD, a classical OFAT analysis was employed to select the best combinations of factors to obtain optimum lipase production with minimum conditions by varying one factor and maintaining the remaining factors at constant. Based on the selection in the DSD, the influential parameters were subjected for OFAT. Additional parameters such as fermentation time, moisture content, temperature and various carbon sources were also included in the OFAT design to explore their effects on lipase production and obtain their optimum level. The ranges selected were: 0.5–1.5% (v/w) inoculum size, 8.0–12.0 initial pH, 1.25–1.75% (v/w) olive oil concentration, 0.6–1.0% w/w peptone concentration, 1–8 days of fermentation time, 0.0–2.0% carbon concentration (methanol, ethanol,

glucose), 50–80% initial moisture content and 35–50 °C incubation temperature.

FCCCD method

Significant factors from the OFAT studies were subjected to the optimization process using response surface methodology (RSM) in the form of FCCCD to obtain the optimum fermentation conditions for maximal lipase production. In the FCCCD, three factors were studied, consisting of olive oil concentration, pH, and inoculum size. Other factors with less deviation toward lipase production were fixed at OFAT optimum concentrations. A set of 20 experimental runs with six center points were generated (Table 2). Each factor was studied in three levels [low (−1), medium (0) and high (+1)]. The results were used to generate a regression model by evaluating the regression coefficient values and analysis of variance (ANOVA). The general representation of the model is designated in Eq. (1):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, \quad (1)$$

where y is the predicted response (lipase activity in U/gds), β_0 term represented the intercept, β_i , β_{ij} and β_{ii} terms are

the regression coefficients for linear, interaction and quadratic, respectively. Meanwhile, the term x_i and x_j denoted the independent variables.

Data analysis

Design Expert 9.0 (Stat-Ease Inc., Minneapolis, USA) was used to design the experiments for the screening, optimization as well as validation stage. For the OFAT analysis, one-way ANOVA post-hoc Tukey's test ($p \leq 0.05$) was performed using IBM SPSS Statistics V.19 to calculate the significant difference in the data means to analyze the contribution of the factors that affect lipase production. To evaluate the optimization model, ANOVA and the response surface regression (RSREG) procedure of SAS/STAT(R) 9.2 were used.

Results and discussion

DSD for screening of the factors

Screening design is used to assess the effects of a large number of factors on a response in several experimental runs. Lipase produced based on the experimental design

Table 2 Faced centered central composite design (FCCCD) for selection of medium components and process conditions for TS-OST lipase production

Run	Factor 1	Factor 2	Factor 3	Response	
	A: inoculum size, %v/w	B: olive oil, %v/w	C: pH	Lipase activity (actual), U/gds	Lipase activity (predicted), U/gds
1	1 (−1)	2.5 (+1)	12.0 (+1)	4.39	3.58
2	1 (−1)	1.5 (−1)	12.0 (+1)	21.53	21.05
3	2 (0)	2 (0)	12.0 (+1)	50.13	51.45
4 ^a	2 (0)	2 (0)	10.0 (0)	52.83	52.36
5 ^a	2 (0)	2 (0)	10.0 (0)	55.93	52.36
6	3 (+1)	2.5 (+1)	8.0 (−1)	18.81	17.93
7 ^a	2 (0)	2 (0)	10.0 (0)	58.63	52.36
8	1 (−1)	1.5 (−1)	8.0 (−1)	44.50	42.69
9 ^a	2 (0)	2 (0)	10.0 (0)	53.23	52.36
10 ^a	2 (0)	2 (0)	10.0 (0)	51.59	52.36
11	3 (+1)	1.5 (−1)	8.0 (−1)	15.76	15.22
12	2 (0)	1.5 (−1)	10.0 (0)	37.79	41.12
13	3 (+1)	2 (0)	10.0 (0)	39.60	41.06
14	3 (+1)	1.5 (−1)	12.0 (+1)	21.58	21.09
15	2 (0)	2.5 (+1)	10.0 (0)	31.66	33.73
16	3 (+1)	2.5 (+1)	12.0 (+1)	42.60	43.06
17 ^a	2 (0)	2 (0)	10.0 (0)	52.78	52.36
18	2 (0)	2 (0)	8.0 (−1)	45.62	49.70
19	1 (−1)	2 (0)	10.0 (0)	31.11	35.05
20	1 (−1)	2.5 (+1)	8.0 (−1)	6.81	5.95

^a Center points

showed activity fluctuating between 4.60 to 30.91 U/gds (Table 1). The effect of each factor on the extracellular lipase production was illustrated in a Pareto chart shown in Fig. 1.

The most contributing factor affecting the lipase production was the inoculum size, followed by pH, an inorganic nitrogen source $(\text{NH}_4)_2\text{SO}_4$, peptone and olive oil concentration. Since KH_2PO_4 , Na_2HPO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and Tween 80 affected production negatively and considered insignificant at a low level, these factors were screened from the subsequent OFAT experimental analysis. Yeast extract was also excluded as it has a negligible influence on lipase production. Both $(\text{NH}_4)_2\text{SO}_4$ and peptone were studied to search for the most suitable nitrogen source. As for the lipid source, olive oil was a better lipase inducer than Tween 80. Additional minerals, such as $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and KH_2PO_4 were not needed. Imandi et al. (2010) reported similar findings for lipase production in SSF by *Yarrowia lipolytica* from PKC, in which inoculum size and peptone were among the most influential factors. In another study, Yele and Desai (2015) reported that out of 15 medium components screened for TS-OST lipase from *Staphylococcus warneri*, olive oil, peptone, maltose, and K_2HPO_4 had the highest contribution.

OFAT experimental analysis

OFAT experimental analysis was further conducted to obtain optimum design conditions for the enzyme production prior to the optimization stage (Elgharabawy et al. 2014). The parameters subjected to OFAT analysis include the inoculum size, pH, olive oil, $(\text{NH}_4)_2\text{SO}_4$ and peptone concentration, various carbon sources, fermentation time, moisture content, as well as temperature.

Fig. 1 Pareto chart illustrates the order of significance of the factors affecting the TS-OST lipase production

Effect of fermentation time

All proteins suffer denaturation and loss of catalytic activity over time. Thus, attaining the right incubation time for the enzyme production is important to obtain optimum production. From Fig. 2a, maximum lipase activity was observed after 3 days (72 h) of incubation time with 34.20 U/gds. A longer fermentation time caused declination in the lipase production. Thus, the fermentation time was maintained to 3 days for the optimization process. The decrease in activity might be related to the depletion of the nutrients or denaturation of the enzyme at longer fermentation time (Toscano et al. 2013). The result was in agreement with the study reported by Masomian et al. (2010), Ray (2012) and Ferrarezi et al. (2014).

Effect of carbon sources

Several carbon sources (glucose, ethanol, and methanol) were added to the fermentation medium to study their effect on lipase production. Based on Fig. 2b, ethanol at 2% concentration, was the most effective supplement for lipase production by thermotolerant *Rhizopus* sp. as it increased the activity from 18.24 to 27.13 U/gds. Barth and Gaillardin (1997) reported using ethanol as a carbon source to grow lipolytic yeast *Y. lipolytica* at a concentration up to 3%. However, higher ethanol concentrations were considered toxic to the organism. Meanwhile, methanol was found to increase the lipase production initially at 1% concentration, and 2% methanol was found to inhibit production. As for glucose, although the organism grew on glucose, the amount of lipase secreted was lower than that when in the absence of glucose. Lipase activity decreased by 6.6% from 18.24 U/gds in the absence of glucose, to 17.04 U/gds at 2% glucose concentration. Glucose can inhibit the growth of the organism by a repression effect, which leads to poor growth and thus poor enzyme synthesis (Norouzian 2008). Since only ethanol appeared to influence lipase production, 2% ethanol concentration was selected as the supplementary carbon source in the optimization process.

Effect of olive oil

Olive oil, with a high content of oleic acid, is reported to be one of the most commonly used substrates for lipase production by many bacteria and filamentous fungi as it serves not only as an inducer for lipase secretion but also as a carbon source for microorganism growth (Toscano et al. 2013). Maximum lipase production was attained in the medium supplemented with 1.75% olive oil (Fig. 2c), producing 28.11 U/gds lipase activity. Utilizing olive oil in the medium demonstrated better lipase production because

Fig. 2 Effects of **a** fermentation time, **b** different carbon source concentrations, **c** olive oil concentrations, **d** peptone concentrations, **e** growth temperature, **f** initial moisture contents, **g** inoculum size and **h** pH on TS-OST lipase production by thermotolerant *Rhizopus* sp. strain. Mean values with different letter are significantly different from each other using one-way ANOVA post-hoc Tukey's test ($p \leq 0.05$)

lipases favor hydrolyzing long chain carbon-length, such as olive oil, better than the short chain carbon-length. Since lipase production increased with increasing olive oil concentrations, which was inconsistent with the previous screening result that showed a negative effect, it was studied further in the optimization process to obtain the adequate concentration for optimum lipase production. Karbalaeei-Heidari (2014) used 2% olive oil concentration to obtain maximum solvent-tolerant lipase production from the *Pseudomonas* sp. strain NEB-1 with 83.44 U/ml lipase activity.

Effect of nitrogen source

Lipase production from fungi required somewhat higher concentrations of nitrogen source than the other microbial

enzymes. The previous screening result showed that peptone and $(\text{NH}_4)_2\text{SO}_4$ were determined to be effective nitrogen sources for lipase production by thermotolerant *Rhizopus* sp. However, the OFAT study for $(\text{NH}_4)_2\text{SO}_4$ showed an insignificant effect on the lipase production (Online Resource 1). Therefore, this factor was excluded from the subsequent fermentation medium. Meanwhile, peptone as the complex media rich in nitrogen (about 14%) could adequately provide the bio-element needed by the microorganism to secrete optimal lipase production (Rahman et al. 2006). As shown in Fig. 2d, the highest lipase activity was obtained (40.03 U/gds) at 0.6% w/w peptone concentration. Therefore, peptone concentration was fixed at a minimum level with 0.6% w/w in the subsequent optimization process. This finding was consistent with

previous similar studies reported by Cihangir and Sarikaya (2004) and Rahman et al. (2006).

Effect of growth temperature

The microbial growth temperature is another crucial factor that varies from organism to organism and small changes in growth temperature might influence the enzyme production. The maximum lipase production (Fig. 2e) was achieved at 40 °C with 34.97 U/gds and drastically declined at 50 °C with only 1.99 U/gds. According to Pirt (1975), temperature affects the secretion of the extracellular enzymes by altering the physical properties of the cell membrane. The decrease in lipase activity at higher temperatures might be due to the large amounts of metabolic heat that are produced, causing the fermenting substrate temperature to increase and inhibit the microbial growth as well as the enzyme formation (Bhatti et al. 2007). However, since growth at elevated temperatures is preferable to attain thermostable property of enzyme, growth temperature of 45 °C was used in the subsequent optimization study. This result was consistent with the previous findings by Hernández-Rodríguez et al. (2009) and Ferrarezi et al. (2014).

Effect of initial moisture content

In SSF, the optimum moisture content for growth and substrate utilization depends on the nature of the microorganism and the substrate used for cultivation. High initial moisture content leads to the accumulation of substrate particles, poor aeration, and possible anaerobic conditions, while very low moisture content restricts the fungal growth due to a reduction in solubility of the nutrients of the substrate (Kamath et al. 2015). In this study, an initial moisture content of 70% was needed to yield the highest enzyme production of 19.82 U/gds (Fig. 2f). Further increases or decreases in moisture content yield lower enzyme production. By comparing the results obtained from DSD, where moisture content had a positive contribution to lipase production, it was clear that the optimum moisture content was 70%. Therefore, 70% moisture content was fixed during the subsequent fermentation. In another study, 70% of initial moisture content gave the highest yield of whole-cell synthetic lipase (WCSL) by *Rhizopus chinensis* (Sun and Xu 2008) and *Penicillium simplicissimum* lipase (Gutarra et al. 2009).

Effect of inoculum size

Inoculum size is important in determining the productivity of the enzyme. Lower inoculum levels result in lesser amounts of biomass in the production medium. High

inoculum size is predicted to shorten the lag phase of fungal growth thereby achieving maximum enzyme production in a shorter time. However, in some circumstances, inoculum size only influences the microorganism growth and does not influence the enzyme production (Kumar and Kanwar 2012). Therefore, the effect of inoculum size on lipase production was studied (Fig. 2g). Lipase activity was found to increase as the inoculum size increased. The highest lipase activity was found at the high inoculum concentration of 1.5% with 25.93 U/gds. Since the results showed inconsistency with the previous DSD results, this factor was considered in the optimization stage by extending the inoculum size from 1 to 2%. In another study, maximum activity (18.58 U/gds) was accomplished using 2.0 ml [20.0% (v/w)] of inoculum by *Y. lipolytica* on PKC (Imandi et al. 2010).

Effect of initial pH

Each microorganism owns a unique optimum pH and pH range suitable for its growth and activity. Filamentous fungi are thought to be able to thrive over a broad range of pH under solid-state culture as the solid substrate has a better buffering capacity (Shaheen et al. 2008). As the screening design showed the high positive effect of lipase production with pH of the medium, fermentation medium at alkaline pH between 8.0 and 12.0 was selected for the OFAT study. The results (Fig. 2h) demonstrated that lipase production increased with increasing pH, and maximum activity was achieved at pH 12.0 with 26.0 U/gds. This showed that the lipase production from thermotolerant *Rhizopus* sp. was more favorable at alkaline pH. However, considering that very high alkaline pH might cause a problem in the industry, the initial pH of the growth medium was considered for further investigation in the optimization process using the same range to ensure the optimum pH suitable for the lipase production. Alkaline pH 9.5 (Romdhane et al. 2010) and pH 10.0 (Aruna and Karim 2014) were previously used to attain maximum lipase production.

FCCCD for optimization of medium component and process condition

Significant factors from the OFAT studies were subjected to the optimization process using RSM in the form of FCCCD. Table 2 listed the experimental and predicted response of the lipase activity for each experimental run taken from the regression equation of twenty runs. The highest lipase activity achieved was 58.63 U/gds at the center point of the design, and the lowest activity observed was 4.39 U/gds at run 1.

Several regression analyses of the experimental data were employed to calculate the regression coefficients of the equation and the fitted equation was used to predict the enzyme activity. The quadratic polynomial equation provided levels of lipase activity as a function of inoculum size, olive oil concentration and pH, which can be presented in terms of coded factors as shown in the following Eq. (2):

$$Y = 52.36 + 3.00A - 3.69B + 0.87C + 9.86AB + 6.88AC + 4.81BC - 14.31A^2 - 14.94B^2 - 1.79C^2, \quad (2)$$

where Y is the lipase activity (U/gds) produced as a function of the coded levels of inoculum size (A), olive oil concentration (B) and pH (C), respectively.

ANOVA was used to assess the adequacy of the statistical model, which were summarized in Table 3. The F value of 53.12 and p value of <0.0001 of the model indicated that the selected quadratic model was significant. P value was also used to determine the significance of each coefficient and used as an indication to examine the

interaction strength between each coefficient that was independent. P value of less than 0.05 implies that model terms are significant, while values greater than 0.1 means insignificant model terms. In this case, the terms A , B , AB , AC , BC , A^2 , and B^2 were found to be a significant model and influenced the overall lipase production remarkably. Factor C and C^2 were found to be insignificant. Based on the F values of the main factors studied, the olive oil concentration showed the highest value, denoting that it presented the strongest influence on the lipase production, while the pH showed the least pronounced effect. The lack of fit, with F value of 2.26 also implied that it was not significant relative to the pure error. Non-significant lack of fit indicates that the model fits adequately. In addition, the efficiency of the model was demonstrated by the high value of R^2 (0.98) and adjusted R^2 (0.96), which indicated a better correlation between the actual and predicted values. The signal-to-noise ratio was measured by the adequate precision, in which a ratio greater than 4 is considered a requirement for desirable models and the model showed a

Table 3 Analysis of variance (ANOVA) and RSREG procedure canonical analysis of response surface based on the FCCCD experimental runs for TS-OST lipase production

ANOVA						
Source	Sum of squares	df	Mean square	F value	p value Prob > F	
Model	5328.97	9	592.11	53.12	<0.0001	Significant
A: inoculum size	90.19	1	90.19	8.09	0.0174	
B: Olive oil	136.22	1	136.22	12.22	0.0058	
C: pH	7.63	1	7.63	0.68	0.4274	
AB	778.00	1	778.00	69.80	<0.0001	
AC	378.22	1	378.22	33.93	0.0002	
BC	185.45	1	185.45	16.64	0.0022	
A ²	563.14	1	563.14	50.52	<0.0001	
B ²	613.80	1	613.80	55.07	<0.0001	
C ²	8.84	1	8.84	0.79	0.3942	
Residual	111.47	10	11.15			
Lack of fit	77.28	5	15.46	2.26	0.1958	Not significant
Pure error	34.19	5	6.84			
Cor total	5440.44	19				
RSREG procedure canonical analysis of response surface						
Eigenvalues	Eigenvectors					
	Inoculum		Olive oil		pH	
0.0066	0.3064		0.2491		0.9187	
−11.4612	0.6505		0.6499		−0.3931	
−19.5880	−0.6950		0.7181		0.0371	
Stationary point is a saddle point						

$R^2 = 0.98$, adjusted $R^2 = 0.96$, CV = 9.06, predicted $R^2 = 0.92$, adequate precision = 20.67

Fig. 3 Three-dimension response surface plot of the interaction effects of: **a** olive oil concentration and inoculum size, **b** pH and inoculum size, **c** pH and olive oil concentration on TS-OST lipase production

Table 4 Validation of experimental model for optimization of TS-OST lipase production

Experiment	Inoculum size (% v/w)	Olive oil (% v/w)	pH	Activity, U/gds (theoretical)	Activity, U/gds (experimental)	Error (%)
1	2.21	2.21	12.0	51.63	51.22	+0.79
2	1.00	1.50	12.0	26.72	26.26	+1.73
3	1.00	2.50	8.0	10.05	10.65	−5.92

ratio of 20.67. Meanwhile, the coefficient of variation (CV) is a measure expressing standard deviation as a percentage of the mean and small value of CV gives better reproducibility (Liyana-Pathirana and Shahidi 2005). The CV for the lipase production was 9.06%, which was within the acceptable range.

The regression equation was used to construct the response surface (three-dimensional) plots used to investigate the interaction between variables and to determine the optimum concentration of each factor for maximum lipase production. The plots were based on the concentration functions of two variables while fixing the third variable at its optimum concentration. The significance of the interactions between the corresponding variables is indicated by an elliptical or saddle nature of the contour plots (Muralidhar et al. 2001). Based on the coded data obtained using RSREG procedure of

statistical analysis system (SAS) software, canonical analysis for lipase activity (Table 3) demonstrated a saddle point as the stationary point for the enzyme production. Canonical analysis is used to evaluate the overall shape of the curvature and determine the stationary point of response surface model.

In Table 3, eigenvalues and eigenvectors denoted the shape of the response surface. The larger absolute eigenvalue implies the higher significance in the curvature of the response surface. The largest eigenvalue (−19.5880) corresponds to the eigenvector (−0.6950, 0.7181, 0.0371) with the largest component of which (0.7181) is associated with factor olive oil concentration. Similarly, the second-largest eigenvalue (−11.4612) is associated with inoculum size and the third eigenvalue (0.0066) associated with pH is relatively smaller than the other two indicating that the response surface is

particularly unaffected by changes in this factor, which were consistent with the ANOVA results. A mixture of positive and negative signs of eigenvalues indicated that the stationary point for lipase activity was a saddle point. Thus, ridge analysis was required to locate the region of optimum response. The method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the center of the original design. The ridge max analysis indicated that maximum lipase activity was 53.565 U/gds obtained at 2.36% (v/w) inoculum size, 2.07% (v/w) olive oil concentration and pH of 11.8 at the distance of the coded radius 1.0. Three-dimensional contour plots of the predicted response surface shown in Fig. 3 confirmed this conclusion. This predicted result was similar to the experiment, where the optimum response was observed at the center point level (Table 2).

Figure 3a depicted the interaction between inoculum size and olive oil concentration on lipase production when the pH was set at the central point. The response plot showed a somewhat elliptical contour as both factors exhibited quadratic effects on lipase production. This suggested that the optimum operating conditions were well-defined and the effect of the interaction between these factors was significant. Thus, it is evident that the increase in lipase activity resulted from the increase in inoculum size and olive oil concentration to optimum values, and further increase led to a decrease in the activity. Both of these factors were the important variables for lipase production and considered indicators of effectiveness and economic performance since their overdose will increase unnecessary cost and decrease the activity of lipase. Figure 3b showed the effect of pH and inoculum size on lipase activity at the central point of olive oil concentration. The shape of the response surface curves showed a moderate interaction between these tested variables. Inoculum size exhibited quadratic effects on the response, in which lipase activity improved as the inoculum size increased up to 2% (v/w) concentration, followed by a decline in activity with its further increase. However, pH demonstrated a linear effect on lipase activity. The pH was shown to have an inconsequential effect on lipase activity as compared to the inoculum size, for the range covered in this study. Figure 3c represented the interaction between pH and olive oil concentration at fixed inoculum size of 2% (v/w). Similarly, lower and higher levels of both the pH and olive oil did not result in higher enzyme activity. Olive oil concentration exhibited a quadratic effect on the lipase activity, while the effect of pH was linear regardless of the proportion of olive oil in the medium. Olive oil compositions toward the lower design point of the variable produced a greater response compared to

that produced at the higher design point. Statistical optimization for thermostable lipase production was also reported by Abdel-Fattah (2002) and Dandavate et al. (2009). In all cases, statistical optimization analysis enhanced the enzyme production.

Validation of the statistical 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 triplicate to test three combinations of media constituents in determining the lipase production and the results were compared with the predicted values, as shown in Table 4. The results showed that experimental response was reasonably close to the predicted values (percentage error <10%), confirming the validity and adequacy of the predicted models.

Conclusions

Combining DSD, OFAT and FCCCD experimental models, a successful improvement in the lipase production was achieved. The inoculum size and olive oil concentration were the most influential factors for maximum lipase production. The optimum parameters that correspond to highest lipase activity of 58.63 U/gds were 2% (v/w) inoculum size, 2% (v/w) olive oil, 0.6% w/w peptone, 2% (v/w) ethanol, 70% moisture content, initial pH of 10.0, 45 °C fermentation temperature as well as 72 h of fermentation time. The predicted optimum lipase activity obtained from the ridge analysis using RSREG procedure of SAS software was 53.565 U/gds at 2.36% (v/w) inoculum size, 2.07% (v/w) olive oil concentration, and pH of 11.8, which was in good agreement with the experimental result.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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