Statistical optimization of lipase production in solid-state fermentation by *Aspergillus tamarii* NDA03a and application of the fermented solid as a biocatalyst for biodiesel production

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Background and objective
Biodiesel, an attractive alternative fuel, is defined by the American Society for Testing and Materials (ASTM) as fatty acid methyl esters (FAME). Biodiesel is an ecofriendly fuel compared with many other transportation fuels. The aim of this study was to implement the statistical approaches for optimization of *Aspergillus tamarii* NDA03a mutant G lipase produced in solid-state fermentation (SSF), and then application of the dried fermented solid as a biocatalyst for biodiesel production from waste frying oil (WFO).
Materials and methods
A. tamarii NDA03a mutant (3G) was previously selected as a good lipase producer. Five oil residue meals were evaluated in the presence of wheat bran (WB) for their potential as enzyme inducers and substrates for the production of 3G lipase by SSF. The best oil residue meal was selected and used in subsequent experiments. The fermented solid thus obtained was collected, lyophilized, and used as a biocatalyst for waste frying oil transesterification to FAME. To optimize SSF conditions for lipase production using 3G, a Plackett–Burman design was used at first to screen the critical factors from several process variables, and finally, a central composite design was applied to further estimate the relationship between the variables and response as well as optimize the levels. Response was measured in terms of FAME yield. To verify the adequacy and accuracy of the model, validation experiments were also carried out.
Results and conclusion
The most favorable oil residue meal that enhances 3G lipase production by SSF was black cumin meal. Results of the Plackett–Burman design revealed that the factors contributing to the main effect were incubation temperature, incubation period, and moisture content. The optimal SSF conditions for lipase production were WB 10 g, black cumin meal 6% (w/w of WB), pH 8, temperature 28°C, moisture content 40%, molasses 1% (w/w of WB), and incubation period 3 days. Under these optimized conditions, produced FAME yield (65.55%) increased by 58% compared with the basal medium (41.46%). A good agreement between the experimental (65.55%) and predicted (65.03%) values was detected. The significance of this model was confirmed by its probability value and lack of fit ($P<0.05$) and clearly showed that the model was sufficient to describe the correlation between the FAME yield and the tested variables. The obtained results ascertained the success of response surface methodology as an efficient technique to optimize the lipase production in SSF and consequently the ability of application of the dried fermented solid as a biocatalyst for biodiesel production.

Keywords:
central composite design, fermented solid, lipase, transesterification, waste frying oil

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Introduction

Biodiesel, an attractive alternative fuel, is defined by the American Society for Testing and Materials as fatty acid methyl esters (FAME). Biodiesel is an ecofriendly fuel compared with many other transportation fuels. It is a biodegradable, renewable, carbon neutral, land nontoxic fuel [1]. It can be used without any modification in diesel engines and can be blended with any ratio [2]. FAME can be produced through esterification of free fatty acids or transesterification of glycerides in the presence of alcohol [3]. Waste frying oil (WFO) is regarded as a potential alternative feedstock for biodiesel production [1]. Its use for biodiesel production is very important to reduce the production cost of this cost-sensitive energy product

and to eliminate the environment pollution and human health risk caused by WFO inappropriate disposal [1,4].

Lipase-catalyzed transesterification is an ecofriendly alternative to chemical process using acid or base catalyst owing to an improved selectivity, a nontoxic catalyst, a lower process temperature, pressure and waste, a simultaneous esterification of free fatty acids and transesterification of glycerides, and an easier separation of biodiesel and glycerol [3]. However, the high cost of the lipase enzyme is the main obstacle for a commercially feasible enzymatic production of biodiesel fuel. To overcome this problem, production of lipase by solid-state fermentation (SSF), as well as usage of the dried fermented solid with active lipase activity as a biocatalyst instead of purified enzyme is a potential way to reduce enzyme production cost because the extra-extraction, purification, and immobilization steps are not necessary [5,6].

Among the evaluation of various fungi for their ability in lipase production, Aspergilli are devoted to being efficient and potential producers [7,8]. Several renewable, cheap, and agro-industrial residues such as babassu cake, soybean, rice husk, and wheat bran (WB) have been used to produce lipase by *Aspergillus* species in SSF process [9–11]. Many advantages have been offered by SSF as compared with submerged fermentation like low production cost, high productivity, saving of water and energy, and the use of various agro-industrial residues as substrates [12].

To produce high-quality biodiesel products, an optimization strategy for lipase production in SSF process should be established. Response surface methodology (RSM) is an efficient and imperative tool for the optimization of various fermentation processes and multivariable systems [13]. RSM is considered as a combination of statistical and mathematical protocols where no complex calculations are required to analyze the resulting data [14]. Other advantages of using RSM are to search for relations between factors and the most suitable conditions for desirable response, and to be cost effective than traditional variation of one parameter at a time [15].

The use of dry fermented solid with lipase activity as a biocatalyst for biodiesel production has been reported by many researchers [6,16,17]. *Aspergillus tamarii* NDA03a mutant G (3G) has been recently identified as a potential producer of lipase, which effectively transesterifies WFO to FAME [18]. Cultivation conditions determining the production of lipase by this mutant in SSF with WB as a substrate have not been studied. Hence, the present work aims to implement the statistical approaches for optimization of 3G lipase production in SSF and application of the obtained fermented solid as a biocatalyst for biodiesel production from WFO. The optimization process involved two steps: Plackett–Burman (PB) design and RSM considering FAME yield as a response for 3G lipase production ability.

Materials and methods

Materials

WFO was collected from local restaurants. WB was purchased from a local market in Egypt. Methyl heptadecanoate standard was purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Methanol, hexane, diethyl ether, and acetic acid were purchased from Merck Chemical Co. (Darmstadt, Germany). potato dextrose agar (PDA) and medium ingredients were purchased from Fisher Scientific (Hampton, New Hampshire, USA). All other chemicals were of analytical grade.

Isolation, molecular identification, and mutation of *Aspergillus* isolate

A. tamarii NDA03a was previously isolated, identified, and selected as a good lipase producer [18]. It was isolated by suspension of 5 g of a soil in Egypt (30° 08′ 25.6″ N 31° 16′ 20.6″ E) in 50-ml sterile physiological saline solution. Isolation was carried out using the pour plate dilution method [19] on PDA in which carbon source was replaced by olive oil. The plates were incubated at 28°C for 3–5 days. This isolate was molecularly identified using rRNA gene sequence ITS1 and ITS2 as *A. tamarii* NDA03a (Genbank Accession Number MK849615) as reported by Elhussiny *et al.* [18]. This identified isolate was exposed to ethyl methanesulfonate for producing hyperlipolysis mutants [18]. The mutagenesis process was carried out as follows: 10 ml of 200 mM ethyl methanesulfonate solution was applied to freshly prepared 5-day-old fungal spore suspension (7×10^8) for 1 h. Phosphate buffer pH 7, 0.2 M, was used to dilute the mixture to cease the mutagenesis process. Samples were cultivated on PDA at 28°C for 3–5 days. *A. tamarii* NDA03a mutant G (3G) was previously selected based on its high ability to transesterify WFO [18].

Preparation of oil residue meals

Sesame, almond, watercress, black mustard, and black cumin meals were prepared from *Sesamum indicum*,

Prunus amygdalus, *Eruca sativa*, *Brassica nigra*, and *Nigella sativa* seeds, respectively, by oil extraction using hydraulic press extraction method. The extraction procedure was performed as follows: the dried seeds were milled to a fine powder. The samples of seed powder were wrapped in a thick heavy duty cloth, and then the oil extraction was carried out using simple hydraulic press with a maximum pressure of 3500 psi for 1 h at room temperature [20]. The extracted oil was collected, and the extracted meals or cakes which remained after oil extraction from the seeds were milled into a fine powder and packed in polyethylene bags and stored in deep freezer at −20±2°C until analyzed. All extractions were performed in triplicate.

Chemical analysis of prepared meals

Moisture content, total lipids, crude protein (N×6.25), crude fiber, and ash of prepared oil residue meals were determined according to the methods outlined in the Association of Official Analytical Chemists [21]. Total carbohydrate content was calculated by the difference in weights.

Lipase production by solid-state fermentation

Five oil residue meals were evaluated for their potential as enzyme inducers and substrates for 3G lipase production by SSF and application of produced fermented solid for WFO transesterification. Initially, 10 g of WB and 6% (w/w of WB) of each oil meal as a substrate were taken individually, moistened with distilled water, and autoclaved for 30 min. The medium was inoculated with 3G of 3-day-old fresh PDA culture and incubated at 30°C for 6 days under static conditions. Each fermentation test was repeated in triplicate. The best oil residue meal was selected and used in the subsequent experiments. The fermented matter thus obtained was collected, lyophilized, and used as a biocatalyst. The lipase activity in the fermented solid was evaluated by its ability to transesterify WFO to biodiesel (FAME).

Methanolysis of waste frying oil

Methanolysis of WFO was carried out in 100-ml Erlenmeyer flasks at 35°C with constant shaking at 250 rpm for 72 h. The reaction mixture consisted of 5 g WFO, 10% (w/w of WFO) dried fermented solid, 15% (w/w of WFO) 0.2 M Tris buffer (pH 7.5), and 3 : 1 methanol to WFO molar ratio (added stepwise to the reaction mixtures three times at 0, 24, and 48 h reaction time). WFO was previously emulsified with Tris buffer (pH 7.5) before the addition of the biocatalyst using ultrasonication. At the end of reaction time, fermented solid biocatalyst was separated from the reaction mixture by centrifugation at 10 000 rpm for 15 min. The upper oil phase containing esters was analyzed qualitatively by thin-layer chromatography [22] and quantitatively by capillary gas chromatography (GC) [23].

Analyses of fatty acid methyl esters

Thin-layer chromatography was performed on pre-coated silica gel plate (Merck, Kenilworth, New Jersey, USA). The plate was chromatographed for FAME with a solvent system of hexane : diethyl ether : acetic acid (80 : 20 : 1, v/v/v). The chromatograms were developed with iodine vapor. FAME in the oil phase was analyzed by an Agilent Technologies (Santa Clara, California, USA) 6890 N GC equipped with flame ionization detector and a fused silica capillary column (30 mm×0.32 mm×0.25 mm). The GC temperature condition was oven temperature of 210°C using helium as a carrier gas, flame ionization detector temperature of 250°C, and injector temperature of 250°C. Overall, 10 mg/ml of methyl heptadecanoate solution was used as an internal standard, and the FAME content expressed as a mass fraction in percent was calculated by the use of the equation 1. The peak identification was made by comparing the retention time between the sample and the standard compound.

$$C = \frac{\sum A - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m} \times 100\% \quad (1)$$

Where ΣA=total peak area of FAME; A_{IS}=peak area of internal standard (methyl heptadecanoate); C_{IS}=concentration of the internal standard solution (mg/ml); V_{IS}=volume of the internal standard solution used (ml); and m=mass of the sample (mg).

Experimental design for 3G lipase production by solid-state fermentation

A PB design was used at first to screen critical factors from several process variables, and finally, a central composite design (CCD) was applied to further estimate the relationship between the variables and response as well as optimize the levels. Response was measured in terms of FAME yield. The PB design with the name and level of the variables is shown in Table 1. Each independent variable is represented in two levels, high and low, which are denoted by +1 and −1, respectively. The design comprised 13 experiments with two replicates at the center point (0). Fermentation was carried out in duplication, and the average value was taken as the response. Usually, the variable with a P value of less than or equal to 0.05 was considered to have a significant effect on the response [24] and was selected for further CCD optimization.

Table 1 Variables and levels for Plackett–Burman experimental design

Variables	Symbol	Units	Levels	
			−1	+1
Substrate (black cumin meal)	X_1	%	6	12
Medium pH	X_2	–	5	8
Temperature	X_3	°C	28	40
Moisture content	X_4	%	60	100
Time	X_5	days	4	6
Inoculum size	X_6	disc	1	3
Molasses	X_7	%	0	1
Fodder yeast	X_8	%	0	1

To determine the optimum level of selected variables (temperature, moisture, and time) as shown in Table 2, that were screened from the PB design and to investigate their interactions, RSM using CCD was applied. These variables were tested at three levels. An experimental design of 16 experiments was formulated. Response was measured in terms of FAME yield. To represent the graphical examination or descriptions of the experimental results, three-dimensional response surfaces were developed using JMP8 statistical software (SAS Institute Inc. Cary, North Carolina, USA). Depending on this design, the effect of the independent significant variables ($P \leq 0.05$) on the response (FAME yield) can be evaluated. These 3D plots were showing the relation between any two independent variables and response by maintaining the other independent variable at a constant value.

Results and discussion

Effect of different oil meals on 3G lipase production in solid-state fermentation

Different oil extraction residues were tested as an inducer for the production of lipase in the presence of WB as a substrate/support material. WB was reported as the best substrate for lipase production by dos Santos *et al.* [12], as it contained 3.55% of lipid, rich in carbohydrates and fibers, and it could be used with dual function as a carbon source and as a physical support for fungal growth. Supplementation of SSF medium with oil extraction residues is attractive, as they are abundant, cheap, renewable, and nutrient sources (contain nitrogen, carbon, and minerals). Moreover, each residue could serve as a physical support and as an inducer for the production of lipase [25,26]. As shown in Table 3, some tested oil extraction residues supported WFO transesterification using 3G dried fermented solid to different degrees. The most favorable oil residue meal that enhance 3G lipase production in SSF was black cumin meal that resulted in the highest FAME yield of 41.46%. This could be attributed to its relatively high

Table 2 Selected variables and levels for central composite design experimental design

Variables	Symbol	Unit	Experimental level		
			−1	0	+1
Fermentation temperature	X_3	°C	25	28	31
Moisture content	X_4	%	40	50	60
Fermentation time	X_5	days	2	3	4

Table 3 Screening of different oil meals for 3G lipase production in solid-state fermentation

Substrate	FAME (%)
Watercress meal	27.77
Almond meal	25.38
Black cumin meal	41.46
Sesame meal	32.84
Black mustard meal	37.87
Control (no meal)	33.15

FAME, fatty acid methyl esters.

oil and carbohydrate contents as shown in Table 4. Black cumin meal could be served as an enzyme inducer and a carbon source for microbial growth [26]. On the contrary, watercress and almond meals were accompanied by inferior FAME yields (27.77 and 25.38%, respectively) compared with the control (with no meals). This may be attributed to the inhibition effect caused by the high amounts of protein (42.9%) found in almond meal and high amounts of fiber found in watercress meal (21.77%). The carbohydrate contents were approximately similar among used oil meals; this indicated that the carbohydrate content was a nonsignificant factor. Another explanation was related to Halldorsson *et al.* [27], who reported that all of the lipases prefer the presence of more saturated fatty acids as substrates. As black cumin meal contains suitable amounts of saturated fatty acid (14.66 g/100 g of total fatty acid) as reported by Thilakarathna *et al.* [28], it could enhance lipase production, leading to the highest yield of FAME (41.46%). However, the watercress seed oil exhibited the greatest variety of fatty acids represented by the majority of unsaturated fatty acids such as linolenic acid (34%) and oleic acid (22%), followed by saturated fatty acids, such as palmitic 10.1%), stearic acids (2.9%), and arachidic acid (3.4%), which were presented in low amounts [29]. Moreover, nuts are natural foods rich in monounsaturated or polyunsaturated fatty acids, and no saturated fatty acids are present [30].

Screening of significant variables by Plackett–Burman design

PB design was used to screen eight different medium components and fermentation conditions as 13 run

Table 4 Chemical analysis of used residual oil meals

	Constituent %					
Oil residue meals	Moisture	Crude oil	Crude protein (Nx6.25)	Total carbohydrates	Crude fiber	Ash
Watercress	6.55	17.98	25.61	22.90	21.77	5.19
Almond	7.50	12.14	42.91	24.53	7.4	5.52
Black cumin	6.75	25.37	24.13	27.30	11.05	5.40
Sesame	7.10	14.65	39.50	23.41	6.53	8.81
Black mustard	7.43	17.60	36.42	21.00	12.50	5.05

Table 5 Experimental design for evaluation of factors affecting 3G lipase production in solid-state fermentation

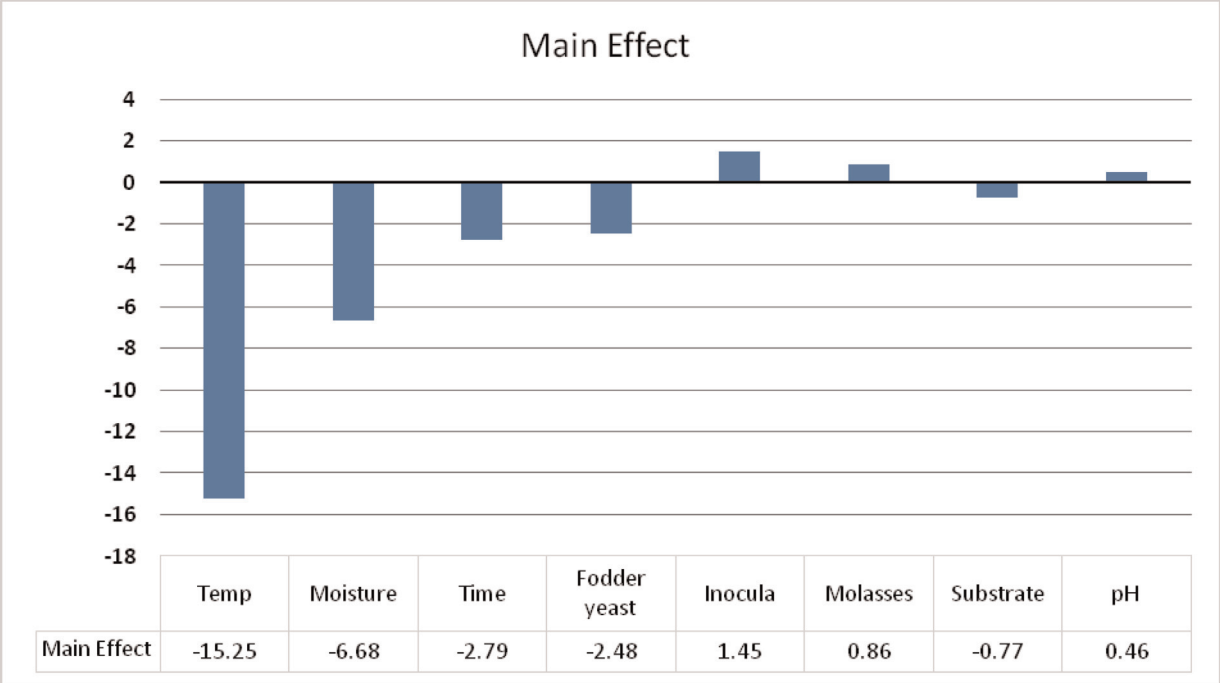
Run	Pattern	Substrate (%)	pH	Temperature (°C)	Moisture (%)	Time (days)	Inoculum size (disc)	Molasses (%)	Fodder yeast (%)	FAME (%)
		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	
1	−−−+−−+−	6	5	28	100	4	1	1	0	37.12
2	−−+−−+−+	6	5	40	60	4	3	0	1	14.05
3	−−+−+++−	6	5	40	60	6	3	1	0	20.38
4	−+−−+−++	6	8	28	60	6	1	1	1	31.77
5	−+−+++−−	6	8	28	100	6	3	0	0	27.39
6	−+++−−+	6	8	40	100	4	1	0	1	13.53
7	00000000	9	6.5	34	80	5	2	0.5	0.5	33.10
8	+−−−+−−+	12	5	28	60	6	1	0	1	41.15
9	+−−+−+++	12	5	28	100	4	3	1	1	23.10
10	+−+++−−−	12	5	40	100	6	1	0	0	43.71
11	++−−−+−−	12	8	28	60	4	3	0	0	19.36
12	+++−−−+−	12	8	40	60	4	20	1	0	14.54
13	++++++++	12	8	40	100	6	60	1	1	6.39
Ctrl		6	7	28	40	6	40	0	0	41.46

FAME, fatty acid methyl esters.

experiments with two levels of each variable. The FAME yield response (dependent variable) of PB experimental design for 13 trials is given in Table 5. Based on the obtained results, the highest and lowest FAME yield of 43.71 and 6.39%, respectively, were observed in runs 10 and 13, respectively. The main effect of each variable on 3G lipase production in SSF is represented in Figure 1. Results revealed that the factors contributing to the main effect were incubation temperature, incubation period, and moisture content. The other factors (medium pH, substrate concentration, molasses concentration, and inoculum size) had insignificant effects on lipase production in SSF by 3G. As shown in Figure 1, relatively high negative effects of the incubation temperature (15.25%) and moisture content (6.68%) were observed in the tested ranges, whereas fodder yeast, incubation time, and substrate concentration (black cumin meal) had low negative effects. On the contrary, the other factors (inoculum size and medium pH) had positive effects on FAME yield. This indicated that the moisture content must be lowered than 60% because a high concentration of moisture results in great decreases in the production of microbial metabolites in SSF and can cause agglomeration of medium particles and lead to oxygen transfer limitations [31]. On the contrary, Nema *et al.* [32] reported a positive influence of the moisture content on lipase production by *Aspergillus niger* MTCC 872. The observed negative effect of the incubation temperature could be explained by the denaturation of the tertiary structure of the enzyme protein caused by excessive heat [33].

The other factors like fodder yeast and substrate concentration (black cumin meal) had low negative effects (−2.48 and −0.77%, respectively) on lipase production assayed by FAME yields; hence, they were maintained at their low levels. However, a slight positive effect of molasses (0.86%) on FAME yield was observed (Figure 1); thus, it was maintained at its high level. Molasses, a by-product of the sugar industry, is presenting characteristics such as low cost, abundance, and easy storage at room temperature. So, it was used as a carbon source in the SSF medium. These results were similar to those obtained by Melissa *et al.* [34] who found that higher molasses concentrations did not further increase enzyme production but also did not repress enzyme synthesis. Moreover, Vasiee *et al.* [35] found that molasses had a slightly high positive effect on lipase production.

Figure 1



The main effect of each variable on 3G lipase production in SSF.

Based on PB results, the insignificant variables were ignored and the values of the incubation temperature, moisture content, and incubation time were selected for the further study by a CCD to attain their optimal levels.

Response surface methodology using central composite design

Once the ranges of the three independent variables, that is, incubation temperature, moisture content, and incubation period, were selected through the PB screening, a 3-factor-3-level CCD was employed to estimate the relationship between the variables and response (FAME yield) as well as optimize their levels. The highest FAME of 66.99% was observed at run 8 (Table 6). As shown in Figure 2, the predicted and experimental values were significant, which suggested that the model gave a good fit.

Model fitting and analysis of variance

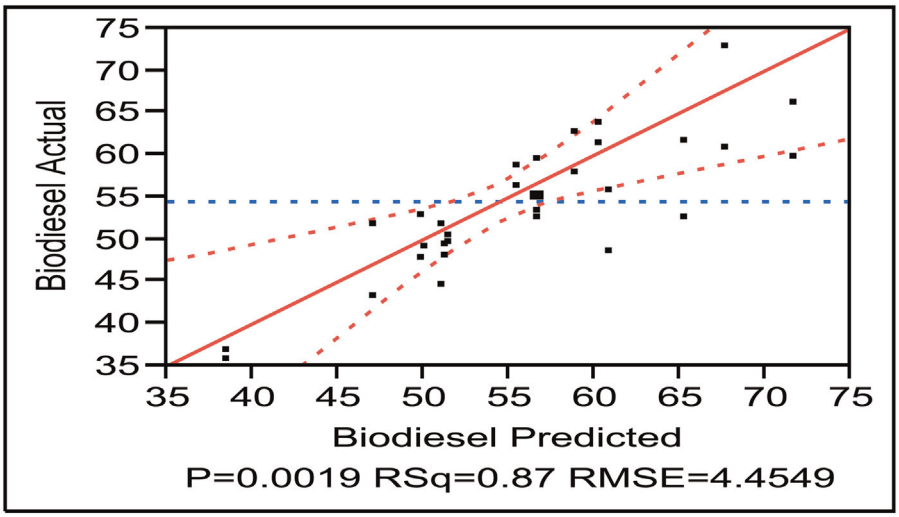
Analysis of variance (ANOVA) was used to evaluate the significance of the quadratic polynomial model [35]. So, the results obtained from CCD were then analyzed by standard ANOVA, as shown in Table 7. The model R^2 value of 0.87 indicates that the statistical model can explain 87% of variability in the response (a value of R^2>0.75 indicated the correctness of the model). Colla *et al.* [36] studied optimization of lipase production under submerged fermentation by filamentous fungi using RSM and found that a coefficient of determination (R^2) was 0.89 and an adjusted coefficient of determination (R^2 adjusted) was 0.86. Moreover, the higher model F value (24.91), small P value of 0.0019, and lower lack of fit (7.68) indicated the accuracy of the model and implied its significance in describing the relation between the FAME yield and the given variables (Figure 3). These results are similar to

Table 6 Experimental plan for optimization of lipase production in solid-state fermentation using central composite design

Run	Temperature (X_3) (°C)	Moisture (X_4) (%)	Time (X_5) (days)	FAME (%)
1	25	40	2	56.84
2	25	60	2	62.26
3	25	50	3	61.95
4	25	40	4	51.64
5	25	60	4	47.67
6	28	50	2	49.44
7	28	40	3	66.17
8	28	50	3	66.99
9	28	50	3	61.25
10	28	60	3	64.47
11	28	50	4	53.77
12	31	40	2	43.91
13	31	60	2	56.15
14	31	50	3	57.72
15	31	40	4	66.15
16	31	60	4	37.02
Ctrl				41.46

Where the other factors (substrate 6%, molasses 1%, inoculum 1 disc/10 g wheat bran, pH 8) were fixed. FAME, fatty acid methyl esters.

Figure 2



Actual by predicted plot CCD design for 3G lipase production in SSF.

Table 7 Analysis of variance for the fitted quadratic polynomial model

Source	DF	Sum of squares	Mean square	*F* ratio	*P*>*F*
Model	9	1371.41	152.38	24.91	0.0019*
Lack of fit	5	190.80	38.16	7.68	0.0015*
Pure error	5	7.66	1.53		
Total	19	1569.87	192.07		
R^2=0.87					

*Significant at "*P* value" less than 0.05.

those obtained by Kaushik *et al.* [8] and Ebrahimi *et al.* [37], who indicated that the high *F* value and small low *P* value indicated the accuracy of the model.

The *P* value is used as a tool to investigate the significance of each regression coefficient [38]. So, the significance of each parameter that was evaluated by the *P* value is listed in Table 8. The most significant parameters ($P<0.05$) were the incubation time (X_5), square of incubation time $(X_5)^2$, and the interactive effect of the incubation time (X_5), and moisture content (X_4). Meanwhile, the other terms were insignificant ($P>0.05$). For example, the effect of moisture content (X_4) only is very negligible. Furthermore, the interactions of X_3 (temperature) with X_4 (moisture content) and X_3 (temperature) with X_5 (incubation time) have a positive interaction.

Moreover, the model matched with a full second-order polynomial equation as described by equation 2:

$$Y(\%) = -274.83 + 16.198X_3 + 0.94X_4 + 68.472\ X_5 - 0.0764X_3X_4 + 0.954X_3X_5 - 0.634X_4X_5 0.283(X_3)^2 + 0.0293(X_4)^2 10.78(X_5)^2 . \quad (2)$$

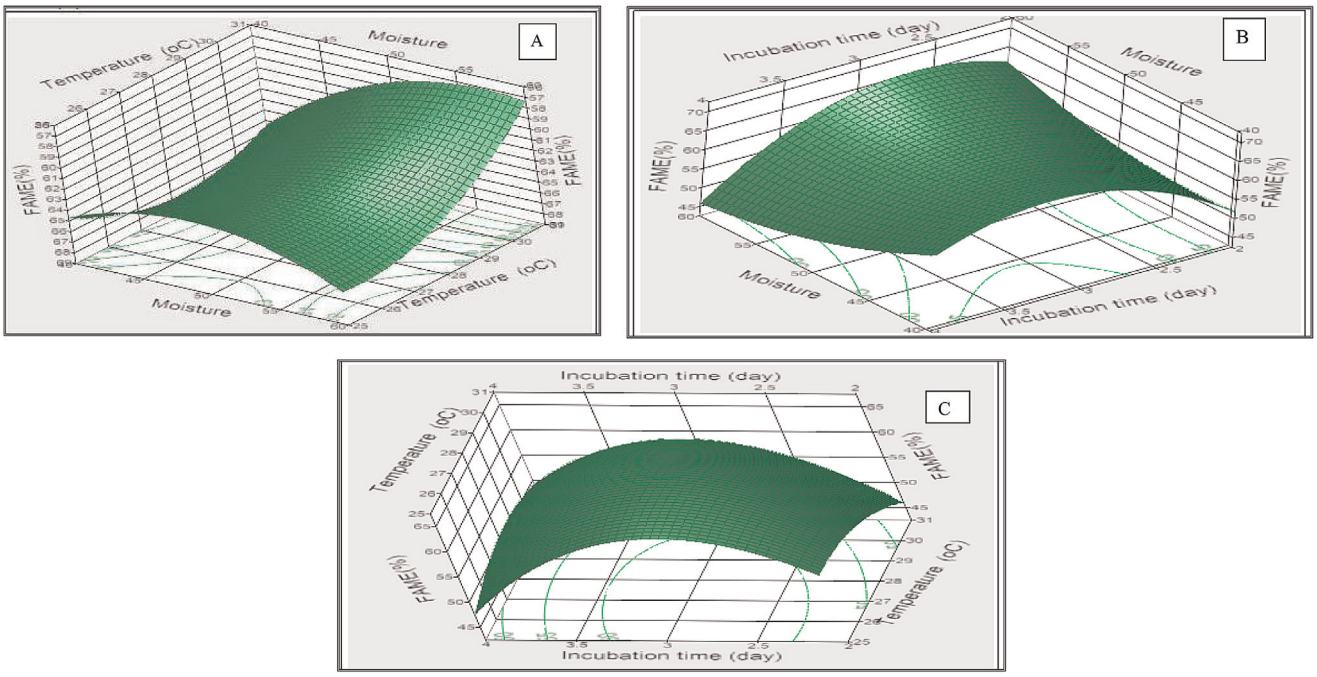
Where X_3 is temperature, X_4 is moisture content, X_5 is incubation time, and *Y* is the response.

The largest coefficient with negative value was given by $(X_5)^2$. This means that the long incubation period, the less lipase production in SSF, and consequently, low FAME yield is detected. So, 3-day incubation period was the best, as shown in runs 3,7, 8, 9, and 10, in which these optimized dried fermented solids gave a high range of FAME (61.25–66.99%) at 28°C, and 40–60% moisture contents. So, the incubation period (X_5) was the most significant variable, as it had the largest coefficient and its *P* value of 0.055.

The effect of solid-state fermentation variables on lipase production in solid-state fermentation

Three-dimensional curves presented in Figure 3a–c show the effects of independent SSF variables on lipase production in SSF as represented by FAME yield. Figure 3a represents the contour plot and response surface curves of the effect of the incubation temperature and moisture content on FAME yield, where the incubation period was constant. Initially, FAME yield had raised with the increase in both the incubation temperature and

Figure 3



Contour and 3D diagram of the effect of (a) incubation temperature and moisture content, (b) moisture content and incubation period, and (c) incubation temperature and incubation period on FAME yield.

Table 8 Regression analysis of factors for 3G lipase production in solid-state fermentation

Term	SE	Coefficient	*t* ratio	*P* value
$X_4 \times X_5$	0.193642	−0.6345	−3.2766596	0.0169*
X_5^2	3.373204	−10.78328	−3.20	0.0187*
X_5	28.86196	68.472989	2.37	0.0553
$X_3 \times X_5$	0.645474	0.9541667	1.48	0.1898
$X_3 \times X_4$	0.064547	−.076417	−1.18	0.2812
X_4^2	0.033732	0.029317	0.87	0.4182
X_3	21.33143	16.198383	0.76	0.4764
X_3^2	0.37481	0.283697	−.0.76	0.4778
X_4	3.874586	0.9400425	0.24	0.8164

X_3, temperature; X_4, moisture content; X_5, incubation time.
*Significant at "*P* value" less than 0.05.

moisture content. However, this rising profile was stopped and converted to a descending one at incubation temperatures higher than 28°C and moisture content higher than 40%.

Figure 3b shows the significant interaction between the moisture content and incubation period and response where the incubation temperature was constant. By increasing the incubation period to more than 3 days and moisture content to more than 40 or 45%, there was an observed decline in FAME yield. Figure 3c showed the interaction between the independent variables (incubation temperature and incubation period) and response, where the moisture content is constant. These variables had a slightly positive effect on FAME yield. Maximum FAME yield ~60% was produced upon increasing the incubation period up to 3 days, with an increase in the incubation temperature between 28 and 30°C.

The contour plot and response surface curves of the effect of each two independent variables and the response (FAME yield) (Figure 3a–c) suggested that FAME yield declined at incubation temperature above 28°C, incubation period higher than 3 days, and moisture content more than 40%. This means that the temperature of 31°C was not suitable for the growth of 3G and consequently bad lipase production, leading to the low yield of FAME. Moreover, heat creates water condensation which returned to the fermented solid causing heterogeneity in the solid substrate. Mohseni *et al.* [39] reported that maximum lipase activity by *A. niger* from agricultural residues was achieved after 4 days of inoculation. However, Mahadik *et al.* [9] has achieved highly active lipase by *A. niger* strain after 5 days of incubation. Beyond this period (3 days), lower enzyme production was obtained probably owing to the decrease of the required nutrient substance for the growth of the microorganism. Moreover, at high moisture content (50 and 60%), the FAME yield decreased, which could be attributed to the reduction of lipase production ability in SSF as a result of oxygen transfer limitations [31] or reduction of surface to volume ratio of solid material [40] caused by agglomeration of medium particles in SSF at high moisture content. In contrast to our results, the optimum

Table 9 Validation of central composite design for 3G lipase production in solid-state fermentation

Test type	Temperature (°C)	Time (days)	Moisture (%)	FAME (%)		Valid (%)
				Mathematical	Experimental	
Test validation	25	2	60	71.55581	58.45	81.69
	25	2	55	63.4	39.16	61.76
	25	3	60	67.179	53.35	79.41
	28	3	40	65.03	65.55	100.
	28	4	40	65.0378	50.54	77.71
Control	28	6	60	–	41.46	

FAME, fatty acid methyl esters.

initial moisture content for lipase production from wheat Rawa by *Aspergillus sp*. was 80% [41].

Verification experiment

The verification experiment was done by five random set of experiments using the predicted optimal conditions for 3G lipase production in SSF, whereas the basal SSF medium was used as a control (Table 9). According to these results, there was a good agreement between the experimental and predicted values. The maximum FAME yield of 65.55% was obtained at moisture content of 40%, temperature of 28°C, and after 3 days of incubation period. The two steps of optimization resulted in a formula of the following fermentation conditions: 10 g WB, black cumin meal 6%, molasses 1%, adjusted at initial pH 8 with moisture content of 40%, inoculated by 2 disc/10 g WB, and incubated at 28°C for 3 days. Under SSF with these optimized conditions, FAME yield by 3G fermented solid increased by ~58% (1.58 times) compared with the basal medium (41.46%). These results were close to those obtained by Vasiee *et al.* [35] who optimized the cultivation conditions for lipase production from rice flour through PB design and RSM by *Bacillus cereus*, which was 1.83 times more than the nonoptimal conditions.

Conclusion

The application of dry fermented solids containing naturally immobilized enzymes as catalysts in synthesis reactions is one of the biotechnological interests in the field of biotechnology. So, *A. tamarii* NDA03a mutant G (3G) fermented solid containing lipase used as a biocatalyst for biodiesel production by transesterification of WFO.

An optimization study for the production of 3G lipase by SSF was undergone using PB design and RSM. The obtained results ascertained the success of RSM as an efficient technique to optimize 3G lipase production in SSF and consequently the ability of application of obtained dried fermented solid for biodiesel production. Incubation temperature of 28°C, moisture content of 40%, and incubation period of 3 days were the optimum conditions, which increased lipase production in SSF by 1.58 times as compared with the basal medium (41.46%) as indicated by FAME yield. The ANOVA results showed that the most significant variables affected the FAME yield were the interaction between the incubation period and incubation temperature and the square of the incubation period showing P value of 0.0169 and 0.0187, respectively. A good agreement between the experimental (65.55%) and predicted (65.03%) values was detected. A suitable coefficient of determination (R^2=0.87) clearly showed that the model was highly significant and sufficient to describe the correlation between the FAME yield and the tested variables.

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Conflicts of interest

There are no conflicts of interest.

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