Optimization of parameters for lipase production by *Aspergillus niger* NRRL-599 using response surface methodology

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Background and objective
Lipases are characterized to catalyze precise chemical transformation. That is they have widespread applications in detergents, cosmetics, food, organics synthesis, and pharmaceutical industries. In recent years, the study and development of lipase production in solid state fermentation, also using sequential statistical strategy as optimization for some vital factors are gaining more attention. This may be attributed to several advantages using techniques such as simplicity of fermentation media, no need for complex machinery.
Equipment and control system, compactness of fermentation vessel due to lower water volume, high yields, less energy needed, and lower capital.
Materials and methods
Aspergillus niger NRRL-599 strain was tested for lipase production using different agroindustrial wastes including wheat bran, wheat germ cake oil, jojoba cake oil, almond cake oil, and olive oil as substrates under solid state fermentation. For further optimization, lipase activity was studied using two sequential optimization.
Results and conclusion
Olive oil waste was the most suitable substrate for lipase production (125 U/ml). Using one variable at a time, maximum lipase activity (200 U/ml) and specific enzyme activity (357.1 U/mg) were recorded in the presence of 5% w/v olive oil, 48 h inoculum age, 4% v/v inoculum size after 14 days fermentation at 30°C. The screening of the seven physiological factors using Plackett–Burman design showed that only three variables; that is tween 80, moisture content, and inoculum age affected significantly lipase production.
Optimization by Box–Behnken design resulted in the highest lipase activity (420 U/ml) in which the most effective variables were inoculum size, inoculum age, moisture content, and tween 80 1%.

Keywords:
Aspergillus *niger*, lipase production, olive oil: agroindustrial wastes, Plackett–Burman, solid state fermentation

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Introduction

Enzymes are important for their various industrial applications. The demand for the industrial market for enzymes continues to grow due to the improvement of new technologies for production, the use of genetic engineering and continuous emergence of new fields of applications [1]. According to a new research report by Global Market Insights Inc. the enzyme market is expected to cross USD 9.5 billion by 2024. This is due to the wide range of biotechnological applications of microbes and their enzymes in antibiotic synthesis, biopolymers, food, paper, vitamins, and textile industry. Lipases have widespread applications in detergents, cosmetic, food, organic synthesis, and pharmaceutical industries [2].

In recent years, the study and development of lipase production in solid state fermentation (SSF) are gaining more attention. This may be attributed to several advantages using techniques such as simplicity of fermentation media, no need for complex machinery, equipment and control system, compactness of fermentation vessel due to lower water volume, high yields, less energy needed [3].

Lipases are produced from different sources, however, the most suitable of which are from microbes including bacteria [4], fungi [5], and yeast [6]. High-quality lipases can be produced in lower cost and shorter time [7,8].

In addition, SSF has attracted great attention because of the availability of the agroindustrial wastes used as a

cheap carbon source instead of the commercial synthetic media [9].

Lipase production is influenced by several environmental factors such as the composition of fermentation media, pH, temperature as well as the microbial strain [10]. Classical methods of optimization do not define the relation between factors and their effects on each other; in addition, the classical methods require a large number of experiments [11]. Plackett–Burman design was used to distinguish the vital variables and their main effects [12]. Box–Behnken design shows the interaction between the independent variables [13].

Our objective was to produce extracellular lipase by *Aspergillus niger* NRRL-599 from available agroindustrial waste using SSF technique by applying two sequential factorial designs.

Materials and methods

Chemicals

All the chemicals used during this study were of analytical grade and were obtained from Sigma Aldrich Chemical Co. (St Louis, Missouri, USA).

Different substrates, namely jojoba oil cake, wheat germ oil cake, almond oil cake, wheat bran, and olive oil waste were obtained from Industries and Nutrition Division, Fats and Oils Department.

Microorganism

A. niger NRRL-599 was obtained from Natural and Microbial Products Department (National Research Centre, Cairo, Egypt) and was routinely grown on potato-dextrose agar medium (Sigma Aldrich Chemical Co.) at 30°C and preserved at 80°C in 50% (v/v) glycerol.

Inoculum preparation

In order to prepare the inoculum, 4 ml spore suspension of 7-day old cultures (after adjusting the amount of spores otherwise stated) was transferred into 250 ml Erlenmeyer flasks containing 100 ml of sterilized nutrient broth medium which has the following composition (g/l): a beef extract, 5.0; glucose, 5; peptone, 5.0; and sodium chloride, 5.0. The pH of the medium was initially adjusted at pH 7.0. The flasks were incubated on a reciprocal shaker (MAXQ 481 R HP) at 200 rpm for 4 days at 30°C [14].

Solid state fermentation

Different substrates were used, namely jojoba oil cake, wheat bran, wheat germ oil cake, almond oil cake, and olive oil waste. Fermentation was carried out with 5 g of a substrate in 250 ml Erlenmeyer flasks. The moisture content was adjusted with 20 ml tap water/5 g waste. After sterilization (121°C, 15 min) the flasks were allowed to cool down then each flask was inoculated with 4 ml of inoculum and incubated at 30°C at 7, 11, 14, and 18 days [15].

Enzyme extraction

At the end of fermentation, the crude enzyme was extracted by mixing the fermented substrate with 100 ml of tap water, then shaking the mixture in a reciprocal shaker at 200 rpm for 15 min, the obtained extract was filtered, and the supernatant was used as crude lipase enzyme [5].

Lipase activity assay

Lipase hydrolysis activity was assayed using 1 ml of culture filtrate mixed with 3 ml emulsion of olive oil in Arabic gum (10% w/v) and 2.5 ml of deionized water in 1 ml of 0.1 M tris-HCL buffer (pH 7.5). After incubation for 2 h at 37°C and 160 rpm, the reaction was stopped by addition of 10 ml of 99% acetone or ethanol solution. The fatty acids produced due to hydrolysis were titrated with 0.05 N NaOH. A control assay was carried out by boiling the enzyme mixture [16].

One unit of lipase activity was defined as the amount of enzyme preparation necessary to produce 1 μm of free fatty acids under the assay conditions.

Protein determination

The protein content was determined according to Lowry method [17].

Statistical design

Plackett–Burman design

Effect of levels of seven variables (time course, moisture content, pH, waste concentration, temperature, inoculum age, and emulsifying agent) were studied for optimization of maximum lipase activity using the Box–Behnken design [18]. The most critical parameters by *A. niger* were studied with JMP 8 software (SAS Institute Inc. Cary,North Carolina, USA) using the Plackett–Burman design. For each variable, high (+), low (−), and medium (0) levels were tested. Each trial represents the average value of enzyme activity (U/ml) and the protein level (mg/ml) which was taken as an independent response.

Response surface methodology

For further optimization of lipase activity, significant parameters were studied using the Box–Behnken.

The experimental design was comprised of 13 runs at three levels. Each run with three replicates and the average value of enzyme activity and protein content were represented as a dependent response.

The significance of the model was determined by analysis of variance, the regression equation was obtained, a P value less than 0.05 indicates that the model term is significant. The fit of the model (R^2) was studied and the results closer to show a better correlation between experimental and predicted values. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP.8 software.

Validation model

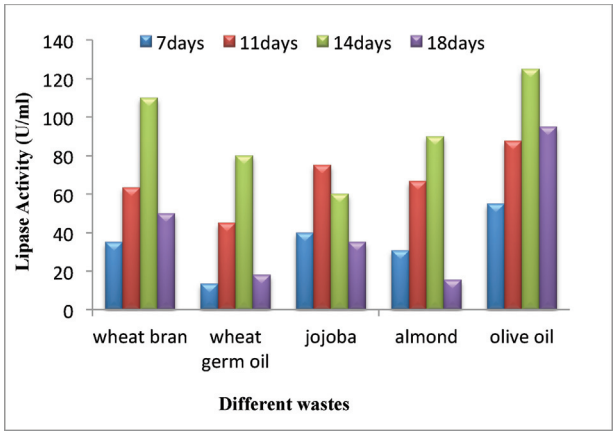
It was performed under conditions predicted by the experiment model. The experiments were examined in triplicate at an interval of 14 days.

Results and discussion

The main objective was to utilize agroindustrial waste for lipase production. Maximum lipase activity and specific activity were detected in presence of olive oil waste (125 U/ml and 62.1 U/mg protein, respectively) followed by wheat bran (110 U/ml and 73.3 U/mg protein, respectively) after 14 days of fermentation (Fig. 1).

Rapp [19], studied the effect of natural fats on stimulation of lipase production by *Fusarium oxysporum*. Similar results were shown by Ali *et al.* [20], who stated that lipase could be produced by *Exiguobacterium* spp. BBXS-7 using vegetable oil as a carbon source. Burkert *et al.* [21], reported that olive oil and soybean oil showed higher lipase activity using *Geotrichum* spp.

Figure 1



Effect of different wastes on lipase activity at different time.

Effect of inoculum age

The influence of inoculum age on lipase production was analyzed using olive oil cake, after 72 h of fermentation (data not shown). The maximum lipase activity and specific activity (200 U/ml) were determined after 72 h inoculum age showed the best results in terms of lipase activity and specific activity (200 U/ml and 357.1 U/mg protein, respectively). These results agreed with Vardanega *et al.* [22], who found that 72 h was suitable for lipase production by SSF of soybean meal using *Penicillium* spp. Similarly, *Penicillium notatum* of 72 h old was used for the production of lipase using different agricultural wastes [9]. However, the age of inoculum depends on the process, cultivation conditions, medium composition, and the microorganism [23].

Effect of inoculum size on lipase production

In the present investigation, different inoculum sizes (1, 2, 3, 4, 5, and 6) from 72 h old culture were used to study the effect of inoculum size in lipase production (data not shown). The lipase activity and specific activity (200 U/ml and 181.8 U/mg protein, respectively) were increased with increasing the inoculum size until it reached a maximum at 4% v/v. Increasing the inoculum size than 4% v/v decreases the production of enzyme occurred. This may be attributed to the fact that there is an increase in cell mass formation and a decrease in nutrients [24].

Optimization of lipase production by Plackett–Burman design

Extracellular lipase produced by different microorganisms is greatly influenced by the physical factors such as temperature, pH, and incubation period. Several studies were carried out to increase the enzyme production by optimization of several variables at the same time [7,25]. Thus, the experimental design with seven variables (time course, moisture content, pH, olive oil waste, temperature, inoculums age, and tween 80) was carried out to study the main effect of each factor on lipase production by *A. niger*. The results in Table 1 showed a variation in lipase activity from 0 to 250 U/ml. The results indicated that the medium optimization is necessary for high enzyme production. The maximum enzyme activity and Specific enzyme activity (SEA) (250 U/ml and 1315.8 U/mg protein, respectively) were observed in trial number 2 after 16 days of fermentation, whereas no enzyme activity in trial numbers 1, 5, and 8.

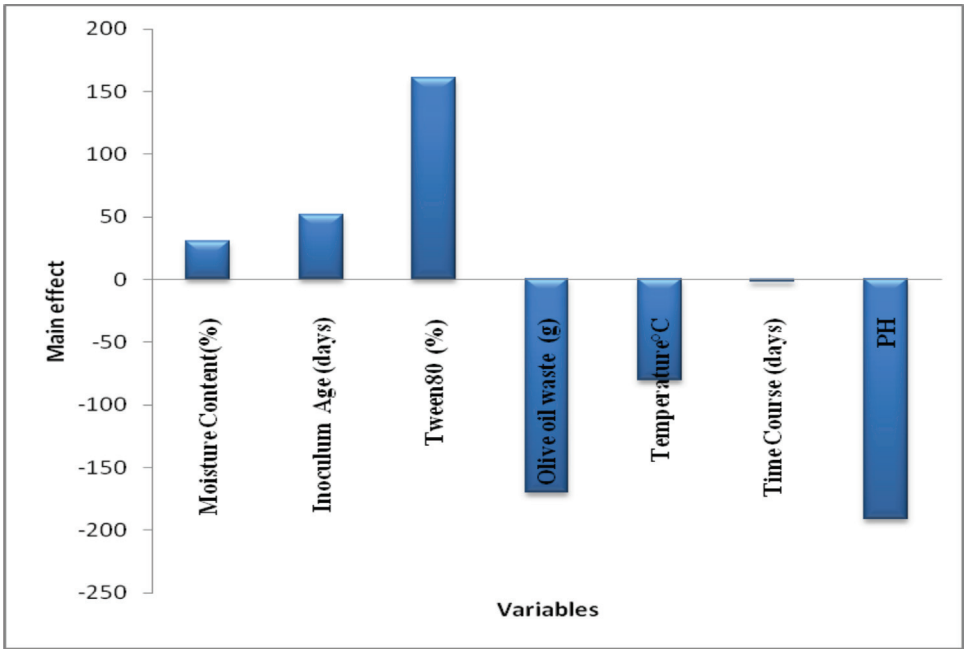
The main effects of the seven examined variables on lipase production were calculated and shown graphically

Table 1 Matrix of the Plackett–Burman design experiments on nine variables

Trials	Independent variables							Lipase activity (U/ml)	Protein (mg/ml)	SEA (U/mg protein)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7			
1	–(12)	–(2.5)	–(6)	+(7)	+(35)	+(4)	–(0.5%)	0	0.23	0
2	+(16)	–1 (2.5)	–1 (6)	–1 (3)	–1 (25)	+1 (4)	+1 (1.5%)	250	0.19	1315.8
3	–1 (12)	+1 (7.5)	–1 (6)	–1 (3)	+1 (35)	–1 (2)	+1 (1.5%)	200	0.2	1000
4	+1 (16)	+1 (7.5)	–1 (6)	+1 (7)	–1 (25)	–1 (2)	–1 (0.5%)	40	0.24	166.6
5	–1 (12)	–1 (2.5)	+1 (8)	+1 (7)	–1 (25)	–1 (2)	+1 (1.5%)	0	0.25	0
6	+1 (16)	–1 (2.5)	+1 (8)	–1 (3)	+1 (35)	–1 (2)	–1 (0.5%)	7.1	0.23	30
7	–1 (12)	+1 (7.5)	+1 (8)	–1 (3)	–1 (25)	+1 (4)	–1 (0.5%)	89.2	0.26	377.6
8	+1 (16)	+1 (7.5)	+1 (8)	+1 (7)	+1 (35)	+1 (4)	+1 (1.5%)	0	0.27	0
9	0 (14)	0 (5)	0 (7)	0 (5)	0 (30)	0 (3)	0 (1%)	200	0.1	2000

SEA, Specific enzyme activity; X_1, time course (days); X_2, moisture content (%); X_3, pH; X_4, olive oil waste (g); X_5, temperature (°C); X_6, inoculum age (days); X_7, tween 80 (%).

Figure 2



Effect of culture conditions and medium composition on lipase (U/ml) produced by *Aspergillus niger* NRRL-599.

in Fig. 2. The results obtained showed that emulsifying agent tween 80, moisture content, inoculum age had positive effects on lipase production whereas the other variables had negative effects.

Increase in moisture content causes a decrease in the substrate porosity enhances the substrate stickiness, changes the particle structure of substrate, and decreases both the gas volume and exchange. On the other hand, the decrease in moisture contents results in a decrease in nutrients solubility and improper swelling as well as higher water tension [26,27].

Tween 80 at 1.5% was found to be the most effective variable during lipase production by *A. niger* NRRL-599 because it served as a carbon source and an inducer. These results agreed with Salihu *et al.* [28] who found that the presence of tween 80 led to higher lipase production from *Penicillium citrinum*. The straight line of regression with data points across indicates the stability of the model, the agreement between predicted and actual values and thus the assumption of data point presentation is confirmed (data not shown).

Box–Behnken design

The three variables [inoculums age (days), moisture content (ml), and tween 80 (%)] identified by Plackett–Burman design experiment having positive main effects on enzyme activity and SEA were further tested and optimized through Box–Behnken design methodology [18]. This method allows the

Table 2 Experimental range and levels of the independent variables by using Box–Behnken design

Variables	Symbol	–	0	+1
		Range and levels		
Tween 80 (%)	X_1	1	1.5	2
Moisture content (ml)	X_2	1	2.4	4
Inoculum age (days)	X_3	3	4	5

Table 4 Analysis of variance analysis for Box–Behnken design

Terms	Estimate	SE	*t* ratio	*P*>|*t*|
Intercept	250	18.91957	13.21	<0.0001*
X_1	–38.5	11.58582	–3.32	0.0209*
X_2	68.625	11.58582	5.92	0.0020*
X_3	20.125	11.58582	1.74	0.1429
X_1×X2	20	16.38483	1.22	0.2766
X_1×X_3	–17.5	16.38483	–1.07	0.3343
X_2×X_3	62.75	16.38483	3.83	0.0123*
X_1×X_1	39.875	17.05387	2.34	0.0665
X_2×X_2	–24.875	17.05387	–1.46	0.2045
X_3×X_3	42.625	17.05387	2.50	0.0545

A value of P < 0.05 indicates that the model term is significant.

interaction of three independent variables at three different levels [low (-1), medium (0), high (+1)] were listed in Table 2 with 13 trials.

Box–Behnken designed results were shown in Table 3. Maximum lipase activity (420 U/ml) was shown in trial number 12 with tween 80 1%, moisture content 4 ml(v/v), and inoculum age 5 days. The *F* value (Fisher's statistical analysis) and *P* value (>0.0001) were used for determining the significance of the model. Low values of *P* indicate the high significance of the corresponding coefficient whereas large *t* and *F* values indicate the significance of corresponding coefficients [29]. Subjecting our model to analysis of variance analysis in Table 4. The correlation coefficient (R^2) measures how much the variability of the observed response can be explained by the experimental parameters and their interactions [30].

In Fig. 3 R^2 of the model is 0.94, that is 94%. The predicted R^2 is in acceptable agreement with the adjusted R^2 as shown by the actual predicted plot.

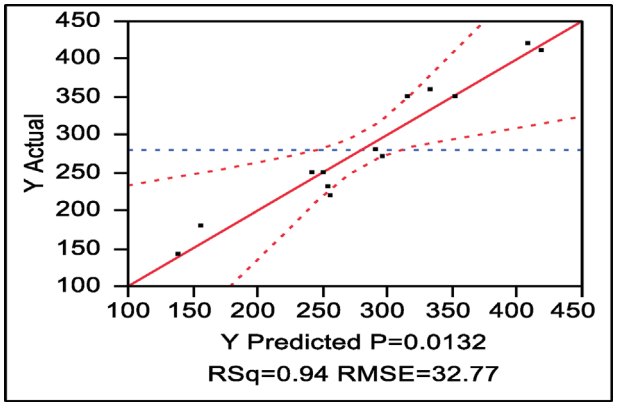
The model *P* value of 0.013 is significant for lipase model and similarly, the model *F* value of 1.6 the effect of each parameter on lipase activity and interaction between the three variables were illustrated in Fig. 4.

Three-dimensional aided in the visual determination of the maximum levels of each of the three parameters

Table 3 Experimental design of Box–Behnken design

Trials	Independent variables			
	X_1	X_2	X_3	Lipase activity (U/ml)
1	– (1)	– (1)	(0) 4	250
2	+ (2)	– (1)	(0) 4	200
3	– (1)	+ (4)	(0) 4	300
4	+ (2)	+ (4)	(0) 4	150
5	– (1)	0 (2.5)	–1 (3)	400
6	+ (2)	0 (2.5)	– (3)	280
7	– (1)	0 (2.5)	+ (5)	420
8	+1 (2)	0 (2.5)	+ (5)	240
9	0 (1.5)	–1 (1)	– (3)	195
10	0 (1.5)	+ (4)	– (3)	270
11	0 (1.5)	– (1)	+ (5)	180
12	0 (1.5)	+ (4)	+ (5)	400
13	0 (1.5)	0 (2.5)	0 (4)	250

Figure 3

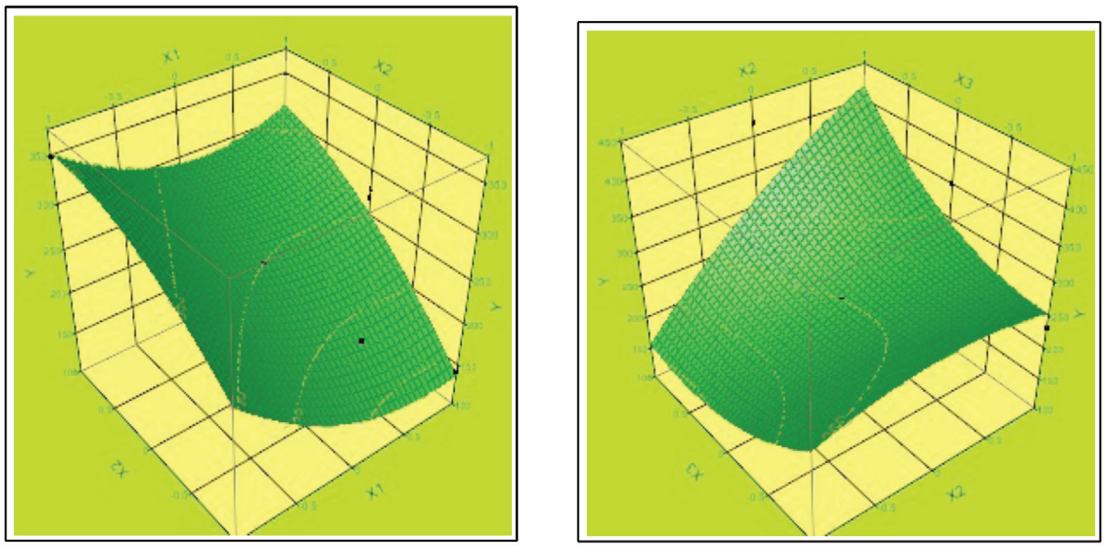


Predicted lipase activities versus experimentally observed lipase activity for the Box–Behnken design.

when they interact. It is well known that the three-dimensional responses have either elliptical or circular representation, which is considered either significance or negligible [31]. Figure 4 showed that, as the level of these parameters changes, their interactive effect on the response also varies. The interaction between tween 80 (X_1) and moisture content (X_2) was examined while maintaining the inoculum age (X_3) was constant (Fig. 4a) at maximum tween 80 concentration and moisture content, lipase production was 350 U/ml. In Fig. 4b, lipase activity was found to be maximum (420 U/ml) revealing the effect of the interaction between the moisture content and inoculum age. Although Fig. 4c showed the effect of interaction between tween 80 concentrations and inoculum age on lipase activity (400 U/ml).

The quadratic model represents $Y_{activity}$ (U/ml) as a function of tween 80 (X_1), moisture content (X_2), and inoculum age (X_3). The production of lipase enzyme

Figure 4



a **b**

Contour plot of lipase production by *Aspergillus niger* showing the interactive effects of different concentrations of X_1 and X_2 (a), X_3 and X_2 (b), and X_1 and X_3 (c), Y: lipase activity (U/ml).

Y_{activity} (U/ml) was predicted by the following model equation:

$$Y_{\text{activity(U/ml)}} = 250 - 38.5(X_1) + 68.63(X_2) + 20.13(X_3) - 20(X_1)(X_2) - 7.5(X_2)(X_3) + 62.75(X_2)(X_3) + 39.9(X_1)^2 - 24.9(X_2)^2 + 42.6(X_3)^2.$$

A verification experiment was performed in triplicates, the results showed an actual maximum lipase production 125% when compared with that produced by the basal condition.

Conclusion

This study was investigated the lipase activity of *A. niger* spp. on agroindustrial residues as a substrate, which expressed that olive oil waste was the best for lipase production on solid medium. By optimizing the culture parameters using Plackett–Burman design, the lipase activity was further increased. This paper throws light on the exploitation of agroindustrial waste for production of an important industrial enzyme using a low cost method as SSF.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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