Artificial Neural Networks and Response Surface Methodology Approach for Optimization of an Eco-Friendly and Detergent-Stable Lipase Production from *Actinomadura Keratinilytica* Strain Cpt29

Noura Semache,[1] Fatiha Benamia,[1,*] Bilal Kerouaz,[2] Inès Belhaj,[3] Selma Bounour,[1] Hafedh Belghith,[3] Ali Gargouri,[3] Ali Ladjama[2] and Zeineddine Djeghaba[1]

[1] *Laboratory of Applied Organic Chemistry, Chemistry Department, Faculty of Sciences, Badji Mokhtar University, P.O. Box 12, 23000 Annaba, Algeria*

[2] *Laboratory of Applied Biochemistry and Microbiology, Biochemistry Department, Faculty of Sciences, Badji Mokhtar University, P.O. Box 12, 23000, Annaba, Algeria*

[3] *Laboratory of Molecular Biotechnology of Eukaryotes, Centre of Biotechnology of Sfax, University of Sfax, Road of Sidi Mansour Km 6, P.O. Box 1177, Sfax 3018, Tunisia*

* *Corresponding author: E-mail: fatiha.benamia@univ-annaba.org*

Abstract

This work mainly focused on the production of an efficient, economical, and eco-friendly lipase (AKL29) from *Actinomadura keratinilytica* strain Cpt29 isolated from poultry compost in north east of Algeria, for use in detergent industries. AKL29 shows a significant lipase activity (45 U/mL) towards hydrolyzed triacylglycerols, indicating that it is a true lipase. For maximum lipase production the modeling and optimization of potential culture parameters such as incubation temperature, cultivation time, and Tween 80 (v/v) were built using RSM and ANN approaches. The results show that both the two models provided good quality predictions, yet the ANN showed a clear superiority over RSM for both data fitting and estimation capabilities. A 4.1-fold increase in lipase production was recorded under the following optimal condition: incubation temperature (37.9 °C), cultivation time (111 h), and Tween 80 (3.27%, v/v). Furthermore, the partially purified lipase showed good stability, high compatibility, and significant wash performance with various commercial laundry detergents, making this novel lipase a promising potential candidate for detergent industries.

Keywords: Lipase; *Actinomadura keratinilytica*; Optimization; RSM; ANN; Detergent.

1. Introduction

Lipases are glycerol ester hydrolases that catalyzes the hydrolysis of triacylglycerols to release diacylglyceride, monoacylglycerol, long-chain fatty acids and glycerol at the interface of oil and water.[1] It has been reported that the first lipases were obtained from *Penicillium oxalicum* and *Aspergillus flavus*.[2] Since, the lipases were considered as a great biotechnological and industrial catalyst after carbohydrases and proteases.[3] Lipases are prevalent in nature and are produced by plants, animals, and microorganisms including fungi, bacteria, and actinomycetes.[4] Recently, it has been reported that several actinomycete isolates are able to hydrolyze fats and oils.[5] Microbial lipases are mainly extracellular and their production is significantly influenced by the culture medium parameters. Generally, the major factor influencing the lipase activity was the carbon source. The production of these lipases is generally conducted in the presence of oil, triacylglycerols, fatty acids, esters, glycerol, or Tweens.[6,7] Microbial lipases play a major role in various fields such as the synthesis of organic chemicals and industrial applications. Development of lipase-based technologies for the synthesis of novel compounds increased their use.[8] The main commercial

application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales.[7] Approximately, sixty percent of industrial enzymes are hydrolytic in nature and are used by the detergent, dairy and leather industries.[9]

The main objective of this work is the production of an efficient, natural, and economical lipase for use in industrial applications and in particular the laundry detergent industry. For this purpose a novel lipase (AKL29) from *Actinomadura keratinilytica* strain Cpt29 isolated from poultry compost in local farm north east of Algeria, was produced. To our knowledge, the lipase cultivation from this strain has never been described. This study mainly focuses on the optimization of the parameters of culture medium to increase the production of AKL29. For this purpose artificial neural networks (ANN) and response surface methodology (RSM) have been investigated to build a predicted effects model and optimization of culture parameters of lipase production.

The last decade has seen a multitude of data analysis tools based on biological phenomena develop into well-established modeling techniques, such as artificial intelligence and evolutionary computing. Artificial neural network (ANN) is now the most popular machine learning tool in biotechnology.[10] On the other hand, the statistical optimization of processes has advantages compared to the classical one.[11] Numerous researchers have reported the use of statistical methods for the production of lipases by microorganisms.[12]

The classical method of optimization involves varying one parameter at time and ignoring the combined interactions between experimental conditions of the process. In recent years, the artificial neural network (ANN) has been used as a highly powerful and flexible method in various processes. It is expected to reveal functions representing phenomena but it cannot clarify the interaction among variables and the significance of each variable. Response surface methodology (RSM) is an effective statistical technique for developing, improving, and optimizing complex process.[11]

For an exhaustive study on the improvement of lipase production from *Actinomadura keratinilytica* strain Cpt29, both ANN and RSM as statistical approaches have been performed in the present work. After finding the most influential factors (incubation temperature, cultivation time, and Tween 80, v/v), for composition of production medium among several parameters of culture medium screened in our previous study, the optimization of these three significant parameters for maximum lipase production was carried out using RSM and ANN. In this work, also a partial purification was investigated to improve the lipolytic activity efficiency of the produced lipase. Furthermore, the stability, compatibility, and wash performance of the partially purified AKL29 with various laundry detergents were carried out to evaluate its potential as bio-additive in various detergents formulations.

2. Experimental

2. 1. Materials

Candida rugosa lipase (CRL, Type VII, 760 U/mg), Bradford reagent, bovine serum albumin (99%) were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Benzamidine were from Fluka (Buchs, Switzerland); Gum Arabic was from Merck (D-6100 Darmstadt, Germany), pH-stat was from Metrohm (Switzerland). Unless specified otherwise, all substrates, chemicals, and reagents were of the analytical grade or highest available purity purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephacryl S-75 was from Pharmacia (Pharmacia, Uppsala, Sweden).

2. 2. Microorganism Source

The organism required for the lipase production is a thermophilic actinomycetes *Actinomadura keratinilytica* strain Cpt29 [GenBank accession no. KC447297]. The strain Cpt29 was isolated from poultry compost collected from a local farm in north-east region of Annaba, Algeria as previously described elsewhere.[13] Stock culture of *Actinomadura keratinilytica* strain Cpt29 [C.p.t: Compost poultry thermophilic] was maintained by periodic subculture and stored at 4 °C.

2. 3. Lipase Production

The lipase production from *Actinomadura keratinilytica* strain Cpt29 was carried out as follows. Firstly, the bacterial isolate strain Cpt29 was subjected to a qualitative screening for lipase activity, using agar plates containing a slightly modified basal medium (g/L): K_2HPO_4, 0.8; KH_2PO_4, 6; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.5; NaCl, 3; $FeSO_4$, 0.001, supplemented with 3% (v/v) Tween 80; and bacteriological agar, 20.[14] The medium was adjusted to different pH (4-10) in order to set the optimum pH of the culture medium, and the plates were incubated at 35 °C for three days. Lipase producing strain Cpt29 was identified by monitoring clear zone formation around the bacterial colony. Halo zones seen were considered as positive for lipase production.

Lipase production was carried out using a method similar to that described in previous work.[15] 10mL of inoculums culture was added in 100 mL of culture medium in 500 mL Erlenmeyer flasks and incubated at 35 °C on a rotary shaker at 150 rpm for three days. The culture broths were centrifuged at 4830 xg for 20 min to remove mycelia and medium debris, and the cell-free supernatant was used as a crude enzyme solution for determination of the lipolytic activity.

2. 4. Lipase Activity Assay

Lipase activity was measured titrimetrically using olive oil hydrolysis. According to previous works[15-17] the

experiments were performed using olive oil emulsion obtained by mixing 5 mL of olive oil with 45 mL of 10% (w/v) of gum arabic (GA) in 30 mL of 25 mM Tris–HCl buffer (pH 8) in the presence of 2 mM CaCl$_2$, and 200 µl of enzyme solution. The quantity of free fatty acids (FFAs) released was titrated adding 0.1N sodium hydroxide to the reaction medium. Hydrolysis of olive oil emulsion was monitored by pH-stat (718 Stat Titrino, Metrohm, Switzerland). One unit (1U) of lipase activity corresponds to 1 µmol of fatty acid released per minute under the assay conditions used. All determinations were performed in triplicate.

2. 5. Estimation of Total Extracellular Protein

The total extracellular protein content was measured by Bradford method using Coomassie blue assay procedure and bovine serum albumin (BSA) as standard. Samples were analyzed in spectrophotometer (Jenway 6405 UV/Vis) at 595 nm and the protein concentration was determined using the calibration curve of BSA.[18]

2. 6. Partial Purification of AKL29

The partial purification of lipase from the isolate strain Cpt29 was carried out by two steps including acetone precipitation strategy and gel filtration on Sephacryl S-75. The experiments were performed at 4 °C using a method similar to that described in previous work.[19]

2. 6. 1. Acetone Precipitation

After the incubation period, the culture of strain Cpt29 (200 mL) grown on Tween 80 under optimal cultivation conditions was centrifuged for 20 min at 4830 xg to remove the microbial cells. The crude enzyme solution (188 U/mL) was submitted to the partial purification using acetone precipitation as important step. Four volumes of ice-cold acetone (−20 °C) were added to one volume of cell-free culture supernatant to remove most of the other proteins. To minimize the impurities as much as possible, the experiment was repeated with gradual increments of 3% acetone saturation under a gentle stirring. The precipitate was then recovered by centrifugation at 12000 xg for 25 min, and then was suspended in a minimal volume of 20 mM Tris-HCl buffer (pH 8) containing 1mM benzamidine, and the protein content was estimated.

2. 6. 2. Filtration on Sephacryl S-75

After the acetone precipitation step, the obtained supernatant was applied onto a column (3 cm × 160 cm) of gel filtration Sephacryl S-75 equilibrated with 20 mM Tris-HCl buffer (pH 8) containing 1mM benzamidine. The elution of lipase was performed with the same buffer solu-

tion at a rate of 45 mL/h. All the elute fractions (2mL each) were collected, and then were checked for lipase activity by titrimetric method. The fractions containing the lipase activity were pooled and the protein content was measured spectrophotomerically at 280 nm.[15]

2. 7. Optimization of Lipase Production

The optimization of lipase production from strain Cpt29 was carried out using two steps including the choice of the best carbon source and the culture medium optimization for maximum lipase production. The optimization of culture medium was investigated using RSM and ANN methodologies.

2. 7. 1. Selection of Carbon Source

To select the significant substrate for AKL29 production, a screening study was performed using four different sources of carbon (Tween 80, Tween 20, olive oil and wheat bran). Each substrate was added to the basal medium at various concentrations to evaluate its effect on the lipase production. Kinetics of the lipase production were monitored by inoculation of 10^6 spores/mL in basal medium followed by incubation at 35 °C, under shaking condition. Samples were taken aseptically every day and enzyme activity was measured using a pH-stat.

2. 7. 2. Statistical Analysis

In order to study the effects of culture conditions on the lipase production, two different statistical approaches were performed for the modeling and the optimization of the lipase activity which is the response (Y) of the experimental design and function of independent variables in RSM and training of the artificial neuron in ANN.

For this purpose a Box-Behnken design was performed to evaluate the effects of culture conditions on the activity of the produced lipase (U/mL), which is the response (Y) of experimental design. A total of fifteen runs with different combination of the significant experimental factors were performed (Table 1). The culture medium conditions are expressed by the following factors (Xi): incubation temperature X_1 (20–50 °C), cultivation time X_2 (2–5 days), and Tween 80 (v/v) as carbon source X_3 (2–4%). A quadratic polynomial regression model (Eq. 1) was assumed to predict the optimal response (Y). The proposed model for response (Y) was:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad (1)$$

Where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients (β_0 is the intercept term, β_i is the linear effect term, β_{ii} is the quadratic effect term, β_{ij} is the interaction effect term, and Y is the predicted response value); X_1, X_2,..., X_k are the input variables that explain the response Y.[11]

2. 8. Influence of Laundry Detergents on Partially Purified Lipase Activity

In this work, the enzymatic performance of partially purified AKL29 towards laundry detergents was verified by studying the effect of various brand commercial detergents on the stability and compatibility of lipase using the commercial *Candida rugosa* lipase (CRL) for comparison. Furthermore, wash performance of the partially purified AKL29 as a green additive for detergent, was evaluated. Effect of laundry detergents on the lipase activity was carried out using a method similar to that described in previous work.[20]

2. 8. 1. Influence of Detergents on the Lipase Stability and Compatibility

The enzyme compatibility and stability of the partially purified AKL29 with laundry detergents were assessed using a variety of commercial detergents including, ARIEL (Procter and Gamble, Switzerland), ISIS (Henkel, Algeria), OMO (Unilever, Algeria), LE CHAT (Henkel, Algeria),

and NICE (Sarl Nice Plus, Algeria). All experiments were assessed using CRL as commercial lipase for comparative evaluation. In order to simulate the washing conditions, the selected commercial detergents were diluted in tap water to give a final concentration of 7 mg / mL. All pre-existing endogenous lipases contained in these detergents were inactivated by heating the diluted detergents for 60 min at 70 °C before addition of the enzyme. The effect of lipase stability as well as its compatibility with commercial laundry detergents were studied by incubating each of the two lipases tested (AKL29 or CRL) with the various modified detergents at 40 °C for 60 min. The residual activities were determined at pH 8 and 45 °C and the enzymatic activity in the absence of any detergent was taken as 100%.

2. 8. 2. Wash Performance Analysis of Partially Purified Lipase

The wash performance evaluation of partially purified AKL29 as eco-friendly additive in laundry detergent was investigated using small white cotton cloth pieces
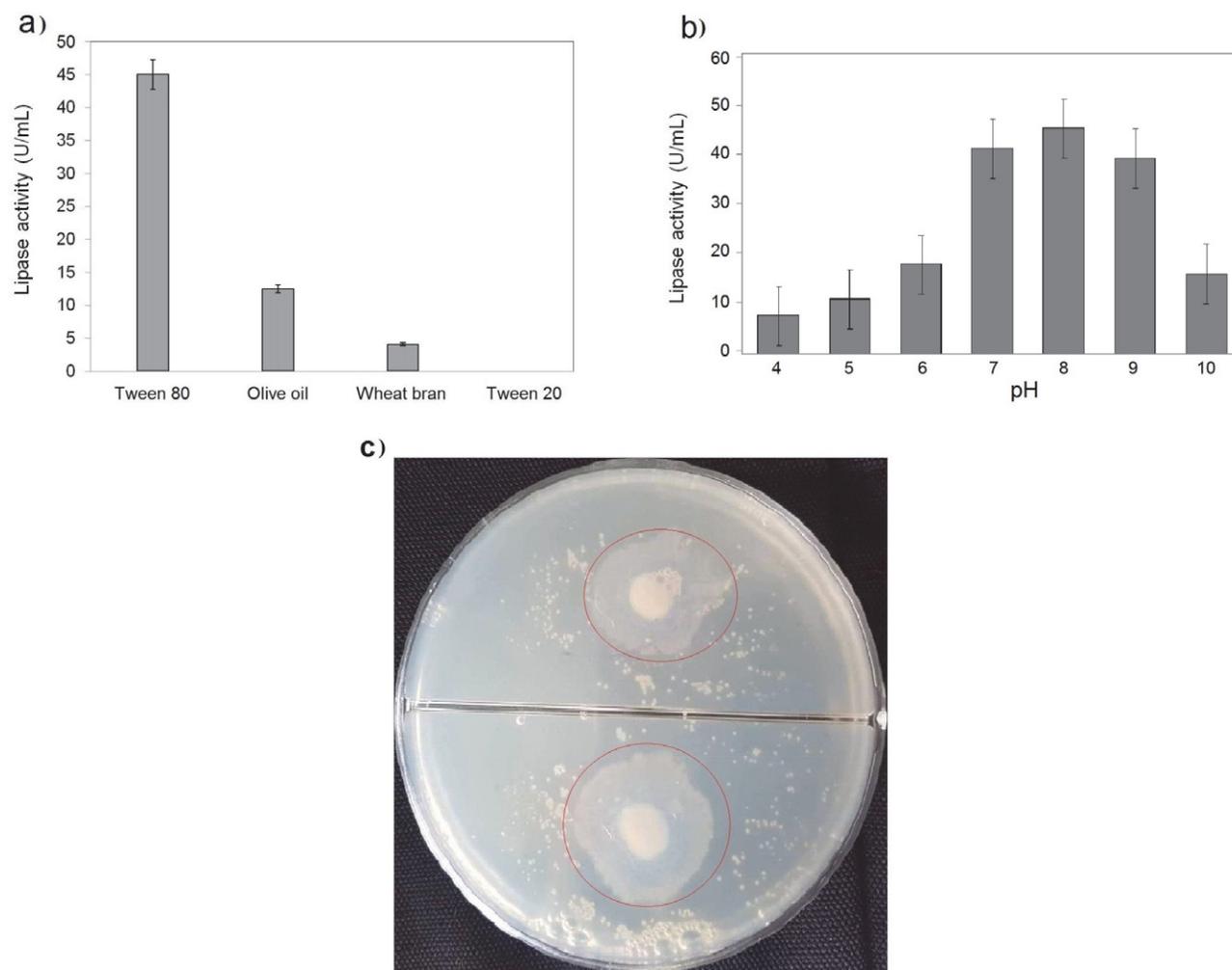
Figure 1. Influence of various carbon sources on the AKL29 activity (a). Effect of pH on the AKL29 activity (b). The lipase activities were measured at 35 °C, pH 8, after three days using different carbon sources, and different pH. The experiments were conducted three times and the error bars represent standard deviation. Hydrolysis zone formed by the lipolytic strain Cpt29 (c).

stained with a mixture of sauce sample and fat/greasy material. The cloth pieces were then incubated in different wash treatments at 40 °C and stirred at 100 rpm for 30 min. Incubation process was carried out using 100 mL of modified detergent at final concentration of 7 mg / mL (heat inactivated). After incubation the modified detergent was added to the lipase solution (50 U/mL). After that, the cloth pieces were removed, rinsed with distilled water, dried and submitted to visual observation to examine the stain removal effects of the lipase. Olive oil was extracted with petroleum ether for 6h using a Soxhlet extractor after complete evaporation of petroleum ether from the extract. The percentage of the removed olive oil was calculated by the following formula:[20]

$$Removal\,(\%) = \frac{W_1 - W_2}{W_2} \; X \; 100 \qquad (2)$$

Where, W_1 and W_2 denoted the weights of total olive oil before and after washing (mg), respectively.

3. Results and Discussion

3. 1. Selection of Carbon Sources

To select the appropriate carbon source that maximizes the activity of lipase produced by strain Cpt29, various carbon sources such as Tween 80, Tween 20, Olive oil, and Wheat bran were tested. The screened carbon sources were supplemented at 3%, v/v to the basal Mendel's medium. The effects of carbon sources on the lipase activity are illustrated by Figure 1a.

Figure 1a shows that the highest level of lipase activity (45 ± 2 U/mL) was produced in presence of Tween 80 emulsion as substrate after three days of growth. The

lower lipase activity of 4 ± 1.5 U/mL was observed on media supplemented with oil olive as carbon source. Only Tween 20, present in the medium as carbon source, permits to decline significantly the lipase activity. Based on these results, Tween 80 was selected as the best carbon source for lipase production by isolate Cpt29. The produced lipase was assayed for its ability to hydrolyze Tween 80 as an exclusive carbon source, in agar plates incubated at 35 °C for three days. Lipase produced by strain Cpt29 was found more active over a range of pH from 7 to 9, with an optimum at pH 8 (Figure 1b). Colonies with a large clear zone formed by the hydrolysis of Tween 80 indicated the presence of lipolytic activity (Figure 1c).

3. 2. Optimization of AKL29 Activity

Response surface methodology (RSM) using Box-Behnken design was applied to determine the optimal levels of the activity of lipase produced by strain Cpt29. RSM allows the analysis of the effects of significant culture parameters and also generates a mathematical model which makes it possible to predict the optimal response (Y).[11] The evaluation of the resulting model (Table 2) was conducted by the statistical theory and analyzing data in Table 1 using Minitab 16 as statistical software.

The results of the analysis of variance (ANOVA, Table 2) show that the interaction terms are not significant (*P*-value > 0.05). So to improve data fit, these terms were excluded from this analysis. The final model is expressed in terms of linear terms (X_i) and quadratic terms (X_i^2) (Eq.3):

$$Y = 765 + 13.95\,X_1 + 124.10\,X_2 + 216.54\,X_3$$
$$- 0.197\,X_1^2 - 14.69\,X_2^2 - 34.54\,X_3^2 \qquad (3)$$

Table 1. Experimental design used in RSM and ANN studies with the values of selected independent variables and the corresponding observed and predicted responses (Y).

X_1 (°C)	X_2 (days)	X_3 (%)	Observed values	Y^a (U/mL) RSM predicted values	ANN predicted values
50	5.0	3	147	142.63	146.71
35	5.0	4	157	155.50	157.44
20	3.5	2	51	49.88	51.25
35	5.0	2	122	121.75	122.33
35	2.0	4	57	61.75	57.13
35	3.5	3	158	159.33	159.05
50	3.5	2	73	77.38	73.44
35	3.5	3	156	159.33	159.05
35	2.0	2	31	28.00	30.74
20	2.0	3	24	21.38	24.25
50	2.0	3	48	48.88	48.05
20	3.5	4	86	83.63	85.88
35	3.5	3	164	159.33	159.05
50	3.5	4	112	111.13	111.99
20	5.0	3	109	115.13	108.60

[a]: values represent means of three replicates. Y: lipase activity (U/mL)

Table 2. All estimated regression coefficients for response (Y).

Term	Coefficient	*P*-value
X_1	13.95	0.000
X_2	124.10	0.000
X_3	216.54	0.000
X_1^2	–0.197	0.000
X_2^2	–14.69	0.000
X_3^2	–34.54	0.000
$X_1 X_2$	0.156	0.153
$X_1 X_3$	0.067	0.651
$X_2 X_3$	1.500	0.328

The coefficient of determination (R^2) of the model adjusted to 99.21% tested the fit of the model and indicates the real relationship among the selected parameters. These values showed that the best model that fits our data is the quadratic one. The results (Table 2) show that the carbon source (Tween 80) and the culture time exhibite high influence on the production of lipase. This significance was evaluated by the high values of their effect compared to that of incubation temperature. Also the very meaningful Fisher's coefficient ($F_{0.95}$ = 3.58 <<< 292.42) with the high coefficient of determination (R^2 = 99.6%), and the study of the linear regression between the observed values of response (Y_{obs}) and the predicted ones (Y_{pred}) confirm the validity of this model (Figure 2).
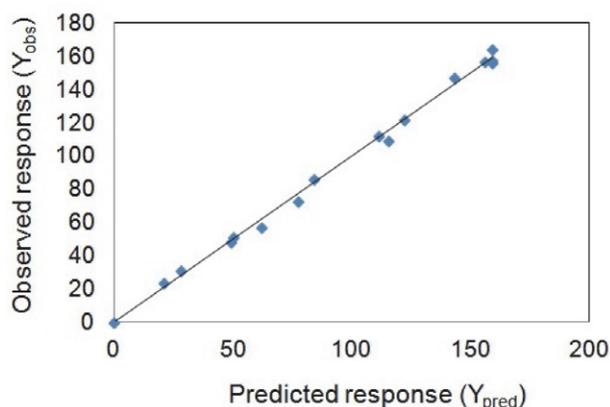
Figure 2. Parity plot showing the goodness-of-fit for RSM model (R^2 = 99.5%).

The fitting quality of RSM model is also shown by the results of analysis of variance (ANOVA, Table 3) which indicate that the model is adequate to represent the actual relationship between response (Y) and the significant variables.[11] The low values of residual errors and the no significance of the lack of fit, show that the quadratic model obtained by the RSM approach using Box-Behnken design can be accepted to describe the studied phenomenon (Table 3).

The best way to predict the relationship between response and parameters of the interactions is to analyze the contour plots and the response surface graphs that give a detailed presentation of the optimum value predicted from the results.[21,22] Each plot and graph that gives the variation of the lipase activity (Y) with independent variables represent an infinite number of combinations of two test variables with the other two fixed at their respective zero level (Figure 3). The contour plots and 3D response surface graphs represented in Figure 3 show that the lipase activity (U/mL) was enhanced by the values near the middle of the input variables.

## 3. 3. Artificial Neural Network (ANN) Analysis

The ANN architectures used in this purpose was a multilayer forward neural network trained with a Multi Layer Perceptron (MLP) incremental back propagation network with linear transfer function for output and TanH transfer function for hidden neurons. The input layer consists of incubation temperature (X_1), cultivation time (X_2), and Tween 80 (v/v) as carbon source (X_3). The output is represented by the activity of lipase produced by strain Cpt29. In order to select the optimal neural network architecture, which is an important test for a successful application, several ANN architectures (the number of hidden layers and the type of transfer functions), the top three ANN models are summarized in Table 4.

According to the values of coefficient of determination (R^2) and absolute average deviation (AAD) which indicate the significance of the model, we have used the three hidden layers to evaluate the ANN performance analysis compared with RSM one (Figure 4a). ANN analysis was

Table 3. ANOVA for the obtained model of RSM (Eq. 3).

Source	DF	Sum of squares	Least square mean	F	*P*-value
Regression	6	35018	5896	292.42	0.000
Linear	3	21369	5766	289.00	0.000
Square	3	13649	4550	228.00	0.000
Residual Error	8	160	20	221.00	0.000
Lack of fit	6	125	21	1.20	0.520 >> α = 0.05
Pure Error	2	35	17		
Total	14	35177			

DF: degree of freedom. *P* significant if value less than 0.05. R^2 = 99.6 %
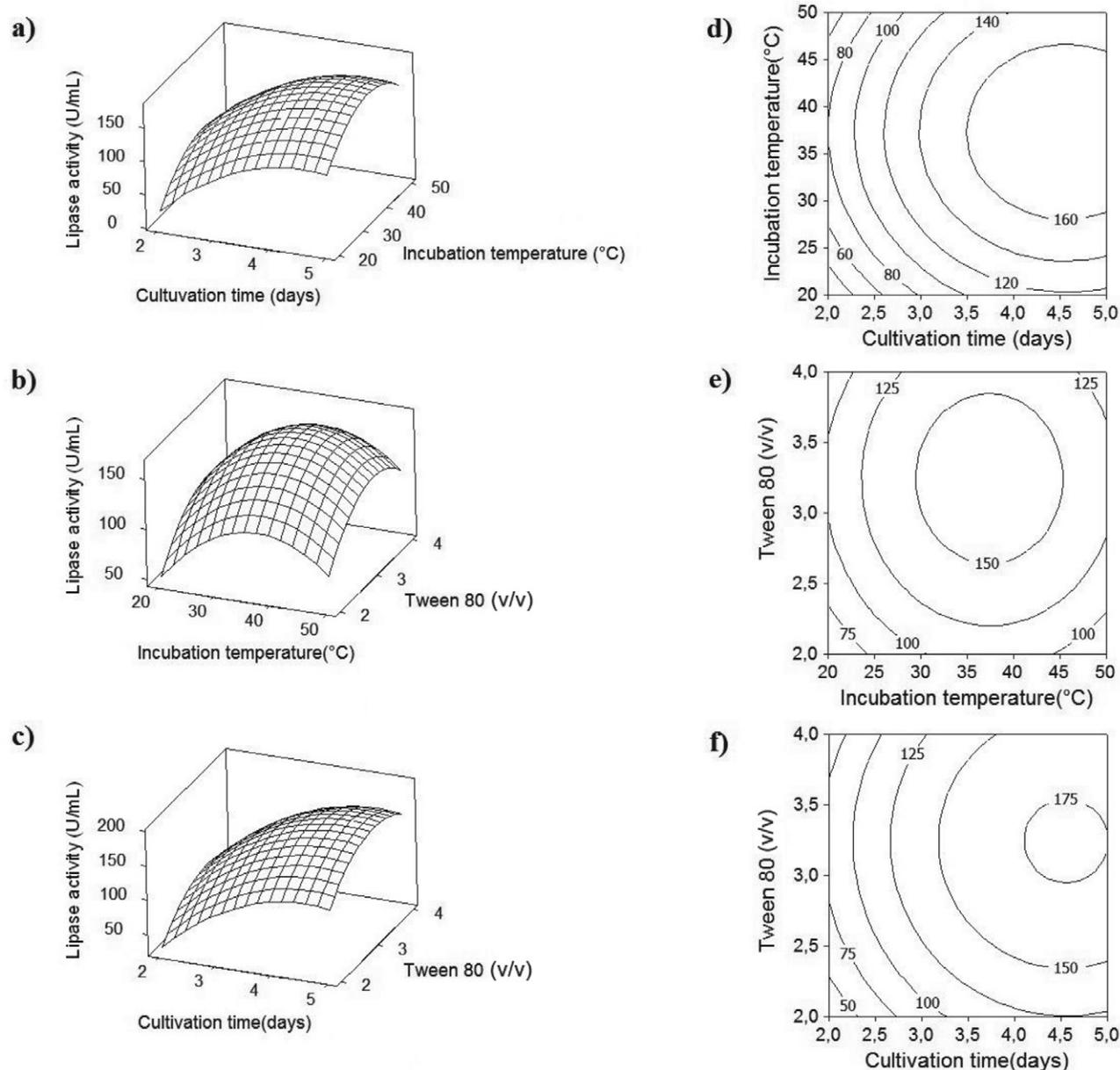
Figure 3. Response surface graphs with contour plots for the effects of independent variables on lipase activity (U/mL), and their mutual interactions, respectively: incubation temperature and cultivation time (a) and (d), incubation temperature and Tween 80 (v/v) (b) and (e), cultivation time and Tween 80 (v/v) (c) and (f).

conducted using SAS software (SAS software, version 9.0, SAS Institute, Inc., Cary, NC). The validity of the ANN (3-3-1) model for testing data is confirmed by the goodness of fit between predicted and experimental response values (Figure 4b, R^2 = 99.9%).

3. 4. Comparison of RSM and ANN Models

The observed values of the lipase activity (Y) along with the predicted ones calculated by ANN and RSM (Table 1) show the goodness of fit for the corresponding

Table 4. Significant ANN architectures and their effects on the estimation and prediction of lipase activity (Y).

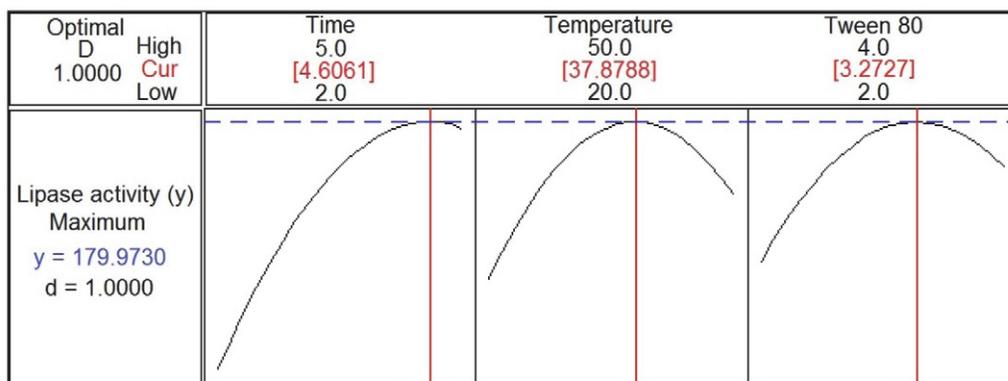| Model	Learning Algorithm	Transfer function, output	Transfer function, hidden neurons	Training set, R^2 (%)	Validation set, R^2 (%)	Training set, AAD	Validation set, AAD
3-3-1	MLP	Linear	Tanh	99.73	99.80	0.013	0.0002
3-2-1	MLP	Linear	Tanh	95.12	95.10	0.055	0.0007
3-1-1	MLP	Linear	Tanh	79.24	76.60	0.114	0.0021
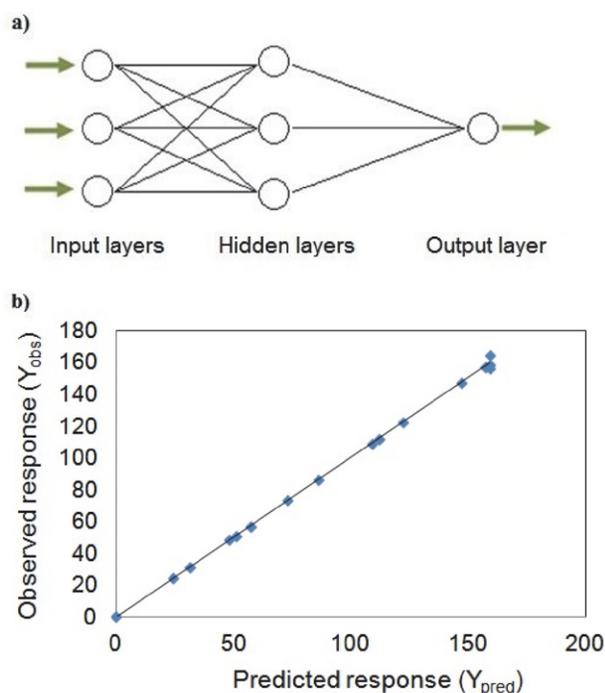
Figure 4. Neural network architecture used for the prediction of lipase activity (a). Parity plot showing the goodness-of-fit for RSM model (R² = 99.9%) (b).

models (R^2 = 99.9% and 99.5%, for ANN and RSM, respectively). Both performed models provided good quality predictions, yet the ANN showed a clear superiority over RSM for both data fitting and estimation capabilities (Figures 2 and 4b).

3. 5. Optimization of the Response (Y)

The optimal point of culture parameters for maximum lipase production was determined by the desirability function of Minitab software. The optimal lipase production expressed by lipase activity (U/mL) corresponds relatively to the middle values of the three powerful factors (Figure 5). Experimental validation of enzyme activity was performed using optimal operating variables which were

set to incubation temperature of 37.9 °C, 111 h of cultivation time, and 3.27% (v/v) Tween 80.The experimental obtained value of the lipase activity (188 U/mL) is upper than the theoretical value (179.9 U/mL).

Furthermore, the lipase activity from *Actinomadura keratinilytica* strain Cpt29 as a thermophilic actinomycete, was found to be significantly superior than several ones reported previously for the most of the other actinomycetes (Table 5).

3. 6. Partial Purification of AKL29

Extracellular lipase was partially purified to homogeneity from the culture filtrates of *Actinomadura keratinilytica* strain Cpt29 grown on Tween 80 under optimal culture conditions. The crude enzyme was precipitated using acetone to yield as an active pellet. Next the obtained supernatant was applied onto a column (3 cm × 160 cm) of gel filtration Sephacryl S-75 equilibrated with buffer solution of Tris–HCl (20 mM; pH 8) containing 1mM benzamidine. Figure 6 presents the proteine elution profile
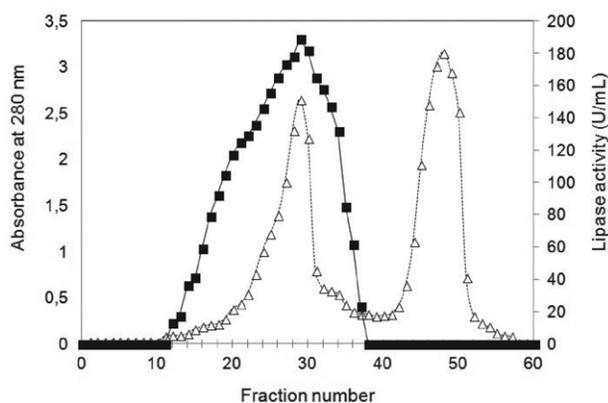
Figure 6. Elution profile of AKL29 obtained by gel filtration using chromatography on Sephacryl S-75. The column (3 cm × 160 cm) was equilibrated with buffer: 20 mM Tris–HCl, pH 8, and 1 mM benzamidine. The elution of lipase (2 mL) was performed at a rate of 45 ml/h. Lipase activity (■) was measured as described in materials and methods section, and the protein (Δ) was monitored by absorbance at 280 nm.

Figure 5. Composite desirability and optimization plot for maximum lipase activity.

Table 5. Lipase activity recorded in some Actinomycetes.

Organism	Substrate	Optimum pH and temperature	Cultivation time (h)	Lipase activity (U/mL)	Reference
Actinomadura keratinilytica strain Cpt29	Tween 80	8 and 37.9 °C	111	188	This work
Streptomyces sp. Al-Dhabi-49	Glucose	8 and 35 °C	120	162	[5]
Streptomyces variabilis NGP 3	Lactose	8.5 and 45 °C	168	39.4	[23]
Streptomyces exfoliates	Triacylglycerides	6 and 37 °C	72	6.9	[24]
Streptomyces sp. TEM 33 strain	*p*-nitrophenyl palmitate	9 and 37 °C	36	3	[25]

Table 6. Flow sheet of the AKL29 partial purification.

Purification step	Total activity[a] (U)	Protein amount[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Culture supernatant	31800 ± 1200	450 ± 36	70 ± 1.5	100	1
Acetone precipitation	30000 ± 700	89 ± 4	337 ± 2.7	94	5
S-75 chromatography	7800 ± 165	4 ± 0.4	1880 ± 79	24.5	27

[a] 1U corresponds to 1μmol of fatty acid released per minute using olive oil emulsion as substrate. [b] Protein amounts were estimated using Bradford's method.

recorded at the final step of the lipase patial purification. The specific activity of the pure lipase increased 27-fold compared to the crude extract (Table 6).
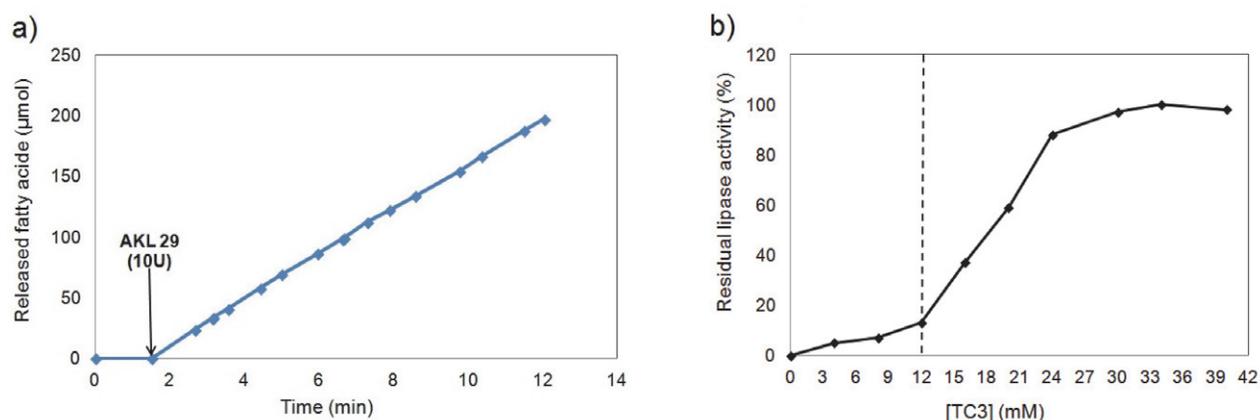
3. 7. Kinetic Study of AKL29

The ability of our partially purified lipase to hydrolyse its substrate without any surfactant, was also tested using the linear kinetic of FAAs release up on olive oil emulsion hydrolysis biocatalyzed by the partially purified lipase (AKL29) for 12 min. The results (Figure 7a) indicate that the lipase was efficient and seems to be resistant to interfacial denaturation at lipids-water interfaces. The presence of interfacial activation phenomenon of AKL29 was assessed by studying the rate of hydrolysis of TC3 emulsified in 0.33% gum arabic and 0.15 M NaCl by AKL29 as a function of the concentration of the substrate. As shown

in Figure 7b, when TC3 was in the water-soluble state, AKL29 hydrolyzed slowly his substrate. However, the lipase activity increased rapidly above the solubility limit of TC3 to reach 100% (300 U/mg) at 34 mM. This result indicates that the AKL29 presents the interfacial activation phenomenon. AKL29 which hydrolyses olive oil emulsion, can be considered as a true lipase.[26]

3. 8. Compatability and Stabilty of Partially Purified AKL29 with Laundry Detrgents

On the basis of the lipase kinetic study, the stability and compatibility of AKL29 with various commercial laundry detergents have been investigated. Both the compatibility and the stability of AKL29 as a bio-additive in laundry detergents were evaluated by comparison with commercial *Candida rugosa* lipase (CRL). The results (Figure 8) show

Figure 7. Parity plot showing kinetic study of lipase using olive oil emulsions as substrate (a). Hydrolysis rate of TC3 by AKL29 as function of substrate concentration. The TC3 solutions were systematically prepared by mixing (3 × 30 s) in a warring blender agiven amount of TC3 in 30 ml of 0.33% GA and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH 8 and 45 °C using a pH-stat. The solubility limit of TC3 (12 mM) is indicated by a vertical dotted line (b).

that the lipase exhibited high stability and significant compatibility at 40 °C after 60 min of incubation in various laundry detergents compared to CRL. In fact, 100% of residual activity of AKL29 was recorded in the presence of NICE and ISIS. However, the results show a decrease in the residual activity of AKL29 with the other detergents tested. About 91%, 85% and 73% of residual activity was retained in the presence of OMO, ARIEL, and LE CHAT respectively. On the other hand, a slightly better compatibility and stability of commercial CRL were observed with OMO compared with AKL29 (Figure 8). According to these results, the lipolytic activity of AKL29 could be a potential candidate as a bio-additive for laundry detergent formulations.
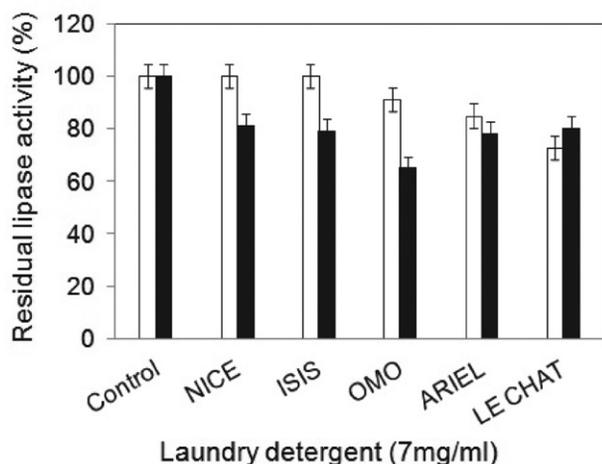
Figure 8. Residual activities of AKL29 (□) and CRL (▪) in the presence of various commercial laundry detergents: enzymes were incubated for 60 min at 40 °C in the presence of detergents at a final concentration of 7 mg/mL. The residual activities were determined at pH 8 and 45 °C using olive oil emulsion as a substrate. Enzymes activities determined without any detergent and incubated under the similar conditions, were taken as 100% (control). The experiments were conducted three times and standard errors are reported. Vertical bars indicate standard error of the mean.

3. 9. Wash Performance Test of Partially Purified AKL29

Partially purified lipase AKL29 was added in various laundry detergents to verify its effect on cleaning grease stains (Table 7). AKL29 showed high rates of olive oil removal compared with detergent alone which gives this novel lipase the advantage of its inclusion in some of laundry detergent formulation.

Table 7. Effect of partially purified AKL29 on removing sample stains from fat / fat (olive oil) sauce from cotton fabric with various commercial laundry detergents.

Laundry detergent	Oil removal (%)	
(7mg/mL)	Detergent	Detergent + AKL29
ISIS	40 ± 0.7	98 ± 1.4
ARIEL	28 ± 1.2	81 ± 1,3
LE CHAT	35 ± 1.9	79 ± 1.7
OMO	32 ± 0.6	70 ± 0.8
NICE	30 ± 1.3	62 ± 0.7

The washing performance of the partially purified AKL29 was evaluated using visual examination of the removal of fat/greasy material stains on cotton fabrics. The washing performance of AKL29 as a bio-additive in several brand laundry detergents was evaluated compared to the control (Figure 9).

4. Conclusion

This work describes the optimization process of a new lipase produced from a thermophilic Actinomycete *Actinomadura keratinilytica* strain Cpt29 isolated from poultry compost in north-east of Algeria. The optimization
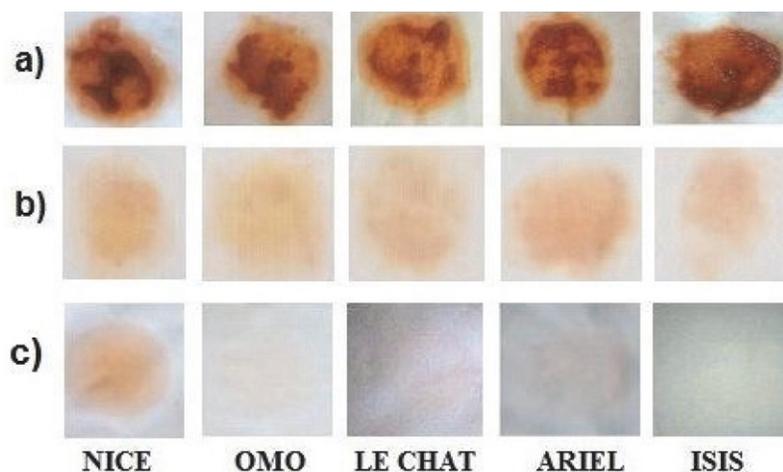
Figure 9. Cloth pieces stained with mixture of sauce sample and fat/greasy material, were washed with commercial laundry detergent (7 mg/mL) added with AKL29 (50 U/mL). Untreated cloth pieces taken as control (a), and treated cloth pieces by detergent alone (b), and treated cloth pieces by detergent with lipase (c).

study using artificial neural network (ANN) and response surface methodology (RSM) approaches, revealed significant improvement in lipase activity with optimal culture conditions. Maximum lipase production of 188 ± 3 U/ml was obtained in Tween 80 (3.27%, v/v), at 37.9 °C, and after 111 h of cultivation. An overall 4.1-fold increase in lipase production was recorded under optimized culture medium. Partial purification using acetone precipitation, and gel filtration on Sephacryl S-75, increased significantly the specific activity of AKL29. Finally, as commercial application, AKL29 exhibited good stability, high compatibility, and significant wash performance with various brand laundry detergents that make this novel lipase a suitable bio-additive for various detergents and a potential biocatalyst for subsequent industrial applications. According to these results, additional studies are underway by the laboratory team to better assess the lipase efficiency.

Acknowledgements

5. References

1. O. Ilesanmi, A. E. Adekunle, J. A. Omolaiye, E. M. Olorode, A. L. Ogunkanmi, *Scientific African*, **2020**, *8*, e00279. **DOI**:10.1016/j.sciaf.2020.e00279

2. D. Kirsh, *Bot Gaz*, **1935**, *97*, 321–333. **DOI**:10.1086/334555

3. F. Hasan, A. A. Shah, A. Hameed, *Enzym Microb Technol*, **2006**, *39*, 235–251. **DOI**:10.1016/j.enzmictec.2005.10.016

4. N. A. Al-Dhabi, A. K. M. Ghilan, G. A. Esmail, M. V. Arasu, V. Duraipandiyan, K. Ponmurugan, *J Infect Public Health*, **2019**, *12*, 549–556. **DOI**:10.1016/j.jiph.2019.01.065

5. N .A. Al-Dhabi, G. A. Esmail, A. K. M. Ghilan, M. V. Arasu, *Saudi J Biol Sci*, **2020**, *27*, 474–479. **DOI**:10.1016/j.sjbs.2019.11.011

6. R. Gupta, N. Gupta, P. Rathi, *Appl Microbiol Biot*, **2004**, *64*, 763–781. **DOI**:10.1007/s00253-004-1568-8

7. R. Sharma, Y. Chisti, U. Banerjee, *Biotechnol Adv*, **2001**, *19*, 627–662. **DOI**:10.1016/S0734-9750(01)00086-6

8. B. Andualema, A. Gessesse, *Biotechnol*, **2012**, *11*, 100–118. **DOI**:10.3923/biotech.2012.100.118

9. R. Malathu, S. Chowdhury, M. Mishra, S. Das, P. Moharana, J. Mitra, U.K. Mukhopadhyay, A. R. Thakur, S.R, *Am J Appl Sci*, **2008**, *5*, 1650–1661. **DOI**:10.3844/ajassp.2008.1650.1661

10. M. Basri, R. N. Zaliha Raja Abd Rahman, A. Ebrahimpour, A. B. Salleh, E. R. Gunawan, M.B. Abd Rahman, *BMC Biotechnol*, **2007**, *7*, 1472. **DOI**:10.1186/1472-6750-7-53

11. F. Benamia, S. Bouchagra, Y. Saihi, Z. Djeghaba, N. Rebbani, *Prep Biochem Biotechnol*, **2013**, *43*, 33–47. **DOI**:10.1007/s11696-016-0080-9

12. L. M. Colla, A. L. Primaz, S. Benedetti, R. A. Loss, M. De Lima, C.O. Reinehr, T.E. Bertolin, J.A.V. Costa, *Braz J Microbiol*, **2016**, *47*, 461–467. **DOI**:10.1016/j.bjm.2016.01.028

13. A. Habbeche, B. Saoudi, B. Jaouadi, S. Haberra, B. Kerouaz, M. Boudelaa, A. Badis, A. Ladjama, *J Biosci Bioeng*, **2014**, *117*, 413–421. **DOI**:10.1016/j.jbiosc.2013.09.006

14. M. Mandels, J. Weber, *Advances in Chemistry*, **1969**, *95*, 391–413. **DOI**:10.1021/ba-1969-0095.ch023

15. I. Belhaj-Ben Romdhane, A. Fendri, Y. Gargouri, A. Gargouri, H. Belghith, *Biochem Eng J*, **2010**, *53*, 112–120. **DOI**:10.1016/j.bej.2010.10.002

16. A. Tiss, F. Carriere, R. Verger, *Anal Biochem*, **2001**, *294*, 36–43. **DOI**:10.1006/abio.2001.5095

17. J. Rathelot, R. Julien, P. Canioni, C. Coeroli, L. Sarda, *Biochimie*, **1975**, *57*, 1117–1122. **DOI**:10.1016/S0300-9084(76)80572-X

18. M. M. Bradford, *Anal Biochem*, **1976**, *72*, 248–254. **DOI**:10.1016/0003-2697(76)90527-3

19. E. Fic, S. Kedracka-Krok, U. Jankowska, A. Pirog, M. Dziedzicka-Wasylewska, *Electrophoresis*, **2010**, *31*, 3573–3579. **DOI**:10.1002/elps.201000197

20. S. Akmoussi-Toumi, S. Khemili-Talbi, I. Ferioune, S. Kebbouche-Gana, *Int J Biol Macromol*, **2018**, *116*, 817–830. **DOI**:10.1016/j.ijbiomac.2018.05.087

21. A. Guvenc, N. Kapucu, H. Kapucu, O. Aydogan, U. Mehmetoglu, *Enzym Microb Technol*, **2007**, *40*, 778–785. **DOI**:10.1016/j.enzmictec.2006.06.010

22. J. C. Santos, H. F. De Castro, *World J Microbiol Biotechnol*, **2006**, *22*, 1007–1011. **DOI**:10.1007/s11274-005-2818-3

23. K. Selvam, B. Vishnupriya, *IJPSR*, **2013**, *4*, 4281–4289. **DOI**:10.13040/IJPSR.0975-8232.4(11)

24. M. M. Aly, S. Tork, S. M. Al-Garni, L. Nawar, *Afr J Microbiol Res*, **2012**, *6*, 1125-1137. **DOI**:10.5897/AJMR11.1123

25. J. B. C. Dos Santos, R. G. Da Silva Cruz, P. W. Tardioli, *Appl Biochem Biotechnol*, **2017**, *183*, 218–240. **DOI**:10.1007/s12010-017-2440-5

26. F. Ferrato, F. Carriere, L. Sarda, R. Verger, *Methods in Enzymol*, **1997**, *286*, 327–347. **DOI**:10.1016/S0076-6879(97)86018-1

Povzetek

V tem delu smo se osredotočili na učinkovito, ekonomično ter okolju prijazno pridobivanje lipaze (AKL29), potencial-no uporabne v pralni industriji, iz *Actinomadura keratinilytica* sev Cpt29, ki je bil izoliran iz perutninskega komposta severovzhodne Alžirije. AKL29 kaže visoko lipazno aktivnost (45 U/mL) pri hidrolizi triacilglicerolov, kar potrjuje, da gre za pravo lipazo. Da bi dosegli maksimalno proizvodnjo lipaze smo s pomočjo RSM in ANN modelov optimirali kultivacijske parametre kot so temperatura inkubacije, čas kultivacije in koncentracijo Tween 80 (v/v). Rezultati so po-kazali, da oba modela dajeta dobre kvalitativne napovedi, da pa kaže ANN model znatno boljše napovedi in ujemanje z eksperimentalnimi podatki. Pod optimalnimi pogoji (temperatura inkubacije 37.9 °C, čas kultivacije 111 h, koncentra-cija Tween 80 3.27 % v/v) smo dosegli 4.1-kratni porast proizvodnje lipaze. Delno očiščena lipaza kaže dobro stabilnost in visoko kompatibilnost ter zmožnost spiranja v kombinaciji s pralnimi detergent, zaradi česar je obetaven kandidat za uporabo v pralni industriji.