Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue4-fulltext-8 *RESEARCH ARTICLE*

Lipase production and growth modeling of a novel thermophilic bacterium: *Aneurinibacillus thermoaerophilus* strain AFNA

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Received November 27, 2010 / Accepted April 25, 2011
Published online: July 15, 2011

Abstract *Aneurinibacillus thermoaerophilus* strain AFNA as a novel isolated extracellular thermostable organic solvent tolerant lipase producing bacterium was employed in the present study. The lipase production of strain AFNA and its correlation with bacterial growth was studied via a modeling assessment by response surface methodology (RSM) and artificial neural network (ANN) techniques. The best achieved models were multilayer full feed forward incremental back propagation network and modified cubic response surface model (mRSM) using backward elimination. The highest lipase specific activity (13.1 Umg^{-1}) and bacterial growth (OD_{600} = 3.0) were obtained at technically similar: growth temperature (53 and 53ºC), inoculum size (2.6 and 3.0%), agitation rate (118 and 115 rpm) and initial pH (7.0 and 7.2) but different medium volume (139 and 87 ml) and incubation period (48 and 38 hrs), respectively. In addition, the importance of effective parameters on the bacterial growth and lipase production was studied where pH and inoculum size were the most and the least effective factors, respectively. Significant correlation between lipase production and bacterial growth was observed when Bivariate correlation was employed to analyse the data. As a conclusion, lipase production was the result of a synergistic combination of effective parameters interactions and these parameters were in equilibrium.

Keywords: artificial neural network, modified response surface methodology, thermostable, organic solvent tolerant

INTRODUCTION

Nowadays, thermostable organic solvents tolerant bacterial lipases (EC 3.1.1.3) gain wide industrial and biotechnological interest due to their novel, multifold applications and resistance to harsh industrially conditions. They are capable of catalyzing an abroad range of novel and important reactions in both aqueous and nonaqueous media. Thereby these lipases present an attractive field for future research and create interest to isolate and study of novel thermostable organic solvents tolerant lipase producing bacteria and the factors affecting their lipase production (Sharma et al. 2001; Svendsen, 2004; Hasan et al. 2006).

Predictive models have been accepted as informative tools for rapid and cost effective study of microbial growth, their products development, risk assessment and scientific purposes (Ross, 1999). Response surface methodology (RSM) is a mathematical modelling system, which assesses the relationships between the response(s) and the independent variables (Manohar and Divakar, 2004), and defines the importance of the independent variables, alone or in combination, in the model. Although RSM is the most frequently used techniques for modelling the biological processes, it is not applicable to all modelling studies (Baş and Boyaci, 2007b). Artificial neural networks (ANNs) occupy a place of prominence, in the past decade, among modeling systems in biological studies (Dutta et al. 2004). Indeed, an artificial neural network is an adaptive data processing paradigm that is inspired by

the way biological nervous systems process information. ANNs learn by examples via a parallel processing style, improve their performance and, therefore, produce reasonable response(s).

In the present investigation, the lipase production behaviour of a newly isolated thermophilic bacterium; *Aneurinibacillus thermoaerophilus* strain AFNA and its correlation with bacterial growth was studied via a modelling assessment by RSM and ANN techniques. In addition, the importance of effective parameters on the bacterial growth and lipase production was studied.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain used in this study was a novel thermostable, organic solvent tolerant lipase producer, isolated from cooking oil contaminated soil from Selangor, Malaysia. It was identified as *Aneurinibacillus thermoaerophilus* strain AFNA via 16S rDNA analysis and deposited in DSMZ, Germany (DSM 21497) and NCIMB, UK (NCIMB 41584). The 16S rDNA sequence of this bacteriumhas been assigned GenBank accession numberGenBank: EF032876. This strain was preserved in sterile 16% (v/v) glycerol in Tryptic Soy Broth (TSB) at -80ºC.

Inoculum preparation

The inoculum was prepared by transferring a loopfull of bacterial cells from a fresh pure culture into 10 ml TSB and incubated at 55ºC, overnight, under 150 rpm agitation. The cells were harvested by centrifugation at 12,000 x g and 4ºC for 10 min. The bacterial pellet was resuspended in sterile normal saline solution (8.5 g/l NaCl) to give an absorbance of 0.5 at 600 nm.

Lipase production medium

In order to select the best lipase production medium, eight different media were tested. The compositions of the media were (% w/v): M1: peptone (3), yeast extract (1), NaCl (0.5), olive oil (1% v/v) (Hun et al. 2003); A1 (modified M1): M1 + $CaCl_2.2H_2O$ (0.05) + gum arabic (1) (Ebrahimpour et al. 2008); A2 (modified M1): A1 + $MgSO_4.7H_2O$ (0.01), $FeCl_3.6H_2O$ (0.004) (Ebrahimpour et al. 2008); GYP: glucose (2), yeast extract (1), peptone (1), $CH_3COONa{\cdot}3H_2O$ (1), $MgSO_4{\cdot}7H_2O$ (0.03), $MnSO_4$ (0.01), KCl (0.05), olive oil (2% v/v) (Hun et al. 2003); M3: nutrient broth (0.325), gum arabic (1), $CaCl_2{\cdot}2H_2O$ (0.05), Tween 80 (1% v/v), olive oil (1% v/v) (Hun et al. 2003); M5: nutrient broth (0.8), triolein (1% v/v) (Hun et al. 2003); TYEM: tryptone (0.6), yeast extract (0.2), $CaCl_2.2H_2O$ (0.02), $MgSO_4.7H_2O$ (0.01), $FeCl_3.6H_2O$ (0.04), olive oil (1.5% v/v) (Lee et al. 1999); MTYEM (modified TYEM): TYEM + gum arabic (1) (Ebrahimpour et al. 2008).

The media were sterilized for 15 min at 121ºC after pH adjustment to 7.0. Bacterial inoculum (2%) was then inoculated into 50 ml production medium and incubated by agitation under 150 rpm, for 48 hrs at 60ºC. The cell free supernatant was obtained by centrifugation at 12,000 x g, 4ºC for 15 min prior to lipase assay.

Data collection; lipase activity, protein content and bacterial growth

Lipase activity was assayed according to Kwon and Rhee (1986) method using olive oil as substrate. The reaction mixture, consisting of 1 ml crude enzyme (culture filtrate), 2.5 ml olive oil emulsion (properly mixed of an equal volume olive oil with sodium phosphate buffer, 50 mM, pH 7.0), and 0.02 ml of 20 mM $CaCl_2$, was incubated in a water bath shaker for 30 min at 50ºC under 200 rpm agitation. The enzyme reaction in the emulsion system was stopped by adding HCl (1 ml, 6 M) and isooctane (5 ml), followed by mixing for 1 min. The upper isooctane layer (4 ml) containing the free fatty acid was transferred to a test tube and properly mixed with 1 ml copper reagent. The reagent was prepared by adjusting the solution of 5% (w/v) copper (II) acetate-1-hydrate to pH 6.1 with pyridine. The free fatty acid dissolved in isooctane was determined by measuring the absorbance of the upper layer at 715 nm after mixture settlement. Lipase activity was determined by measuring the amount of free fatty acid released based on the standard curve of oleic acid in isooctane. The within-run (n = 20) coefficient of

variation was < 8.0%. One unit of lipase activity was defined as 1.0 µmol of free fatty acid liberated min^{-1} and reported as Uml^{-1}.

Protein concentration was determined according to the Bradford method using the Bio-Rad assay reagent (catalogue number 500-0006) and bovine serum albumin as standard, according to the manufacturer's instructions. In order to determine the biomass, 10 ml sample of culture was filtered (cellulose acetate filter, pore size of 0.22 µm, Sartorius) and washed properly with acetone followed by distilled water. The filter was dried at 80ºC to a constant weight. Cell growth was monitored by turbidity measurement at 600 nm (Garcia-Gimeno et al. 2005). Each sample (5 ml) was centrifuged (12,000 x g and 4ºC for 10 min) and the precipitate was washed twice by resuspending in distilled water after removing the supernatant and repeating the centrifugation process. Optical density was measured at 600 nm after resuspending the precipitate.

Experimental design

Response surface method (RSM) offers a large amount of information from a small number of experiments because of using special designs those help the appropriate model be fitted to the response(s). We chose a central composite rotatable design (CCRD), as the most popular RSM design, for the experiments due to its obvious advantages of rotability and the ability to analyse the interaction effects. A central composite design (CCD) includes three groups of design points. Factorial points that consists of all possible combinations of the +1 and -1 levels of the factors; star or axial points that have all of the factors set to 0, the midpoint, except one factor, which has the value +/- Alpha; and center points, which are points with all levels set to coded level 0, the midpoint of each factor range. Typically, the center point of the design is repeated, often four or more times to get a good estimate of experimental error (pure error variance). This gives an adequate estimate of the variation of the response and provides the number of degrees of freedom needed for an adequate statistical test of the model. To summarize, central composite rotatable designs require 5 levels of each factor: -Alpha, -1, 0, 1, and +Alpha. Rotatable designs provide the desirable property of constant prediction variance at all points that are equidistant from the design center, thus improving the quality of the prediction (Montgomery, 2004; Baş and Boyaci, 2007a; Hill and Lewicki, 2007).

Although, CCRD is appropriate for calibration the full quadratic models, a complete description of the process behaviour might require a cubic or higher order model. Design-Expert allows creating the higher order models (up to the fifth order) by adding the appropriate terms. On the other hand, rarely all of the terms of the full models are needed in an application. Model reduction (modification) consists of eliminating those terms that are not desired (Montgomery, 2004; Baş and Boyaci, 2007a; Hill and Lewicki, 2007).

A five-levels-six-factors central composite rotary design (CCRD) was employed throughout the study, requiring 33 experiments (Cochran and Cox, 1992). The fractional factorial design consisted of 16 factorial points, 12 axial points and 5 center points. The following variables and levels were incorporated: growth temperature (45-65ºC); medium volume (50-200 ml); inoculum size (1-5%); agitation rate (0-200 rpm); incubation period (24-72 hrs) and initial pH (5-9) (Table 1). The experimental data [40 points include CCRD design (Table 1) and optimization data (Table 2)] were divided into three sets: training set, testing set and validating set. Each of different combinations was tested in triplicate.

RSM modelling

The CCRD design experimental data were analyzed using Design Expert version 6.06 (Stat Ease Inc. Minneapolis, USA) and then interpreted. The predicted values obtained from RSM models were compared with actual values for testing the models (Table 3) and predicted optimal conditions (Table 2) were used as validating sets.

ANN modelling

A commercial ANN software, NeuralPower version 2.5 (CPC-X Software) was used throughout the study. Multilayer normal feedforward and multilayer full feedforward neural networks were used to predict the responses. The networks were trained by different learning algorithms (incremental back propagation, IBP; batch back propagation, BBP; quickprob, QP; genetic algorithm, GA; and Levenberg-

Marquardt algorithm, LM). In order to determine the optimal network topology, only one hidden layer was used and the number of neurons in this layer and the transfer functions of hidden and output layers (sigmoid, hyperbolic tangent function, Gaussian, linear, threshold linear and bipolar linear) were iteratively determined by developing different networks. Each network was trained until the network root of mean square error (RMSE) was lower than 0.0001, and average correlation coefficient (R) and average determination coefficient (DC) were equal to 1. At the start of the training, weights were initialized with random values and adjusted through a training process in order to minimize network error.

Since the replicates at center point do not improve the prediction capability of the network (Baş and Boyaci, 2007a), the models were improved by using mean of center points. The experimental data (Table 1 and Table 3) were divided into two sets: Training set and testing set, and experimental values of predicted optimal conditions (Table 2) were used as validating set.

Verification of estimated data and correlation study

In order to test the estimation capabilities of the techniques, the predicted responses obtained from RSM and ANNs were compared with the actual responses. The coefficient of determination (R^2) and absolute average deviation (AAD) were determined for finding the best models. The AAD and R^2 were calculated by Equation 1 and Equation 2, respectively.

$$AAD = \left\{ \left[\sum_{i=1}^{p} \left(\left| y_{i,\,exp} - y_{i,\,cal} \right| / y_{i,\,exp} \right) \right] / p \right\} \times 100$$

[Equation 1]

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and p is the number of the experimental run.

$$R^2 = 1 - \frac{\sum_{i=1-n} \left(\text{model prediction}_i - \text{experimental value}_i \right)^2}{\sum_{i=1-n} \left(\text{average experimental value} - \text{experimental value}_i \right)^2}$$

[Equation 2]

where n is the number of experimental data.

R^2 is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model. Because R^2 alone is not a measure of the model's accuracy, it is necessary to use absolute average deviation (AAD) analysis, which is a direct method for describing the deviations. Evaluation of R^2 and AAD values together would be better to check the accuracy of the model. R^2 must be close to 1.0 and the AAD between the predicted and observed data must be as small as possible. The acceptable values of R^2 and AAD values mean that the model equation defines the true behavior of the system and it can be used for interpolation in the experimental domain (Baş and Boyaci, 2007a).

Bivariate correlation was used to test the correlation between lipase production and bacterial growth using SPSS software (Version 18).

Table 1. Five-levels-six-factors central composite rotary experimental design employed throughout the study consisted of 16 factorial points, 12 axial points and 5 center points along with responses (lipase specific activity and bacterial growth).

Growth Temperature (°C)	Medium volume (ml)	Inoculum size (%)	Agitation rate (rpm)	Incubation period (hrs)	Initial pH	Lipase specific activity (Umg^{-1}) (Mean ± SD)	Bacterial growth (OD_{600}) (Mean ± SD)
****60.0**	**162.5**	**4**	**150**	**36**	**6**	**5.11 ± 0.16**	**0.25 ± 0.03**
60	162.5	4	50	60	6	7.11 ± 0.31	1.10 ± 0.03
60	162.5	2	150	36	8	9.23 ± 0.23	0.56 ± 0.04
60	87.5	4	50	60	8	5.57 ± 0.33	0.50 ± 0.03
50	162.5	2	150	60	8	9.21 ± 0.23	0.77 ± 0.01
60	87.5	4	150	36	8	8.06 ± 0.28	0.39 ± 0.02
****50.0**	**162.5**	**4**	**50**	**36**	**6**	**7.28 ± 0.31**	**1.05 ± 0.03**
60	162.5	2	50	60	8	7.15 ± 0.31	2.00 ± 0.02
60	87.5	2	150	36	6	6.84 ± 0.36	0.27 ± 0.03
50	87.5	4	50	36	8	7.18 ± 0.30	1.55 ± 0.05
50	162.5	2	50	36	8	6.30 ± 0.36	1.90 ± 0.01
****60.0**	**87.5**	**2**	**50**	**60**	**6**	**5.65 ± 0.29**	**1.50 ± 0.03**
****50.0**	**87.5**	**2**	**150**	**60**	**6**	**7.36 ± 0.30**	**0.26 ± 0.02**
50	87.5	4	150	60	8	7.21 ± 0.33	0.40 ± 0.03
50	162.5	4	150	60	6	7.05 ± 0.30	0.30 ± 0.04
50	87.5	2	50	36	6	7.79 ± 0.40	0.75 ± 0.03
45	125	3	100	48	7	9.32 ± 0.21	1.42 ± 0.02
65	125	3	100	48	7	5.11 ± 0.50	0.62 ± 0.04
55	50	3	100	48	7	5.50 ± 0.33	2.88 ± 0.03
55	200	3	100	48	7	9.26 ± 0.27	2.68 ± 0.06
55	125	1	100	48	7	9.29 ± 0.27	1.91 ± 0.02
55	125	5	100	48	7	8.79 ± 0.25	1.86 ± 0.08
55	125	3	0	48	7	9.42 ± 0.28	0.77 ± 0.02
55	125	3	200	48	7	9.90 ± 0.29	1.10 ± 0.04
55	125	3	100	24	7	6.37 ± 0.31	1.88 ± 0.02
55	125	3	100	72	7	4.44 ± 0.33	0.60 ± 0.05
55	125	3	100	48	5	4.42 ± 0.33	0.79 ± 0.03
55	125	3	100	48	9	0.86 ± 0.41	1.60 ± 0.07
*55.0	125	3	100	48	7	12.75 ± 0.21	2.57 ± 0.06
*55.0	125	3	100	48	7	12.33 ± 0.17	2.61 ± 0.04
*55.0	125	3	100	48	7	11.99 ± 0.16	2.49 ± 0.05
*55.0	125	3	100	48	7	12.50 ± 0.17	2.75 ± 0.03
*55.0	125	3	100	48	7	12.27 ± 0.24	2.65 ± 0.02

ANN training set: normal numbers; ANN training set, center points: *****numbers; ANN testing set: ******bold numbers.

Table 2. The optimal conditions for lipase production and bacterial growth.

Growth temperature (°C)	Medium volume (ml)	Inoculum size (%)	Agitation rate (rpm)	Incubation period (hrs)	Initial pH	ANN predicted	Actual	RSM predicted
						Specific activity (Umg^{-1})	Specific activity (Umg^{-1}) (Mean ± SD)	Specific activity (Umg^{-1})
53	139	2.6	118	48	7.0	13.0	13.1 ± 0.21	12.9
53	133	2.9	102	48	6.9	12.8	12.9 ± 0.25	12.8
55	125	3.0	100	48	7.0	12.1	12.1 ± 0.19	12.4
51	146	2.5	57	45	7.0	11.8	11.9 ± 0.30	11.9
53	130	3.4	97	54	6.9	11.7	11.6 ± 0.17	11.8
						Growth (OD_{600})	Growth (OD_{600}) (Mean ± SD)	Growth (OD_{600})
53	87	3.0	115	38	7.2	3.00	3.00 ± 0.03	2.98
54	130	3.2	112	38	7.8	2.69	2.70 ± 0.02	2.79
51	94	3.3	142	46	6.0	2.36	2.41 ± 0.03	2.32
55	120	2.4	65	41	7.8	2.27	2.32 ± 0.03	2.33
58	123	3.8	95	50	7.0	2.08	2.10 ± 0.05	2.14

ANN R^2 = 0.98; ANN AAD (%) = 1.21; RSM R^2 = 0.97; RSM AAD (%) = 2.1.

Table 3. Actual and predicted lipase specific activity and bacterial growth by RSM an ANN models along with coefficient of determination, R^2, and absolute average determination, AAD.

Actual specific activity (Umg^{-1}) (Mean ± SD)	ANN predicted specific activity (Umg^{-1})	RSM predicted specific activity (Umg^{-1})	Actual bacterial growth (OD_{600}) (Mean ± SD)	ANN predicted bacterial growth (OD_{600})	RSM predicted bacterial growth (OD_{600})
7.11 ± 0.31	7.11	7.15	1.10 ± 0.03	1.10	1.08
9.23 ± 0.23	9.22	8.85	0.56 ± 0.04	0.56	0.65
5.57 ± 0.33	5.57	6.17	0.50 ± 0.03	0.50	0.55
9.21 ± 0.23	9.21	9.32	0.77 ± 0.01	0.77	0.80
8.06 ± 0.28	8.06	7.98	0.39 ± 0.02	0.39	0.42
7.15 ± 0.31	7.15	7.18	2.00 ± 0.02	2.00	1.94
6.84 ± 0.36	6.84	7.69	0.27 ± 0.03	0.27	0.21
7.18 ± 0.30	7.18	6.27	1.55 ± 0.05	1.55	1.49
6.30 ± 0.36	6.30	6.66	1.90 ± 0.01	1.90	1.95
7.21 ± 0.33	7.23	7.69	0.40 ± 0.03	0.40	0.49
7.05 ± 0.30	7.03	6.82	0.30 ± 0.04	0.30	0.24
7.79 ± 0.40	7.79	8.16	0.75 ± 0.03	0.75	0.73
9.32 ± 0.21	9.32	9.32	1.42 ± 0.02	1.42	1.39
5.11 ± 0.50	5.13	5.11	0.62 ± 0.04	0.62	0.59
5.50 ± 0.33	5.48	5.50	2.88 ± 0.03	2.88	2.77
9.26 ± 0.27	9.26	9.26	2.68 ± 0.06	2.67	2.57
9.29 ± 0.27	9.29	9.29	1.91 ± 0.02	1.91	1.88
8.79 ± 0.25	8.79	8.79	1.86 ± 0.08	1.86	1.83
9.42 ± 0.28	9.42	9.42	0.77 ± 0.02	0.77	0.74
9.90 ± 0.29	9.91	9.90	1.10 ± 0.04	1.10	1.07
6.37 ± 0.31	6.38	6.37	1.88 ± 0.02	1.88	1.85
4.44 ± 0.33	4.43	4.44	0.60 ± 0.05	0.60	0.57
4.42 ± 0.33	4.42	4.42	0.79 ± 0.03	0.79	0.81
0.86 ± 0.41	0.84	0.86	1.60 ± 0.07	1.60	1.51
12.36 ± 0.19	12.36	12.36	***2.61* ± 0.04**	***2.61***	***2.67***
5.11 ± 0.16	5.11	4.97	**0.25 ± 0.03**	**0.25**	**0.25**
7.28 ± 0.31	7.27	7.59	**1.05 ± 0.03**	**1.05**	**1.14**
5.65 ± 0.29	5.66	4.83	**1.50 ± 0.03**	**1.50**	**1.59**
7.36 ± 0.30	7.36	6.99	**0.26 ± 0.02**	**0.26**	**0.26**

ANN training set R^2 = 1; ANN training set AAD (%) = 0.2; RSM R^2 = 0.99; RSM AAD (%) = 3.11; ANN testing set R^2 = 1; ANN testing set AAD (%) = 0.081; ANN training set: normal and italic (center points) numbers; ANN testing set: bold numbers.

RESULTS AND DISCUSSION

Effect of media composition on lipase production

The production of lipases is mostly inducer-dependent (Lotti et al. 1998). Different media have different stimulation effects on lipase production (He and Tan, 2006) based on the physiological and biochemical pathways of the bacterium. In order to select the best lipase production medium, the ability of bacterium to produce lipase was tested in eight different liquid media (Figure 1) those were appropriate for the bacterium growth. These media could be categorized in four groups.

The first group included M1, A1 and A2 media. The media of this group were established based on the M1 basal medium, which was composed of peptone and yeast extract as organic nitrogen sources, olive oil as oil carbon source and Na^+ as metal ion. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract, which have been used for lipase production by various thermophilic *Bacillus* sp. (Ghanem et al. 2000; Sharma et al. 2002). Yeast extract is one of the most important nitrogen sources for high level lipase production by different microorganisms (Bora and Kalita, 2007). In addition, yeast extract supplies vitamins and trace

elements for the growth of bacteria and increases their lipase production (Gupta et al. 2007). A1 was a modified M1 by adding Ca^{2+} as second metal ion and gum arabic as emulsifier those increased the lipase production by 2.43 times compared to M1. A2 was a modified medium of A1 with additional metal ions, Mg^{2+} and Fe^{3+}. Although the lipase production in this medium was higher than the basal medium (M1) most probably due to existence of Ca^{2+} and gum arabic, additional metal ions mentioned seems to suppress the lipase production compared to A1. Different microorganisms have different requirements for metal ions. Calcium ions play essential roles for many microbial species. They are important in maintaining cell wall rigidity, stabilizing oligomeric proteins and covalently bounding protein peptidoglycan complexes in the outer membrane (Macció et al. 2002). Lipase production by various *Bacillus* sp. was stimulated in the presence of Ca^{2+} alone (Sharma et al. 2002; Alkan et al. 2007) or in combination with other ions such as Mg^{2+}, and Fe^{2+} (Janssen et al. 1994). The lipase production induction by this group was the highest among different groups, where A1 was the best lipase production medium.

The second group includes TYEM and its modified form, MTYEM. This group, similar to first group was composed of organic nitrogen sources, tryptone and yeast extract, and oil carbon source, olive oil. These media had Ca^{+2}, Mg^{+2} and Fe^{+3} as metal ions. MTYEM with extra emulsifier, gum arabic, showed 2.34 times higher lipase production stimulation compared to TYEM. It has been reported by different researchers that highly branched, helically configurated, non-metabolizable polysaccharides such as gum arabic are able to enhance the lipase production. This might probably be due to the emulsification of culture media containing oil to increase the lipid surface (interfacial area between oil and water) for lipase action, detachment of lipase from the oil surface, and from binding sites at the outer membrane of Gram-negative bacteria (Winkler and Stuckman, 1979; Lee et al. 1999).

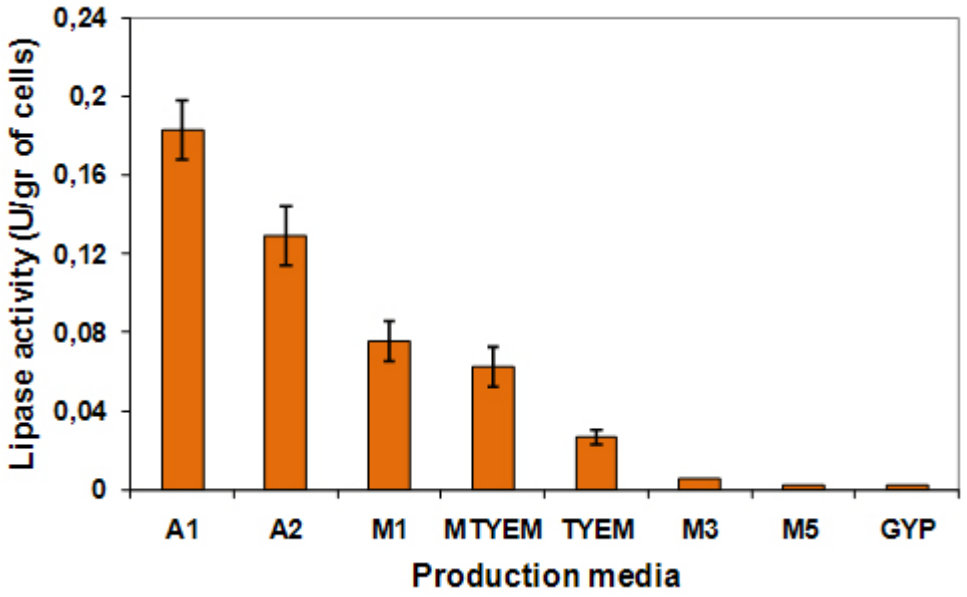

Fig. 1 Lipase production (U/gr of cells) in different composition of production media. Bacterial inoculum (2% v/v; OD_{600} = 0.5 of overnight culture in TSB) was individually inoculated into 50 ml of different production media and incubated by agitation under 150 rpm, for 48 hrs at 60ºC.

The bacterium in the last 3 tested media produced very low amount of the lipase. M3 and M5 media as the third group composed of peptone, meat extract and yeast extract as organic nitrogen sources, olive oil (triolein) as oil carbon source and Na^{+} as metal ion. In addition, M3 had Tween 80 as surfactant and extra carbon source, gum arabic as emulsifier and Ca^{+2} as additional metal ion. The roles of Ca^{+2} and gum arabic as lipase production stimulators, beside Tween 80, could be seen as previously was mentioned for A1, A2 and MTYEM. M3 showed 2.6 times higher lipase production compared to M5. The last medium, GYP, was made of peptone and yeast extract as organic nitrogen sources, olive oil as oil carbon source, sodium acetate and glucose as simple non oil carbon sources, and Na^{+}, K^{+}, Mg^{2+} and Mn^{2+} as metal ions. The lipase production in this medium almost was zero. Difference between this

medium and M1 as one of the effective media on the lipase production, in spite of some additional metal ions that could act as the lipase production suppressors, was sodium acetate and glucose as simple carbon sources those can be easily used by bacteria for growth. High levels of lipase production were reported from various thermophilic *Bacillus* sp. in the presence of olive oil as carbon source in the culture medium (Lee et al. 1999; Eltaweel et al. 2005; Bora and Kalita, 2007). Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production (He and Tan, 2006; Kaushik et al. 2006), while carbon sources that are easily broken down and used by bacteria play an inhibitory role (Lee et al. 1999). It seems when bacteria cannot easily find the suitable carbon sources, try to produce extracellular hydrolytic enzymes such as lipases, proteases and amylases and use of other sources. Then oils not only are important for higher lipase production via increase the number of lipase producers as carbon sources but also act as lipase production inducers.

As a result, A1 production medium was chosen as the medium to be used in the further study.

Table 4. The analysis of variance (ANOVA) used to evaluate the adequacy of the modified cubic response surface fitted model for bacterial growth.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	24.19	22	1.1	73.63	< 0.0001	**significant**
A	0.32	1	0.32	21.43	0.0009	
B	0.02	1	0.02	1.34	0.2741	
C	1.25E-03	1	1.25E-03	0.084	0.7782	
D	0.054	1	0.054	3.65	0.0853	
E	0.82	1	0.82	54.86	< 0.0001	
F	0.74	1	0.74	49.45	< 0.0001	
A2	5.34	1	5.34	357.56	< 0.0001	
C2	1.26	1	1.26	84.29	< 0.0001	
D2	5.89	1	5.89	394.62	< 0.0001	
E2	4.03	1	4.03	270.11	< 0.0001	
F2	4.29	1	4.29	287	< 0.0001	
AC	0.18	1	0.18	12.24	0.0057	
AD	0.57	1	0.57	38.16	0.0001	
AE	1.49	1	1.49	100.08	< 0.0001	
AF	0.23	1	0.23	15.59	0.0027	
BD	0.089	1	0.089	5.93	0.0352	
BF	0.11	1	0.11	7.19	0.023	
CD	0.13	1	0.13	8.56	0.0152	
CE	0.25	1	0.25	16.57	0.0022	
CF	0.2	1	0.2	13.49	0.0043	
DE	0.16	1	0.16	10.86	0.0081	
EF	0.15	1	0.15	10.32	0.0093	
Residual	0.15	10	0.015			
Lack of Fit	0.11	6	0.019	2.02	0.2593	**not significant**
Pure Error	0.037	4	9.28E-03			
Cor Total	24.34	32				

A: growth temperature; B: medium volume; C: inoculum size; D: agitation rate; E: incubation period; F: initial medium pH.

Predictive models

In this research we tried to analyse, model and interpret the experimental data using two completely different processing views; response surface methodology (RSM) as a mathematical modeling system, and artificial neural network (ANN) as an adaptative data processing method based on learning and interpretation.

RSM is a collection of statistical and mathematical techniques and shows several advantages over the ANNs (Manohar and Divakar, 2004). This method offers a large amount of information from a small number of experiments and provides mathematical model and equation for system studied. In addition, RSM has been furnished by ANOVA analysis, which can help to statistically analyse the whole model produced, every single parameters involved and their interactions. Although, RSM is based on the use of a second order equation as its major drawback, the biggest mistake is fitting the all-experimental data to the quadratic model. In fact, it is possible to predict a model equation with a higher degree than the second order and modified it by removing non significant terms. We employed the ability of Design Expert (Stat Ease Inc. Minneapolis, USA) mathematically packed program throughout the study to find the best modified fitted models for the experimental data analysis (Montgomery, 2004; Baş and Boyaci, 2007a).

On the other hand, in most cases an ANN is an adaptive system that changes its structure based on external or internal information that flows through the network during the learning phase. ANNs gather their knowledge by detecting the patterns and relationships in data and learn through experience, not from programming. An ANN is formed from hundreds of single units, artificial neurons or processing elements, connected with coefficients (weights), which constitute the neural structure and are organised in layers. The power of neural computations comes from connecting neurons in a network. The ability of ANNs to learn the process characteristics with little prior knowledge is desirable and eases their implementation and heightens their modelling potential. This property makes ANNs powerful and flexible tools that are well suited to model complex relationships between inputs and outputs or to find patterns in data via non-linear, distributed, parallel and local processing and adaptation (Agatonovic-Kustrin and Beresford, 2000; Baş and Boyaci, 2007b).

Table 5. The analysis of variance (ANOVA) used to evaluate the adequacy of the modified cubic response surface fitted model for lipase production.

Source	Sum of squares	DF	Mean square	F Value	Prob > F	
Model	230.69	23	10.03	100.21	< 0.0001	**significant**
A	8.86	1	8.86	88.48	< 0.0001	
B	7.07	1	7.07	70.62	< 0.0001	
C	0.12	1	0.12	1.21	0.2992	
D	0.11	1	0.11	1.13	0.3147	
E	1.86	1	1.86	18.56	0.002	
F	6.32	1	6.32	63.19	< 0.0001	
A2	37.93	1	37.93	378.93	< 0.0001	
B2	35.56	1	35.56	355.3	< 0.0001	
C2	15.83	1	15.83	158.19	< 0.0001	
D2	10.47	1	10.47	104.63	< 0.0001	
E2	69.28	1	69.28	692.17	< 0.0001	
F2	135.28	1	135.28	1351.52	< 0.0001	
AD	0.81	1	0.81	8.06	0.0194	
AE	0.36	1	0.36	3.58	0.091	
AF	1.48	1	1.48	14.77	0.0039	
BC	8.28	1	8.28	82.72	< 0.0001	
BE	2.8	1	2.8	27.98	0.0005	
BF	0.19	1	0.19	1.87	0.2044	
CD	1.87	1	1.87	18.68	0.0019	
CF	3.14	1	3.14	31.33	0.0003	
DE	3.1	1	3.1	30.92	0.0004	
DF	5.06	1	5.06	50.51	< 0.0001	
ADE	29.6	1	29.6	295.78	< 0.0001	
Residual	0.9	9	0.1			
Lack of fit	0.59	5	0.12	1.48	0.3623	**not significant**
Pure error	0.32	4	0.079			
Cor total	231.59	32				

A: growth temperature; B: medium volume; C: inoculum size; D: agitation rate; E: incubation period; F: initial medium pH.

Indeed ANN is a superior and more accurate modeling technique when compared to the RSM as it represents the nonlinearities in much better way (Dutta et al. 2004). On the other hand, neural networks also have the disadvantage of requiring large amounts of training data in comparison with RSM that offers a large amount of information from a small number of experiments. This advantage of RSM is because of its experimental design (Montgomery, 2004). To overcome this ANN problem, in present study, we used the RSM idea, and then a statistical experimental design, CCRD, was employed to reduce the number of experiments.

RSM models

The best fitting models were determined through multiple linear regressions with backward elimination. Finally, the modified cubic polynomial model with very small "model P-values" (< 0.0001) and large "lack of fit P-values" (0.2593 for growth and 0.3623 for specific activity) from the analysis of ANOVA (Table 4 and Table 5) and a suitable coefficient of determination (R^2 = 0.99) and adjusted coefficient of determination ($R^2_{adjusted}$ = 0.98), was highly significant to represent the actual relationships between the responses and the significant variables (Equation 3 and Equation 4).

Lipase specific activity (Umg^{-1}) = -18.9 + 0.6 T + 0.3 V + 4.3 IS - 2.5 Ag - 4.1 t + 25.8 pH - 0.05 T2 -8.9 E-4 V2 - 0.8 IS2 - 2.7 E-4 Ag2 - 0.01 t2 - 2.4 pH2 + 0.05 T.Ag + 0.1 T.t + 0.06 T.pH - 0.03 V.IS + 9.3 E-4 V.t + 5.0 E-3 V.pH - 6.8 E-3 IS.Ag + 0.8 IS.pH + 0.05 Ag.t + 0.01 Ag.pH - 9.9 E-4 T.Ag.t **[Equation 3]**

Bacterial growth (OD_{600}) = -76.77 + 1.74 T - 0.02 V + 4.08 IS + 0.12 Ag - 0.15 t + 7.29 pH - 0.02 T^2 - 0.2 IS^2 - 1.8 E-4 Ag^2 - 2.5 E-3 t^2 - 0.38 pH^2 - 0.02 T.IS - 1.3 E-3 T.Ag + 8.8 E-3 T.t - 0.02 T.pH - 4 E-5 V.Ag + 3.8 E-3 V.pH + 1.8 E-3 IS.Ag - 0.01 IS.t - 0.2 IS.pH - 2.9 E-4 Ag.t - 8.1 E-3 t.pH **[Equation 4]**

Where T, V, IS, Ag and t are symbols for growth temperature, medium volume, inoculum size, agitation rate and incubation time, respectively.

ANN models

The best produced ANN models in the present study were multilayer full feed forward incremental back propagation networks with Gaussian transfer function and one hidden layer consisted of 16 neurons. The optimized values of networks for learning rate and momentum were 0.15 and 0.8, respectively. In the case of training data set, the coefficient of determination (R^2) and absolute average deviation (AAD) were 1 and 0.20%, respectively, whereas for the testing data set, R^2 was 1.0 and AAD was 0.08% (Table 3) and for validating data sets R^2 and AAD were 0.98 and 1.21%, respectively (Table 2). Comparison of predicted and experimental values in training, testing and validating data sets, not only revealed capability of ANN in prediction of known data responses (the data that have been used for training) but also showed the ability of generalization for unknown data (the data that have not been used for training) and implying that empirical models derived from ANN can be used to adequately describe the relationship between the input factors and responses.

Main effects and interactions between parameters

The interaction of the parameters and optimal value of each variable is clearly represented in the three dimensional response surface plots. Figure 2a i and Figure 2a ii, represent the three dimensional plots as function of growth temperature and inoculum size on lipase specific activity and bacterial growth, respectively, when other parameters were kept at optimum point. Maximum lipase specific activity of 13.1 Umg^{-1} was obtained at the 53ºC and 2.6% inoculum size that was technically similar to the condition for maximum bacterium growth, 53ºC and 3.0% inoculum size. Further increase or decrease in these parameters led to the decrease in the responses. For extracellular enzymes, temperature influences their secretion, possibly by changing the physical properties of the cell membrane (Rahman et al. 2005). On the other hand, though higher temperature causes higher reaction rates and higher solubility of substrate and products, yet according to Henry's law (Atkins and De Paula, 2001) oxygen solubility is decreased.

Suitable inoculum size provides sufficient nutrient and oxygen levels for enough growth of bacteria and therefore, enhance the lipase production. If the inoculum size is too small, insufficient number of

bacteria will lead to reduced amount of secreted lipase. High inoculum size can result in the lack of oxygen and nutrient depletion in the culture media (Rahman et al. 2005; Shafee et al. 2005).

Figures 2b i and ii, depict the interaction between medium volume and agitation rate. The maximum lipase specific activity and bacterial growth were obtained at different culture volume of 139 and 87 ml, respectively but technically similar agitation rate (118 and 115 rpm). Generally, suitable agitation lead to sufficient supply of dissolved oxygen in the culture medium (Kumar and Takagi, 1999). Agitation also promotes a reduction in nutrient particle size, favouring the nutrient homogenization in the culture medium, providing additionally a rise in mass transfer rates and nutrient uptake by bacteria, which favouring microbial growth (Beg et al. 2003).

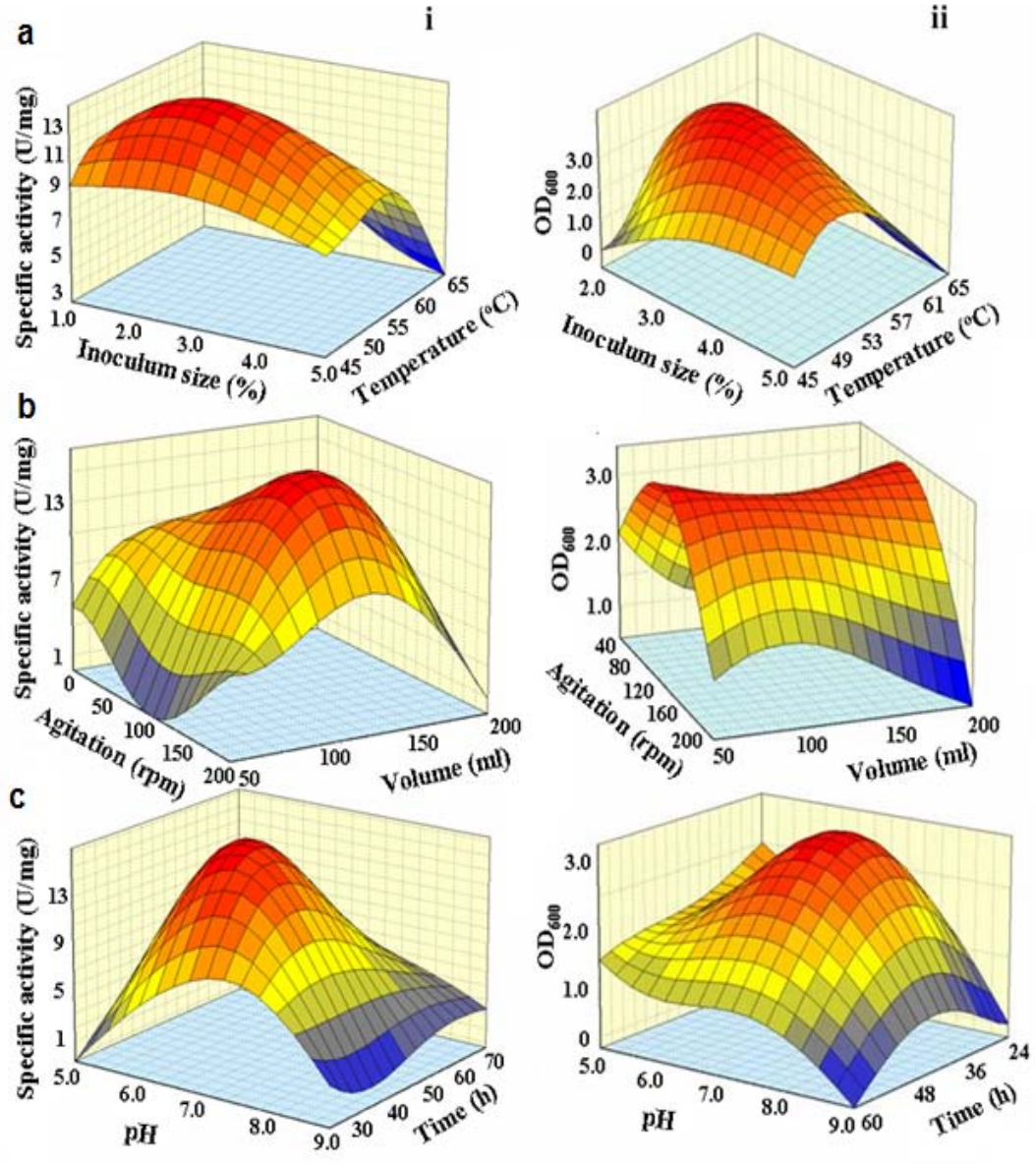

Fig. 2 Three dimensional plots showing the effect of: (a) growth temperature and inoculum size; **(b)** medium volume and agitation rate and **(c)** growth medium initial pH and incubation time, on lipase production **(i)** and bacterial growth **(ii)**, respectively.

Though medium volume may have a great effect on the enzyme production, yet Figure 2b i reveals that medium volume had no significant effect on bacterial growth at optimum condition within the chosen range (50-200 ml). Although a larger medium volume initially contains more oxygen, nutrients and space for growth of bacteria, the void in the container and subsequently oxygenation of the medium will be decreased. On the other hand, it seems that ratio of surface area to volume (A/V) is important for lipase production where higher ratio cause higher oxygenation and lipase production (Woolley and Petersen, 1994).

The combined effect of growth medium initial pH and incubation time on lipase production and bacterial growth is shown in Figure 2c i and Figure 2c ii, respectively. According to the plot, a neutral initial pH (7.0 and 7.2) caused maximum lipase production and bacterial growth. Maximum bacterial growth was achieved after 38 hrs incubation period, while the lipase production needed longer incubation time (48 hrs).

Lipases are produced throughout bacterial growth, with peak production being obtained by late exponential growth phase (Gupta et al. 2004). Figure 2c i reveals that after 30 hrs incubation (near to end of the bacterial growth phase), lipase production was stimulated and drastically increased to reach a maximum amount at 48 hrs following by decrease of lipase specific activity due to reduction of active cells, and enzymes most probably because of proteolysis by present proteases as well as physical damages.

To obtain the maximum lipase production, achievement of maximum possible number of bacteria (lipase producers) and enough lipase production stimulation is necessary. Significant correlation between lipase production and bacterial growth was observed when Bivariate correlation was used to analyse the data. Pearson correlation coefficient of 0.566 was statistically significant at the 0.01 level (2-tailed). It means that there was 32% correlation between lipase production and bacterial growth.

On the other hand, Figure 3 shows the importance percentage of effective parameters on the bacterial growth and lipase production based on the ratio of optimized ANN weights. An ANN is formed from hundreds of artificial neurons, connected with coefficients (weights). The weights are the adjustable parameters and, in that sense, a neural network is a parameterized system. The weighed sum of the inputs constitutes the activation of the neuron. The activation signal is passed through transfer function to produce a single output of the neuron. During training, the weights are optimized until the error in predictions is minimized and the network reaches the specified level of accuracy. Once the network is trained and tested it can be given new input information to predict the output. In this stage, the ratio of optimized weights shows the incorporation percentage of each input parameter in final output that can be computed and presented as an importance value. pH with 18.2% and 21% of importance on bacterial growth and lipase production, respectively was the most effective factor, while inoculum size was the least effective factor with 13.5% and 13.7%, respectively. Incubation period (18.5%), Growth temperature (16.8%), agitation rate (15.2%) and medium volume (15%) were subsequent degrees of importance for bacterial growth. This sequence was different for lipase production as follows: Growth temperature (17.7%), medium volume (17.4%), incubation period (16.9%) and agitation rate (16%).

Optimization

The optimal conditions for lipase production and bacterial growth are presented in Table 2. Among the various optimum conditions, highest lipase specific activity (13.1 Umg^{-1}) and bacterial growth (OD_{600} = 3.0) were obtained at technically similar: growth temperature (53 and 53ºC), inoculum size (2.6 and 3.0%), agitation rate (118 and 115 rpm) and initial pH (7.0 and 7.2) but different medium volume (139 and 87 ml) and incubation period (48 and 38 hrs), respectively. Since medium volume was not an effective factor on bacterial growth (Figure 1b ii), duration of incubation, when other parameters were kept at optimum values, was critical to achieve firstly a maximum bacterial growth, then maximum lipase induction.

In the current study, ANN and modified RSM were carried out and compared to study the bacterial growth and lipase production of a newly isolated thermophilic lipolytic bacterium, *Aneurinibacillus thermoaerophilus* strain AFNA. The artificial neural network (ANN) method was improved by using the statistical experimental design to overcome the ANN disadvantage of requiring large amounts of training data and reduce the number of experiments. On the other hand, although as an adaptative system, ANN represents the nonlinearities in much better way than other modelling methods and,

therefore it is capable to analyse more complex nonlinear data sets, the improved RSM by using equation modification (modified RSM) was comparable to ANN to provide high quality modelling for bacterial growth and lipase production. Finally, although each of the methods could be separately used to study the system behaviour, a combination of their information provided a powerful and flexible tool for modelling, interpretation and optimization of bacterial growth and enzyme production.

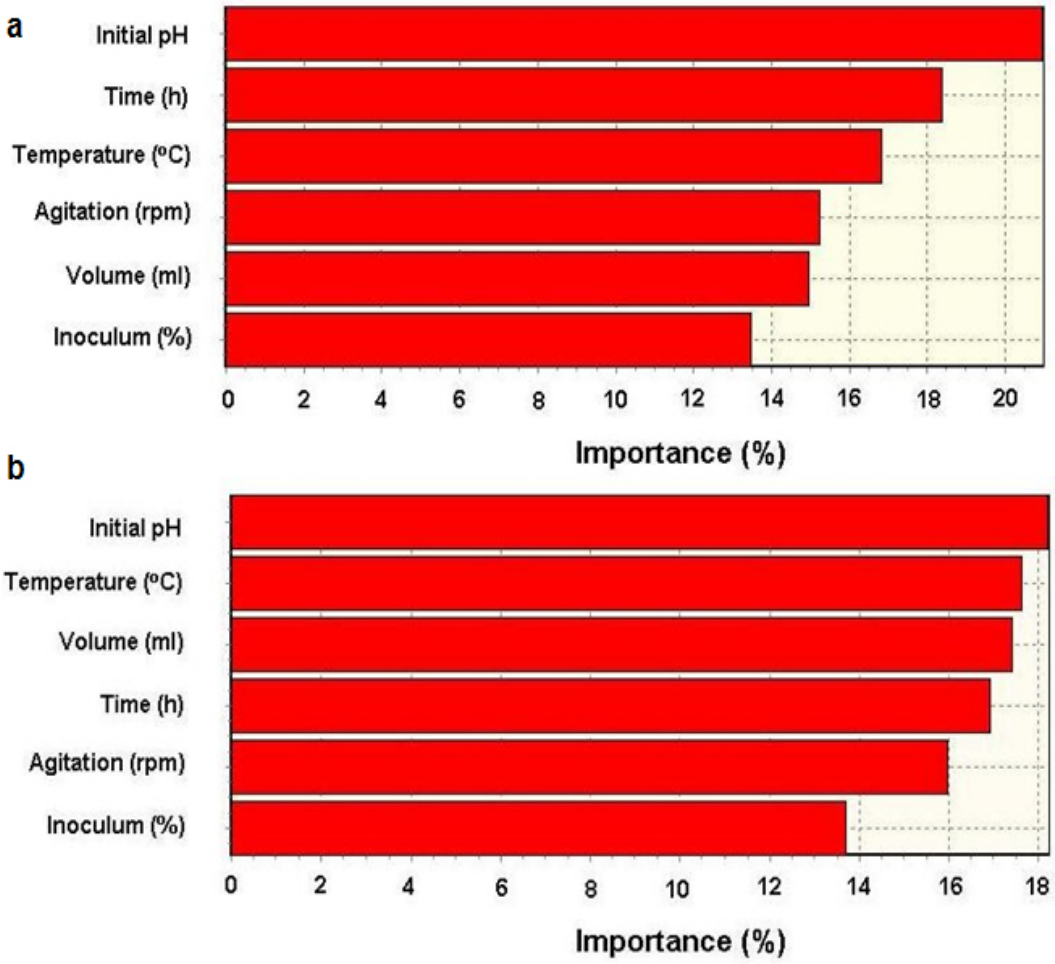

Fig. 3 Importance of effective parameters on (a) bacterial growth and (b) lipase production.

On the other hand, this study was a good example to support the following inference. Lipase production is the result of a synergistic combination of effective parameters interactions and these parameters are in equilibrium. Achievement of maximum possible number of bacteria as well as enough lipase production stimulation is necessary to obtain the maximum lipase production. In addition, some nutritional factors can act as "inducer" for production of extracellular hydrolytic enzymes. In the case of lipases, most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production, while carbon sources that are easily broken down and used by bacteria play an inhibitory role.

Financial support: The financial support by Universiti Putra Malaysia is gratefully acknowledged.

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How to reference this article:

EBRAHIMPOUR, A.; RAHMAN, R.N.Z.R.A.; KAMARUDIN, N.H.A.; BASRI, M. and SALLEH, A.B. (2011). Lipase production and growth modeling of a novel thermophilic bacterium: *Aneurinibacillus thermoaerophilus* strain AFNA. *Electronic Journal of Biotechnology*, vol. 14, no. 4. http://dx.doi.org/10.2225/vol14-issue4-fulltext-8