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Enhancement of BLIS production by *Pediococcus acidilactici* kp10 in optimized fermentation conditions using artificial neural network

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Running title: BLIS Production by *Pediococcus acidilactici* kp10

Abstract

The present work was aimed at enhancing the production of BLIS produced by *Pediococcus acidilactici* Kp10 through optimization of fermentation parameters. M17 was chosen in preliminary study as a culture medium since the production of BLIS was nine times higher (1,427.7 AU/mL) compared to that produced by MRS (160 AU/mL). The fermentation parameters such as temperature, inoculum size, buffer strength, concentration of tween 80 and agitation speed were screened using 2 level half-factorial design. BLIS production influenced by three most significant factors which identified as temperature, inoculum size and agitation speed were further optimized using artificial neural network (ANN). ANN predicted maximum activity of 5,262.64 AU/mL was obtained at optimum conditions of 120 rpm, 3% and 28.5 °C. The observed BLIS activity at the predicted optimum levels of the tested variables in ANN was 5,118.5 AU/mL which was close to the predicted BLIS activity. Increased BLIS activity in the final solution resulted from the optimized process would reduce the downstream step such as concentrating purified product during purification.

Keywords: Bacteriocin-like inhibitory substance, optimization, artificial neural network, natural preservative, antibacterial spectrum, biological origin

Introduction

Current consumer demands for commercial natural preservative processed foods has created considerable interest in the search for safe and food-grade preservatives of biological origin. Antibacterial peptides or bacteriocins produced by many strains of lactic acid bacteria (LAB) have been used as food preservatives for many years without any known adverse effects. Bacteriocins, especially those with broad antibacterial spectrum are bactericidal against food spoilage and many pathogenic bacteria.

Specific requirements with reference to the production of bacteriocins have been reported [1-3]. Bacteriocin titres can be modified by altering the cultivation conditions of the producing bacterium and certain combinations of influencing factors need to be optimized to enhance bacteriocin production [4]. With regards to the complexity of the factors within the food environments itself an in-depth knowledge of the interactions of these factors influencing the production of bacteriocin need to be understood for subsequent application in the optimization process. Most studies carried out to date claimed validation by statistical analysis and the combination of variables, their values and limits were arbitrarily chosen based primarily on personal experience [5].

Conventional methods in fermentation optimization require treating each factor separately which are laborious, incomplete and time consuming. If several factors are to be considered simultaneously their interactions are not discernible even for the dominant ones. These conventional approaches did not yield reliable results either. In this respect experimental factorial design have been successfully applied for

optimization of various biomanufacturing processes [6], which could also be used to investigate the interacting factors [7, 8].

Artificial neural network (ANN) has recently emerged as one of the most efficient methods for empirical modelling and prediction in solving complex systems such as bacteriocin production [7, 9]. Several studies have demonstrated that the accuracy of prediction of ANN models were far more superior compared to RSM using the same experimental design [10, 11]. ANN does not require prior specification of suitable fitting function. ANN has universal approximation capability which means it can approximate almost all kinds of non-linear functions including quadratic functions [12]. The ability of ANN to predict the process characteristics with little prior knowledge is desirable which simplify their implementation and increases their modeling potential. This property makes ANN a powerful and flexible tool that is well-suited to modeling biochemical processes [13].

Reports on multiple factors affecting the production of bacteriocins are relatively scarce and the optimization of bioprocess parameters for enhanced production of bacteriocin remain elusive [14]. Improvement of bacteriocin production would therefore require a comprehensive understanding of the factors affecting their production [15]. Thus the objective of this study was to optimize the fermentation parameters for enhancement of production of BLIS by *P. acidilactici* Kp10. This strain has demonstrated a high antimicrobial activity against *Listeria monocytogenes*, a virulent food-borne pathogen [16]. The effects of fermentation parameters such as temperature, inoculum size, buffer strength, tween 80 concentration and agitation speed on BLIS production were evaluated using the half factorial design. The most

significant factors were then optimized using the ANN to predict the enhanced bacteriocin production by *P. acidilactici* Kp10.

Materials and methods

Bacterial strain, medium selection and inoculum preparation

P. acidilactici Kp10, isolated from dried curd was used in this study [16]. Two types of medium, MRS and M17 were initially tested in this study. MRS broth (Merck, Darmstadt, Germany) consisted of peptone (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), glucose (20 g/L), di-potassium hydrogen phosphate (2 g/L), Tween 80 (1 g/L), di-ammonium hydrogen citrate (2 g/L), sodium acetate (5 g/L), magnesium sulphate (0.2 g/L) and manganese sulphate (0.04 g/L). M17 broth (Merck, Darmstadt, Germany) consisted of peptone (10 g/L), yeast extract (2.5 g/L), meat extract (5 g/L), lactose (5 g/L), ascorbic acid (0.5 g/L), sodium β-glycerophosphate (19 g/L) and magnesium sulphate (0.25 g/L). Three different temperatures (30 ^{o}C, 35 ^{o}C and 37 ^{o}C) were employed to select the preferred complex medium for BLIS production by *P. acidilactici* Kp10.

Primary cultivation was prepared by taking a single bacterial colony from the agar plate and grown in 10 mL tubes each containing M17 broth and incubated at 37 ^{o}C for 24 h. This initial culture (1 % v/v) was then inoculated into a 50 mL Falcon ^{TM} tube containing 10 mL of M17 medium and incubated at 37 ^{o}C for 24 h. This culture was used as a standard inoculum throughout the study.

Determination of antimicrobial spectrum

Antimicrobial activity of the isolates was determined by the agar well diffusion method [17] using cell-free culture supernatants (CFCS). The isolates were grown in

M17 broth at 30 °C for 24 h and the cultures were centrifuged at 12,000 ×*g* for 20 min at 4°C (rotor model 1189, Universal 22R centrifuge, Hettich AG, Switzerland). Aliquots of supernatant in two-fold dilution (100 µL) were placed in wells (6 mm diameter) of cooled soft agar plates (25 mL) previously seeded (1% v/v) with the actively growing test strain (*Listeria monocytogenes* ATCC 15313). The plates were incubated at 37 °C for 24 h for the growth of *L. monocytogenes* as the target microorganism. After 24 h, the growth inhibition zones were measured, and antimicrobial activity (AU/mL) was determined as described by Parente, Brienza, Moles, & Ricciardi, [18]. It is based on results from 2-fold dilutions of CFCS, which are either as a CDA (with the critical dilution being the last dilution which produced a zone larger than well diameter) or as a quantitative assay. A standard curve is prepared using a series of 2-fold dilutions of BLIS solution. The response (*R*) is calculated either as the diameter or as the area of the inhibition zone corrected for the diameter or area of the well. The dose (*d*) is the amount of BLIS pipetted into each well (100 µL l/D). The dose/response curve is calculated according to equation 1:

$$R = a + b \log (d) \quad (1)$$

The critical dose (*CD*) is defined as the amount of BLIS solution (in mL) corresponding to a null inhibition zone and calculated by extrapolating the dose/response curve. The titer, in AU/mL, is calculated as the reciprocal of the CD. The standard curve, relating dose in AU to R, had a 0 intercept and the same slope of Equation 1. The activity in AU/mL of a sample can be calculated using equation 2.

$$\text{AU/mL} = (1000/100)\ D\ 10^{(R/b)} \quad (2)$$

Experimental design

Two types of media (MRS and M17), normally used for LAB cultivation were initially tested for the growth of *P. acidilactici* Kp10 and BLIS production. The two-level half factorial design was initially used to screen important factors that affect BLIS production by *Pediococcus acidilactici* Kp10. The five factors considered were temperature (20 °C and 37 °C), inoculum size (1% and 10%), buffer strength (sodium β-glycerophosphate concentration, 60 μM and 180 μM), Tween 80 concentration (0.16 g/L and 1.6 g/L) and agitation speed (0 rpm and 200 rpm). Each factor was tested with an equal number of repetitions at high and low levels. A total of 16 experiments were run in duplicates (Table 1) .

For further optimization of the culture conditions, a total of 34 experimental runs were designed according to Box-Wilson (BW) 2^3 full factorial central composite design (CCD) with three most significant factors (temperature, inoculum size and agitation speed) selected from the initial screening results (Table 2). In the application of ANN, the experimental data obtained from the two-level half factorial design were analyzed using Intelligent Problem Solver (STATISTICA software version 7) to construct the regression based networks from the data. A total of 175 different trained networks were observed for selection on the basis of the highest coefficient of correlation determination (R^2) and the lowest selection error. A Multilayer Perceptron Network (MPN) was selected from the above. Back Propagation (BP) and Conjugate Gradient Descent (CGD) logarithms were used in the training of neural network on the basis of varying input/output pair data sets. The eight experiments were carried out for the selection group, 18 for the training group and 8 for the testing group (Table 3). The network developed in this study comprised of three layers - an input

182 layer with three neurons, one hidden layer with nine neurons and an output layer with
183 one neuron. Determination of the optimum topology was based on minimum error of
184 testing. The ideal network was used for the prediction and optimization of BLIS
185 production.
186
187 **Optimization capability of ANN**

188 The analysis of variance (ANOVA) was employed to determine the
189 significance of the model parameters. Adjusted R^2, absolute average deviation (AAD)
190 and root mean square error (RMSE) were calculated in addition to the R^2 for
191 prediction of bacteriocin production by the ANN. R^2 was calculated using equation
192 (3):

193
$$R^2 = \frac{\sum_{i=1-n}\left(X_i - y_{i,exp}\right)^2}{\sum_{i=1-n}\left(\bar{y}_i - y_{i,exp}\right)^2}$$

194 (Equation 3)
195
196 Where;
197 X_i: BLIS production
198 $y_{i,exp}$: experimental BLIS production and
199 $\bar{y}_i$: average observed BLIS production

200 The adjusted R^2 was calculated using equation (4):

201
$$Adjusted\ R^2 = 1 - [(1 - R^2) \times \frac{N-1}{N-K-1}]$$

202

(Equation 4)

Where;

N: total number of observations and

K: number of input variables

AAD was calculated using equation (5).

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

(Equation 5)

Where;

$y_{i,exp}$ and $y_{i,cal}$: experimental and calculated responses and

p: number of experiments

RMSE was calculated using equation (6).

$$RMSE = \sqrt{\frac{\sum(y_{i,exp} - y_{i,cal})^2}{n}}$$

(Equation 6)

Where;

y_i, exp: experimental response

$y_{i,\,cal}$ is the calculated response and p is the number of experiments

Analytical procedures

BLIS production by *P. acidilactici* Kp10 was tested by the agar well diffusion method [19] using CFCS. CFCS was prepared by growing *P. acidilactici* Kp10 in M17 broth at

30 °C for 24 h. Cells produced were subsequently separated by centrifugation at 12,000 × *g* for 20 min at 4 °C. Each aliquot of supernatant in two-fold dilution (100 µL) were placed in wells (6 mm diameter) of cooled soft agar plates (25 mL) previously seeded (1% v/v) with the actively growing *L. monocytogenes* ATCC 15313. The plates were then incubated at 37 °C for 24h. After that, the diameters of the growth inhibition zones were measured and the antimicrobial activity, defined as the mean reciprocal of the highest dilution showing inhibition of the indicator lawn, was expressed in Activity Units (AU) per mL (AU/ mL). Cell growth was measured by optical density of the culture at 650_{nm} using UV/VIS spectrophotometer (Perkin Elmer, Lambda 25, USA).

Result and discussion

LAB are fastidious microorganisms with respect to their nutrient requirement which require a rich medium containing yeast extract and protein hydrolysates for good growth and bacteriocin production [16, 20]. Therefore, a suitable medium is essentially for a good growth and bacteriocin production. For all temperatures tested, BLIS production by *P. acidilactici* Kp10 in M17 medium was substantially higher compared to that produced in MRS medium (Table 4). For both media, the highest BLIS production was obtained at 30 °C and a drastic reduction of BLIS production was observed at 37 °C. BLIS production using M17 medium was nine times higher (1,427.7 AU/mL) than that obtained using MRS medium (160 AU/mL). Hence, M17 medium was selected as a basal medium for the subsequent experiments.

Results from the screening of culture conditions using two-level half factorial design showed that the highest BLIS production (run 9) was at 20 °C with 10 g/L of

tween 80 while the lowest production (run 8) was obtained at 37 ^{o}C with 0.16 g/L of tween 80 (Table 1). Other parameters - inoculum size (10%) buffer strength (60 μM) and agitation speed (200 rpm) remained the same for the highest and the lowest production of BLIS. The culture pH which ranged between 6 (experimental run 6) and 7.01 (experimental run 11) was well correlated with respect to the buffer strength. Analysis of variance (ANOVA) showed that temperature, agitation speed and inoculum size were the most significant factors influencing BLIS production by *P. acidilactici* Kp10 (Table 5). On the other hand, buffer strength and tween 80 showed no significant effects on BLIS production.

The presence of Tween 80 (polyoxyethylene sorbitan mono-oleate) enhanced the production of some bacteriocins by preventing aggregation of bacteriocins molecules (Nissen-Meyer *et al*., 1992; Parente and Hill, 1992; Moretro *et al*., 2000). This could be brought about by the formation of micelles in the medium which stabilized the production of bacteriocin [21] and facilitated the discharge of bacteriocin from the cell surface [22]. However, the effect of Tween 80 is greatly dependent on the type of bacteriocin and the producing strain. As observed in the present study, Tween 80 used in the half factorial design did not affect BLIS production which was similarly reported for sakacin A [23], pediocin A [24], lactocin S [25], plantaricin 423 [26] and pediocin AcH [27, 28].

Although the buffering capacity of the system due to sodium β-glycerophosphate influenced the growth of bacterium, its role to exclude the effect of organic acids was not significant from the result of this study. The possible explanation on this could be the BLIS from *P. acidilactici* Kp10 was stable at acidic

pH [16] and reduced BLIS activity during the fermentation could not be explained by the reduction in the culture pH. The enzymatic reactions are controlled by pH and the decrease in culture pH results to a decrease in enzymatic reactions with concomitant reduction in growth rate.

Among the three significant factors analyzed, temperature appeared to exhibit the most pronounced effect compared to agitation speed and inoculum size. Temperature plays an important role in bacteriocin production [28] where elevated temperature may completely suppress bacteriocin synthesis and sometimes leads to an irreversible loss of its property. The influence of temperature on bacteriocin production was strain dependent. Most of the strains isolated to date, require a temperature ranging from 30 °C to 37 °C for bacteriocin production [29]. At high temperatures (44 ^{o}C), microorganisms were unable to synthesize or secrete bacteriocin and degradation or inactivation could not be increased [30]. Conversely, high cell yield and high bacteriocin activities at low temperatures could be due to different rate-limiting reactions which are temperature dependent. This resulted in better utilization of carbon and/or energy at low growth rates and increased availability of essential metabolites (ATP included) for bacteriocin synthesis [31].

Multilayer perceptron networks (MPN) with back-propagation and conjugate gradient descent (CGD) logarithms were used in the training of neural network on the basis of varying input/output pair data sets. The topology of the network consisted of three layers (3:9:1)- an input layer consisting of three variables, middle hidden layer of 9 neurons and one output layer was used for the prediction and optimization of BLIS production. The neurons activation for ANN processing is represented by

different colors (Figure 1). The optimum levels predicted from response graphs, made by ANN for temperature, agitation and inoculum size were 28.5 ^{o}C, 120 rpm and 3% respectively. The highest effect was from the temperature, followed by agitation as calculated by the sensitivity analysis (Table 6). From the sensitivity analysis conducted in this study, temperature (ratio = 136.8833) gave the highest effect on BLIS production by *P. acidilactici* Kp10. The ratio parameter described the behavior of the neural network when a variable was removed from the input data. The interaction effects were represented by surface plots (Figure 2). The surface plot between temperature and inoculum size obtained from ANN is as shown in Figure 2a. However, the surface plot between temperature and agitation (Figure 2b) is portrayed as a combination of maximum hill and stationary ridge while the surface plot between inoculum size and agitation (Figure 2c) is depicted as a combination of saddle and stationary ridge.

Before optimization, maximum BLIS activity obtained experimentally was 888.56 AU/mL. Following optimization BLIS activity increased to 5,118.49 AU/mL. The response surface curves were plotted to understand the interaction of the variables and to determine the optimum level of each variable for maximum response [32]. The response surface and contours of ANN models for BLIS production are shown in Figure 2 (a, b, c). From the data obtained, it was observed that the lower and higher levels of all variables did not result in higher BLIS production. The shape of the response surface curves showed a moderate interaction between the variables. Comparison of BLIS production by *P. acidilactici* Kp10 before and after optimization and the verification experiments including ANN-predicted optimum levels for the tested variables are presented in Table 7. ANN predicted activity of 5,262.64 AU/mL

was obtained at 120 rpm, 3% and 28.5 ℃. Experiments in triplicate were conducted on these optimum levels to calculate the observed response. The observed BLIS activity at the predicted optimum levels of the tested variables at ANN predicted levels was 5,118.5. The BLIS activity predicted by ANN was close to the observed BLIS activity at all experimental levels of three variables. ANN results showed a high values of R^2 (1) and adjusted R^2 (1) and low values of AAD (0.49) and RMSE (0.02).

Analysis of experimental data using ANOVA provided the statistical relationships of the output. The probability decreases as the value of the *F* value increases. If this probability is less than 0.05 the terms are significant and their inclusion improves the model [33]. The present study showed that the effect of temperature (*F* value: 46.419) was more pronounced than the effect of agitation (*F* value: 6.419) where both have the probability of less than 0.05. *P*-values were used as a tool to check significance of each variable which also showed the interaction strength between each independent variable [34]. The smaller the *P*-values, the higher is the significance of the corresponding variable [33]. Larger values of RMSE and AAD mean higher chances of errors in prediction. In this study, lower RMSE and ADD values obtained from ANN were more reliable and accurate. Experimental results from ANN were in good agreement with the predicted values indicating that actual and predicted values confirmed each other and that the models were reasonable and of high accuracy in predicting the values of the dependent variables.

Conclusions

ANN provided a confident estimation capability through the range of variables in the optimization of BLIS production by *P. acidilactici* Kp10. The ability of ANN to

predict the process characteristics with little prior knowledge is desirable which simplify its implementation and increase its modeling potential. This property makes ANN a powerful and flexible tool well-suited to modeling of complex bioprocesess. As observed from the present study, BLIS production by *P. acidilactici* Kp10 in optimized fermentation (5,118.49 AU/mL) was about 6 times higher than that obtained in non-optimized fermentation (888.56 AU/mL).

Competing interests

The authors declare that they have no competing interests.

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Figure Captions

Fig. 1

The topology of neural network for estimation of BLIS production by *Pediococcus acidilactici* Kp10. Triangles represent the inputs (Neurons added for ANN processing); inoculum size temperature and agitation. Squares represent the hidden and output layer (neurons generated during ANN processing). Small open circles indicate the input and output layers (the neurons that can be observed in the form of numerical values). Colours indicate the activation level of neurons; (red) positive activation; (green) negative activation. Intensity of colour represents the activation intensity for ANN processing.

Fig. 2

Surface plot obtained from optimization using ANN for the combined effect of (a) temperature and inoculum size (by keeping agitation at center point); (b) temperature and agitation (by keeping inoculum size at center point); (c) inoculum size and agitation (by keeping temperature at center point) on BLIS production by *Pediococcus acidilactici* Kp10. The different colours in the legend represent the respective range of BLIS production.

Table 1 Half factorial screening experimental design used to test the effect of several fermentation parameters on the production of BLIS by *P. acidilactici* Kp10.

Run no.	Temp (X_1)	Inoc. size (X_2)	Buffer strength (X_3)	Tween 80 conc. (X_4)	Agit. speed (X_5)	Response		
	(^{o}c)	%	µM	g/L	rpm	OD (650 nm)	Activity (AU/mL)	pH
1	1(37)	-1(1)	1(180)	-1(0.16)	1(200)	1.81±0	573.55	6.5
2	1(37)	1(10)	1(180)	-1(0.16)	-1(0)	0.5±0.04	1738.58	6.5
3	1(37)	1(10)	1(180)	1(1.6)	1(200)	1.59±0	767.92	6.7
4	-1(20)	1(10)	1(180)	1(1.6)	-1(0)	0.89±0	1028.15	6.8
5	-1(20)	1(10)	-1(60)	-1(0.16)	-1(0)	0.84±0.16	1897.65	6.3
6	1(37)	-1(1)	-1(60)	1(1.6)	1(200)	2.44±0	1417.34	6.0
7	-1(20)	1(10)	1(180)	-1(0.16)	1(200)	3.31±0.02	3208.88	6.3
8	1(37)	1(10)	-1(60)	-1(0.16)	1(200)	1.61±0	428.38	6.1
9	-1(20)	1(10)	-1(60)	1(1.6)	1(200)	3.51±0.06	3822.95	6.6
10	-1(20)	-1(1)	1(180)	-1(0.16)	-1(0)	0.73±0.07	663.66	7.0
11	-1(20)	-1(1)	1(180)	1(1.6)	1(200)	1.58±0	1738.58	7.01
12	-1(20)	-1(1)	-1(60)	-1(0.16)	1(200)	3.19±0.3	1592.83	6.3
13	1(37)	-1(1)	-1(60)	-1(0.16)	-1(0)	0.95±0.02	1122.22	6.1
14	-1(20)	-1(1)	-1(60)	1(1.6)	-1(0)	0.83±0.01	863.00	6.5
15	1(37)	-1(1)	1(180)	1(1.6)	-1(0)	0.33±0.01	1089.95	6.5
16	1(37)	1(10)	-1(60)	1(1.6)	0	0.84±0.01	1189.67	6.1

±: Standard deviation of duplicate data
Value of factors in pranthesis are actual level with respect to the coded values.

Table 2 Analysis of variance (ANOVA) for the screening of BLIS production by *Pediococcus acidilactici* Kp10.

Source	Coefficient estimate	Standard Error	Sum of squares	DF	Mean square	F Value	Prob>F
Model			1.282E+007	13	9.860E+005	109.32	0.0091
Intercept	1446.46	23.74					
A	*-405.51*	*23.74*	*2.631E+006*	*1*	*2.631E+006*	*291.70*	*0.0034*
B	*313.82*	*23.74*	*1.576E+006*	*1*	*1.576E+006*	*174.70*	*0.0057*
C	-95.30	23.74	*1.453E+005*	1	*1.453E+005*	16.11	0.0568
D	43.24	23.74	2991.57	1	2991.57	3.32	0.2102
E	*247.35*	*23.74*	*9.789E+005*	*1*	*9.789E+005*	*108.53*	*0.0091*
AB	*-323.63*	*23.74*	*1.676E+006*	*1*	*1.676E+006*	*185.80*	*0.0053*
AC	96.85	23.74	1.501E+005	1	1.501E+005	16.64	0.0552
AD	32.03	23.74	16415.38	1	16415.38	1.82	0.3098
AE	*-491.50*	*23.74*	*3.865E+006*	*1*	*3.865E+006*	*428.54*	*0.0023*
BD	-101.34	23.74	1.643E+005	1	1.643E+005	18.22	0.0508
BE	49.41	23.74	39066.51	1	39066.51	4.33	0.1729
CD	*-238.25*	*23.74*	*9.082E+005*	*1*	*9.082E+005*	*100.69*	*0.0098*
DE	*199.66*	*23.74*	*6.378E+005*	*1*	*6.378E+005*	*70.71*	*0.0138*
Residual			18038.83	2	9019.41		
Cor Total			1.284E+007	15			

A: temperature
B: inoculum size
C: buffer strength
D: tween 80
E: agitation
italic= significant term

Table 3 Box-Wilson 2^3 factorial central composite design for optimization of BLIS production by *P.acidilactici* using ANN.

Exp. No.	Temperature (X_1) (°C)	Inoculum Size (X_2)(%)	Agitation speed (X_5) (rpm)	AU/mL		Final culture pH
				Observed	Predicted by ANN (% difference[a])	
1	*-α(20)*	*0(5.5)*	*0(100)*	*888.6*	*879.1(-1.07)*	*6.4*
2	**-α(20)**	**0(5.5)**	**0(100)**	**888.6**	**879.1(-1.07)**	**6.4**
3	-1(23.45)	+1(8.18)	+1(159.46)	1592.8	1586.2(-0.42)	6.24
4	-1(23.45)	+1(8.18)	-1(40.54)	888.6	923.2(3.90)	6.53
5	**-1(23.45)**	**-1(2.82)**	**-1(40.54)**	**1592.8**	**1577.3(-0.97)**	**6.56**
6	**-1(23.45)**	**+1(8.18)**	**-1(40.54)**	**888.6**	**923.2(3.90)**	**6.54**
7	**-1(23.45)**	**+1(8.18)**	**+1(159.46)**	**1592.8**	**1586.1(-0.42)**	**6.27**
8	*-1(23.45)*	*-1(2.82)*	*+1(159.46)*	*2855.3*	*2876.2(0.73)*	*6.19*
9	**-1(23.45)**	**-1(2.82)**	**+1(159.46)**	**2855.3**	**2876.2(0.73)**	**6.18**
10	**-1(23.45)**	**-1(2.82)**	**-1(40.54)**	**1592.8**	**1577.3(-0.97)**	**6.55**
11	0(28.5)	+α(10)	0(100)	5118.5	5096.2(-0.44)	6.5
12	*0(28.5)*	*0(5.5)*	*0(100)*	*5118.5*	*5123.8(0.10)*	*6.41*
13	0(28.5)	0(5.5)	0(100)	5118.5	5123.8(0.10)	6.41
14	**0(28.5)**	**0(5.5)**	**0(100)**	**5118.5**	**5123.8(0.10)**	**6.41**
15	**0(28.5)**	**0(5.5)**	**-α(0)**	**5118.5**	**5098.9(-0.38)**	**6.27**
16	0(28.5)	0(5.5)	+α(200)	1592.8	1587.0(-0.35)	6.24
17	**0(28.5)**	**0(5.5)**	**+α(200)**	**1592.8**	**1587.0(-0.35)**	**6.21**
18	*0(28.5)*	*-α(1)*	*0(100)*	*5118.5*	*5135.0(0.32)*	*6.36*
19	**0(28.5)**	**-α(1)**	**0(100)**	**5118.5**	**5135.0(0.32)**	**6.32**
20	**0(28.5)**	**+α(10)**	**0(100)**	**5118.5**	**5096.2(-0.44)**	**6.49**
21	*0(28.5)*	*0(5.5)*	*0(100)*	*5118.5*	*5123.8(0.10)*	*6.43*
22	0(28.5)	0(5.5)	-α(0)	5118.5	5098.9(-0.38)	6.26
23	**0(28.5)**	**0(5.5)**	**0(100)**	**5118.5**	**5123.8(0.10)**	**6.45**
24	*0(28.5)*	*0(5.5)*	*0(100)*	*5118.5*	*5123.8(0.10)*	*6.41*

25	+1(33.55)	+1(8.18)	+1(159.46)	1592.8	1619.9(1.70)	6.29
26	**+1(33.55)**	**+1(8.18)**	**+1(159.46)**	**1592.8**	**1619.9(1.70)**	**6.29**
27	**+1(33.55)**	**-1(2.82)**	**+1(159.46)**	**1592.8**	**1582.9(-0.62)**	**6.3**
28	**+1(33.55)**	**-1(2.82)**	**-1(40.54)**	**1592.8**	**1604.3(0.72)**	**6.41**
29	**+1(33.55)**	**-1(2.82)**	**+1(159.46)**	**1592.8**	**1582.9(-0.62)**	**6.33**
30	**+1(33.55)**	**+1(8.18)**	**-1(40.54)**	**1592.8**	**1593.8(0.06)**	**6.41**
31	*+1(33.55)*	*+1(8.18)*	*-1(40.54)*	*1592.8*	*1593.8(0.06)*	*6.41*
32	*+1(33.55)*	*-1(2.82)*	*-1(40.54)*	*1592.8*	*1604.3(0.72)*	*6.4*
33	+α(37)	0(5.5)	0(100)	2132.6	2122.0(-0.50)	6.4
34	**+α(37)**	**0(5.5)**	**0(100)**	**2132.6**	**2122.0(-0.50)**	**6.4**

The italic, bold and normal values represent the experiments used for selection, training and testing, respectively, by the selected ANN.
[a] % difference was calculated as the % difference between the observed value and corresponding predicted value over the observed value.

Table 4 Effect of two types of medium and cultivation temperature on growth of *P. acidilactici* Kp10 and BLIS production.

Fermentation time (h)	30 °C				35 °C				37 °C			
	MRS		M17		MRS		M17		MRS		M17	
	OD (650 nm)	AU/mL	OD (650 nm)	AU/mL	OD (650 nm)	AU/mL	OD (650 nm)	AU/mL	OD (650 nm)	AU/mL	OD (650 nm)	AU/mL
0	0.07	57.6	0.08	103.3	0.07	57.6	0.08	103.3	0.07	57.6	0.08	103.3
3	0.09	62.1	0.23	103.3	0.08	57.6	0.25	214.2	0.09	57.6	0.3	103.3
6	0.09	66.7	0.60	220.5	0.09	69.8	0.72	331.8	0.09	65.8	0.69	322.3
9	0.09	83.2	0.67	545.0	0.09	72.8	0.73	594.8	0.09	72.8	0.79	594.8
12	0.09	124.3	0.72	921.5	0.09	75.1	0.77	1035.6	0.09	73.0	0.72	688.3
15	0.09	160.0	0.75	1386.6	0.09	90.0	0.81	1035.6	0.09	74.9	0.72	921.5
18	0.09	138.3	0.75	1427.7	0.09	83.2	0.78	1187.2	0.08	43.0	0.71	854.2
21	0.09	90.1	0.75	967.2	0.09	65.4	0.7	765.3	0.08	36.2	0.69	796.4
24	0.08	43.0	0.62	456.3	0.09	37.2	0.66	523.1	0.08	32.1	0.66	624.1

Size of inoculum: 10% (v/v)

Initial pH for:
1) **M17 broth:** 7.2 ± 0.2
2) **MRS broth:** 5.7 ± 0.2

Table 5 Analysis of variance for BLIS optimization using Box-Wilson 2^3 factorial central composite design.

Variables	Analysis of variance				Parameter estimates				
	SS	DF	MS	F	Estimates	t values	*P* values	Confidence limits	
								-95%	+95%
Intercept	**39150376**	**1**	**39150376**	**33.1865**	**-50107.3**	**-5.7607**	**0.0000**	**-68059.1**	**-32155.5**
Temperature (X_1)	**54761709**	**1**	**54761709**	**46.4197**	**3621.8**	**6.8132**	**0.0000**	**2524.6**	**4718.9**
Temperature^2 (X_1^2)	**59264102**	**1**	**59264102**	**50.2362**	**-63.5**	**-7.0877**	**0.0000**	**-82.0**	**-45.0**
Inoculum size (X_2)	23	1	23	0.0000	3.1	0.0043	0.9965	-1436.0	1442.1
Inoculum size^2 (X_2^2)	2694169	1	2694169	2.2837	-48.3	-1.5112	0.1437	-114.2	17.7
Agitation (X_5)	**7254150**	**1**	**7254150**	**6.1491**	**75.7**	**2.4797**	**0.0205**	**12.7**	**138.6**
Agitation^2 (X_5^2)	**21167884**	**1**	**21167884**	**17.9433**	**-0.3**	**-4.2359**	**0.0002**	**-0.4**	**-0.1**
Temperature(X_1) * Inoculum size(X_2)	966977	1	966977	0.8196	18.2	0.9053	0.3742	-23.3	59.6
Temperature(X_1) * Agitation(X_5)	966977	1	966977	0.8196	-0.8	-0.9053	0.3742	-2.7	1.0
Inoculum size (X_2) * Agitation(X_5)	77925	1	77925	0.0660	-0.4	-0.2570	0.7993	-4.0	3.1
Error	28312989	24	1179708						

Bold letters represented significant variables and their calculated values. SS stands for sum of squares and MS stands for mean sum of squares.

Table 6 Sensitivity Analysis by ANN.

Parameter	Temperature (X_1) (°C)	Inoculum Size (X_2)(%)	Agitation speed (X_5) (rpm)
Ratio	136.88	15.91	70.1
Rank	1	3	2

Ratios are values given by ANN as a result of sensitivity analysis to input variables.
Rank is the order according to the ratios.

Table 7 Predicted optimal levels by ANN and observed and predicted response on these levels.

Sr. No.	Method	Agitation Speed (rpm)(X_5)	Inoculum Size (%)(X_2)	Temperature (°C)(X_1)	BLIS production (Y)(AU/mL)	
					Predicted by ANN	Experimental
1	Base case	0	10	37	1,912.2	888.6
2	ANN predicted optima	120	3	28.5	5,262.6	5,118.5

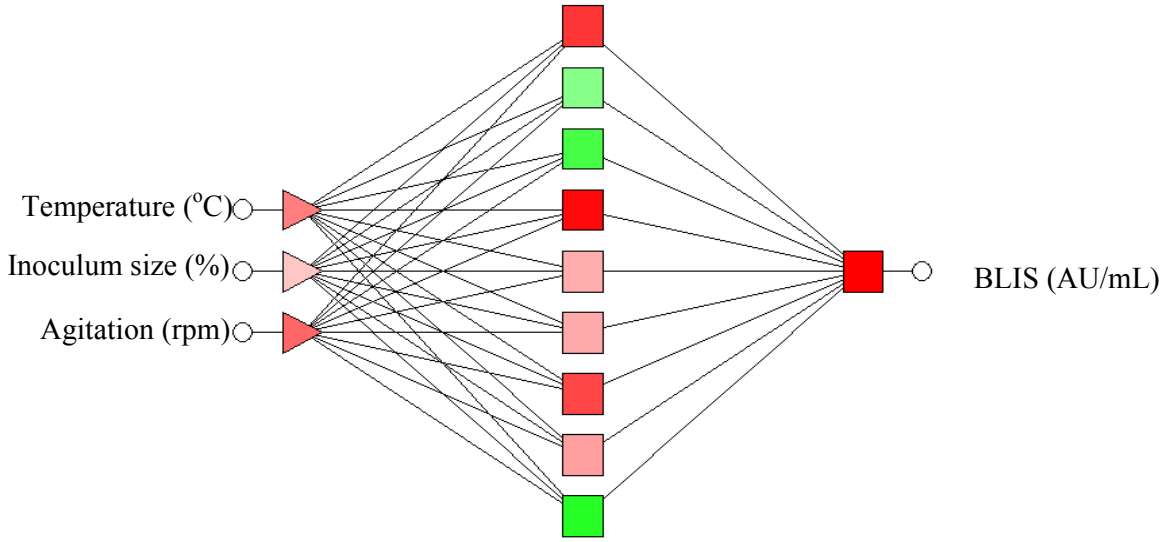

Fig. 1.

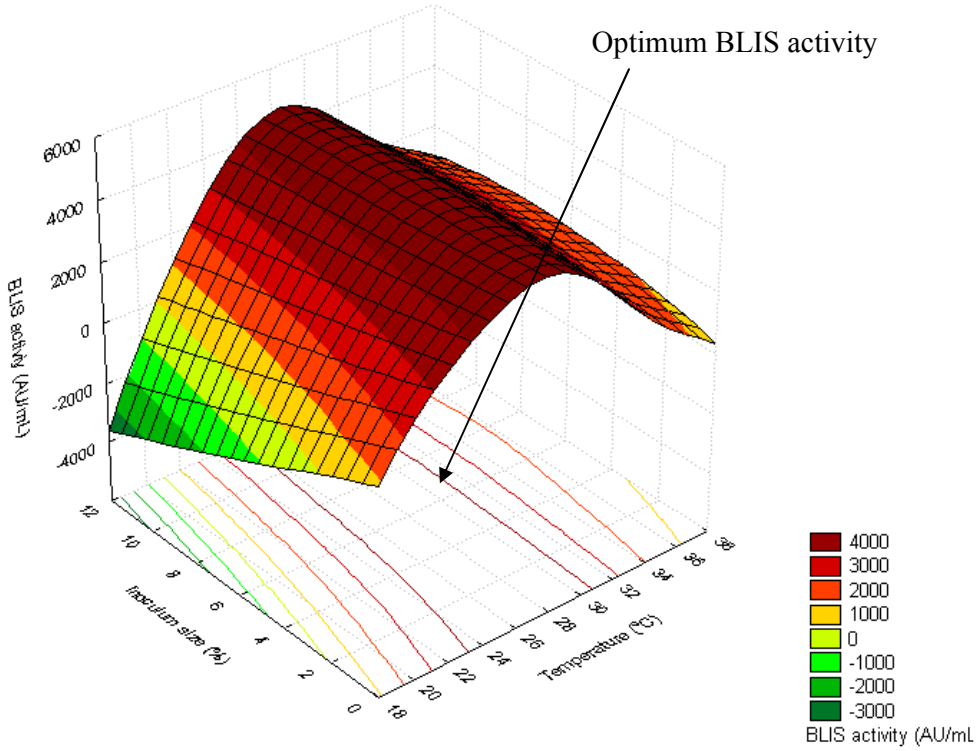
Optimum BLIS activity
BLIS activity (AU/mL)
6000
4000
2000
0
-2000
-4000
Inoculum size (%)
12
10
8
6
4
2
0
Temperature (°C)
18
20
22
24
26
28
30
32
34
36
38
4000
3000
2000
1000
0
-1000
-2000
-3000
BLIS activity (AU/mL

Fig. 2a.

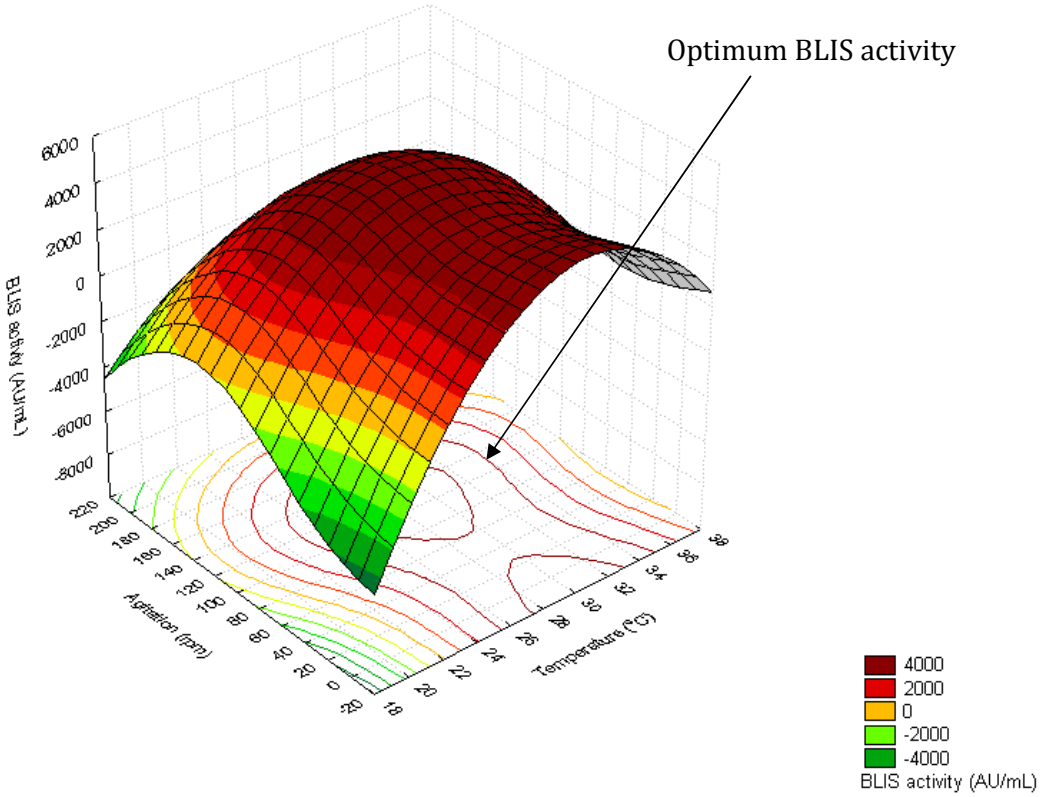
Optimum BLIS activity
BLIS activity (AU/mL)
6000
4000
2000
0
-2000
-4000
-6000
-8000
220
200
180
160
140
120
100
80
60
40
20
0
-20
Agitation (rpm)
18
20
22
24
26
28
30
32
34
36
38
Temperature (°C)
4000
2000
0
-2000
-4000
BLIS activity (AU/mL)

Fig. 2b.

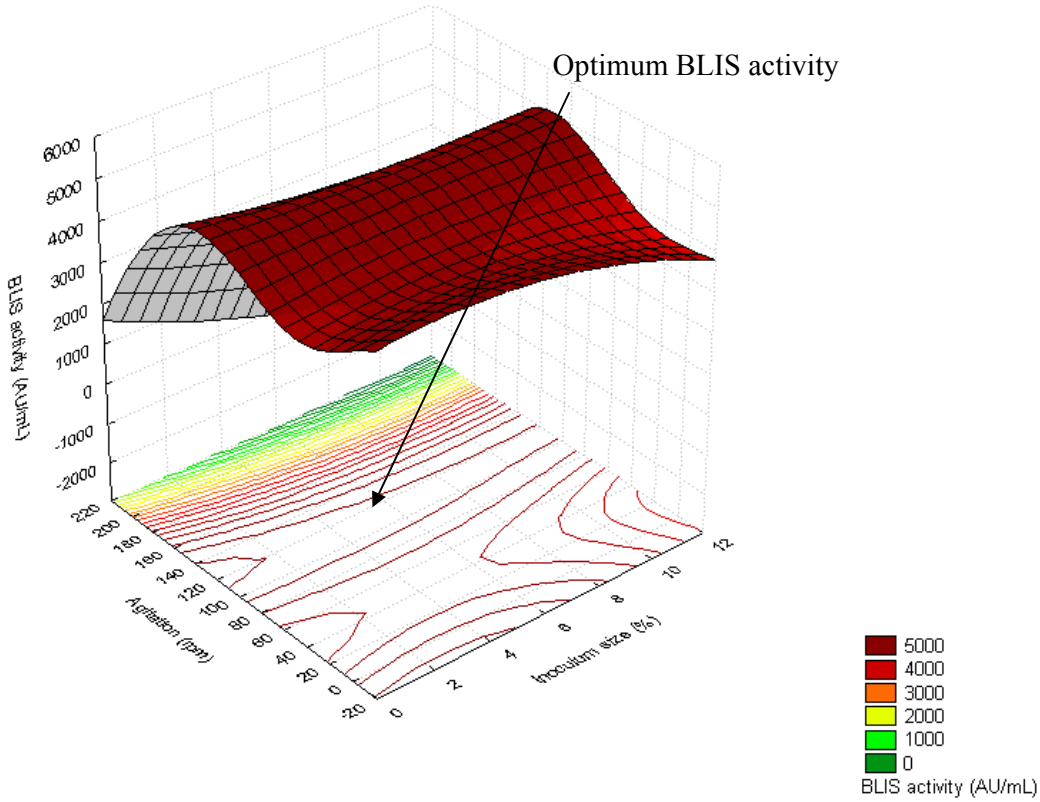
Optimum BLIS activity
BLIS activity (AU/mL)
6000
5000
4000
3000
2000
1000
0
-1000
-2000
Agitation (rpm)
220
200
180
160
140
120
100
80
60
40
20
0
-20
Inoculum size (%)
0
2
4
6
8
10
12
5000
4000
3000
2000
1000
0
BLIS activity (AU/mL)

Fig. 2C.