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

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The present study was aimed at enhancing the production of bacteriocin-like inhibitory substance (BLIS) produced by *Pediococcus acidilactici* Kp10 through optimizing the fermentation parameters. M17 was chosen in preliminary study as a culture medium because BLIS production was nine times higher (1427.7 AU mL^{-1}) compared to that produced by MRS (160 AU mL^{-1}). The fermentation parameters such as temperature, inoculum size, buffer strength, concentration of tween 80 and agitation speed were screened using two level half-factorial design. BLIS production is influenced by three most significant factors identified as temperature, inoculum size and agitation speed, which were further optimized using an artificial neural network (ANN). ANN predicted that a maximum activity of 5262.64 AU mL^{-1} would be 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 5118.5 AU mL^{-1}, which was close to the predicted BLIS activity. Increased BLIS activity in the final solution, which resulted from the optimized process, would reduce downstream steps such as concentrating the product during purification.

Introduction

Current consumer demands for commercial natural preservative processed foods have attracted 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 since many years without any known adverse effects. Bacteriocins, especially those with a 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 a combination of variables, and their values and limits were arbitrarily chosen based primarily on personal experience.[5]

Conventional methods in fermentation optimization require treating each factor separately, which is 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 has been successfully applied for the 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 for the prediction of ANN models was far more superior compared to RSM using the same experimental design.[10,11] ANN does not require prior specification of a suitable fitting function. ANN has universal approximation capability, which means that it can approximate almost all types of non-linear functions including quadratic functions.[12] The ability of ANN

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to predict the process characteristics with little prior knowledge is desirable, which simplifies their implementation and increases their modeling potential. This property makes ANN a powerful and flexible tool that is well-suited for modeling biochemical processes.[13]

Reports on the multiple factors that affect the production of bacteriocins are relatively scarce and the optimization of bioprocess parameters for enhanced bacteriocin production remain elusive.[14] Improvement of bacteriocins 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 enhancing the production of bacteriocin-like inhibitory substance (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 BLIS 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^{-1}), meat extract (8 g L^{-1}), yeast extract (4 g L^{-1}), glucose (20 g L^{-1}), di-potassium hydrogen phosphate (2 g L^{-1}), Tween 80 (1 g L^{-1}), di-ammonium hydrogen citrate (2 g L^{-1}), sodium acetate (5 g L^{-1}), magnesium sulphate (0.2 g L^{-1}) and manganese sulphate (0.04 g L^{-1}). M17 broth (Merck, Darmstadt, Germany) consisted of peptone (10 g L^{-1}), yeast extract (2.5 g L^{-1}), meat extract (5 g L^{-1}), lactose (5 g L^{-1}), ascorbic acid (0.5 g L^{-1}), sodium β-glycerophosphate (19 g L^{-1}) and magnesium sulphate (0.25 g L^{-1}). Three different temperatures (30 °C, 35 °C and 37 °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 growing in 10 mL tubes each containing M17 broth with incubation at 37 °C for 24 h. This initial culture (1% v/v) was then inoculated into a 50 mL Falcon™ tube containing 10 mL of M17 medium and incubated at 37 °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^{-1}) was determined as described by Parente, Brienza, Moles, and Ricciardi.[18] The measurement is based on the results obtained from 2-fold dilutions of CFCS, which are either as a CDA (with the critical dilution being the last dilution that produced a zone larger than well diameter) or as a quantitative assay. A standard curve was prepared using a series of 2-fold dilutions of BLIS solution. The response (R) was 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) was the amount of BLIS pipetted into each well (100 μL l/D). The dose/response curve was calculated according to eqn (1) as follows:

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

The critical dose (CD) is defined as the amount of BLIS solution (in mL) corresponding to a null inhibition zone and was calculated by extrapolating the dose/response curve. The titer, in AU mL^{-1}, was 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 eqn (1). The activity in AU mL^{-1} of a sample was calculated using eqn (2) as follows:

$$\text{AU mL}^{-1} = (1000/100)D10^{(R/b)} \quad (2)$$

Experimental design

Two types of medium (MRS and M17) that are 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 *P. 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^{-1} and 1.6 g L^{-1}) 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 duplicate (Table 1).

For further optimization of the culture conditions, a total of 34 experimental runs were designed according to the 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

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[a]

Run no	Temp (X_1) (°C)	Inoc. size (X_2)%	Buffer strength (X_3), μM	Tween 80 conc. (X_4), g L^{-1}	Agit. speed (X_5), rpm	Response: OD (650 nm)	Response: Activity (AU mL^{-1})	Response: 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

[a] ±: Standard deviation of duplicate data values of the factors in parenthesis are the actual level with respect to the coded values.

abovementioned analysis. Back propagation (BP) and conjugate gradient descent (CGD) logarithms were used in the neural network training 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 layer with three neurons, one hidden layer with nine neurons and an output layer with one neuron. Determination of the optimum topology was based on the minimum error of testing. The ideal network was used for the prediction and optimization of BLIS production.

Optimization capability of ANN

The analysis of variance (ANOVA) was employed to determine the significance of the model parameters. Adjusted R^2, absolute average deviation (AAD) and root mean square error (RMSE) were calculated in addition to the R^2 for the prediction of BLIS production by ANN. R^2 was calculated using eqn (3) as follows:

$$R^2 = \frac{\sum_{i=1-n} \left(X_i - y_{i,\exp}\right)^2}{\sum_{i=1-n} \left(y_i^- - y_{i,\exp}\right)^2} \quad (3)$$

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

Source	Coefficient estimate	Standard error	Sum of squares	DF	Mean square	*F* value	Prob > *F*
Model			1.282×10^7	13	9.860×10^5	109.32	0.0091
Intercept	1446.46	23.74					
A	*−405.51*	*23.74*	*2.631×10^6*	*1*	*2.631×10^6*	*291.70*	*0.0034*
B	*313.82*	*23.74*	*1.576×10^6*	*1*	*1.576×10^6*	*174.70*	*0.0057*
C	−95.30	23.74	*1.453×10^5*	1	*1.453×10^5*	16.11	0.0568
D	43.24	23.74	2991.57	1	2991.57	3.32	0.2102
E	*247.35*	*23.74*	*9.789×10^5*	*1*	*9.789×10^5*	*108.53*	*0.0091*
AB	*−323.63*	*23.74*	*1.676×10^6*	*1*	*1.676×10^6*	*185.80*	*0.0053*
AC	96.85	23.74	1.501×10^5	1	1.501×10^5	16.64	0.0552
AD	32.03	23.74	16 415.38	1	16 415.38	1.82	0.3098
AE	*−491.50*	*23.74*	*3.865×10^6*	*1*	*3.865×10^6*	*428.54*	*0.0023*
BD	−101.34	23.74	1.643×10^5	1	1.643×10^5	18.22	0.0508
BE	49.41	23.74	39 066.51	1	39 066.51	4.33	0.1729
CD	*−238.25*	*23.74*	*9.082×10^5*	*1*	*9.082×10^5*	*100.69*	*0.0098*
DE	*199.66*	*23.74*	*6.378×10^5*	*1*	*6.378×10^5*	*70.71*	*0.0138*
Residual			18 038.83	2	9019.41		
Cor total			1.284×10^7	15			

[a] 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 the optimization of BLIS production by *P. acidilactici* using ANN[a]

Exp. no	Temperature (X_1) (°C)	Inoculum Size (X_2) (%)	Agitation speed (X_5) (rpm)	AU mL^{-1} Observed	AU mL^{-1} Predicted by ANN (% difference[b])	Final culture pH
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**

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

where X_i is BLIS production, $y_{i,\mathrm{exp}}$ is experimental BLIS production and y_i^- is the average observed BLIS production.

The adjusted R^2 was calculated using eqn (4) as follows:

$$\text{Adjusted } R^2 = 1 - \left[\left(1 - R^2\right) \times \frac{N-1}{N-K-1}\right] \quad (4)$$

where N is the total number of observations and K is the number of input variables.

AAD was calculated using eqn (5) as follows:

$$\mathrm{AAD} = \left\{\left[\sum_{i=1}^{p}\left(\left|y_{i,\mathrm{exp}} - y_{i,\mathrm{cal}}\right|/y_{i,\mathrm{exp}}\right)/P\right]\right\} \times 100 \quad (5)$$

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

RMSE was calculated using eqn (6) as follows:

$$\mathrm{RMSE} = \sqrt{\frac{\sum\left(y_{i,\mathrm{exp}} - y_{i,\mathrm{cal}}\right)^2}{n}} \quad (6)$$

where $y_{i,\mathrm{exp}}$ is the experimental response and $y_{i,\mathrm{cal}}$ 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) containing 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 24 h. Subsequently,

the diameters of the growth inhibition zones were measured and the antimicrobial activity, defined as the mean reciprocal of the highest dilution showing clear zones of inhibition, was expressed in Activity Units (AU) per mL (AU mL^{-1}). Cell growth was measured from the optical density of the culture at 650 nm obtained using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 25, USA).

Results 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 essential for good growth and bacteriocin production. For all the temperatures tested, BLIS production by *P. acidilactici* Kp10 in M17 medium was substantially higher compared to that produced in the 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 the M17 medium was nine times higher (1427.7 AU mL^{-1}) than that obtained using the MRS medium (160 AU mL^{-1}). Thus, the M17 medium was selected as a basal medium for the subsequent experiments.

Results from the screening of culture conditions using a two-level half factorial design showed that the highest BLIS production (run 9) was at 20 °C with 10 g L^{-1} of Tween 80, while the lowest production (run 8) was obtained at 37 °C with 0.16 g L^{-1} of Tween 80 (Table 1). Other parameters—inoculum size (10%), buffer strength (60 μM) and agitation speed (200 rpm)—

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

	30 °C				35 °C				37 °C			
	MRS		M17		MRS		M17		MRS		M17	
Fermentation time (h)	OD (650 nm)	AU mL^{-1}	OD (650 nm)	AU mL^{-1}	OD (650 nm)	AU mL^{-1}	OD (650 nm)	AU mL^{-1}	OD (650 nm)	AU mL^{-1}	OD (650 nm)	AU mL^{-1}
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

[a] 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[a]

	Analysis of variance				Parameter estimates				
								Confidence limits	
Variables	SS	DF	MS	*F*	Estimates	*t* values	*P* values	−95%	+95%
Intercept	**39 150 376**	**1**	**39 150 376**	**33.1865**	**−50 107.3**	**−5.7607**	**0.0000**	**−68 059.1**	**−32 155.5**
Temperature (X_1)	**54 761 709**	**1**	**54 761 709**	**46.4197**	**3621.8**	**6.8132**	**0.0000**	**2524.6**	**4718.9**
Temperature^2 (X_1^2)	**59 264 102**	**1**	**59 264 102**	**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)	2 694 169	1	2 694 169	2.2837	−48.3	−1.5112	0.1437	−114.2	17.7
Agitation (X_5)	**7 254 150**	**1**	**7 254 150**	**6.1491**	**75.7**	**2.4797**	**0.0205**	**12.7**	**138.6**
Agitation^2 (X_5^2)	**21 167 884**	**1**	**21 167 884**	**17.9433**	**−0.3**	**−4.2359**	**0.0002**	**−0.4**	**−0.1**
Temperature (X_1) × inoculum size (X_2)	966 977	1	966 977	0.8196	18.2	0.9053	0.3742	−23.3	59.6
Temperature (X_1) × agitation (X_5)	966 977	1	966 977	0.8196	−0.8	−0.9053	0.3742	−2.7	1.0
Inoculum size (X_2) × agitation (X_5)	77 925	1	77 925	0.0660	−0.4	−0.2570	0.7993	−4.0	3.1
Error	28 312 989	24	1 179 708						

[a] Bold letters represent significant variables and their calculated values. SS stands for the sum of squares and MS stands for the mean sum of squares.

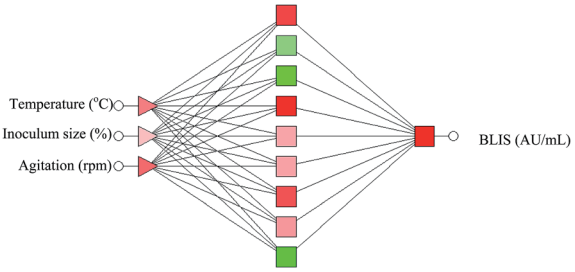

Fig. 1 The topology of neural network for the estimation of BLIS production by *Pediococcus acidilactici* Kp10. Triangles represent the inputs (neurons added for ANN processing): inoculum size, temperature and agitation. Squares represent the hidden and output layer (neurons generated during ANN processing). 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 and (green) negative activation. Intensity of colour represents the activation intensity for ANN processing.

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 concentration showed no significant effects on BLIS production.

The presence of Tween 80 (polyoxyethylene sorbitan monooleate) enhanced the production of some bacteriocins by preventing the aggregation of bacteriocin molecules (Nissen-Meyer *et al.*, 1992; Parente and Hill, 1992; Moretro *et al.*, 2000). This could be observed due to the formation of micelles in the medium that stabilized the production of bacteriocin[21] and facilitated the discharge of bacteriocin from the cell surface.[22] However, the effect of Tween 80 greatly depends 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 results of this study. The possible explanation on this could be the fact that the BLIS from *P. acidilactici* Kp10 was stable at

Table 6 Sensitivity analysis by ANN[a]

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

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

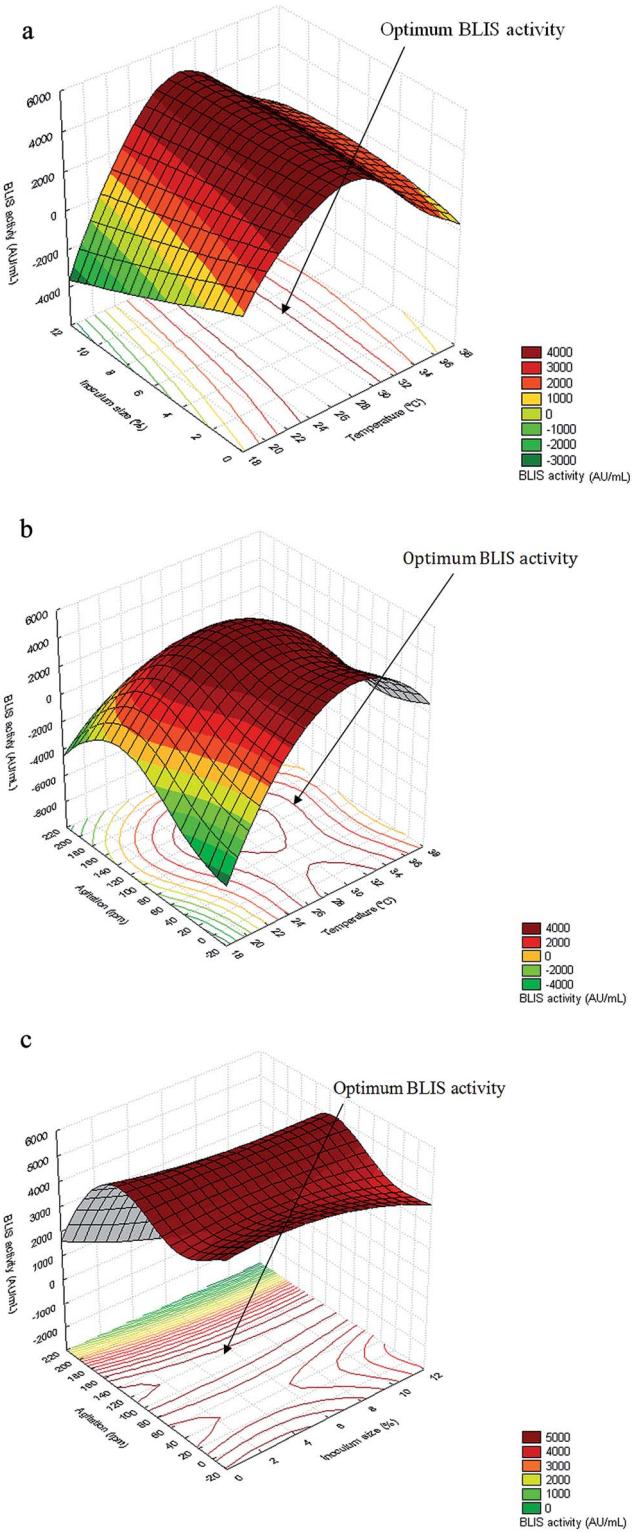

Fig. 2 Surface plot obtained from optimization using ANN for the combined effect of (a) temperature and inoculum size (by keeping agitation at center point); (b) temperature and agitation (by keeping inoculum size at center point); (c) inoculum size and agitation (by keeping temperature at center point) on BLIS production by *Pediococcus acidilactici* Kp10. The different colours in the legend represent the respective range of BLIS production.

Table 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^{-1})	
					Predicted by ANN	Experimental
1	Base case	0	10	37	1912.2	888.6
2	ANN predicted optima	120	3	28.5	5262.6	5118.5

acidic pH[16] and the reduced BLIS activity during the fermentation could not be explained by the decrease in the pH of the culture. The enzymatic reactions are controlled by pH and the decrease in pH of the culture results in a decrease in enzymatic reactions with a 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 lead 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 °C), microorganisms are 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 the 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 consisting of three layers (3 : 9 : 1): an input layer consisting of three variables, middle hidden layer containing 9 neurons and one output layer, was used for the prediction and optimization of BLIS production. The activation of neurons for ANN processing is represented by different colors (Fig. 1). The optimum levels predicted from response graphs, made by ANN for temperature, agitation and inoculum size were 28.5 °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) exhibited the highest effect on the 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 (Fig. 2). The surface plot between temperature and inoculum size obtained from ANN is shown in Fig. 2a. However, the surface plot between temperature and agitation (Fig. 2b) is portrayed as a combination of maximum hill and stationary ridge, whereas the surface plot between inoculum size and agitation (Fig. 2c) is depicted as a combination of saddle and stationary ridge.

Before optimization, maximum BLIS activity obtained experimentally was 888.56 AU mL^{-1}. Following optimization BLIS activity increased to 5118.49 AU mL^{-1}. 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 Fig. 2a–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 5262.64 AU mL^{-1} was obtained at 120 rpm, 3% and 28.5 °C. 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 the ANN predicted levels was 5118.5. The BLIS activity predicted by ANN was close to the observed BLIS activity at all the experimental levels of three variables. ANN results showed high values of R^2 (1) and adjusted R^2 (1), while low values of AAD (0.49) and RMSE (0.02).

Analysis of the experimental data using ANOVA provided the statistical relationships of the output. The probability decreased as the F value increased. 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 indicate 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 the 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 simplifies its implementation and increases the modeling potential. This property makes ANN a powerful and flexible tool that is well suited for modeling of complex bioprocesses. As observed from the present study, BLIS production by *P. acidilactici* Kp10 in optimized fermentation (5118.49 AU mL^{-1}) was about six times higher than that obtained in non-optimized fermentation (888.56 AU mL^{-1}).

Conflict of interest

The authors declare that they have no competing interests.

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