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Optimization of Bacteriocin Production from *Lactobacillus gasseri* NBL 18 through Response Surface Methodology

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A B S T R A C T

Response surface methodology (RSM) is a combination of statistical and mathematical techniques used to create the model and to analyze a response influenced by several factors. The present research was carried out to enhance bacteriocin production by the Lactic acid bacteria *Lactobacillus gasseri* NBL 18 isolated in our lab from infant fecal samples. The influence of physical parameters *viz.* temperature (37-42°C), pH (4.0-8.0), incubation time (6-24h) and inoculum level (1- 3%) on bacteriocin production was analyzed through RSM. Maximum bacteriocin production of 2.56 X 10^4 AU/ml was obtained at temperature 37°C, pH 8.0, inoculum size 3% and incubation time of 24 h. Statistical analysis showed that all the four factors had significant effects on bacteriocin production. RSM proved to be a powerful tool in the optimization of bacteriocin production by *L. gasseri* NBL 18.

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides, which are produced by a wide variety of bacteria (De Vugst and Vandamme, 1994). They were originally defined as proteins characterized by lethal biosynthesis, predominantly intra-species killing activity and adsorption to specific receptors on the surface of bacteriocin sensitive cells (Joerger and Klaenhammer, 1990). Bacteriocins produced by Lactic Acid Bacteria (LAB) have presented a potential use in food industries as biopreservatives as they are able to inhibit the growth of a wide variety of bacteria, including many food spoilage bacteria and pathogens. In order to use a bacteriocin as a food preservative, either the bacteriocin producing strain is used as a starter culture or the bacteriocin in its pure form is used as a food additive. Direct application of bacteriocin for food preservation requires optimization of their production which is dependent on multiple strain-specific factors such as incubation time, temperature, pH and composition of the media (Zamhir *et al.*, 2016). Therefore, it is necessary to conduct research to find out the optimum condition of bacteriocin production. Optimization culture conditions by conventional methods involve

changing one independent variable while keeping constant all other variables. This method may lead to unreliable and wrong conclusions and also extremely time consuming and expensive (Oh *et al.,* 1995).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 1997). It is well suited to study the interaction of different factors on bacteriocin production (Cladera-Olivera *et al.,* 2004; Leães *et al.,* 2011; Kumar *et al.,* 2012). In the present study the production of bacteriocin from *L. gasseri* NBL 18 was optimized through RSM for maximal bacteriocin production.

Materials and Methods

Bacterial cultures

The bacteriocin producing strain *Lactobacillus gasseri* NBL 18 was isolated from 0-6 months old infant fecal samples and identified by PCR analysis of its 16S-23SrRNA gene as described by Song *et al.,* (2000). The nucleotide sequence has been deposited with NCBI data base under the accession number JQ809334.1. The indicator organism *Enterococcus faecalis* NCDC 114 was obtained from National Dairy Research Institute (NCDC), Karnal, Haryana, India.

Bacteriocin Production

MRS medium was inoculated with 1.0% of the inoculum (*L. gasseri*) and incubated at 37^{o}C for 18-24hrs. The cell free culture supernatants (CFCS) was obtained by centrifuging at 12000 g for 10 min at 4^{o}C and heating the supernatant to 90^{o}C for 5-7 min to kill live cells and to inactivate the proteases. Further its pH was adjusted to 6.5 with 1N NaOH. This was used as crude bacteriocin.

Antimicrobial activity assay

The antimicrobial activity was evaluated by spot on lawn assay as described by Ulhmann *et al.,* (1992). Antimicrobial activity was expressed in arbitrary units (AU/ml). Crude bacteriocin was two-fold serially diluted and one arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn (Ivanova *et al.,* 2000).

Response surface optimization of the cultivation conditions for maximal bacteriocin production by *Lactobacillus gasseri* NBL 18

The central composite rotatable design (CCRD), one of the most important experimental designs used in process optimization studies was applied in this study with the objective to develop an empirical model of the process and to obtain a precise estimate of the optimum operating conditions for the factors involved. To describe the nature of the response surface in the optimum region, a four factor (five levels at each factor) second order central composite rotatable design (CCRD) was adopted. The independent factors *viz:* pH (A), Incubation temperature (B), Inoculation level (C) and Incubation Time (D) were considered for optimization of processing variables for bacteriocin production. The selected range for the variables was 4-8 for pH, 37-42^{o}C for incubation temperature, 1-3% inoculum level and 6-24 h of incubation period. For the four factors, the CCRD design constituted of 30 experiments as shown in Table 2. This design was made up of 24 factorial design, six replications of the center points and the eight axial design. The axial distance α was chosen to be 1.68 to make this design rotatable. A center point is a point in which all variables are set at their mid value. Six center

experiments were included in factorial designs as repetition so as to minimize the risk of missing non- linear relationships in the middle of the intervals, and also for the determination of confidence intervals. The response function (Y) was bacteriocin produced (AU/ml). The response was related with the coded factors by a second –degree polynomial equation Eq. (1) using the least square method.

$$Y = bo + b1A + b2B + b3C + b4D + b11A2 + b22B2 + b33C2 + b44D2 + b12AB + b13AC + b14AD + b23BC + b24BD + b34CD + \varepsilon \quad \ldots\ldots\ldots\ldots (1)$$

The coefficient of the polynomials were represented by bo (constant terms), b1, b2, b3 b4 (linear terms), b11, b22, b33, b44 (quaratic terms), b12, b23, b33 b14, b24 b34 (interactive terms) and ε (random error). Thus the optimization of bacteriocin production was achieved using a central composite design and surface modelling method.

The results were analyzed by Design-Expert 8.0.7.1 package (StatEase, Inc., Minneapolis, MN, USA). Adequacy of the model was evaluated using F ratio, model was considered adequate when F-calculated was more than table-F. The analysis of variance (ANOVA) tables were generated and the effect of variables at linear, quadratic and interactive level on individual response was described using significance at 1 and 5% levels of confidence. The magnitude and sign of coefficients in the model indicated the effects of variables on response. The magnitude of coefficient described the extent of dependency of variables on increasing or decreasing the response depending on positive or negative sign of coefficient terms. In the case of negative interaction, the level of one factor could be increased while decreasing the level of other variable. All negative coefficients of quadratic terms indicate maximum response at stationary point, all positive coefficients of quadratic terms indicate minimum response at origin of stationary point, whereas mixed sign of quadratic terms indicate mini-max response (middle point) at origin of stationary point (Table 1).

Results and Discussion

The design matrix representing different combinations of the four factors along with response (experiments were performed in triplicate) are delineated in Table 2. Regression coefficient and ANOVA of fitted quadratic model for bacteriocin production are shown in Table 3.

Diagnostic check of the quadratic model

The quadratic model for response Bacteriocin activity (AU/ml) was obtained through successive regression analysis. The dependence of the response with respect to levels of four factors (pH, Temperature, Inoculum level and Incubation time) in the form of correlation is presented in Table 3. The model F values for all attributes were more than the Table F values at 5% level of confidence and it indicated the significance of model terms. The lack of fit test, which measure the fitness of the model obtained, did not result in a significant F value, indicating that the model is sufficiently accurate for predicting the bacteriocin production by *L. gasseri* NBL 18 from any combination of factor levels within the range evaluated.

Effect of pH, incubation time, inoculum level and incubation temperature on bacteriocin activity

Bacteriocin activity after growth was highly significantly positively ($p<0.01$) affected by pH of the broth and inoculums level and negatively affected by incubation temperature and positively affected by incubation time, but statistically non-significant at linear level. At quadratic level, all parameter had positive effect, but all are non-significant. The interactive effect of pH* incubation time had

highly positive significant effect, pH and incubation temperature, and incubation temperature and incubation time had negative effect, but statistically non-significant. Other parameters were found to have no interactive effect. Multiple regression equation generated to predict the bacteriocin production as affected by different factors in terms of actual factors is as follows:

Bacteriocin activity = + 95.25000 + 10.42333 * pH - 6.84000 * Temperature -2.33333 * Inoculum level + 1.44222 * Incubation time - 0.32000 * pH * Temperature + 3.24740E-015* pH * Inoculum level +0.22222* pH* Incubation time -2.66454E-015* Temperature * Inoculum level -0.071111 * Temperature * Incubation time - 2.59052E-016 * Inoculum level * Incubation time +0.062500 * pH2+0.12000 * Temperature2 +0.65000* Inoculum level2+0.012963* Incubation time 2 (Fig. 1).

Optimization of pH, incubation temperature, inoculation level and incubation time for maximum bacteriocin production

The optimization of levels of pH, incubation temperature, inoculation level and incubation time was attempted using CCRD response surface design and conditions were set as presented in Table 4.

The optimum solution obtained as a result of numerical optimization was verified and the optimum level of pH (8.00), incubation temperature (37^{o}C), inoculation level (3%) and incubation time (24h) were used for maximum bacteriocin production. The actual values of the optimization were compared with the predicted values given by the software using t-test as shown in Table 5. The t-test indicated that there were no significant differences between the predicted and the observed values of bacteriocin produced. This indicated that the model was significant and fitted to the date perfectly, so the bacteriocin produced was maximum from possible combinations of variables.

Environmental conditions such as pH, inoculum level, incubation time and temperature not only affect the growth and biomass production of the culture but also determine the bacteriocin production in the medium. Moreover, these environmental conditions may interfere with bacteriocin stability (Leroy *et al.*, 2005). Our experimental results are in agreement with the earlier reports of several researchers. Maximum production of bacteriocin from NBL 18 occurred at high cell densities (3% inoculum level), which is supported by the reports for bacteriocins produced by other LAB (Van-Laack *et al.*, 1992; Keppler *et al.*, 1994).

Table.1 Coded and actual values of variables in RSM experiment

Level	Coded				Actual			
	Factor 1	Factor 2	Factor 3	Factor 4	pH	Incubation temperature	Inoculum level	Incubation time
-2.0	-2.0	-2.0	-2.0	-2.0	2	34.5	0	**-3**
-1.0	-1.0	-1.0	-1.0	-1.0	4	37	1	**6**
0	0	0	0	0	6	39.5	2	**15**
+1.0	+1.0	+1.0	+1.0	+1.0	8	42	3	**24**
+2.0	**+2.0**	**+2.0**	**+2.0**	**+2.0**	**10**	**44.5**	**4**	**33**

Table.2 Bacteriocin activity of the culture NBL 18 cultivated with different levels of pH, Temperature, Inoculum level and Incubation time

S.N.	Factors				
	pH	Temperature	Inoculum level	Time	Bacteriocin activity
	(A)	(B)	(C)	(D)	($X10^4$ AU/ml)
1	4.00	37.00	3.00	24.00	**0**
2	8.00	42.00	1.00	24.00	**1.28**
3	4.00	42.00	3.00	6.00	**0**
4	6.00	39.50	2.00	15.00	**0.16**
5	8.00	42.00	3.00	24.00	**1.28**
6	4.00	37.00	3.00	6.00	**0**
7	2.00	39.50	2.00	15.00	**0**
8	4.00	42.00	3.00	24.00	**0**
9	8.00	37.00	3.00	6.00	**0.32**
10	6.00	39.50	4.00	15.00	**0.32**
11	4.00	42.00	1.00	6.00	**0**
12	6.00	39.50	2.00	15.00	**0.16**
13	6.00	34.50	2.00	15.00	**0.08**
14	6.00	39.50	2.00	15.00	**0.16**
15	4.00	42.00	1.00	24.00	**0**
16	4.00	37.00	1.00	24.00	**0**
17	4.00	37.00	1.00	6.00	**0**
18	8.00	42.00	1.00	6.00	**0.32**
19	6.00	39.50	2.00	-3.00	**0**
20	8.00	37.00	1.00	24.00	**2.56**
21	8.00	37.00	3.00	24.00	**2.56**
22	8.00	42.00	3.00	6.00	**0.32**
23	6.00	44.50	2.00	15.00	**0.32**
24	6.00	39.50	2.00	33.00	**0.64**
25	6.00	39.50	2.00	15.00	**0.16**
26	6.00	39.50	2.00	15.00	**0.16**
27	6.00	39.50	2.00	15.00	**0.16**
28	6.00	39.50	0.00	15.00	**0**
29	8.00	37.00	1.00	6.00	**0.32**
30	10.00	39.50	2.00	15.00	**0**

Table.3 Regression coefficients and ANOVA of fitted quadratic model for maximum bacteriocin production

Partial coefficients	Bacteriocin activity
Intercept	**1.60**
A-pH	**3.73****
B-Incubation temperature	**-0.87**
C-Inoculation level	**0.27****
D- Incubation time	**3.20**
A^2	**0.25**
B^2	**0.75**
C^2	**0.65**
D^2	**1.05**
AB	**-1.60**
AC	**0.00**
AD	**4.00****
BC	**0.00**
BD	**-1.60**
CD	**0.00**
Model F value	**3.02***
R^2	**0.74**
APV	**7.16**
Lack of fit	**NS**

**Significant at 1% level (P<0.01)
*Significant at 5% level (P<0.05)

Table.4 Conditions during optimization of bacteriocin production in CCRD

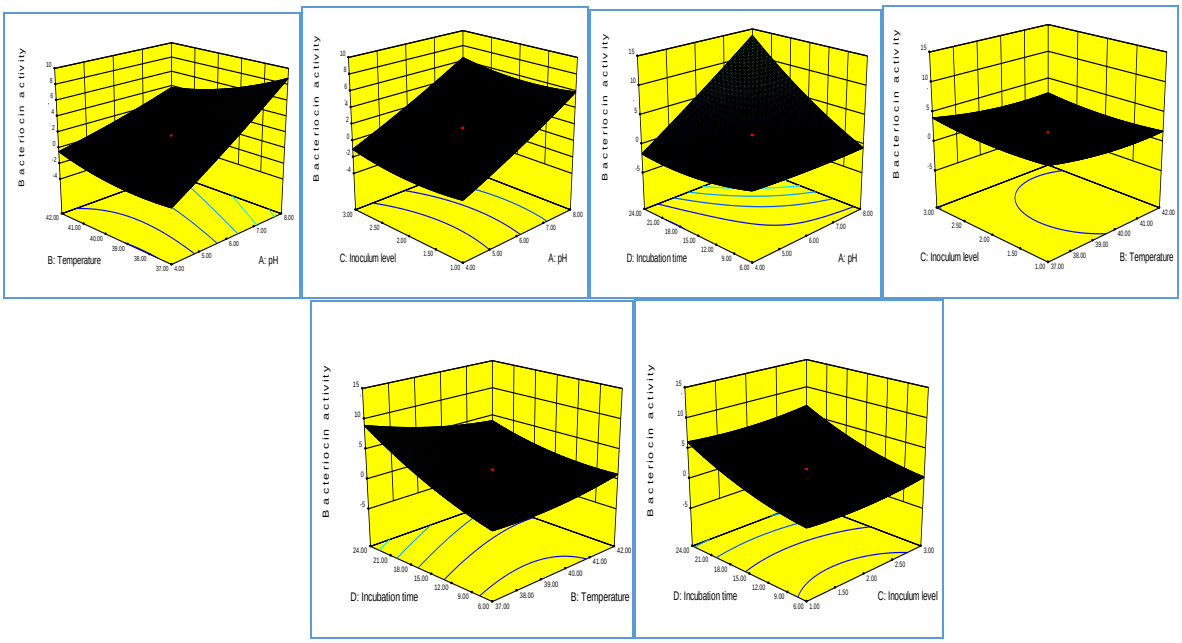
Name	Goal	Low limit	Upper limit
pH	In range	4	**8**
Incubation temperature	In range	37	**42**
Inoculation level	In range	1	**3**
Incubation time	In range	6	**24**
Bacteriocin activity	**Maximize**	**0**	**25.6**

Table.5 Optimized values as compared to predicted values

Responses	Predicted score*	Observed score@	t-value#
Bacteriocin production	**19.56**	**21.33**	**0.41NS**

*predicted values of Design Expert 8.0.7.1 package
@actual values (mean of three trials); #p<0.05; NSnonsignificant

Fig.1 Response surface curves for bacteriocin activity as influenced by the level of pH, incubation temperature, inoculum level and incubation time



Various workers have reported that growth and bacteriocin production by a strain occurs at optimum levels in the neutral and slightly alkaline pH range (De Vugst and Vandamme, 1994; Franz *et al.*, 1996; Kang and Lee, 2005). This is in support to our finding where a maximal bacteriocin production was obtained at a pH of 8.00. The decrease in bacteriocin production at very low pH values in most of the cases has been attributed to the reduced cell mass.

Although the bacteriocin production is detected over a wide range of temperature, the production is maximum at the optimum temperature of growth of the producer strain and a relatively longer incubation times are needed to achieve the highest bacteriocin titres at low temperatures (Biswas *et al.*, 1991; Schved *et al.*, 1993; Todorov and Dicks, 2004). Similar results are obtained in our study with a maximal bacteriocin production at 37^{o}C (optimum growth temperature for lactobacilli) for 24h. Some workers have also reported contradictory results where they found that the optimum temperature for the production of bacteriocins by a strain was lower than that of growth (Mataragas *et al.*, 2003). RSM results indicated all the four factors studied have significant effect on bacteriocin production and proved to be a powerful tool in optimizing the culture conditions.

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