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Optimization of medium components for maximizing the bacteriocin production by *Lactobacillus plantarum* ATM11 using statistical design

[1*]Thirumurugan, A., [2]Ramachandran, S. and [3]Gobikrishnan, S..

[1]*Department of Biotechnology, Kumaraguru College of Technology, Coimbatore -641 049, Tamilnadu, India*
[2]*Department of Mechanical Engineering, Thermal Engineering, Jayam College of Engineering and Technology, Dharmapuri – 636 813, Tamilnadu, India*
[3]*Department of Biotechnology, Karunya University, Coimbatore – 641 114, Tamilnadu, India*

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Abstract

In the present study, statistically based experimental design was applied for the optimization of bacteriocin production by *Lactobacillus plantarum* ATM11 isolated from goat slaughter house soil. Seven variables were chosen for Plackett-Burman design, in which three variables were screened as influencing factors: yeast extract, Tween 80 and K_2HPO_4. These three factors were subsequently optimized using Box-Behnken design and the optimal conditions were found to be 12.1 g/L for yeast extract, 2.5 g/L for Tween 80 and 1.99 g/L for K_2HPO_4. The validity of the optimal conditions was verified in a separate experiment.

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Introduction

Bacteriocins of Lactic Acid Bacteria (LAB) have been the focus of research because of their potential use as biopreservatives (Kleanhammer *et al*., 1993). Bacteriocins are ribosomally synthesized antibacterial peptides, which are regarded as potential alternatives to conventional antimicrobial agents (Cleveland *et al*., 2001). One of the major reasons for their worldwide use is the production of a wide range of antimicrobial substances that efficiently contribute to the preservation of the fermented products (Piard and Desmazeaud, 1992). Bacteriocins are among the most promising preservatives in the food industry, and are a family of microbial defense system, which meant they may prohibit the invasion of other strains or the change of the environment, both biotic and abiotic (Riley and Wertz, 2002).

In the recent years, different kinds of bacteriocins have been found from different bacteria; however, only one bacteriocin named nisin has been used for food preservation. Earlier in 1969, WHO announced that nisin was a kind of food preservative with high efficiency and safety, and later in 1983, FDA declared that nisin was generally recognized as a safe food preservative (Han *et al*., 2011). Except nisin, most work related to bacteriocins have been focused on isolation and purification on a laboratory scale, without or much consideration to industrial-scale up studies and production or towards application to human life.

Generally, bacteriocin is closely associated with the growth of bacterial culture because bacteriocin is released during the growth of bacteriocin-producing cultures, and at the end of the bacterial growth, bacteriocin efficiency decreases very slowly due to the protease degradation (Hur *et al.*, 2000). Production of bacteriocins can be influenced by various factors, including medium composition and culture conditions such as pH and temperature, as well as the carbon and nitrogen sources and inorganic salts (Keren *et al*., 2004; Mataragas *et al*., 2004). Importantly, for application, it is necessary to optimize the fermentation conditions and medium composition. De Man Rogosa and Sharpe (MRS) is the standard culture media for lactic acid bacteria (LAB), but its high cost limits its suitability for industrial-scale production. MRS has ever been used for large scale fermentation, while the purpose was to produce enzymes or other metabolites from LAB (Lu *et al*., 2003; Hummel *et al*., 1983). Studies on lowering the cost of culture media have already been reported for bacteriocin production (Dominiquez *et al*., 2007; Trinetta *et al*., 2008; Wiese *et al.*, 2010).

Optimization of the variables that affect product formation can be performed by statistical experimental designs such as Plackett-Burman and response surface methodology (RSM), which

*Corresponding author.
Email: *biotechthiru@gmail.com*

eliminates the limitations of one variable-at-a-time approach (Stanbury *et al*., 1997)). The use of RSM in biotechnological processes is gaining immense importance for the optimization of production of microbial products and biomolecules (Tiwari and Srivastava, 2008).

Some reports have already been reported on the statistical optimization, i.e., bacteriocin production using *L. plantarum* YJG (Han *et al.*, 2001), bacteriocin production using *L. plantarum* LPC010 (Leal-Sánchez *et al*., 2002), bacteriocin production using *L. plantarum* LR/14 (Tiwari and Srivastava, 2008), nisin production using *L. lactis* subsp. *lactis* (Guo *et al*., 2010), bacteriocin production using *L.brevis* DF01 (Lee *et al*., 2012) and bacteriocin production using *P. acidilactin* (Neera *et al*., 2013). However, many studies have been investigated, since variables are varied while optimizing the medium for bacteriocin preoduction by *L. plantarum* ATM11 strain; therefore, this work differed from above mentioned studies.

In our previous study, bacteriocin produced by *Lactobacillus plantarum* ATM11 was combined with gold nanoparticles for increasing the shelf-life of food against food spoiling bacteria (Thirumurugan *et al*., 2013).Therefore, in the present study, we have made an attempt in the optimization of medium components for enhancing the production of bacteriocin using *Lactobacillus plantarum* strain ATM11 by statistical designs. Here, we have used Plackett-Burman design for screening the influencing variables in the media and Box-Behnken design to find the optimum concentration of screened variables and their interactions.

Materials and Methods

Bacterial strain and growth conditions

Bacteriocin producing isolate of *Lactobacillus plantarum* ATM11 is grown in De Man Rogosa and Sharpe (MRS) broth at 37°C, 180 rpm in an incubator shaker. *Micrococcus luteus* (MTCC 1272) was used as indicator organism for this study.

Production of bacteriocin

Bacteriocin production was evaluated in the culture grown aerobically at 37°C and 180 rpm over a period of 26 h. After incubation, cells were removed from the growth medium by centrifugation (10,000 x g for 30 min, 4°C) and passed through 0.22 µm filters. The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin.

Determination of bacteriocin activity

Bacteriocin activity was determined by the agar well-diffusion method using above mentioned organism as an indicator strain. Aliquots (20 µl) of the sterile supernatant were placed in 6-mm-diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with the indicator organism. After 24 h of incubation, the diameters of the zones of growth inhibition were measured. The activity of cell-free supernatant was expressed in arbitrary units per ml (AU/mL). Data are the means of duplicates, and standard error was ±5% of the mean. A unit activity of the bacteriocin was defined as arbitrary unit (AU); 1 AU is a unit area of inhibition zone per unit volume, in this case mm^2/ml. The bacteriocin activity (Usmiati and Marwati, 2011) was calculated using the following formula:

Bacteriocin activity (mm^2/ml) = Lz-Ls/V

Lz = clear zone area (mm^2), Ls = well area (mm^2

V= volume of sample (ml)

Optimization of bacteriocin production

The optimization of medium components for bacteriocin production by *Lactobacillus plantarum* strain ATM11 was carried out in following two stages:

Screening of significant variables by Plackett-Burman design (PBD)

To identify and screen the influencing variables in the critical medium components, Plackett-urman design was followed (Plackett and Burman, 1946) as shown in Table 1. The total number of experiments to be carried out according to Plackett-Burman is n+1, where n is the number of variables. Each variable is represented at two levels, high and low denoted by (+1) and (−1), respectively. Each column contained equal number of positive and negative signs. A total of 11 variables (7 culture variables and 4 dummy variables) were screened in 12 trials with duplicates each and the response was assessed in terms of bacteriocin production (AU/mL) (Table 1). The dummy variables, which are not assigned any values, introduce some redundancy required by the statistical procedure. Incorporation of the dummy variables into an experiment allows an estimation of the variance (experimental error) of an effect. The effect of each factor was assessed in terms of the difference between the average of the measurements made at the high level of that factor and the average of the measurements made at the low level of that factor, which was determined by the following equation:

$$E_{(Xi)} = \frac{2(\Sigma P_{i+} - \Sigma P_{i-})}{N} \quad (1)$$

Where, $E_{(Xi)}$ is the effect of the tested variable. *Pi*+ and *Pi*− are the activities from the trials where the variable (Xi) measured were present at high and low level, respectively, and N is the number of trials (experiments).

Standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (*p* value) of the effect of each concentration was determined using student's -test as given by the equation: where *E(Xi)* is the effect of variable *Xi*.

$$t_{(Xi)} = E_{(Xi)} / SE \quad (2)$$

Optimization of screened variables using Box-Behnken experimental design and response surface methodology

As screened through the Plackett-Burman design, three independent variables, i.e., yeast extract, Tween80 and K_2HPO_4 were studied at three different levels (1, 0, +1), and the factors were coded as shown in Table 3. All experiments were carried out at 37°C and pH 6.5 for 24 h. To derive the optimum level of these screened variables and to study their interactions, response surface methodology (RSM) using Box-Behnken experimental design (BBD) was performed. It provides an economical alternative to central composite design. The Box-Behnken experimental design was performed using the statistical software package Design-Expert 7.0.0 (State Ease Inc., Minneapolis, MN, USA). The Box-Behnken design matrix was constructed as shown in Table 3. A total of 17 experiments were formulated with 5 centre points.

Analysis of variance (ANOVA) was used to estimate the statistical significance for the medium formulation. The limit of significance was given as values of Prob>F less than 0.05. The response in Table 5 was correlated by non linear regression using the quadratic polynomial model:

$$Y = b_0 + b_1A + b_2B + b_3C b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \quad (3)$$

Where, *Y* is the bacteriocin activity, bi are the coefficients, *A, B* and *C* are the three factors in terms of normalized variables, as −1 and +1 and not in real values.

Results and Discussion

In the present study, we have made an attempt to design the medium constituents for maximizing the production of bacteriocin by *L. plantarum* ATM11 isolated from goat slaughter house soil. Some reports have already been reported on screening of medium constituents by Plackett-Burman design. Though already it has been studied, since factors varied among different strains, our study also differed from previous studies (Guo *et al*., 2010; Kumar *et al*., 2010; Han *et al.*, 2011).

Screening of significant variables by Plackett-Burman design (PBD)

Medium constituents for bacteriocin production by *Lactobacillus plantarum* ATM11 was optimized using two statistical tools: Plackett- Burman design and RSM using Box-Behnken design. Plackett-Burman design is a widely used statistical design for the screening of the media constituents (Plackett and Burman, 1946). The design screens important variables that may influence the production of a microbial compound as well as their significant levels, but does not consider the interactive effects among the variables as in BBD. In Box-Behnken matrix, each selected variable is studied at three different levels along with other variables, and therefore, the interactions among the variables at their different levels could be studied.

Experiments were designed to screen the influencing variables using Plackett-Burman experimental design as shown in Table 1. It represents for 7 variables and their corresponding responses in terms of bacteriocin production. Table 2 represents the higher and lower concentration of each variables and regression analysis of the effect of each variable along with the coefficient, F and p -value. A p-value less than 0.05 indicate that the model terms are significant. When the concentration effect value $(E_{(Xi)})$ of the tested variable was positive, the influence of the variable was greater at the high concentration tested, and when negative, the influence of the variable was greater at low concentration. The variation in bacteriocin production in different sets ranged from 105 to 4250 AU/mL, reiterating the importance of selection and identification of important factors. From the regression analysis, it was found that bacteriocin production was affected by yeast extract, Tween 80, and K_2HPO_4. Other variables had no significant influence towards bacteriocin production. From the regression analysis, it was found that bacteriocin production was affected by these factors as indicated by their corresponding F and p-values. These variables had confidence level above 95% in comparison to other variables and thus, were considered to be significant for bacteriocin production by *L. plantarum* ATM11. Similarly, in

Table 1. Plackett-Burman design matrix for eleven variables with responses

Run	A	B	C	D	E	F	G	D_1	D_2	D_3	D_4	Bacteriocin activity (AU/mL)
1	1	-1	-1	-1	1	1	1	-1	-1	1	-1	3200
2	1	1	-1	1	1	-1	1	-1	1	-1	1	5400
3	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	2250
4	-1	1	1	1	-1	1	1	1	1	-1	-1	5400
5	1	-1	1	-1	-1	-1	1	1	1	1	-1	6650
6	-1	1	-1	-1	-1	1	1	-1	1	1	1	5400
7	1	1	-1	1	-1	-1	-1	1	-1	1	1	8000
8	-1	-1	-1	-1	-1	-1	-1	1	1	-1	1	4250
9	1	1	1	-1	1	1	-1	-1	1	1	-1	5400
10	-1	1	1	-1	1	-1	-1	1	-1	1	1	2250
11	-1	-1	1	1	1	-1	1	-1	1	-1	1	5400
12	1	-1	1	1	-1	1	-1	-1	-1	-1	-1	9450

D_1, D_2, D_3 and D_4 are designated as dummy variables

Table 2. Variables and ranges used in Plackett-Burman design and regression analysis

Variables	Factors	Concentration (g/L)		Coefficient estimate	*F*-value	*p* value
		+1	**- 1**			
A	Glucose	25	15	-504.17	3.87	0.1204
B	Yeast extract	15	7.5	-937.50	13.40	**0.0216***
C	$MgSO_4$	2.5	1.5	-695.83	7.38	0.0532
D	Tween 80	2.5	0.75	-1270.83	24.62	**0.0077***
E	Triammonium citrate	2	0.5	420.83	2.70	0.1757
F	K_2HPO_4	2.5	1.0	729.17	8.10	**0.0465***
G	Sodium acetate	1.0	0.5	54.17	0.045	0.8429

Significant *$P \leq 0.05$

the previous studies (Tiwari and Srivastava, 2008) obtained such variables as influencing factors by *L. plantarum* LR/14 and also for chbacteriocin production by *L. lactis* (Li *et al.*, 2002). In a recent study (Kumar *et al*., 2010) from *E. faecium* LR/6, authors have found that Tween 80 did not influence the bacteriocin production, and in our study also, it was found to be insignificant.

Optimization of screened variables using Box-Behnken design and response surface methodology

Response surface methodology has been successfully used in many studies for optimization of bacteriocin production (Delgado *et al*., 2007; Han *et al*., 2011; Lee *et al*., 2012; Neera *et al*., 2013). However, since factors varied among different strains, this work differed from previous studies (Li *et al*., 2002; Cladera-Olivera *et al*., 2004; Delgado *et al.*, 2007; Kumar and Srivastava, 2010; Tiwari and Sivastava, 2010; Lee *et al*.,2012) in choosing factors before RSM. An effect of physical factors on bacteriocin production, including temperature and pH has already been studied (Delgado *et al*., 2007). The composition of the medium was also shown to have an important role in bacteriocin production, reported by Li *et al*. 2002. Also, studies

Table 3. Box-Behnken design of variables (coded and real) and their responses

Run	A	B	C	Bacteriocin activity (AU/mL)		Residuals
				Observed	Predicted	
1.	-1(5)	-1(0.0)	0 (1.75)	1350	1365.02	-15.02
2.	1(15)	-1(0.0)	0 (1.75)	2300	2248.27	51.73
3.	-1(5)	1(2.5)	0 (1.75)	3347	3442.02	-95.02
4.	1(15)	1(2.5)	0 (1.75)	4089	4117.27	28.02
5.	-1(5)	0(1.25)	-1(1)	105	145.64	-40.64
6.	1(15)	0(1.25)	1(2.5)	1368	1475.39	-107.39
7.	-1(5)	0(1.25)	1(2.5)	881	816.89	64.11
8.	1(15)	0(1.25)	1(2.5)	1043	1045.64	-2.64
9.	0 (10)	-1(0.0)	-1(1)	2181	2146.98	34.02
10.	0 (10)	1(2.5)	-1(1)	2843	2728.98	114.02
11.	0 (10)	-1(0.0)	1(2.5)	806	876.73	-70.73
12.	0 (10)	1(2.5)	1(2.5)	4250	4240.73	9.27
13.	0 (10)	0(1.25)	0 (1.75)	1245	1165.68	79.32
14.	0 (10)	0(1.25)	0 (1.75)	1190	1165.68	24.32
15.	0 (10)	0(1.25)	0 (1.75)	1098	1165.68	-67.68
16	0 (10)	0(0.125)	0 (1.75)	1189	1165.68	23.32
17	0 (10)	0(1.25)	0 (1.75)	1193	1165.68	27.32

to reduce the cost of the medium have been recently conducted by Dominguez *et al*., 2007. Tween80 used in Plakett-Burman design did not significantly affect bacteriocin production; similar to the reports for other bacteriocins (Dominiques *et al*., 2001). In contrast, surfactant could stimulate the production of bacteriocins in other studies (Huot *et al*., 1996; Rajaram *et al.*, 2010).

The variables so identified by Plackett-Burman design were further optimized by RSM using Box-Behnken design experimental plan. The results obtained were fed into the Design-Expert software and analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used (Table 3). The maximum experimental value for bacteriocin production was 4250 AU/mL, while the predicted response based on RSM was estimated to be 4240.73 AU/mL. The close correlation between the experimental and predicted data indicates the appropriateness of the model. The quality of the model can also be checked using various criteria. The calculated regression equation for the optimization of media constituents assessed the bacteriocin (Y) as a function of these variables. By applying quadratic regression analysis on the experimental data, the following equation was found to explain bacteriocin production.

$$\text{Bacteriocin} = +1165.68 + 389.63 * A + 986.50 * B + 60.37 * C - 52.00 * A * B - 275.25 * A * C + 695.50 * B * C + 1627.46 * B^2 - 294.79 * C^2 \quad (4)$$

The coefficient of determination in terms of predicted R^2 is 0.9973 that is in close agreement with adjusted R^2 of 0.9945. This validated the experimental and predicted levels of bacteriocin production. The closeness of R^2 value to 1.0 reflected the strength of the model and predicted the response better. This statistical analysis also allowed us to determine the contribution of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise (Kumar *et al.*, 2010). Thus, the estimated adequate precision of 63.91 for bacteriocin production, representing the signal to noise ratio, is an adequate signal. The model F value was 364.94 and the P > F (<0.0001) indicating that the model terms are significant. In other words, A, B, AC, BC, B2, C2 are significant model in terms of bacteriocin production. Since C, AB and A^2 are insignificant, AB and A^2 are removed from the model; hence, it is called model reduction, and since C (K_2HPO_4) is parent term, it could not be removed from the model as reported by Gobikrishnan *et al*., 2013. The corresponding ANOVA is presented in Table 4.

The contour and three-dimensional plots based on the interactions between the variables showed an increase in bacteriocin production as the concentration of each variable increased to optimum level, beyond which a decline could be observed. High concentration of yeast extract and low concentration of K_2HPO_4 favors the production of bacteriocin. The

Table 4. Analysis of variance (ANOVA) and regression analysis for selected model

Source	Sum of squares	df	Mean square	F-value	p-value Prob>F	
Model	2.264E+007	8	2.830E+006	364.94	< 0.0001	Significant
A:Yeast extract	1.214E+006	1	1.214E+006	156.63	< 0.0001	
B:Tween 80	7.785E+006	1	7.785E+006	1004.07	< 0.0001	
C:K_2HPO_4	29161.13	1	29161.13	3.76	0.0884	
AB	10816.00	1	10816.00	1.39	0.2715	
AC	3.031E+005	1	3.031E+005	39.08	0.0002	
BC	1.935E+006	1	1.935E+006	249.54	< 0.0001	
B^2	1.118E+007	1	1.118E+007	1442.25	< 0.0001	
C^2	3.669E+005	1	3.669E+005	47.32	0.0001	
Residual	62031.37	8	7753.92			
Lack of fit	50777.37	4	12694.34	4.51	0.0868	Not significant
Pure Error	11254.00	4	2813.50			
Cor Total	2.270E+007	16				

Significant $^{*}P \leq 0.05$

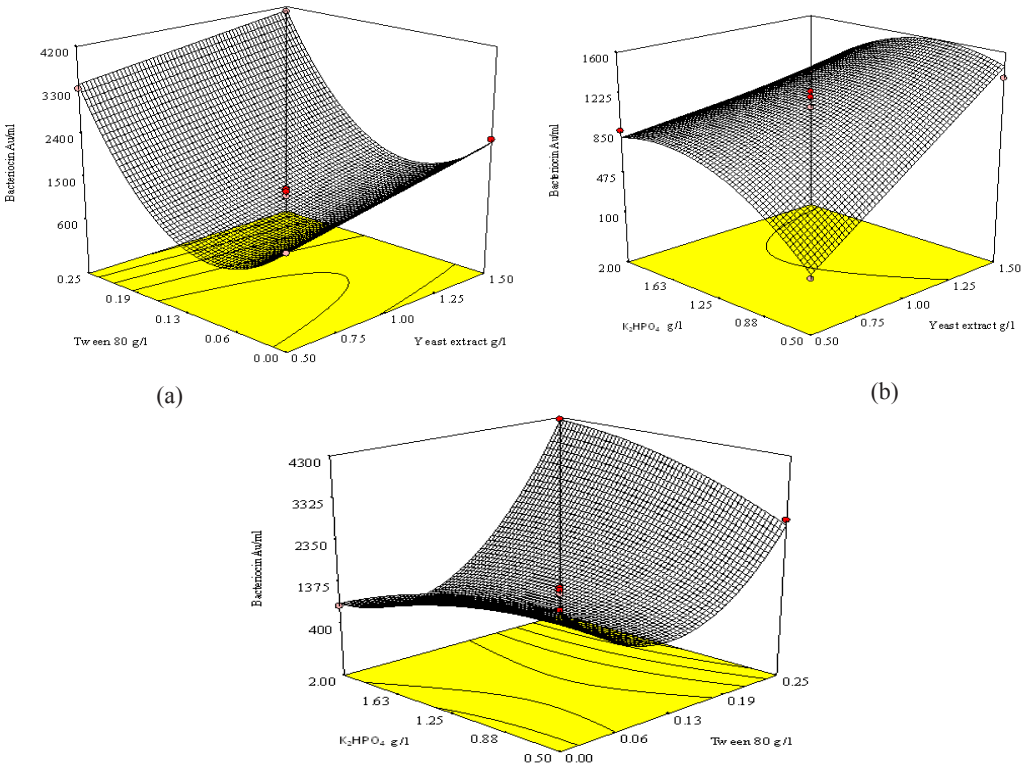

Figure 1. Three dimensional graphs showing the effect of Tween 80, Yeast extract and K_2HPO_4 (a), Tween 80 and yeast extract (b), K_2HPO_4 and yeast extract (c) K_2HPO_4 and Tween 80 on bacteriocin production.

optimal values obtained from the three-dimensional plot (Figure.1a-c) were almost equal to the results obtained by the regression analysis (Eq.4).

The optimum values of the tested variables derived were: yeast extracts 12.1 g/L, Tween 80 2.5 g/L and K_2HPO_4 1.99 g/L .The model was further validated by conducting the experiment under these optimized conditions, which resulted in bacteriocin production of 4265.92 AU/mL closely similar to predicted response of 4185.53 AU/mL, thus proving the validity of the model. The bacteriocin production was, therefore, enhanced by 1.15 fold while comparing the obtained yield in commercially available MRS medium.

Conclusion

Statistical designs (Plackett-Burman and RSM using Box-Behnken experimental design) helped in identification and optimization of important medium constituents for bacteriocin production by slaughter house soil isolate of *L. plantarum* ATM11. The important factor thus identified was the concentration of yeast extract, tween 80 and K_2HPO_4. When these conditions were employed, it led to 1.15-fold higher bacteriocin production than in normal commercially available MRS medium.

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