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Original Contribution

ENHANCED PRODUCTION OF ENTEROCIN FROM ENTEROCOCCUS FAECIUM LR/6 BY STATISTICAL OPTIMIZATION OF THE GROWTH MEDIUM

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ABSTRACT

Statistical screening of media constituents for enhancement of enterocin production from a soil isolate *Enterococcus faecium* LR/6 has been carried out. Sodium acetate, dipotassium hydrogen phosphate, triammonium citrate and tween-80 were identified as the most important factors for enterocin production in the first step, using Plackett-Burman design. Response surface methodology (RSM) was further applied to study their interaction and to determine their optimum levels. The analysis indicated that the maximum enterocin production was achieved in a TGYE medium supplemented with 11 g/l sodium acetate, 3 g/l dipotassium hydrogen phosphate, 1 g/l triammonium citrate and 1 g/l tween-80 after an incubation period of 18 h. Approximately 2-fold improvement in enterocin production was achieved due to this medium optimization. The results indicated the importance of statistical tools in identifying and optimizing various culture components for enhanced enterocin production from *E. faecium* LR/6. Such an improved production will facilitate the purification and various applications of enterocin, especially in food biopreservation.

Keywords: Enterocin, *enterococcus faecium* LR/6, medium optimization, plackett-Burman design, response surface methodology.

INTRODUCTION

Lactic acid bacteria (LAB) are well known for their capacity to produce bacteriocins. Given the high prevalence and pivotal roles that this group of bacteria plays in food and health, the structure, biosynthesis, genetics, and food applications of LAB bacteriocins have been studied extensively [1-4]. Bacteriocins are ribosomally synthesized bacterial peptides or proteins, which show antimicrobial activity generally against related species. Bacteriocins have been classified into four classes (class I, II, III and IV) based on their genetic and biochemical characteristics [5]. Many kinds of bacteriocins produced by enterococcal strains have also been identified. Most of them were discovered from strains belonging to Enterococcus faecium and E. faecalis [6]. LAB bacteriocins have been investigated extensively for further application as food preservatives pharmaceuticals. The potential of and preservation could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive. Both and more so the latter will necessarily require optimization of their production that may be dependent on multiple factors, and are usually strain specific [7, 8]. Thus, these factors need to be optimized in achieve order to higher production. Productivity of microbial metabolites can be increased by nutritional requirements, physical parameters and genetic make up of the producing strain. Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time keeping the others at fixed level. In comparison, the statistical methods offer several advantages over conventional methods in being rapid and reliable, and that shortlists significant nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time and material [9]. Identification of the

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variables that affect product formation by statistical experimental designs such as Plackett-Burman and RSM can eliminate the limitations of 'one variable at a time' approach [10]. The use of RSM in biotechnological processes is gaining immense importance for the optimization of the production of microbial products and biomolecules [11-14]. There are very few reports on the statistical optimization of bacteriocin production [14,15,16]. In this investigation, optimization of media constituents for enhanced enterocin production was carried out in two steps. First, a Plackett-Burman (PB) design was used to determine the effects media likely of constituents. Subsequently, the factors that had significant effects were optimized using a central composite design and response surface analysis.

MATERIALS AND METHODS

1. Bacterial strains, Culture media, Growth Conditions and Chemicals

Strain LR/6 isolated from soil and identified as Enterococcus faecium (manuscript submitted) was routinely propagated in normal TGYE medium (5 g/l tryptone, 1 g/l glucose, 3 g/l yeast extract, and pH 7.0) (14) at 37°C and 200 in an incubator shaker (Kuhner, rpm This medium was further Switzerland). modified (15 g/l tryptone, 20 g/l glucose, 20 g/l yeast extract, 0.1 g/l MnSO₄, and pH 7.0) to assess the production of bacteriocin by strain LR/6. Micrococcus luteus used as an indicator organism was grown in nutrient broth (5 g/l peptone, 3 g/l beef extract, 5 g/l NaCl, and pH 7.0) [14] under similar conditions. Growth was measured turbidometrically as A₆₃₀ (Genesys Thermospectronic, USA). 10 vis. All chemicals were obtained from Sigma-Aldrich. USA, and all media were purchased from Himedia, Mumbai, India.

2. Bacteriocin Preparation

A log-phase grown culture of strain LR/6 was used to inoculate ($\sim 10^6$ CFU/ml) the modified which TGYE broth. was subsequently incubated at 37°C and 200 rpm over a period of 24 h. Samples were taken at regular time intervals to check the growth, pH and enterocin production. Cells were harvested bv centrifugation at 12000 x g for 10 min at room temperature and the culture filtrate was filtered through 0.2-um collected and membrane. The filtered culture supernatant served as the source of bacteriocin that was labeled as enterocin.

3. Antimicrobial Assay

The antimicrobial activity was quantified by microtitre plate assay [14]. Each well of the plate contained 200-µl of nutrient broth with the indicator organism ($A_{630} = 0.02$), to which was added enterocin fractions at two-fold dilution in 50-µl of nutrient broth. The plates were incubated for 6 h at 37°C and the growth of the indicator organism was measured spectrophotometrically as A_{630} with Microplate Reader (Bio-Rad, USA). One bacteriocin unit (AU/ml) was arbitrarily defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50% in comparison to an untreated control.

In parallel, antimicrobial activity was also checked by agar-well diffusion assay (AWDA) method [14]. Aliquots (100- μ l) of the sterile enterocin were placed in 6-mm diameter wells that had been cut in 0.8% soft agar plates seeded with the indicator bacteria (~10⁸ CFU/ml). After overnight incubation, diameter of the zone of growth inhibition was measured.

4. Formulation of an Optimum Medium using Plackett-Burman Design

The present study was aimed at the designing of an optimum medium for an enhanced production of the enterocin. For this purpose, the modified TGYE was used as the basal medium and was further supplemented with some simple media constituents of MRS (de Man-Rogosa-Sharpe) medium, a medium commonly used for the growth of LAB. In order to study the effects of these constituents on enterocin production from E. faecium LR/6, a Plackett-Burman design was used [17] as shown in Table 1. The PB design has proved very effective and is widely used to identify significant variables (>5) with minimum of testing [18]. The total number of experiments to be carried out according to Plackett-Burman design is n+1, where n is the number of variables. Each variable is represented at two levels, high and low denoted by (+) and (-), respectively. Each column should contain equal number of positive and negative signs. A total of eleven factors or variables were screened in twelve trials. Thus, the effect of each variable is the difference between the average of the measurements made at the high and the average of the measurements made at the low level of that factor, which was determined by the equation:

	Variables							Response				
Run	Glucose	Yeast	Tryptone	Tween-	Triammonium	Sodium	MgSO ₄	MnSO ₄	K ₂ HPO ₄	pН	Incubation	Bacteriocin
no.	(g/l)	extract	(g/l)	80 (g/l)	citrate (g/l)	acetate	(g/l)	(g/l)	(g/l)		period (h)	production
		(g/l)				(g/l)						(AU/ml)
1	30 (+)	30 (+)	5 (-)	2.5 (+)	0.3 (-)	1 (-)	0.1 (-)	0.05 (-)	2.5 (+)	8 (+)	24 (+)	204 ± 8.84
2	30 (+)	30 (+)	5 (-)	2.5 (+)	2.5 (+)	1 (-)	1.0 (+)	0.25 (+)	0.5 (-)	6 (-)	12 (-)	257 ± 10.42
3	10 (-)	30 (+)	25 (+)	0.5 (-)	2.5 (+)	1 (-)	0.1 (-)	0.25 (+)	0.5 (-)	8 (+)	24 (+)	201 ± 7.89
4	10 (-)	10 (-)	2.5 (+)	2.5 (+)	2.5 (+)	1 (-)	1.0 (+)	0.05 (-)	2.5 (+)	8 (+)	12 (-)	208 ± 5.30
5	30 (+)	30 (+)	25 (+)	0.5 (-)	2.5 (+)	10 (+)	0.1 (-)	0.05 (-)	2.5 (+)	6 (-)	12 (-)	502 ± 6.80
6	10 (-)	10 (-)	5 (-)	0.5 (-)	0.3 (-)	1 (-)	0.1 (-)	0.05 (-)	0.5 (-)	6 (-)	12 (-)	196 ± 4.90
7	30 (+)	10 (-)	25 (+)	0.5 (-)	0.3 (-)	1 (-)	1.0 (+)	0.25 (+)	2.5 (+)	6 (-)	24 (+)	241 ± 7.02
8	10 (-)	10 (-)	5 (-)	2.5 (+)	2.5 (+)	10 (+)	0.1 (-)	0.25 (+)	2.5 (+)	6 (-)	24 (+)	465 ± 22.8
9	30 (+)	10 (-)	25 (+)	2.5 (+)	0.3 (-)	10 (+)	0.1 (-)	0.25 (+)	0.5 (-)	8 (+)	12 (-)	248 ± 6.66
10	10 (-)	30 (+)	25 (+)	2.5 (+)	0.3 (-)	10 (+)	1.0 (+)	0.05 (-)	0.5 (-)	6 (-)	24 (+)	159 ± 4.30
11	10 (-)	30 (+)	5 (-)	0.5 (-)	0.3 (-)	10 (+)	1.0 (+)	0.25 (+)	2.5 (+)	8 (+)	12 (-)	416 ± 20.50
12	30 (+)	10 (-)	5 (-)	0.5 (-)	2.5 (+)	1.0 (+)	1.0 (+)	0.05 (-)	0.5 (-)	8 (+)	24 (+)	470 ± 18.80

Table 1. Plackett-Burman design for bacteriocin production by Enterococcus faecium LR/6

$$E_{(Xi)} = \underbrace{\frac{2(\Sigma Pi^{+} - \Sigma Pi^{-})}{N}}_{N}$$
(1)

Where $E_{(xi)}$ is the concentration effect of the tested variables. P_{i+} and P_{i-} represent the enterocin activities of the trials where the variable (*Xi*) measured was present at the high or low level, respectively, and the 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 constituent was determined using student's t-test as given by the equation:

$$t(x_i) = E(x_i) / SE$$
(2)

Where E (x_i) is the effect of variable X_i

5. Optimization of Critical Variables using Response Surface Methodology

The critical variables so identified, sodium acetate. dipotassium hydrogen phosphate, triammonium citrate and tween-80 were further optimized by response surface methodology (RSM) based on central composite design (CCD) experimental plan. The statistical software package Design-Expert (Version 6.0.7, Stat-Ease, Minneapolis, Minn., USA) was used to study their interactions and to find out their optimal values. Each variable in the design was studied at five different levels as presented in **Table 3.** A 2^4 factorial design, with eight axial points and six replicates at the centre point with a total number of 30 experiments were employed as shown in Table 4. The behavior of the system was explained by the following quadratic equation:

 $KUMAR \ M., \ et \ al.$ $Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_1 \beta_2 A B + \beta_1 \beta_3 A C + \beta_1 \beta_4 A D + \beta_2 \beta_3 B C + \beta_2 \beta_4 B D + \beta_3 \beta_4 C D$ (3)

Where *Y* is predicted response, β_0 is the intercept, β_1 , β_2 , β_3 , β_4 are linear coefficients, β_{11} , β_{22} , β_{33} and β_{44} are squared coefficients, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$ and $\beta_3\beta_4$ are interaction coefficients and *A*, *B*, *C*, *D*, *A*², *B*², *C*², *D*², *AB*, *AC*, *AD*, *BC* and *CD* are independent variables.

Table 2.	Results	of re	egression	analysis	for	the
Plackett-	Burman	desi	gn.			

Terms	Coefficient	F-value	P-value
Intercept	297.25	37.78	0.0063
А	23.08	11.06	0.0449
В	-7.42	1.14	0.3637
С	-37.42	29.05	0.0125
D	-40.42	33.90	0.0101
E	53.25	58.84	0.0046
F	79.42	130.87	0.0014
G	-5.42	0.61	0.4921
Н	-8.49	1.42	0.4444
Ι	42.08	36.75	0.0090
J	-5.10	0.67	0.5627
Κ	-7.92	1.49	0.4373
0 00 1	614	(\mathbf{D}^2) 0.00	

Coefficient of determination $(R^2) = 0.99$

A- glucose, B- yeast extract, C- tryptone, D- tween-80, E- triammonium citrate, F- sodium acetate, G-MgSO₄, H-MnSO₄, I-K₂HPO₄, J-pH, K-incubation period.

Independent					
Variables –	-α	-1	0	+1	$+\alpha$
А	2.5	6.9	11.3	15.6	20
В	1.0	2.0	3.0	4.0	5
С	1.0	2.0	3.0	4.0	5
D	0.1	0.5	1.0	1.5	2

Table 3. Range of variables used for the response surface methodology. A-sodium acetate (g/l), B-dipotassium hydrogen phosphate (g/l), C-triammonium citrate (g/l), D-tween-80 (g/l).

6. Large Scale Production of Bacteriocin

The feasibility of enhanced bacteriocin production was tested in shake flasks of varied culture volumes (0.01-0.50 l) under optimized conditions. Strain LR/6 was grown in

optimized TGYE medium at 37°C and 200 rpm, for 18h. The cell free culture supernatant was used for the determination of antimicrobial activity.

Run no.	А	В	С	D	Bacteriocin p (AU/n	roduction nl)
					Experimental	Predicted
1	20.0	3.0	3.0	1.0	258.00 ± 8.80	266.37
2	11.3	3.0	3.0	1.0	225.00 ± 10.20	242.25
3	11.3	3.0	3.0	2.0	136.00 ± 4.80	131.17
4	2.5	3.0	3.0	1.0	217.00 ± 6.60	225.79
5	15.6	2.0	4.0	1.5	358.00 ± 18.80	378.83
6	15.6	4.0	4.0	1.5	309.00 ± 20.10	324.46
7	6.9	4.0	4.0	1.5	278.00 ± 4.90	272.38
8	15.6	4.0	2.0	0.5	343.00 ± 12.00	336.75
9	11.3	3.0	3.0	0.1	382.00 ± 10.80	391.83
10	11.3	3.0	3.0	1.0	302.00 ± 16.80	327.46
11	11.3	1.0	3.0	1.0	302.00 ± 12.40	306.38
12	11.3	3.0	3.0	1.0	378.00 ± 20.40	360.75
13	11.3	3.0	1.0	1.0	350.00 ± 18.90	361.04
14	11.3	3.0	5.0	1.0	258.00 ± 5.50	266.42
15	15.6	2.0	2.0	1.5	318.00 ± 12.80	304.33
16	6.9	2.0	2.0	1.5	317.00 ± 19.75	328.46
17	15.6	4.0	2.0	1.5	367.00 ± 25.08	334.29
18	15.6	2.0	2.0	0.5	325.00 ± 14.70	334.29
19	11.3	3.0	3.0	1.0	596.00 ± 30.41	549.37
20	15.6	4.0	4.0	0.5	453.00 ± 22.80	476.21
21	11.3	5.0	3.0	1.0	130.00 ± 6.80	115.71
22	6.9	4.0	2.0	1.5	205.00 ± 10.50	195.88
23	15.6	2.0	4.0	0.5	160.00 ± 8.98	144.71
24	11.3	3.0	3.0	1.0	270.00 ± 12.70	261.88
25	6.9	2.0	4.0	1.5	379.00 ± 20.40	385.50
26	6.9	4.0	4.0	0.5	380.00 ± 25.80	385.50
27	6.9	4.0	2.0	0.5	394.00 ± 18.21	385.50
28	6.9	2.0	2.0	0.5	380.00 ± 30.40	385.50
29	11.3	3.0	3.0	1.0	390.00 ± 18.30	385.50
30	6.9	2.0	0.40	0.05	390.00 ± 18.40	385.50

Table 4. Experimental design and results of central composite design of RSM A- sodium acetate (g/l), B- dipotassium hydrogen phosphate (g/l), C-triammonium citrate (g/l), D-tween-80 (g/l)

7. Statistical Analysis

Each value is expressed as mean along with respective standard error of mean. Each data point is the average of three repeated measurements from two independent replicates.

RESULTS AND DISCUSSION

Bacteriocin production by *E. faecium* LR/6 was studied in TGYE medium, which was further, optimized using two statistical tools (Plackett-Burman design and RSM). Plackett-Burman design is a widely used statistical design for the screening of the media constituents [17]. The design screens important variables that may affect the production of a microbial compound as well as their significant levels, but does not consider the interactive effects among the variables as in RSM. In RSM, each selected variable is studied at five different levels along with other variables, and therefore, the interactions among the variables at their different levels could be studied. When grown in modified TGYE, *E. faecium* LR/6 produced 300 AU/ml of enterocin (**Figure 1**). In order to enhance the

enterocin production, experiments were designed to optimize media constituents using Plackett-Burman experimental design. Table 1 presents the Plackett-Burman design for 11 culture variables and their corresponding response in terms of bacteriocin production, while Table 2 presents the 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 159 to 502 AU/ml, reiterating the importance of selection and identification of important factors. The pareto graph was drawn to show the effect of all variables on bacteriocin production (Figure 2). As is clear from this figure, sodium acetate had the major influence on the bacteriocin production, followed by dipotassium hydrogen phosphate, and triammonium citrate. Tween-80 and tryptone, on the other hand, had a significant negative influence. Other variables had only a minor contribution towards bacteriocin production. From the regression analysis also, it was found that bacteriocin production was affected by these factors as indicated by their corresponding F and *p*-values (**Table 2**). Though this analysis identified tween-80 as a significant factor, its inclusion was based on the surfactant nature of tween-80, allowing a better dissipation of the bacteriocin molecules in the culture supernatant as suggested earlier [7]. These variables had confidence level above 95% in comparison to

other variables and thus, were considered to be highly significant for bacteriocin production by E. faecium LR/6. In literature, such factors have similarly identified been for bacteriocin production from Lactobacillus plantarum LR/14 [14] and L. lactis [19]. These factors are highly variable, reiterating the fact that they are strainand product-specific. The variables so identified Plackett-Burman design were further bv optimized by RSM using CCD experimental plan (Table 3). 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. Based on the central composite design, the experimental levels of bacteriocin production under each set of condition was determined and compared with the corresponding predicted levels (Table 4). The maximum experimental value for bacteriocin production was 596 AU/ml, while the predicted response based on RSM was estimated to be 549.37 AU/ml. The close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. 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 multiple regression analysis on the experimental data, the following equation was found to explain bacteriocin production:

 $Y = +385.50 - 18.29B + 20.04C + 29.29D - 12.80A^{2} + 31.82B^{2} - 57.43C^{2} - 45.55D^{2} + 29.69AB - 7.56AC - 10.06AD + 7.19BC + 12.44BD - 35.81CD (4)$



Figure 1. Time course of bacteriocin production and growth of *Enterococcus faecium* LR/6 in optimized (closed square, closed inverted triangle) and modified (open circle, open triangle) TGYE medium.



Figure 2. Pareto graph showing the contribution of different variables on enterocin production

The coefficient of determination in terms of predicted R^2 is 0.8683 that is in close agreement with adjusted R^2 of 0.9505. 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. Thus, the estimated adequate precision of 29.408 for bacteriocin production, representing the signal to noise ratio, is an adequate signal. The model *F* value was 43.87 and the P > F (<0.0001) indicating that the model terms are significant. In other words, *A*, *B*, *C*, *D*, *A*², *B*², *C*², *D*², *AB*, *AC*, *AD*, *BC*, *BD*, *CD* are significant model terms for bacteriocin production. The corresponding ANOVA is presented in **Table 5**.

Source	Sum of	Degree of	Mean square	F value	P value
	square	freedom			
Model	2.657E+005	13	20442.05	43.87	< 0.0001
Residual	7455.96	16	466.00		
Pure Error	215.50	5	43.10		
Cor Total	2.732E+005	29			

Table 5. Analysis of variance (ANOVA) and regression analysis for selected model.

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 reached the optimum level, beyond which a decline could be observed (**Figure 3 a-c**). The optimal values obtained from the contour plots were almost equal to the results obtained by the regression analysis (equation 4). The optimum values of the tested variables derived were: sodium acetate 11 g/l, dipotassium hydrogen phosphate 3.0 g/l, triammonium citrate 1.0 g/l, and tween-80 1.0 g/l. The model was further validated by repeating the experiment under

these optimized conditions, which resulted in bacteriocin production of 606 ± 30.82 AU/ml (closely similar to predicted response of 549.37 AU/ml). Therefore, bacteriocin production could be enhanced ~2-fold by media constituent's optimization using statistical tools as compared to the modified TGYE as shown in **Figure 1**. Similarly, enhancements in bacteriocin production by L. plantarum LR/14 [14] and Lactococcus lactis [19] through medium optimization have been reported. Increase in bacteriocin production by statistical optimization of culture parameters

Bacteriocin production (AU/ml

has also been reported in L. plantarum LPCO10 [15]. These authors have reported a 3.2×10^4 times more bacteriocin when grown in a fermentor using a different set of factors. This suggests the strain-to-strain differences as well as the conditions applied for such an optimization and, therefore, needs to be determined for each strain. Moreover, the strong correlation between experimental and predicted levels of bacteriocin production by E. faecium LR/6 under the optimized conditions also reflected the efficacy of such statistical tools.







Figure 3. Three-dimensional graph showing the effect and interaction of sodium acetate and triammonium citrate (a), K_2 HPO₄ and tween-80 (b) and K_2 HPO₄ and sodium acetate (c) on enterocin production from E. faecium LR/6

Enterocin production from E. faecium LR/6 was sustainable in Erlenmeyer flasks of varied culture volumes (0.02-0.5 l), suggesting a good scope for maintenance of enhanced production (Table 6).

The present work, therefore, has established the utility of the PB and RSM tools in optimizing the media constituents for enhancing the production of enterocin from E. faecium LR/6.

meatum		
Flask volume (l)	Culture volume (l)	Bacteriocin production (AU/ml±SD)
0.100	0.02	606 ± 30.82
0.250	0.05	590 ± 18.91
0.500	0.10	602 ± 28.30
1.000	0.20	596 ± 40.31
2 000	0.50	610 ± 31.8

Table 6. Bacteriocin productions in shake flasks carrying different volumes of the culturemedium

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