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ORIGINAL ARTICLE

Statistical optimization of fermentative medium for enhanced bacteriocin production by *Lactococcus lactis* subsp. lactis R10

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ABSTRACT
Complex growth medium such as deMan Rogosa Sharpe (MRS) medium, commonly used for cultivation of fastidious lactic acid bacteria. In the present study, composition of the MRS medium for the production of bacteriocin by Lactococcus lactis subsp. lactis R10, an isolate from radish (Raphanus sativus) was optimized using response surface methodology. Temperature, K_2HPO_4 and $(NH_4)_3C_6H_5O_7$ were identified as the most important factors for bacteriocin production using Plackett- Burman design. Therefore, these three foremost factors were further optimized by response surface methodology to achieve efficient yield. The optimum MRS composition was found to be sucrose 8 g/l, peptone 3 g/l, beef extract 10 g/l, yeast extract 8 g/l, sodium acetate 8 g/l, triammonium citrate 4 g/l, K_2HPO_4 2 g/l, tween 80 0.5 ml/l, magnesium sulphate 0.2 g/l, manganese sulphate 0.05 g/l, pH 6.5 and temperature 37°C. The optimized growth media allowed higher amount of bacteriocin activity (838.00 AU/ml) production which were 1.3 fold higher than the unmodified MRS media.
Key words: *Bacteriocin, Response surface methodology, Central composite design, Lactococcus lactis subsp. lactis R10.*

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INTRODUCTION

In recent years, the consumption of foods formulated with chemical preservatives has increased consumer concern due to health effects, and created a demand for more natural and minimally processed foods. As a result, there has been a great interest in naturally produced antimicrobial agents for their application in food preservation (1). Lactic acid bacteria (LAB) are an industrially important group of microorganism with GRAS (Generally Recognized As Safe) status and are associated with meat, dairy and vegetable fermentation. LAB produces a wide variety of antagonistic factors that include metabolic end products such as lactic acid, antibiotic-like substances and antimicrobial proteins or bacteriocins. Bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial peptides, which display a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria. LAB bacteriocins have attracted growing interest in recent years because of their potential usage as biopreservatives in the food industry to eradicate food spoilage and food-borne pathogenic bacteria (2). The potential could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive. Both will necessarily require optimization of their production, which may be dependent on multiple factors, and are usually strain specific (3).The bacteriocin, nisin (or group N inhibitory substance),discovered in England by Rogers and Whittier in 1928, is produced by certain strains of *Lactococcus lactis* subsp. *Lactis* that effectively inhibits Gram-positive and Gram-negative bacteria and also the out growth of spores of Bacilli and Clostridia. It is composed of 34 amino acids and approved in more than 50 countries as a natural food preservative such as processed cheese, beverages and canned foods (4). Nisin is the only bacteriocin approved for food applications being considered to be safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969. As a result, the field has developed increasingly, resulting in the discovery and detailed characterization of a great number of bacteriocins from LAB in the last few decades (5). Bacteriocin producing species have now been identified among all the genera that comprise the LAB including *Lactobacillus, Pediococcus, Lactococcus, Streptococcus, Leuconostoc* and *Carnobacterium* as well as several *Enterococcus* species.

Optimal bacteriocin production in batch fermentation usually requires complex media and well-controlled physical conditions, including temperature and pH. However, optimization of a medium by the classical method involves changing one independent variable at a time while keeping others at a fixed level. This is extremely time-consuming and expensive for a large number of variables and may also result in unacceptable conclusions (6). Studies on multiple factors affecting the production of bacteriocins are relatively scarce and it is difficult to optimize them for biotechnological processes. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses. RSM has been successfully applied in many areas of biotechnology, including some reports on bacteriocin production (7). Therefore, the aim of this work was to optimize a culture media for the production of bacteriocin by *Lactococcus lactis* subsp. *lactis* R10. For this reason, Central Composite design was applied in order to determine the effects of MRS media components on bacteriocin production.

MATERIALS AND METHODS

Bacterial strains, culture media, Growth conditions

The bacteriocinogenic strain *L. lactis* subsp. *lactis* R10 was isolated from fermented radish (*Raphanus sativus)*. The strain was identified by sequencing of 16S rRNA gene followed by blast homology search. Modified MRS media containing sucrose 12 g/l, peptone 10 g/l, beef extract 10 g/l, yeast extract 5 g/l, dipotassium hydrogen phosphate 2 g/l, tween 80 1ml/l, triammonium citrate 2 g/l, sodium acetate 5 g/l, magnesium sulphate 0.2 g/l, manganese sulphate 0.05 g/l and pH 6.5 were used for maximum bacteriocin production by *L. lactis* subsp. *lactis* R10. *Bacillus subtilis* was used as test organism and grown in Nutrient agar (NA) media at 30°C.

Bacteriocin assay

Bacteriocin activity was determined by using agar well diffusion assay (8). The supernatant of 36 h grown culture (from modified sucrose MRS broth) was centrifuged at 12, 000 x g for 15 min at 4°C, then supernatant was neutralized with sterile 5M NaOH. Aliquots (50 µl) of culture supernatants were applied to disks on agar plates previously inoculated with a cell suspension of *B. subtilis* (10^6 CFU/ml). The NA plates were incubated at 30˚C for 24 to 48 h and the diameter of inhibition zone around the wells was measured with a calliper. Bacteriocin activity was expressed as arbitrary units (AU ml^{-1}) and defined as the reciprocal of the highest two fold serial dilution showing a distinct zone of inhibition.

Bacteriocin activity (AU/ml) = 2^n x100

n- Highest dilution showing growth inhibition zone

Central composite design (CCD)

Based on the results of Plackett-Burman Design, 3 variables were chosen in this experiment were K_2HPO_4, $(NH_4)_3C_6H_5O_7$ and temperature. Taking the above factors into consideration, central composite design based on 5 levels and 3 variables (K_2HPO_4, $(NH_4)_3C_6H_5O_7$ and temperature) were used to study their combined influence on bacteriocin production by *L. lactis* subsp. *lactis* R10 (Table 1). The design consisted of 20 experiments with 8 factorial points, 6 axial points with α = 1 .682 and 6 center points for replication. In developing the regression equation, the test factors were coded according to the following equation;

$$x_i = \frac{Xi - Xo}{\delta Xi}$$

where, x_i is the dimensionless coded value of the *i*th independent variable; X_i the natural value of the *i*th independent variable; X_0 the natural value of the *i*th independent variable at the center point and δX_i the step-change value. The experimental results were fitted with a second order polynomial function

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

where, *Y* is the predicted response, b_0 the intercept, b_1 , b_2 , b_3 the linear coefficients, b_{11},b_{22}, b_{33} the squared coefficients and b_{12}, b_{13}, b_{23} the interaction coefficients.

Data was analyzed by the statistical software package Design Expert (8.0.6, Stat-Ease, Minneapolis, Minn. USA). The fit quality of the model was evaluated by R^2 and Analysis of variance (ANOVA). Statistical testing of the model was done by Fisher's statistical test. The robustness of the model and optimal values of parameters was assessed by the determination coefficient, correlation coefficient (R) or F- test (9).

Table 1- Natural levels, codes and intervals of variation of the independent variables in the design of experiments

Independent variables	Symbol	Range and levels				
		-α	-1	0	+1	+ α
Di-potassium hydrogen phosphate (g/l) (K_2HPO_4)	A	1.32	2.00	3.00	4.00	4.68
Triammonium citrate (g/l) ($(NH_4)_3C_6H_5O_7$)	B	1.32	2.00	3.00	4.00	4.68
Temperature (°C)	C	30.30	32.00	34.50	37.00	38.70

RESULTS AND DISCUSSION

Bacteriocin production depends on temperature, $K_2HPO_4$and $(NH_4)_3C_6H_5O_7$ based on the results of the Plackett-Burman design were further optimized by RSM using the CCD experimental plan. The results obtained were fed into the Design Expert software and analyzed using Analysis of Variance (ANOVA) as appropriate to the experimental design used. Based on the central composite design, the experimental levels of bacteriocin production was determined and compared with the corresponding predicted levels (Table 2). The maximum experimental value for bacteriocin production was 838.00 AU/ml, while the predicted response based on RSM was estimated to be 832.36 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 medium components assessed the bacteriocin activity as a function of the variables. By applying multiple regression analysis on the experimental data, the following coded and actual final equations were found to explain bacteriocin activity.

Bacteriocin activity (AU/ml) = 815.30 + 8.854 * A + 20.492 * B + 33.126 * C + 5.75 * A * B - 29.75 * A * C + 3.5 * B * C - 14.75 * A^2 - 7.5 * B^2 - 32.95 *C^2 ----------2

Bacteriocin activity (AU/ml) = - 7241.71 + 490.6542* K_2HPO_4 - 0.0438* $(NH_4)_3C_6H_5O_7$+ 408.6085* temperature + 5.75 * K_2HPO_4 * $(NH_4)_3C_6H_5O_7$-11.9 * K_2HPO_4 * temperature + 1.4 * $(NH_4)_3C_6H_5O_7$* temperature - 14.7501 * $K_2HPO_4^2$ - 7.50229 * $(NH_4)_3C_6H_5O_7^2$ - 5.2733 * $temperature^2$-----3

The quadratic model in Eqs. (2) and Eqs. (3) with 9 terms contain 3 linear terms, 3 two-factor interactions and 3 quadratic terms. Adequacy and fitness of bacteriocin activity was evaluated by standard analysis of variance (ANOVA). From the ANOVA, the linear terms A, B, C and interaction term AC and quadratic terms A^2, B^2 and C^2 were statistically significant at ($p<0.05$) in bacteriocin activity. The corresponding ANOVA is presented in Table 3. The *p*-value of "lack of fit" was 0.0690 ($p>0.01$) which indicated that it was insignificant relative to the pure error. The value of the adjusted determination coefficient (Adj R^2 = 0.9704) also confirmed that the model was highly significant. The significance of each co-efficient was determined by student's t-test (Table 4). Analysis of variance and Fischer's F-test showed that the $F_{(9, 10)}$= 70.27 which is greater than the tabulated value of 3.02 thereby demonstrating significance for the regression model. The smaller the p- value and larger the t- value, the more significant is the corresponding co-efficient[9]. Student's t- test showed that all the linear co-efficients were significant. These values suggest that these factors have a direct relationship on the production of bacteriocin. The sign and magnitude of co-efficients indicate the effect of the variable on the response

The fitness of the model was examined by determination coefficient (R^2) for bacteriocin activity as (0.9844), which implied that more than 98.44% of the sample variation was attributed to the variables and only 1.6% of the total variance could not be explained by the model. The high F value (70.27) and a very low probability (p>F=0.005) indicated that the present model for bacteriocin activity showed good agreement between predicted and experimental results. 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 27.24 for bacteriocin production, representing the signal to noise ratio, is an adequate signal.

Two-dimensional contour surface graphs were generated for the pair-wise combination of the three factors while keeping the other one at its centre levels for the bacteriocin production (Fig. 1-3). The 2D contour surface plot in (Fig. 1) exhibits the behaviour of bacteriocin production (AU/ml) with respect to changes in the K_2HPO_4 and $(NH_4)_3C_6H_5O_7$. The graph suggests that increased levels of bacteriocin activity were obtained by increasing concentrations of K_2HPO_4 and $(NH_4)_3C_6H_5O_7$. Similarly, the response surface plot in (Fig. 2) describes bacteriocin production with respect to changes in K_2HPO_4 and temperature, while $(NH_4)_3C_6H_5O_7$ was constant (3.00 g/l). Higher bacteriocin production was observed with increasing

temperature from 35-37°C, while decreasing K_2HPO_4 from 4-2 g/l. The response surface plot in (Fig.3) shows the effect on bacteriocin production with respect to changes in $(NH_4)_3C_6H_5O_7$ and temperature, while K_2HPO_4 was constant (3.00 g/l). The maximum bacteriocin production occurred at increasing concentration of $(NH_4)_3C_6H_5O_7$ and temperature. A plotting of the normal values versus residuals of bacteriocin production by *L. lactis* subsp. *lactis* R 10 showed that data were very close to the straight line and situated at both sides of its indicating that model is fairly good (Fig.4). Generally, bacteriocin production by LAB is reported as a temperature-sensitive process, whereby the optimal temperature for bacteriocin production does not necessarily coincide with the optimal growth temperature (10). It has been suggested that bacteriocin production by LAB is enhanced by suboptimal temperatures (11-12). Inorganic phosphate was also previously shown to be a positive factor for lacticin LLC518 production by *Lactococcus lactis* subsp. *lactis* LLC518 using response surface methodology (13), which is consistent with the results of bacteriocin production by *Lactobacillus rhamnosus* PEN (14). De Vuyst and Vandamme reported that potassium dihydrogen phosphate was able to improve cell growth and nisin synthesis (15). Nilsson et al., studied that acetate induced the antilisterial bacteriocin production by *Carnobacterium piscicola* A9b (16).

The shape of the response surface plots indicated the mutual interaction effects between the test variables. An elliptical and saddle nature of the surface plots indicates the significance of the interactions between the corresponding variables; otherwise, if it is circular, the mutual interaction effect is non-significant. From the elliptical response surface plot in Fig.5, the mutual interaction effect between K_2HPO_4 and temperature was significant for bacteriocin production. Similarly, enhancements in bacteriocin production by *Lactobacillus plantarum* LR/14 (17) and *Lactococcus lactis* (18) through medium optimization have been reported. 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 *Lactococcus lactis* subsp*. lactis* R10 under the optimized condition also reflected the efficacy of such statistical tools.

Table 2 - Central composite design matrix of MRS media component of independent variables and their corresponding experimental and predicted values of bacteriocin activity

Standard order	Coded level			Bacteriocin activity (AU/ml)	
	A	B	C	Observed	Predicted
1	-1	-1	-1	670.00	677.12
2	+1	-1	-1	738.00	742.82
3	-1	+1	-1	694.00	699.6
4	+1	+1	-1	792.00	788.31
5	-1	-1	+1	793.00	795.87
6	+1	-1	+1	749.00	742.58
7	-1	+1	+1	838.00	832.36
8	+1	+1	+1	810.00	802.06
9	-1.682	0	0	765.00	758.69
10	+1.682	0	0	781.00	788.47
11	0	-1.682	0	765.00	759.62
12	0	+1.682	0	822.00	828.54
13	0	0	-1.682	675.00	666.37
14	0	0	+1.682	768.00	777.79
15	0	0	0	811.00	815.30
16	0	0	0	819.00	815.30
17	0	0	0	807.00	815.30
18	0	0	0	818.00	815.30
19	0	0	0	821.00	815.30
20	0	0	0	816.00	815.30

Table 3 - Analysis of variance (ANOVA) for optimization of MRS media component for bacteriocin production by *L. lactis* subsp. *lactis* R10

Source	Sum of Squares	Degree of Freedom	Mean Square	F- Value	p-value
Model	46956.34	9	5217.371	70.27168	<0.0001**
K_2HPO_4 (A)	1070.445	1	1070.445	14.4176	0.0035*
$(NH_4)_3C_6H_5O_7$ (B)	5735.058	1	5735.058	77.24429	<0.0001**
Temperature (C)	14986.75	1	14986.75	201.8534	< 0.0001**
AB	264.5	1	264.5	3.562495	0.0884
AC	7080.5	1	7080.5	95.36577	< 0.0001**
BC	98	1	98	1.319942	0.2773
A^2	3135.416	1	3135.416	42.23026	< 0.0001**
B^2	811.1312	1	811.1312	10.92496	0.0079*
C^2	15654.1	1	15654.1	210.8419	<0.0001**
Residual	742.4571	10	74.24571		
Lack of Fit	601.1238	5	120.2248	4.253235	0.0690
Pure Error	141.3333	5	28.26667		
Corrected Total	47698.8	19			
R^2= 98.44%; Table $F_{9.10(1\%)}$ = 3.02					

Table 4 - Regression coefficient for optimization of media component for bacteriocin production by *L. lactis* subsp. *lactis* R10

Term	Co efficient	Standard error	T - value
Intercept	815.30	3.514	232.279
A	8.85	2.332	3.798
B	20.49	2.332	8.794
C	33.13	2.332	14.218
AB	5.75	3.046	1.885
AC	-29.75	3.046	-9.754
BC	3.50	3.046	1.147
A^2	-14.75	2.269	-6.497
B^2	-7.50	2.269	-3.304
C^2	-32.96	2.269	-14.519

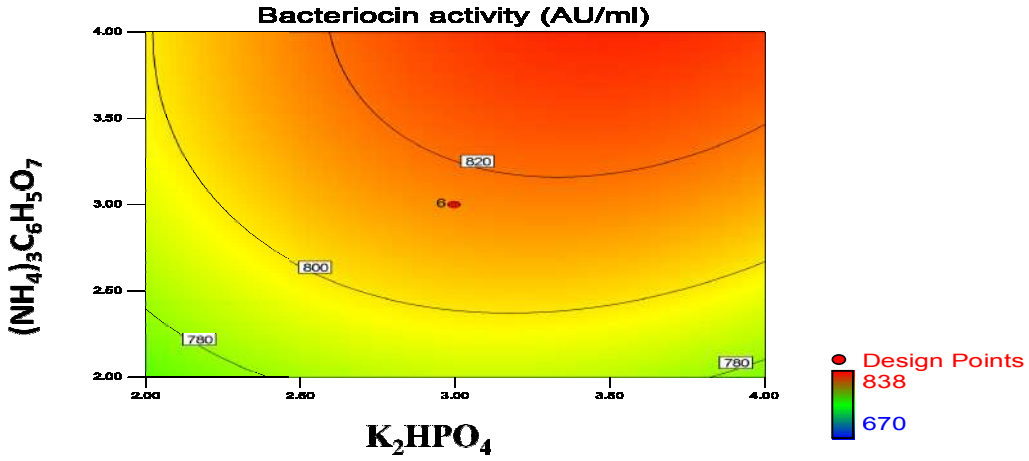

Fig. 1 - 2D contour plot showing the effect of K_2HPO_4 and $(NH_4)_3C_6H_5O_7$ on bacteriocin production by *L. lactic* subsp. *lactis* R10

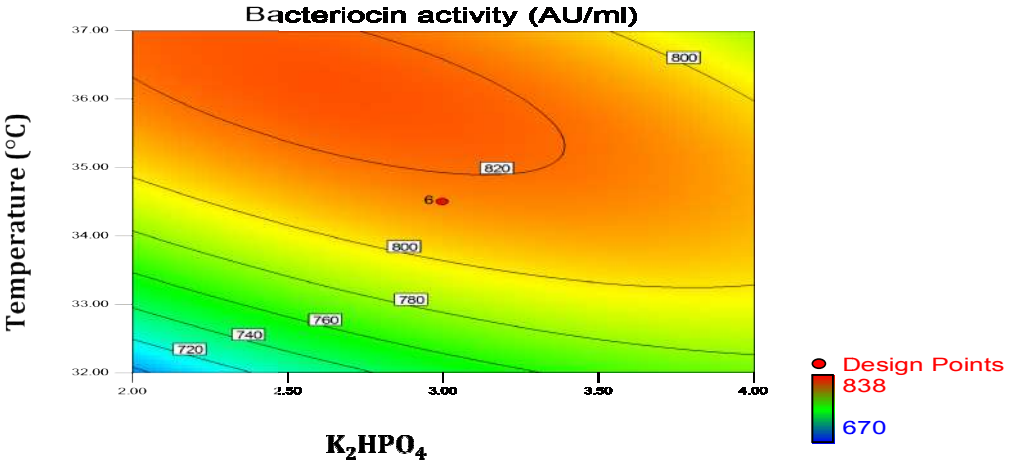

Fig. 2- 2D contour plot showing the effect of K_2HPO_4 and temperature on bacteriocin production by *L. lactic* subsp. *lactis* R10

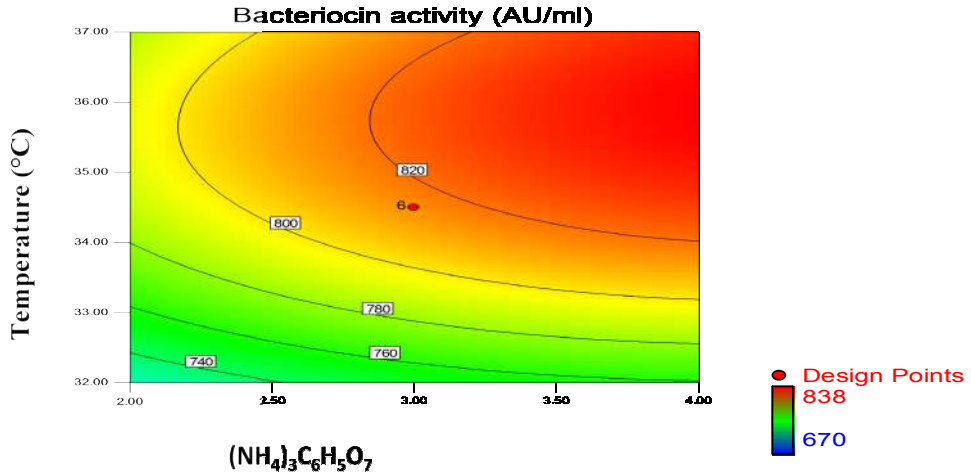

Fig. 3- 2D contour plot showing the effect of triammonium citrate and temperature on bacteriocin production by *L. lactic* subsp. *lactis* R10

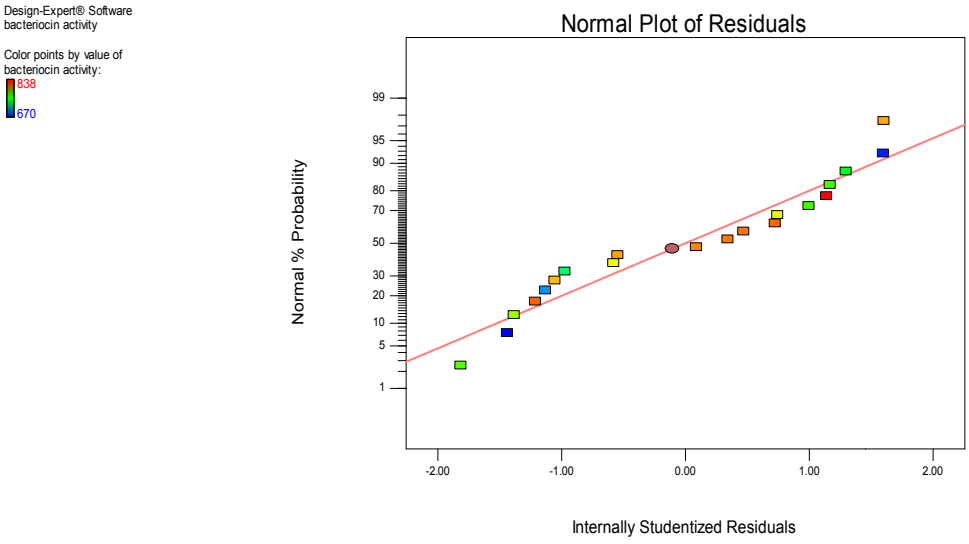

Fig. 4- Plot between expected normal values versus residuals of bacteriocin production by *L. lactic* subsp. *lactis* R10

CONCLUSION

Using the methods of response surface analysis, it was possible to determine optimal operating conditions to obtain more bacteriocin production. The bacteriocin activity was found to be enhanced with optimal concentrations of K_2HPO_4, $(NH_4)_3C_6H_5O_7$ and temperature. The bacteriocin activity predicted was 832.36AU/ml in the optimized MRS media composition of 2 g/l K_2HPO_4, $(NH_4)_3C_6H_5O_7$ 4 g/l and temperature 37ºC. RSM proved to be a powerful tool in optimizing bacteriocin production by *L. lactis* subsp*. lactis* R10.

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