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Research Article

OPTIMIZATION OF BACTERIOCIN PRODUCTION IN *PEDIOCOCCUS ACIDILACTICI* BA28 USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

To enhance production of pediocin BA28, produced by *Pediococcus acidilactici*, cultivation conditions and medium composition were optimized using response surface methodology (RSM). The Plackett Burman experimental design was effective in searching for the significant variables that influence bacteriocin production. From PB Design, three factors peptone, beef extract and initial pH were found to be significant factors and had positive effect on bacteriocin production. The effects of three main factors on bacteriocin production were further investigated by central composite design (CCD). RSM revealed that the maximum bacteriocin production was achieved at peptone concentration of 5% w/v, beef extract concentration of 5% w/v and an initial pH of 6.0. After RSM, the titer of bacteriocin was all most same as obtained in MRS medium which is much costlier than the designed media. Bacteriocin production in a cost effective medium might facilitate industrial scale production of lactic acid bacteriocin and their use as a natural food biopreservative.

Keywords: *Pediococcus acidilactici*, pediocin BA28, response surface methodology, media optimization

INTRODUCTION

Bacteriocin of lactic acid bacteria (LAB) are biologically active, ribosomally synthesized antibacterial peptides that display an antagonistic activity against taxonomically related species and other bacteria of health[1] and pathogenic bacteria such as *Bacteroides, Candida, Escherichia, Enterococcus, Helicobacter, Gardnerella, Klebsiella, Listeria, Neisseria, Propionibacterium, Staphylococci, Streptococci* and *Vibrio*[2]. These biological antimicrobial substances have attached increasing research attention owing to their potential in food biopreservation as purified metabolites, through the use of their producer strains in the starter culture or as adjunct starter culture[3]. Therefore, there has been a continuous need to define the most appropriate condition for bacteriocin production in fermentation media and food systems.

Pediococci are homo-fermentative, Gram positive, non-motile, catalase negative facultative anaerobes of the family *Streptococcaceae.* The *Pediococci* are saprophytes often found in fermenting vegetable material. Many of these organisms are important to the food industry in that they are involved in the variety of food fermentations[4]. These are probiotics involved in reduction of oxygen and inhibition of plant metabolism. These specialized lactic acid producing strain helps to keep a proper balance of micro flora in the digestive system. *Pediococci* are found in foods, plants and as beer-spoilage agents. They grow at a temperature ranging 10° C to 45°C and between pH ranging from 4.5 to 8.0[5].

Bacteriocin production in *Pediococcus* sp. may be dependent on multiple factors and is usually a strain specific phenomenon[6]. Thus, these factors need to be optimized in order to achieve higher productivity of microbial metabolites that can be increased by nutritional supplementations and providing congenial physical environment. Nutritional requirements 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 nutrient, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time and material[7]. The effect of medium composition on bacteriocin production has been extensively studied and there is general agreement that De man/Rogosa sharp (MRS) broth is one of the most suitable media for maximizing growth and bacteriocin production in LAB[8].

Keeping in view the industrial importance of pediocin and advantages of RSM for optimization of critical medium components over conventional method, the present study was undertaken to optimize production media for pediocin BA28 of *Pediococcus acidilactici* BA28. The effect of peptone, beef extract and initial pH were studied.

MATERIALS AND METHODS

Procurement and maintenance of cultures

Pediococcus acidilactici was revived and maintained in MRS medium; pH 6.5 (*Lactobacillus* Heteroferm Screen Broth, Himedia) containing 0.1% Tween-80 at 37ºC. Standard indicator strain of *Listeria monocytogenes* MTCC 657 was procured from MTCC, Chandigarh, India. It was maintained as broth and agar cultures in Brain Heart Infusion broth (Himedia) at 37ºC. *Enterococcus faecalis* lab isolate procured from Prof. R. K. Malik (NDRI, Karnal, India) was grown in MRS medium at 37ºC and maintained as glycerol stocks was also used as indicator strain.

Bacteriocin activity assay

The antimicrobial activity of all bacteriocin preparations was confirmed by the well diffusion assay[9] and disc diffusion method[10]. Bacteriocin activity was expressed as arbitrary unit (AU) and expressed as AU/ml as per standard protocol of Pucci and coworkers[11]. Well diffusion assays were performed using 50 µl of each dilution in the wells that had been cut in 0.75% soft agar plates seeded with indicator strains such as *E. faecalis* and *L. monocytogenes* MTCC 657. After overnight incubation, the antimicrobial activity was demonstrated by clear zone around the wells.

Experimental design

The statistical analysis of the data was performed using Design Expert statistical software version 8.0.7.1 (State–Ease Inc, Minneapolis, MN). Previous reports revealed a constant requirement of dextrose (5% w/v) and tween-80 (0.1% v/v) in the minimal media, an initial pH of 6.5 and incubation temperature of 37°C as constant factors that support bacteriocin production in hetero-fermentative lactic acid bacteria[12].

Plackett Burman experimental design (PBD)

The purpose of the first optimization step was to identify important ingredients of the culture medium. PB design was applied for screening of the significant variables that influence pediocin production. PBD are very useful in identifying the important nutrients and interactions between 2 or more nutrients in relatively few experiments as compared to the one factor at a time technique.

11 media components i.e. yeast extract, peptone, beef extract, meat extract, malt extract, tryptone, ammonium oxalate, tri-ammonium citrate, potassium dihydrogen phosphate, ammonium sulphate and potassium sodium tartarate were selected which were expected to have influence on bacteriocin production[13]. Each of the 11 factors were examined in two levels: low (−1) and high (+1) levels based on Plackett Burman matrix design, which is a fraction of a two-level factorial design and allows the investigation of n−1 variables in at least n experiment. The lower and higher levels of each variable and the design matrix are shown in Table 1.

Table 1:Experimental design and responses of Plackett Burman matrix

S. No.	Peptone (% w/v)	Yeast extract (% w/v)	Beef extract (% w/v)	Meat extract (% w/v)	Tryptone (% w/v)	Malt extract (% w/v)	Amm. Oxalate (% w/v)	Amm. Citrate (% w/v)	KH_2PO_4 (% w/v)	$(NH_4)_2SO_4$ (% w/v)	KNa tartarate (% w/v)	Predicted response (cm)	Experimental response (cm)
1	1	0	0	0	1	0	0.5	0.2	0	0.5	0.2	1.2	1.3
2	1	0	1	1	0	1	0.5	0.2	0	0	0	1.2	1.4
3	0	0	1	0	1	1	0	0.2	0.2	0.5	0	1.2	1.4
4	0	1	1	1	0	0	0	0.2	0	0.5	0.2	1.25	1.4
5	0	0	0	0	0	0	0	0	0	0	0	-	-
6	1	0	1	1	1	0	0	0	0.2	0	0.2	1.3	1.5
7	0	1	0	1	1	0	0.5	0.2	0.2	0	0	0.9	1
8	1	1	0	0	0	1	0	0.2	0.2	0	0.2	0.8	1
9	0	1	1	0	1	1	0.5	0	0	0	0.2	1.2	1.5
10	1	1	0	1	1	1	0	0	0	0.5	0	1.1	1.3
11	1	1	1	0	0	0	0.5	0	0.2	0.5	0	1.3	1.4
12	0	0	0	1	0	1	0.5	0	0.2	0.5	0.2	1.2	1.3

Central Composite Rotatable Design (CCRD)

The second step of media optimization was carried out with three constituents i.e. peptone, beef extract and pH. A rotatable design was used for the investigation CCRD with the full replicate was used to determine the combination of variable levels in each experiment that consisted of 20 experimental sets (Table 2). The relationship among the variables was determined by fitting the second order polynomial equation to bacteriocin responses obtained from 20 experiments.

$$Y = \beta_0 + \Sigma^{k}_{i=1}\beta_1 X_i + \Sigma^{k}_{i=1}\beta_{ii} X^2_i + \Sigma_i \Sigma_j \beta_{ij} X_i X_j$$

Where, Y is the predicted response, K is the number of factor variables, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coeffient a, β_{ij} is the interaction coefficient and X_i is the factor variable in the coded form. If the difference is significant between the mean of the centre points and that of the variable points ($p<0.05$), the optimum will be near or within the experimental design.

Table 2: Experimental design and bacteriocin responses of CCRD matrix

S. No.	Peptone (% w/v)	Beef extract (% w/v)	pH	Predicted response (cm)	Experimental response (cm)
1	5	5	6	1.2	1.4
2	10	10	4	1.1	1.2
3	0	10	4	0.8	-
4	5	5	6	1.2	1.3
5	-3.4	5	6	0.8	-
6	0	0	8	0	-
7	5	13.4	6	1.1	1.1
8	5	-3.4	6	0.9	-
9	10	0	8	0.9	-
10	0	10	8	0.8	-
11	5	5	6	1.2	1.3
12	5	5	6	1.2	1.3
13	5	5	9.36	1.1	1.3
14	10	10	8	1.1	-
15	5	5	2.6	0	-
16	13.40	5	6	1	1.1
17	0	0	4	0	-
18	10	0	4	0.9	-
19	5	5	6	1.2	1.4
20	5	5	6	1.2	1.4

RESULTS AND DISCUSSION

The growth of bacteria and accumulation of cellular metabolites are strongly influenced by growth environment and medium composition such as carbon sources, nitrogen sources, growth factors, and minerals. Search for the major factors and their optimization for biotechnological processes including multi-variables is difficult. The traditional 'one-factor-at-a-time approach' that was used in medium optimization to obtain high yields of the desired metabolites disregards the complex interactions among various physicochemical parameters[14]. Statistically based experimental designs such as Placket Burman design and response surface analysis fulfill this requirement. RSM, an experimental strategy for finding out the optimum conditions among the multivariable system, is a much more efficient technique for optimization of microorganism's metabolites production[15]. This method has been successfully applied to the optimization of medium composition, conditions of bacteriocin production[15-17].

RSM has been successfully used in many studies for optimization of bacteriocin production[17-18]. However, since factors varied among different strains, this work differs from previous studies[17-18] in choosing factors before RSM. Effects of physical factors on bacteriocin production, including temperature and pH[17] were recently studied. The composition of the medium was also shown to have an important role in bacteriocin production[18]. However, studies to reduce the cost of the medium have only been recently conducted[17].

Bacteriocin production by *P. acidilactici* was studied in MRS medium, which was further optimized using two statistical tools. The designs screened important variables that might 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 the software, each selected variable is studied at five different levels along with other variables and therefore, the interaction among the variables at their different levels could be studied.

Plackett Burman responses

In order to enhance the production of pediocin BA28, experiments were designed to optimize media constituents. Table 1 presents the PB design matrix and the corresponding bacteriocin response in term of zone of inhibition and concentration for each ingredient in optimized media was appropriately enlarged as the ranges for the variables.

Out of designed media compositions, sets 1, 2, 3, 4, 6, 9, 10 and 11 showed antimicrobial activity (table 1) and set 6 gave highest yield of bacteriocin production containing peptone (1% w/v), beef extract (1% w/v), meat extract (1% w/v), tryptone (1% w/v), KH_2PO_4 (0.2% w/v), potassium sodium tartarate (0.2% w/v), dextrose (5% w/v) and Tween 80 0.01% w/v (Fig 1).

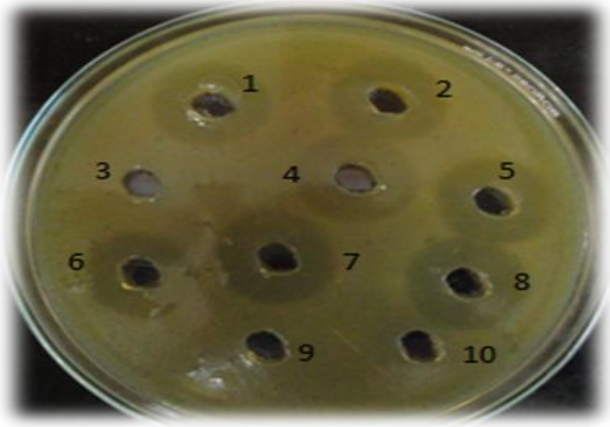

Figure 1: Well diffusion assay showing antimicrobial activity of pediocin BA28; wells 1, 2, 4, 5, 6, 7 and 8 shows zone of inhibition

KH_2PO_4 was further taken as constant factor because it improves the yield of bacteriocin production. According to Todorov and Dicks[19], factors influencing the growth of *Lactobacillus rhamnosus* bacteriocins ST461BZ and ST462BZ which has lead to the conclusion that bacteriocin activity can be increased to 51,200 AU/ml in MRS broth supplemented with 20g/l KH_2PO_4 which gives 25 folds higher production of bacteriocins as compared to the MRS broth without supplementation.

CCRD responses

The results of CCRD experiments for studying the effects of three independent variables peptone, beef extract and pH on pediocin production by *P. acidilactici* are presented in Table 2. The experimental results were fitted to a quadratic model that will enable the prediction of the output response (bacteriocin activity) under optimized medium conditions. The twenty experiments based on CCRD were carried out with different combinations of variables and the results were presented in table 2.

Comparison of predicted and experimental values revealed a good correspondence between them implying that empirical model derived from RSM can be used to adequately describe the relationship between the variable and response (Table 2). Maximum desirability of 100% for bacteriocin production was achieved with composition: peptone (5% w/v), beef extract (5% w/v), KH_2PO_4 (0.2% w/v), dextrose (5% w/v), Tween 80 0.01% w/v and pH 6 at 37˚C for 24 h.

The three dimensional response surface curves were then plotted to graphically show the interaction of factors as function of peptone, beef extract and pH in Fig 2. It showed that the regression coefficients of all the linear term and all Quadratic Coefficients of X1, X2 and X3 were significant at $p>0.001$. It can be observed from the figure that quantities of peptone, beef extract and pH play vital role in the antimicrobial activity of pediocin BA28 as with the increase in quantity of peptone beyond 5% w/v and beef extract, the activity decreases and with the same of pH and peptone. Same was observed with function of pH and beef extract which lead to the conclusion that high quantity of organic nitrogen sources and pH effects the antimicrobial activity of bacteriocin BA28. CCRD run 1, 19 and 20 gave maximum bacteriocin responses 33,000 AU/ml which is in equivalence to standard MRS media proposed for bacteriocin production in lactic acid bacteria.

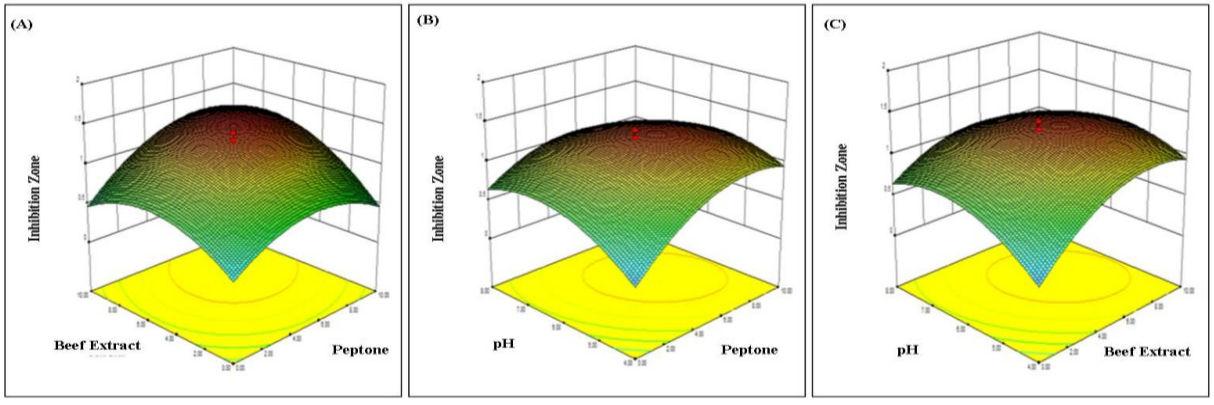

Figure 2: Response surface of pediocin production by *Pediococcus acidilactici* BA28 estimated by zone of inhibition; (A) as function of peptone and beef extract; (B) as function of pH and peptone; (C) as function of pH 6 and beef extract

Results are in agreement with the earlier reports of several researchers. Earlier, Li and co-workers[20] shows that peptone and KH_2PO_4 are the two significant factors for bacteriocin production and have the positive effect. The optimal medium made peptone decreased by 0.5% and allowed bacteriocin yield to increase from 1074 to 2150 IU ml^{-1} compared to Cooked Meat Medium. Whereas from the results, it was observed that bacteriocin yield increases from 31,000 to 33,000 AU ml^{-1} with optimized medium composition.

The ANOVA results of regression model for Y is described in Table 3. ANOVA of the regression model for Y demonstrated that the model was significant due to an F-value of 3.50 and a very high probability value ($p<0.001$). The P-values are used as a tool to check the significance of each of the coefficients, which in turn indicate the pattern of the then it, was more significant to the corresponding coefficient. As shown in table 3, R^2 is 0.8590, which indicates that the model as fitted explained 86 % of the variability in bacteriocin responses. These results show that the model chosen can satisfactorily explain the effects of optimization pH, peptone and beef extract on bacteriocin production by *P. acidilactici* BA28 using optimized medium. The following model was fitted for bacteriocin production,

$$Y= 9888.9 + 616.2\ X1+1101.9X^2 +1893.6\ X1\ X2$$

Conventional method of optimization 'one at a time approach' does not lead to substantial increase in bacteriocin production. In addition, this approach is time consuming and limitation of ignoring the importance of interaction of various parameters. The RSM result indicated that the three variables selected by CCRD have significant impact on pediocin BA28 production by *P. acidilactici* BA28. The quadratic effect of peptone was more significant that is followed by quadratic effect of KH_2PO_4, beef extract and pH.

Table 3: Analysis of variance among bacteriocin responses

Source	Sum of squares	Df	Mean square	F-value	p-value
Model	5.91	9	0.66	3.50	0.0320
A-peptone	0.68	1	0.68	3.63	0.0860
B-beef extract	0.68	1	0.68	3.63	0.0860
C-pH	0.071	1	0.71	0.38	0.5517
AB	0.18	1	0.18	0.96	0.3506
AC	0.18		0.18	0.96	0.3506
BC	0.18	1	0.18	0.96	0.3506
A_2	1.68	1	1.68	8.96	0.0135
B_2	1.68	1	1.68	8.96	0.0135
C_2	1.35	1	1.35	7.21	0.0229
Residual	1.88	10	0.19	-	-
Lack of fit	1.83	5	0.37	37.85	0.0006
Pure error	0.048	5	9.667E-003	-	-
Core total	7.79	19	-	-	-

Standard Deviation 0.43, Mean 0.62, C.V% 69.89, PRESS 14.23,R-Squared 0.8590, Pred R-Squared -0.8957, Adeq Precisior 5.414

The experiment reduced the number of components from 1 to 5 for the preparation of media for production of pediocin BA28 as compared to MRS media (heterofermetative *Lactobacillus* broth) which contains total ten components for supporting bacterial growth and bacteriocin production. It is more costly than the media optimized with the help of RSM. From the above results, the final medium composition was selected as significant for the production of pediocin BA28 from *P. acidilactici* BA28.

CONCLUSION

RSM was used to determine the effects of three important factors (peptone, beef extract and pH) on bacteriocin production from broth. Linear, quadratic and interaction effects of these variables on bacteriocin production were determined. The statistical approach proved to be beneficial in optimizing a medium for bacteriocin production by *P. acidilactici*. The model generated in this study satisfied all the necessary arguments for its use in optimization. By fitting the experimental data to a second-order polynomial equation, the optimum levels of important response variables were determined. Using the optimum levels of fermentation parameters, a maximum bacteriocin production of 33,000 AU/ml was obtained. This study indicates that the medium design using statistical technique such as RSM can be very useful in improving the production of pediocin BA28 by *P. acidilactici* BA28 and in similar bioprocesses. The optimized medium not only allowed the increase in bacteriocin activity, but also reduced the cost of the medium.

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