Brazilian Journal of Microbiology (2011) 42: 716-720
ISSN 1517-8382

OPTIMIZATION OF MEDIA COMPONENTS FOR ENHANCED PRODUCTION OF *STREPTOCOCCUS PHOCAE* PI80 AND ITS BACTERIOCIN USING RESPONSE SURFACE METHODOLOGY

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Submitted: April 20, 2010; Returned to authors for corrections: October 13, 2010; Approved: January 13, 2011.

ABSTRACT

The standard MRS components were optimized using response surface methodology for increasing yield of *Streptococcus phocae* PI80 viable cells and its bacteriocin. The highest amounts of bacteriocin activity and viable cells were recorded from prediction point of optimized MRS medium and achieved two fold higher (33049.8 AU.mL^{-1} and 14.05 LogCFU.mL^{-1}) than un-optimized counterpart.

Key words: *Streptococcus phocae* PI80, total viable cells, bacteriocin, response surface methodology

INTRODUCTION

Probiotics are defined as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance (1). During the fermentation period, the probiotic bacterium is able to produce some antimicrobial metabolites like lactic acid, diacetyl, hydrogen peroxide and bacteriocin or bacteriocin like compounds (5). Bacteriocins are proteinaceous antimicrobial peptides or protein which is synthesized ribosomally by lactic acid bacteria to inhibit the growth of diverse Gram positive and Gram negative bacterial strains (4). Bacteriocins are of great interest to food industry in recent years because they are used as natural food preservatives to control food born and spoilage pathogen especially *Listeria monocytogenes* etc. The cell growth and bacteriocin production by lactic acid bacteria were strongly influenced by carbon, nitrogen sources and growth factors. It is difficult to find major factors and optimize them for biotechnological processes including multi variables.

Commonly, the non-statistical technology 'one-factor at a time' (OVAT) method was used by many researchers for media optimization. But, OVAT is a time-consuming method and may also conclude wrong results (6). Thus, recently the optimum culture condition was achieved by statistical based experimental designs to investigate the large number of experimental variables without having to increase the number of experiments to an extreme value (8). In this study, the traditional statistical technology based experimental design was used to improve the standard MRS medium by replacing dextrose and beef extract with lactose and meat extract as carbon and nitrogen sources. The response surface methodology (RSM) was employed to build models to evaluate the effective factors and study their interactions and select optimum growth condition.

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MATERIALS AND METHODS

Bacterial cultures and growth media

The strain *Streptococcus phocae* PI80 was previously isolated from the gut of marine shrimp *Penaeus indicus* (2). *L. monocytogenes* (MTCC 657) was used as indicator strain and it was cultured routinely in BHI agar (HiMedia, Mumbai) and a stock is maintained at -20^{o}C. The bacteriocin activity was assayed by agar well diffusion method and expressed as arbitrary units (AU.mL^{-1}) (10).

Experimental designs

Six major components of the MRS medium [peptone (A), meat extract (B) instead of beef extract, malt extract (C) instead of yeast extract, lactose (D) instead of dextrose, tween 80 (E), and K_2HPO_4 (F) instead of dipotassium phosphate] were used as variables. The two level factorial design facilitated to find out the variable having most significant effects among the aforementioned variables on bacteriocin and viable cell production. The concentration of each component in the medium was changed to match the ranges for the variables (Table 1A). According to the 2-level factorial design, 2^{6-2} experiments were carried out with sixteen factorial runs and three runs were center point runs for statistical reasons. Totally nineteen experimental runs were performed in fractional factorial design. The effect of each significant variable on bacteriocin and viable cells production were identified by the probability levels above 95% ($P< 0.05$). The method of steepest ascent path was performed along with the factorial design so that the optimum would be outside the factorial design space and to achieve the maximum increase of responses. In order to achieve the increasing yield production, the significant factors (peptone, meat extract and lactose) were selected for second step optimization by the empirical model of RSM. It is one of the most important experimental designs to gain a quadratic model, contains trials plus a star configuration to estimate quadratic effects and central points to evaluate the original variability and assure gross curvature, with bacteriocin and viable cells production as response. To describe the nature of the response surface in the optimum region, a RSM with five coded levels ($-\alpha$, –1, 0, +1, $+\alpha$) was adapted. Three key variables (peptone, meat extract and lactose) were selected for this model, based on their significance in the factorial design analysis. The CCD experiments contained a total of 22 experimental trials that included ten trials for factorial design: six trials for axial points and six trials for the replication of the central points for estimating the pure experimental variance in triplicate. The statistical software package Design-Expert 7.1.6 (StatEase, Inc., Minneapolis, USA) was used for the analysis of experimental data and to plot response surfaces.

Table 1. A) Experimental ranges and levels of the independent variables

Independent variables (g.L^{-1})	**Low level (–1)**	**Center point (0)**	**High level (+1)**
MRS medium replacement			
A – Peptone	10.0	20.0	30.0
B – Meat extract instead of beef extract	10.0	20.0	30.0
C – Malt extract instead of yeast extract	2.5	5.0	7.5
D – Lactose instead of dextrose	6.0	12.0	18.0
E – Tween 80	3.0	6.0	9.0
F – K_2HPO_4 instead of dipotassium PO_4	2.5	5.0	7.5

RESULTS AND DISCUSSION

Two level factorial design

Table 1B shows the experimental design and the results of two-level factorial design for MRS medium. The bacteriocin activity varied markedly from 5900 to 26833.3 AU.mL^{-1}, and total viable cell counts varied from 8.07 to 13.11LogCFU.mL^{-1} at different levels of various components in the medium. The concentration of all factors were strongly affected bacteriocin activity with *P*-values of <0.0001. Among these variables, peptone, meat extract, malt extract and lactose affected total viable cells with *P*-values of <0.0001. The variables tween 80 and K_2HPO_4 did not significantly influence total viable cells production. Regression coefficients were calculated and the response variables could be expressed according to the experimental data:

Bacteriocin activity = + 15539.57 + 2606.24A + 3047.91B + 1747.92C + 1927.09D – 2043.75E – 347.92F+139.58AB – 1427.08AC + 860.42AD – 1193.75AE –181.25AF – 831.25BD – 22.92BF **(1)**

Total viable cells = +11.26 + 0.55A + 0.70B + 0.54C + 0.42D – 0.34E + 0.028F – 0.048AB – 0.32AC +0.063AD – 0.34AE – 0.10AF – 0.30BD – 0.065BF **(2)**

The adequacy, fitness of bacteriocin activity and viable cells were evaluated by analysis of variance (ANOVA). From the ANOVA, the linear terms A, B, C, D, E , F and interaction terms AB, AC, AD, AE, AF and BD were found to be statistically significant at (*P*<0.05) in bacteriocin activity. For total viable cell production, all linear and interaction terms except A, B, C, D, E, AC, AE and BD were statistically significant at (*P*<0.05). The steepest ascent path was performed to identify the most significant variables among the variables used in this study.

Table 1. B) Experimental runs and the results of two-level factorial design

Run	A	B	C	D	E	F	Bacteriocin activity (AU.mL^{-1})		Total viable cells (LogCFU.mL^{-1})	
							Actual	Predicted	Actual	Predicted
1	+1	–1	–1	+1	+1	+1	14466.7	14452.07	10.86	10.87
2	–1	–1	–1	–1	–1	–1	5900.0	5885.41	8.07	8.09
3	+1	–1	–1	–1	+1	–1	8333.3	8347.89	9.65	9.65
4	+1	+1	+1	–1	+1	–1	16966.7	16952.07	11.80	11.81
5	+1	+1	–1	+1	–1	–1	26833.3	26818.74	13.11	13.11
6	–1	+1	–1	–1	+1	+1	11466.7	11452.07	10.45	10.45
7	–1	+1	–1	+1	+1	–1	12166.7	12181.25	10.75	10.76
8	–1	+1	+1	+1	–1	+1	19866.7	19852.07	12.28	12.29
9	0	0	0	0	0	0	16966.7	16966.66	12.00	12.09
10	+1	–1	+1	–1	–1	+1	14466.7	14452.07	11.12	11.12
11	0	0	0	0	0	0	16966.7	16966.66	12.16	12.09
12	+1	+1	+1	+1	+1	+1	19866.7	19881.25	12.22	12.22
13	–1	–1	+1	–1	+1	+1	10233.3	10247.92	10.51	10.52
14	–1	+1	+1	–1	–1	–1	19866.7	19881.25	12.35	12.35
15	–1	–1	–1	+1	–1	+1	9500.0	9514.59	10.11	10.11
16	–1	–1	+1	+1	+1	–1	14466.7	14452.07	11.12	11.12
17	+1	–1	+1	+1	–1	–1	22566.7	22581.25	13.00	13.02
18	+1	+1	–1	–1	–1	+1	21666.6	21681.19	12.75	12.76
19	0	0	0	0	0	0	16966.7	16966.66	12.11	12.09

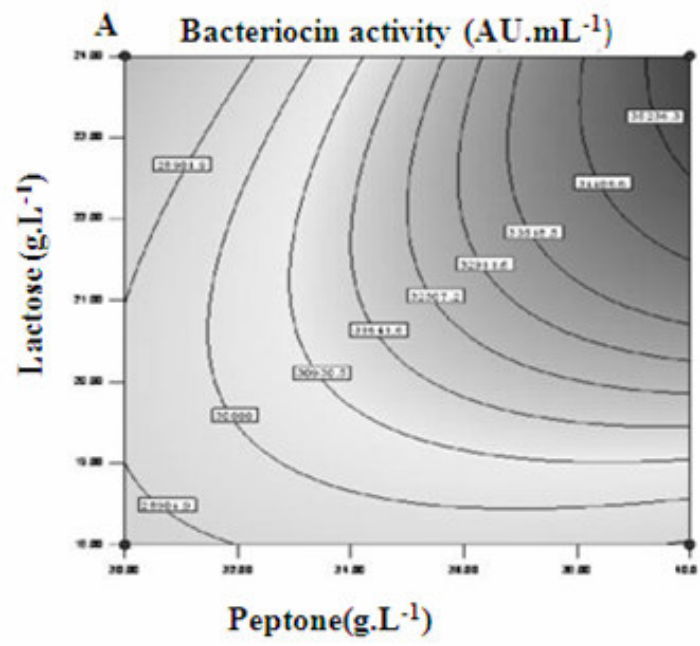
Response surface methodology

The most sensitive variables such as peptone *(*A), meat extract (B) and lactose (D) were further optimized by RSM, using a central composite design. The ranges of the variables were 30-40 g.L^{-1} for peptone and meat extract and 18-24 g.L^{-1} for lactose. The maximum experimental value for bacteriocin activity and total viable cell count were 36,836.31AU.mL^{-1}and 14.26 LogCFU.mL^{-1}, while the predicted responses were estimated to be 37,139.7AU.mL^{-1} and 14.44 LogCFU.mL^{-1}. By applying multiple regression analysis to the experimental data, the following coded final equation was found to explain bacteriocin activity and total viable cell production:

Bacteriocin activity = +31803.92 +1406.21A – 495.58B + 3193.29C + 512.50AB + 912.50AC – 1412.50BC – 366.81A2 + 57.45B2 – 1262.48C2 **(3)**

Total viable cells = +13.14 + 0.37A – 0.034B + 0.28C – 0.16AB + 0.35AC – 0.067BC + 7.599 – 0.03A2 – 0.084B2 + 0.12C2 **(4)**

The interaction between the peptone, meat extract, lactose and their effects on bacteriocin activity and total viable cell production are plotted in Figure 1. The response surface plot of the model equation suggests that increased levels of bacteriocin activity were obtained by increasing concentrations of peptone and lactose. From the model equations, derived by differentiation of equations 3 and 4, we can obtain the maximum prediction point of the model, which was 40 g.L^{-1} of peptone, 30 g.L^{-1} of meat extract and 24 g.L^{-1} of lactose. The model predicted a maximum response for bacteriocin activity (37139.7 AU.mL^{-1}) and total viable cell count (14.44 LogCFU.mL^{-1}). To confirm the predicted results of the model, experiments were performed and maximum bacteriocin activity (36166.6 AU.mL^{-1}) was obtained. The bacteriocin produced by *S. phocae* PI80 had antagonistic activity against food pathogen *L. monocytogenes*, whereas previous reports on bacteriocin production by *B. licheniformis* MKU3 didn't show the antagonistic activity against *Listeria* sp (3). Also, *B. licheniformis* 26 L-10/3RA showed highest (2600 AU.mL^{-1}) of antibacterial activity against *Streptococcus bovis* (7). Preetha *et al*. (8) obtained maximum bacteriocin activity (24.33mm and 101.33 mg.L^{-1}) and biomass (1.83 g.L^{-1} and 1.44 g.L^{-1}) by *Micrococcus* MCCB104 at 24 h of fermentation in the presence of glucose, lactose and glycerol. In contrast, *S. phocae* PI80 produced maximum viable cells and bacteriocin activity with in 16 h of incubation which being a shortest incubation time reported thus far. These observations suggested that bacteriocin and total viable cell production were stimulated by peptone, meat extract and lactose. Moreover, the optimal composition of MRS medium for bacteriocin production and growth of *S. phocae* PI80 consists of: peptone (40.0 g.L^{-1}), meat extract (30.0 g.L^{-1}), malt extract (5.0 g.L^{-1}), lactose (24.0 g.L^{-1}), tween 80 (6.0 g.L^{-1}), K_2HPO_4 (2.5 g.L^{-1}), triammonium citrate (1.0 g.L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.10 g.L^{-1}) and $MnSO_4{\cdot}7H_2O$ (0.05 g.L^{-1}). This observation was strengthened by the arguments of Rodrigues *et al*. (9), who reported probiotics *S. thermophilus* A and *Lactobacillus lactis 53* showed maximum biomass 3.123 g.L^{-1} productions in basal MRS medium with 38.6 g.L^{-1} peptone, 43.0 g.L^{-1} lactose and 5 g.L^{-1} meat extract.



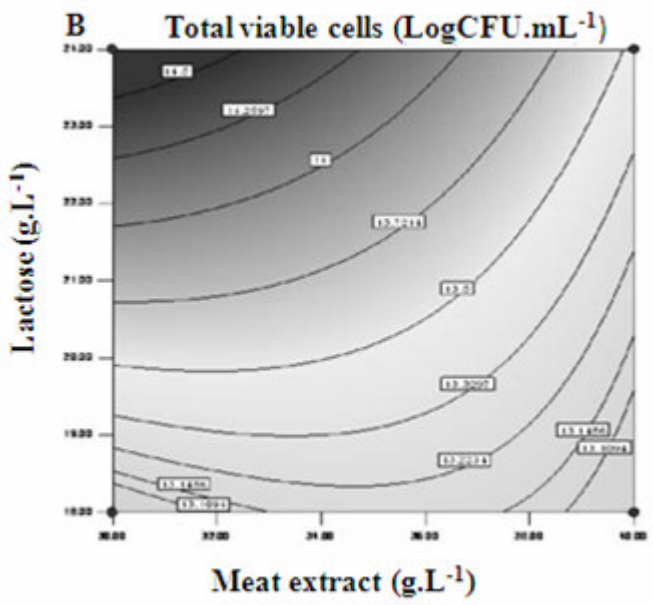


Figure 1. Response surface contour plots of bacteriocin activity (AU.mL^{-1}) and total viable cells (LogCFU.mL^{-1}) for *S. phocae* PI80. Bacteriocin activity and total viable cells are the response variable of interest. The counter plots represent the effect of the significant variables and their interaction with the response variable. The effects of peptone, meat extract and lactose and their mutual interaction on bacteriocin activity and viable cells production are expressed in plots (A) and (B).

In conclusion, the method of experimental factorial design and response surface analysis were possible to determine optimal operating conditions to obtain higher bacteriocin activity and total viable cell production. Under optimized condition the probiotic *S. phocae* PI80 produced higher amount of bacteriocin that effectively inhibited the growth of Gram negative and Gram positive bacteria including shrimp and food born pathogens.

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