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The role of environmental factors and medium composition on bacteriocin production by an aquaculture probiotic *Enterococcus faecium* MC13 isolated from fish intestine

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Abstract−The aim of this study was to optimize medium composition for higher yield of total viable cells and bacteriocin by *Enterococcus faecium* MC13. The factors such as peptone, meat extract, yeast extract, lactose, glycerol, tween 80, triammonium citrate and K_2HPO_4 were selected based on the Lactobacillus MRS medium composition. Two level factorial designs (FD) and steepest ascent path were performed to identify vital factors among the variables. Through the 2^{-8} FD, peptone, yeast extract and lactose were found to be significant factors involved in the enhanced production of viable cells and bacteriocin. Therefore, these three foremost factors were further optimized by central composite design to achieve efficient yield. The optimum MRS composition was found to be peptone (40.0 g/L), meat extract (30.0 g/L), yeast extract (40.0 g/L), lactose (24.0 g/L), glycerol (5.8 g/L), Tween 80 (3.0 g/L), triammonium citrate (1.0 g/L), K_2HPO_4 (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.10 g/L), $MnSO_4 \cdot 7H_2O$ (0.05 g/L) and dipotassium PO_4 (2.0 g/L). The optimized growth medium allowed higher amount of bacteriocin activity (36,100 $AUml^{-1}$) and total viable cells (14.22 $LogCFUml^{-1}$) production which were two-times higher than the commercial MRS medium.

Key words: *Enterococcus faecium* MC13, Total Viable Cells, Bacteriocin, Response Surface Methodology, Optimization

INTRODUCTION

Probiotics are defined as "live microorganisms when administered in adequate amounts confer beneficial health effect on the host" [1] which has been shown to supply a number of therapeutic health benefits such as reduction of cholesterol, improve the immune system, alleviate lactose intolerance, maintain remission of Crohn's disease, give faster relief from diarrhea, and prevent urogenital infections [1]. Their mechanistic action is not fully understood, but the capacity of the organisms to compete for nutrients, space and adherence sites along with production of antimicrobial agents such as organic acid, hydrogen peroxide and bacteriocin towards bacterial pathogen has been well documented [1].

Bacteriocin production has been described for several genera of lactic acid bacteria (LAB), including *Lactobacillus*, *Carnobacterium*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc* [2]. Bacteriocins are a heterogeneous group of protein ribosomally synthesized by lactic acid bacteria, which can display a broad spectrum of antimicrobial activity against gram positive and gram negative pathogenic bacteria [3]. 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. Nisin is one of the most important bacteriocins produced by bacteria such as *Lactococcus lactis* subsp. *lactis*, which are the most intensively studied lantibiotics, due to their potential application in many areas. It has a broad range of inhibitory activity against bacterial pathogen, especially towards food spoilage bacteria and is heat stable at very low pH of the surrounding environment [4]. Nisin is commercially produced by bacterial culture as it is known for its safety as rightly approved by FAD and is widely used as a food preservative, especially in canned foods and dairy products in most countries [5].

Generally, bacteriocin production is closely associated with the growth of bacterial culture because bacteriocin is released during the growth of bacteriocin producing cultures; and at end of the bacterial growth, bacteriocin efficiency decreases very slowly due to the protease degradation [6]. Subsequently, bacteriocin production is also significantly affected by changes in growth condition such as growth medium, pH and temperature [7]. Bacterial growth phase can be prolonged to enhance the tenure of bacteriocin production and medium components such as NaCl and ethanol; increasing the concentration of carbon and nitrogen sources can stabilize the bacteriocin production [8]. Also, the pH of the medium has been shown to significantly affect the bacteriocin activity [9]. Therefore, it is clear that the optimization of environmental factors is very essential for increasing the amount of bacteriocin production. The effect of medium components on bacteriocin production, such as enterocin P from *Enterococuus faecium* P13 [10], bacteriocin like inhibitory substances from *Enterococcus mundtii* WGWT1-1A [11] and bacteriocin from *Bacillus licheniformis* AnBa9 [12] has been previously reported.

MATERIALS AND METHODS

1. Bacterial Cultures and Growth Media

Enterococcus faecium MC13 (AY751463) strain was previously isolated from the gut of marine fish *Mughil cephalus* [13]. Bacteriocin-producing strain was maintained at −4 °C in Lactobacilli MRS and their bacteriocin activity was estimated using the agar well dif-

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fusion method and expressed in arbitrary units (AU/ml), calculated as $a^b \times 100$, where "a" represents the dilution factor, and "b" represents the last dilution that produces an inhibition zone of 2 mm in diameter [14]. One arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest two fold dilution showing a clear zone of growth inhibition of the indicator strains and activity is expressed per ml after multiplication by 100.

2. Effect of Temperature, pH, Salinity on Growth and Bacteriocin Production

Optimization of temperature for the growth and bacteriocin production of *E. faecium* MC13 in MRS broth was determined by inoculation of early log phase cultures at different incubation temperature (25 °C, 30 °C, 35 °C, 40 °C and 45 °C); pH (5.5, 6.0, 6.5 and 7.0) and varied concentrations of NaCl (0%, 0.5%, 1%, 2%, and 3%). The cells were harvested at two-hour intervals for a period of 24 h by centrifugation (8,000 g for 10 min at 4 °C). Total viable cells were counted using the plate-count method in MRS agar medium. Prior to the experiment, the pH of the cell-free supernatant was neutralized to 6.5 with 1 N NaOH and filter-sterilized (pore size 0.22 μm) for estimation of bacteriocin activity ($AUml^{-1}$) by the agar well diffusion method. *L. monocytogenes* 657 was used as indicator strain for estimation of bacteriocin activity for all experiment.

3. Two Level Factorial Design

To find which components of the medium have significant effects on cell growth and bacteriocin production, a first optimization step was developed. Eight major components in MRS medium (Peptone, meat extract, yeast extract, lactose, glycerol, Tween 80, triammonium citrate and K_2HPO_4) were selected to be set as factors in the factorial design analyses, and the different levels of these factors used are shown in Table 1. According to the factorial design, 2^8 experiments have to be performed. If the experimenter can reasonably assume that certain higher-order interactions are negligible, information on the main effects and lower-order interactions may be obtained by running only a fraction of the complete factorial set. The number of experiments can then be reduced by using only a part of the factorial design without loss of information about the major effects. For a moderately large number of factors, smaller fractions are frequently useful. Therefore, for a 2^{-6} factorial design with eight factors at two levels, only 16 experimental runs were performed (Table 2). All experiments were carried out at 37 °C and pH 6.5 for 16 h.

Table 1. Experimental ranges and levels of the independent variables (A, B, C, D, E, F, G and H) used in the two-level factorial design

Independent variables (g/L)	Low level	Center point	High level
	(–)	(0)	(+1)
MRS medium modification			
A - Peptone	10.0	20.0	30.0
B - Meat extract instead of Beef extract	10.0	20.0	30.0
C - Yeast extract	2.5	5.0	7.5
D - Lactose instead of Dextrose	6.0	12.0	18.0
E - Glycerol as a carbon sources	6.0	12.0	18.0
F - Tween 80	3.0	6.0	9.0
G - Triammonium citrate	1.0	2.0	3.0
H - K_2HPO_4 instead of Dipotassium PO_4	2.5	5.0	7.5

4. Response Surface Methodology

The idea of this second experiment is to develop an empirical model to acquire a more precise estimate of the optimum operating conditions for the factors involved. This approach of optimization is called RSM, and the second design component is termed as cen-

Table 2. Experimental design and results of the two-level factorial design for optimization of MRS fermentation medium

Run	A	B	C	D	E	F	G	H	Bacteriocin activity ($AUml^{-1}$)		Total viable cells ($LogCFUml^{-1}$)	
									Actual	Predicted	Actual	Predicted
1	+1	+1	−1	−1	−1	+1	+1	−1	16900	16787	12.22	12.21
2	+1	+1	+1	+1	+1	+1	+1	+1	19000	19525	12.44	12.46
3	−1	−1	−1	−1	−1	−1	−1	−1	4900	4825	10.86	10.85
4	+	−1	−1	+1	+1	+1	−1	−1	16900	17012	12.24	12.24
5	−1	+1	−1	+1	+1	−1	+1	−1	10000	10075	11.80	11.80
6	0	0	0	0	0	0	0	0	16900	16900	12.24	12.26
7	−1	+1	−1	−1	+1	+1	−1	+1	12100	12025	11.96	11.97
8	+1	+1	−1	+1	−1	−1	−1	+1	22500	22612	12.75	12.75
9	−1	−1	+1	−1	+1	+1	+1	−1	8100	8212	11.84	11.85
10	+1	−1	−1	−1	+1	−1	+1	+1	14400	14287	12.14	12.16
11	−1	+1	+1	−1	−1	−1	+1	+1	16900	17012	12.16	12.16
12	0	0	0	0	0	0	0	0	16900	16900	12.28	12.26
13	−1	−1	−1	+1	−1	+1	+1	+1	6400	6475	11.12	11.12
14	0	0	0	0	0	0	0	0	16900	16900	12.24	12.26
15	+1	−1	+1	+1	−1	−1	+1	−1	22500	22425	12.94	12.94
16	−1	+1	+1	+1	−1	+1	−1	−1	19600	19487	12.60	12.60
17	−1	−1	+1	+1	+1	−1	−1	+1	14400	14287	12.01	12.02
18	+1	−1	+1	−1	−1	+1	−1	+1	19600	19675	12.33	12.33
19	+1	+1	+1	−1	+1	−1	−1	−1	22500	22575	12.46	12.47

tral composite design (CCD), 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 active substances production as response. To describe the nature of the response surface in the optimum region, a CCD with five coded levels ($-\alpha$, −1, 0, +1, $+\alpha$) was performed. Three factors (peptone, yeast extract and lactose) were selected for this model, based on their significance in the factorial design analysis. All experiments were carried out at 37 ºC and pH 6.5 for 16 h. The CCD experiments contained a total of 20 experimental trials that included eight trials for factorial design: six trials for axial points and six trials for the replication of the central points (Table 4). The statistical software package Design-Expert 7.1.4 (StatEase, Inc., Minneapolis, USA) was used for the analysis of experimental data and to plot response surfaces. Analysis of variance (ANOVA) was used to estimate the statistical parameters for the optimization of MRS medium (Table 3). The level of significance was set at a value of *P* less than 0.05. A differential calculation was then employed for predicting the optimum point.

Table 3. Analysis of variance (ANOVA) for the first order models evaluated by two level factorial design

Values	Bacteriocin production	Total viable production
Model F-values	1046.24	612.93
Model P-values	<0.0001	<0.0001
Curvature F-value	144.02	82.63
Curvature P-value	0.0003	0.0008
R-squared	0.9997	0.9995
Adj R-squared	0.9988	0.9979
Pred R-squared	0.9812	0.9885
Adeq precision	104.696	99.750

5. Bacteriocin Susceptibility

The stability of bacteriocin was determined by exposing of cell free supernatant (CFS) to various pH, temperature and ultraviolet radiation conditions or to proteinase K (25 µg/ml), and bacteriocin activity was determined by the agar well diffusion method.

RESULTS AND DISCUSSION

E. faecium MC13 was isolated from fish intestines, which have the tendency to produce bacteriocin showing broad spectrum of inhibitory activity against closely related gram positive and gram negative bacterial strains. Agar well diffusion method was used to detect the bacteriocin activity.

1. Effect of Temperature, pH, NaCl on Bacteriocin and Total Viable Cell Production

The effect of temperature on growth and bacteriocin production by *E. faecium* MC13 was investigated in an MRS fermentation broth at temperatures ranging from 25 ºC to 45 ºC. At all temperatures, bacteriocin production started in late log phase (after 2 h of incubation), and the maximum production of 16,900 $AUml^{-1}$ was observed in the mid-stationary phase at 35 ºC. At 30 ºC and 35 ºC, bacteriocin activity decreased to 12,100 $AUml^{-1}$, which was observed in 18 and 6 h of incubation and it was stable till the end of the incubation period. Initially, viable cell count increased after 6 h of incubation at all temperatures, subsequently stabilizing until the end of

Table 4. Experimental design and results of central composite design for the optimization of MRS fermentation medium

Run	A	B	D	Bacteriocin activity ($AUml^{-1}$)		Total viable cells ($LogCFUml^{-1}$)	
				Actual	Predicted	Actual	Predicted
1	−1	−1	−1	19600	19298	12.23	11.73
2	0	0	0	25600	23172	13.30	13.13
3	+1	−1	+1	32400	31205	13.60	13.40
4	+1	+1	−1	25600	27424	12.94	13.06
5	−1	−1	+1	22500	20777	12.24	12.19
6	−1	+1	+1	25600	24838	13.11	13.05
7	0	0	0	22500	22716	13.11	13.13
8	−1	+1	−1	19600	20896	12.11	12.37
9	+1	+1	+1	32400	35266	13.46	14.02
10	+1	−1	−1	22500	23363	12.54	12.65
11	0	0	0	22500	22716	13.14	13.13
12	0	0	0	22500	22716	13.16	13.13
13	1.682	0	0	36100	33556	14.22	13.91
14	0	1.682	0	32400	29342	13.75	13.27
15	0	0	1.682	25600	26132	13.40	13.29
16	−1.682	0	0	16900	16836	12.07	12.32
17	0	0	0	22500	22716	12.94	12.95
18	0	0	0	22500	22716	13.14	13.15
19	0	−1.682	0	19600	22513	11.80	12.21
20	0	0	−1.682	16900	16223	12.05	12.09

the incubation period. The pH of the medium decreased from an initial pH of 6.6 to 4.58. The effect of culture pH on growth and bacteriocin production by the probiotic *E. faecium* MC13 was investigated. The initial pH of the medium was adjusted to 5.5, 6.0, 6.5 or 7.0 with 1 N HCl or 1 N NaOH. The maximum bacteriocin activity of 16,900 $AUml^{-1}$ was obtained after 8 to 12 h of incubation at pH 6.5, but the activity was decreased at pH 5.5, 6.0 or 7.0. Hence, the optimum pH was found to be 6.5. The maximum total viable cell counts (12.123, 12.607 and 12.502 $LogCFUml^{-1}$) were found to be at pH 6.0, 6.5 and 7.0 during the incubation period. The pH of the medium started decreasing and continued to decrease until the end of the incubation period at each pH. These results suggest that the optimum pH for maximum bacteriocin activity and total viable cell production is 6.5. The results are in accord with the production of the bacteriocins warnerin in *S. warneri* [15], thermophilin 1277 in *S. thermophilus* SBT1277 [16], bacteriocin in *L. pentosus* ST71BZ [17], antibacterial proteins in *B. licheniformis* AnBa9 [12] and antibacterial compounds in *B. coagulans* [18], in which high levels of bacteriocin and total viable cell production were achieved at a suboptimal temperature and pH. Based on these results, the optimum incubation temperature, culture pH for bacteriocin production and maximal viable cell count for *E. faecium* MC13 were determined to be at 35 °C and 6.5 (Fig. 1(a), (b)).

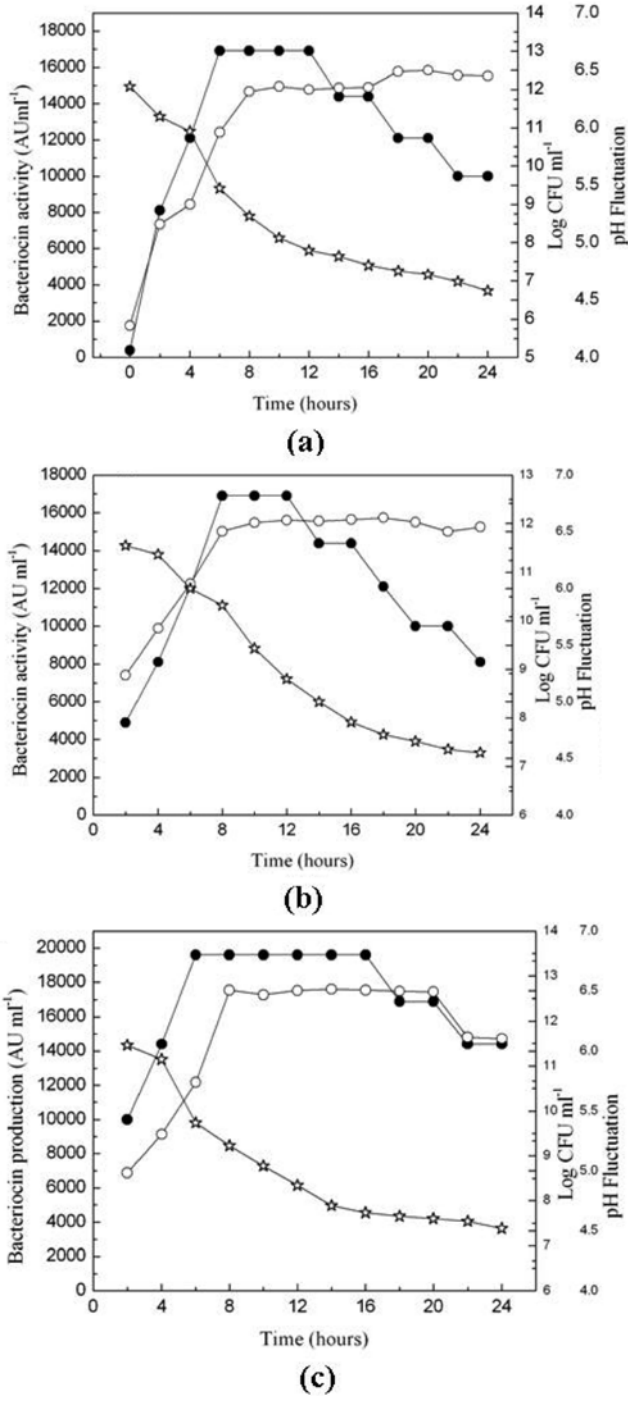

Fig. 1. Bacteriocin activity (●) and total viable cells of *E. faecium* MC13 (○) and pH variation (☆) in MRS broth at optimum temperature 35 °C (a), pH 6.5 (b) and salinity 2% (c).

Bacteriocin production by *E. faecium* MC13 is influenced by the presence of NaCl in the MRS fermentation medium. Therefore, *E. faecium* MC13 cells were grown in MRS broth containing different concentrations of NaCl (0%, 0.5%, 1%, 2% or 3%). The optimal salinity for the production of bacteriocin was 2%, with the maximum production of 19,600 $AUml^{-1}$ obtained after 6 h of incubation (Fig. 1(c)). However, bacteriocin production decreased in the presence of 0%, 0.5% and 1% NaCl. Although NaCl affects the growth and bacteriocin production of several bacterial strains [19]. Delgado et al. [20] have reported that the addition of NaCl enhances the production of the bacteriocin plantaricin by *L. plantarum* LPCO10 and *L. pentosus* B96. The addition of NaCl enhanced maximum viable cell counts, but no difference was found among the different concentrations of NaCl. The pH of the medium decreased until the end of the incubation, as in the previous experiments.

2. Effects of MRS Components on Bacteriocin Production and Cell Growth

Bacteriocin and total viable cell production generally depend on environmental factors and the composition of the growth medium. Therefore, optimization of culture parameters like temperature, pH, salinity and medium components is important for the enhancement of bacteriocin and viable cell production [21-23]. Accordingly, in the present study, primary screening of nutrients was done by a conventional screening method. The first optimization step (I) was conducted by two-level factorial design, and the final optimization step was done by means of central composite design of RSM. Factorial design has capacity to find the medium components that play significant roles in synthesizing bacteriocin activity and cell growth. For MRS medium optimization, eight components were selected for a one-variable-at-a-time approach. The concentration of each component in the medium was changed to match the ranges for the variables. The independent variables, experimental ranges and levels investigated in this study are given in Table 1. The results of the experiments to achieve MRS medium optimization are presented in Table 2. The bacteriocin activity varied markedly from 4,900 to 22,500 $AUml^{-1}$, and total viable cell counts varied from 10.85 to 12.94 $LogCFUml^{-1}$ at different levels of various components in the medium. The concentration of peptone, yeast extract and lactose strongly influenced bacteriocin activity and total viable cells with *P*-values of <0.0001. Glycerol, Tween 80 and K_2HPO_4 did not significantly affect the bacteriocin activity. Regression coefficients were calculated, and the response variables Y1 and Y2 could be expressed

according to the experimental data:

$$Y1=+15456.25+3906.25A+2056.25B+2443.75C+1031.25D-706.25E-556.25F-1106.25G+281.25H-1043.75AB-756.25AC-306.25AE-556.25AF-618.75AH \quad (1)$$

$$Y2=+12.12+0.32A+0.18B+0.23C+0.12D-0.023F+0.034G-015AB-0.13AC+0.032AD-0.11AE-0.11AF+0.030AG-0.021AH \quad (2)$$

Where Y1 is bacteriocin activity ($AUml^{-1}$) and Y2 is total viable cell production ($LogCFUml^{-1}$).

The first-order model in Eqs. (1) and (2) with thirteen terms contains eight linear terms, five two factorial interaction. Adequacy and fitness of bacteriocin activity and viable cells production were evaluated by standard analysis of variance (ANOVA). Table 3 shows the ANOVA of the regression models explained that the models were highly significant ($P<0.0001$) for both bacteriocin and viable cells production. The fitness of the model was analyzed by determination coefficient ($R2=0.9997$ and 0.9995), which implied that the sample variation more than 99.5% was attributed to the variables and only 0.5% of the total variance could not be explained by the model. Moreover, the adjusted determination coefficient (Adj $R2=0.9988$ and 0.9988) favorably confirmed the model significant and showed statiscally to not significant lack of fit, though the model was supposed to be adequate for prediction within the variables.

3. Steepest Ascent Path for Optimization

The steepest ascent path was determined by Eqs. (1) and (2) and from analyzed regression results. The first-order model Eqs. (1) and (2) predicted a higher amount of peptone (A), yeast extract (C), lactose (D) and lower concentration of meat extract (B), glycerol (E), tween 80 (F), triammonium citrate (G) and K_2HPO_4 (H) significantly increased the bacteriocin (26635.6 $AUml^{-1}$) and viable cell (13.0 Log $CFUml^{-1}$) production. Therefore, significant main effects such as peptone, yeast extract, lactose concentration were increased and decreased, in order to get a positive consequence in response variables. Consequently, this medium was selected for further optimization.

4. Response Surface Methodology

The second step optimization was carried out by RSM using central composite design with selected three significant variables such as peptone (A), yeast extract (C) and lactose (D). The ranges of the variables were 30-40 g/L for peptone and 30-40 g/L yeast extract and 18-24 g/L for lactose. The experimental design and results are presented in Table 4. The maximum experimental value for bacteriocin activity and total viable cell count were 36,100 $AUml^{-1}$ and 14.22 $LogCFUml^{-1}$, while the predicted responses, based on RSM, were estimated to be 33,556 $AUml^{-1}$ and 13.91 $LogCFUml^{-1}$. 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 bacteriocin activity (Y3) and total viable cell count (Y4) as functions of these variables. 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:

$$Y3=+22944.78+4238.93A+2030.26B+2945.89C+0.000AB+975.00AC+0.000BC+1312.06A2+1135.28B2-544.10C2 \quad (3)$$

$$Y4=+13.14+0.47A+0.32B+0.36C-0.060AB+0.071AC+0.054BC-0.015A2-0.15B2-0.16C2 \quad (4)$$

Where Y3 is bacteriocin activity ($AUml^{-1}$) and Y4 is total viable cell production ($LogCFUml^{-1}$).

The statistical significance of the model equation was evaluated by an *F*-test, which showed that the regression is statistically significant ($P<0.05$) for bacteriocin activity. The model *F*-value of 7.4 implies that the model for bacteriocin activity is significant. A value of *p* less than 0.05 indicates that the model's terms are also significant. The high-level factorial points (+1) of the peptone, yeast extract and lactose factors improved the level of bacteriocin activity (32,400 $AUml^{-1}$) and the number of total viable cells (13.46 $LogCFUml^{-1}$).

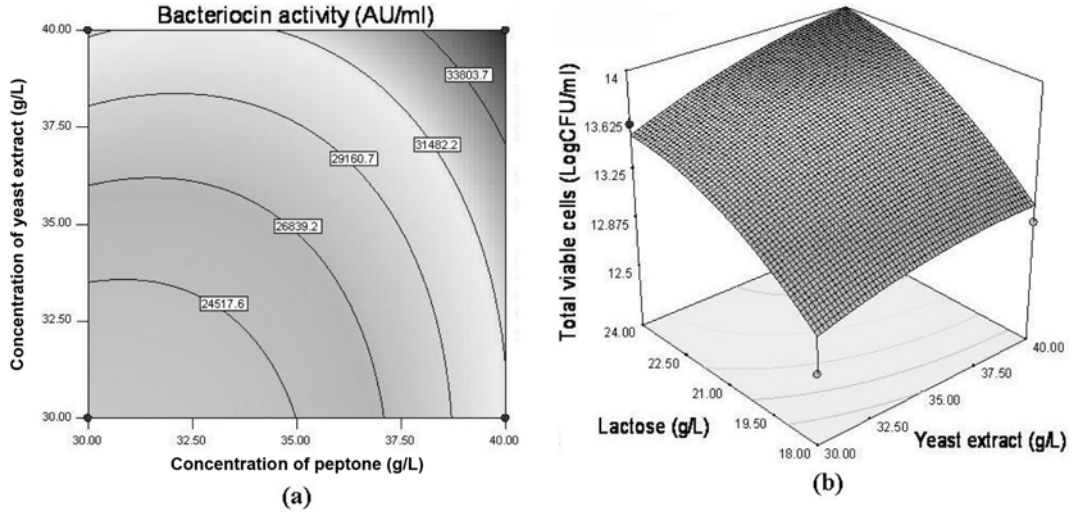

Fig. 2. Response surface contour plots of bacteriocin activity ($AUml^{-1}$) and total viable cells ($LogCFUml^{-1}$) for *E. faecium* MC13. Bacteriocin activity and total viable cells are the response variable of interest. The counter plots represent the effect of the significant variables and their interaction with the response variable. The effects of peptone and lactose and their mutual interaction on bacteriocin activity and viable cells production are expressed in plots (a) and (b).

Bacteriocin activity and total viable cell production were low under the low-level (−1) star point of each factor. Furthermore, the interaction of low-level factorial points (−1) of all factors did not significantly affect bacteriocin activity or the total viable cell count. However, the interaction of any single environmental factor at a low-level factorial point with other factors at high-level factorial points supported higher bacteriocin activity and total viable cell production, suggesting high-level factorial levels of any two environmental factors are necessary for bacteriocin activity and total viable cell production. Perturbation graphs showed that all factors played important roles in bacteriocin activity, and bacteriocin activity increased to its maximum in stationary growth phase. Production of bacteriocin is usually associated with primary metabolite kinetics [24]. However, bacteriocin has been reported as a secondary metabolite in *Lactobacillus plantarum* LPCO10 [25], *B. licheniformis* 26 L-10/3RA [26], *L. lactis subsp. lactis* [27] and *L. pentosus* B96 [20].

The interaction between the nutrients and their effects on bacteriocin activity and total viable cell production are plotted in Fig. 2. The response surface plot of the model equation suggests that increased levels of bacteriocin activity and total viable cell production were obtained by increasing the concentrations of all factorial factors. This result was similar to that of Anthony et al. [12]. From the model equations, derived by differentiation of Eqs. (3) and (4), we can obtain the maximum prediction point of the model, which was 40 g/L of peptone, 40 g/L of yeast extract and 24 g/L of lactose. The model predicted a maximum response for bacteriocin activity (35038.1 $AUml^{-1}$) and total viable cell count (14.02 $LogCFUml^{-1}$). To confirm the predicted results of the model, experiments were performed and maximum bacteriocin activity (36,100 $AUml^{-1}$) was determined. This observation suggested that, in the case of bacteriocin activity and total viable cell production, peptone, yeast extract and lactose exhibited significant effects. Moreover, the optimal composition of MRS fermentation medium for bacteriocin production and growth of *E. faecium* MC13 consists of peptone (40.0 g/L), meat extract (30.0 g/L), yeast extract (40.0 g/L), lactose (24.0 g/L), glycerol (5.8 g/L), tween 80 (3.0 g/L), tri-ammonium citrate (1.0 g/L), K_2HPO_4 (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.10 g/L), $MnSO_4 \cdot 7H_2O$ (0.05 g/L) and dipotassium PO_4 (2.0 g/L). This observation is supported by previous studies [21,28].

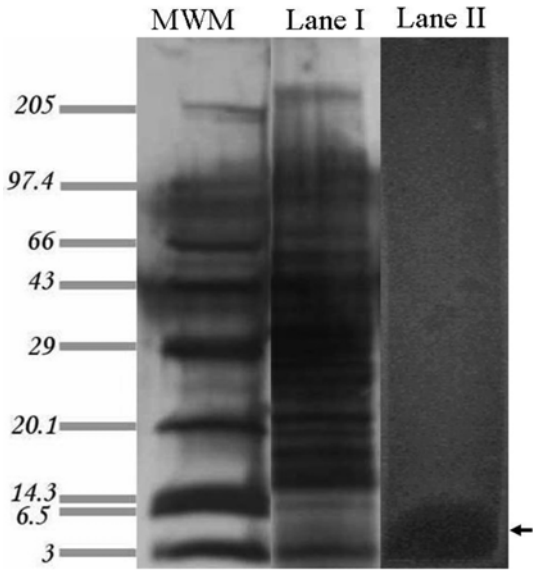

Fig. 3. Tricine-SDS-PAGE and *in vitro* activity assay of crude bacteriocin. MWM: molecular weight marker (kDa). Lane I: protein profile of the crude bacteriocin-containing fraction of *E. faecium* MC13. MWM and Lane I were stained by silver staining. Lane II: results of the *in vitro* assay to detect bacteriocin activity. The arrow indicates the inhibition of bacterial growth.

E. faecium MC13 produced bacteriocin with a molecular mass of 3.5 kDa, exhibiting a zone of inhibition against the indicator strain *L. monocytogenes* 657 in SDS-PAGE (Fig. 3). This bacteriocin is smaller than enterocin M from *E. faecium* AL14 [29], the bacteriocin from *B. licheniformis* MKU3 [22] and the antimicrobial protein from *B. licheniformis* AnBa9 [12]. Furthermore, the bacteriocin activity was completely inactivated after treatment of the cell-free supernatant with proteinase K, while bacteriocin was found to be stable at 80 ºC for 10 min and still active (300 $AUml^{-1}$). Bacteriocin lost its activity after incubation at 100 ºC for 10 min. The bacteriocin activity derived from *E. faecium* AL14 is reported to be resistant at 100 ºC for up to 1 hr, but sensitive to proteolytic enzymes [29]. Bacteriocin was also found to be active over a pH range of 2-12 (1,600-400 $AUml^{-1}$) and at a wide range of ultraviolet exposures of 1-12 min (1,600 $AUml^{-1}$).

CONCLUSIONS

Using the methods of experimental factorial design and response surface analysis, it was possible to determine optimal operating conditions to obtain high bacteriocin activity and total viable cell production. The validity of the model was confirmed by fitting the values of the variables in the model equation and by carrying out experiments at those values. The highest amounts of bacteriocin activity and viable cells were observed in increased concentrations of peptone, yeast extract and lactose. The optimum temperature, pH and salinity have also improved the bacteriocin production as well as viable cell count. Under optimized conditions, the probiotic *E. faecium* MC13 produced high amounts of bacteriocin, effectively inhibiting the growth of gram-negative and gram-positive bacteria, including the food-borne pathogen *L. monocytogenes* 657, *P. aeruginosa*, *V. parahaemolyticus* and *V. harveyi*. Thus, *E. faecium* MC13 can be used for seafood biopreservation as well as for controlling vibriosis, a bacterial shrimp disease.

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