CHAPTER 4

OPTIMIZATION OF CULTURE PARAMETERS FOR ENHANCED BACTEROICIN AND VAILABLE CELLS PRODUCTION BY PROBIOTICS STREPTOCOCCUS PHOCAE PI80 AND ENTEROCOCCUS FAECIUM MC13

4.1. Introduction

In general, the bacteriocin production is closely associated with growth of bacterial culture as 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 (Hur et al., 2000). Bacteriocin production is often regulated by growth temperature and medium pH, as shown for thermophilin 1277 produced by Streptococcus thermophilus 1277 (Kabuki et al., 2007), macedocin produced by S. macedonicus ACA-DC 198 (Georgalaki et al., 2002); enterocin 1146 (Parente and Ricciardi, 1994); enterocin AS-48 (Abriouel et al., 2001) and bacteriocinST4SA produced by Enterococcus mundtii ST4SA (Todorov and Dicks, 2009). Studies conducted on bacteriocin production by other lactic acid bacteria, eg. Pediocin AcH (Biswas et al., 1991), pediocin PD-1 (Nes et al., 2001), plantaricin ST13BR (Todorov et al., 2004) and bacteriocin ST23LD produced by Lactobacillus plantarum ST23LD (Todorov and Dicks, 2006) have shown that the production is also regulated by growth temperature and pH. In some cases, higher levels of bacteriocin production have been recorded at suboptimal growth conditions (Parente and Ricciardi, 1994; Audisto et al., 2001). Bacterial growth phase can also be prolonged to enhance the tenure of bacteriocin production (Lv et al., 2005).

Although, medium components such as NaCl, ethanol, increasing concentration of carbon and nitrogen sources can stabilizes the bacteriocin production (Leroy et al., 2003). Therefore, it
is cleared that the optimization of environmental factors is very essential for increasing amount of bacteriocin and biomass production by lactic acid bacteria. The effect of medium components on the bacteriocin production, such as enterocin P from *E. faecium* P13 (Herranz et al., 2001), bacteriocin like inhibitory substances from *E. mundtii* WGWT1-1A (Settanni et al., 2008), pediocin from *Pediococcus acidilactici* NRRL B5627 (Anastasiadou et al., 2008a) and bacteriocin from *Bacillus licheniformis* AnBa9 (Anthony et al., 2009) has been previously reported. Moreover, Todorov and Dicks (2005) studied that the effect of carbon, nitrogen sources and growth factors on bacteriocin production by *E. faecium* ST311LD in lactobacillus MRS medium.

The Lactobacillus MRS broth is one of the most important growth medium for all lactic acid bacteria. Being growth media for wide range of bacteria they are also not optimal in composition and concentration for use in specialized processes. Growth of *L. sakei* CTC 494 in MRS broth was inhibited because of nutrition depletion, which suggesting that the MRS was not fully optimized for bacteriocin and biomass production (Leroy and De Vuyst, 2001). Growth associated nature of bacteriocin production suggests that optimization of growth media can be used for increasing level of bacteriocin by improving the cell growth (Mataragas et al., 2004). Therefore media optimization plays a crucial role with respect of bacteriocin and biomass production by lactic acid bacteria.

The aim of the present study was to improve the standard MRS medium using factorial central composite design with surface modeling method for extreme bacterial cell growth and bacteriocin production by aquaculture probiotics *S. phocae* PI80 and *E. faecium* MC13. After evaluation, it revealed that bacteriocin production is growth-associated. In this chapter, we
present the optimization of culture conditions and medium composition for production of viable cells and antimicrobial peptides in detail.

4.2. Materials and methods

4.2.1. Bacterial cultures and growth media

The probiotic strains *Streptococcus phocae* PI80 (EU117220) and *Enterococcus faecium* MC13 (AY751463) was previously isolated from the gut of marine shrimp (*Penaeus indicus*) and fish (*Mughil cephalus*) (Gopalakannan, 2006). Bacteriocin producing strains were maintained at -4°C in Lactobacilli MRS and their bacteriocin activity was estimated using the agar well diffusion method and expressed in arbitrary units (AU ml⁻¹). The method was described detail in general materials and methods (Chapter 3).

4.2.2. Batch fermentation

The probiotic cultures *S. phocae* PI80 and *E. faecium* MC13 were prepared by inoculating single colony into 10 ml of MRS broth and incubated at 37°C for 16 h with agitation (200 g). Batch fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium (pH 6.5) inoculated with 5% (v/v) 16 h old culture and incubated at 37°C with agitation (200 g) for 16 h.

4.2.3. Effect of temperature on growth and bacteriocin production

Optimization of temperature for the growth and bacteriocin production of *S. phocae* and *E. faecium* in MRS broth were determined by inoculation of early log phase cultures and incubation at different temperature 25°C, 30°C, 35°C, 40°C and 45°C. The cells were harvested at every 2 h 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 medium. The pH of the cell-free
supernatant was adjusted 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.

4.2.4. Effect of pH on growth and bacteriocin production

To evaluate the effect of pH on growth and bacteriocin production, *S. phocae* PI80 and *E. faecium* MC13 were grown in MRS broth at different pH (5.5, 6.0, 6.5 and 7.0). The pH of the MRS broth was adjusted to required value by addition of 1 N HCl or 1 N NaOH; afterward, the medium was inoculated with *S. phocae* PI80 and *E. faecium* MC13 and incubated at 37°C. Total viable cells were counted, and bacteriocin activity was measured at prescribed intervals, as described above.

4.2.5. Effect of salinity on growth and bacteriocin production

The strains *S. phocae* PI80 and *E. faecium* MC13 were isolated from marine shrimp and fish, and hence, experiments were conducted to determine the optimum concentration of NaCl in the medium for enhanced growth and bacteriocin production. *S. phocae* PI80 and *E. faecium* MC13 were cultured in MRS broth containing different concentrations of NaCl (0%, 0.5%, 1%, 2%, and 3%) and incubated at 37°C. Total viable cells were counted, and bacteriocin activity was recorded at prescribed intervals, as described previously. Cell-free supernatant of *S. phocae* PI80 and *E. faecium* MC13 were collected after 16 h of growth and neutralized with 1 N NaOH. The cell-free supernatant was precipitated with 70% ammonium sulfate and analyzed by tricine-SDS-polyacrylamide gel electrophoresis (PAGE) to detect the presence of bacteriocin.

4.2.6. Effect of surfactant on growth and bacteriocin production

To evaluate the effect of surfactant on growth and bacteriocin production, *S. phocae* PI80 and *E. faecium* MC13 were grown in MRS broth containing various concentration of surfactant Tween 80 (0.2-1.%). After 16 h of incubation period, the cells were harvested by centrifugation
(8,000 g for 10 min at 4°C). Total viable cells were counted using the plate-count method in MRS medium. The pH of the cell-free supernatant was adjusted 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.

4.2.7. Experimental design

RSM is a good mathematical and statistical technique that is useful for the modeling and analysis of problems in which the response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 1997).

4.2.7.1. Two level factorial design

To find out 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 K₂HPO₄) were selected to be set as factors in the factorial design analyses, and the different levels of these factors used are shown in Table 4. According to the factorial design, 2⁸ 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 2⁶ factorial design with eight factors at two levels, only 16 experimental runs were performed. All experiments were carried out at 37°C and pH 6.5 for 16 h.
4.2.7.2. Response surface methodology

The concept 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 central composite design (CCD), one of the most important experimental designs to gain a quadratic model, contains of 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 (−α, −1, 0, +1, +α) was performed. Three factors (peptone, meat extract and lactose for PI80; peptone, yeast extract and lactose for MC13) were selected for this model, based on their significance in the factorial design analysis. All experiments were carried out at 37°C 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.

4.3. Results

Bacteriocin activity of S. phocae PI80 and E. faecium MC13 was detected against all the listed indicator strains except the gram negative bacterial strains, E. coli CSH57 and SK-39 (Table 3).

4.3.1. Effect of temperature, pH, salinity and Tween 80 on bacteriocin and total viable cell production by S. phocae PI80 and E. faecium MC13

Optimum temperature, pH, salinity and surfactant Tween 80 for the mass culture of probiotics Streptococcus phocae PI80 and Enterococcus faecium MC13 were found to be temperature 30 - 35°C (Fig. 1 and 2), pH 6.5 (Fig. 3 and 4), salinity 1 - 2% (Fig. 5 and 6), and surfactant 0.6% (Fig. 7 and 8). The cells free extracts of sixteen hours culture S. phocae PI80
and *E. faecium* MC13 were neutralized with 1 N NaOH and analyzed in Tricine SDS-PAGE to find out the presence of bacteriocin. Figure 9 shows the production of bacteriocin in the order of increasing concentration of salinities. The lower molecular weight proteins found to be more in the concentration of 0.5%, 1% and 2% NaCl. This shows that probionts *S. phocae* PI80 and *E. faecium* MC13 can tolerate high salinities as well as grow well and produce bacteriocin.

**Table 3. Growth medium and incubation temperature of indicator strains and antimicrobial spectrum of the cell free supernatant of *S. phocae* PI80 and *E. faecium* MC13**

<table>
<thead>
<tr>
<th>Indicator strains</th>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>PI80- Activity (AU/ml)</th>
<th>MC13-Activity (AU/ml)</th>
</tr>
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<tbody>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>TSA</td>
<td>37</td>
<td>10000</td>
<td>12100</td>
</tr>
<tr>
<td>V. <em>vulnificus</em> 1145</td>
<td>TSA</td>
<td>37</td>
<td>12100</td>
<td>10000</td>
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<tr>
<td>V. <em>fischeri</em> 1738</td>
<td>TSA</td>
<td>37</td>
<td>12100</td>
<td>14400</td>
</tr>
<tr>
<td>V. <em>anguillarum</em></td>
<td>TSA</td>
<td>37</td>
<td>10000</td>
<td>12100</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
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<td>37</td>
<td>10000</td>
<td>10000</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5-α</td>
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<td>16900</td>
<td>16900</td>
</tr>
<tr>
<td><em>E. coli</em> KL-16</td>
<td>BHI</td>
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<td>16900</td>
<td>16900</td>
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<tr>
<td><em>E. coli</em> KL-96</td>
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<td>12100</td>
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<td>0</td>
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<td><em>E. coli</em> SK-39</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>BHI</td>
<td>37</td>
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<td>10000</td>
</tr>
<tr>
<td>Klebsiella pneumonia 30</td>
<td>BHI</td>
<td>37</td>
<td>6400</td>
<td>8100</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
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<td>37</td>
<td>10000</td>
<td>12100</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
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<td>37</td>
<td>6400</td>
<td>6400</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
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<td>2500</td>
<td>6400</td>
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<tr>
<td><em>Listeria monocytogenes</em> 657</td>
<td>BHI</td>
<td>37</td>
<td>14400</td>
<td>16900</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>BHI</td>
<td>37</td>
<td>2500</td>
<td>6400</td>
</tr>
<tr>
<td>L. <em>acidophilus</em></td>
<td>BHI</td>
<td>37</td>
<td>1600</td>
<td>900</td>
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<tr>
<td>L. <em>rhamnosus</em></td>
<td>BHI</td>
<td>37</td>
<td>1600</td>
<td>16900</td>
</tr>
</tbody>
</table>

TSA, Tryptone soya agar; BHI, Brain heart infusion broth.

Antimicrobial activity was determined for each strain after serial dilution of crude bacteriocin by the agar well diffusion method.
Figure 1. Growth of *Streptococcus phocae* PI80 (○), bacteriocin production (●) and pH variation (△) in MRS broth at 25°C (A), 30°C (B), 35°C (C), 40°C (D) and 45°C (E).
Figure 2. Growth of *Enterococcus faecium* MC13 (○), bacteriocin production (●) and pH variation (▲) in MRS broth at 25°C (A), 30°C (B), 35°C (C), 40°C (D) and 45°C (E).
Figure 3. Growth of *Streptococcus phocae* PI80 (○–○), bacteriocin production (●–●) and pH variation (△–△) in MRS broth at pH 5.5 (A), 6.0 (B), 6.5 (C) and 7.0 (D).
Figure 4. Growth of Enterococcus faecium MC13 (○-○), bacteriocin production (●-●) and pH variation (×-×) in MRS broth at pH 5.5 (A), 6.0 (B), 6.5 (C) and 7.0 (D).
Figure 5. Growth of *Streptococcus phocae* PI80 (○○), bacteriocin production (●●) and pH variation (✈✈) in MRS broth with NaCl-0% (A), 0.5% (B), 1% (C), 2 (D) and 3% (E).
Figure 6. Growth of *Enterococcus faecium* MC13 (○), bacteriocin production (●) and pH variation (△) in MRS broth with NaCl-0% (A), 0.5% (B), 1% (C), 2 (D) and 3% (E).
Figure 7. Tricine SDS-PAGE showing the effect of salinity on bacteriocin production by *S. phocae* PI80 (A) and *E. faecium* MC13 (B). Lane 1- MWM and Lane2 – Lane 6 shows the order of increasing concentration salinities (0, 0.5, 1, 2, and 3%).
Figure 8. Growth of *S. phocae* PI80 (○), bacteriocin production (●) and pH variation (△) in MRS broth with Tween 80.

Figure 9. Growth of *E. faecium* MC13 (○), bacteriocin production (●) and pH variation (△) in MRS broth with Tween 80.
4.3.4. Statistical optimization of MRS medium

4.3.4.1. Effects of MRS components on bacteriocin production and cell growth of *S. phocae* PI80

4.3.4.1.1. Two level factorial design

The factorial design enables the identification of the medium components that play a significant role on bacteriocin activity and cell growth as well as the ranges within the medium components varies. For MRS medium optimization, eight components were selected by one-variable at a time (OVAT) approach. The concentration of the each component in the medium was appropriately enlarged as the ranges for the variables. The independent variables, experimental range and levels investigated in this study are given in Table 4. Results of the experimental design performed to achieve MRS medium optimization are presented in Table 5. The bacteriocin activity varied markedly from 6,400 to 22,500 AUml⁻¹ as well as total viable cells varied from 9.65 to 12.62 LogCFUml⁻¹ with the different levels of components in the medium. The concentration of peptone, meat extract and lactose strongly induced the bacteriocin activity with p-values of 0.0003, <0.0001 and 0.0015 as well as total viable cells with p-values of 0.0002, <0.0001 respectively. Whereas yeast extract, tri-ammonium citrate and K₂HPO₄ didn’t significantly influence bacteriocin activity. Furthermore, it was found that the glycerol and Tween 80 is more important for bacteriocin activity and total viable cells production. The values of the regression coefficients were calculated and the response variables bacteriocin activity and total viable cells could be written as experimental data:

\[
\text{Bacteriocin activity} = + 1531.25 + 1843.75A + 2618.75B + 93.75C + 1006.25D - 1881.25E + 1031.25F - 368.75G - 343.75H - 743.75AC - 931.25AD - 218.75AE - 331.25AF - 1631.25AG - 1031.25AH
\]  \quad (1)
Total viable cells = + 11.69 + 0.25A + 0.55B + 0.26C + 0.36D – 0.15E + 0.33F + 0.044G + 0.070H – 0.12AB – 0.048AD – 0.11AE – 0.034AF – 0.053AG – 0.33AH – (2)

Adequacy and fitness of bacteriocin activity and total viable cells were evaluated by ANOVA and calculation of regression coefficients are presented in Table 6. ANOVA analysis of bacteriocin activity and total viable cell production indicated that the confidence levels were greater than 95%. The high F-value and a very low probability ($P>F= 0.0001$) indicated that the present model for both bacteriocin activity and total viable cell production showed good agreement between predicted and experimental results. From the ANOVA analysis for bacteriocin activity, linear terms C, G and H, interaction terms AE and AF were statistically insignificant at probability level ($P<0.05$). It is evident from the Table 6, the linear terms A, B, D, E and F, interaction terms AC, AD, AG and AH were statistically significant ($P<0.05$). Whereas for total viable cell production, except linear terms G and interaction terms AD and AC all other linear, interaction terms were statistically significant at probability level ($P<0.05$). It is confirmed from the Table 6. The steepest ascent path was determined by Eqs. (1 & 2) and from analyzed regression results. The first order model Eqs. (1 & 2) predicted the higher amount of peptone (A), meat extract (B), lactose (D) and lower concentration of yeast extract (C), glycerol (E), Tween 80 (F), triammonium citrate (G) and $K_2HPO_4$ (H) significantly increased the bacteriocin (22,343 AUml$^{-1}$) and viable cell (12.61 LogCFUml$^{-1}$) production. Therefore, significant main effects such as peptone, meat extract and lactose concentration were increased and decreased, in order to get a positive consequence in response variables. Consequently, this medium was selected for further optimization.
4.3.4.1.2. Response surface methodology

The objective of this second experiment is to develop an empirical model of the process and to obtain a more precise estimate of the optimum operating conditions for the factors involved. This approach to process optimization is called response surface methodology and the second design is a central composite design (CCD), one of the most important experimental designs used in process optimization studies. In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed. Three factors (peptone, meat extract and lactose) were selected for this model based on the factorial design. The CCD experiments contained a total of 20 experimental trials that include eight trials for factorial design, six trials for axial points and six trials for replication of the central points (Table 7). The variables were selected based on the results of 2 level factorial designs. For MRS medium optimization, the levels of the three significant variables, peptone (A), meat extract (B) and lactose (D) were further optimized by RSM using central composite design. The ranges of the variables are 30-40g/L for peptone, meat extract and 18-24 g/L for lactose. The experimental design and the results are presented in Table 7. The maximum experimental value for bacteriocin activity and total viable cells was (32,400 AUml\(^{-1}\) and 13.28 LogCFUml\(^{-1}\)), while the predicted response based on RSM was estimated to be 30,196 AUml\(^{-1}\) and 13.27 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 the bacteriocin activity and total viable cells as a function of these variables. By applying multiple regression analysis on the experimental data, the following coded final equation was found to explain bacteriocin activity and total viable cells production:
Bacteriocin activity = + 22147.13 + 3780.64A + 439.34B + 2520.20C + 750.0AB + 1775.0AC – 750.0BC + 187.44A_2 – 395.93B_2 – 360.57C_2 

Total viable cells = + 12.85 + 0.34A – 0.069B + 0.16C – 0.032AB – 0.059AC + 0.019BC – 0.054A_2 – 0.057B_2 – 0.17C_2

The statistical significance of the model equation was evaluated by F-test which showed that the regression is statistically significant at probability level (P<0.05) for bacteriocin activity. The model F-value of 9.42 implies the model for bacteriocin activity is significant. The value of p > F less than 0.05 indicates that the model terms are also significant. Although, the linear terms A and D, interaction terms AD are significant (Table 8). The high level factorial point (+1) of factors peptone, meat extract and lactose have improved the level of bacteriocin activity (32,400 AU/mL) and total viable cells (13.27 LogCFU/mL) production. The bacteriocin activity and total viable cell production was low under low level (−1) star point of all factors. Further, interaction of low level factorial points (−1) of all factors was not significantly affect the bacteriocin activity and total viable cell production. However, the interaction of any one environmental factor at low level factorial point with other factors at high level factorial point supported the higher bacteriocin activity and total viable cell production, suggesting high level factorial level of any two environmental factors is necessary for bacteriocin activity and total viable cell production.

The interaction between the nutrients and their effects on bacteriocin activity and total viable cell production was plotted in Figure 10. The response surface plot of the model equation suggested that increased level of bacteriocin activity and total viable cells production was obtained from the increasing concentration of all factorial factors. From the model, we can obtain the maximum prediction point of the model, which was 40 g/L of peptone, 35 g/L of meat extract and 24 g/L of lactose.
Table 4. Experimental range and levels of the independent variables (A, B, C, D, E, F, G and H) used in the 2 level factorial design (FD)

<table>
<thead>
<tr>
<th>Independent variables (g/L)</th>
<th>Low level (-1)</th>
<th>centre point (0)</th>
<th>High level (+1)</th>
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<tr>
<td>MRS medium optimization</td>
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<tr>
<td>A – Peptone</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
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<tr>
<td>B – Meat extract</td>
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<td>20.0</td>
<td>30.0</td>
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<tr>
<td>C – Yeast extract</td>
<td>2.5</td>
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<tr>
<td>D – Lactose</td>
<td>6.0</td>
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<tr>
<td>E – Glycerol</td>
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<td>12.0</td>
<td>18.0</td>
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<tr>
<td>F – Tween 80</td>
<td>3.0</td>
<td>6.0</td>
<td>9.0</td>
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<tr>
<td>G – Tri ammonium citrate</td>
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<td>H – K₂HPO₄</td>
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<td>5.0</td>
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Table 5. Experimental design and results of the 2 level factorial design (FD) for optimization of MRS fermentation medium for bacteriocin and total viable cells production by S. phocae PI80

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>Bacteriocin activity (AU/ml)</th>
<th>Total viable cells (LogCFU/ml)</th>
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Table 6. Regression analysis (ANOVA) of two level factorial for bacteriocin activity and *S. phocae* PI80 total viable cells in optimization of MRS fermentation medium

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Table 7. Experimental design and results of central composite design (CCD) for bacteriocin and total viable cells production by *S. phocae* PI80

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Table 8. Regression analysis (ANOVA) of CCD for bacteriocin activity and *S. phocae* PI80 total viable cells in optimization of MRS medium

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Figure 10. Response surface contour plots of bacteriocin activity (AUml\(^{-1}\)) and total viable cells (LogCFUml\(^{-1}\)) for *S. phocae* PI80. The counter plots represent the effect of the significant variables and their interaction with the response variable. The effects of peptone, lactose and their mutual interaction on bacteriocin activity and viable cells production are expressed in plots A and B.
The model predicted a maximum response for bacteriocin activity (33,049.8 AU/ml) and total viable cells (14.05 LogCFU/ml). In order to confirm the predicted results of the model, the maximum bacteriocin activity (32,400 AU/ml) was obtained from the experiments. This observation suggested that in the case of bacteriocin activity and total viable cell production, peptone, meat extract and lactose exhibited significant interaction. This indicated that these three factors played an important role for both bacteriocin and total viable cell production. Moreover, the optimum MRS fermentation medium composition for the bacteriocin production and the growth of *S. phocae* P180 consists of: peptone (40 g/L), meat extracts (35 g/L), yeast extract (7.5 g/L), lactose (24 g/L), glycerol (6 g/L), Tween 80 (6 g/L), tri ammonium citrate (1 g/L) and K$_2$HPO$_4$ (2.5 g/L).

4.3.4.2. Effects of MRS components on bacteriocin production and cell growth of *E. faecium* MC13

4.3.4.2.1. Two level factorial design

*E. faecium* MC13 results of the experiments to achieve MRS medium optimization are presented in Table 9. The bacteriocin activity varied markedly from 4,900 to 22,500 AU/ml, and total viable cell counts varied from 10.85 to 12.94 LogCFU/ml 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, tween80 and K$_2$HPO$_4$ did not significantly affect the bacteriocin activity. Regression coefficients were calculated, and the response variables bacteriocin activity and total viable cells could be expressed according to the experimental data:
Table 9. Experimental design and results of the 2-level factorial design for optimization of MRS medium components for bacteriocin and total viable cells production by *E. faecium* MC13

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</table>

Total viable cells = + 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 - (2)

The first order model in Eq. (1&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 10 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 of 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 0.9979) were favourably to confirm the model significant and showed statiscally not significant lack of fit (Table 11). Though the model was supposed to be adequate for prediction within the variables. ANOVA of bacteriocin activity and total viable cell production indicated that the confidence levels were greater than 99%. The high F-value and low P-value (P<0.0001) indicate that the present model for both bacteriocin activity and total viable cell production shows good agreement between predicted and experimental results. From the ANOVA for bacteriocin activity, linear terms A, B, C, D and G and interaction terms AB and AC were statisticaly significant at probality level (P<0.05). As well as in the total viable cell production, all linear and interaction terms except E, F, G, H, AD, AG and AH were statisticaly signifiant (P<0.05) (Table 10).
Table 10. Regression analysis (ANOVA) for bacteriocin activity and total viable cells in the optimization of MRS fermentation medium

<table>
<thead>
<tr>
<th>Source</th>
<th>Bacteriocin activity</th>
<th>Total viable cells</th>
<th>Bacteriocin activity</th>
<th>Total viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>1046.24</td>
<td>612.93</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A</td>
<td>6677.35</td>
<td>3028.57</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B</td>
<td>1850.27</td>
<td>958.71</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C</td>
<td>2613.35</td>
<td>1552.12</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>D</td>
<td>465.38</td>
<td>420.70</td>
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<td>&lt;0.0001</td>
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<tr>
<td>E</td>
<td>218.27</td>
<td>-</td>
<td>0.0001</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>135.40</td>
<td>15.53</td>
<td>0.0003</td>
<td>0.0169</td>
</tr>
<tr>
<td>G</td>
<td>535.40</td>
<td>33.02</td>
<td>&lt;0.0001</td>
<td>0.0045</td>
</tr>
<tr>
<td>H</td>
<td>34.62</td>
<td>-</td>
<td>0.0042</td>
<td>-</td>
</tr>
<tr>
<td>AB</td>
<td>476.74</td>
<td>685.56</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
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<td>470.02</td>
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<td>&lt;0.0001</td>
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<tr>
<td>AD</td>
<td>-</td>
<td>29.92</td>
<td>-</td>
<td>0.0054</td>
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<tr>
<td>AE</td>
<td>41.04</td>
<td>375.78</td>
<td>0.0030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AF</td>
<td>135.40</td>
<td>358.69</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AG</td>
<td>-</td>
<td>25.89</td>
<td>-</td>
<td>0.0070</td>
</tr>
<tr>
<td>AH</td>
<td>167.54</td>
<td>12.66</td>
<td>0.0002</td>
<td>0.0236</td>
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<tr>
<td>Curvature</td>
<td>144.02</td>
<td>82.63</td>
<td>0.0003</td>
<td>0.0008</td>
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Table 11. Analysis of variance (ANOVA) for the first order models evaluated by two level factorial design.

<table>
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<tr>
<th>Values</th>
<th>Bacteriocin production</th>
<th>Total viable cells production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model F-values</td>
<td>1046.24</td>
<td>612.93</td>
</tr>
<tr>
<td>Model P-Values</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Curvature F-Value</td>
<td>144.02</td>
<td>82.63</td>
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<tr>
<td>Curvature P-Value</td>
<td>0.0003</td>
<td>0.0008</td>
</tr>
<tr>
<td>R-Squared</td>
<td>0.9997</td>
<td>0.9995</td>
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<tr>
<td>Adj R-Squared</td>
<td>0.9988</td>
<td>0.9979</td>
</tr>
<tr>
<td>Pred R-Squared</td>
<td>0.9812</td>
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</tr>
<tr>
<td>Adeq Precision</td>
<td>104.696</td>
<td>99.750</td>
</tr>
</tbody>
</table>
4.3.4.2.2. Steepest ascent path for optimization

The steepest ascent path was determined by Eqs. (1 & 2) and from analyzed regression results. The first order model Eqs. (1 & 2) were predicted the higher amount peptone (A), yeast extract (C), lactose (D) and lower concentration of meat extract (B), glycerol (E), Tween 80 (F), triammonium citrate (G) and K₂HPO₄ (H) were significantly increased the bacteriocin (26635.6 AUml⁻¹) and viable cell (13.0 LogCFUml⁻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.

4.3.4.2.3. 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 12. 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. 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 = 22944.78 + 4238.93A + 2945.89C + 0.000AB + 975.00AC + 0.000BC + 1312.06A² + 1135.28B² - 544.10C²

Total viable cells = 13.14 + 0.47A + 0.32B + 0.36C - 0.060AB + 0.071AC + 0.054BC - 0.015A² - 0.15B² - 0.16C²
The statistical significance of the model equation was evaluated by an F-test, which showed that the regression was statistically significant \((P<0.05)\) for bacteriocin activity. The model F-value of 7.35 and 5.11 implies that the models for bacteriocin activity and viable cell production were significant. The value of \(p > F\) less than 0.05 indicates that the model terms are also significant. Table 13 showed regression analysis (ANOVA) of CCD for bacteriocin activity and total viable cell production by \(E.\ faecium\) MC13. The linear terms A and D; interaction terms AC are significant. The high-level factorial points (+1) of the peptone, yeast extract and lactose factors improved the level of bacteriocin activity (32,400 AU ml\(^{-1}\)) and the number of total viable cells (13.46 LogCFU ml\(^{-1}\)). The interaction between the nutrients and their effects on bacteriocin activity and total viable cell production are plotted in Figures 11. 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 of peptone, 40 g/L of yeast extract and 24 g/L of lactose. The model predicted a maximum response for bacteriocin activity (35,038.1 AU ml\(^{-1}\)) and total viable cell count (14.02 LogCFU ml\(^{-1}\)). To confirm the predicted results of the model, experiments were performed and maximum bacteriocin activity (36,100 AU ml\(^{-1}\)) 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. In addition, the optimal composition of MRS fermentation medium for bacteriocin production and growth of \(E.\ faecium\) MC13 consists of: peptone (40 g/L), meat extract (30 g/L), yeast extract (40 g/L), lactose (24 g/L), glycerol (5.8 g/L), Tween 80 (3 g/L), triammonium citrate (1 g/L) and \(K_2\)HPO\(_4\) (2.5 g/L).
Table 12. Experimental design and results of central composite design for bacteriocin and total viable cell production by *E. faecium* MC13

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>C</th>
<th>D</th>
<th>Bacteriocin activity (AU/ml)</th>
<th>Total viable cells (LogCFU/ml)</th>
<th>Actual</th>
<th>Predicted</th>
<th>Actual</th>
<th>Predicted</th>
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</table>

Table 13. Regression analysis (ANOVA) of CCD for bacteriocin activity and *E. faecium* MC13 viable cells in optimization of MRS medium

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
<th>Total viable cells</th>
<th>p-Value (Prob&gt; F)</th>
<th>Bacteriocin activity</th>
<th>Total viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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</tr>
<tr>
<td>C</td>
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<tr>
<td>D</td>
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</tr>
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Figure 11. Response surface contour plots of bacteriocin activity (AU/ml) and total viable cells (LogCFU/ml) for *E. faecium* MC13. The counter plots represent the effect of the significant variables and their interaction with the response variable. The effects of peptone, yeast extract, lactose and their mutual interaction on bacteriocin activity and viable cells production are expressed in plots A and B.
4.4. Discussion

The production of bacteriocin or bacteriocin like substances by many lactic acid bacteria such as *Streptococcus thermophilus*, *Enterococcus faecium*, *Micrococcus* spp, and *Lactobacillus* species has been reported (Kabuki et al., 2007; Kanmani et al., 2011b; Preetha et al., 2007a). Several bacterial strains produced bacteriocins which were exhibited narrow spectrum of inhibitory activity against pathogenic bacteria (Kayalvizhi and Gunasekaran, 2008). But some bacterial strains produce bacteriocin with broad spectrum of antimicrobial activity against diverse group of Gram positive and Gram negative bacterial pathogens especially food born, shrimp and fish pathogens such as *Listeria monocytogenes* and *Vibrio* and *Aeromonas* species (Kanmani et al., 2011a). *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 were able to produce bacteriocins which also exhibited broad spectrum of inhibitory activity against various Gram positive and Gram negative bacterial strains including *Lactobacillus plantarum*, *L. acidophilus* and *L. rhamnosus* (Kanmani et al., 2011b). Similarly, lactic acid bacterium *S. thermophilus* SBT1277 isolated from raw milk produced bacteriocin which showed broad range of inhibitory activity against diverse group of bacterial strains particularly *Lactobacillus* spp., and food spoilage bacteria *Clostridium* species (Kabuki et al., 2007). Sensitivity of the *S. phocae* PI80 and *E. faecium* MC13 bacteriocins to proteolytic enzyme (Proteinase K) confirmed their proteinaceous nature. Moreover these bacteriocins were found to have UV, pH and heat stability. As all these findings are identical to the characteristics of many bacteriocins produced by lactic acid bacteria, antimicrobial substances from *S. phocae* PI80 and *E. faecium* MC13 were suggested to be a bacteriocin.

Many research works have demonstrated that physiological factors such as temperature, pH, salinity and media components play an important role in the production of bacteriocins by
bacterial strains (Anthony et al., 2009; Preetha et al., 2007b; Kabuki et al., 2007). The kinetics of the bacteriocin and total viable cells production by *S. phocae* PI80 and *E. faecium* MC13 were investigated in MRS broth at various temperatures. At all temperatures, bacteriocin production started in late log phase (after 2 h of incubation), and the maximum production of 16,900 AU/ml was observed in the mid-stationary phase at 35°C. At 45°C, bacteriocin production *S. phocae* PI80 and *E. faecium* MC13 were decreased to 14,400 and 10,000 AU/ml, which were observed between 10-12 h and 6-12 h of incubation. Thereafter, bacteriocin production decreased slowly to the end of the incubation period. This has been occurred due to the several possible mechanisms such as protein aggregation, proteolytic degradation by a specific and nonspecific enzymes and adsorption to producer cells (Kabuki et al., 2007). Initially, the PI80 and MC13 viable cell counts increased after 6 h of incubation at all temperatures, subsequently stabilizing until the end of the incubation period. The pH of the medium decreased from an initial pH of 6.6 to 4.5, indicating the production of lactic acid. Similarly, production of bacteriocin by *S. thermophilus* SBT1277 increased when temperature increased form 25°C to 35°C (Kabuki et al., 2007). *Enterococcus faecium* MC13 produced higher amount bacteriocin when cells grown at 35°C than at 20°C (Leory and De Vuyst, 1999). The production of bacteriocin by *S. thermophilus* 81 incubated at 30°C which was higher than that incubated at 42°C (Ivanova et al., 1998). Moreover, Kim et al. (2006) reported that the bacteriocin production by *Micrococcus* spp. GO5 was higher when the temperature was increased from 25°C to 37°C.

Bacteriocin and total viable cell production were mainly dependent on the medium pH during the fermentation period. The use of pH controlled fermentation can lead to enhance the growth of bacterial cells and subsequently increase the bacteriocin production (Herranz et al., 2001). So the effect of culture pH on growth and bacteriocin production by *S. phocae* PI80 and
E. faecium MC13 were investigated in MRS fermentation broth. The maximum bacteriocin activity (16,900 AUml⁻¹) and total viable cells (12.622 and 12.607 LogCFUml⁻¹) production by S. phocae PI80 and E. faecium MC13 were observed when bacteria incubated at pH 6.5, but the activity was decreased at pH 5.5, 6.0 and 7.0. These results indicated that the culture pH had a strong effect on bacteriocin and viable cell production by probiotics S. phocae PI80 and E. faecium MC13. Similarly, the optimal pH for the production of bacteriocin was 6.5, with the maximum bacteriocin activity of 640 AUml⁻¹ obtained after 5-6 h of incubation (Kabuki et al., 2007). Aktypis et al. (1998) reported that the thermophilin T production by S. thermophilus ACA-DC was much higher under the controlled medium pH of 6.2 than 6.8. Production of bacteriocin by E. faecium P13 have increased four fold when cells grown at controlled pH 6.0 than uncontrolled pH (Herranz et al., 2001). Moreover, 6.5 is the optimal pH for bacteriocin production in S. warneri (Prema et al., 2006), L. pentosus ST71BZ (Todorov and Dicks, 2007) and B. coagulans (Emad, 2008). Bacteriocin production by S. phocae PI80 and E. faecium MC13 was influenced by the presence of NaCl in the MRS fermentation medium. The optimal salinity for the production of bacteriocin by S. phocae PI80 and E. faecium MC13 were 1% and 2%, with the maximum production of 19,600 AUml⁻¹. However, higher concentration (3%) decreased the bacteriocin production by S. phocae PI80. In common, NaCl affect the growth and bacteriocin production in several bacterial strains (Delgado et al., 2005). This negative effect of NaCl on bacteriocin production may attribute duo to its interference with the inducer receptor interaction (Nilsen et al., 1998). Delgado et al. (2005) have reported that the addition of NaCl enhances the production of the bacteriocin plantaricin S 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.
Bacteriocin and total viable cells production generally depend on environmental factors and the concentration of the growth medium. Therefore, optimization of media components were also important culture parameters for the enhancement of bacteriocin and total viable production (Preetha et al., 2007b; Kayalvizhi and Gunasekaran, 2008; Anthony et al., 2009). Bacteriocin production by probiotic bacteria *S. phocae* PI80 and *E. faecium* MC13 were found to be growth associated and the yield of bacteriocin was increased using a response surface optimization of MRS medium components. One factor at a time (OVAT) approach is a classical method which generally used for media optimization, but a time consuming and a labour intensive method. Rodriguez et al. (2006) reported that the fractional factorial design is an important and valuable tool for the rapid evaluation of the effects of different media components. Optimization through factorial design and response surface analysis is a general practice in biotechnology and various researchers used these techniques for the optimization of media components (Preetha et al 2007b; Kayalvizhi and Gunasekaran 2008; Anthony et al., 2009). Hence in this study, optimization of MRS media components were accomplished by two consecutive steps; first a two level factorial design was performed to identify the significant variables that affect the bacteriocin and viable cell production by *S. phocae* PI80 and *E. faecium* MC13. Later, response surface methodology using central composite design with five coded levels (−α, −1, 0, +1, +α) was performed to estimate linear and quadratic effects and their corresponding response surfaces. Peptone (4%), meat extracts (3.5%), and lactose (2.4%) have significantly influenced the bacteriocin and viable cell production by probiotic *S. phocae* PI80. Similarly, the bacteriocin activity and viable cells of *E. faecium* MC13 were also enhanced by addition of peptone (4%), yeast extract (4%) and lactose (2.4%). These results are in agreement with the reported bacteriocin production by *Micrococcus* GO5 spp., (Preetha et al., 2007a) and antimicrobial
peptide from *B. licheniformis* AnBa9 (Anthony et al., 2009) and bacteriocin form *L. plantarum* LPCO10 (Leal-Sanchez et al., 2002). The production of bacteriocin from *B. licheniformis* MKU3 was significantly enhanced by the presence of peptone at the concentration of 5 g/L (Kayalvizhi and Gunasekaran, 2008). In addition, peptone was found to be significantly increasing the nisin production by *Lactococcus lactis* ATCC 11454 (Li et al., 2002). Anthony et al. (2009) reported that the yeast extract play an important role in antimicrobial peptide production by *B. licheniformis* AnBa9 when compared with other chemicals. High concentrations of yeast extract (4.5%) and lactose (1.7%) influenced antimicrobial peptide production by *B. licheniformis* AnBa9. This possible effect could be due to the availability of high quantity of free amino acids and short peptides and more growth factors from yeast extract that induce bacteriocin production by bacterial strains (Cheigh et al., 2002). The lactose and lactose rich substances like sausage, whey and skimmed milk powder induced bacteriocin production in LAB and other bacteriocin producing bacteria (Cheigh et al., 2002; Todorov and Dicks, 2004). Todorov et al. (2000) found the higher concentrations of K$_2$HPO$_4$ repressed the bacteriocin activity of plantaricin ST31, which also agrees well with our results of bacteriocin production.