Korean J. Food Sci. Ani. Resour.
Vol. 30, No. 1, pp. 66~72(2010)

ARTICLE

Medium Optimization for Pediocin SA131 Production by *Pediococcus pentosaceus* SA131 against Bovine Mastitis Using Response Surface Methodology

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Abstract

Pediococcus pentosaceus SA131 was isolated from *jeotgal*, is the bacteriocin producer against bovine mastitis pathogens, *Streptococcus uberis* E290, *Enterococcus gallinarum* E362, and *Staphylococcus epidermidis* ATCC 12228. The medium composition for pediocin SA131 production by *P. pentosaceus* SA131 was optimized using response surface methodology. Component of medium was studied as carbon source (glucose, fructose, lactose, glycerol, sucrose, maltose, and mannitol), nitrogen source (beef extract, yeast extract, peptone, malt extract, and tryptone), mineral and surfactant ($MgSO_4$, KH_2PO_4, $(NH_4)_2SO_4$, $MnSO_4$, NaCl, sodium acetate, and Tween 80). Through one factor-at-a-time experiment, glucose, fructose, yeast extract, malt extract, NaCl, $MgSO_4$, and Tween 80 were determined as the good ingredient. The effects of major factors for pediocin SA131 production were investigated by two-level fractional factorial designs (FFD). By a 2^4 FFD, fructose, yeast extract, and $MnSO_4$ were found to be the important factors for the bacteriocin production. Subsequently, a 2^3 central composite design (CCD) was adopted to derive a statistical model for optimizing the composition of the fermentation medium. The estimated optimum composition for the production of pediocin SA131 by *P. pentosaceus* SA131 was as follows; 0.13% fructose, 1% glucose, 1.8% yeast extract, 2.58% $MnSO_4$, 0.2% NaCl, and 0.2% Tween 80. The pediocin production under optimized medium was increased to 1,000 AU/mL, compared to the 400 AU/mL in MRS medium.

Key words: *Pediococcus pentosaceus* SA131, bacteriocin, pediocin SA131, medium optimization, bovine mastitis, response surface methodology

Introduction

Bovine mastitis is an inflammation of the mammary glands due to a microbial infection and is one of the most important causes of economic losses to the dairy industry due to rejected milk, degraded milk quality, and drug costs (Nascimento *et al*., 2005). Although several bacterial pathogens can cause bovine mastitis, *Staphylococcus* spp. and *Streptococcus* spp. are the major causative agents and it is difficult to eradicate (Coelho *et al*., 2007; Ochoa-Zarzosa *et al*., 2008).

The treatment of mastitis makes use of antimicrobial substances. These methods include management of immediate release procedures during lactation or long-acting procedures during the non-lactating period. Most of drugs have been utilized for non-lactating cow therapy. This treatment is considered inadequate because of limited success, cure rates vary considerably. In addition, public authorities advise careful use of antibiotics as their use that may promote bacterial antibiotic resistance and leave antibiotic residues in the food chain. Moreover, it has been verified that the indiscriminate treatment with antibiotics, without either a technical prescription or identification tests of the pathogen, can contribute to an increased resistance of their microorganisms, making the cure of mastitis more difficult. Thus, the alternative methods for controlling mastitis are necessary and one of these methods could be the use of bacteriocins (Coelho *et al*., 2007).

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Bacteriocins are antimicrobial peptides produced by various lactic acid bacteria (LAB) including lactobacilli, lactococci, leuconostoc, and pediococci (Choi *et al*., 2000). LAB have been utilized as starter strains, probiotics, foods, and feed additives for therapeutic, prophylactic acid growth promotion in animal. Therefore, bacteriocins produced by LAB have presented a potential use either in food industries as biopreservatives or in the prevention and treatment of some infectious diseases, having medical and veterinary applications. Nisin from *Lactococcus lactis* is representative bacteriocin, has activity against mastitis and has been widely used as a food preservative in many countries (Barboza-Corona *et al*., 2009). The staphylococcal bacteriocins showed antibacterial activity against *Staphylococcus aureus* and *Streptococcus agalactiae* involved in bovine mastitis (Coelho *et al*., 2007). Also, lacticin NK34 was indentified as a bacteriocin having inhibitory activity against mastitis (Lee *et al*., 2008).

The production of bacteriocins is influenced by bacterial growth because bacteriocins are synthesized during the growth of the producing organism. Bacteriocin production is also affected by the medium compositions such as carbon sources, nitrogen sources, growth factors, and inorganic salts. Therefore, the medium compositions are very important for the optimization of bacteriocin production. It is difficult to search for the major factors and to optimize them for biotechnological processes including multivariable. For optimizing multivariables, the classical 'one factor-at-a time' method has utilized. This method involves changing independent variable while fixing all others at a fixed level. However, it is extremely time-consuming and expensive for a large number of variables and also may result in wrong conclusions. One of the valuable techniques to identify the explanatory variable in the system is response surface methodology (RSM). This is a collection of statistical techniques for designing experiments, building models, evaluation the effects of factors, and searching optimal conditions of factors for desirable response (Cheigh *et al*., 2002; Delgado *et al*., 2007; He *et al*., 2004; Li *et al*., 2002).

The aim of this study was to optimize culture medium for maximum production of pediocin SA131 using RSM.

Materials and Methods

Bacterial strains and culture media

P. pentosaceus SA131 was isolated from *jeotgal*, cultured in lactobacilli MRS medium (Difco Laboratories, Detroit, MI, USA) at 35°C. The indicator strain was *Staphylococcus epidermidis* ATCC 12228. *S. epidermidis* ATCC 12228 was cultured in tryptic soy broth (TSB, Difco) at 35°C. These strains were stored at -70°C in medium with 20%(v/v) glycerol.

Antimicrobial activity assay

Pediocin SA131 activity was determined by the spot-on-lawn method (Lee *et al.*, 2008). Soft MRS agar seeded (1%, v/v) with the indicator microorganisms (ca. 10^7 CFU) was overlayed on the plate, and was solidified. Bacteriocin solution was diluted serially using two-fold dilution, and 10 μL of each dilution was spotted on the plate. The plates were incubated at 35°C overnight. The bacteriocin activity was determined as arbitrary unit (AU) as follow; Bacteriocin activity (AU/mL) = $2^N \times 100$, where N = dilution number with the smallest zone of inhibition.

Selection of the best medium for bacteriocin production

Carbon, nitrogen source, mineral and surfactant for selection of the best source have been screened by one factor-at-a time method. Firstly, for selection of carbon source, 1%(w/v) carbon source (glucose, fructose, lactose, glycerol, sucrose, maltose, and mannitol) was added in 200 mL flask containing 100 mL of medium with 1% yeast extract. After selection of carbon source, the cultivation medium with 1%(w/v) nitrogen source (beef extract, yeast extract, peptone, malt extract, and tryptone) and 1% fructose was cultured. Subsequently, the medium with mineral and surfactant ($MgSO_4$, KH_2PO_4, $(NH_4)_2SO_4$, $MnSO_4$, NaCl, sodium acetate, and Tween 80) and 1% fructose and yeast extract was cultured. Cultivation was performed for 20 h of incubation at 35°C. The number of viable cells of the culture was determined as colony forming unit (CFU) and antimicrobial activity (AU/mL) of pediocin SA131 was observed after 20 h.

Fractional factorial design

Seven variables affecting production of pediocin SA131 were selected by one factor-at-a time method. The variables having significant effects were identified using a fractional factorial design (FFD). This method is very useful in identifying the important compounds and interactions between two and more nutrients in few experiments (Kim *et al*, 2004; Wang and Liu, 2008). FFD at the given range of the above parameters in terms of coded and actual values is presented in Table 1. A 2^4 FFD with seven factors at two levels, 2^4=16 runs and four runs were center point runs for statistical reasons. Data analy-

Table 1. The concentration of variables of different levels of the 2^4 fractional factorial design (%, w/v)

Variable code	Independent variables	Levels		
		-1	0	1
X_1	Fructose	0	0.5	1
X_2	Glucose	0.5	1	1.5
X_3	Yeast extract	0.5	1	1.5
X_4	Malt extract	0	0.5	1
X_5	NaCl	0.1	0.2	0.3
X_6	$MnSO_4$	0	0.1	0.2
X_7	Tween 80	0.1	0.2	0.3

sis was generated by the software SAS (Statistical Analysis Program, version 9.1). The major effects of variables on bacteriocin production were identified for the isolates on the basis of confidence levels above 95% (p<0.05). From the FFD experimental results, a first-order model was represented by;

$$Y = \Sigma a_i x_i + c_o + \varepsilon \quad (1)$$

Where Y is the predicted response (bacteriocin activity). The parameters a_i are functions of x_i, c_o is the intercept term and the remaining term, ε, represents random errors in the yield values.

Central composite design and statistical analysis

To describe the nature of the response surface in the optimum region, a 2^3 factorial central composite design (CCD) was performed. CCD with six star points ($\alpha = 2$) and three replications of the central points was used at five levels. The levels for each factor are given in Table 4. For predicting the optimal point, a second-order polynomial function was;

$$Y = c_0 + \Sigma_{i=1}^{n} a_i x_i + \Sigma_{j \le i}^{n} b_{ij} x_i x_j \quad (2)$$

Where Y, predicted response, stands for bacteriocin activity (AU/mL). Subscripts i and j takes values from 1 to the number of variables (n); the c_o is the intercept term; the a_i values are linear coefficient; the b_{ij} values are quadratic coefficients; x_i and x_j are the level of the independent variables (Liu and Tzeng, 1998; Purama and Goyal, 2008). The data analysis was generated by the software SAS (Statistical Analysis Program, version 9.1).

Results and Discussion

Pediocin SA131 production in MRS broth

The growth of *P. pentosaceus* SA131 and the production of pediocin SA131 were observed in MRS broth at 35°C for 24 h (Fig. 1). The cells reached stationary phase after 18 h of fermentation and the number of viable cell was 9.46 Log CFU/mL. The production of pediocin SA131 approximately followed the growth curve and increased to 400 AU/mL during the exponential phase of growth and remained at this level throughout the stationary phase. For selection of the best components, one factor-at-a time method was taken by 20 h of incubation at 35°C.

Effect of different source in cell growth and pediocin SA131 production

The effect of carbon source was studied using medium with 1%(w/v) different carbon source (glucose, fructose, lactose, glycerol, sucrose, maltose, and mannitol) and 1% yeast extract. As shown in Fig. 2A, *P. pentosaceus* SA131 in media contained gluocose or fructose showed the high bacteriocin activity of pediocin SA131. At 1%(w/v) concentration of carbon source, the bacteriocin activity was 40 and 80 AU/mL for glucose and fructose, respectively. However, maximum growth was observed in 1% sucrose. Accordingly, glucose and fructose were selected as the potential carbon source for production of pediocin SA131. It was also reported to be suitable carbon source for pediocin SA131 production by *P. acidilactici* NRRL B5627 (Anastasiadou *et al*., 2008).

In order to assess the influence of different nitrogen source on bacteriocin production, the cultivation medium with 1%(w/v) nitrogen source (beef extract, yeast extract, peptone, malt extract, and tryptone) and 1% fructose was cultured. As shown in Fig. 2B, the bacteriocin activity in the medium contained 1% yeast extract or 1% malt extract was higher than others. Yeast extract played an important role on the production of the bacteriocin. Due to the use of larger quantity of free amino acids, short peptides and more growth factors from yeast extract that induced bacteriocin production (Anthony *et al*., 2009; Cheigh *et al*., 2002; Kim *et al*., 2006).

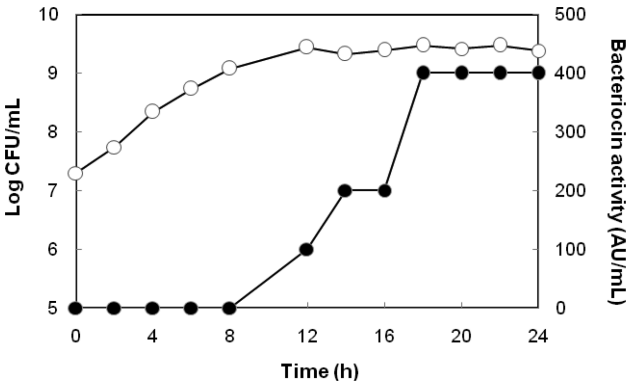

Fig. 1. Growth of *P. pentosaceus* SA131 (○) and production of pediocin SA131 (●) in MRS broth.

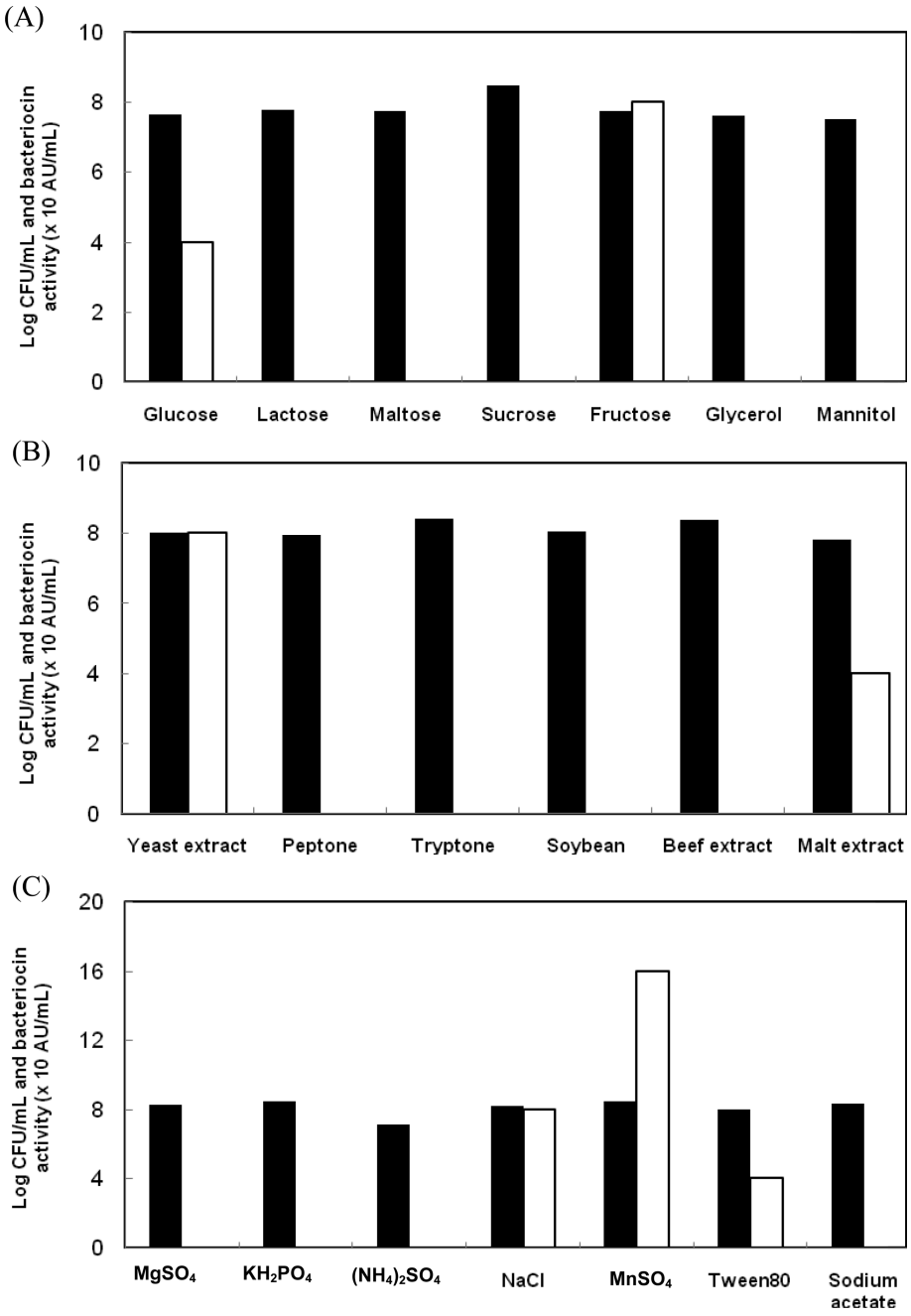

Fig. 2. Effect of different sources on the growth (■) and production of pediocin SA131 (□) at 20 h. (A) effect of carbon sources, (B) effect of nitrogen sources, (C) effect of minerals and surfactants.

The effect of mineral and surfactant was tested using medium with 0.2%(w/v) mineral and surfactant ($MgSO_4$, KH_2PO_4, $(NH_4)_2SO_4$, $MnSO_4$, NaCl, sodium acetate, and Tween 80), 1% fructose and 1% yeast extract. As shown in Fig. 2C, pediocin SA131 activity was observed in $MnSO_4$, NaCl, and Tween 80. An increase in $MnSO_4$ and Tween 80 concentration results in the increase of plantaricin 423 (Verellen *et al*., 1998) and pediocin PD-1 (Nel *et al*., 2001). Therefore, seven variables (glucose, fructose, yeast extract, malt extract, $MnSO_4$, NaCl, and Tween 80) supposed to affect pediocin SA131 production were selected for the two-level FFD.

Optimization for pediocin SA131 production by FFD

A two-level FFD was employed and Table 2 shows the seven independent variables and their concentrations at the different coded levels of the FFD and the corresponding response. The corresponding first-order model equation fitted to the experimental data has the formula;

Table 2. Experimental design and results of 2^4 fractional factorial design

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Bacteriocin activity (AU/mL)
1	-1	-1	-1	-1	-1	-1	-1	0
2	1	-1	-1	-1	1	1	1	200
3	-1	1	-1	-1	1	1	-1	300
4	1	1	-1	-1	-1	-1	1	0
5	-1	-1	1	-1	1	-1	1	300
6	1	-1	1	-1	-1	1	-1	300
7	-1	1	1	-1	-1	1	1	600
8	1	1	1	-1	1	-1	-1	200
9	-1	-1	-1	1	-1	1	1	200
10	1	-1	-1	1	1	-1	-1	0
11	-1	1	-1	1	1	-1	1	0
12	1	1	-1	1	-1	1	-1	0
13	-1	-1	1	1	1	1	-1	200
14	1	-1	1	1	-1	-1	1	0
15	-1	1	1	1	-1	-1	-1	0
16	1	1	1	1	1	1	1	150
17	0	0	0	0	0	0	0	100
18	0	0	0	0	0	0	0	100
19	0	0	0	0	0	0	0	100
20	0	0	0	0	0	0	0	100

$$Y = -41.87 - 93.75X_1 + 6.25X_2 + 131.25X_3 - 168.75X_4 + 156.25X_5 + 906.25X_6 + 281.25X_7 \quad (3)$$

From Eq. (3), $MnSO_4$ (X_6) was shown to be the most significant factor for pediocin SA131 production. Manganese have roles as constituents of important enzymes involved in glucose hydrolysis and oxidative stress response in lactic acid bacteria (Coenye and Vandamme, 2003; Miyoshi *et al.*, 2003). A main protective role of manganese ions from oxidation is expected to enhance overall culture productivity. In addition, an improved glucose conversion rate in the presence of manganese should be expected to increase the flux towards end-products synthesis in the metabolic machinery of *Pediococcus* (Anastasiadou *et al*., 2008). Regression analysis of the FFD in Table 3 presented fructose, yeast extract, malt extract, and $MnSO_4$ were significant at the probability levels of 95% and proved to be the most important components. Glucose, NaCl, and Tween 80 were not found to be statistically significant.

Optimization for pediocin SA131 production by steepest ascent path

Steepest ascent method utilizes the magnitude and sign of the linear effects to determine the direction toward a predictive higher response. The path begins at the center of the current design space and stretched well outside the design space. The path of steepest ascent was determined by Eq. (3) and regression analysis for pediocin SA131 production. Glucose (X_2), NaCl (X_4) and Tween 80 (X_7) were fixed at the center level of the FFD because they were not significant at the probability level of 95% for pediocin SA131 production. For four significant factors, increasing the concentration of yeast extract (X_3), $MnSO_4$ (X_5) and decreasing the concentration of fructose (X_1), malt extract (X_4) according to the signs of their main effects should have a positive consequence for pediocin SA131 production by *P. pentosaceus* SA131. The highest pediocin SA131 production was achieved with 0.2% fructose, 1.42% yeast extract, 3.1% $MnSO_4$ and this medium was chosen for the further optimization (data not shown).

Table 3. Results of the FFD regression analysis for bacteriocin activity

Term	Coefficient	t-Value	Significant level
Intercept	-41.87	-0.46	0.6536
X_1	-93.75	-2.29	0.0408[a]
X_2	6.25	0.15	0.8811
X_3	131.25	3.21	0.0075[a]
X_4	-168.75	-4.13	0.0014[a]
X_5	156.25	0.76	0.4596
X_6	906.25	4.43	0.0008[b]
X_7	281.25	1.38	0.1941

[a] Significant at 5% level.
[b] Significant at 1% level.

Optimization for pediocin SA131 production by RSM

The central composite design, with five settings for three factors (fructose, yeast extract, and $MnSO_4$) was run to optimize the process. The experimental design and results of ANOVA for the 17 trials are presented in Tables 4 and 5. Corresponding second-order response model for Eq. (2) that was found after SAS RSREG analysis for the regression is presented below;

$$Y = 5013 - 2246.43X_1 + 1645.60X_2 - 3475.71X_3 + 4004.34X_1^2 - 90X_1X_2 - 139.82X_2^2 + 150X_1X_3 - 350X_2X_3 + 628X_3^2 \quad (4)$$

Statistical significance of the second-order model equation was checked by *F*-test. The fit of model was presented by the coefficient of determination R^2, which was found to be 0.87, indicating 87% of the variability in the response can be explained by the model. The two dimensional contour plot is generally the graphical representa-

Table 4. The central composite design of three variables in real and coded units (parenthesis) and the response of bacteriocin activity

Run	Fructose (X_1)	Yeast extract (X_2)	$MnSO_4$ (X_3)	Bacteriocin activity (AU/mL)
1	0.1 (-1)	0.92 (-1)	2.8 (-1)	572
2	0.3 (1)	0.92 (-1)	2.8 (-1)	572
3	0.1 (-1)	1.92 (1)	2.8 (-1)	800
4	0.3 (1)	1.92 (1)	2.8 (-1)	668
5	0.1 (-1)	0.92 (-1)	3.4 (1)	668
6	0.3 (1)	0.92 (-1)	3.4 (1)	572
7	0.1 (-1)	1.92 (1)	3.4 (1)	572
8	0.3 (1)	1.92 (1)	3.4 (1)	572
9	0.0 (-2)	1.42 (0)	3.1 (0)	800
10	0.4 (2)	1.42 (0)	3.1 (0)	668
11	0.2 (0)	0.42 (-2)	3.1 (0)	200
12	0.2 (0)	2.42 (2)	3.1 (0)	668
13	0.2 (0)	1.42 (0)	2.5 (-2)	800
14	0.2 (0)	1.42 (0)	3.7 (2)	800
15	0.2 (0)	1.42 (0)	3.1 (0)	572
16	0.2 (0)	1.42 (0)	3.1 (0)	572
17	0.2 (0)	1.42 (0)	3.1 (0)	572

X_1, X_2, and X_3 are mentioned in %, w/v.

Table 5. ANOVA results for central composite design

Regression	DF[1)]	Type sum of squares	*R*-square	*F*-value	*P* > *F*
Linear	3	103059	0.3095	5.78	0.0262
Quadratic	3	165912	0.4983	9.30	0.0077
Crossproduct	3	22374	0.0672	1.25	0.3608
Total model	9	291345	0.8750	5.44	0.0180

[1)]DF : Degree of freedom.

tion of the regression equation and it is easy to understand the interaction between two nutrients (Fig. 3). Fig. 3A describes the combined effects for fructose and yeast extract. An increase in pediocin SA131 production could be significantly achieved with the increase of yeast extract. The two dimensional contour plot of the combined effects of yeast extract and $MnSO_4$ on the pediocin SA131 production was shown in Fig. 3B. It was evident from the calculated response surface that pediocin SA131 activity (AU/mL) reached its maximum at a concentration of coded level; 0.13% fructose, 1.8% yeast extract, 2.58% $MnSO_4$. The model predicted a maximum response of 911 AU/mL for this point. To confirm these results, experiments using the maximum point were performed and a value of 1,000 AU/mL was obtained. This value also was about doubled than that in MRS medium. The good correlation between these two results confirmed the validity of response model and the model was proven to be adequate.

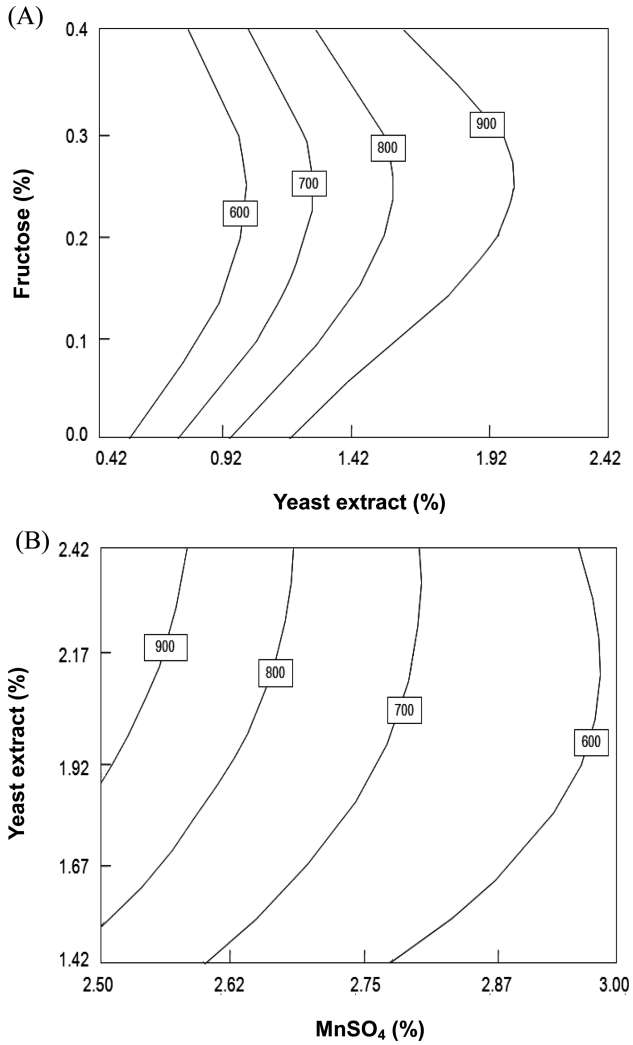

Fig. 3. Contour plot of pediocin SA131 production. (A) effect of fructose and yeast extract, (B) effect of yeast extract and $MnSO_4$.

Acknowledgements

This study was funded by a research grant from National Veterinary Research & Quarantine Service and supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093824), Korea.

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(Received 2009.10.30/Revised 2010.2.16/Accepted 2010.2.17)