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Full Length Research Paper

Optimization of bacteriocin production by *Lactobacillus plantarum* YJG, isolated from the mucosa of the gut of healthy chickens

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The objective was to develop an optimal, albeit low-cost medium for bacteriocin production, thereby facilitating industrial production. Soybean meal and peptide, two low-cost nitrogen sources often applied in industrial fermentation, were used to replace their expensive counterparts in De Man Rogosa and Sharpe (MRS). Two factors were first chosen from the 11 considered in the Plankett-Burman (PB) design. Then, the path of steepest ascent and central composite design (CCD) were used to approach the optimum region of the response and determine the maximum activity of the bacteriocin. Optimal concentration of glucose (36.3 g/l) and NaCl (1.41 g/l) stimulated the production of bacteriocins. And the optimal equation was then verified by 50 L fermentor. Under optimized conditions, *Lactobacillus plantarum* YJG produced a 1.4 fold higher production of bacteriocin than the common MRS, with 40.6% cost savings relative to non-optimized conditions.

Key words: *Lactobacillus plantarum* YJG, bacteriocin, response surface methodology, central composite design, optimization.

INTRODUCTION

Bacteriocins, ribosomally synthesized antibacterial peptides, are regarded as potential alternatives to conventional antimicrobials (Klaenhammer, 1988; Cleveland et al., 2001). For example, the bacteriocin produced by *Lactobacillus plantarum* YJG in this study inhibited some strains of pathogenic bacteria, including S*taphylococcus aureus* and *Salmonella typhi* (Integrated Vocational Development Centre, IVDC, Beijing, China).

Bacteriocins were one family of microbial defense system, which meant they may prohibit the invasion of other strains or the change of the environment, both biotic and abiotic (Riley and Wertz, 2002). Now many kinds of bacteriocins have been found from different bacteria; however, only one bacteriocin named nisin has been really applicated. Early in 1969, WHO announced that nisin was a kind of food preservative with safety and high efficiency, and after that, in 1983 FDA declared that nisin was general recognized as safe. Except nisin, most of other bacteriocins are focused on isolation and purification on a laboratory scale, with little or no consideration to industrial-scale production or being applicated to human life.

Production of bacteriocins can be influenced by many things, including medium composition(Zhou et al., 2008), environmental factors (Leal-Sánchez et al., 2002; Motta and Brandelli, 2008), and other growth conditions. De Man Rogosa and Sharpe (MRS) is the standard culture media for lactic acid bacteria (LAB), but its high cost limits its suitability for industrial-scale production. MRS has ever been used for large scale fermentation, while the purpose was to produce something, like some enzymes, or other metabolin (Lu et al., 2003; Hummel et al., 1983). Studies lowering the cost of culture media have been published (Dominguez et al., 2007; Trinetta et al., 2008; Wiese et al., 2010), not referring to industrial fermentation.

Response surface methodology is a statistical method

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to verify optimal conditions of factors for a required response. This technique was successfully applied in many fields of biotechnology, including bacteriocin production (Dominguez et al., 2007; Wiese et al., 2010; Delgado et al., 2007; Anthony et al., 2009). The objective of the present study was to increase the efficiency of bacteriocin production and reduce the cost of fermentation, thereby increasing the viability of industrial-scale production.

MATERIALS AND METHODS

Bacterial strains and media

The producer strain used in this work was *L. plantarum* YJG, was previously isolated from the mucosa of the gut of healthy chickens (Zhu et al., 2009). The indicator strain was *S. aureus* IVDC C56005 used for the bacteriocin activity assay. The MRS broth and the Beef extract-Peptone-Yeast extract (BPY) (Xie et al., 2009) broth, both containing 20% (v/v) glycerol, were separately used as the stock culture for the producer strain and indicator organism at -20°C, respectively. Bacterial stocks were propagated twice before expectation. Strong colony was first picked out and inoculated in sterile liquid MRS, and the inoculum was cultured at 37°C statically for 24 h, then the initial pH was adjusted to 7.0 with 1M sterile NaOH. The second operation was repeated in the same way. The cells biomass reached 10^9 cfu / ml.

Selection of optimal sources of nitrogen and carbon substrates for bacteriocin production

There are three organic nitrogen sources in MRS medium, including beef extract, soybean peptone and yeast extract. Due to the high cost of the first two nitrogen sources, several nitrogen sources, commonly used in industry, namely peptide powder (products from the fermentation of soybean), soybean meal (by-products from soybean oil extraction), urea, and $(NH_4)_2SO_4$ were studied. The unit of the soybean meal should be less than 0.2 mm. The yeast extract was as a control group. Corn flour (<0.2 mm) and molasses were chosen as the carbon sources. Both KH_2PO_4 and $CaCO_3$ were added to regulate the pH of the culture media, NaCl was also in consideration, whereas the remaining ingredients of MRS were retained.

Determination of the levels of the 11 factors (Plankett-Burman design)

The levels of NaCl was determind by single-factor experiment (Table 1a), while CaCO3, K2HPO4 and KH2PO4 were determined by the orthogonal experimental design (Table 1b).

Cultivation

Aliquot (2 ml) inoculum was added to the 100 ml sterile improved MRS broth for optimization. The initial pH of every treatment above (including in PB, the steepest ascent and CCD) was adjusted to 7.0 with 1M NaOH. The media inoculated was cultured at 37°C for 44 to 48 h without shaking. Aliquots (1 ml) were centrifuged at 12000 g for 10 min. The supernatants were filter-sterilized using 0.22 m microfilters (Pall Corparation, Massachusetts, USA) and adjusted to pH 5.0 with 1 M sterile NaOH for bacteriocin activity assay.

Table 1a. The single-factor experiment (NaCl).

Treatment	Concentration of NaCl (g/l)	Initial pH
1	0	6.0
2	0.2	6.0
3	2.0	6.0
4	20	6.0
5	0	6.5
6	0.2	6.5
7	2.0	6.5
8	20	6.5
9	0	7.0
10	0.2	7.0
11	2.0	7.0
12	20	7.0

Four concentrations of NaCl at different initial pH value were considered to find out the proper one.

Table 1b. The orthogonal experimental design.

Treatment	$CaCO_3$ (g/l)	K_2HPO_4 (g/l)	KH_2PO_4 (g/l)
x1	5	1.0	1.0
x2	5	2.0	1.5
x3	5	3.0	2.0
x4	10	1.0	1.0
x5	10	2.0	1.5
x6	10	3.0	2.0
x7	15	1.0	1.0
x8	15	2.0	1.5
x9	15	3.0	2.0

Three salts ($CaCO_3$, K_2HPO_4, KH_2PO_4) were referred in this experiment to find the proper concentration of each salt in PB design.

Bacteriocin activity assay

The bacteriocin activity was detected by agar disk diffusion assay. An aliquot of 200 µl cell-free supernatant was added to Oxford cup on the plates, and incubated at 30°C. The bacteriocin bioactivity was indicated using the square of the inhibition area (mm^2) of the indicator bacteria (Delgado et al., 2007; Xie et al., 2009).

Experimental designs

A Plackett-Burman (PB) design was used to determine the most influential of the 11 factors for bacteriocin production and the low and high level were shown (Table 1c) . The PB design replicated for three times (each time included 12 treatments). This method is often used for an experiment involving many factors, the significance of which is not certain to the response variable.

Table 1c. The coded number and the two levels of the factors in Plackett-Burman design.

Factors	Number	Coded level		Factors	Number	Coded level	
		-1	1			-1	1
Glucose (g/l)	A	30	45	NaCl (g/l)	G	1	1.5
soybean peptone (g/l)	B	15	25	K_2HPO_4 (g/l)	H	2	3
yeast extract (g/l)	C	10	20	KH_2PO_4 (g/l)	J	1.5	2.5
peptide powder (g/l)	D	20	30	Culture temperature (°C)	K	30	37
Tween 80 (g/l)	E	0.1	0.15	Inoculum (%)	L	2.0	3.0
$CaCO_3$ (g/l)	F	1	1.5				

The eleven factors were expressed by capital letters A to G, with each factor having two levels.

Table 2. The level of the two factors in central composite design (CCD).

Variable	Symbol	Coded level				
		-1.414	-1	0	1	1.414
Glucose (g/l)	A	1.72	10	30	50	58.28
NaCl (g/l)	G	0. 086	0. 5	1.5	2.5	2.914

Each variable has five levels.

In this design, significance is determined by comparing the difference of two levels. Relative to a single-factor study, a PB design can be conducted more quickly and easily. Then the path of steepest ascent was used to approach the central point of the response.

To determine the response surface in the optimum region, a central composite design, was used to study the interaction of x1 and x2 on the bacteriocin production and Table 2 indicated the levels of both factors. The two variables were coded at five levels (± , ±1, 0). Actually, this design was made up of a 2^2 factorial design with its four points augmented with five central points (both factors at 0 level). The axial distance was chosen to be 1.414 to make the design orthogonal. The CCD design included 13 treatments, which were all carried out at 37°C without shaking. For two factors the equation model was:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \quad (1)$$

Where Y, predicted response, b_0, intercept; b_1, b_2, linear coefficients; b_{11}, b_{22}, squared coefficients and b_{12}, interaction coefficients.

The results were analyzed by Design-Expert 7.1.3 (StatEase, Inc., Minneapolis, MN, USA) and SPSS 10.0 (SPSS Corp., Chicago, IL, USA). In addition, ANOVA was used to assess the statistical variables for optimization of fermentation medium, with an F- test used to verify the statistical significance. The coefficient of determination (R^2) was employed for the quality of the fit of the polynomial model equation.

Validation of the model

To verify the accuracy of the model and the optimum results, the experiment was conducted on the base of an optimum medium with a 50 L fermentor. The experiment was repeated three times.

RESULTS

Determination of the levels of some factors (Plankett-Burman design)

Levels of $CaCO_3$, K_2HPO_4, KH_2PO_4 were determined by an orthogonal experimental design, and the result was as follows (as shown in Figure 1, x1-x9), whereas levels of NaCl and Tween 80 were determined by single-factor experiments and the result was shown in Figure 1 (x1-x9).

Selection of the best nitrogen and carbon source substrates

Bacteriocin activity and biomass were tested on different nitrogen and carbon substrates. Glucose was remained the best carbon source; Meanwhile, yeast extract, soybean meal and peptide were all chosen as the proper nitrogen sources for the high bacteriocin production and biomass (Figure 2).

Response surface methodology

Plackett-Burman design

Variables were separated into two groups: variables (A, L, K) had the negative effect on bacteriocin production, whereas the other ones (factors except A, L, K) had the positive impact (shown in Figure 3). Two variables, glucose and NaCl were identified (ANOVA) as the most important factors (data not shown).

The path of steepest ascent

According to the two significant factors on bacteriocin production, A (Glucose) had a negative effect, whereas the effects of G (NaCl) were positive (Figure 3); as a result, the design of the two factors should have a positive consequence for bacteriocin production. The outcome of changing directions of these two factors was

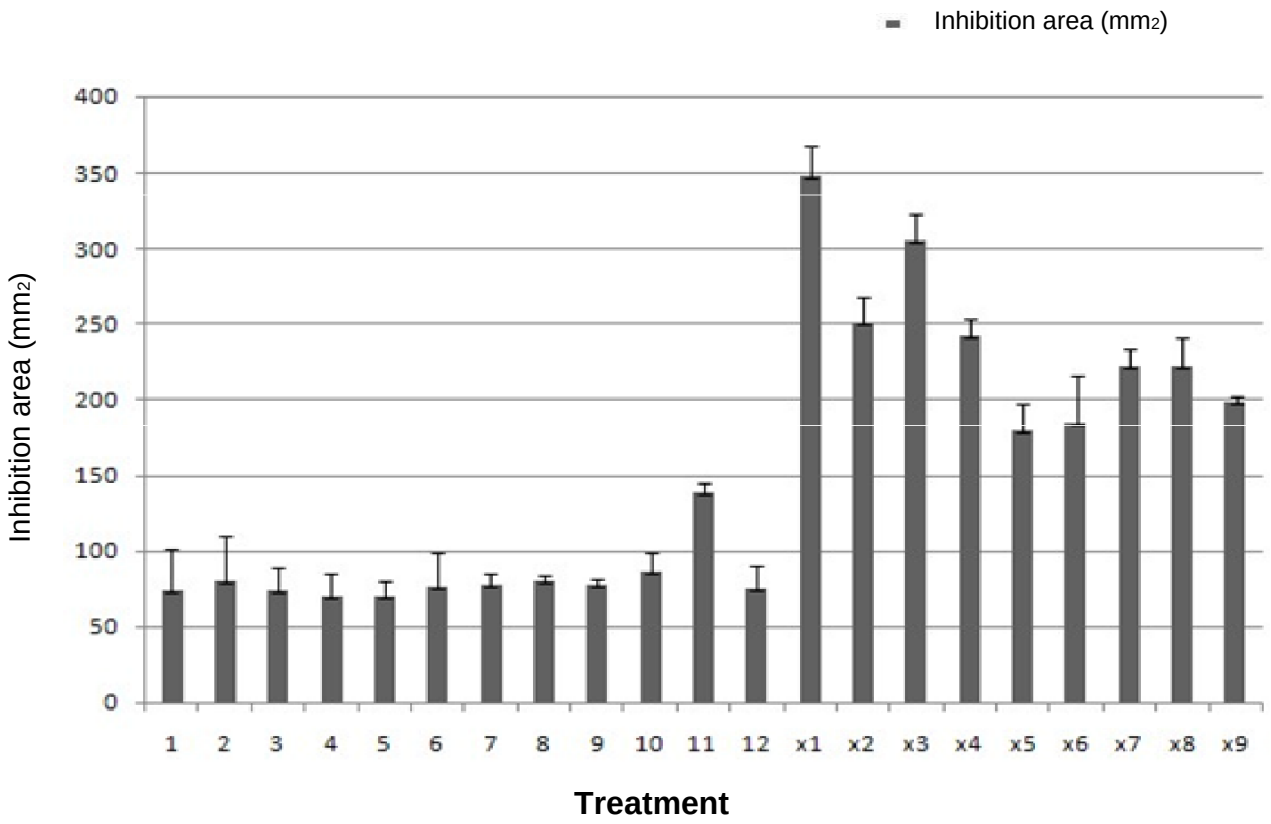

Figure 1. Different salt on bacteriocin production-NaCl on bacteriocin production(1-10) ; The orthogonal experimental design of $CaCO_3$, K_2HPO_4, KH_2PO_4 on bacteriocin production (x1-x9).

shown in Table 3. Apparently, the optimum medium was the third treatment.

Central composite design and optimization of the medium

The level of the central point was determined through the path of the steepest ascent. The effects of levels of the two variables in the central composite design are shown (Table 2), including the experimental and predicted values of bacteriocin activity (Table 4). Based on the data analyzed by design-expert 7.1.3, the model was in accordance with a second-order polynomial function, with the following form:

$$Y= 439.69+82.16\ A-14.46\ G-21.71\ AG-133.38A^2-123.87G^2 \quad (2)$$

where Y is bacteriocin activity (inhibition area, mm^2), A is Glucose (g/l); and G is NaCl (g/l) . The statistical significance of the model equqtion was evaluated by the ANOVA; since, R^2 = 0.9404 (R^2 0.75 indicated the aptness of the model), 94.04% of the variability could be explained by the mode. The levels of the impact of the A, A^2, G^2 on the value of Y were significant. Therefore, the quadratic coefficient had a significant effect on the response, whereas the coefficient of AG was not significant. Consequently, the interaction of glucose and NaCl had a small influence on bacteriocin production. Since both of the quadratic coefficients in equation (2) were negative, the parabola opened downwards and had a maximum point (Figure 4a).

Validation of the model

By derivation of equation (2), the maximum point of the response model could be calculated; that was 36.3 g/l (glucose) and 1.41 g/l (NaCl). When the concentration of gulcose was over 36.3 g/l, the bacteriocin production was reduced (Figure 4). Furthermore, the model predicted the maximum response of 465.16 mm^2. To validate the maximum point, experiments using the best concentration of glucose and NaCl were performed. Under these conditions, the mean value of bacteriocin activity was 519.84 mm^2, which was close to the predicted value. Therefore, the response model was appropriate for the expected, and the bacteriocin activity was improved by 1.4 fold relative to that using conventional MRS.

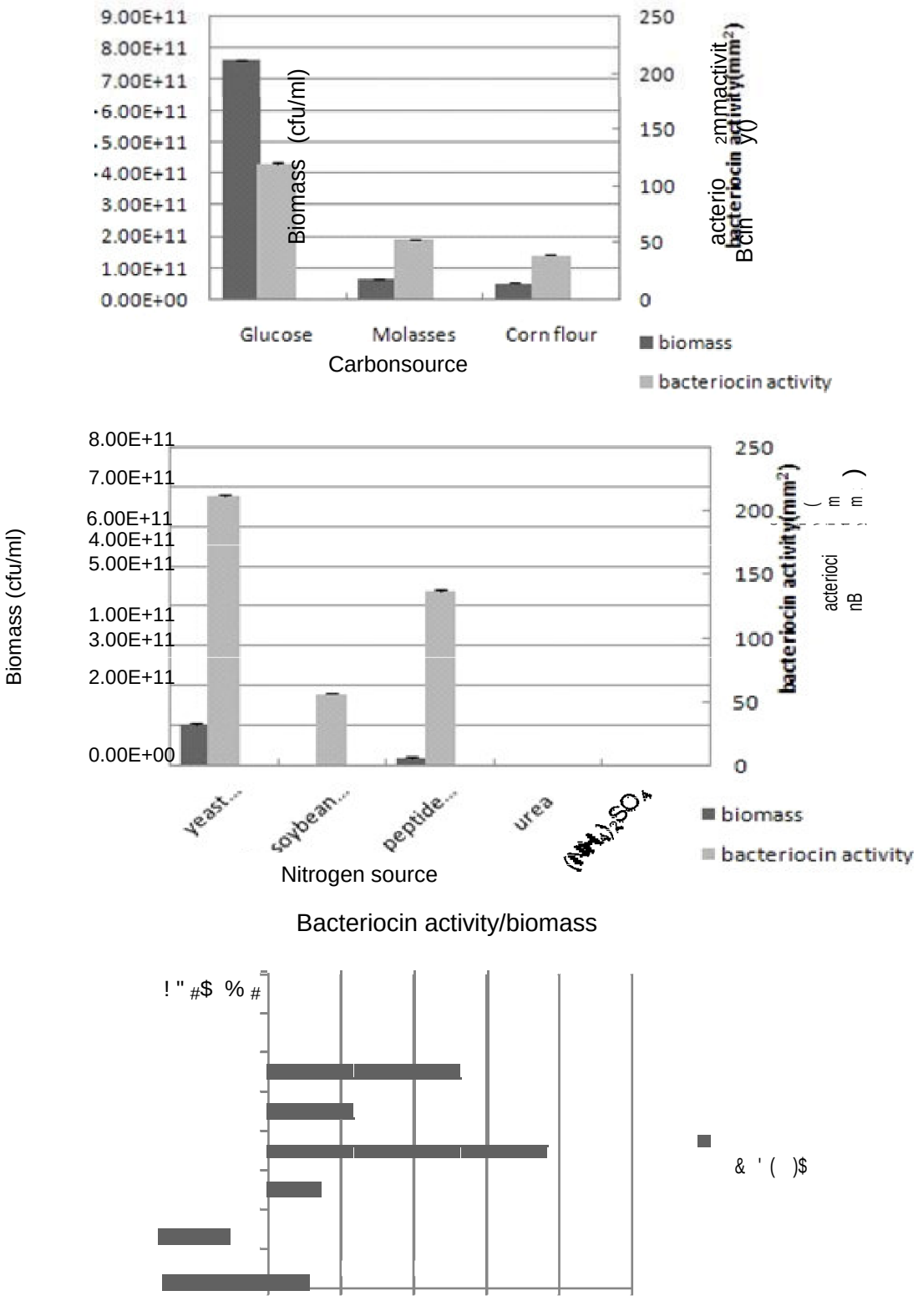

Figure 2. The effects of different nitrogen sources and carbon sources on the bacteriocin production and biomass. Different carbon sources experiment was implemented when every treatment was the same (initial pH 6.5, 37°C, without shaking, the ingredients of MRS except carbon sources); The nitrogen sources experiment was tried out the same as the carbon sources (the same conditions including culture conditions and the ingredients of MRS except nitrogen sources).

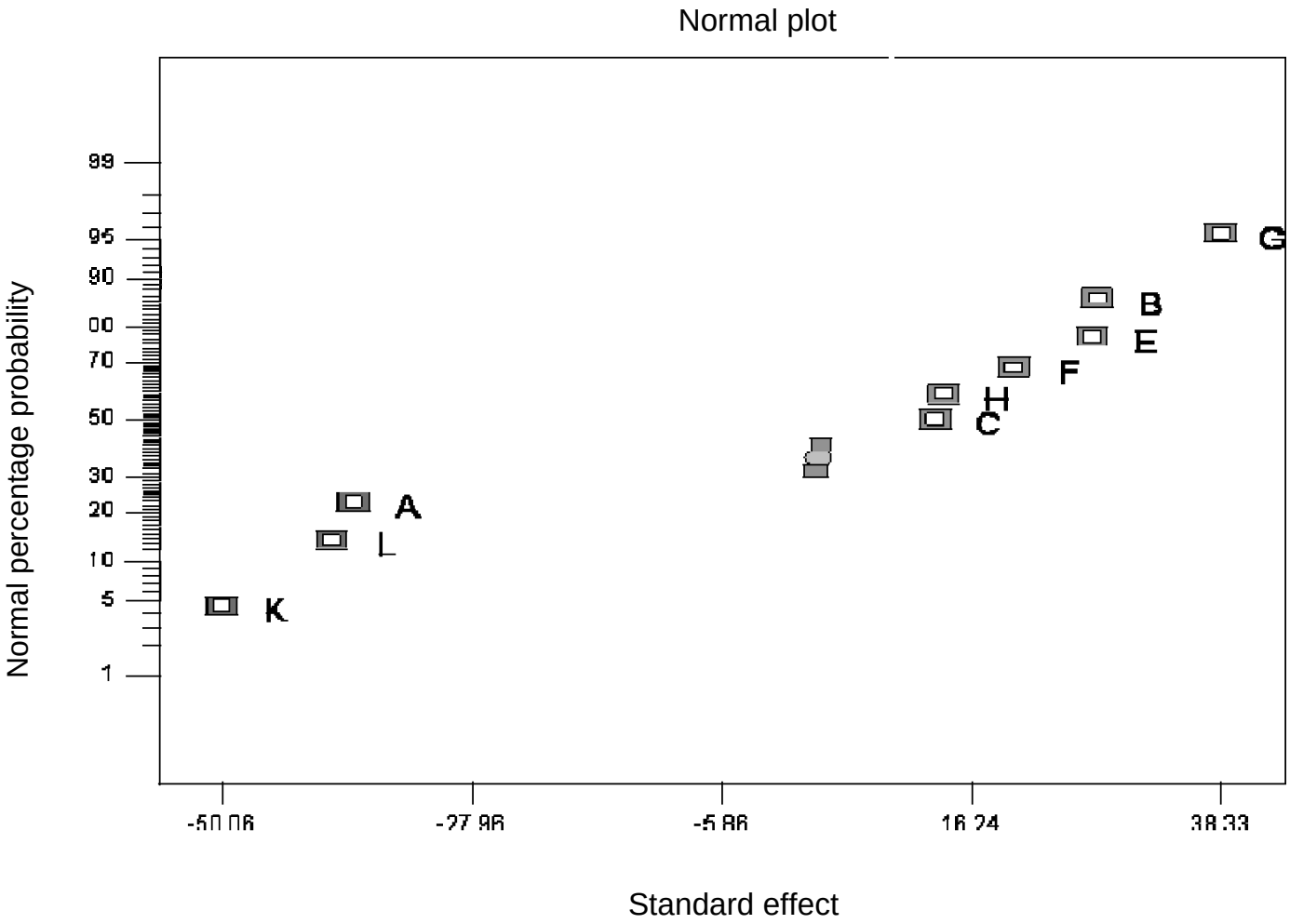

Figure 3. The influence of the factors on the production of bacteriocin s by PB design. From this figure, it was concluded that A, L, K showed the negative effects on bacteriocin production; Meanwhile, the other factors showed positive.

Table 3. The path of steepest ascent and the value of the response variable.

Run no.	Glucose (g/l)	NaCl (g/l)	Bacteriocin activity (mm^2)
1	40	0.5	378.79ab
2	35	1.0	369.65b
3	30	1.5	442.17a
4	25	2.0	322.43bc
5	20	2.5	266.73cd
6	15	3.0	246.86d
7	10	3.5	161.28e
8	5	4.0	66.44f

The data of bacteriocin activity was analysed by multiple comparison, and the lowercase letters stand for the significance between the groups. The third group, not the first group, was chosen as the level of the central point of CCD, because of the cost problem.

The calculation of fermentation cost

The cost of conventional MRS and optimal MRS was separately calculated (according to the prices in our country). Both of the cost of salt addicted to the medium was almost the same. The main difference was the cost

Table 4. The design and the result of CCD.

Run no.	A (g/l)	G (g/l)	Bacteriocin activity (mm^2)		Bacteriocin activity/Biomass[b]
			Observed[a]	Predicted	
1	-1	-1	117.29	93.03	20.22
2	1	-1	251.75	300.76	25.93
3	-1	1	166.41	107.53	35.99
4	1	1	214.13	228.43	21.68
5	-1.414	0	0.00	56.74	0
6	1.414	0	336.11	289.11	28.16
7	0	-1.414	232.05	212.40	26.73
8	0	1.414	142.09	171.50	14.30
9	0	0	466.56	439.69	42.77
10	0	0	412.09	439.69	37.83
11	0	0	408.04	439.69	37.50
12	0	0	445.21	439.69	40.85
13	0	0	439.60	439.69	40.32

[a] means the measured value. [b] means the specific activity, and the denominator was actually the log10 of biomass.

of the nitrogen sources. The cost of substrates was reduced by 57.9%, but considering the amount of glucose, the cost of optimal medium was reduced by about 40.6%.

DISCUSSION

Response surface methodology has been successfully used in many studies for optimization of bacteriocin production (Delgado et al., 2007; Cladera-Olivera et al., 2004; Li et al., 2002). However, since factors varied among different strains, this work differed previous studies (Delgado et al., 2007; Cladera-Olivera et al., 2004; Li et al., 2002) in choosing factors before RSM.

Effects of physical factors on bacteriocin production, including temperature and pH (Delgado et al., 2007) were recently studied. The composition of the medium was also shown to have an important role in bacteriocin production (Li et al., 2002). However, studies to reduce the cost of the medium have only been recently conducted (Dominguez et al., 2007). In the present study, some nitrogen sources in MRS were replaced to reduce costs. However, some the nitrogen substrates, could not be used by *L. plantarum* YJG, such as urea (Figure 2), perhaps due to lack of required enzymes. It proved *L. plantarum* YJG could not grow well only with inorganic nitrogen sources possibly for absence in vitamins and DNA precursors, which was rich in yeast extract.

Low bacteriocin production was caused when using the corn flour as carbon source (Figure 2), perhaps because the bacteriocins were adsorbed to the surface of the corn. Since both molasses and corn flour reduced biomass, we inferred that the bacteria could not make good use of polysaccharides, although monosaccharide were readily utilized. From Figure 4, it could be concluded that the concentration of glucose over 3.63 g/l could possibly inhibit the bacteriocin.

Tween 80 used in Plankett- Burman design did not significantly affect bacteriocin production, similar to reports for other bacteriocins (Trinetta et al., 2008). In contrast, surfactant could stimulate the production of bacteriocins in other studies (Rajaram et al., 2010; Huot et al., 1996).

It was well known that NaCl is required by many bacteria, for Na^+ is important to the osmotic pressure to the cells. But NaCl was not needed for other bacteriocin production (Delgado et al., 2007). In this study, NaCl played an important part to the growth of the bacteria (Figure 4). But high concentration over 1.41 g/l could inhibit the bacteriocin production when glucose concentrations were low. The similar result was found for enterocin P production (Herranz et al., 2001). In contrast, when the concentrations of glucose were high, the effects of changes in NaCl concentrations were not as marked. It was noteworthy that the interaction of the two factors was obvious when both concentrations were low.

In addition to the nitrogen sources substrates and carbon substrates, salt was also in considered in the present study (Table1, Figure 1) . The salt, including $CaCO_3$, K_2HPO_4, KH_2PO_4, could regulate pH of the fermentation broth, with substantial effects on biomass production. Previous studies to identify cheaper media focused primarily on nitrogen substrates to some extent (Dominguez et al., 2007; Trinetta et al., 2008; Wiese et al., 2010) . However, in the present study, the effects of salt were also considered. The lowest concentration of $CaCO_3$ could stimulate the bacteriocin production, perhaps because it was insoluble, and large amount of could possibly prevent the bacteria production. Also, it seemed that low amount of phosphate could improve the formation of bacteriocins, maybe because the bacteriocin

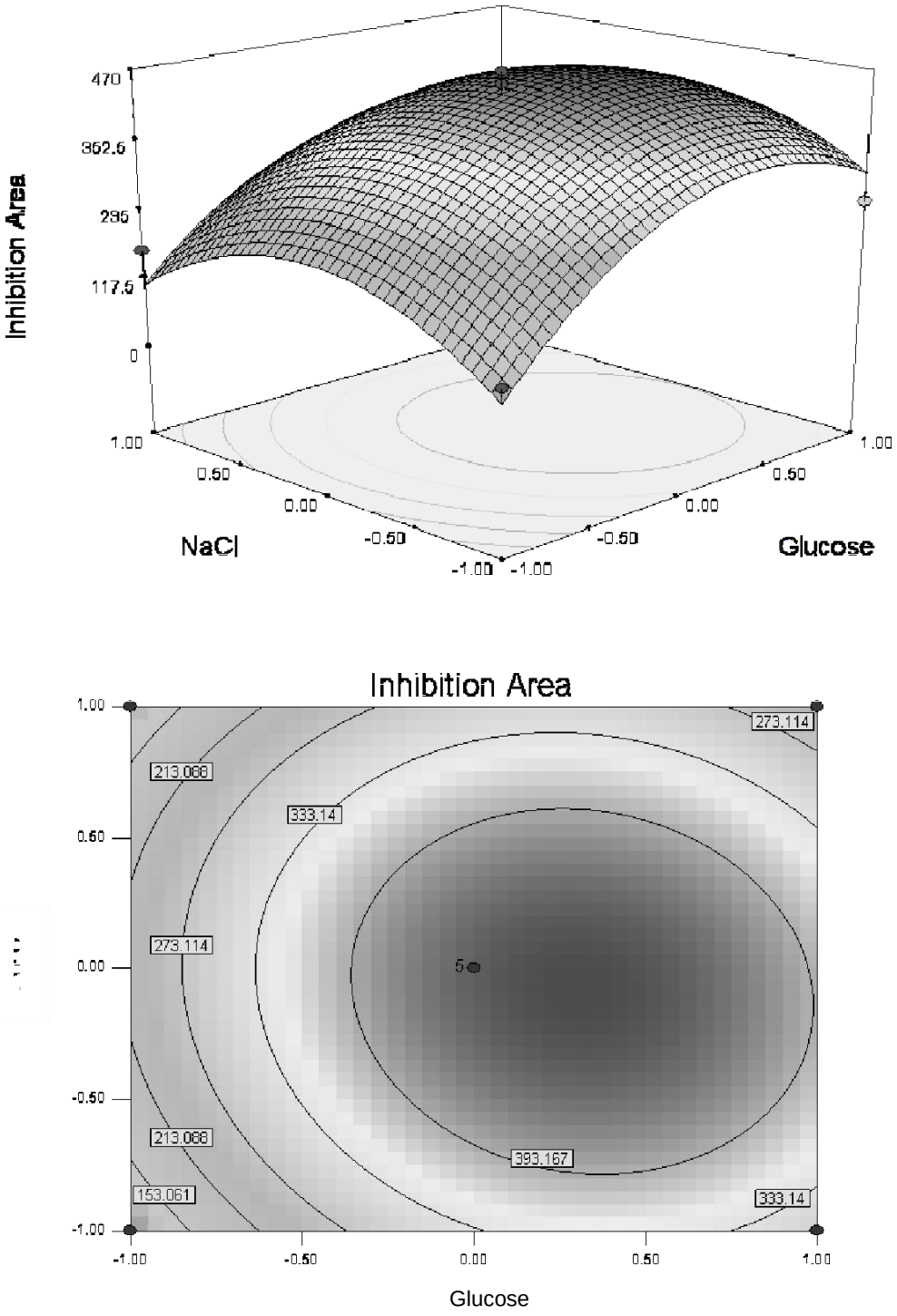

Figure 4. Response surface of production of bacteriocin(inhibition area) by *L. plantarum* YJG(a) and the contour map of CCD (b). Bacteriocin activity was expressed using inhibition area (mm^2).

would not be secreted unless pH reached regular value. The main use of salts here was to improve the amount of the bacteria, indirectly leading to higher bacteriocin production. For the cost of these buffer substances was not high, so it is necessary to apply these buffer substances in industrial processes to prolong the growth period.

In summary, response surface methodology proved to

be a useful tool to optimize the medium by *L. plantarum* YJG. The concentrations of optimal medium ingredients were as follows: Glucose was 3.6 g/l, NaCl was 1.41 g/l, $CaCO_3$ was 1 g/l, KH_2PO_4 was g/l, soybean peptone was 10.0 g/l, peptide powder was 10.0 g/l, yeast extract was 10.0 g/l, ammonium citrate was 2.0 g/l, $MgSO_4$ was 0.575 g/l, $MnSO_4$ was 0.14 g/l. The optimized medium not only allowed the bacteriocin activity increase from 376.36 to 443.85 mm^2, but reduced the cost of the medium by 40.6%.

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