Optimization of antimicrobial substances produced from *Lactobacillus paracasei* subsp. *paracasei* NTU 101 (DSM 28047) and *Lactobacillus plantarum* NTU 102 by response surface methodology

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Abstract Response surface methodology (RSM) was used to study the effects of medium composition and growth conditions on antimicrobial substances produced by *Lactobacillus paracasei* subsp. *paracasei* NTU 101 and *Lactobacillus plantarum* NTU 102. These strains are used in the food and pharmaceutical industries because of their beneficial effects on the human gastrointestinal tract and in immune modulation. The production of antimicrobial substances was optimized by the Box-Behnken experimental design. An empirical model was developed through RSM to describe the relationship between variables (molasses, soymilk, and incubation temperature). The maximum antimicrobial activity of *L. paracasei* subsp. *paracasei* NTU 101 was 12.50 AU/mL with 18.33 g/L molasses and 14.53 g/L soymilk at a temperature of 35.00 °C, whereas in MRS (de Man, Rogosa, and Sharpe) broth, the activity was 14.00 AU/mL. Similarly, the maximum antimicrobial activity of *L. plantarum* NTU 102 was 15.00 AU/mL with 20.56 g/L molasses and 13.54 g/L soymilk at a temperature of 36.50 °C, whereas in MRS, the activity was 13.50 AU/mL. The antimicrobial activities visualized in response surface plots were 12.26 and 15.59 AU/mL for NTU 101 and 102, respectively. The results derived from RSM regression were close to those obtained with the experimental design treatments. These results revealed that an alternative medium could be used for large-scale commercial production of the antimicrobial substances, with potential prospects for application.

Keywords Response surface methodology · *Lactobacillus paracasei* subsp. *paracasei* · *Lactobacillus plantarum* · Antimicrobial activity

Introduction

Lactic acid bacteria (LAB) are widely distributed in nature. They appear to reside in dairy and fish as well as vegetables and grains. Over the last few decades, lactic acid fermentation has been used to suppress microbial spoilage and prolong shelf life in food preservation. In fact, lactic acid fermentation was believed to be the oldest means of food preservation (Ross et al. 2002). Protection of food from spoilage and pathogenic microorganisms by LAB is achieved through the production of organic acids, hydrogen peroxide, diacetyl (Menssens and De Vugst 2002), antifungal compounds such as fatty acids (Corsetti et al. 1996), or phenyllactic acid (Lavermicocca et al. 2000) and bacteriocins (Larsen et al. 1993).

Consumers have been consistently concerned about possible adverse health effects from chemical food additives. As a result, they are drawn to natural and “fresher” foods without chemical preservatives. This has stimulated research interest in finding natural but effective preservatives and increased the demand for minimally processed foods with long shelf life. Bacteriocins, which are peptides ribosomally synthesized by LAB, are considered natural preservatives or bio-preservatives that fulfill these requirements (Savadoago et al. 2006). Bio-preservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf life. Three approaches are commonly used in the

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Table 1 Experiment data based on Box-Behnken statistical design for the antimicrobial activity of *L. paracasei* subsp. *paracasei* NTU 101

Runs	Variables			Code value			Antimicrobial activity (AU/mL)
	Molasses (g/L)	Soymilk (g/L)	Incubation temperature (°C)	X ₁	X ₂	X ₃	
01	40.00	17.50	40	1	0	1	8.00
02	10.00	17.50	40	-1	0	1	8.50
03	40.00	25.00	35	1	1	0	10.50
04	25.00	10.00	40	0	-1	1	8.50
05	25.00	17.50	35	0	0	0	12.00
06	25.00	10.00	30	0	-1	-1	8.90
07	40.00	10.00	35	1	-1	0	9.50
08	25.00	17.50	35	0	0	0	12.50
09	10.00	25.00	35	-1	1	0	9.50
10	40.00	17.50	30	1	0	-1	9.50
11	25.00	17.50	35	0	0	0	12.00
12	10.00	17.50	30	-1	0	-1	8.90
13	25.00	25.00	30	0	1	-1	9.200
14	25.00	25.00	40	0	1	1	7.60
15	10.00	10.00	35	-1	-1	0	12.50

application of bacteriocins for the bio-preservation of food (Schillinger et al. 1996): (i) Inoculation of bacteriocin-producing strains in the products, (ii) addition of purified or semi-purified bacteriocins as food preservatives, and (iii) use of food fermented by bacteriocin-producing strain as an ingredient in food processing.

Lactobacillus paracasei subsp. *paracasei* NTU 101 and *Lactobacillus plantarum* NTU 102 were isolated from infant feces and homemade Korean-style cabbage pickles, respectively, in our laboratory (Lin et al. 2004; Chiu et al. 2009). In our previous studies, these strains showed good survival at low pH, tolerance to high bile concentration, pathogen inhibition activities (Pan et al. 2002), and decreased ratio of low-density lipoprotein cholesterol (LDL-C) to high-density lipoprotein cholesterol (HDL-C) (Chiu et al. 2009; Tsai et al. 2009). These strains also showed the ability to induce immune modulation and enhance the immune ability of *Lactobacillus vannamei* while increasing resistance to *Vibrio alginolyticus* infection (Chiu et al. 2007; Tsai et al. 2010; Chiang et al. 2012).

The bio-preservative effect of LAB and their positive effect on human health explain the great interest of scientists in their growth and physiology. As Lactobacilli are fastidious about nutrient requirements, an enriched medium is required for growth (Liew et al. 2005). The most common medium for LAB is the MRS medium (de Man, Rogosa, and Sharpe) (de Man et al. 1960; Rogosa et al. 1961). Several studies have focused on the optimization of the LAB growth medium. Research has been carried out to examine the influence of various carbon and

Table 2 Experiment data based on Box-Behnken statistical design for the antimicrobial activity of *L. plantarum* NTU 102

Runs	Variables			Code value			Antimicrobial activity (AU/mL)
	Molasses (g/L)	Soymilk (g/L)	Incubation temperature (°C)	X ₁	X ₂	X ₃	
01	30.00	20.00	35	1	1	0	14.80
02	20.00	5.00	40	0	-1	1	13.20
03	30.00	5.00	35	1	-1	0	13.90
04	10.00	12.50	40	-1	0	1	13.60
05	20.00	20.00	40	0	1	1	13.30
06	20.00	5.00	30	0	-1	-1	14.20
07	30.00	12.50	40	1	0	1	11.80
08	20.00	20.00	30	0	1	-1	14.80
09	20.00	12.50	35	0	0	0	15.80
10	10.00	5.00	35	-1	-1	0	13.70
11	10.00	20.00	35	-1	1	0	13.50
12	20.00	12.50	35	0	0	0	16.10
13	10.00	12.50	30	-1	0	-1	13.40
14	20.00	12.50	35	0	0	0	15.80
15	30.00	12.50	30	1	0	-1	14.50

nitrogen sources, other factors affecting growth (amino acids, vitamins, and minerals), and culture conditions such as temperature, pH, and the level of aeration (Liew et al. 2005).

Response surface methodology (RSM) is commonly used to explore nonlinear relationships between studied factors and the dependent variables. It also provides information about the optimal values of these factors to determine the expected largest (or smallest) extreme values for the dependent variables. RSM has been used increasingly to optimize growth-related

Table 3 Regression coefficients, mean square, and P values derived by multivariate analysis for the antimicrobial activity of *L. paracasei* subsp. *paracasei* NTU 101

Source	Coefficient	Degree of freedom (df)	Mean squares	P value
Intercept	37.73	9	4.19	0.0022
A ₁	0.45	1	0.45	0.2057
A ₂	0.72	1	0.72	0.1257
A ₃	2.10	1	2.10	0.0257
A ₁₂	4.00	1	4.00	0.0075
A ₁₃	0.30	1	0.30	0.2873
A ₂₃	0.25	1	0.25	0.3286
A ₁ ²	1.87	1	1.87	0.0314
A ₂ ²	3.25	1	3.25	0.0114
A ₃ ²	27.17	1	27.17	< 0.0001
R ²	0.92			

Fig. 1 Response surface methodology plot for antimicrobial activity produced by *L. paracasei* subsp. *paracasei* NTU 101. Effects of molasses and soymilk (a), molasses and incubation temperatures (b), and soymilk and incubation temperatures (c) on antimicrobial activity at center point level

factors in different fermentative and biotechnological processes. The optimization of medium formulation is the primary step in bioprocess technology. The aim of our study is to evaluate a molasses-based medium for cost-effective production of antibacterial substances from *L. paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU 102.

Materials and methods

Microorganism

In our previous study, *L. paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU 102 were isolated from infant feces and pickles respectively and showed several functional properties (Lin et al. 2004; Pan et al. 2002). *L. paracasei* subsp. *paracasei* NTU 101 (DSM 28047) was maintained in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), while *L. plantarum* NTU 102 was maintained in the Bioresource Collection and Research Center (BCRC). These strains were grown in MRS broth (Difco Laboratories Inc., Detroit, MI, USA) at 37 °C for 24 h. *Vibrio parahaemolyticus* BCRC 12864 (ATCC 27519) was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). It was cultured on Tryptic Soy Broth (TSB) (Difco Laboratories Inc., Detroit, MI, USA) and incubated at 37 °C for 24 h.

Preparation of microbial growth media

The molasses (50 g/L, w/v) (Taiwan Sugar Corp., Taiwan) in deionized water was heated at 100 °C for 30 min. Soymilk was prepared using a previously published method (Chou and Hou 2000). Whole soybeans were presoaked overnight in deionized water. The drained and rinsed soaked beans were put in a large pot and ground into slurry in a blender with a volume of distilled water that was ten-times the weight of soaked beans for 3 min. The resultant slurry was filtered through double-layered cheesecloth to obtain soymilk. The soymilk was heated in water baths at 90 °C for 1 h.

Antimicrobial activity

Antimicrobial activity was quantified using an assay on the basis of a modified well diffusion method (Schillinger and Lucke 1989; Fooks and Gibson 2002). The well diffusion method assay has been widely used to determine bactericidal activity against different microorganisms. The cultures, containing approximately 10^9 colony-forming units (CFU)/mL of indicator bacteria, were inoculated on the Mueller–Hinton agar plates (Difco Laboratories Inc.). The antimicrobial activities were determined by measuring the diameter of the inhibition zone around the wells. The antimicrobial activity was

defined as the reciprocal of the highest dilution showing at least a 1 mm zone definite inhibition of the indicator strain around the well and was expressed as activity units per milliliter (AU/mL).

RSM experimental design

For the purpose of the experimental design, the production medium was prepared in different formulae in Tables 1 and 2. After 24 h the cells were removed by centrifugation and the supernatants were used for the measurement of antimicrobial activity. In this study, the effects of molasses, soymilk, and incubation temperature on antimicrobial activity were evaluated. Levels of these factors were optimized for maximum diameter of inhibition zone (the response) using one of the response surface methodologies, the Box–Behnken statistical design (Box and Behnken 1960). Table 1 represents a 15-trial experimental design for *L. paracasei* subsp. *paracasei* NTU 101, where each variable was tested in three different coded levels: low (−1), middle (0) and high (+1). The coded values correspond for molasses: −1 (10.00 g/L), 0 (25.00 g/L), +1 (40.00 g/L); for soymilk: −1 (10.00 g/L), 0 (17.50 g/L), +1 (25.00 g/L) and for incubation temperature: −1 (30 °C), 0 (35 °C), +1 (40 °C). Table 2 represents a 15-trial experimental design for *L. plantarum* NTU 102, where each variable was tested in three different coded levels: low (−1), middle (0) and high (+1). The coded values correspond for molasses: −1 (10.00 g/L), 0 (20.00 g/L), +1 (30.00 g/L); for soymilk: −1 (5.00 g/L), 0 (12.50 g/L), +1 (20.00 g/L) and for incubation temperature: −1 (30 °C), 0 (35 °C), +1 (40 °C). The polynomial equation is in the following form:

Table 4 Regression coefficients, mean square, and P values derived by multivariate analysis for the antimicrobial activity of *L. plantarum* NTU 102

Source	Coefficient	Degree of freedom (df)	Mean squares	P value
Intercept	18.25	9	2.03	0.0039
A ₁	0.08	1	0.08	0.4731
A ₂	0.24	1	0.24	0.2328
A ₃	3.12	1	3.12	0.0047
A ₁₂	0.30	1	0.30	0.1919
A ₁₃	2.10	1	2.10	0.0106
A ₂₃	0.06	1	0.06	0.5235
A ₁ ²	5.65	1	5.65	0.0013
A ₂ ²	1.75	1	1.75	0.0152
A ₃ ²	6.61	1	6.61	0.0009
R ²	0.90			

Fig. 2 Response surface methodology plot for antimicrobial activity produced by *L. plantarum* NTU 102. Effects of molasses and soymilk (a), molasses and incubation temperatures (b), and soymilk and incubation temperatures (c) on antimicrobial activity at center point level

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

Where Y is the predicted response, A_0 is the model constant; X_1 , X_2 , and X_3 are independent variables; A_1 , A_2 , and A_3 are linear coefficients and A_{11} , A_{22} , and A_{33} are the quadratic coefficients, respectively.

Data analysis and optimization

Design-expert 7.0 was used to fit the response surface model to the experimental data through the multiple regression analysis. Optimization of the culture conditions in terms of molasses, soymilk, and incubation temperature was analyzed using the predictive polynomial model. Statistical comparisons were performed using one-way analysis of variance with Duncan's test. Statistically significant differences were defined as $P < 0.05$.

Results and discussion

Application of Box-Behnken design and data analysis

The experimental designs and the results of antimicrobial activity are showed in Tables 1 and 2. The antimicrobial activities were determined by measuring the diameter of the inhibition zone around the wells at 24 h. As shown in Table 1, the antimicrobial activity of *L. paracasei* subsp. *paracasei* NTU 101 ranged from 7.60 to 12.50 AU/mL. In Table 3, the R^2 value of 0.92 indicates the correlation between the experimental and predicted values. The analysis of variance for the three variables (molasses, soymilk, and incubation temperature) indicated that antimicrobial activity could be described by a polynomial model with a relatively high coefficient of determination. The coefficient of incubation temperature (A_3) was significant with a lower P value ($P = 0.0257$). These results showed that incubation temperature was the primary factor in influencing the antimicrobial activities. The response surface model was:

$$Y = 37.73 + 0.45X_1 + 0.72X_2 + 2.10X_3 + 4.00X_1X_2 + 0.30X_1X_3 + 0.25X_2X_3 + 1.87X_1^2 + 3.25X_2^2 + 27.11X_3^2$$

The response surface plots represented the expected response and correlation between variables in three-dimensional plots. The relationship between variables and the response is illustrated in Fig. 1. Based on the regression equation, the optimum values were 18.32 g/L molasses and 14.52 g/L soymilk at an incubation temperature of 35 °C.

As shown in Table 2, the antimicrobial activity of *L. plantarum* NTU 102 ranged from 11.80 to 16.10 AU/mL. The R^2 value of 0.90 indicates the correlation between the experimental and predicted values (Table 4). The linear coefficient of incubation temperature was significant with a lower P value ($P < 0.05$). These results showed that the incubation temperature was the most important factor influencing the antimicrobial activities. The response surface model was:

$$Y = 18.25 + 0.08X_1 + 0.24X_2 + 3.12X_3 + 0.30X_1X_2 + 2.10X_1X_3 + 0.06X_2X_3 + 5.65X_1^2 + 1.75X_2^2 + 6.61X_3^2$$

The response surface plot for antimicrobial activity revealed that the optimum value was obtained with 20.56 g/L molasses and 13.54 g/L soymilk at an incubation temperature of 36.5 °C (Fig. 2).

In Table 5, the antimicrobial activities of *L. paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU 102 were 12.26 and 15.59 AU/mL, respectively, which were lower than the visualized values in the response surface plot. The error between the predicted and observed values was expressed in terms of the prediction interval, which was utilized as the error metric for the response surface. Using the prediction interval of the response surface, the upper and lower limits of reliability were compared with the target reliability to obtain an optimum design (Kim and Choi 2008).

In this study, an empirical model was developed through RSM to describe the relationship between variables (molasses, soymilk, and incubation temperature). The results revealed that incubation temperature was the primary factor in influencing the antimicrobial activity. Molasses and soymilk, as an alternative medium, could be used for LAB growth. Previous studies focused on the influence of various carbon and nitrogen sources, growth-related factors (such as amino acids and vitamins), and incubation conditions on lactobacilli growth.

Table 5 The comparisons of predicted and experimental data in optimum operation conditions

	Critical values of factor			Response (AU/mL)		Error (%)
	Molasses (g/L)	Soymilk (g/L)	Incubation temperature (°C)	Predicted	Observed	
<i>L. paracasei</i> subsp. <i>paracasei</i> NTU 101	18.33	14.53	35.0	12.26	12.50	1.96
<i>L. plantarum</i> NTU 102	20.56	13.54	36.5	15.59	15.00	3.70

The studies found that supplements of yeast extract, glucose, and vitamins as well as changes in pH could enhance the biomass production of *Lactobacillus rhamnosus* (Liew et al. 2005). Molasses, a by-product of sugar refining, is produced during the crystallization of sucrose from cane juice and is generally used as an animal feed or a biomass resource for fermentation (Takara et al. 2007). Previous studies showed that molasses enhanced the biomass and lactic acid produced by *Lactobacillus delbrueckii* subsp. *delbrueckii* (Dumbrepatil et al. 2008). The present study revealed that molasses and soymilk medium could support the growth and resulted in appreciable antimicrobial activity.

MRS broth is used for the growth and enumeration of cultures of *Lactobacillus* strains. This medium contains proteose peptone No. 3, beef extract, yeast extract, dextrose, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and dipotassium phosphate in Difco formula (Difco Laboratories Inc.). The price per kg of MRS broth is 233.64 USD. The MRS solution was prepared by dissolving 55 g of MRS powder in 1 L of sterilized water, resulting in a cost of 12.85 USD per kg of medium for the incubation of *Lactobacillus*. In our study, the molasses and soymilk medium provided an alternative to the incubation of LAB. Molasses and soybean were low priced compared with MRS broth. The costs of molasses and soybean are 0.52 and 0.62 USD per kg, respectively. Only 250 g of whole soybeans is needed to make 1 L of soymilk; therefore, the molasses and soymilk medium would cost approximately 0.18 USD per liter. In replacing the MRS broth with the molasses and soymilk medium, the cost is reduced by more than seventy-fold.

Conclusion

The molasses and soymilk medium enabled the production of appreciable antimicrobial activities in the culture broth of *L. paracasei* subsp. *paracasei* NTU 101 and *L. plantarum* NTU 102. These results revealed that the alternative medium could be used for large-scale commercial production of the antimicrobial substances, with potential prospects for application.

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