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반응표면분석법을 이용한 *Lactobacillus* 균주 배양조건의 통계적 최적화

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Statistical Optimization of Culture Conditions for *Lactobacillus* Strains using Response Surface Methodology

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ABSTRACT - The demand for probiotic products has been steadily increasing, and *Lactobacillus* strains are widely used and are currently the most popular probiotics. Optimizing culture conditions for *Lactobacillus* production for use as probiotics will enhance their profitability by reducing production costs and time. Statistical analysis using response surface methodology revealed the following optimal sets of independent variables: 22.55 h (cultivation time), 25°C (cultivation temperature), and 3.41% (w/w, prebiotics concentration) for *Lactobacillus acidophilus*; 24 h, 30.86°C, and 2% (w/w) for *Lactiplantibacillus plantarum*; 66.67 h, 35°C, and 3.41% (w/w) for *Lacticaseibacillus rhamnosus*. Actual outcomes using predicted optimal conditions for *Lactobacillus* strains have been confirmed to closely match predicted results. This study will provide valuable guidelines for high yield *Lactobacillus* production.

Keywords: *Lactobacillus*, Culture condition, Optimization, Response surface methodology

Lactobacillus are Gram-positive, microaerobic or anaerobic essential fermenting organisms that produce lactic acid as the main end product of sugar fermentation[1)]. *Lactobacillus* are considered as probiotics because they play an important role in the production of various fermented products, and are naturally associated with the mucous membranes of the gastrointestinal tract of humans and animals[2)]. According to the lists compiled by George Kerry et al.,[3)] *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Escherichia coli*, *Saccharomyces*, and *Enterococcus* are all acknowledged as probiotics. Probiotics offer a range of benefits, including the maintenance of intestinal flora, alleviation of lactose intolerance[4)], enhancement of the immune system[5-7)], and relief from symptoms associated with constipation and diarrhea[8,9)]. Furthermore, it hinders the proliferation of detrimental bacteria in the intestinal tract and mitigates the production of harmful substances by these bacteria[10-12)]. The significance of personal health care is emerging due to the recent global pandemic of COVID-19, and the demand for probiotics products that help 'intestinal health' and 'improvement of immunity' is increasing[13)]. An analysis of the intestinal microbiome of Koreans revealed a decrease in beneficial bacteria, specifically *Lactobacillus* and *Bifidobacterium*, as individuals aged[14)]. In addition, as a result of the Korea Consumer Agency's probiotics product quality test (2020), it was reported that most of the lactic acid bacteria types were biased to 1 or 2 species, and in particular, the proportion of *Lactobacillus* strains was high. To utilize probiotics as health functional food, it is crucial to ensure the appropriate count of lactic acid bacteria that meets the standard requirements.

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Additionally, the development of a culture method that reduces manufacturing costs and time is necessary. The culture process holds significant importance in enhancing the yield of lactic acid bacteria from both industrial and economic perspectives. Using conventional single-factor approaches to optimize culture media is time consuming and can lead to misinterpretation of results due to interactions between factors[15]. In contrast, statistical optimization methods such as the response surface methodology can be used to study relationships among many independent variables, as those methods are very efficient and economical[15,16].

Thus, in this study, after setting the incubation temperature, incubation time, and prebiotics concentration as variables, we aimed to explore the optimal conditions for the culture of *Lactobacillus* by using the central composite design among the response surface methodology.

Materials and Methods

Strain and cultivation

The *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and prebiotics employed in this study were procured from Maeil (Maeil Dairies Co., Ltd., Pyeongtaek, Korea) and utilized for experimental purposes. The powdered form of the strain was activated by culturing in de Man, Rogosa, and Sharp (MRS) broth (MB cell, Seoul, Korea) at 37°C for 24-48 h, followed by subculturing under the same conditions to prepare stock cultures for each experiment. The prebiotics were stored in a desiccator and used for each experiment.

Determination of experimental parameter ranges of *Lactobacillus* strains

Since the culture media, culture conditions, unit price, and recovery time as important variables during the probiotics culture process, the culture time, culture temperature, and type and concentration of prebiotics were set as independent variables.

Incubation time

To assess the growth of *Lactobacillus* strains at different incubation times, they were inoculated into brain heart infusion (BHI) broth (MB cell) at a concentration of 5-8 log CFU/mL. The cultures were incubated for 72 h at 37°C in a shaking incubator (C-SKI-3, Changshin-lab, Seoul, Korea) at 180 rpm, after which the optical density and total bacterial counts were measured. The culture media was recovered at intervals of 0, 3, 6, 12, 18, 24, 48, and 72 h and used in the experiment. Total bacterial count was measured by serially diluting 1 mL of the culture media in 9 mL of peptone water, followed by pouring 1 mL of the diluted solution onto MRS agar (MB cell).

Type of prebiotics

To assess the growth of *Lactobacillus* strains according to different types of probiotics, they were inoculated into BHI broth supplemented with galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), and 2'-fucocyl lactose (2'-FL) at a concentration of 5 log CFU/mL. The cultures were incubated for 72 h at 37°C in a shaking incubator at 180 rpm after which the total bacterial counts were measured. The culture media was recovered at intervals of 24, 48, and 72 h and used in the experiment. Total bacterial count was measured by serially diluting 1 mL of the culture media in 9 mL of peptone water, followed by pouring 1 mL of the diluted solution onto MRS agar.

Incubation temperature

To assess the growth of *Lactobacillus* strains according to different incubation temperatures, they were inoculated into BHI broth supplemented with GOS, FOS, and 2'-FL at a concentration of 5 log CFU/mL. The cultures were incubated for 72 h at 20 to 35°C in a shaking incubator at 180 rpm after which the total bacterial counts were measured. The culture media was recovered at intervals of 24, 48, and 72 h and used in the experiment. Total bacterial count was measured by serially diluting 1 mL of the culture media in 9 mL of peptone water, followed by pouring 1 mL of the diluted solution onto MRS agar.

Prebiotics concentration

To assess the growth of *Lactobacillus* strains according to the concentration of prebiotics, 5 log CFU/mL was inoculated into BHI broth supplemented with GOS, FOS, and 2'-FL ranging from 0% to 4%. The cultures were incubated for 72 h at 37°C in a shaking incubator at 180 rpm after which the total bacterial counts were measured. The culture media was recovered at intervals of 24, 48, and 72 h and used in the experiment. Total bacterial count was measured by serially diluting 1 mL of the culture media in 9 mL of peptone water, followed by pouring 1 mL of the diluted solution onto MRS agar.

Experimental design for response surface methodology

Response surface methodology (RSM) was used for experimental design and optimization to determine culture optimal conditions for *Lactobacillus* strains. The experiment was designed through central composite design (CCD) by setting three independent variables as incubation time (X_1), incubation temperature (X_2), and prebiotics concentration (X_3). Coded independent variables and uncoded independent variables are shown in Table 1 and 2. Central composite design consisting of 3 factors and 3 levels was designed with 6 central points and a total of 20 experiments with the addition of experiments at axial points (α=1). Experiments were

Table 1. Central composite design arrangement and response for production amounts of *L. acidophilus* and *L. plantarum*

Sample No.	Variables				
	Incubation time (h)	Incubation temp. (°C)	Prebiotics conc. (%)	*L. acidophilus* (log CFU/mL)	*L. plantarum* (log CFU/mL)
1	18	30	3	9.16±0.06	8.79±0.05
2	18	30	4	9.19±0.16	8.95±0.17
3	12	25	4	8.39±0.22	7.20±0.15
4	12	25	2	8.36±0.04	7.13±0.20
5	12	35	2	9.15±0.05	8.85±0.05
6	12	35	4	8.92±0.17	9.01±0.11
7	18	30	3	9.17±0.11	8.90±0.04
8	18	30	2	9.18±0.09	8.91±.0.06
9	24	30	3	8.86±0.09	8.93±0.08
10	18	30	3	9.29±0.06	8.87±0.07
11	24	35	4	8.85±0.15	9.08±0.07
12	18	30	3	9.28±0.09	8.98±0.09
13	18	25	3	9.26±0.04	7.99±0.05
14	24	25	2	9.23±0.07	8.90±0.16
15	24	25	4	9.23±0.05	8.98±0.11
16	24	35	2	8.56±0.29	9.15±0.08
17	18	35	3	9.15±0.03	9.06±0.15
18	18	30	3	9.09±0.06	8.88±0.09
19	18	30	3	9.17±0.06	8.96±0.08
20	12	30	3	9.09±0.07	8.42±0.08

Table 2. Central composite design arrangement and response for production amounts of *L. rhamnosus*

Sample No.	Variables			
	Incubation time (h)	Incubation temp. (°C)	Prebiotics conc. (%)	*L. rhamnosus* (log CFU/mL)
1	48	30	3	8.98±0.17
2	48	30	2	8.89±0.06
3	48	30	3	9.08±0.06
4	24	25	4	8.92±0.06
5	48	35	3	9.15±0.02
6	24	35	2	8.85±0.23
7	48	30	3	8.89±0.05
8	48	30	3	9.06±0.15
9	72	35	4	9.22±0.09
10	48	30	3	8.93±0.12
11	72	30	3	9.08±0.11
12	72	35	2	9.02±0.09
13	24	35	4	8.94±0.04
14	48	30	4	8.87±0.07
15	72	25	4	9.09±0.09

Table 2. (Continued) Central composite design arrangement and response for production amounts of *L. rhamnosus*

Sample No.	Variables			
	Incubation time (h)	Incubation temp. (°C)	Prebiotics conc. (%)	*L. rhamnosus* (log CFU/mL)
15	72	25	4	9.09±0.09
16	24	30	3	8.70±0.22
17	72	25	2	9.00±0.02
18	48	30	3	8.92±0.02
19	24	25	2	8.96±0.12
20	48	25	3	9.26±0.05

conducted in a randomized order. The RSM application conditions were determined based on previous studies. For *L. acidophilus* and *L. plantarum*, the ranges were set as follows: 12-24 h, 20-35°C, and 2-4% concentration. For *L. rhamnosus*, the ranges were set as: 24-72 h, 20-35°C, and 2-4% concentration.

Statistical analysis

All data, shown as mean ± SD, were analyzed using one-

way ANOVA. Differences with P-values less than 0.05 were considered statistically significant. 3D surface graph of the response surface were plotted using Minitab 18 software.

Results

Model fitting

The culture conditions of *Lactobacillus* strains were modeled using the response surface methodology (RSM), incorporating the variables of incubation time, incubation temperature, and prebiotics concentration, which experimental ranges have been selected through preliminary study. The total bacterial count of *Lactobacillus* strains in each of the 20 experimental designs generated by RSM is shown in the Table 1 and 2. Statistical significance of the polynomial coefficients derived from the experimental results is shown in Table 3 and 4. The fits of a quadratic polynomial model were evaluated by R^2 and adjusted R^2 along with analysis of variance (ANOVA).

L. acidophilus

The interaction term of incubation time with incubation time as well as the interaction term of incubation time with incubation temperature were significant (P<0.05) effect with

Table 3. Regression coefficients of the quadratic polynomials as functions of the conditions for the amounts of *Lactobacillus* strain

	Variables	Coefficients	*P*-values
L. acidophilus	Intercept	-0.28	
	Incubation time	0.5463	0.084
	Incubation temp.	0.274	0.722
	Prebiotics conc.	0.184	0.834
	Incubation time×Incubation time	-0.00741	0.009
	Incubation temp.×Incubation temp.	-0.00158	0.642
	Prebiotics conc.×Prebiotics conc.	-0.0628	0.465
	Incubation time×Incubation temp.	-0.00987	0.000
	Incubation time×Prebiotics conc.	0.01013	0.238
	Incubation temp.×Prebiotics conc.	0.00065	0.948
L. plantarum	Intercept	-14.23	
	Incubation time	0.6298	0.000
	Incubation temp.	1.063	0.000
	Prebiotics conc.	-0.476	0.472
	Incubation time×Incubation time	-0.00401	0.071
	Incubation temp.×Incubation temp.	-0.01201	0.002
	Prebiotics conc.×Prebiotics conc.	0.1065	0.167
	Incubation time×Incubation temp.	-0.01325	0.000
	Incubation time×Prebiotics conc.	-0.00462	0.524
	Incubation temp.×Prebiotics conc.	-0.00173	0.841
L. rhamnosus	Intercept	16.45	
	Incubation time	0.0087	0.002
	Incubation temp.	-0.563	0.872
	Prebiotics conc.	0.414	0.212
	Incubation time×Incubation time	-0.000168	0.064
	Incubation temp.×Incubation temp.	0.00886	0.001
	Prebiotics conc.×Prebiotics conc.	-0.1034	0.050
	Incubation time×Incubation temp.	0.000261	0.277
	Incubation time×Prebiotics conc.	0.00130	0.278
	Incubation temp.×Prebiotics conc.	0.00587	0.306

Table 4. Analysis of variance for predictive response surface quadratic models for the culture condition of *Lactobacillus* strains

	Variables	Sum of squares	Degrees of freedom	Mean square	*P*-values
L. acidophilus	Model	1.36554	9	0.151726	0.002
	Residual	0.18762	10	0.018762	
	Lack of fit	0.15582	5	0.031164	0.053
	Pure error	0.03180	5	0.006359	
	R^2	0.8792			
	Adjusted R^2	0.7705			
L. plantarum	Model	6.29277	9	0.69920	< 0.0001
	Residual	0.14056	10	0.01406	
	Lack of fit	0.11670	5	0.02334	0.053
	Pure error	0.02386	5	0.00477	
	R^2	0.9782			
	Adjusted R^2	0.9585			
L. rhamnosus	Model	0.119429	9	0.031297	0.008
	Residual	0.059398	10	0.005940	
	Lack of fit	0.029024	5	0.005805	0.519
	Pure error	0.015950	5	0.006075	
	R^2	0.8258			
	Adjusted R^2	0.6691			

P-values of 0.009 and 0.000, respectively. The relative significance of the independent variables for *L. acidophilus* production could be ranked as follows: incubation time > incubation temperature > prebiotic concentration, with *P*-values of 0.084, 0.722, and 0.834, respectively. The R^2 along with adjusted R^2 values for *L. acidophilus* were 0.8792 and 0.7705, respectively, indicating no lack of fit with a *P*-value of 0.053 at the 95% significance level. Furthermore, since the observed regression probability is < 0.01, these data indicate that the proposed model was significant.

L. plantarum

The interaction terms of incubation time, incubation temperature, as well as incubation time with incubation temperature, exhibited significant ($P < 0.05$) effects, as evidenced by their respective *P*-values of 0.000, 0.000, 0.002, and 0.000. The significance of the independent variables for *L. plantarum* production can be ranked as follows: incubation time, incubation temperature > prebiotics concentration, with *P*-values of 0.000, 0.000, and 0.472, respectively. The R^2 along with adjusted R^2 values for *L. plantarum* were 0.9782 and 0.9585, respectively, indicating no lack of fit with a *P*-value of 0.053 at the 95% significance level. Furthermore, since the observed regression probability is < 0.0001, these data indicate that the proposed model was significant.

L. rhamnosus

The interaction terms of incubation time, incubation temperature with incubation temperature exhibited significant ($P < 0.05$) effects, as evidenced by their respective *P*-values of 0.002 and 0.001. The significance of the independent variables for *L. rhamnosus* production can be ranked as follows: incubation time > prebiotics concentration > incubation temperature, with *P*-values of 0.002, 0.212, and 0.872, respectively. The R^2 along with adjusted R^2 values were 0.8258 and 0.6691 for *L. rhamnosus*, respectively, indicating no lack of fit with a *P*-value of 0.0519 at the 95% significance level. Furthermore, since the observed regression probability is < 0.01, these data indicate that the proposed model was significant.

Optimal culture conditions by RSM

A response surface plots for bacterial counts of *Lactobacillus* strains are shown in Fig. 1. The optimized culture conditions of independent variables affecting the response variables were 22.55 h, 25°C and 3.41% (w/w) for *L. acidophilus*; 24 h, 30.86°C and 2% (w/w) for *L. plantarum*; 66.67 h, 35°C and 3.41% (w/w) for *L. rhamnosus*.

Discussion

To commercialize probiotics, achieving economical

production of bacteria is crucial, necessitating optimization processes due to the presence of various variables in each production process[17]. Carbon source plays a vital role in microbial growth, and the metabolic rate of the carbon source affects the production of primary or secondary metabolites as well as the proliferation of microorganisms[18,19]. Among the

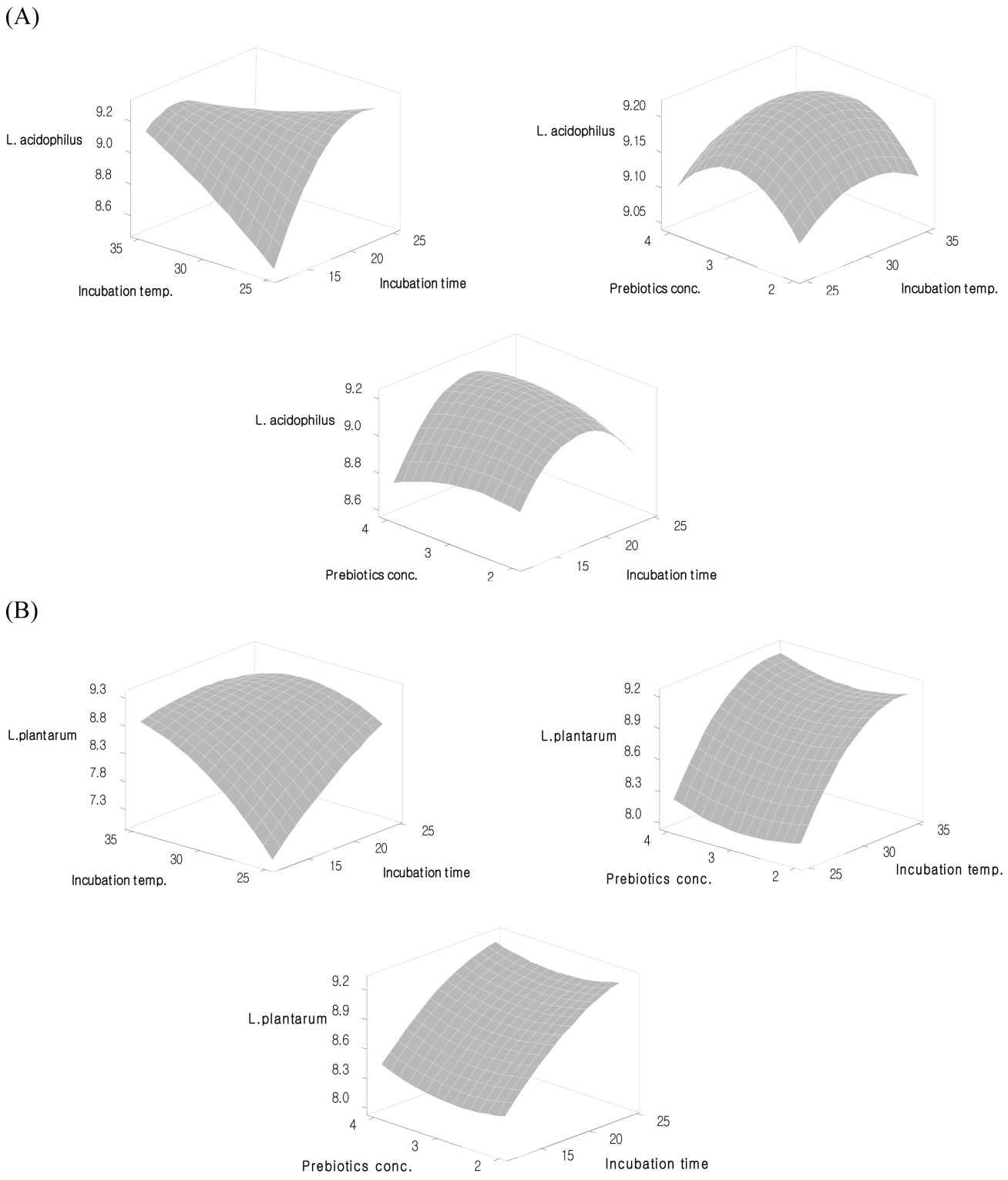

Fig. 1. Optimal culture condition for production of *L. acidophilus* (A), *L. plantarum* (B) and *L. rhamnosus* (C) as a function of incubation time, incubation temperature, and prebiotics concentration.

(C)

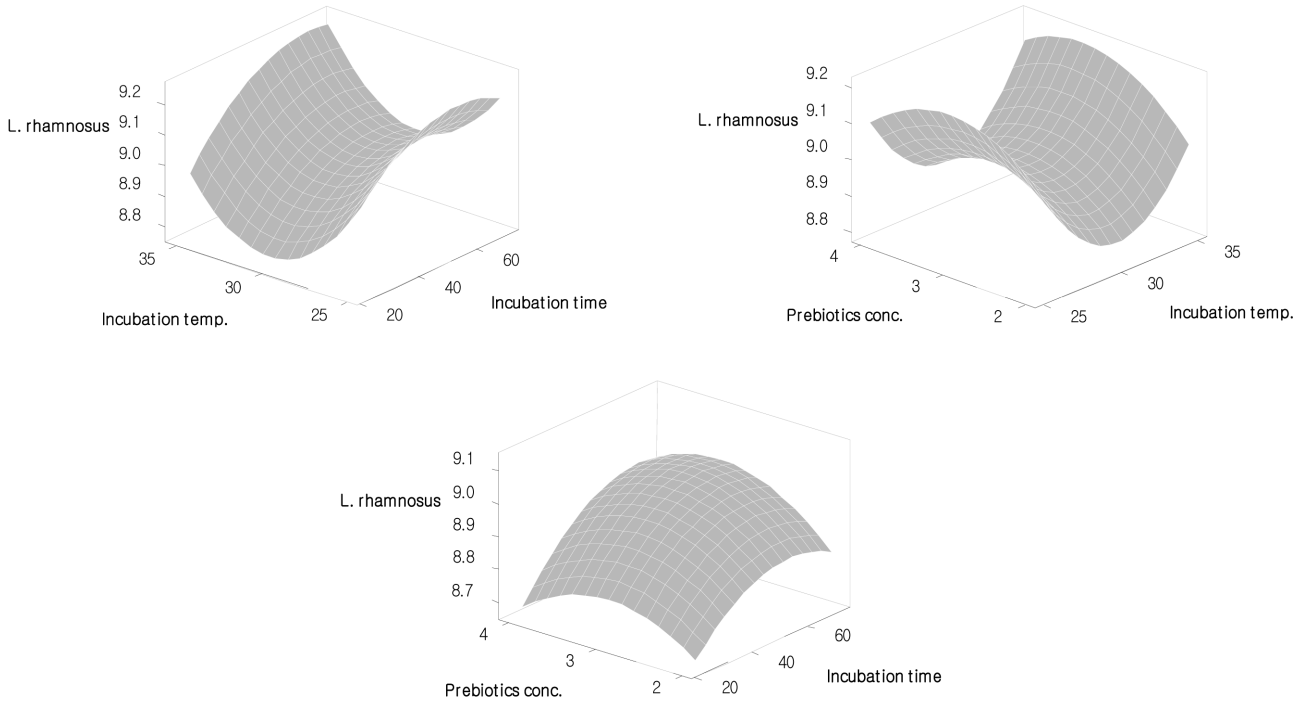

Fig. 1. Continued.

Table 5. Optimum conditions and comparison of predicted and observed values for verification

Responses	Optimum culture condition			Predicted (log CFU/mL)	Observed (log CFU/mL)	Existing (log CFU/mL)
	Incubation time (h)	Incubation temp. (°C)	Prebiotics conc. (%)			
L. acidophilus	22.55	25.00	3.41	9.30	9.26	8.93±0.08
L. plantarum	24.00	30.86	2.00	9.28	9.15	8.71±0.05
L. rhamnosus	66.67	35.00	3.41	9.26	9.31	8.93±0.04

carbon sources used, GOS exhibited the most favorable effect as a carbon source for *Lactobacillus* strains, indicating that the growth of *Lactobacillus* strains can vary depending on the type of carbon source. Moreover, it was observed that the growth of *Lactobacillus* strains can be influenced by temperature, with environmental factors, including temperature control, playing a vital role in the growth of lactic acid bacteria[20-22]. In this study, the culture conditions of *L. acidophilus*, *L. plantarum*, and *L. rhamnosus* were modeled as a function of incubation temperature, incubation time, and prebiotics concentration using the principles of RSM. RSM is an effective experimental design method that predicts the minimum and maximum values through the multilateral interaction results of various independent variables that must be considered for the purpose of bacteria quantity proliferation, and uses them to confirm the optimal values of each variable[23]. By applying RSM, the optimal culture conditions for *Lactobacillus* strains were calculated, considering the advantage of reducing experimental expenses. Previous research has confirmed that the activity and growth of *Lactobacillus* species are enhanced when prebiotics are present at a concentration of 2%[24,25], which aligns with the findings for *L. plantarum*. Messens et al[21]. found that the temperature range between 20 and 35°C is the ideal temperature for growth. This matched the outcomes of three *Lactobacillus* strains. The optimized culture conditions for the *Lactobacillus* strains differed for each strain, with the models predicting maximum yields of 9.30, 9.28, and 9.26 log CFU/mL for *L. acidophilus*, *L. plantarum*, and *L. rhamnosus*, respectively. When the *Lactobacillus* strains were cultured using the established optimal conditions, the measured responses closely matched the predicted results, with approximate log CFU/mL values of 9.26, 9.15, and 9.31 for *L. acidophilus*, *L. plantarum*, and *L. rhamnosus*,

respectively (Table 5). The existing culture conditions specified in the Korean Food Code indicate a range of 48 to 72 ± 3 h at 35 to 37°C. However, the optimized culture conditions in this study were found to require lower temperatures and less time compared to conventional conditions. These results are considered to be because the existing conditions are conditions for similarly simulating the human body environment, and required nutritional and physicochemical factors are not considered.

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국문요약

프로바이오틱스 제품에 대한 수요가 지속적으로 증가하고 있으며, *Lactobacillus* 균주가 가장 대중적인 프로바이오틱스로 널리 사용되고 있다. 프로바이오틱스는 기준에 적합한 균수의 확보가 중요하며 제조원가나 시간 등을 낮추기 위해 배양법의 개발이 필요하므로 *Lactobacillus* 생산을 위한 배양 조건이 최적화되었다. 반응표면방법론에 의한 통계적 최적화에서 반응 변수에 영향을 미치는 독립변수의 최적 조건은 *Lactobacillus acidophilus*의 경우 22.55시간(배양시간), 25°C(배양온도), 3.41%(프리바이오틱스 농도); *Lactiplantibacillus plantarum*의 경우 24시간, 30.86°C, 2.00%; *Lacticaseibacillus rhamnosus*의 경우 66.67시간, 35°C, 3.41%이었다. *Lactobacillus*의 최적 배양조건은 예측한 결과와 실제 결과가 밀접하게 일치하는 것을 확인하였다. 이러한 데이터는 수율 높은 *Lactobacillus*를 생산하는데 중요한 포인트를 제공할 것이다.

Conflict of interest

The authors declare no potential conflict of interest.

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