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Optimizing Conditions for the Growth of *Lactobacillus casei* YIT 9018 in Tryptone-Yeast Extract-Glucose Medium by Using Response Surface Methodology

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This study was undertaken to find optimum conditions of tryptone, yeast extract, glucose, Tween 80, and incubation temperature for the growth of *Lactobacillus casei* YIT 9018 and to assess the effects of these factors by use of response surface methodology. A central composite design was used as an experimental design for allocation of treatment combinations. A second-order polynomial regression model, which was used at first for analysis of the experiment, had a significant lack of fit. Therefore, cubic and quartic terms were incorporated into the regression model through variable selection procedures. Effects involving incubation temperature, yeast extract, glucose, and tryptone were significant, whereas the only significant effect involving Tween 80 was the interaction effect between temperature and Tween 80. It turned out that growth of *L. casei* YIT 9018 was most strongly affected by the incubation temperature. Estimated optimum conditions of the factors for growth of *L. casei* YIT 9018 are as follows: tryptone, 3.04%; yeast extract, 0.892%; glucose, 1.58%; Tween 80, 0%; incubation temperature, 35°C.

In 1907, Metchnikoff hypothesized that the lactic acid bacteria in Bulgarian yogurt could provide a potential human health benefit. Currently, lactobacilli being used as probiotics include *Lactobacillus casei*, *L. acidophilus*, *L. bulgaricus*, *L. fermentum*, *L. plantarum*, *L. lactis*, and *L. reuteri*. Probiotics also contain bacteria belonging to the genera *Bifidobacterium*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Bacillus* (3). The large-scale fermentation of lactobacilli is very useful in the manufacture of commercial starter cultures and pharmaceuticals.

Conditions of fermentation, such as temperature, pH, the types of growth media, oxygen, and the type of neutralizer, have a large effect on the growth activity of lactobacilli (4). Among these, the types of growth media used play an important role in the growth activity. Various growth media for lactic acid bacteria, such as MRS broth, M-17, Elliker's broth, skim milk, and whey permeates, have been widely used (6). Some factors to consider in the choice of growth medium are costs, ability to produce a large number of cells, and harvesting method. Simple synthetic media, with these factors taken into account, have been studied (14). For the preparation of concentrated lactic acid cultures, the tryptone-yeast extract-lactose medium and the tryptone-meat extract-glucose medium have been used because they were not only inexpensive but also easy to harvest (1, 9, 13).

A conventional method that has been used for multifactor experimental design is the "change-one-factor-at-a-time" method. This is an experimentation method in which a single factor is varied while all other factors are kept fixed at a specific set of conditions. This method may lead to unreliable results and wrong conclusions, and it is inferior to the factorial design method (5).

Response surface methodology (RSM), which includes factorial designs and regression analysis, can better deal with multifactor experiments. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses (7, 8).

In our experiment, the response is the growth of *L. casei* YIT 9018 represented by $\log_{10}$ (number of viable cells) and the factors are tryptone, yeast extract, glucose, Tween 80 (these four are medium components) and incubation temperature. Our research objectives are to find the optimum conditions of these factors and to assess their effects.

MATERIALS AND METHODS

Microorganism and growth media. *L. casei* YIT 9018, which is used in the manufacture of Yakult in Korea, was used in this experiment. Pure culture was cultivated in MRS broth at 37°C. *L. casei* was stored at −20°C and thawed just before the experiment. The growth experiment was done with 500-ml volumes of medium in a 1-liter flask. The inoculum consisted of 1% (vol/vol) of culture incubated for 18 h at 2% tryptone–0.7% yeast extract–2.5% glucose medium (pH 6.8) to obtain an initial biomass concentration of 10^6 CFU/ml. After incubation for 12 h with various treatment combinations (see Table 2), the number of the viable cells was measured.

Experimental design. A central composite design in two blocks was used to allocate treatment combinations in this experiment (see Table 2). The experiment was conducted for 2 days. The first block, representing the first day of the experiment, contains the 32 factorial runs and 4 center runs. The second block, representing the second day of the experiment, contains 10 axial runs and 4 center runs.

In this experiment, the response, i.e., the amount of growth of *L. casei* YIT 9018 as measured by $\log_{10}$ (number of viable cells), was assumed to be under the influence of five factors described in Introduction. To set up a statistical model, we let Y denote $\log_{10}$ (number of viable cells) and we determined code factor levels as follows: X_1 = (tryptone − 2)/0.845, X_2 = (yeast extract − 0.7)/0.296, X_3 = (glucose − 2.5)/1.057, X_4 = (Tween 80 − 0.1)/0.042, and X_5 = (temperature − 37)/5.1. Table 1 contains actual factor levels corresponding to coded factor levels. For each factor, a conventional level was set to zero as a coded level. Treatment combinations and observed responses are presented in Table 2. Using

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TABLE 1. Actual factor levels corresponding to coded factor levels

Factor	Symbol	Actual factor level at coded factor level of:				
		−2.366	−1	0	1	2.366
Tryptone (%)	X_1	0	1.155	2	2.845	4
Yeast extract (%)	X_2	0	0.404	0.7	0.996	1.4
Glucose (%)	X_3	0	1.443	2.5	3.557	5
Tween 80 (%)	X_4	0	0.058	0.1	0.142	0.2
Temp (°C)	X_5	25	31.9	37	42.1	49

this design, we can fit a second- or higher-order polynomial regression model to the data.

Statistical analysis. Data were analyzed by the SAS system. SAS/STAT procedures were used for regression analysis (11). In our regression model, the response variable is $\log_{10}$ (number of viable cells) and candidates for explanatory variables are linear, interaction, quadratic, cubic, and quartic terms of coded levels of tryptone, yeast extract, glucose, Tween 80, and temperature. The α-level at which every term in the selected model should be significant was set as 5%. Optimum conditions were found through SAS data-step programming. Response surface plots were generated by SAS/GRAPH (12).

RESULTS AND DISCUSSION

Developing a regression model. First, the second-order polynomial regression model containing 5 linear, 5 quadratic, and 10 interaction terms plus 1 block term was employed by using the RSREG procedure of SAS/STAT. Analysis of variance for evaluation of the second-order model is presented in Table 3.

Table 3 shows that the second-order model was significant and that $r^2 = 0.809$. However, the lack of fit was significant ($P = 0.0061$). This suggests that the model does not accurately represent data in the experimental region. This indicates that higher-order terms might have to be included in the regression model. Since each factor has five levels, up to quartic terms could be included the model (2).

Therefore, variable selection techniques were used to find a better model. Among variable selection techniques available in the REG procedure of SAS/STAT, the smallest Mallows' C_p selection method, the maximum R^2 improvement technique, and the stepwise method were used to select good predictors from the following candidates for model terms:

$$\text{Block},$$
$$X_1, X_2, X_3, X_4, X_5$$
$$X_1X_2, X_1X_3, X_1X_4, X_1X_5, X_2X_3, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5$$
$$X_1^2, X_2^2, X_3^2, X_4^2, X_5^2$$
$$X_1^3, X_2^3, X_3^3, X_4^3, X_5^3$$
$$X_1^4, X_2^4, X_3^4, X_4^4, X_5^4$$

The same 13-variable model was identified by application of all three of the variable selection methods mentioned above. The functional form of this model is as follows:

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_5X_5 + b_{12}X_2X_3 + b_{45}X_4X_5 + b_{55}X_5^2 + b_{222}X_2^3 + b_{333}X_3^3 + b_{555}X_5^3 + b_{1111}X_1^4 + b_{2222}X_2^4 + b_{3333}X_3^4$$

Tables 4 and 5 show how the above model was fitted to the data. The fourth-order subset model in Tables 4 and 5, which was to be used as the response surface model for subsequent analyses, was superior to the second-order full model in Table 3; it has larger r^2 (0.946 > 0.809) and smaller coefficient of variation (2.32% < 4.95%), with the lack of fit being insignificant ($P = 0.2660$), the number of explanatory variables being smaller (13 < 21), and all regression coefficient estimates being significant at the 5% or lower level.

In the model described in Tables 4 and 5, effects involving incubation temperature, yeast extract, glucose, and tryptone were significant whereas the only significant effect involving Tween 80 was the interaction effect between temperature and Tween 80. Note that the t value of the quadratic term of temperature was a two-digit number, which showed that the quadratic effect of temperature was the strongest effect. The intercept b_0 is the estimated response at the center point (X_1, X_2, X_3, X_4, X_5) = (0, 0, 0, 0, 0).

TABLE 2. Treatment combinations[a] and responses

Run	Block[b]	Coded variable level					Response[c] (Y)
		X_1	X_2	X_3	X_4	X_5	
1	−1	−1	−1	−1	−1	−1	7.85
2	−1	1	−1	−1	−1	−1	8.23
3	−1	−1	1	−1	−1	−1	8.11
4	−1	1	1	−1	−1	−1	8.15
5	−1	−1	−1	1	−1	−1	7.74
6	−1	1	−1	1	−1	−1	7.53
7	−1	−1	1	1	−1	−1	7.96
8	−1	1	1	1	−1	−1	8.15
9	−1	−1	−1	−1	1	−1	7.88
10	−1	1	−1	−1	1	−1	8.08
11	−1	−1	1	−1	1	−1	7.93
12	−1	1	1	−1	1	−1	7.85
13	−1	−1	−1	1	1	−1	7.38
14	−1	1	−1	1	1	−1	7.58
15	−1	−1	1	1	1	−1	7.7
16	−1	1	1	1	1	−1	7.85
17	−1	−1	−1	−1	−1	1	7.45
18	−1	1	−1	−1	−1	1	7.6
19	−1	−1	1	−1	−1	1	7.48
20	−1	1	1	−1	−1	1	7.81
21	−1	−1	−1	1	−1	1	6.48
22	−1	1	−1	1	−1	1	7.3
23	−1	−1	1	1	−1	1	7.48
24	−1	1	1	1	−1	1	7.45
25	−1	−1	−1	−1	1	1	7.7
26	−1	1	−1	−1	1	1	7.78
27	−1	−1	1	−1	1	1	7.65
28	−1	1	1	−1	1	1	8.04
29	−1	−1	−1	1	1	1	7.34
30	−1	1	−1	1	1	1	6.7
31	−1	−1	1	1	1	1	7.34
32	−1	1	1	1	1	1	7.88
33	−1	0	0	0	0	0	8
34	−1	0	0	0	0	0	8.04
35	−1	0	0	0	0	0	8.26
36	−1	0	0	0	0	0	8.34
37	1	−2.366	0	0	0	0	7.62
38	1	2.366	0	0	0	0	8.04
39	1	0	−2.366	0	0	0	7.53
40	1	0	2.366	0	0	0	7.15
41	1	0	0	−2.366	0	0	7.51
42	1	0	0	2.366	0	0	7.89
43	1	0	0	0	−2.366	0	7.98
44	1	0	0	0	2.366	0	8.28
45	1	0	0	0	0	−2.366	7.34
46	1	0	0	0	0	2.366	4.04
47	1	0	0	0	0	0	8.32
48	1	0	0	0	0	0	8.15
49	1	0	0	0	0	0	8.34
50	1	0	0	0	0	0	8.12

[a] All combinations were adjusted to pH 6.8.
[b] −1, first day of the experiment; 1, second day of the experiment.
[c] $\log_{10}$ (number of viable cells).

Finding the optimum point of the factors. Our response surface model can be written as

$$\hat{Y} = b_0 + f_1(X_1) + f_{23}(X_2, X_3) + f_{45}(X_4, X_5)$$

TABLE 3. Analysis of variance for evaluation of the second-order model[a]

Source of variation	No. of degrees of freedom	Sum of squares	Mean square	*F* value	*P* value
Model	21	17.1813	0.8182	5.66	0.0001
Residual	28	4.0533	0.1448		
Lack of fit	22	3.9322	0.1787	8.86	0.0061
Pure error	6	0.1211	0.0202		
Total	49	21.2346			

[a] $r^2 = 0.809$, coefficient of variation = 4.95%.

where

$$f_1(X_1) = b_1X_1 + b_{1111}X_1^4$$

$$f_{23}(X_2, X_3) = b_2X_2 + b_3X_3 + b_{23}X_2X_3 + b_{222}X_2^3 + b_{333}X_3^3 + b_{2222}X_2^4 + b_{3333}X_3^4$$

and

$$f_{45}(X_4, X_5) = b_5X_5 + b_{45}X_4X_5 + b_{55}X_5^2 + b_{555}X_5^3$$

We searched for the optimum value of X_1 that maximizes $f_1(X_1)$, the optimum values of X_2 and X_3 that maximize $f_{23}(X_2, X_3)$, and the optimum values of X_4 and X_5 that maximize $f_{45}(X_4, X_5)$. $f_1(X_1)$ was maximized through differentiation. $f_{23}(X_2, X_3)$ was maximized through calculation and sorting of $f_{23}(X_2, X_3)$ values on a grid of points for X_2 and X_3. $f_{45}(X_4, X_5)$ also was maximized through calculation and sorting of $f_{45}(X_4, X_5)$ values on a grid of points for X_4 and X_5. The search was done with computer programs written in SAS. An illustrative SAS program to search for optimum response values on a grid of points of explanatory factors in the range of interest is given in the chapter for the RSREG procedure (11).

The optimum point we obtained this way was $(X_1, X_2, X_3, X_4, X_5) = (1.225, 0.650, -0.870, -2.366, -0.402)$. Recoding the coded levels back to the original levels, we obtained the following results: tryptone = 3.04%, yeast extract = 0.892%, glucose = 1.58%, Tween 80 = 0%, and incubation temperature = 35°C. Notice here that the optimum levels of glucose (X_3) and Tween 80 (X_4) were lower than conventional levels; in particular, the optimum level of Tween 80 was found to be zero. These lower levels imply a reduction of the cost—an economic gain.

Here, we explain how zero was obtained as the optimum level of Tween 80. Note that the coefficient estimate of the Tween 80-temperature interaction term has a positive sign ($b_{45} = 0.089063$). This implies that for an increase of the response, the coded levels of Tween 80 and temperature must have the same signs—both greater than zero or both smaller than zero. So, in order for the value of $b_{45}X_4X_5$ to be maximized, if X_5 is lower than zero, X_4 must be the lowest possible value, and if X_5 is higher, X_4 must be the highest possible value. Here, $(X_4, X_5) = (-2.366, -0.402)$ produced the maximum value of $f_{45}(X_4, X_5)$.

TABLE 4. Analysis of variance in the regression model selected through variable selection[a]

Source of variation	No. of degrees of freedom	Sum of squares	Mean square	*F* value	*P* value
Model	13	20.0912	1.5455	48.66	0.0001
Residual	36	1.1434	0.0318		
Lack of fit	30	1.0223	0.0341	1.69	0.2660
Pure error	6	0.1211	0.0202		
Total	49	21.2346			

[a] $r^2 = 0.946$, coefficient of variation = 2.32%.

TABLE 5. Coefficient estimates in the regression model selected through variable selection

Variable	Coefficient estimate	Standard error	*t* value	*P* value
Intercept	$b_0 = 8.173146$	0.03907252	209.179	0.0001
X_1	$b_1 = 0.081112$	0.02711625	2.991	0.0050
X_2	$b_2 = 0.177641$	0.04006769	4.434	0.0001
X_3	$b_3 = -0.235472$	0.04006769	−5.877	0.0001
X_5	$b_5 = -0.095250$	0.04006769	−2.377	0.0229
X_2X_3	$b_{23} = 0.103437$	0.03150474	3.283	0.0023
X_4X_5	$b_{45} = 0.089063$	0.03150474	2.827	0.0076
X_5^2	$b_{55} = -0.445748$	0.02460326	−18.117	0.0001
X_2^3	$b_{222} = -0.046078$	0.01345869	−3.424	0.0016
X_3^3	$b_{333} = 0.056409$	0.01345869	4.191	0.0002
X_5^3	$b_{555} = -0.107562$	0.01345869	−7.992	0.0001
X_1^4	$b_{1111} = -0.011019$	0.00428762	−2.570	0.0145
X_2^4	$b_{2222} = -0.026656$	0.00428762	−6.217	0.0001
X_3^4	$b_{3333} = -0.015168$	0.00428762	−3.538	0.0011

The estimated maximum response corresponding to the optimum factor levels was 8.504, which is larger than 8.173, the estimated response at the center point. This is an improvement claimed by the regression model. A validation experiment will ascertain whether there is a real improvement.

Assessing factor effects with the partial-effects plot. The partial-effect functions and plot (10) were used to assess the effect of each factor graphically. The partial-effect function of a certain factor is a function that describes how the response moves as the level of that factor changes when the other factors are fixed at their optimum levels. Let $\hat{Y} = f(X_1, X_2, X_3, X_4, X_5)$ denote our response surface model described in Tables 4 and 5 and $(X_1^*, X_2^*, X_3^*, X_4^*, X_5^*)$ denote the optimum point of the factors which is, in our case, $(1.225, 0.650, -0.870, -2.366, -0.402)$. Then, the partial-effect function of X_1 is defined as

$$\hat{Y}(X_1) = f(X_1, X_2^*, X_3^*, X_4^*, X_5^*)$$

Similarly, the partial-effect functions of X_2, X_3, X_4, and X_5 are defined as

$$\hat{Y}(X_2) = f(X_1^*, X_2, X_3^*, X_4^*, X_5^*)$$

$$\hat{Y}(X_3) = f(X_1^*, X_2^*, X_3, X_4^*, X_5^*)$$

$$\hat{Y}(X_4) = f(X_1^*, X_2^*, X_3^*, X_4, X_5^*)$$

$$\hat{Y}(X_5) = f(X_1^*, X_2^*, X_3^*, X_4^*, X_5)$$

The partial-effect curve is a curve drawn with the vertical axis representing $\hat{Y}(X_j)$ and the horizontal axis representing X_j. By overlaying all partial-effect curves, we get the partial-effects plot. In the partial-effects plot, since all X_j have common coded levels, we let the horizontal axis represent the common coded-factor level. Figure 1 is the partial-effects plot of our factors.

In Fig. 1, the curve with the most conspicuous change is the partial-effect curve of temperature; the estimated response increases gradually until the coded level of temperature

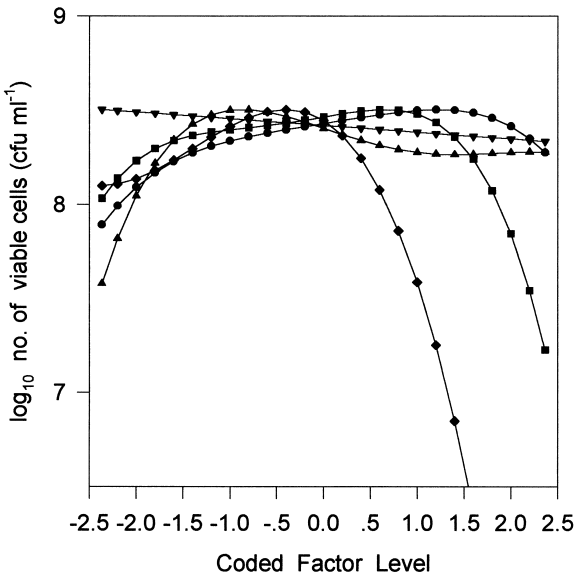

FIG. 1. Partial-effects plot of tryptone (●), yeast extract (■), glucose (▲), Tween 80 (▼), and incubation temperature (◆).

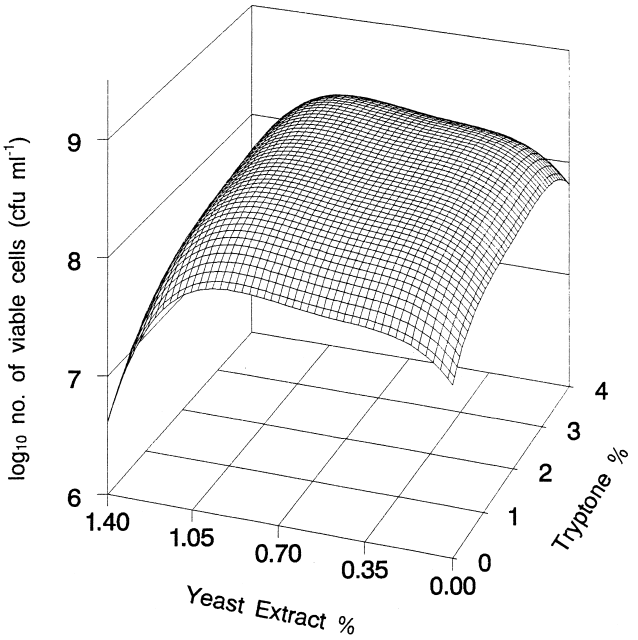

FIG. 2. Response surface for the effects of tryptone and yeast extract on the growth of *L. casei* at glucose = 1.58% and temperature = 35°C.

reaches −0.402 and decreases rapidly after the temperature becomes higher than its coded level of −0.402 when the other factors are fixed at their optimum levels. From this, we can ascertain that temperature was the most significant factor, with its quadratic effect being most pronounced.

The second most conspicuous change was found in the response curve of yeast extract; the estimated response increases gradually until the coded level of yeast extract reaches 0.65 and decreases rapidly after the yeast extract percentage becomes higher than its coded level of 0.65 when the other factors are fixed at their optimum levels.

The partial-response curve of glucose also shows a pronounced change; the estimated response increases rapidly until the coded level of glucose reaches −0.87 and decreases gradually after the glucose percentage becomes higher than its coded level of −0.87 when the other factors are fixed at their optimum levels.

The partial effect of tryptone seemed moderate; the estimated response increases gradually until the coded level of tryptone reaches 1.225 and decreases gradually after the tryptone percentage becomes higher than its coded level of 1.225 when the other factors are fixed at their optimum levels.

As for Tween 80, its partial effect seemed weak; the estimated response shows a very slow linear decrease according to the change of the coded level of Tween 80 from −2.366 to 2.366 when the other factors are fixed at their optimum levels. Actually, with the other factors being fixed at their optimum levels, the estimated maximum and minimum responses corresponding to the minimum and maximum coded levels of Tween 80 were 8.504 and 8.335; the difference between them is small. Therefore, we concluded that the partial effect of Tween 80 was not practically significant.

Plotting three-dimensional response surface plots. For any two of the four significant factors, a three-dimensional response surface plot was drawn with the vertical axis representing $\log_{10}$ (number of viable cells) and two horizontal axes representing the actual levels of two explanatory factors. In each plot, the factors not represented by the two horizontal axes are fixed at their optimum actual levels. All six plots were produced. Figures 2 through 7 are such plots.

In Fig. 2 through 7 (except for Fig. 5), we see that the effects of pairs of factors were additive since there are no interactions except the yeast extract-glucose interaction. By additivity of the two-factor effects, we mean that the effect of one factor on the response does not depend on the level of the other factor.

Figure 5 shows nonadditive effects of yeast extract and glucose that are due to the significant interaction between them. The coefficient estimate of this interaction term has a positive sign (b_{23} = 0.103437). Considering this interaction only, a

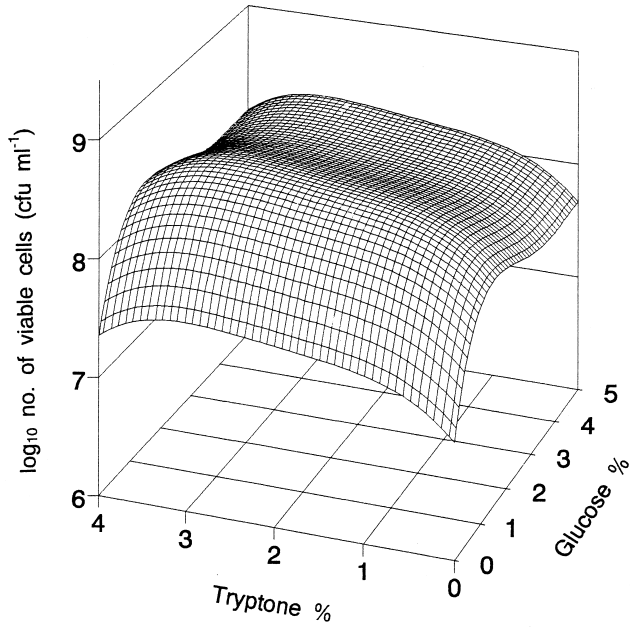

FIG. 3. Response surface for the effects of tryptone and glucose on the growth of *L. casei* at yeast extract = 0.892% and temperature = 35°C.

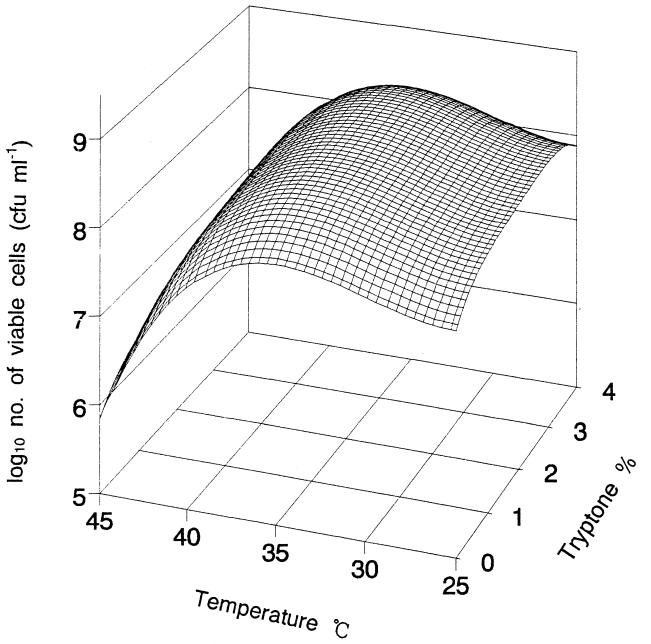

FIG. 4. Response surface for the effects of tryptone and temperature on the growth of *L. casei* at yeast extract = 0.892% and glucose = 1.58%.

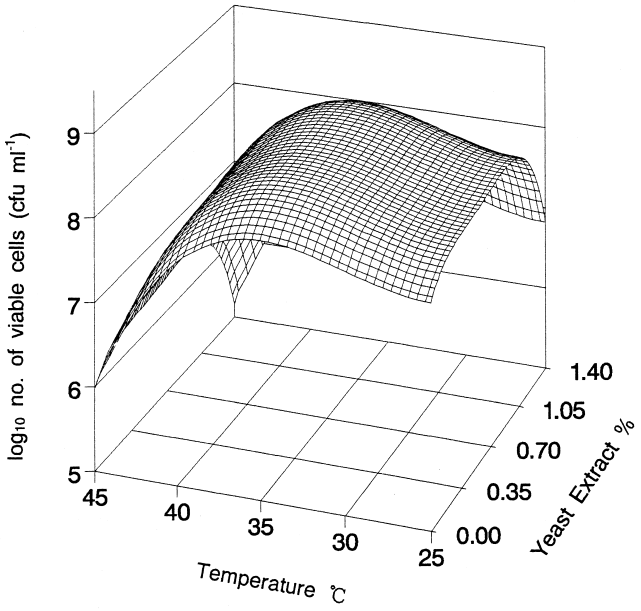

FIG. 6. Response surface for the effects of yeast extract and temperature on the growth of *L. casei* at tryptone = 3.035% and glucose = 1.58%.

positive sign may imply that for an increase of the response, the coded levels of yeast extract and glucose must have the same signs—both greater than zero or both smaller than zero. However, the three-dimensional plot does not show this feature and, at the optimum point, X_2 and X_3 do not have the same sign—$(X_2, X_3) = (0.650, -0.870)$. This is considered to be due to the other terms (linear, cubic, and quartic terms) dominating the interaction term.

Validating the optimum point of the factors. An experiment was conducted to validate the optimum point of the factors found in this study. Here, we compared three growth media: the MRS medium, the optimum-point medium, and the center-point medium. The compositions of these three media are given in Table 6. Figure 8 shows three growth curves at the three media drawn with the vertical axis representing $\log_{10}$ (number of viable cells) and the horizontal axis representing the elapsed time in hours.

The MRS medium produced the largest number of viable cells at every hour (Fig. 8). However, the MRS medium is an expensive, luxuriant medium with complicated components. As

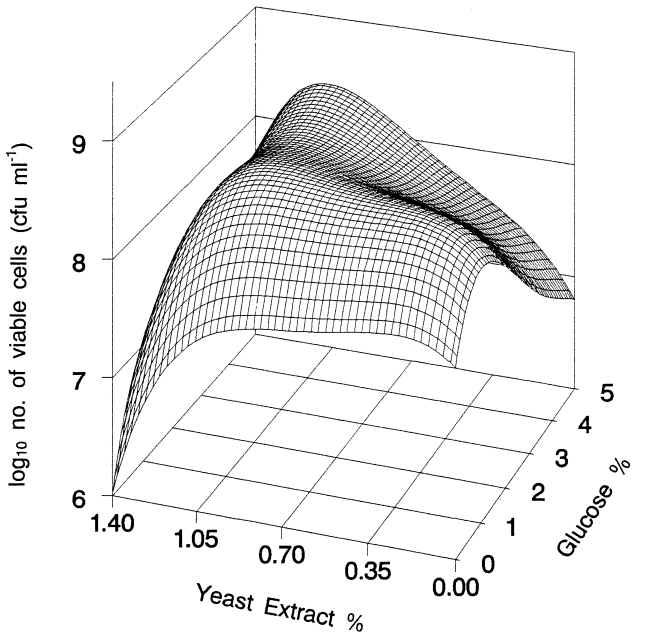

FIG. 5. Response surface for the effects of yeast extract and glucose on the growth of *L. casei* at tryptone = 3.035% and temperature = 35°C.

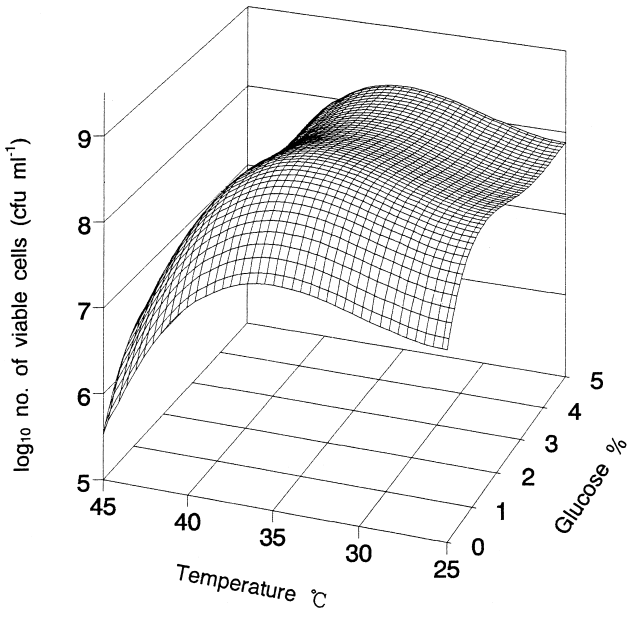

FIG. 7. Response surface for the effects of glucose and temperature on the growth of *L. casei* at tryptone = 3.035% and yeast extract = 0.892%.

TABLE 6. Compositions of three media for the growth of *L. casei* YIT 9018

Component	Amt of component (%) in:		
	Optimum-point medium	Center-point medium	MRS medium
Tryptone	3.04	2.0	—[a]
Yeast extract	0.892	0.7	0.5
Glucose	1.58	2.5	2.0
Tween 80	—	0.1	0.1
Proteose peptone	—	—	1.0
Beef extract	—	—	1.0
Ammonium citrate	—	—	0.2
Sodium acetate	—	—	0.5
Magnesium sulfate	—	—	0.01
Manganese sulfate	—	—	0.005
Dipotassium phosphate	—	—	0.2
Incubation temp	35°C	37°C	37°C

[a] —, absence of constituent.

for the optimum-point medium, even though its productive performance was second to that of the MRS medium, it is cheaper and produced more viable cells than the center-point medium at every hour. For example, at 12 h, log values of the viable cells are 8.991 in the MRS medium, 8.75 in the optimum-point medium, and 8.423 in the center-point medium. The optimum-point medium was found to be more productive than the center-point medium and more economical than the MRS medium.

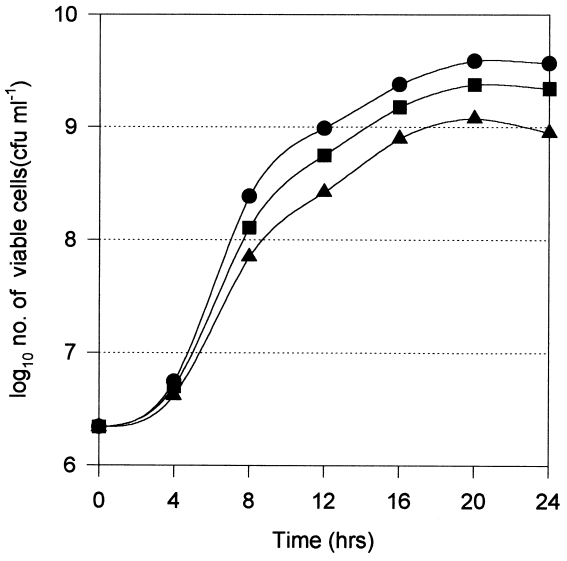

FIG. 8. Growth curves of *L. casei* in the MRS broth (●), optimum-point (■), and center-point (▲) media as obtained from the validation experiment.

Conclusions. RSM including experimental design and regression analysis was effective in developing an analysis model, finding the optimum point of the factors, and assessing the effects of the factors. The optimum conditions of the factors were as follows: tryptone = 3.04%, yeast extract = 0.892%, glucose = 1.58%, Tween 80 = 0%, and incubation temperature = 35°C. Through the validation experiment, we could ascertain that this optimum-point medium gave better productivity than the center-point medium. In this paper, the methodological points we stress are (i) using variable selection techniques to choose higher-order terms, (ii) finding the optimum point based on the calculation on a grid of points of factor levels, and (iii) plotting the partial-effects plot to assess the effect of each factor. We found that RSM could be successfully used for design and analysis of fermentation experiments involving microorganisms.

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