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# New Response Surface Approach to Optimize Medium Composition for Production of Bacteriocin by *Lactobacillus acidophilus* ATCC 4356

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**Abstract** The objective of this study was to optimize medium composition of initial pH, tryptone, glucose, yeast extract, and mineral mixture for production of bacteriocin by *Lactobacillus acidophilus* ATCC 4356, using response surface methodology. A response surface approach including new statistical and plotting methods was employed for design and analysis of the experiment. An interiorly augmented central composite design was used as an experimental design. A normal-distribution log-link generalized linear model based on a subset fourth-order polynomial ($R^2$=0.94, Mean Error Deviance=0.0065) was used as an analysis model. This model was statistically superior to the full second-order polynomial-based generalized linear model ($R^2$=0.80, Mean Error Deviance= 0.0140). Nonlinear programming determined the optimum composition of the medium as initial pH 6.35, tryptone 1.21%, glucose 0.9%, yeast extract 0.65%, and mineral mixture 1.17%. A validation experiment confirmed that the optimized medium was comparable to the MRS medium in bacteriocin production, having the advantage of economy and practicality.

**Key words:** *Lactobacillus acidophilus*, bacteriocin, response surface methodology, optimization

Bacteriocins produced by lactic acid bacteria (LAB) are usually active against closely related Gram-positive bacteria including pathogenic microorganisms. Among them, class II bacteriocins produced by LAB are low molecular weight peptides with heat and pH stability [7, 10]. For these reasons, bacteriocins may have useful applications in food as natural preservatives [6, 8, 11]. The effectiveness of some bacteriocin producers including *Pediococcus acidilactici* PAC 1.0 and *Lactobacillus sake* Lb 706 has been studied in several food systems [12, 17, 18].

In a previous study, *Lactobacillus acidophilus* ATCC 4356, a potential probiotic strain, exhibited bacteriocin activity against various LAB and foodborne pathogens. The bacteriocin produced by *L. acidophilus* ATCC 4356 was a peptide with heat and pH stability, which indicates it to be categorized as a class II bacteriocin [4].

In general, the growth medium of LAB is comprised of sugars, peptone, and growth promoters such as yeast extract and minerals. The concentration of these ingredients can be altered to maximize the growth and to promote the subsequent performance of the culture, since the composition of the medium has a profound effect on the growth of bacteria. However, the bacteriocin production may not correlate with cell growth. Thus, optimization of medium composition is needed to enhance bacteriocin production.

Several studies have been conducted for the development of predictive equations to describe the effects of various factors on the bacterial growth [3]. Response surface methodology (RSM) has been used successfully for modeling how the factors influence the growth of bacteria. In a previous study, medium composition was optimized to obtain the maximum viability of *L. casei* YIT 9018 using RSM [13].

In this study, optimal medium composition of initial pH, yeast extract, tryptone, mineral mixture, and glucose for bacteriocin production by *L. acidophilus* ATCC 4356 were determined and the effects of these medium components on the bacteriocin activity were assessed, using a response surface approach that includes newly-developed statistical and plotting methods for experimental design and analysis.

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## MATERIALS AND METHODS

### Bacteria

*L. acidophilus* ATCC 4356 and *L. delbrueckii* subsp. *lactis* ATCC 4797 strains were obtained from American Type Culture Collection and were grown for 18 h at 37°C in MRS broth (Oxoid, Unipath Ltd., Basingstoke, Hants, U.K.). The viable cell counts of *L. acidophilus* ATCC 4356 were determined by the pour plate method on MRS agar (Oxoid, U.K.).

### Bacteriocin Activity

The spot-on-the-lawn method was used to determine the antimicrobial activity of *L. acidophilus* ATCC 4356 against *L. delbrueckii* subsp. *lactis* ATCC 4797 [9]. A 10 μl aliquot of cell-free supernatant of *L. acidophilus* ATCC 4356 culture was spotted onto MRS agar and then was incubated at 37°C for 18 h. The arbitrary units (AU) of bacteriocin were defined as the reciprocal of the highest dilution showing a clear zone of inhibition.

### Experimental Design

An interiorly augmented central composite design (CCD) was used to allocate factor-level combinations in this experiment. The response, denoted by Y in Table 2, is log(bacterial number of *L. delbrueckii* subsp. *lactis* ATCC 4797), which indicates the degree of viability of *L. delbrueckii* subsp. *lactis* ATCC 4797 by bacteriocin treatment. A smaller Y value indicates higher bacteriocin activity. The aim of the experiment was to determine the factor-level combination that minimizes the Y value.

The response was assumed to be under influence of five factors, which are initial pH, yeast extract, tryptone, mineral mixture, and glucose. Table 1 shows the unit and range of levels for each factor. Mineral mixture consisted of 0.1% tween 80, 11 mM $K_2SO_4$, 36 mM sodium acetate, 25.4 mM ammonium acetate, 0.8 mM $MgSO_4$, and 0.2 mM $MnSO_4$.

For statistical design and analysis of the experiment, factor levels were coded as

$X_1$=(pH−6.8)/0.36
$X_2$=(Yeast Extract−0.5)/0.18
$X_3$=(Tryptone−1.0)/0.22
$X_4$=(Mineral Mixture−1.0)/0.22
$X_5$=(Glucose−1.3)/0.36

**Table 1.** Unit and range of levels for each factor.

| Factor | Unit | Minimum level | Median level | Maximum level |
|---|---|---|---|---|
| pH | | 6.0 | 6.8 | 7.6 |
| Yeast Extract | % | 0.1 | 0.5 | 0.9 |
| Tryptone | % | 0.5 | 1.0 | 1.5 |
| Mineral Mixture | % | 0.5 | 1.0 | 1.5 |
| Glucose | % | 0.5 | 1.3 | 2.1 |

Factor-level combination arrangement and observed responses are presented in Table 2. The actual factor levels needed to be expressed as numbers with two decimal places.

The design in Table 2 is not an ordinary central composite design. It is a central composite design augmented by interior axial runs that are runs 43 through 62. In these runs, each interior axial point was replicated twice. In this design, each factor has 7 levels. Compared to an ordinary central composite design, this design allows us to do two more things, which are (1) exploring the interior of the design region more thoroughly, and (2) using a polynomial of order up to 4 as the linear component of a general linear model. This design augmentation will of course increase expenses and time for experimentation, but allow better optimization through more sophisticated modeling. If experimentation-generated products are produced on a large scale, total profit due to better optimization will be much greater than additional cost due to design augmentation.

This design is as spherical as possible, the squared radius of the design in terms of coded variables being as close to 5 as possible. In terms of coded variables, the design region is the surface and interior of the 5-th dimensional sphere with a radius of the square root of 5. The interior axial points in runs 43-62 are located as close as possible to the surface of the 5-th dimensional sphere with a radius of half of the square root of 5.

### Statistical Analysis

Data were analyzed using the SAS System. Procedures of SAS/STAT and SAS/OR were used to perform analyses including generalized linear modeling and nonlinear optimization [16]. As a link function for the response mean in generalized linear modeling, the natural log function was selected. A subset fourth-order polynomial was chosen to be the linear component of the generalized linear model. The optimum factor-level combination was determined by nonlinear optimization. Response surface plots were generated using Minitab [15].

## RESULTS AND DISCUSSION

### Developing a Generalized Linear Model

A polynomial in $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ was to be used for generalized linear modeling, and the values of the original responses (Y in Table 2) are small and close to 0, yet greater than 0. In this case, the value of a polynomial fitted to the data could be below 0, but a negative value cannot be a predicted value of Y. Here, if an appropriate function of the mean of the original responses is modeled as a polynomial, the negative values of a polynomial can be legitimate values from the model. The natural log is such a function, because even though log(some value) is negative,

**Table 2.** Factor-level combinations in coded and actual levels and responses.

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | pH | Yeast extract | Tryptone | Mineral mixture | Glucose | Y* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −1.000 | −1.000 | −1.000 | −1.000 | −1.000 | 6.44 | 0.32 | 0.78 | 0.78 | 0.94 | 0.375 |
| 2 | 1.000 | −1.000 | −1.000 | −1.000 | −1.000 | 7.16 | 0.32 | 0.78 | 0.78 | 0.94 | 0.980 |
| 3 | −1.000 | 1.000 | −1.000 | −1.000 | −1.000 | 6.44 | 0.68 | 0.78 | 0.78 | 0.94 | 0.249 |
| 4 | 1.000 | 1.000 | −1.000 | −1.000 | −1.000 | 7.16 | 0.68 | 0.78 | 0.78 | 0.94 | 0.412 |
| 5 | −1.000 | −1.000 | 1.000 | −1.000 | −1.000 | 6.44 | 0.32 | 1.22 | 0.78 | 0.94 | 0.235 |
| 6 | 1.000 | −1.000 | 1.000 | −1.000 | −1.000 | 7.16 | 0.32 | 1.22 | 0.78 | 0.94 | 0.990 |
| 7 | −1.000 | 1.000 | 1.000 | −1.000 | −1.000 | 6.44 | 0.68 | 1.22 | 0.78 | 0.94 | 0.146 |
| 8 | 1.000 | 1.000 | 1.000 | −1.000 | −1.000 | 7.16 | 0.68 | 1.22 | 0.78 | 0.94 | 0.313 |
| 9 | −1.000 | −1.000 | −1.000 | 1.000 | −1.000 | 6.44 | 0.32 | 0.78 | 1.22 | 0.94 | 0.266 |
| 10 | 1.000 | −1.000 | −1.000 | 1.000 | −1.000 | 7.16 | 0.32 | 0.78 | 1.22 | 0.94 | 0.340 |
| 11 | −1.000 | 1.000 | −1.000 | 1.000 | −1.000 | 6.44 | 0.68 | 0.78 | 1.22 | 0.94 | 0.123 |
| 12 | 1.000 | 1.000 | −1.000 | 1.000 | −1.000 | 7.16 | 0.68 | 0.78 | 1.22 | 0.94 | 0.459 |
| 13 | −1.000 | −1.000 | 1.000 | 1.000 | −1.000 | 6.44 | 0.32 | 1.22 | 1.22 | 0.94 | 0.131 |
| 14 | 1.000 | −1.000 | 1.000 | 1.000 | −1.000 | 7.16 | 0.32 | 1.22 | 1.22 | 0.94 | 0.243 |
| 15 | −1.000 | 1.000 | 1.000 | 1.000 | −1.000 | 6.44 | 0.68 | 1.22 | 1.22 | 0.94 | 0.119 |
| 16 | 1.000 | 1.000 | 1.000 | 1.000 | −1.000 | 7.16 | 0.68 | 1.22 | 1.22 | 0.94 | 0.580 |
| 17 | −1.000 | −1.000 | −1.000 | −1.000 | 1.000 | 6.44 | 0.32 | 0.78 | 0.78 | 1.66 | 0.244 |
| 18 | 1.000 | −1.000 | −1.000 | −1.000 | 1.000 | 7.16 | 0.32 | 0.78 | 0.78 | 1.66 | 0.720 |
| 19 | −1.000 | 1.000 | −1.000 | −1.000 | 1.000 | 6.44 | 0.68 | 0.78 | 0.78 | 1.66 | 0.230 |
| 20 | 1.000 | 1.000 | −1.000 | −1.000 | 1.000 | 7.16 | 0.68 | 0.78 | 0.78 | 1.66 | 0.272 |
| 21 | −1.000 | −1.000 | 1.000 | −1.000 | 1.000 | 6.44 | 0.32 | 1.22 | 0.78 | 1.66 | 0.210 |
| 22 | 1.000 | −1.000 | 1.000 | −1.000 | 1.000 | 7.16 | 0.32 | 1.22 | 0.78 | 1.66 | 0.610 |
| 23 | −1.000 | 1.000 | 1.000 | −1.000 | 1.000 | 6.44 | 0.68 | 1.22 | 0.78 | 1.66 | 0.232 |
| 24 | 1.000 | 1.000 | 1.000 | −1.000 | 1.000 | 7.16 | 0.68 | 1.22 | 0.78 | 1.66 | 0.150 |
| 25 | −1.000 | −1.000 | −1.000 | 1.000 | 1.000 | 6.44 | 0.32 | 0.78 | 1.22 | 1.66 | 0.274 |
| 26 | 1.000 | −1.000 | −1.000 | 1.000 | 1.000 | 7.16 | 0.32 | 0.78 | 1.22 | 1.66 | 0.455 |
| 27 | −1.000 | 1.000 | −1.000 | 1.000 | 1.000 | 6.44 | 0.68 | 0.78 | 1.22 | 1.66 | 0.810 |
| 28 | 1.000 | 1.000 | −1.000 | 1.000 | 1.000 | 7.16 | 0.68 | 0.78 | 1.22 | 1.66 | 0.248 |
| 29 | −1.000 | −1.000 | 1.000 | 1.000 | 1.000 | 6.44 | 0.32 | 1.22 | 1.22 | 1.66 | 0.136 |
| 30 | 1.000 | −1.000 | 1.000 | 1.000 | 1.000 | 7.16 | 0.32 | 1.22 | 1.22 | 1.66 | 0.449 |
| 31 | −1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 6.44 | 0.68 | 1.22 | 1.22 | 1.66 | 0.131 |
| 32 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 7.16 | 0.68 | 1.22 | 1.22 | 1.66 | 0.166 |
| 33 | −2.222 | 0.000 | 0.000 | 0.000 | 0.000 | 6.00 | 0.50 | 1.00 | 1.00 | 1.30 | 0.275 |
| 34 | 2.222 | 0.000 | 0.000 | 0.000 | 0.000 | 7.60 | 0.50 | 1.00 | 1.00 | 1.30 | 0.755 |
| 35 | 0.000 | −2.222 | 0.000 | 0.000 | 0.000 | 6.80 | 0.10 | 1.00 | 1.00 | 1.30 | 0.700 |
| 36 | 0.000 | 2.222 | 0.000 | 0.000 | 0.000 | 6.80 | 0.90 | 1.00 | 1.00 | 1.30 | 0.175 |
| 37 | 0.000 | 0.000 | −2.273 | 0.000 | 0.000 | 6.80 | 0.50 | 0.50 | 1.00 | 1.30 | 0.738 |
| 38 | 0.000 | 0.000 | 2.273 | 0.000 | 0.000 | 6.80 | 0.50 | 1.50 | 1.00 | 1.30 | 0.134 |
| 39 | 0.000 | 0.000 | 0.000 | −2.273 | 0.000 | 6.80 | 0.50 | 1.00 | 0.50 | 1.30 | 0.209 |
| 40 | 0.000 | 0.000 | 0.000 | 2.273 | 0.000 | 6.80 | 0.50 | 1.00 | 1.50 | 1.30 | 0.172 |
| 41 | 0.000 | 0.000 | 0.000 | 0.000 | −2.222 | 6.80 | 0.50 | 1.00 | 1.00 | 0.50 | 0.170 |
| 42 | 0.000 | 0.000 | 0.000 | 0.000 | 2.222 | 6.80 | 0.50 | 1.00 | 1.00 | 2.10 | 0.230 |
| 43 | −1.111 | 0.000 | 0.000 | 0.000 | 0.000 | 6.40 | 0.50 | 1.00 | 1.00 | 1.30 | 0.163 |
| 44 | −1.111 | 0.000 | 0.000 | 0.000 | 0.000 | 6.40 | 0.50 | 1.00 | 1.00 | 1.30 | 0.123 |
| 45 | 1.111 | 0.000 | 0.000 | 0.000 | 0.000 | 7.20 | 0.50 | 1.00 | 1.00 | 1.30 | 0.603 |
| 46 | 1.111 | 0.000 | 0.000 | 0.000 | 0.000 | 7.20 | 0.50 | 1.00 | 1.00 | 1.30 | 0.458 |
| 47 | 0.000 | −1.111 | 0.000 | 0.000 | 0.000 | 6.80 | 0.30 | 1.00 | 1.00 | 1.30 | 0.787 |
| 48 | 0.000 | −1.111 | 0.000 | 0.000 | 0.000 | 6.80 | 0.30 | 1.00 | 1.00 | 1.30 | 0.458 |
| 49 | 0.000 | 1.111 | 0.000 | 0.000 | 0.000 | 6.80 | 0.70 | 1.00 | 1.00 | 1.30 | 0.408 |
| 50 | 0.000 | 1.111 | 0.000 | 0.000 | 0.000 | 6.80 | 0.70 | 1.00 | 1.00 | 1.30 | 0.353 |
| 51 | 0.000 | 0.000 | −1.136 | 0.000 | 0.000 | 6.80 | 0.50 | 0.75 | 1.00 | 1.30 | 0.624 |
| 52 | 0.000 | 0.000 | −1.136 | 0.000 | 0.000 | 6.80 | 0.50 | 0.75 | 1.00 | 1.30 | 0.775 |

**Table 2.** Continued.

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | pH | Yeast extract | Tryptone | Mineral mixture | Glucose | Y* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 53 | 0.000 | 0.000 | 1.136 | 0.000 | 0.000 | 6.80 | 0.50 | 1.25 | 1.00 | 1.30 | 0.323 |
| 54 | 0.000 | 0.000 | 1.136 | 0.000 | 0.000 | 6.80 | 0.50 | 1.25 | 1.00 | 1.30 | 0.218 |
| 55 | 0.000 | 0.000 | 0.000 | -1.136 | 0.000 | 6.80 | 0.50 | 1.00 | 0.75 | 1.30 | 0.367 |
| 56 | 0.000 | 0.000 | 0.000 | -1.136 | 0.000 | 6.80 | 0.50 | 1.00 | 0.75 | 1.30 | 0.195 |
| 57 | 0.000 | 0.000 | 0.000 | 1.136 | 0.000 | 6.80 | 0.50 | 1.00 | 1.25 | 1.30 | 0.310 |
| 58 | 0.000 | 0.000 | 0.000 | 1.136 | 0.000 | 6.80 | 0.50 | 1.00 | 1.25 | 1.30 | 0.271 |
| 59 | 0.000 | 0.000 | 0.000 | 0.000 | -1.111 | 6.80 | 0.50 | 1.00 | 1.00 | 0.90 | 0.280 |
| 60 | 0.000 | 0.000 | 0.000 | 0.000 | -1.111 | 6.80 | 0.50 | 1.00 | 1.00 | 0.90 | 0.304 |
| 61 | 0.000 | 0.000 | 0.000 | 0.000 | 1.111 | 6.80 | 0.50 | 1.00 | 1.00 | 1.70 | 0.531 |
| 62 | 0.000 | 0.000 | 0.000 | 0.000 | 1.111 | 6.80 | 0.50 | 1.00 | 1.00 | 1.70 | 0.518 |
| 63 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 6.80 | 0.50 | 1.00 | 1.00 | 1.30 | 0.295 |
| 64 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 6.80 | 0.50 | 1.00 | 1.00 | 1.30 | 0.398 |
| 65 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 6.80 | 0.50 | 1.00 | 1.00 | 1.30 | 0.320 |
| 66 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 6.80 | 0.50 | 1.00 | 1.00 | 1.30 | 0.351 |

*log(bacterial number of *L. delbrueckii* subsp. *lactis* ATCC 4797 which survived by the bacteriocin treatment, CFU $ml^{-1}$).

exp[log(some value)] is always positive. Thus, log(mean of Y) was modeled using generalized linear modeling with an assumption that Y follows a normal distribution.

As a polynomial for this normal-distribution log-link generalized linear model, an appropriate subset fourth-order polynomial was selected using the GENMOD (GENeralized linear MODels) procedure of SAS/STAT [16] in the following way: First, a generalized linear model with a normal distribution and the log link was fitted using a fourth-order polynomial that contains 5 linear terms, 10 two-way interactions, 10 three-way interactions, 5 four-way interactions, 5 quadratic terms, 5 cubic terms, and 5 quartic terms. Then, starting with higher-order terms, backward elimination of insignificant terms was repeatedly performed to reduce the model to its final form presented in Table 3.

This subset fourth-order polynomial-based generalized linear model was found to be statistically superior to the full second-order polynomial-based generalized linear model. As criteria for comparison between these two models, R-square and the Mean Error Deviance were used.

Here, R-square has been defined as the squared correlation between the actual value and the predicted value from the model. In this case, because log(mean of Y) was modeled as a polynomial, R-square was defined as the square of the correlation coefficient between the actual value of Y and exp(fitted value of a polynomial). For the subset fourth-order polynomial-based generalized linear model given in Table 3, R-square was 0.94, whereas R-square was 0.80 for the full second-order polynomial-based generalized linear model with the same distribution and the same link.

The Mean Error Deviance is the error deviance divided by its associated degrees of freedom, where the error deviance is the sum of the squared differences between actual values and predicted values from a generalized linear model. For a generalized linear model, if the distribution is normal and the link is the identity, the Mean Error Deviance in this generalized linear model becomes identical with the Error Mean Square, which is an estimate of error variance, in an ordinary regression model. For the subset fourth-order polynomial-based generalized linear model given in Table 3, the Mean Error Deviance was 0.0065, whereas the Mean Error Deviance was 0.0140 for the full second-order polynomial-based generalized linear model with the same distribution and the same link.

**Determining the Optimum Factor-Level Combination**

Nonlinear optimization was performed to determine the factor-level combination that minimizes log(predicted Y) under the restriction that $X_1^2+X_2^2+X_3^2+X_4^2+X_5^2 \leq 5$, using the NLP (NonLinear Programming) procedure of SAS/OR [5]. Table 4 shows the optimum factor-level combination in coded and actual levels.

At this optimum point, the value of log(predicted Y) was -5.72, and accordingly, the value of predicted Y was exp(-5.72)=0.003, which is very close to 0. Here, let ($x_1^*$, $x_2^*$, $x_3^*$, $x_4^*$, $x_5^*$)=(-1.237, 0.836, 0.969, 0.771, -1.113), the optimum point in coded units. Then, $x_1^{*2}+x_2^{*2}+x_3^{*2}+x_4^{*2}+x_5^{*2}=5$, which implies this point lies on the perimeter of the design region.

**Assessing Factor Effects Using the Partial Effects Plot**

Here, to assess the effect of each factor graphically, the partial effect functions and the partial effects plot [14] were used. 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 $f(X_1, X_2, X_3, X_4, X_5)$ denote log(predicted Y) given in Table 3 and ($x_1^*$, $x_2^*$, $x_3^*$, $x_4^*$, $x_5^*$) denote the optimum point of coded factor levels given in

**Table 3.** A generalized linear model based on a subset fourth-order polynomial fitted to the data.

| log(predicted Y[a])= | | | | |
|---|---|---|---|---|
| | | -0.9097 | (0.0378, 578.83, | <0.0001) |
| $+X_1$ | * | 0.5734 | (0.0606, 89.51, | <0.0001) |
| $+X_2$ | * | -0.2931 | (0.0312, 88.46, | <0.0001) |
| $+X_3$ | * | -0.3783 | (0.0404, 87.48, | <0.0001) |
| $+X_4$ | * | -0.1318 | (0.0342, 14.86, | 0.0001) |
| $+X_5$ | * | 0.1633 | (0.0383, 18.19, | <0.0001) |
| $+X_1X_3$ | * | 0.2854 | (0.0489, 34.02, | <0.0001) |
| $+X_1X_5$ | * | -0.3312 | (0.0481, 47.38, | <0.0001) |
| $+X_2X_3$ | * | -0.1825 | (0.0458, 15.90, | <0.0001) |
| $+X_2X_4$ | * | 0.1431 | (0.0414, 11.96, | 0.0005) |
| $+X_2X_5$ | * | 0.1544 | (0.0491, 9.89, | 0.0017) |
| $+X_3X_4$ | * | -0.0866 | (0.0390, 4.95, | 0.0261) |
| $+X_3X_5$ | * | 0.1287 | (0.0515, 6.24, | 0.0125) |
| $+X_4X_5$ | * | 0.1632 | (0.0391, 17.46, | <0.0001) |
| $+X_1^2$ | * | -0.3589 | (0.0819, 19.21, | <0.0001) |
| $+X_2^2$ | * | 0.1422 | (0.0645, 4.86, | 0.0276) |
| $+X_3^2$ | * | 0.1094 | (0.0625, 3.07, | 0.0799) |
| $+X_4^2$ | * | -0.4118 | (0.0874, 22.21, | <0.0001) |
| $+X_1X_2X_3$ | * | 0.1360 | (0.0432, 9.91, | 0.0016) |
| $+X_1X_2X_4$ | * | 0.1186 | (0.0415, 8.16, | 0.0043) |
| $+X_1X_2X_5$ | * | -0.3331 | (0.0526, 40.11, | <0.0001) |
| $+X_1X_3X_4$ | * | 0.1435 | (0.0371, 14.94, | 0.0001) |
| $+X_1X_3X_5$ | * | -0.1991 | (0.0524, 14.47, | 0.0001) |
| $+X_1X_4X_5$ | * | -0.0906 | (0.0408, 4.92, | 0.0265) |
| $+X_2X_3X_5$ | * | 0.0885 | (0.0521, 2.89, | 0.0892) |
| $+X_1^3$ | * | -0.0698 | (0.0174, 16.08, | <0.0001) |
| $+X_1X_2X_3X_4$ | * | 0.0735 | (0.0337, 4.75, | 0.0293) |
| $+X_1X_2X_3X_5$ | * | -0.1379 | (0.0523, 6.95, | 0.0084) |
| $+X_1X_2X_4X_5$ | * | -0.1305 | (0.0350, 13.91, | 0.0002) |
| $+X_1^4$ | * | 0.0777 | (0.0174, 19.96, | <0.0001) |
| $+X_2^4$ | * | -0.0330 | (0.0128, 6.71, | 0.0096) |
| $+X_3^4$ | * | -0.0307 | (0.0114, 7.19, | 0.0073) |
| $+X_4^4$ | * | 0.0482 | (0.0197, 5.97, | 0.0145) |
| $+X_5^4$ | * | -0.0331 | (0.0085, 15.34, | <0.0001) |

For each coefficient estimate, numbers in parentheses are: (Standard Error, Chi-Square from Maximum Likelihood Estimation, P-value).

[a]log(bacterial number of *L. delbrueckii* subsp. *lactis* ATCC 4797 which survived by the bacteriocin treatment, CFU $ml^{-1}$).

*: Multiplication.

Table 4, that is, (-1.237, 0.836, 0.969, 0.771, -1.113). Then, the partial effect function of $X_1$, $PEF(X_1)$, is defined as

$$PEF(X_1)=f(X_1, x_1^*, x_3^*, x_4^*, x_5^*),$$
where $X_1$ is such that $X_1^2 \le 5-(x_2^{*2}+x_3^{*2}+x_4^{*2}+x_5^{*2})$.

Similarly, the partial effect functions of $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are defined as

$$PEF(X_2)=f(x_1^*, X_2, x_3^*, x_4^*, x_5^*),$$
where $X_2$ is such that $X_2^2 \le 5-(x_1^{*2}+x_3^{*2}+x_4^{*2}+x_5^{*2})$,
$$PEF(X_3)=f(x_1^*, x_2^*, X_3, x_4^*, x_5^*),$$
where $X_3$ is such that $X_3^2 \le 5-(x_1^{*2}+x_2^{*2}+x_4^{*2}+x_5^{*2})$,

**Table 4.** Optimum factor-level combination in coded and actual levels.

| | pH | Yeast extract | Tryptone mixture | Mineral | Glucose |
|---|---|---|---|---|---|
| Coded level | -1.237 | 0.836 | 0.969 | 0.771 | -1.113 |
| Actual level | 6.35 | 0.65 | 1.21 | 1.17 | 0.90 |

$$PEF(X_4)=f(x_1^*, x_2^*, x_3^*, X_4, x_5^*),$$
where $X_4$ is such that $X_4^2 \le 5-(x_1^{*2}+x_2^{*2}+x_3^{*2}+x_5^{*2})$,

and

$$PEF(X_5)=(x_1^*, x_2^*, x_3^*, x_4^*, X_5),$$
where $X_5$ is such that $X_5^2 \le 5-(x_1^{*2}+x_2^{*2}+x_3^{*2}+x_4^{*2})$.

The partial effect curve for factor j is a curve drawn with the vertical axis representing $PEF(X_j)$ and the horizontal axis representing $X_j$. By overlaying all partial effect curves, the partial effects plot is obtained. In the partial effects plot, since all $X_j$ are the coded factor levels, the horizontal axis represents the coded factor level. Figure 1 is the partial effects plot of the factors in this study.

Figure 1 shows that for pH and glucose, as their value decreases within a certain range, log(predicted Y) decreases, and that for yeast extract, tryptone, and mineral mixture, as their value increases within a certain range, log(predicted Y) decreases. Also, if the magnitudes of the partial effects are compared apart from their directions for the minimum response, pH has the largest magnitude of the partial effect; yeast extract, tryptone, and glucose have similar second largest magnitudes of the partial effects; and mineral mixture has the smallest magnitude of the partial effect.

As seen in Fig. 1, the partial effects plot shows the strength and the feature of influence of each factor on the

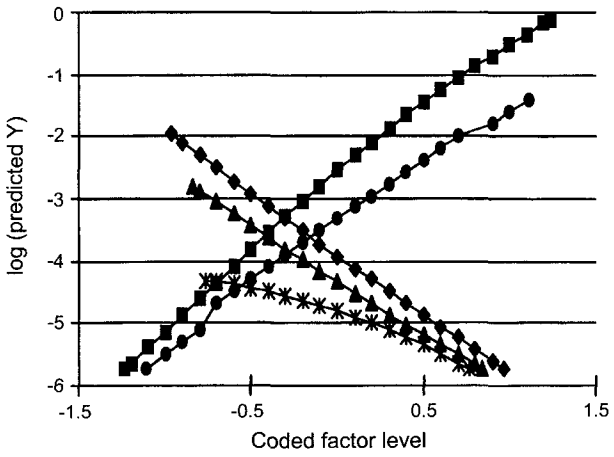


**Fig. 1.** The plot of partial effects of initial pH (-■-), yeast extract (-▲-), tryptone (-◆-), mineral mixture (-∗-), and glucose (-●-) on the number of viable cells of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 which survived the bacteriocin treatment.

predicted response when levels of the other factors in each case are optimal. Visualizing the shape characteristics of the response surface in one plot, this plot allows graphical comparative assessment of the relative importance of the factors in optimizing the response.

**Plotting Three-Dimensional Response Surface Plots**

For each two factors associated with each two-way interaction term in the model in Table 3, a three-dimensional response surface plot was drawn with the vertical axis representing log(predicted Y) and two horizontal axes representing the actual levels of the two factors. In each plot, the factors not represented by the two horizontal axes are fixed at their optimum actual levels.

Because the design region is spherical in each plot, the design region for the two factors is the boundary and interior of the circle in the square on the bottom. Each plot shows the location of the optimum point and the shape of the response surface. For example, in Fig. 2B (three-dimensional response surface plot of log(predicted Y) versus pH and Glucose), the optimum point, (pH, Glucose)= (6.35, 0.90), lies on the perimeter of the circle in the square on the bottom, at which log(predicted Y) has its minimum value -5.72, and the response surface looks like a tilted bridge in the shape of an arch. Plots in Fig. 2 and Fig. 3 were drawn using the 3D Plot procedure of the Graph menu in Minitab [15].

**Validating the Optimum Factor-Level Combination**

An experiment was conducted to validate the optimum factor-level combination found in this study. As seen in Fig. 4, the overall growth of *L. acidophilus* ATCC 4356

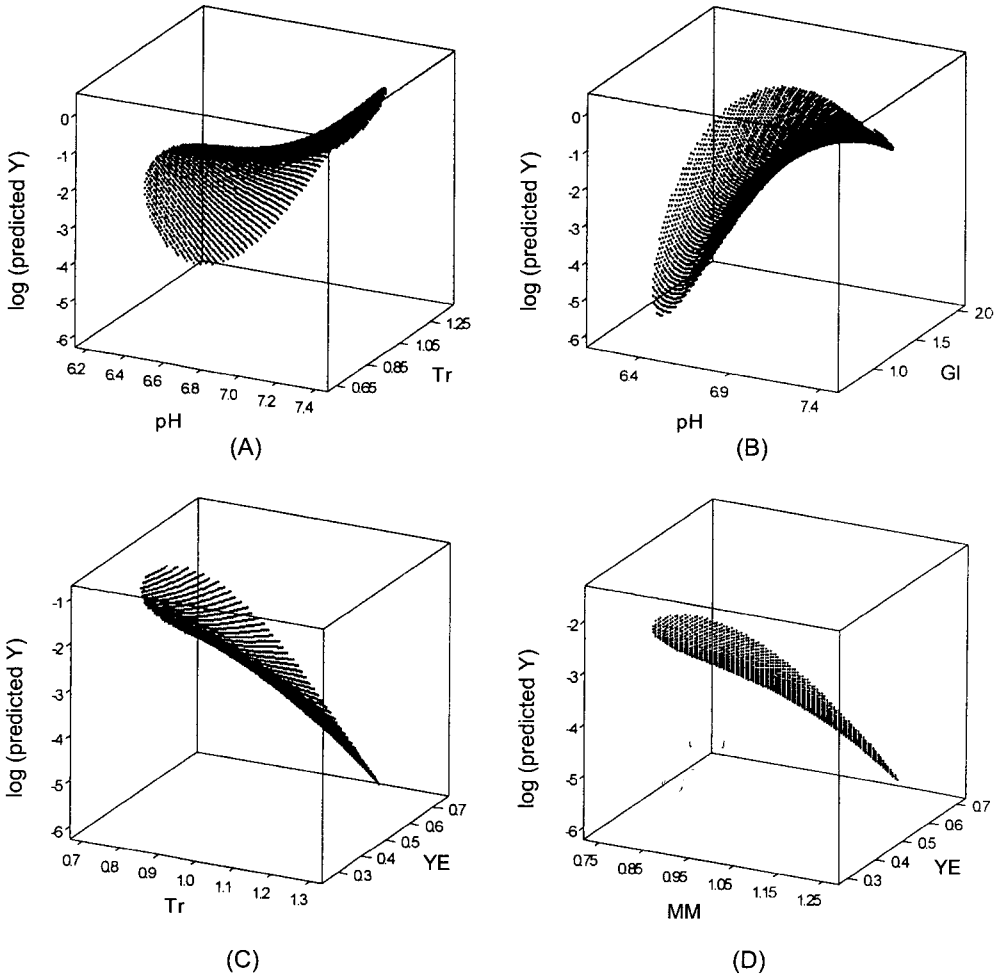


**Fig. 2.** Three-dimensional response surface plots for the effects of medium components on the number of viable cells of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 which survived the bacteriocin treatment.

(A) log (predicted Y) versus pH and tryptone with yeast extract=0.65%, mineral mixture=1.17%, and glucose=0.9%; (B) log (predicted Y) versus pH and glucose (Gl) with yeast extract (YE)=0.65%, tryptone (Tr)=1.21%, and mineral mixture (MM)=1.17%; (C) log (predicted Y) versus tryptone and yeast extract with pH=6.35, mineral mixture=1.17%, and glucose=0.9%; (D) log (predicted Y) versus mineral mixture and yeast extract with pH=6.35, tryptone=1.21%, and glucose=0.9%.

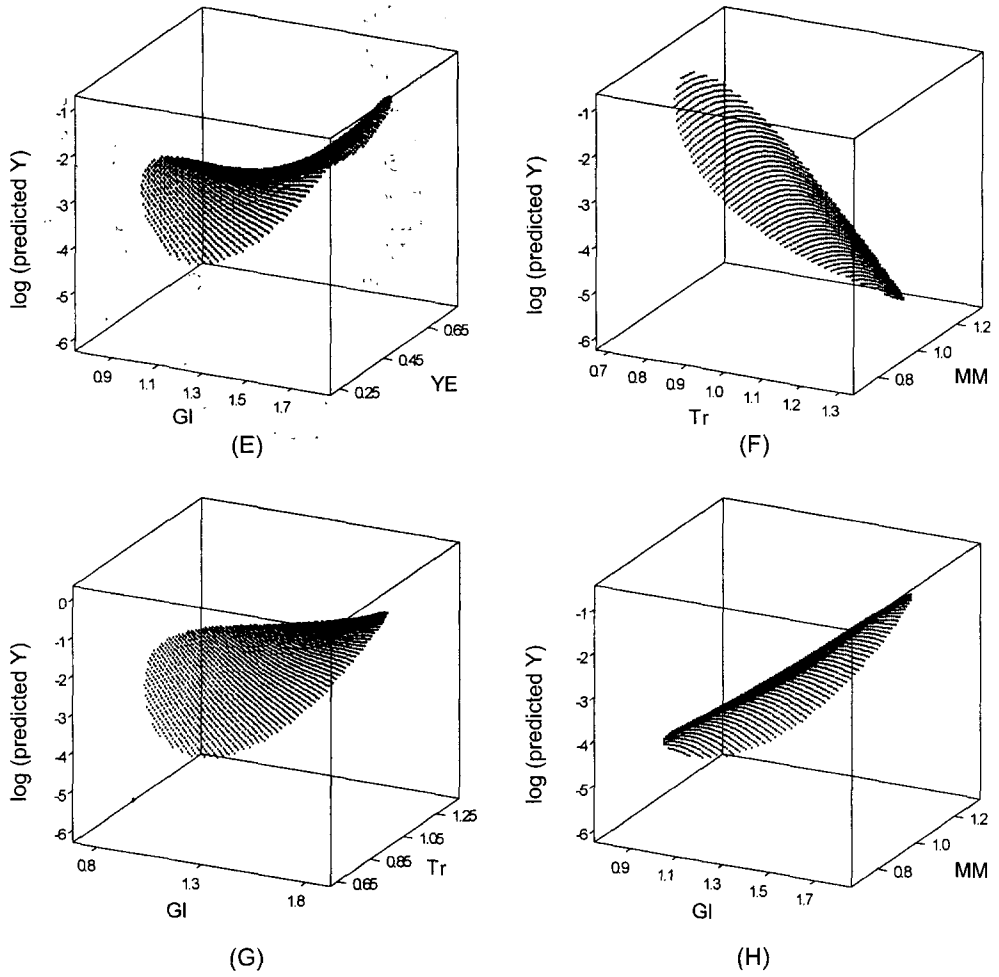


**Fig. 3.** Three-dimensional response surface plots for the effects of medium components on the number of viable cells of*Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 which survived the bacteriocin treatment.
(E) log (predicted Y) versus glucose (Gl) and yeast extract (YE) with pH=6.35, tryptone (Tr)=1.21%, and mineral mixture (MM)=1.17%; (F) log (predicted Y) versus tryptone and mineral mixture with pH=6.35, yeast extract=0.65%, and glucose=0.9%; (G) log (predicted Y) versus glucose and tryptone with pH=6.35, yeast extract=0.65%, mineral mixture=1.17%; (H) log (predicted Y) versus glucose and mineral mixture with pH=6.35, yeast extract=0.65%, and tryptone=1.21%.

and bacteriocin production were slightly better in the MRS medium than in the optimized medium during the incubation period. However, bacteriocin activity of the optimized medium was similar to that of the MRS medium after more than 20 h of incubation. In a preliminary study, the production of bacteriocin during the growth of *L. acidophilus* ATCC 4356 was tested in 3 commercial media such as MRS, M17-glucose, and 10% skim milk containing 1% yeast extract. *L. delbrueckii* subsp. *lactis* ATCC 4797 was used as the indicator strain. The MRS medium showed the highest bacteriocin production whereas M17-glucose medium showed the lowest production. In the 10% skim milk-1% yeast extract medium, the recovery of bacteriocin activity was very low and it was partly due to the difficulty in harvesting cells (data not shown). Therefore, we used the MRS medium as a reference medium and compared it with the optimized medium in bacteriocin activity.

The production of bacteriocin seems to be dependent on the growth and physiological activity of the producing species. Some of the LAB bacteriocins are produced at the late logarithmic phase or stationary phase, since the depletion of nutrients may induce the production of bacteriocin [1, 2]. The production of bacteriocin by *L. acidophilus* ATCC 4356 at an early stationary growth phase in the optimized medium was comparable to that in the MRS medium. Considering the MRS medium is expensive and consists of complicated components, it may not be suitable for industrial production of bacterial metabolites such as bacteriocin, even though it produced higher bacteriocin titers. The optimized medium may be more practical in the food systems, since it may produce less undesirable

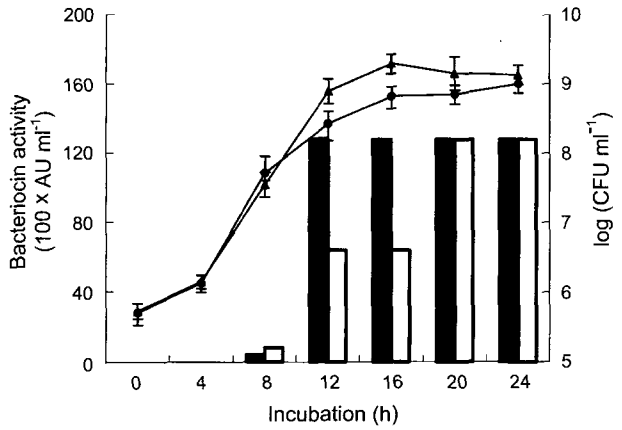


**Fig. 4.** Growth and bacteriocin production of *Lactobacillus acidophilus* ATCC 4356 in the MRS medium and the optimized medium.

Viable cells in the MRS medium (-▲-), viable cells in the optimized medium (-●-); bacteriocin activity in the MRS medium (■), bacteriocin activity in the optimized medium (□).

side effects, including off-flavor and discoloration, than the MRS medium.

## CONCLUSION

A new response surface approach utilizing an interiorly augmented central composite design, a subset fourth-order polynomial-based generalized linear model, nonlinear optimization, and the partial effects plot was effective in developing an analysis model, determining the optimum factor-level combination, and assessing the factor effects. The optimum conditions of the medium in this study were: initial pH 6.35, tryptone 1.21%, glucose 0.9%, yeast extract 0.65%, and mineral mixture 1.17%. The optimized medium was comparable in bacteriocin production to the MRS medium, having the advantage of economy and practicality.

## Acknowledgment

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