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Optimizing Medium Components for the Maximum Growth of *Lactobacillus plantarum* JNU 2116 Using Response Surface Methodology

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Abstract This study was undertaken to find the optimum soy-peptone, glucose, yeast extract, and magnesium sulfate amounts for the maximum growth of *Lactobacillus plantarum* JNU 2116 and to assess the effects of these medium factors through the use of response surface methodology. A central composite design was used as the experimental design for the allocation of treatment combinations. In the analysis of the experiment, due to a significant lack of fit of the second-order polynomial regression model that was used at first, cubic terms were added to the model, and then two-way interaction terms were deleted from the model since they were found to be all statistically insignificant. A relative comparison among the four factors showed that the growth of *L. plantarum* JNU 2116 was affected strongly by yeast extract, moderately by glucose and peptone, and slightly by magnesium sulfate. The estimated optimum amounts of the medium factors for the growth of *L. plantarum* JNU 2116 are as follows: soy-peptone 0.213%, glucose 1.232%, yeast extract 1.97%, and magnesium sulfate 0.08%. These results may contribute to the production of *L. plantarum* L67 as a starter culture that may have potential application in yogurt and fermented meat products.

Keywords response surface methodology, optimization, growth, *Lactobacillus plantarum*

Introduction

Lactobacillus species are the most frequently and safely used probiotics. It is even commonly consumed by children and immunocompromised individuals (Borriello et al., 2003). In particular, *Lactobacillus plantarum*, a member of the hetero-fermentative group of *Lactobacilli*, is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including silage, sourdough, fish, and many fermented vegetables (Seddik et al., 2017). Traditionally, *L. plantarum* was not used in yogurt fermentation, but it was used in fermented sausages and some types of cheese.

According to a recent report, *L. plantarum* L67, which has a high survival rate at low pH, was found to have been mixed with lactic acid bacteria as a starter culture and could be used for making yogurt (Song et al., 2016).

The species of *L. plantarum* exhibits various biological effects, including antitumor, anticoagulant, antiviral, immunomodulatory, anti-inflammatory, antidiabetic, antioxidative, and free radical-scavenging activities (Andersson et al., 2010; Giardina et al., 2014).

In a previous study (Jeong et al., 2015), we determined that *L. plantarum* JNU 2116, isolated from kimchi, has a high survival rate at low pH. Strain JNU 2116 produces an anticarcinogenic peptide (NH₂-XLEXKKAEXITXX) in the presence of mutagens (4-nitroquinoline 1-oxide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and 2-amino-3-methylimidazole quinoline).

The growth of lactic acid bacteria is greatly affected by the fermentation conditions, such as temperature, pH, medium components, and oxygen, among which the types of growth medium play an important role in bacterial viability. For growing lactic acid bacteria, MRS and M17 media, as well as skim milk, are used extensively. Tryptone, a medium component produced by the digestion of milk caseins by trypsin (Fraser and Powell, 1950), provides an excellent source of amino acids and small peptides for growing microorganisms and is therefore commonly incorporated into the growth media of lactic acid bacteria and other microorganisms.

In order to use probiotics as a health supplement, the fermentation process is a critical factor in large-scale production for enhancing the cell yield (Maýra -Mäkinen and Bigret, 1993). Thus, the evaluation of the medium ingredients should be carried out for fermentation optimization. Oh et al. (1995) suggested that a modified response surface (RS) model with cubic and quartic terms could find a more optimal point and thus could assess factor effects more appropriately.

In this study, using a new RS modeling approach, we evaluated the effects of the four medium components that are soy-peptone, glucose, yeast extract, and magnesium sulfate, and estimated the optimum growth conditions for enhancing viable cells of *L. plantarum* JNU 2116.

Materials and Methods

Experimental design

A central composite design (Myers et al., 2009) in 6 blocks was used to allocate treatment combinations in this experiment, which consisted of 36 runs and was conducted for 6 days (Table 1). Each of the first 4 blocks, which represented days 1, 2, 3, and 4 of the experiment, contained 4 factorial runs and 2 center runs. Each of the last 2 blocks, which represented days 5 and 6 of the experiment, contained 4 axial runs and 2 center runs.

In this experiment, the response, Log (CFU), was assumed to be under the influence of the 4 medium factors mentioned in the Introduction. To set up a statistical model, we denoted Log (CFU) by *Y* and determined the coded factor levels as follows:

$$X_1=(\text{peptone}-2)/1, X_2=(\text{glucose}-14)/7, X_3=(\text{yeast extract}-10)/5, \text{ and } X_4=(\text{magnesium sulfate}-1)/0.5.$$

Table 2 contains the actual factor levels corresponding to the coded factor levels. The observed responses of the treatment combinations are presented in Table 1. Using this design, we could fit a second- or higher-order polynomial regression model to the data.

Bacterial strain and the enumeration of viable cells

L. plantarum JNU 2116 was obtained from stock cultures maintained by the Dairy Microbiology Laboratory at Chonnam

Table 1. Treatment combinations and responses

No.	Block	Peptone	Glucose	Yeast extract	Magnesium sulfate	OD	Log (CFU)
		(g/L) [×1]	(g/L) [×2]	(g/L) [×3]	(g/L) [×4]		
1	0	1 [-1]	7 [-1]	5 [-1]	0.5 [-1]	1.532	8.72098574
2	0	3 [1]	7 [-1]	5 [-1]	0.5 [-1]	1.328	8.98944982
3	0	1 [-1]	21 [1]	5 [-1]	0.5 [-1]	1.171	8.6127839
4	0	3 [1]	21 [1]	5 [-1]	0.5 [-1]	1.33	8.61278386
5	0	2 [0]	14 [0]	10 [0]	1 [0]	1.577	8.979093
6	0	2 [0]	14 [0]	10 [0]	1 [0]	1.589	8.960471
7	1	1 [-1]	7 [-1]	15 [1]	0.5 [-1]	1.741	8.90471554
8	1	3 [1]	7 [-1]	15 [1]	0.5 [-1]	1.714	8.95760729
9	1	1 [-1]	21 [1]	15 [1]	0.5 [-1]	1.666	8.9444827
10	1	3 [1]	21 [1]	15 [1]	0.5 [-1]	1.695	8.89762709
11	1	2 [0]	14 [0]	10 [0]	1 [0]	1.524	8.829947
12	1	2 [0]	14 [0]	10 [0]	1 [0]	1.548	8.85309
13	2	1 [-1]	7 [-1]	5 [-1]	1.5 [1]	1.166	8.63346845
14	2	3 [1]	7 [-1]	5 [-1]	1.5 [1]	1.293	8.63346846
15	2	1 [-1]	21 [1]	5 [-1]	1.5 [1]	1.14	8.6263404
16	2	3 [1]	21 [1]	5 [-1]	1.5 [1]	1.322	8.69722934
17	2	2 [0]	14 [0]	10 [0]	1 [0]	1.554	8.878522
18	2	2 [0]	14 [0]	10 [0]	1 [0]	1.566	8.860937
19	3	1 [-1]	7 [-1]	15 [1]	1.5 [1]	1.723	8.85491302
20	3	3 [1]	7 [-1]	15 [1]	1.5 [1]	1.763	8.99694925
21	3	1 [-1]	21 [1]	15 [1]	1.5 [1]	1.678	8.8481891
22	3	3 [1]	21 [1]	15 [1]	1.5 [1]	1.684	8.88817949
23	3	2 [0]	14 [0]	10 [0]	1 [0]	1.457	8.90309
24	3	2 [0]	14 [0]	10 [0]	1 [0]	1.506	8.742725
25	4	0 [-2]	14 [0]	10 [0]	1 [0]	1.101	8.42488163
26	4	4 [2]	14 [0]	10 [0]	1 [0]	1.531	8.53529412
27	4	2 [0]	0 [-2]	10 [0]	1 [0]	0.701	8.3283796
28	4	2 [0]	28 [2]	10 [0]	1 [0]	1.433	8.64443859
29	4	2 [0]	14 [0]	10 [0]	1 [0]	1.423	8.740363
30	4	2 [0]	14 [0]	10 [0]	1 [0]	1.427	8.799341
31	5	2 [0]	14 [0]	0 [-2]	1 [0]	0.409	8.23299611
32	5	2 [0]	14 [0]	20 [2]	1 [0]	1.905	9.14921911
33	5	2 [0]	14 [0]	10 [0]	0 [-2]	1.409	8.8802418
34	5	2 [0]	14 [0]	10 [0]	2 [2]	1.722	9.071882
35	5	2 [0]	14 [0]	10 [0]	1 [0]	1.591	8.888179
36	5	2 [0]	14 [0]	10 [0]	1 [0]	1.596	8.857332

Table 2. Actual factor levels corresponding to the coded factor levels

Actual factor	Coded factor	Actual factor level at the coded factor level of				
		-2	-1	0	1	2
Peptone (g/L)	X ₁	0	1	2	3	4
Glucose (g/L)	X ₂	0	7	14	21	28
Yeast extract (g/L)	X ₃	0	5	10	15	20
Magnesium sulfate (g/L)	X ₄	0	0.5	1	1.5	2

National University (Gwangju, Korea). *L. plantarum* JNU 2116 was grown for overnight at 37°C in de Man Rogosa Sharpe broth (1960; MRS) and was subcultured three times prior to use. Stock culture of *L. plantarum* JNU 2116 was stored at -80°C in 10% (v/v) skim milk containing cryoprotectant. For the enumeration of *L. plantarum* JNU2116, the appropriate diluted-samples were plated by using MRS agar. The plates were incubated at 37°C for 48 h.

Statistical analysis

Data were analyzed using SAS software (SAS Korea, SAS® 9.4). SAS/STAT (2013) procedures were used for regression modeling. Optimum conditions were found through SAS data-step programming. The plots were generated by SAS/GRAPH (2013).

Results and Discussion

Developing a regression model

First, the second-order polynomial regression model containing 4 linear, 4 quadratic, and 6 interaction terms plus 5 dummy variables for 6 blocks was employed by using the RSREG procedure of SAS/STAT. The analysis of variance for evaluation of the second-order model is presented in Table 3.

Table 3 shows that the r^2 value of the second-order model was 0.8768 and the lack of fit was significant ($p=0.0197$). This suggested that this regression model did not accurately represent the data in the experimental region, indicating that cubic

Table 3. Analysis of variance for evaluation of the second-order model

Regression	Degrees of freedom	Type I sum of squares	r^2	F-value	p-value
Covariates	5	0.42846	0.3054	7.94	0.0006
Linear	4	0.56331	0.4016	13.04	<0.0001
Quadratic	4	0.20883	0.1489	4.84	0.0095
Interactions	6	0.02942	0.0210	0.45	0.8318
Total model	19	1.23002	0.8768	6.00	0.0004
Residual	Degrees of freedom	Sum of squares	Mean square	F-value	p-value
Lack of fit	10	0.15710	0.01571	6.02	0.0197
Pure error	6	0.01567	0.00261		
Total error	16	0.17276	0.01080		

terms might have to be included in the model. Thus, a third-order polynomial model containing 4 linear, 4 quadratic, 4 cubic, and 6 interaction terms plus 5 dummy variables for 6 blocks was fitted to the data. Table 4 displays the analysis of variance for this third-order model.

As shown in Table 4, the r^2 value of this third-order model was 0.9444, the lack of fit of the model was barely insignificant ($p=0.0587$) at the 5% level, and interactions were not significant with an F -value less than 1 ($p=0.6179$). Thus, in anticipation of an improvement of the model, we got rid of the interaction terms in Table 4. Table 5 shows the analysis of variance of this third-order model without interaction terms.

From Table 5, we see that the r^2 value of this reduced third-order model was 0.9235, the lack of fit of this model was more insignificant ($p=0.0985$), and each of the covariate, linear, quadratic, and cubic parts contained at least one significant term. This model had fewer terms than the full second-order model in Table 3, yet it produced a larger r^2 value and an insignificant lack of fit. Thus, we used this model as the final one for the medium optimization. The coefficients are given in Table 6, where D_1 , D_2 , D_3 , D_4 , and D_5 were defined as follows: if day=1, then $D_1=1$, $D_2=0$, $D_3=0$, $D_4=0$, $D_5=0$; if day=2, then $D_1=0$, $D_2=1$, $D_3=0$, $D_4=0$, $D_5=0$; if day=3, then $D_1=0$, $D_2=0$, $D_3=1$, $D_4=0$, $D_5=0$; if day=4, then $D_1=0$, $D_2=0$, $D_3=0$, $D_4=1$, $D_5=0$; if day=5, then $D_1=0$, $D_2=0$, $D_3=0$, $D_4=0$, $D_5=1$; and if day=6, then $D_1=0$, $D_2=0$, $D_3=0$, $D_4=0$, $D_5=1$.

Table 4. Analysis of variance for evaluation of the third-order model

Regression	Degrees of freedom	Type I sum of squares	r^2	F -value	p -value
Covariates	5	0.42846	0.3054	13.20	0.0002
Linear	4	0.56331	0.4016	21.69	<0.0001
Quadratic	4	0.20883	0.1489	8.04	0.0022
Cubic	4	0.09484	0.0676	3.65	0.0362
Interactions	6	0.02942	0.0210	0.75	0.6179
Total model	23	1.32486	0.9444	8.87	0.0002
Residual	Degrees of freedom	Sum of squares	Mean square	F -value	p -value
Lack of fit	6	0.06226	0.01038	3.97	0.0587
Pure error	6	0.01567	0.00261		
Total error	12	0.07793	0.00649		

Table 5. Analysis of variance for evaluation of the reduced third-order model

Regression	Degrees of freedom	Type I sum of squares	r^2	F -value	p -value
Covariates	5	0.42846	0.3054	14.37	<0.0001
Linear	4	0.56331	0.4016	23.61	<0.0001
Quadratic	4	0.20883	0.1489	8.75	0.0004
Cubic	4	0.09484	0.0676	3.98	0.0175
Total model	17	1.29544	0.9235	12.78	<0.0001
Residual	Degrees of freedom	Sum of squares	Mean square	F -value	p -value
Lack of fit	12	0.09168	0.00764	2.93	0.0985
Pure error	6	0.01567	0.00261		
Total error	18	0.10734	0.00596		

Table 6. Coefficient estimates from the third-order model without interactions

Variable	Parameter estimate	Standard error	<i>t</i> -value	<i>p</i> -value
Intercept	$b_0=8.83360$	0.04816	183.43	<0.0001
D_1	$a_1=0.15174$	0.06305	2.41	0.0271
D_2	$a_2=0.03152$	0.06305	0.5	0.6232
D_3	$a_3=0.05973$	0.06305	0.95	0.356
D_4	$a_4=0.00487$	0.06305	0.08	0.9393
D_5	$a_5=-0.10291$	0.07722	-1.33	0.1993
X_1	$b_1=0.03475$	0.02730	1.27	0.2193
X_1^2	$b_{11}=-0.05776$	0.01672	-3.45	0.0028
X_1^3	$b_{111}=-0.00179$	0.01115	-0.16	0.8744
X_2	$b_2=-0.07333$	0.02730	-2.69	0.0151
X_2^2	$b_{22}=-0.05618$	0.01672	-3.36	0.0035
X_2^3	$b_{222}=0.03809$	0.01115	3.42	0.0031
X_3	$b_3=0.12919$	0.04550	2.84	0.0109
X_3^2	$b_{33}=-0.03073$	0.01672	-1.84	0.0826
X_3^3	$b_{333}=0.02497$	0.01439	1.74	0.0998
X_4	$b_4=-0.01489$	0.04550	-0.33	0.7472
X_4^2	$b_{44}=0.04051$	0.01672	2.42	0.0262
X_4^3	$b_{444}=-0.01570$	0.01439	1.09	0.2896

Comparing the third-order polynomial effects of the factors

Our response surface model is written as

$$\hat{Y} = b_0 + a_1 D_1 + a_2 D_2 + a_3 D_3 + a_4 D_4 + a_5 D_5 + \hat{f}(X_1) + \hat{f}(X_2) + \hat{f}(X_3) + \hat{f}(X_4)$$

where

$$\hat{f}(X_j) = b_j X_j + b_{jj} X_j^2 + b_{jjj} X_j^3, j=1, 2, 3, 4$$

In the equation, \hat{Y} denotes the predicted value of Y , and for $j=1, 2, 3, 4$, each $\hat{f}(X_j)$ denotes an estimate of the original function $f(X_j) = \beta_j X_j + \beta_{jj} X_j^2 + \beta_{jjj} X_j^3$.

For $k \geq 2$, a k^{th} -order polynomial effect of the factor X_j is defined as the F -value in testing if all the coefficients of the linear term, the quadratic term, ..., and the k^{th} -power term of X_j are zero. To compare the third-order polynomial effects of the factors X_1, X_2, X_3 , and X_4 , we compared the F -values and p -values from testing.

$$H_0: \beta_j = \beta_{jj} = \beta_{jjj} = 0, j=1, 2, 3, 4.$$

Table 7 contains the results from these tests.

From Table 7, we can see that the F -value for X_3 (yeast extract) was the largest (31.67), those for X_2 (glucose) and X_1 (peptone) were similar in the middle group (7.67 and 5.29, respectively), and that for X_4 (magnesium sulfate) was the smallest (2.98). Thus, in relative comparison, the order of the factors according to the magnitude of the third-order polynomial effect is as follows:

Table 7. Test results from comparison of the third-order polynomial effects of the factors

Result from testing $H_0: \beta_1=\beta_{11}=\beta_{111}=0$ (Factor: Peptone)				
Source	Degrees of freedom	Mean square	F-value	p-value
Numerator	3	0.03155	5.29	0.0086
Denominator	18	0.00596		
Result from testing $H_0: \beta_2=\beta_{22}=\beta_{222}=0$ (Factor: Glucose)				
Source	Degrees of freedom	Mean square	F-value	p-value
Numerator	3	0.04572	7.67	0.0017
Denominator	18	0.00596		
Result from testing $H_0: \beta_3=\beta_{33}=\beta_{333}=0$ (Factor: Yeast extract)				
Source	Degrees of freedom	Mean square	F-value	p-value
Numerator	3	0.18887	31.67	<0.0001
Denominator	18	0.00596		
Result from testing $H_0: \beta_4=\beta_{44}=\beta_{444}=0$ (Factor: Magnesium sulfate)				
Source	Degrees of freedom	Mean square	F-value	p-value
Numerator	3	0.01779	2.98	0.0587
Denominator	18	0.00596		

yeast extract>glucose \cong peptone>magnesium sulfate.

Drawing the third-order polynomial effect plots of the factors

Since there were no interaction terms in the final model, we could draw the third-order polynomial effect plots of each factor separately (Fig. 1).

In Fig. 1, the 4 plots depict $\hat{f}(X_j)=b_j X_j+b_{jj} X_j^2+b_{jjj} X_j^3$, $j=1, 2, 3, 4$, respectively, from left to right. The values of b_j , b_{jj} , and b_{jjj} , $j=1, 2, 3, 4$, are given in Table 6. The shapes of these 4 curves were in accordance with the 4 test results in Table 7.

A relative comparison using Fig. 1 also says that the growth of *L. plantarum* JNU 2116 was affected strongly by yeast extract, moderately by glucose and peptone, and slightly by magnesium sulfate.

Finding the optimum point of the factors

Through a search on a grid (Oh et al., 1995), we maximized the predicted response from the model with the coefficients in Table 6, assuming that the values of the block variables were $D_1=1$, $D_2=0$, $D_3=0$, $D_4=0$, and $D_5=0$. The bounds for the factor levels were $-2 \leq X_j \leq 2$, $j=1, 2, 3, 4$, and the radius of the spherical region of the experimental design displayed in Table 1 was 2. Thus, with the bounds of $-2 \leq X_j \leq 2$, $j=1, 2, 3, 4$, we made a search within the spherical region with the radius of 2, for which the constraint was $X_1^2+X_2^2+X_3^2+X_4^2 \leq 2^2=4$. This search, which was conducted using SAS data-step programming, determined the optimum point, described in Table 8.

Experimenting for validation

A validation experiment was performed to verify the optimization results in Table 8. Table 9 shows the medium composition from the optimization results, which was used for the validation experiment.

To verify the validity of the optimum medium with the composition in Table 9, our validation experiment compared it with MRS broth, a frequently used commercial medium.

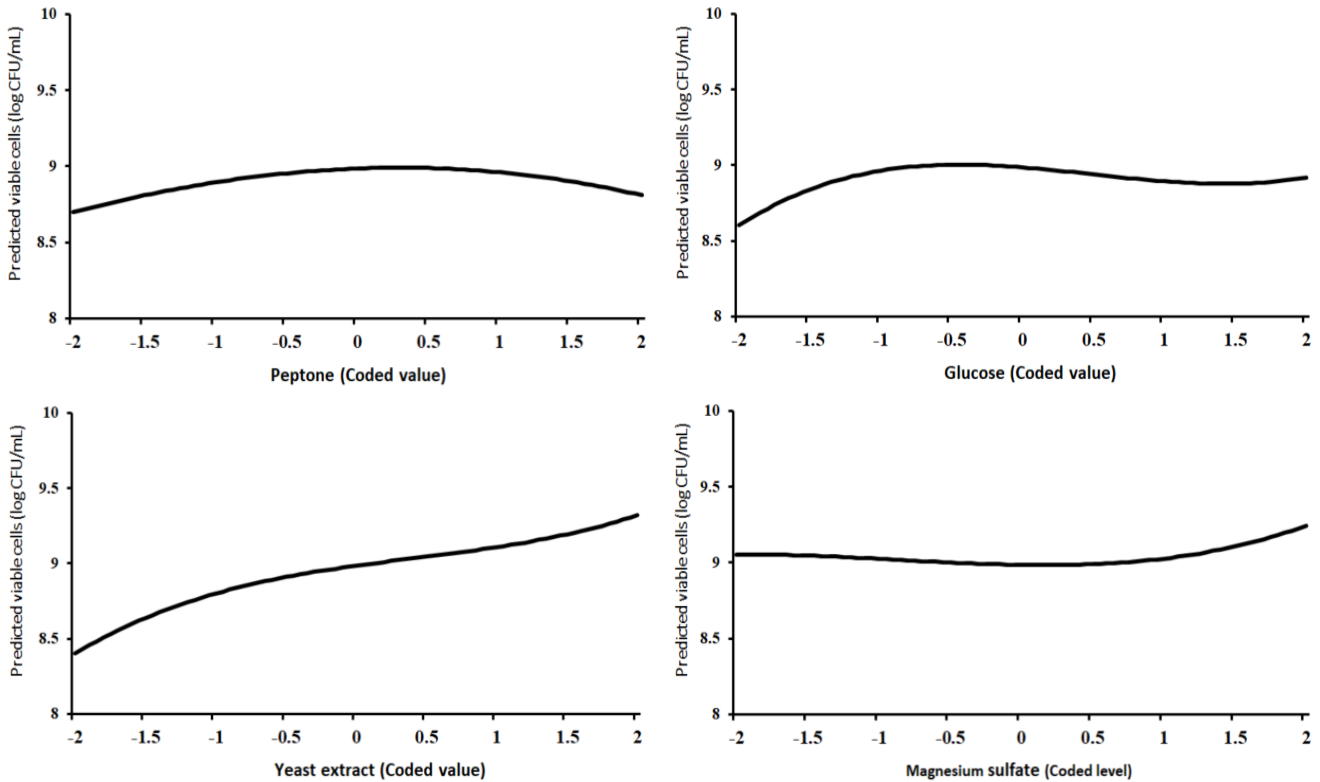


Fig. 1. Third-order polynomial effect plots of peptone, glucose, yeast extract, and magnesium sulfate in the range of -2 to 2 (in coded level), from left to right.

Table 8. Optimization results from an exploration within the hypersphere with a radius of 2

X_1	X_2	X_3	X_4	Distance from the origin	Predicted maximum Log(CFU)	Peptone (g/L)	Glucose (g/L)	Yeast extract (g/L)	Magnesium sulfate (g/L)
0.13	-0.24	1.94	-0.4	1.9995	9.3314	2.31	12.32	19.7	0.8

The data in Table 9 show that for both media, the number of viable cells was more than 10^9 (CFU/mL), which is a good result. Our optimum medium turned out to be economically viable in that the price for producing as much as 300 liters of it is as cheap as a little over 100 US dollars, and its cultivation amount is as much as 96.2% that of MRS broth.

Drawing 3D response surface plots

As done in the paper by Oh et al. (1995), for any 2 of the 4 factors, a three-dimensional response surface plot was drawn, with the vertical axis representing the predicted viable cells (Log CFU) and the 2 horizontal axes representing the actual levels of 2 explanatory factors. In these plots, the values of the block variables were set to be $D_1=1$, $D_2=0$, $D_3=0$, $D_4=0$, and $D_5=0$.

In each plot, the factors not represented by the 2 horizontal axes are fixed at their optimum actual levels. From all 6 plots produced (Figs 2 through 7), we can see that the effects of the pairs of the factors were additive since there were no interactions. By the additivity of the 2-factor effects, we mean that the effect of one factor on the response does not depend on the level of the other factors (Oh et al., 1995).

Table 9. Optimum medium composition and the results of the validation experiment

Component (%)	MRS medium	Optimum medium
Soy peptone	- ^a	0.231
Proteose peptone	1.0	-
Glucose	2.0	1.232
Yeast extract	0.5	1.97
Magnesium sulfate	0.01	0.08
Ammonium citrate	0.2	-
Beef extract	1.0	-
Manganese sulfate	0.005	-
Potassium phosphate dibasic	0.2	-
Sodium acetate	0.5	-
Tween 80	0.1	-
Viable cells (Log CFU/mL)	9.51	9.15

^a absence of constituent.

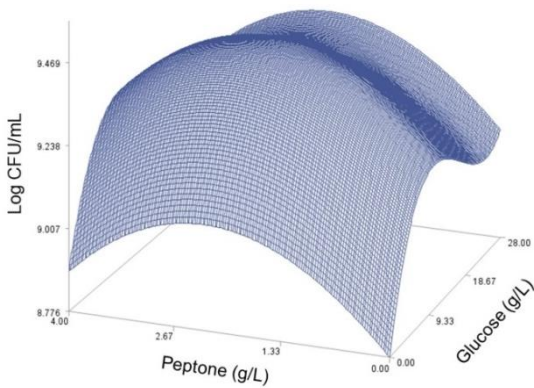


Fig. 2. Response surface plot for the effects of peptone and glucose on the predicted viable cells at yeast extract=17.5 (g/L) and magnesium sulfate=1.99 (g/L).

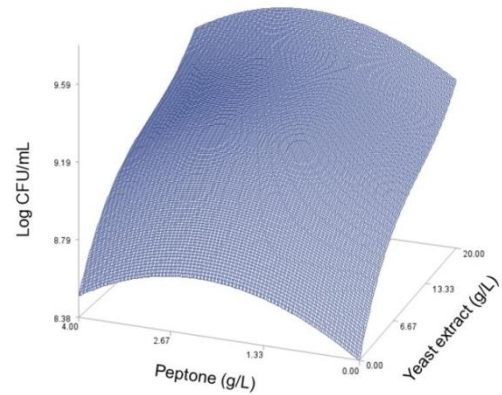


Fig. 3. Response surface plot for the effects of peptone and yeast extract on the predicted viable cells at glucose=12.25 (g/L) and magnesium sulfate=1.99 (g/L).

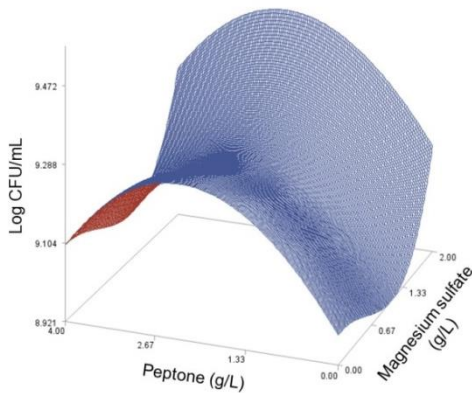


Fig. 4. Response surface plot for the effects of peptone and magnesium sulfate on the predicted viable cells at glucose=12.25 (g/L) and yeast extract=17.5 (g/L).

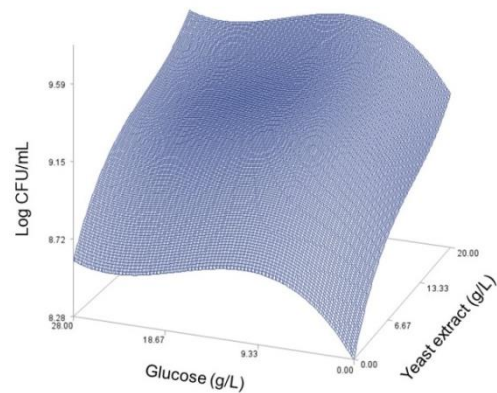


Fig. 5. Response surface plot for the effects of glucose and yeast extract on the predicted viable cells at peptone=2.13 (g/L) and magnesium sulfate=1.99 (g/L).

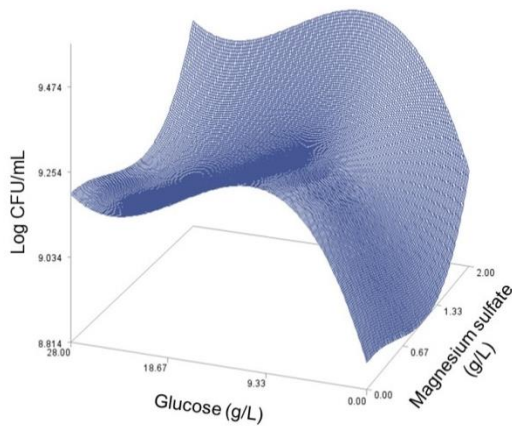


Fig. 6. Response surface plot for the effects of glucose and magnesium sulfate on the predicted viable cells at peptone=2.13 (g/L) and yeast extract=17.5 (g/L).

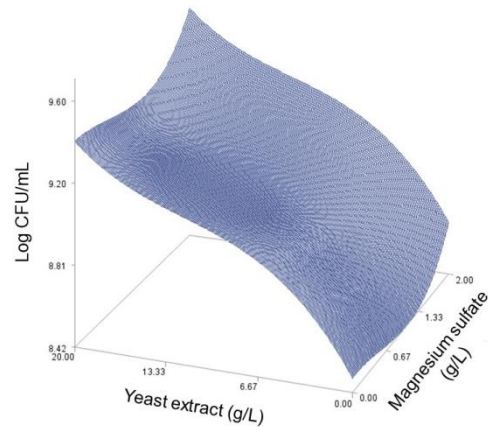


Fig. 7. Response surface plot for the effects of yeast extract and magnesium sulfate on the predicted viable cells at peptone=2.13 (g/L) and glucose=12.25 (g/L).

Conclusion

Using a new response surface approach, we developed a statistical model to assess the third-order polynomial effects of the four medium components on the growth of *L. plantarum* JNU 2116, and established their estimated optimum levels to maximize its growth, which are soy-peptone 0.213%, glucose 1.232%, yeast extract 1.97%, and magnesium sulfate 0.08%, through a validation experiment. Our optimum medium turned out to be economically viable in that its production price is cheaper than that of MRS broth and its cultivation amount is close to that of MRS broth.

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