Korean J. Chem. Eng., **26**(3), 754-759 (2009)

Optimization of culture media for *Bacillus* species by statistical experimental design methods

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(*Received 11 November 2008 • accepted 21 December 2008*)

Abstract−The medium compositions of *Bacillus* species (three strains) were optimized sequentially through two statistical experimental designs, Plackett-Burman and the Box-Behnken, which were determined by representative three-factor response-surface methodology. pH and DO control was found to enhance the viable cell number of *Bacilli*. Most strains grew well in the optimum condition and reached nearly up to of 5×10^9 CFU/mL. Additionally, when three of the *Bacilli* were cultivated in the optimized medium with pH and DO control, the viable cell number increased 3.6 times in *B. subtilis*, 64.1 times in *B. licheniformis* and 8.3 times in *B. coagulans*, respectively, in comparison with the original industrial medium.

Key words: Optimization of Media, *Bacillus* Species, Plackett-Burman Design, Box-Behnken Design, Response Surface Method

INTRODUCTION

Feed additive antibiotics have been extensively used as growing accelerators since the 1950s. However, because of residual antibiotics and resistance the use of antibiotics has been regulated throughout the world [1,2]. Therefore, antibiotics used only for disease control of livestock and probiotics were developed as growing accelerators. Probiotics, live microbial food supplements that beneficially affect the host by improving its intestinal microbial balance, are quickly gaining interest as functional foods in the current era of self-care and complementary medicine. Lactic acid bacteria, *Bacilli*, and yeast are representatively used as probiotics [1,3,4].

Bacilli, which are used as probiotics and biological control agents, should be introduced by viable cells. Sporulation of *Bacillus* species is essential for long-term storage and decrease of the death ratio during commercial preparation. Furthermore, conversion of a vegetative cell to a spore is difficult, so high cell density cultivation is required [5,6].

Medium optimization and convenient cultivation are essential for high cell density cultivation of *Bacillus* species. High cell density cultivation through medium optimization was studied by various statistical experimental designs [7,10,13]. One-factor-at-a-time, a typical method used for optimizing a multivariable system not only is time consuming, but also is difficult to determine the interaction of factors [7-10]. However, a statistical experimental design, named the Plackett-Burman experimental design (PBD), is very useful in identifying the significant nutrients for cell growth or enzyme production. Furthermore, optimum concentrations of medium components, which are selected by PBD, are determined by the Box-Behnken experimental design (BBD) and its representative three-factor response-surface method [11,13,14,16].

In this study, we optimized media for high cell density cultivation of *Bacillus* species using a series of statistical experimental designs, PBD and BBD. In addition, optimum media were compared to initial industrial media and were verified by comparison of computing values.

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MATERIAL AND METHODS

1. Strains and Culture Conditions

Three strains, *B. coagulans*, *B. subtilis* and *B. licheniformis,* were obtained from the Korean Culture Collection. Inoculum cultures of *Bacillus* species were incubated in Luria-Bertani (LB) broth. After the culture broth was transferred to production medium, the cells were incubated in a shaking incubator (30 °C, 250 rpm, 12-14 hr, *B. coagulans*; 37 °C).

2. Assay for Viable Cell Number Counting

Cell growth was measured by counting the viable number of cells because the medium contained insoluble components. Samples were withdrawn aseptically for analysis of viable cell numbers and spore numbers. Colony-forming-units (CFU) were measured by serial plating technique on LB agar. Appropriately diluted samples were plated and incubated for 12 hr at 30 °C.

3. Effect of Initial pH and Working Volume on Cell Growth

Bacillus species were cultivated on LB media with different initial pH of 3, 5, 7, 9 and 11. 6 N NaOH and 3 N HCl were used for pH adjustment. Samples were aseptically withdrawn and analyzed every 2 hrs over a 16hrs time period. Optical densities at 600 nm, were measured by spectrophotometer (UV-160A, Shimadzu Co., Japan) to study the effect of initial pH on cell growth [15].

4. Statistical Media Optimization

Three components of the original production medium, which affected cell growth significantly, were identified by the Plackett-Burman design [11,14]. In addition, the optimum concentration of these media components was determined by using the Box-Behnken design and its representative three-factor response-surface method. Statistical analyses were carried out with Minitab software (Version 14, Minitab Co., PA, USA).

5. Cultivation

Bioreactor experiments were performed in a 2.5 L jar fermenter

with a working volume of 1.0 L. Each medium was steam sterilized. Inoculation was performed with 1% (v/v) inoculum. The fermentation was performed at 30 ℃ with an aeration rate of 1.0 vvm and an agitation rate of 350 rpm for 18 h. The oxygen saturation was controlled 20% by changing the agitation speed. When necessary, cultivation pH was adjusted by automatic titration of 6 N NaOH or/and 3 N HCl. Samples were withdrawn from the fermentation broth in 2-h intervals and analyzed to determine the viable cell number.

RESULTS AND DISCUSSION

1. Effect of Initial pH

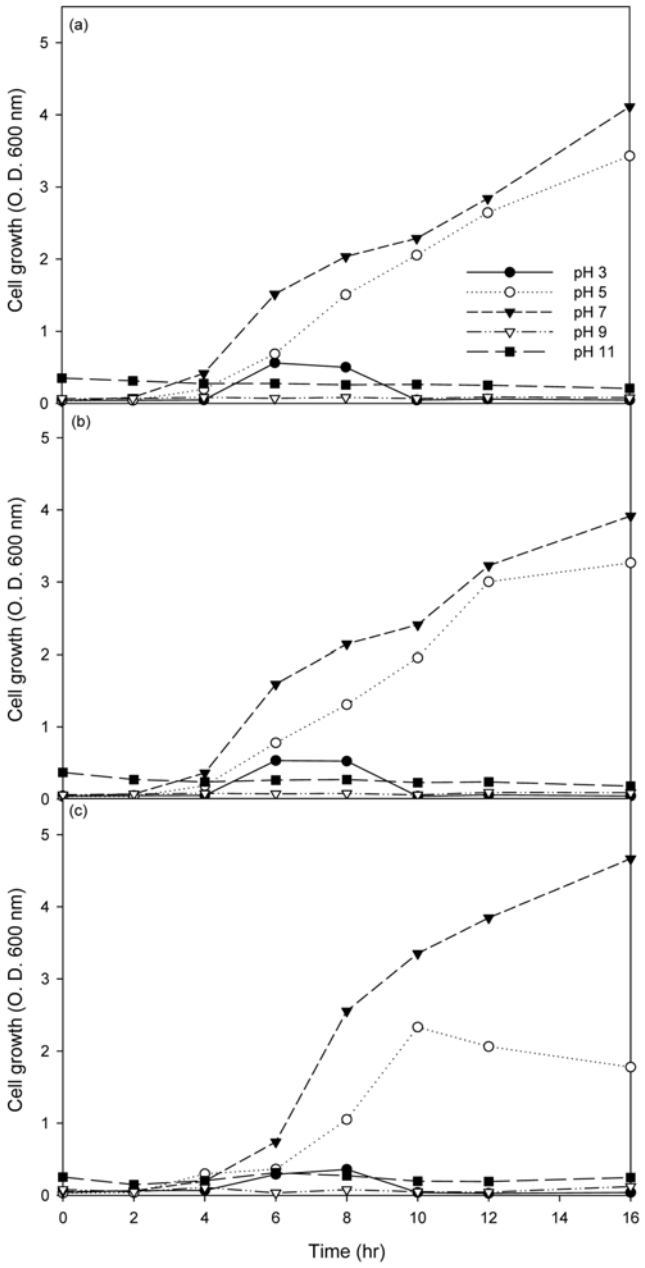

Fig. 1. Effects of initial pH on growth of *Bacillus* species: (a) *B. subtilis*, (b) *B. licheniformis* and (c) *B. coagulans*. *pH control: 6 N NaOH and 3 N HCl were used for pH adjustment.

The highest growth rate was observed at an initial pH of 7. Low and high pH had a negative effect on cell growth (Fig. 1). All strains showed similar growth patterns. A pH of 7 was the most effective, and a pH of 5 also showed potentiality for cell growth. When media for three species of *Bacilli* were optimized, optimum pH could be adopted for increasing the cell number in all of the experiments.

2. Statistical Medium Optimization

Most industrial media are composed by complex components such as yeast extract, peptone, soy bean meal, casein, etc. Therefore, it is very difficult to determine the C/N ratio of these components. One-factor-at-a-time for determination of C/N ratio and media composition, a typical method used for optimizing a multivariable system, not only is time consuming, but is also difficult to find the interaction of factors [7-10]. However, the Plackett-Burman experimental design (PBD) is very useful in identifying the important nutrients for cell growth. Main growth factors were selected by PBD for optimization of *Bacillus* species cell growth. The PBD method was composed of 15 culture flasks: twelve flasks which contained a combination of high and low concentrations of each component and three control flasks which contained a mean concentration of each component. The viable cell number was used as a response calculation of the main effect (Table 1). Results were obtained by calculation by differences of (+) and (−) of individual runs.

Soybean meal, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4$ at a high concentration increased the viable cell number. However, a high concentration of soluble starch and $CaCl_2$ decreased the viable cell number in the case of *B. coagulans*. Table 2 describes the screened main growth factors (X, Y and Z). As a result, soluble starch, yeast extract and ammonium sulfate were screened as the main growth factors for *B. licheniformis*, and soybean meal, yeast extract and magnesium sulfate were the main growth factors for *B. subtilis*, respectively (Fig. 2).

Table 1. Plackett-Burman experimental design for screening of effective components for growth of *Bacillus* species

Factor*	Combinations**														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	−	+	−	+	+	+	−	−	−	+	−	−	0	0	0
B	−	+	+	−	+	+	+	−	−	−	+	−	0	0	0
C	+	−	+	−	−	+	+	+	−	−	−	−	0	0	0
D	−	+	−	+	+	−	+	+	+	−	−	−	0	0	0
E	−	−	+	−	+	+	−	+	+	+	−	−	0	0	0
F	−	−	−	+	−	+	+	−	+	+	+	−	0	0	0
G	+	−	−	−	+	−	+	+	−	+	+	−	0	0	0

'+', '−' and '0' levels indicate the higher, lower and medium levels, respectively, of a factor in that combination and all experimental data were expressed by using the average of triplicate results

*Basic production medium components: A: 8.00 g/L of Soybean meal, B: 10.00 g/L of Soluble starch, C: 5.00 g/L of Yeast extract, D: 2.50 g/L $(NH_4)_2SO_4$, E: 3.00 g/L of KH_2PO_4, F: 1.55 g/L of $CaCl_2 \cdot 2H_2O$, G: 1.50 g/L of $MgSO_4 \cdot 7H_2O$

**Code for component concentrations: +; 200% of each concentration of basic medium, −; 20% of each concentration of basic medium, 0: served as the control, containing the averages of the high (+) and low (−) concentrations of each components

Table 2. Three main factors screened (X, Y, Z) of *Bacillus* species by Plackett-Burman design

Bacilli	Selected three main factors*		
	X	Y	Z
B. subtilis	Soybean meal	Yeast extract	$MgSO_4 \cdot 7H_2O$
B. licheniformis	Soluble starch	Yeast extract	$(NH_4)_2SO_4$
B. coagulans	Soybean meal	$(NH_4)_2SO_4$	$MgSO_4 \cdot 7H_2O$

*The efficiency of cell growth was determined by colony forming units using a 12 hr cultivation broth in each of the strains, and high concentrations of X, Y and Z components were shown to increase the cell growth

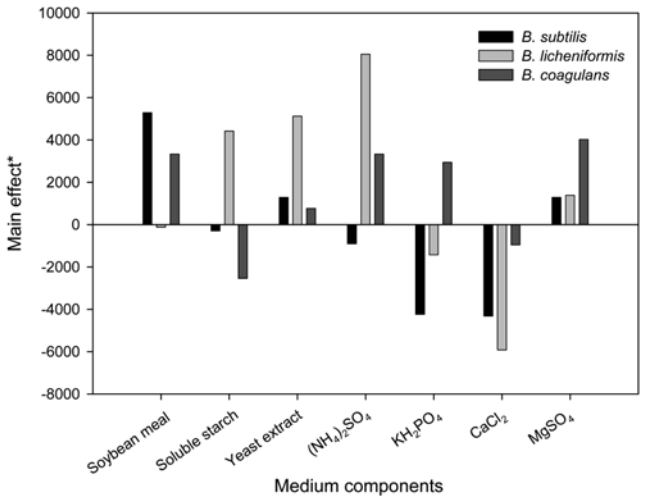

Fig. 2. Results of Plackett-Burman design for screening the main growth factors of *Bacilli*. *Main effect: obtained by detecting the level (×10^5) of colony forming unit with differing combinations and concentrations of the seven media components. **Culture conditions: 50 mL/250 mL flask, 250 rpm (shaking incubator), 30 ºC (*B. coagulans*; 37 ºC) and initial pH 7.0; sampling point: 12 hr; Medium composition: description shown in Table 1.

To find the entire optimum point, concentrations of three selected components were distributed to determine the adequate range for optimization. The highest viable cell number was observed at the combination of 30 g/*l* soybean meal, 6 g/L $(NH_4)_2SO_4$, and 6 g/L $MgSO_4$, respectively, in the case of *B. coagulans*. The pseudo-optimum point was obtained at a designated range in the case of $(NH_4)_2SO_4$, $MgSO_4$. However, the viable cell number increased according to an increase of soybean meal concentration (Fig. 3). These results applied to BBD. The other two *Bacillus* species were also tested by using a similar pattern. Concentrations of '+', '−' and '0' levels for the Box-Behnken design are described in Table 3. The effects of X, Y and Z on the growth of *Bacilli* were evaluated. Levels of these factors were optimized for maximum viable cell production (the response) by using one of the response surface methodologies, the Box-Behnken statistical design. Table 3 represents a 15-trial experimental design, where each variable was tested in three different coded levels: low (−), middle (0) and high (+). The coded values of *B. coagulans* correspond for soybean meal: − (20.0 g/L), 0 (30.0 g/L), + (40.0 g/L). For ammonium sulfate: − (2.0 g/L), 0 (5.0 g/L), + (8.0 g/L). For magnesium sulfate: − (4.0 g/L), 0 (6.0 g/L), + (8.0 g/L). Twelve flasks containing a combination of high and

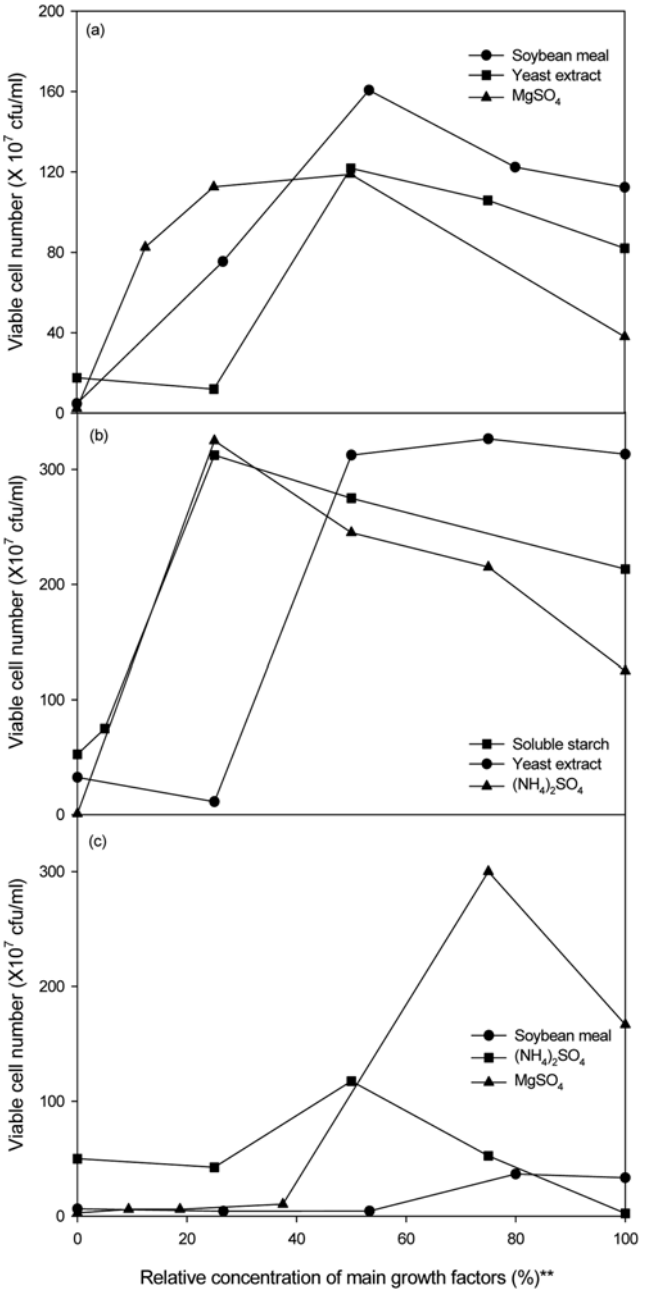

Fig. 3. Determining the adequate ranges of the main growth factors for finding the pseudo-optimum points of *Bacilli*: (a) *B. subtilis*, (b) *B. licheniformis* and (c) *B. coagulans*, as input values of Box-Behnken design. *Culture conditions: 50 mL/250 mL flask, 250 rpm (shaking incubator), 30 ºC (*B. coagulans*; 37 ºC) and initial pH 7.0; sampling point: 12 hr; Main growth factors: description shown in Table 2. **Relative concentration of the main growth factors (%): 100% means 30 g/L of soybean meal, 20 g/L of yeast extract and 6 g/L of $MgSO_4 \cdot 7H_2O$ on (a) *B. subtilis*; 40 g/L of soluble starch, 20 g/L of yeast extract and 10 g/L of $(NH_4)_2SO_4$ on (b) *B. licheniformis*; 30 g/L of soybean meal, 10 g/L of $(NH_4)_2SO_4$ and 8 g/L of of $MgSO_4 \cdot 7H_2O$ on (c) *B. coagulans*.

Table 3. Three factors Box-Behnken design for the medium optimization (code value) through response surface methodology and results by counting of viable cell numbers (cfu/mL) according to strains and running conditions (combinations)

Run	Factors*			*Bacillus subtilis* ($\times 10^9$ cfu/mL)	*B. licheniformis* ($\times 10^9$ cfu/mL)	*B. coagulans* ($\times 10^9$ cfu/mL)
	X	Y	Z			
1	+	0	+	0.72±0.15	3.00±0.50	1.38±0.15
2	–	0	+	0.50±0.05	1.33±0.76	1.18±0.20
3	+	0	–	1.53±0.19	4.33±1.04	0.67±0.08
4	–	0	–	0.95±0.05	3.67±0.29	0.55±0.13
5	0	+	+	1.12±0.15	3.50±0.87	1.12±0.18
6	0	–	+	3.47±0.28	2.33±0.29	0.65±0.10
7	0	+	–	1.23±0.15	5.17±0.76	0.67±0.20
8	0	–	–	4.50±0.10	3.17±1.26	0.92±0.10
9	+	+	0	0.68±0.12	5.67±0.76	0.80±0.28
10	+	–	0	1.42±0.30	3.00±1.32	0.92±0.10
11	–	+	0	0.52±0.10	3.50±1.00	0.68±0.08
12	–	–	0	2.02±0.16	2.33±0.76	0.60±0.13
13	0	0	0	2.60±0.15	3.17±0.29	1.67±0.08
14	0	0	0	2.42±0.29	3.17±0.29	1.73±0.29
15	0	0	0	2.77±0.43	3.50±0.50	1.72±0.06

*Factors were described on Table 2 and screened by P-B Design

'+', '–' and '0' levels indicate the higher, lower and medium levels, respectively, and all experimental data were obtained in triplicate, and code values (g/L) of each component for three factors in the Box-Behnken design are as follows:

Strains	X			Y			Z		
	+	0	–	+	0	–	+	0	–
B. subtilis	30.0	20.0	10.0	22.5	17.5	12.5	4.0	2.5	1.0
B. licheniformis	15.0	12.5	10.0	20.0	15.0	10.0	4	2.5	1.0
B. coagulans	40.0	30.0	20.0	8.0	5.0	2.0	8.0	6.0	4.0

low concentrations of each component and three control flasks containing a mean concentration of each component were used. The interaction and optimum concentration of selected components were obtained through BBD [13]. The highest viable cell number was measured at the combination of 30 g/L soybean meal, 5 g/L $(NH_4)_2SO_4$, 6 g/L $MgSO_4$, respectively, in the case *of B. coagulans*. Also, results of other stains are shown in Table 3 as well.

After BBD application, the optimum concentration of three components, was calculated with Minitab software ver. 14. Coefficients of second-order polynomial functions were obtained by response surface method under 95% of confidence value, and the optimum concentration of major factors (X, Y and Z) was calculated by partial differentiation of second-order polynomial functions and Gaussian elimination. Once the viable cell number ($\times 10^9$ cfu/mL) was measured, a second-order polynomial model was fitted to the response data obtained from the design (Fig. 4). The polynomial equation is in the following form: where K is the predicted response, C is the model constant; X, Y and Z are independent variables; a, b, and c are linear coefficients; g, h and i are cross product coefficients and d, e and f are the quadratic coefficients. The optimum concentrations of the three components, where the maximized viable cell number could be calculated by $\partial K/\partial X=\partial K/\partial Y=\partial K/\partial XZ=0$, were 31.7 g/L soybean meal, 5.2 g/L $(NH_4)_2SO_4$, 6.5 g/L $MgSO_4$. Regression analysis was performed to fit the response function (viable cell number) with the experimental data. The analysis of variance for the four variables (X, Y and Z) indicated that viable cell number can be well described by a polynomial model with a relatively high coefficient of determination. In addition, the interaction coefficients of this variable with other variables were high, which indicates the insignificance of these coefficients. The optimum concentrations of three components were obtained by same method for two other *Bacillus* species. In addition, statistical maximized cell numbers of each strain were calculated through the substitution of X, Y and Z values on second-order polynomial functions, respectively (Table 4).

3. Comparison of Basic Production Media and Optimized Media

After the optimum point of the variables is found, the effect of each factor can be predicted separately. Predicted statistical values of viable cell numbers were not fitted onto the experimental data. On the other hand, when three of *Bacilli* were cultivated in the optimized medium, the viable cell number increased 64.22 times in *B. licheniformis* and 4.14 times in *B. coagulans*, respectively, in comparison with the original industrial medium (Table 5). As this result, improvement of cell growth did not significantly occur in the case of *B. subtilis* because the basic production medium was for this strain.

4. Improvement of Viable Cell Number by pH & D.O. Control

As Fig. 1 illustrates, pH was a significant factor for cell growth and dissolved oxygen was reported as a growth factor [7]. *Bacilli* were cultivated in a 2.5 L jar using the optimized medium for de-

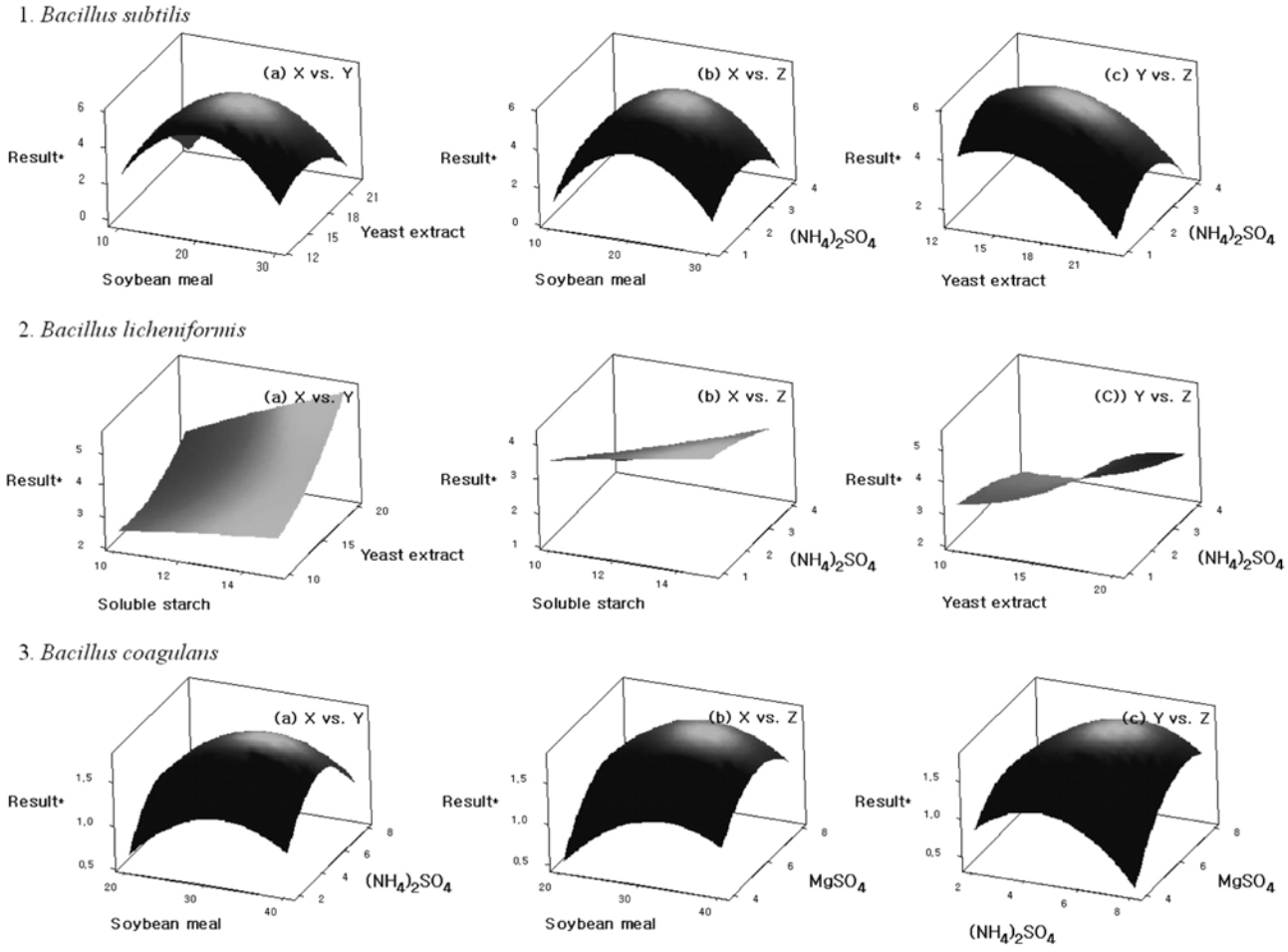

Fig. 4. Response-surface plot of the main growth factors on cell growth. *Result: viable cell number (×10⁹ cfu/mL) and description of the results of each run shown in Table 3. **Surface plots were expressed at the middle setting (0) of hold extra factors.

Table 4. Coefficients of second-order polynomial functions through response surface method under a 95% confidence value and calculation of optimum concentration of major factors (X, Y and Z) by partial differentiation of second-order polynomial functions and Gaussian elimination

Strain**	Constant	a	b	c	d	e	f	g	h	i	X*	Y*	Z*	K*
B. subtilis	–21.9066	1.1706	1.5971	2.9941	–0.0304	–0.0556	–0.7216	0.0038	–0.0061	0.0306	19.0199	15.6525	2.3261	5.7730
B. licheniformis	7.2253	–0.1361	–0.6139	–0.6219	–0.0089	0.0161	–0.0617	0.0300	0.0667	–0.0278	23.2053	3.2729	6.7574	2.5403
B. coagulans	–7.7556	0.2894	0.3660	1.2333	–0.0041	–0.0597	–0.1000	0.0000	–0.0044	0.0396	31.6663	5.2206	6.5079	1.7946

K (Cell number, $\times 10^9$ cfu/mL)=Constant+aX+bY+cZ+dX²+eY²+fZ²+gXY+hXZ+iYZ

*X, Y and Z were concentrations of main factors (g/L). The maximized viable cell numbers could be calculated by $\partial K/\partial X=\partial K/\partial Y=\partial K/\partial Z=0$ and were determined by Gaussian elimination

**Coefficient of regression by R^2; 0.9138 of *B. subtilis*, 0.9796 of *B. licheniformis* and 0.9173 of *B. coagulans*

Table 5. Comparison of basic industrial media and optimized media of *Bacillus* species (×10⁹ cfu/mL)

Strains	Basic production medium	Optimum medium	Increase in cell number*
B. subtilis	2.03±0.21	2.20±0.10	1.1
B. licheniformis	0.11±0.01	7.00±0.78	64.22
B. coagulans	0.99±0.04	4.10±0.56	4.14

*Cell number of optimum medium/Cell number of the basic production medium

termination of pH and DO controlling effects. Fig. 5 shows the time profile of batch cultivation on optimized media with (medium dash) or without (solid) pH and DO control. (a), (b) and (c) are the results of *B. subtilis*, *B. licheniformis* and *B. coagulans*, respectively. In Fig. 5(a), the maximum viable cell number reached up to 7.23 (±0.40) $\times 10^9$(cfu/mL) under controlled condition at 8 hrs, but without a controlled condition, showed the slower growth rate. At the same time, the viable cell number increased 13.2 times at the controlled condition as depicted with an arrow (1). Also, the maximum viable cell number showed an increase of 1.9 times higher than without con-

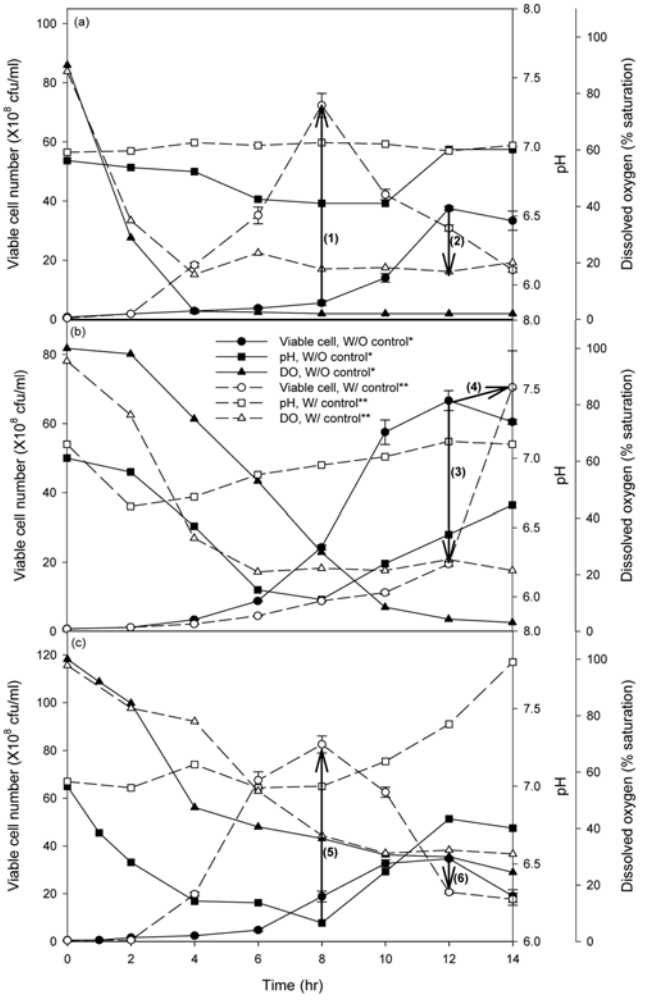

Fig. 5. Batch cultivation of *Bacilli*: (a) *B. subtilis*, (b) *B. licheniformis* and (c) *B. coagulans*, with * or without ** pH and DO controlled in a 2.5 L jar fermenter. Culture conditions: 350 rpm, 1 vvm, 30 °C (*B. coagulans*; 37 °C), initial pH 7.0, pH was controlled by 6 N NaOH and 3 N HCl and DO was controlled by increasing both agitation and aeration. Arrows indicate the maximum differences in viable cell number be- tween cultures with and without pH-DO control.

trolled cultivation. On the other hand, the viable cell number rapidly decreased as depicted by an arrow (2). Therefore, this result showed that controlling pH and DO applied only to increasing the growth rate (Fig. 5(a)). In the case of *B. coagulans*, Fig. 5(c) shows a similar result as in the case of *B. subtilis*.

But the growth rate of *B. licheniformis* was reduced by pH and DO control as depicted by an arrow (3) showing an opposite direction as compared to (1) and (5). However, the viable cell numbers of *Bacilli* increased in the case of pH and DO controlled cultivation. The viable cell number increased 1.93 (p<0.001) times in *B. subtilis*, 1.05 (p>0.50) times in *B. licheniformis* and 2.38 (p< 0.001) times in *B. coagulans*, respectively. As a result of the student t-test, *B. licheniformis* was not significantly increased by pH and DO control. Therefore, optimization of medium was more effective than pH and DO control for increasing of viable cell number in the case of *B. licheniformis* and when three of the *Bacilli* were cultivated in the optimized medium with a pH and DO control, the viable cell number increased 3.56 times in *B. subtilis*, 64.09 times in *B. licheniformis* and 8.33 times in *B. coagulans*, respectively, in comparison with the original industrial medium (Fig. 5).

CONCLUSION

A series of two statistical experimental designs, Plackett-Burman and Box-Behnken, were used as an optimization of industrial complex medium for *Bacilli* cultivation. In addition, the three factors response surface methodology allowed the development of polynomial equations for the growth of *Bacilli* by partial differentiation. Optimized media were compared to basic industrial medium and were verified by comparison of computed values. Viable cell numbers of *Bacilli* were increased 1-65 times through a series of two statistical experimental designs. Therefore, this result suggests that a series of two statistical experimental designs can be used as tool models for optimization of complex media. In addition, application of such models can be of great importance for industrial bioprocesses.

ACKNOWLEDGMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea Government (MEST) R0A-2007-000-10015-0.

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