Statistical Optimization of Medium Components by Response Surface Methodology to Enhance Menaquinone-7 (Vitamin K₂) Production by *Bacillus subtilis*^S

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Optimization of the culture medium to maximize menaquinone-7 (MK-7) production by *Bacillus subtilis* strain KCTC 12392BP in static culture was carried out using statistical experimental methods, including one factor at a time, fractional factorial design, and response surface methodology (RSM). Maltose (carbon source), tryptone (nitrogen source), and glycerol (activator) were identified as the key medium components for MK-7 synthesis by the fractional factorial design, and were selected for statistical optimization by RSM. The statistical analysis indicated that, in the range that was studied, maltose, tryptone, and glycerol were all critical factors having profound effects on the production of MK-7, with their coefficients for linear and quadratic all significant at the $p < 0.05$ level. The established model was efficient and feasible, with a determination coefficient (R^2) of 0.9419. The predicted concentrations of maltose, tryptone, and glycerol in the optimal medium were determined as 36.78, 62.76, and 58.90 g/l, respectively. In this optimized medium, the maximum yield of MK-7 reached a remarkably high level of 71.95 ± 1.00 µg/ml after 9 days of static fermentation, which further verified the practicability of this optimized strategy.

Keywords: Menaquinone-7, vitamin K₂, *Bacillus subtilis*, statistical optimization

Introduction

Vitamin K is essential for the coagulation of blood. In fact, the designated K is an abbreviation of the German word “Koagulationsvitamin” [1]. There are two types of vitamin K isolated from nature. Vitamin K₁ (phylloquinone) is synthesized mainly by plants, whereas vitamin K₂ (menaquinone) is mainly contained in animals and microorganisms [2]. Menaquinone-7 (MK-7), a valuable member of the vitamin K₂ family, has a significant effect in the prevention of cancer [3], osteoporosis [4], and cardiovascular disease [5], which are public health issues with numerous epidemiological links and important economic consequences [6]. MK-7 is available in food products such as meat, cheese, and fermented soybean. The latter has the highest MK-7 concentration formulated as natto in Japan at

12.98 µg/g [7] and cheonggukjang in Korea at 12.47 µg/g [8]. Nevertheless, the MK-7 content from these dietary sources is not as sufficient for therapeutic applications to treat osteoporosis and cardiovascular disease. Therefore, industrial-scale production of MK-7 as a dietary supplement is desirable.

To match the practical application in industry, MK-7 is produced mainly by liquid-state fermentation by using *Bacillus* species, particularly *Bacillus subtilis* [9], *B. amyloliquefaciens* [10], and *B. licheniformis* [11]. Extensive research studies have sought to improve the production of MK-7, by isolation of novel high productivity strains [10], genetic mutations [2, 12], and the optimization of culture medium and operating conditions [8, 9]. However, until recently, the general practice to optimize the medium components for MK-7 production was to use one factor at a time, with

only limited study involving statistical and mathematical techniques [13].

In this study, response surface methodology (RSM) was used to investigate the interactive effects of variable nutrients in the culture medium and their effects on the production of MK-7. RSM reduces the number of experimental trials needed to evaluate multiple parameters and their interactions, and therefore has been used to successfully model and optimize biochemical and biotechnological processes related to food systems [14, 15]. In order to maximize the MK-7 yield, a three-step RSM was applied to obtain the optimal medium compositions. First, three key nutrients having a significant effect on MK-7 production were identified and determined by a fractional factorial design. Second, a factorial central composite rotator design (CCRD) was performed to study the relationship between MK-7 yield and the three key factors to achieve the optimum concentration of each factor. Finally, the validity of this model was determined. The present work describes the successful optimization of a culture medium for the production of MK-7 by *B. subtilis*.

Materials and Methods

Reagents

Authentic MK-7 was purchased from ChromaDex Inc. (USA). Glycerol, glucose, maltose, and Tween 20, 40, 60, and 80 were bought from Junsei Chemical Co. (Japan). Beef extract, tryptone, yeast extract, soytone, and tryptic soy broth were purchased from Difco (USA). 1,4-Dihydroxy-2-naphthoic acid and dipicolinic acid were obtained from Sigma-Aldrich Chemical Co. (USA). All the other chemicals were of analytical or HPLC grade.

Microorganisms and Fermentation Process

The *B. subtilis* strain KCTC 12392BP used in the present study was isolated from commercial cheonggukjang available in the Korean market. The isolate has been deposited at Korea Collection for Type Cultures (Korea Research Institute of Bioscience and Biotechnology, Korea). Soybean extract (boiled soybean soup, 10% (w/v)) was used as the basal liquid medium, in which the MK-7 synthesis was carried out [10]. Twenty milliliters of the soybean extract (pH 7.0) in a 100-ml flask was autoclaved and inoculated with 200 μ l (1% (v/v)) of the precultured cell suspension (10^9 cells/ml). After reciprocal shaking for 6 h, fermentation was conducted statically at 40°C for 5 days.

MK-7 Analysis

The analysis of MK-7 was carried out according to a previous reported procedure [8], with slight modifications. 2-Propanol (2.4 ml) and *n*-hexane (4.8 ml) were mixed with 2 ml of the fermented medium. The mixture was vigorously shaken and kept standing for 1 h. After centrifugation at 3,000 rpm for 10 min

(Union 32R; Hail, Korea), the organic phase was collected and evaporated under nitrogen gas. The oil residue was dissolved in ethanol. After filtering with 0.45- μ m syringe filters (Millipore, USA), the sample solution was injected into a high-performance liquid chromatography system with a model 600 Controller gradient pump equipped with a degasser, and model 717 Plus autosampler (Waters, USA). The analytical column was a Nova-Pak C₁₈ column (WAT086344, 3.9 $\times$ 150 mm; Waters, USA), and the temperature was fixed at 40°C. After reduction by platinum black (reduction column, 4.6 $\times$ 10 mm; JIScience, Korea), the peaks were detected with a model 474 scanning fluorescence detector (Waters, USA) using an excitation and emission wavelength of 320 nm and 430 nm, respectively. The mobile phase was 100% methanol at a flow rate of 1.0 ml/min, and the injection volume of the sample was 20 μ l. The concentration of MK in the medium was expressed as μ g/ml of culture medium.

Experimental Design

Many different medium components could affect the synthesis of MK-7. These include carbon source, nitrogen source, phosphate source, surfactants, and activators [9, 13, 16–18]. In preliminary experiments, the medium constituents were optimized by the one-factor-at-a-time (OFAT) method. It varies only one factor or variable at a time while keeping the other factors constant. The OFAT method can identify the impact of each element of given conditions independently, but it requires a considerable amount of work, and interactions occurring between factors cannot be assessed [19]. Secondly, fractional factorial design was used to screen and evaluate the important medium components that influence the production of MK-7. In order to reduce the number of experimental runs, each factor was set to contain three levels and a 2⁷⁻⁴ fractional factorial design was considered, with one center point run for statistical reasons (Table 1). Finally, factorial CCRD was used to find the optimal conditions of the significant variables in this study. According to the results of the fractional factorial design, the three most important factors were selected, and a factorial CCRD for these three factors with replicates at the center point was performed. The CCRD maximizes the amount of information that can be obtained while limiting the number of individual experiments required [20]. In this study, the experimental design contained 16 trials, and the value of the responses was the mean of triplicate values. The second-order polynomial coefficients were calculated and analyzed using the Design Expert statistical package (ver. 8.0.6; Stat-Ease Inc., USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Model Verification

The optimum concentrations of studied medium components were obtained by solving the regression equation. Subsequently, the MK-7 content synthesized in the optimal culture medium was investigated, and the experimental and predicted values were compared to determine the validity of the model.

Table 1. Fractional factorial design and the corresponding experimental results.

Variables (g/l)								Results
Trial No.	I	II	III	IV	V	VI	VII	MK-7 ($\mu\text{g/ml}$)
1	-(40)	-(10)	-(0.5)	-(4)	-(40)	-(0.33)	-(0.010)	23.19 ± 0.63
2	-(40)	-(10)	-(0.5)	+(6)	+(60)	+(0.99)	+(0.030)	18.84 ± 0.01
3	-(40)	+(30)	+(1.5)	-(4)	-(40)	+(0.99)	+(0.030)	25.99 ± 2.16
4	-(40)	+(30)	+(1.5)	+(6)	+(60)	-(0.33)	-(0.010)	24.43 ± 1.72
5	+(60)	-(10)	+(1.5)	-(4)	+(60)	-(0.33)	+(0.030)	13.74 ± 1.37
6	+(60)	-(10)	+(1.5)	+(6)	-(40)	+(0.99)	-(0.010)	19.30 ± 1.12
7	+(60)	+(30)	-(0.5)	-(4)	+(60)	+(0.99)	-(0.010)	19.53 ± 1.24
8	+(60)	+(30)	-(0.5)	+(6)	-(40)	-(0.33)	+(0.030)	27.31 ± 1.45
9	0(50)	0(20)	0(1.0)	0(5)	0(50)	0(0.66)	0(0.015)	27.43 ± 1.29

Code I, maltose; code II, tryptone; code III, NaH_2PO_4 ; code IV, Tween 80; code V, glycerol; code VI, dipicolinic acid; code VII, 1,4-dihydroxy-2-naphthoic acid.

Results and Discussion

Factor Identification

In the preliminary study, seven factors that displayed profound effects on MK-7 synthesis were selected by using the OFAT method: maltose (Factor I), tryptone (Factor II), NaH_2PO_4 (Factor III), and Tween 80 (Factor IV) were selected as the appropriate candidate carbon source, nitrogen source, phosphate source, and surfactant, respectively. Additionally, glycerol (Factor V), dipicolinic acid (Factor VI), and 1,4-dihydroxy-2-naphthoic acid (Factor VII) had the capability to enhance the production of MK-7 by *B. subtilis*. The production of MK-7 was increased from $7.56 \mu\text{g/ml}$ in the initial medium composition to $27.15 \mu\text{g/ml}$ by OFAT method optimization (Supplementary data). Glycerol has been reported to play an important role in MK-7 production and has been widely used as the optimal carbon source [8, 9, 13]. Compared with glycerol ($15.65 \mu\text{g/ml}$), maltose ($16.45 \mu\text{g/ml}$) showed the most significant effect in this

study, and was selected as the optimal carbon source (Supplementary data). It has been reported that the effect of glycerol is not to promote cell growth, but to increase the yield of MK on a per-cell basis [12]. Moreover, glycerol was used as an additional activator, considering its association with MK-7 production [16].

The fractional factorial design enables the identification of interactions between factors more accurately and allows determining the effect of one factor among the other factors studied [21]. According to Tables 1 and 2, the highest yield of MK-7 was obtained in trial 9 culture medium ($27.43 \mu\text{g/ml}$), where the three most significant factors are maltose, tryptone, and glycerol with the percentage contribution of 11.60%, 35.93%, and 26.56%, respectively, which is in agreement with previous reports; carbon and nitrogen sources can have significant effects on the production of MK-7 [13]. On the basis of these results, maltose, tryptone, and glycerol were identified as the most important medium components for MK-7 synthesis by *B. subtilis* (KCTC 12392BP) and were selected for statistical optimization by RSM.

Table 2. ANOVA table for the experiment in the step of factor identification.

Source of variation	Stdized effects	Sum of square	Percentage contribution (%)
Maltose	-3.16	30.03	11.60
Tryptone	5.57	62.04	35.93
NaH_2PO_4	-1.38	3.80	2.30
Tween 80	1.83	6.69	3.87
Glycerol	-4.79	45.86	26.56
Dipicolinic acid	-1.27	3.24	1.88
DHNA	-0.11	0.03	0.02

DHNA: 1,4-dihydroxy-2-naphthoic acid.

Fitting the Models

A factorial CCRD was employed to analyze the interactive effects of these three major components (factor A: maltose; factor B: tryptone; factor C: glycerol) and to determine the optimal level of each factor. On the basis of the fractional factorial results, the level planning of the CCRD with each factor containing five levels ($-\alpha$, -1, 0, +1, $+\alpha$) was designed as shown in Table 3. Meanwhile, the other four relatively unimportant factors of NaH_2PO_4 , Tween 80, dipicolinic acid, and 1,4-dihydroxy-2-naphthoic acid were fixed at a concentration of 1.0, 5.0, 0.66, and 0.015 g/l, respectively. Each trial in the experimental layout was conducted in

Table 3. Experimental design and central composite design results.

Run	Medium composition (g/l)			Experimental MK-7 (µg/ml)
	Maltose (A)	Tryptone (B)	Glycerol (C)	
1	-1(20)	-1(40)	-1(20)	42.38 ± 0.02
2	+1(60)	-1(40)	-1(20)	44.99 ± 2.18
3	-1(20)	+1(80)	-1(20)	38.55 ± 0.58
4	+1(60)	+1(80)	-1(20)	57.80 ± 0.74
5	-1(20)	-1(40)	+1(60)	51.99 ± 2.16
6	+1(60)	-1(40)	+1(60)	51.72 ± 0.33
7	-1(20)	+1(80)	+1(60)	59.64 ± 0.94
8	+1(60)	+1(80)	+1(60)	57.97 ± 2.18
9	-α(6.36)	0(60)	0(40)	45.79 ± 1.23
10	+α(73.64)	0(60)	0(40)	53.97 ± 1.22
11	0(40)	-α(26.36)	0(40)	47.29 ± 2.60
12	0(40)	+α(93.64)	0(40)	52.18 ± 1.33
13	0(40)	0(60)	-α(6.36)	47.74 ± 1.08
14	0(40)	0(60)	+α(73.64)	62.51 ± 1.87
15	0(40)	0(60)	0(40)	62.28 ± 1.74
16	0(40)	0(60)	0(40)	61.29 ± 2.42

triplicate, and the mean of the three is presented in Table 3.

The results of the second-order response surface model fitting in the form of ANOVA are presented in Table 4. ANOVA of the quadratic regression model demonstrated that the model was highly significant, as evident from the *F*-value of 10.81 with a very low probability value [$(p > F) = 0.0045$], indicating that there was only a small chance (0.45%) that a “model *F*-value” of this size could occur because of noise. The *p*-values are used as a tool to check the significance of each independent variable, with smaller *p*-values indicating a greater significance of the corresponding variable [22]. In this study, the *p*-value was < 0.0100 , which indicated that the model terms were highly significant. This study determined that A, B, C, AC, A², B², and C² were significant model terms. In addition, the linear of glycerol (C), and quadratic of maltose (A²) and tryptone (B²) were more significant ($p < 0.01$) than the other factors. These results suggest that the concentration of glycerol had a direct relationship with the production of MK-7 in this particular complex medium, which is consistent with the previous description that MK-7 production was mainly affected by the glycerol concentration [16]. The concentration of maltose and tryptone were significant at the linear level, and more significant at the quadratic level, meaning that they can act as limiting nutrients and that a little variation in their concentration will alter the product formation rate.

Table 4. ANOVA results for the response surface quadratic model.

Source	Sum of squares	DF ^a	Mean square	<i>F</i> -value	<i>p</i> -value
Model	784.77	9	87.20	10.81	0.0045**
A-Maltose	83.02	1	83.02	10.30	0.0184*
B-Tryptone	70.73	1	70.73	8.77	0.0252*
C-Glycerol	285.54	1	285.54	35.41	0.0010**
AB	29.03	1	29.03	3.60	0.1065
AC	70.82	1	70.82	8.78	0.0252*
BC	3.04	1	3.04	0.38	0.5618
A ²	170.22	1	170.22	21.11	0.0037**
B ²	174.21	1	174.21	21.61	0.0035**
C ²	54.70	1	54.70	6.78	0.0404*
Residual	48.38	6	8.06		
Lack of fit	47.89	5	9.58	19.54	0.1700
Pure error	0.49	1	0.49		
Cor total	833.15	15			
R ²	0.9419		Adj R ²	0.8548	

^aDegrees of freedom.

*Significant at a level of 0.05. **Significant at a level of 0.01.

Multiple regression analysis of the experimental data provided a mathematical model that represented the MK-7 concentration in the experimental region. The relationship that was studied can be expressed by the following second-order polynomial model equation:

$$Y = 61.82 + 2.47*A + 2.28*B + 4.57*C + 1.90*A*B - 2.98*A*C + 0.62*B*C - 4.29*A^2 - 4.34*B^2 - 2.43*C^2$$

where *Y* is the predicted MK-7 concentration (µg/ml); A, B, and C are the coded values of the test variables, maltose, tryptone, and glycerol concentration, respectively.

The model determination coefficient (*R*²) was 0.9419, which indicated that the proposed model could account for 94% of the variability. The value of the adjusted determination coefficient (Adj *R*² = 0.8548) was also high enough to advocate for a high significance of the model [23]. The lack-of-fit test compared the residual error to the “pure error” from replicated design points. The “lack-of-fit *F*-value” of 19.54 also indicated that lack of fit was not significant ($p = 0.17 > 0.05$) relative to the pure error. Thus, the estimated models adequately fit the experimental data. At the same time, a relatively lower value of the coefficient of variation (CV = 5.42%) indicated improved precision and reliability of the conducted experiments [19]. In addition, the “Adeq. Precision” was used to measure the signal (response)-to-

Fig. 1. Response surface and contour plots for the production of MK-7 by *B. subtilis*.

(A) Effects of maltose and tryptone concentration. (B) Effects of maltose and glycerol concentration. (C) Effects of tryptone and glycerol concentration.

noise (deviation) ratio, in which a ratio > 4 was indicative of an adequate signal. In this analysis, the ratio of 9.858 indicated that there was an adequate signal, and that this model can be used to navigate the design space.

To investigate the interactions among the variables and to determine the optimal level of each factor for maximum MK-7 production by *B. subtilis*, response surface plots and contour plots (Figs. 1A–1C) were obtained based on the model equation. Each plot represents an infinite number of combinations of the two test variables, with the other two maintained at their respective zero level. Fig. 1A depicts the effects of interactions that included ambient maltose (20–60 g/l) and tryptone (40–80 g/l) with a constant concentration of glycerol at the zero level of 40 g/l. Both maltose and tryptone demonstrated quadratic effects on the MK-7 yield. Fig. 1B shows that there was a significant mutual interaction between maltose and glycerol, and the optimal maltose concentration was approximately 36–52 g/l (Figs. 1A and 1B). A relative comparison between Fig. 1C and the model equation revealed an insignificant interaction between tryptone and glycerol. These results indicated that MK-7 production was significantly influenced

by maltose, tryptone, and glycerol, and that all three factors were key nutrient materials controlling the biosynthesis of MK-7. Higher concentrations of maltose or glycerol could cause a slight inhibition of MK-7 synthesis. This fact was also suggested during other biosynthesis optimization experiments on carbon and nitrogen repression effects [20]. Glycerol displayed a predominant influence on MK-7 production, but its effect was reported not as a carbon source to promote cell growth, but rather as an increased yield of MK-7 on a per-cell basis [12]. Moreover, the effect of glycerol has been speculated to involve decreased viscosity of the medium to provide the optimum balance between water activity and viscosity, stimulating the uptake of extracellular substrates, and enhancing the mass transfer can consequently increase the MK-7 biosynthesis [16]. The optimal levels of the three investigated factors were calculated by solving the regression equation inside the region of the experiments. The highest MK-7 production of 64.18 $\mu\text{g/ml}$, with a 95% confidence interval between 60.12 and 68.25 $\mu\text{g/ml}$, was predicted by the model for conditions using maltose, tryptone, and glycerol at concentrations of 36.78, 62.76, and 58.90 g/l, respectively.

Model Verification

On the basis of the results of the statistical design, the verification experiments were performed at the predicted medium compositions derived from ridge analysis of RSM. The MK-7 concentration synthesized by *B. subtilis* was at a remarkably high level of 64.34 ± 3.43 µg/ml after 5 days of culture, which was within the 95% confidence interval of the maximum predicted value (64.18 µg/ml). The excellent correlation between the predicted and experimental values verified the validity and adequacy of the predicted model.

In addition, the time course of MK-7 production in the optimized medium (pH 7.0; maltose, tryptone, glycerol, NaH₂PO₄, Tween 80, dipicolinic acid, and 1,4-dihydroxy-2-naphthoic acid at concentrations of 36.78, 62.76, 58.90, 1.0, 5.0, 0.66, and 0.015 g/l, respectively) was monitored in this study. The result is shown in Fig. 2. The MK-7 synthesis profile displayed a similar trend to previous studies [12, 13]. Synthesis was dramatically increased between days 2 and 5 of fermentation, and thereafter steadily approached the maximum level of 71.95 ± 1.00 µg/ml at day 9 during the stationary phase.

This study demonstrated that the use of a factorial CCRD by determining the medium compositions leads to the optimum yield of MK-7 production. Maltose as a carbon source, tryptone as a nitrogen source, and glycerol as an additional activator were the most effective nutrients to enhance MK-7 production by *B. subtilis*. The maximum MK-7 concentration was achieved in the medium with maltose, tryptone, and glycerol at concentrations of 36.78, 62.76, and 58.90 g/l, respectively. A second-order polynomial model

Fig. 2. Time course of MK-7 production by *B. subtilis* in the optimized medium.

Bars that do not share the same superscript letters are significantly ($p < 0.05$) different from each other.

was obtained ($R^2 = 0.9419$) indicating that only 5.81% of the total variations were not explained by the model. The high correlation between the model and the experimental outcomes suggested that RSM offers an efficient and feasible approach to optimize the medium components for maximizing the MK-7 synthesis by *B. subtilis*. The maximum concentration of MK-7 synthesized by *B. subtilis* strain KCTC 12392BP reached a remarkably high level of 71.95 ± 1.00 µg/ml after 9 days of culture with an initial inoculum size of 1% (v/v). This value is much higher than that in previous studies [9, 12, 13].

MK-7 is essential for human health and is highly effective in preventing osteoporosis [4] as well as cardiovascular disease [5]. Therefore, with the increase in yield and productivity, and simultaneous material saving by using RSM, industrial-scale production of MK-7 by *B. subtilis* in the optimized medium is possible and economically attractive. Further work on optimization of process parameters, such as inoculum size, incubation temperature, initial pH, and dissolving oxygen content, is necessary.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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