Food Sci. Biotechnol. Vol. 18, No. 4, pp. 959 ~ 964 (2009)

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

Optimization of Medium Composition for Production of the Antioxidant Substances by *Bacillus polyfermenticus* SCD Using Response Surface Methodology

Jang-Hyun Lee, Mi-Seung Chae, Gooi Hun Choi, Na-Kyoung Lee, and Hyun-Dong Paik*
Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea

Abstract Production of the antioxidant substances by *Bacillus polyfermenticus* SCD was investigated using shake-flask fermentation. The one-factor-at-a-time method was first employed to determine the key ingredients for optimal medium composition, then further investigation of the medium composition was performed using response surface methodology (RSM). The antioxidant activity was measured using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assays. After screening various elements, fructose, tryptone, and $MgSO_4{\cdot}7H_2O$ were chosen as the main factors for study in the statistical experimental design. Central composite design (CCD) was then used to determine the optimal concentrations of these 3 components. Under the proposed optimized medium containing 2.8% fructose, 1.34% tryptone, 0.015% $MgSO_4{\cdot}7H_2O$, 0.5% NaCl, and 0.25% K_2HPO_4, the model predicted an antioxidant activity of 80.5% (R^2=0.9421). The actual experimental results were in agreement with the prediction.

Keywords: *Bacillus polyfermenticus* SCD, antioxidant substance, medium optimization, central composite design, response surface methodology

Introduction

The genus *Bacillus* includes a variety of industrially important species, which are commonly used as hosts in the biotechnology. In particular, *Bacillus polyfermenticus* SCD, which is known as the bispan strain, produces a variety of enzymes which lyse pathogenic strains such as typhoid bacillus, paratyphoid bacillus, shigella, and cholera. The uptake of bispan strains can enhance the appetite and promote digestion in humans by serving as vitamin B_1 and B_2 sources, as well as strengthening protection against non-oral infection and oral immunization (1,2). Also, we have determined that *B. polyfermenticus* SCD has been shown to possess cholesterol-reducing activity as well as antioxidant effects (3).

Reactive oxygen species (ROS), including both radicals such as superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) and non-radicals such as singlet oxygen (O_2^1) and hydrogen peroxide (H_2O_2), are various forms of activated oxygen (4). ROS are produced in living cells as a result of normal cell metabolism but when not adequately removed or formed additionally in the cells by exogenous sources, oxidative stress may occur (5). ROS plays an important role in over a hundred disease conditions in humans, including arthritis, hemorrhagic shock, atherosclerosis, aging, Alzheimer's disease, Parkinson's disease, gastrointestinal dysfunctions, tumor promotion, carcinogenesis, and acquired immune deficiency syndrome (AIDS) (6,7). Therefore, compounds that scavenge ROS may be useful as a preventive tool to control oxidative stress-related diseases.

*Corresponding author: Tel: +82-2-2049-6011; Fax: +82-2-455-3082
E-mail: hdpaik@konkuk.ac.kr
Received February 5, 2009; Revised March 18, 2009; Accepted March 30, 2009

In the food industry, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are often used because they are more effective and cheaper than natural antioxidants. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are restricted due to their side effects such as carcinogenicity (8,9). Most natural antioxidants are phenolic compounds such as flavonoids (quercetin, kaempferol, mycetin, etc.) and vitamin C and E (10). These compounds are found in many plant raw materials, particularly in fruits, seeds, and herbs (11-15), and in many microorganisms (16-18).

The objective of this study was to optimize the cultivation medium by applying response surface methodology (RSM) for production of the antioxidant substance(s) from *B. polyfermenticus* SCD. RSM is a well-known method used in the optimization of medium constituents and other critical variables affecting production of biomolecules. RSM is a much more efficient tool for optimization of production conditions than traditional one-factor-at-a-time approach (19). In our attempt to optimize the cultivation medium for *B. polyfermenticus* SCD, we also employed central composite design (CCD), a well-established, widely used statistical technique for determining the key factors from a large number of potential medium components using only a small number of experiments (20,21).

Materials and Methods

Bacterial strains *Bacillus polyfermenticus* SCD was maintained at −70°C in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) containing 20%(v/v) glycerol. This strain was cultivated in tryptic soy broth with shaking at 37°C.

Inoculum preparation and the antioxidant production Inoculum was prepared in 100 mL of TSB medium as a working volume in 500-mL flask. The temperature was maintained at 37°C and the agitation speed was 150 rpm for 6 hr. A 2% inoculum from the culture ($Abs_{660\ nm}$: 0.8) was added to flasks (500-mL) containing 100 mL of medium with various concentration of carbon and nitrogen sources, and trace elements. After incubation of 12 hr at 37°C under shaking condition (150 rpm), the cultures were harvested. Then, aliquots of 1 mL of culture were centrifuged at 14,000×g for 15 min at 4°C. The supernatant was used as a crude preparation to measure antioxidant activity. In parallel, bacterial suspensions were diluted for determination of bacterial numbers (CFU/mL).

Antioxidant activity on DPPH radicals (electron donating activity) The antioxidant activity of *B. polyfermenticus* SCD was measured in terms of radical scavenging activity, according to the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method (18,22). One mL of 100 μM DPPH ethanol solution was added to 0.2 mL sample supernatant. In parallel, the medium without inoculation was used as a control. The reaction mixture was shaken and incubated for 30 min at room temperature and the absorbance was read at 528 nm using a spectrophotometer (Optizen 2120UV plus; Mecasys Co., Ltd., Daejeon, Korea). The absorbance values were converted into percentage antioxidant activity using the following equation:

$$\text{Antioxidant activity (\%)} = [1-(Abs_{528\ nm\ of\ sample})/(Abs_{528\ nm\ of\ control})]\times 100$$

Microbial cell enumeration One mL from each pooled sample was serially diluted in sterile 0.1% peptone water. The diluted samples were plated on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24-48 hr. The plates were then counted and recorded as colony forming units (CFU)/mL of *B. polyfermenticus* SCD.

Selection of the best carbon source In order to study the effects of different carbon sources (1%, w/v) on antioxidant activity, sorbitol, mannitol, galactose, glycerol, saccharose, lactose, fructose, and dextrose were screened. The basal medium included: 1% peptone, 0.5% NaCl, 0.25% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$. Fermentation was carried out in a basal medium (100 mL) at an initial pH of 7.0± 0.2. The growth and antioxidant activity were monitored after 12 hr at 37°C under shaking conditions (150 rpm). To investigate the influence of the best carbon source, different concentrations (0-4%, w/v) were incorporated into the production medium. The growth and antioxidant activity were monitored after 12 hr at 37°C.

Selection of the best nitrogen source The experiments to determine the influence of nitrogen sources were carried out using a modified basal medium that contained the best carbon source. Organic nitrogen sources included sodium caseinate, soytone, yeast extract, peptone, tryptone peptone, and tryptone, while inorganic nitrogen sources included ammonium sulfate and ammonium thiosulfate. The various nitrogen sources were added as a sole source of nitrogen (1%, w/v), replacing the 1% peptone. After incubation of 12 hr at 37°C, growth and antioxidant activity were monitored. The effect of the best nitrogen source on antioxidant activity was studied by varying the concentrations (0-3%, w/v). The growth and antioxidant activity were monitored after 12 hr at 37°C.

Selection of the best trace element Various trace elements such as $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$ at 0.03%(w/v) were supplemented respectively to investigate their effect on antioxidant activity. The experiments to determine the best trace element concentration (0-0.2%, w/v) were carried out using the medium that contained fructose (2%), tryptone (1%), K_2HPO_4 (0.25%), and NaCl (0.5%).

Central composite design After the critical variables were screened, response surface methodology was performed to optimize the screened medium components for enhanced antioxidant activity. For this purpose, the response surface approach by using central composite design with 5 coded levels was performed. For the 3 factors, this design was made up of a full 2^3 factorial design with its 8 points augmented with 2 replications of the center points (all factors at level 0) and the 6 star points, that is, points having for 1 factor an axial distance to the center of ±α, whereas the other 2 factors are at level 0. The axial distance α was chosen to be 2. For statistical calculations, the relation between the coded values and actual values are described as the following equation:

$$X_i = \frac{A_i - A_0}{\Delta A}$$

where, X_i is a coded value of the variable; A_i the actual value of variable; A_0 the actual value of the A_i at the center point; and ΔA the step change of variable. For a 3-factor system, the model equation was as following:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

Where, Y is the predicted response; β_0 constant; β_i the linear coefficient; β_{ii} the squared coefficient; and β_{ij} the cross-product coefficient.

Results were analyzed by the software, SAS 9.1. The model permitted evaluation of the effects of linear, quadratic, and interactive terms of the independent variables on the chosen dependent variables. The optimum values of the selected variables were obtained by solving the regression equation and also by analyze the contour plots. In order to verify the predicted results, further experiments were performed with the optimized levels of nutrients identified.

Results and Discussion

Effect of carbon sources To select a suitable carbon source for the antioxidant production by *B. polyfermenticus* SCD, 8 carbon compounds including sorbitol, mannitol, galactose, glycerol, saccharose, lactose, fructose, and dextrose were provided at concentrations of 1%(w/v) in the basal medium. Among the various carbon sources studied, fructose was found to be the best carbon source for the antioxidant production and growth of *B. polyfermenticus*

Table 1. Effect of various carbon sources on antioxidant activity and cell growth in *B. polyfermenticus* SCD culture[1)]

Carbon source	Antioxidant activity (%)	Cell growth (CFU/mL)
Dextrose	22.1	1.5×10^8
Fructose	55.1	1.9×10^8
Lactose	12.1	3.8×10^7
Saccharose	19.5	1.1×10^8
Galactose	13.3	1.1×10^7
Sorbitol	18.7	1.0×10^8
Mannitol	20.4	1.3×10^7
Glycerol	23.5	1.2×10^8

[1)]Medium contained 1% peptone, 0.03% $MgSO_4\cdot7H_2O$, 0.5% NaCl, 0.25% K_2HPO_4, and the carbon sources were added at a concentration of 1%(w/v).

Table 2. Effect of various nitrogen sources on antioxidant activity and cell growth in *B. polyfermenticus* SCD culture[1)]

Nitrogen source	Antioxidant activity (%)	Cell growth (CFU/mL)
Peptone	58.0	3.1×10^8
Soytone	60.8	4.8×10^8
Yeast extract	65.5	3.3×10^8
Tryptone	73.6	3.2×10^8
Sodium caseinate	53.1	2.2×10^8
Tryptone peptone	71.8	3.5×10^8
Ammonium sulfate	55.3	2.8×10^8
Ammonium thiosulfate	49.0	1.9×10^8

[1)]Medium contained 2% fructose, 0.03% $MgSO_4\cdot7H_2O$, 0.5% NaCl, 0.25% K_2HPO_4, and the nitrogen sources were added at a concentration of 1%(w/v).

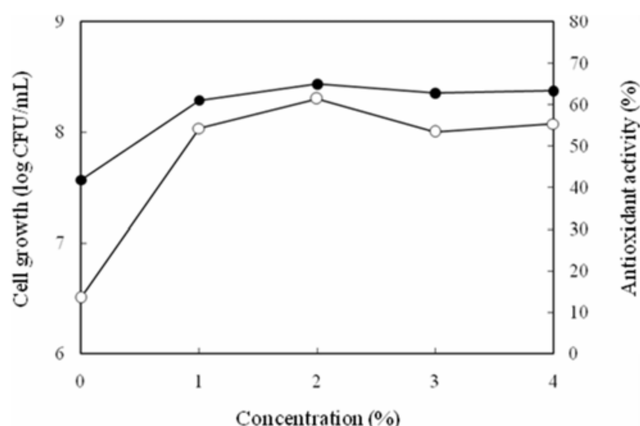

Fig. 1. Effects of fructose concentration on cell growth (●) and antioxidant activity (○) of *B. polyfermenticus* SCD, where the concentrations of peptone and $MgSO_4\cdot7H_2O$ were 1.0 and 0.03%, respectively. Results are mean±SD, *n*=3.

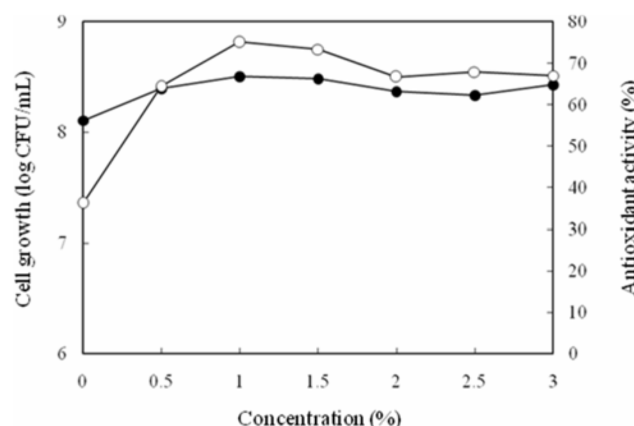

Fig. 2. Effects of tryptone concentration on cell growth (●) and antioxidant activity (○) of *B. polyfermenticus* SCD, where the concentrations of fructose and $MgSO_4\cdot7H_2O$ were 2.0 and 0.03%, respectively. Results are mean±SD, *n*=3.

SCD (Table 1). This is the first report on the positive effect of fructose on antioxidant production in *Bacillus* strains. Liu *et al.* (23) reported that fructose was a suitable carbon source for glutathione production and cell growth in *Saccharomyces cerevisiae*. Subsequently, the effects of fructose at concentrations of 0-4%(w/v) in basal medium were studied on the antioxidant production. The highest antioxidant activity was about 61.4% at concentration of 2% fructose in production medium (Fig. 1).

Effect of nitrogen sources In order to assess the effects of various nitrogen sources on the antioxidant production, various nitrogen sources (sodium caseinate, soytone, yeast extract, peptone, tryptone peptone, tryptone, ammonium sulfate, and ammonium thiosulfate) were added respectively to the basal medium (Table 2). The maximum effect on the antioxidant activity (75.1%) was observed with 1% tryptone while maximum growth was observed in soytone followed by yeast extract (Fig. 2). Liu *et al.* (24) reported that soytone could increase levels of glutamine and other amino acids by enzyme catalysis, all of which are the preferred nitrogen source of *Bacillus subtilis*.

Effect of trace elements The yield of the antioxidant substances under the influence of various trace elements was $MgSO_4\cdot7H_2O>FeSO_4\cdot7H_2O>ZnSO_4\cdot7H_2O>CuSO_4\cdot5H_2O>MnSO_4\cdot6H_2O$ (Table 3). Maximum antioxidant activity (73.8%) and cell growth were observed when $MgSO_4\cdot7H_2O$ was added to the basal medium (Fig. 3). Liu *et al.* (23) reported a similar result that Mg^{2+} was the best trace element for glutathione production and cell growth in *S. cerevisiae*. Also, magnesium deficiency was reported to induce oxidative stress (25,26).

Medium optimization by RSM The range and levels of 3 independent variables, viz., fructose (X_1), tryptone (X_2), and $MgSO_4\cdot7H_2O$ (X_3) are presented in Table 4. The concentrations of other media constituents were kept constant throughout the investigation.

In order to identify the optimum combination of major medium components that enhance the antioxidant production by *B. polyfermenticus* SCD, experiments were performed according to a central composite design experimental plan (Table 5). The experimental results of the CCD were fitted with a second-order polynomial expression. The values of

Table 3. Effect of various trace elements on antioxidant production and cell growth in *B. polyfermenticus* SCD culture[1)]

Trace element	Antioxidant activity (%)	Cell growth (CFU/mL)
$MgSO_4 \cdot 7H_2O$	72.7	2.9×10^8
$MnSO_4 \cdot 6H_2O$	5.6	1.5×10^6
$CuSO_4 \cdot 5H_2O$	12.8	1.2×10^8
$FeSO_4 \cdot 7H_2O$	64.9	2.4×10^8
$ZnSO_4 \cdot 7H_2O$	22.4	1.0×10^8

[1)]Medium contained 2% fructose, 1% tryptone, 0.5% NaCl, 0.25% K_2HPO_4, and the trace element were added at a concentration of 0.03%(w/v).

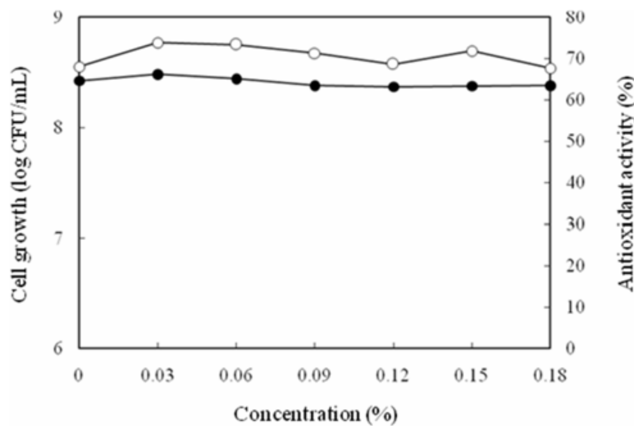

Fig. 3. Effects of $MgSO_4 \cdot 7H_2O$ concentration on cell growth (●) and antioxidant activity (○) of *B. polyfermenticus* SCD. Concentrations of fructose and tryptone were 2.0 and 1.0%, respectively. Results are mean±SD, n=3.

regression coefficients were calculated and the fitted equation for predicting antioxidant production was as following:

$$Y=-15.819513+40.441071X^1+54.848055X_2 -28.368458X_3-5.844802X_1^2-14.811593X_2^2 +74.513107X_3^2-4.558718X_1X_2-15.606873X_1X_3 +7.474992X_2X_3$$

Y represents the response, which is a dependent variable, antioxidant activity (%), and X_1, X_2, X_3 the value of independent variables, fructose, tryptone, and $MgSO_4 \cdot 7H_2O$ in the medium, respectively.

Statistical significance of the second-order model equation was checked by the analysis of variance (ANOVA). The fit of the model was also expressed by the coefficient of determination, R^2, which was found to be 0.9421, indicating that 94% of the variability in the response can be explained by the model (Table 6). The coefficient of variation indicates the degree of precision with which the treatments are compared. The lower the value of the coefficient of variation (10.8728%) the greater is the reliability of the experiments. Also, the lack of fit value of 0.3924 implies that the lack of fit is not significant relative to the pure error.

The p-value serves as a tool for checking the significance of each of the coefficients (Table 7). The smaller the p-value, the more significant is the corresponding coefficient.

Table 4. Experimental range and levels of the independent variables

Variable (%)	Symbol code	Range and levels				
		-2	-1	0	1	2
Fructose	X_1	0	1	2	3	4
Tryptone	X_2	0	0.5	1	1.5	2
$MgSO_4 \cdot 7H_2O$	X_3	0	0.05	0.1	0.15	0.2

Table 5. Experimental design used in RSM studies by using 3 independent variables with 6 center points

Run order	Coded levels			Antioxidant activity (%)	
	X_1	X_2	X_3	Experimental	Predicted
1	-1	-1	-1	43.6	38.4
2	-1	-1	1	42.1	35.9
3	-1	1	-1	62.5	59.4
4	-1	1	1	59.5	57.6
5	1	-1	-1	66.2	66.4
6	1	-1	1	59.2	60.7
7	1	1	-1	73.6	78.3
8	1	1	1	69.8	73.4
9	0	0	0	64.7	68.1
10	0	0	0	70.0	68.1
11	-2	0	0	15.5	22.9
12	2	0	0	72.5	66.6
13	0	-2	0	32.4	36.5
14	0	2	0	72.7	70.2
15	0	0	-2	71.7	72.6
16	0	0	2	64.5	65.2

Table 6. Analysis of variance for the regression model of medium optimization of antioxidant production obtained from the experimental results

Sources	Degree of freedom	Sum of square	Mean square	F value	Pr>F
Regression					
Linear	3	3,107.52	1,035.84	25.36	0.0008
Quadratic	3	831.96	277.32	6.79	0.0235
Crossproduct	3	46.71	15.57	0.38	0.7705
Total regress	9	3,986.20	442.91	10.85	0.0045
Residual					
Lack of fit	5	231.18	46.24	3.34	0.3924
Pure error	1	13.86	13.86		
Total error	6	245.03	40.84		

R^2=0.9421, CV=10.8728%.

The linear coefficient of X_1, X_2, and quadratic coefficient X_1^2 have remarkable effects on the antioxidant production. The linear effect of fructose is more significant than the other factors. These results suggest that the concentrations of fructose have a direct impact on production of the antioxidant. The p-values of quadratic terms show that fructose and tryptone can act as limiting nutrients but fructose affects the antioxidant production more than tryptone.

The antioxidant activities, as predicted by the quadratic model along with the corresponding observed values, are

Table 7. Coefficient estimates by the regression model for optimization of antioxidant activity

Model term	Coefficient	Standard error	*t*-value	*p*-value
Intercept	-15.82	20.40	-0.78	0.4675
X_1	40.34	9.18	4.41	0.0045
X_2	54.84	18.365	2.99	0.0244
X_3	-28.37	183.55	-0.15	0.8822
X_1X_1	-5.84	1.60	-3.66	0.0106
X_1X_2	-4.56	4.52	-1.01	0.3520
X_1X_3	-15.60	45.19	-0.35	0.7416
X_2X_2	-14.81	6.39	-2.32	0.0596
X_2X_3	7.47	90.38	0.08	0.9368
X_3X_3	74.51	639.06	0.12	0.9110

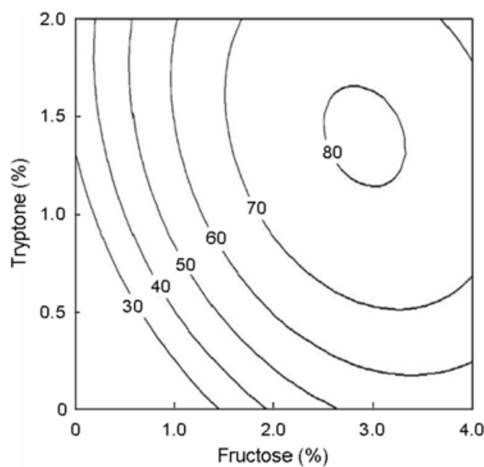

Fig. 4. Contour plot for antioxidant activity at varying concentration of fructose and tryptone with a fixed $MgSO_4 \cdot 7H_2O$ concentration of 0.015%.

shown in Table 5. Comparison of these values indicates that there is an excellent agreement between the model and experimental data.

The contour plots shown in Fig. 4-6 were based on the model where one variable was held constant at its optimum level, while varying the other two within their experimental range. The maximum antioxidant activity was observed with fructose at concentration of 2.5-3.3% (Fig. 4). Tryptone, which was necessary for the maximum activity, was around 1.2-1.8%. Our results also show that fructose has a moderate interaction with tryptone (Fig. 4). However, the effect of $MgSO_4 \cdot 7H_2O$ on antioxidant activity is not significant (Fig. 5 and 6).

Thus, to obtain higher antioxidant activity from *B. polyfermenticus* SCD, the medium composition after optimization was determined to be 2.80% fructose, 1.34% tryptone, 0.015% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, and 0.25% K_2HPO_4. The model predicted that the antioxidant activity achieved in optimized medium was equal to 80.5±6.1%.

The verification of the results using the optimized medium was accomplished by carrying out shake flask experiments. The antioxidant activity reached 75.8% after 12 hr of incubation which was 20% greater than the basal medium alone (Fig. 7). The antioxidant activity of supernatant from

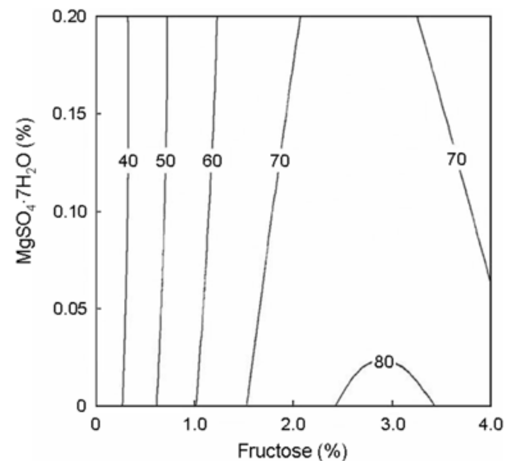

Fig. 5. Contour plot for antioxidant activity at varying concentration of fructose and $MgSO_4 \cdot 7H_2O$ with a fixed tryptone concentration of 1.34%.

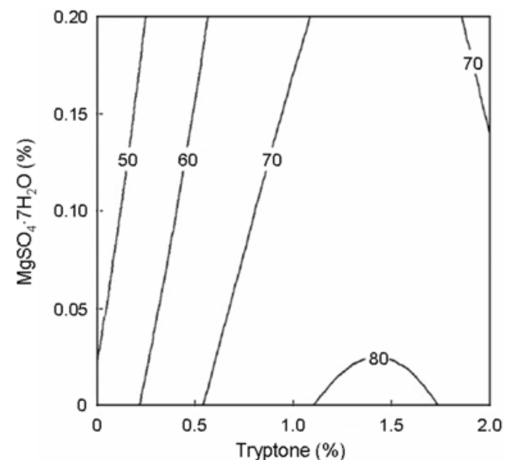

Fig. 6. Contour plot for antioxidant activity at varying concentration of tryptone and $MgSO_4 \cdot 7H_2O$ with a fixed fructose concentration of 2.8%.

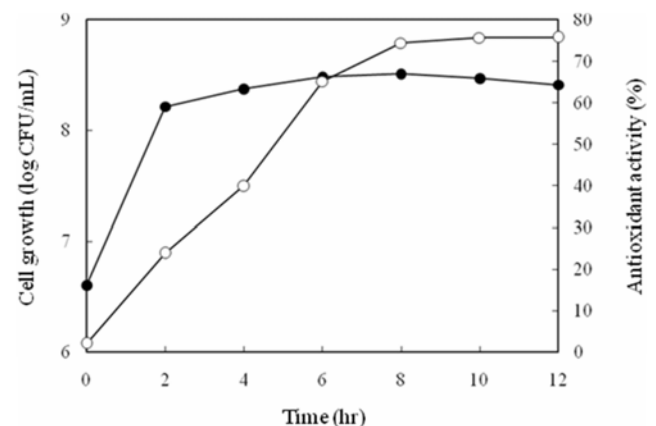

Fig. 7. Time course of cell growth (●) and antioxidant activity (○) during the fermentation by *B. polyfermenticus* SCD. Results are mean±SD, n=3.

tryptic soy broth incubated with *B. polyfermenticus* SCD showed 56.5% DPPH radical scavenging activity. Besides

partially purified extracts isolated by various solvents had the maximum antioxidant with 87.5% radical scavenging activity (18). Although the optimized medium was not purified, the maximum antioxidant activity in this study was similar to that of partially purified extracts in previous study.

The optimized medium enhanced the antioxidant activity successfully and this result was similar to the antioxidant activity predicted by the model.

Acknowledgment

This paper was supported by Konkuk University in 2006.

References

1. Jun KD, Lee KH, Kim WS, Paik H-D. Microbiological identification of medical probiotic bispan strain. J. Korean Soc. Food Sci. Nutr. 28: 124-127 (2000)
2. Paik H-D, Jung MY, Jung HY, Kim WS, Kim KT. Characterization of *Bacillus polyfermenticus* SCD for oral bacteriotherapy of gastro intestinal disorders. Korean J. Food Sci. Technol. 34: 73-78 (2002)
3. Park E, Park JS, Choi SY, Kim KT, Paik H-D. Influence of functional food containing *Bacillus polyfermenticus* SCD on lipid and antioxidant metabolisms in rats fed a high-fat and high-cholesterol diet. Food Sci. Biotechnol. 14: 766-771 (2005)
4. Droge W. Free radicals in the physiological control of cell function. Physiol. Rev. 82: 47-95 (2001)
5. Aherne SA, O'Brien MN. Mechanism of protection by the flavonoids, quercetin and rutin, against *tert*-butylhydroperoxide and menadione induced DNA single strand breaks in Caco-2 cells. Free Radical Bio. Med. 29: 507-514 (2000)
6. Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. Toxicology 148: 187-197 (2000)
7. Jun MR, Jeong WS, Ho CT. Health promoting properties of natural flavor substances. Food Sci. Biotechnol. 15: 329-338 (2006)
8. Gülçin I. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int. J. Food Sci. Nutr. 56: 491-499 (2005)
9. Gülçin I. Antioxidant activity of caffeic acid (3,4-dihydroxy-cinnamic acid). Toxicology 17: 213-220 (2006)
10. Tsaliki E, Lagouri V, Doxastakis G. Evaluation of the antioxidant activity of lupin seed flour and derivatives (*Lupins albus* ssp. *graecus*). Food Chem. 65: 71-75 (1999)
11. Bvochora JM, Danner H, Miyafuji H, Braun R, Zvauya R. Variation of sorghum phenolic compounds during the preparation of opaque beer. Process Biochem. 40: 1207-1213 (2005)
12. Lin YT, Vattem D, Labbe RG, Shetty K. Enhancement of antioxidant activity and inhibition of *Helicobacter pylori* by phenolic phytochemical-enriched alcoholic beverages. Process Biochem. 40: 2059-2065 (2005)
13. Kim SJ, Kim GH. Quantification of quercetin in different parts of onion and its DPPH radical scavenging and antibacterial activity. Food Sci. Biotechnol. 15: 39-43 (2006)
14. Yanishlieva NV, Marinova E, Pokorny J. Natural antioxidants from herbs and spices. Eur. J. Lipid Sci. Tech. 108: 776-793 (2006)
15. Park YG, Kim SH, Choi SH, Han JG, Chung HG. Changes of antioxidant capacity, total phenolics, and vitamin C contents during *Rubus coreanus* fruit ripening. Food Sci. Biotechnol. 17: 251-256 (2008)
16. Spickett CM, Smirnoff N, Pitt AR. The biosynthesis of erythroascorbate in *Saccharomyces cerevisiae* and its role as an antioxidant. Free Radical Bio. Med. 28: 183-192 (2000)
17. Yen GC, Chang YC. Production of antioxidant from *Aspergillus candidus* broth filtrate by fermentor. Process Biochem. 38: 1425-1430 (2003)
18. Kim TH, Lee NK, Chang KH, Park E, Choi SY, Paik H-D. Antioxidant activity of partially purified extracts isolated from *Bacillus polyfermenticus* SCD culture. Food Sci. Biotechnol. 15: 482-484 (2006)
19. Xiong YH, Liu JZ, Song HY, Ji LN. Enhanced production of extracellular ribonuclease from *Aspergillus niger* by optimization of culture conditions using response surface methodology. Biochem. Eng. J. 21: 27-32 (2004)
20. Kalil SJ, Maugeri F, Rodigues MI, Response surface analysis and simulation as a tool for bioprocess design and optimization. Process Biochem. 35: 539-550 (2000)
21. Ooijkaas LP, Wilkinson EC, Tramper J, Buitelaar RM. Medium optimization for spore production of *Coniothyrium minitans* using statistically-based experimental design. Biotechnol. Bioeng. 64: 92-100 (1999)
22. Lee YL, Huang GW, Liang ZC, Mau JL. Antioxidant properties of three extracts from *Pleurotus citrinopileatus*. LWT-Food Sci. Technol. 40: 823-833 (2007)
23. Liu CH, Hwang CF, Liao CC. Medium optimization for glutathione production by *Saccharomyces cerevisiae*. Process Biochem. 34: 17-23 (1999)
24. Liu J, Xing J, Chang T, Ma Z, Liu H. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem. 40: 2757-2762 (2005)
25. Tewari RK, Kumar P, Sharma PN. Magnesium deficiency induced oxidative stress and antioxidant responses in mulberry plants. Sci. Hortic.-England 108: 7-14 (2006)
26. Petrault I, Zimowska W, Mathieu J, Bayle D, Rock E, Favier A, Rayssiguier Y, Mazur A. Changes in gene expression in rat thymocytes identified by cDNA array support the occurrence of oxidative stress in early magnesium deficiency. Biochim. Biophys. Acta 1586: 92-98 (2002)