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# Medium Optimization for Polysaccharide Production of *Cordyceps sinensis*

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## Abstract

As a potential anticarcinogenic agent, polysaccharides from *Cordyceps sinensis* have been demonstrated to possess strong antioxidation activity. The aim of the present research was to study the optimal medium to produce polysaccharides of *C. sinensis* by using response surface methodology (RSM). The composition of optimized medium for polysaccharide production calculated from the regression model of RSM was 6.17% sucrose, 0.53% corn steep powder, 0.5% $(NH_4)_2HPO_4$, and 0.15% $KH_2PO_4$ at pH 4.44, with a predicted maximum polysaccharide production of 3.17 g/L. When applying this optimal medium, the maximum polysaccharide production was 3.05 and 3.21 g/L in a shake flask and a 5-L jar fermentor, respectively. When the pH was controlled at a higher level such as pH 5.0, both cell growth and polysaccharide production were inhibited. A low pH of 2.85 was required for maximum production of polysaccharides.

**Index Entries:** *Cordyceps sinensis*; polysaccharides; medium optimization; response surface methodology; corn steep powder; second-order model.

## Introduction

*Cordyceps sinensis (Berk.) sacc.* is a species of basidiomycetes belonging to polyporacceae (or Ganodermaceae) of Aphyllophorales. *C. sinensis (Berk.)*, a major parasitic fungus, grows on the larvae of *Lepidoptera (1)*. Its fruiting body, with the host larvae named Dong-Chong-Xia-Cao, has been a type of oriental folk medicine used as a tonic to replenish the kidneys

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and soothe the lungs, and for the treatment of impotence, nocturnal emission, night sweats, and chronic cough with hemoptysis. Its medical functions for the treatment of leukocythemia and hepatocirrhosis have been reported in previous studies *(2–4)*. Usually, Dong-Chong-Xia-Cao is found only in the soil of the Himalayas at an elevation of 3000–5000 m. The special growth conditions of Dong-Chong-Xia-Cao limit the available fruiting body; therefore, the cost is very high. As a potential anticarcinogenic agent, the polysaccharides from *C. sinensis* has been demonstrated to possess strong antioxidation activity in assays of xanthine oxidase inhibition, prevention of hemolysis, and inhibition of lipid peroxidation *(5)*. Recently, a number of bioactive constituents from the mycelia and fruiting body of *C. sinensis* have been reported, and a similar pharmacologic efficacy has been found in the mycelia and the fruiting body *(6–8)*. Therefore, the submerged culture for bioactive components from the mycelia of *C. sinensis* has been receiving much attention *(9)*.

The optimal design of culture media for desired metabolic product is important in the development of fermentation processes. Response surface methodology (RSM) is very useful for attaining that purpose, because it can provide information on the influence of factors and the interaction of factors with a relatively small number of experiments. Many articles report that RSM could effectively enhance the production of target product *(10,11)*.

The aim of the present research was to study the optimal medium to produce polysaccharides of *C. sinensis* by using RSM. The factors studied were $X_1$ (sucrose), $X_2$ (corn steep powder [CSP]), $X_3$ (initial pH), and $X_4$ (phosphate). Finally, with optimal medium, the cell growth and polysaccharide production were compared in a shake flask and in a 5-L jar fermentor without pH control and with pH controlled at 5.0.

## Materials and Methods

### *Microorganism*

The fungal strain used (deposited at the Culture Collection & Research Centre, Food Industry Research and Development Institute, Hsinchu, Taiwan) was *C. sinensis* (CCRC 36421). The strain was maintained on potato dextrose agar (PDA) plates at 4°C in a refrigerator and subcultured at 2-wk intervals.

### *Preparation of Inoculum*

The activated mycelium was grown on PDA for 14 d, and experimental inocula were prepared in 250-mL Erlenmeyer flasks containing 100 mL of medium. Four mycelia agar discs (0.5 cm) were obtained with a self-designed cutter and used for liquid inocula in a shake-flask culture *(12)*. The medium consisted of the following components: 25 g/L of sucrose, 4 g/L of $(NH_4)_2HPO_4$, 1 g/L of $KH_2PO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 10 g/L of CSP, 4 g/L of ammonium chloride, and 1 mL/dL of trace elements solution. The trace elements solution contained the following: 100 g/L of $MgSO_4 \cdot$

$7H_2O$, 20 g/L of NaCl, 2 g/L of $CaCl_2$, 5 g/L of $MnSO_4 \cdot H_2O$, 0.5 g/L of $FeCl_3 \cdot 6H_2O$, 0.005 g/L of $CuSO_4 \cdot 5H_2O$, 0.15 g/L of $ZnCl_2$. The pH was adjusted to the desired value by adding 0.1 *N* HCl or 2.5 *M* NaOH. The medium was sterilized at 120°C for 20 min. The flasks were incubated on a New Brunswick rotary shaker (model G24) at 25°C and 150 rpm for 3 d. The mycelium was then homogenized with a sterilized blender for 30 s and used as the inoculum in the jar fermentor.

### *Jar Fermentor*

The fermentations were implemented in a 5-L jar fermentor (Biotop, BTF-A-5*L*; Taichung, Taiwan) with a working volume of 3 L and 10% inoculum at 25°C for 8 d. The composition of the medium was the optimal medium for polysaccharide production calculated from the result of RSM and whose pH was adjusted to 5.0 prior to sterilization. The agitation rate remained at 300 rpm. The culture was aerated at a rate of 1 vvm and the pH was controlled by using 1 *N* NaOH and 1 *N* HCl.

### *Analytical Methods*

#### Cell Concentration

The cell concentration was termed the dry weight per unit volume. A fermentation broth in the amount of 10 mL was obtained and centrifuged at 4185*g* for 15 min (HERMLE, model Z200A). The sediment produced was washed, resuspended, and centrifuged twice with the same volume of distilled water. Then, the sediment was frozen by drying to a constant weight.

#### Polysaccharides

To determine the extracellular polysaccharides (EPSs), the fermentation broth filtrate was added to 4 vol of 95% ethanol at 4°C to precipitate the crude polysaccharides overnight. The precipitated polysaccharides were collected by centrifuging at 10290*g* for 10 min (HERMLE, model Z160M) and then dried to remove residual ethanol by using a freeze-dryer. The total polysaccharide content was determined with a phenol-sulfuric acid assay *(13)*.

#### Experimental Design

RSM was used for the optimization by four independent variables—$X_1$ (sucrose), $X_2$ (CSP), $X_3$ (initial pH), and $X_4$ (phosphate)—to yield the maximum EPSs (dependent variable) of *C. sinensis*. The optimization process started with the identification of important factors in the medium. Then, the steepest ascent design used crucial factors to determine the direction toward the neighborhood of the optimum process response. Two levels of $2^{4-1}$ factorial designs were applied, and the ranges of the variables tested are given in Tables 1 and 2. The results of the factorial design experiment underwent statistical regression to yield a first-order polynomial followed by the path of steepest ascent toward the neighborhood of the optimum process response (Table 2). Then, a central composite design was employed to elucidate fully the optimum location. In Table 3, experiments

Table 1
Assigned Concentrations of Each Variable at Different Levels of $2^{4-1}$ Factorial Design and Results[a]

| | Coded level of medium composition | | | | |
|---|---|---|---|---|---|
| Trial no. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | EPS (g/L) |
| 1 | –1 | –1 | –1 | –1 | 0.48 |
| 2 | 1 | –1 | –1 | 1 | 1.68 |
| 3 | –1 | 1 | –1 | 1 | 0.73 |
| 4 | 1 | 1 | –1 | –1 | 1.85 |
| 5 | –1 | –1 | 1 | 1 | 0.36 |
| 6 | 1 | –1 | 1 | –1 | 0.96 |
| 7 | –1 | 1 | 1 | –1 | 0.5 |
| 8 | 1 | 1 | 1 | 1 | 1.45 |

| | Coded level | | |
|---|---|---|---|
| Independent variable | –1 | 0 | +1 |
| $X_1$ (%) | 0.5 | 2.5 | 4.5 |
| $X_2$ (%) | 0.3 | 0.5 | 0.7 |
| $X_3$ (pH) | 3.0 | 5.0 | 7.0 |
| $X_4$ (%) | 0.125 | 0.5 | 0.875 |

Table 2
Experimental Results Along Path of Steepest Ascent

| | $X_1$ | $X_2$ | $X_3$ | EPS (g/L) |
|---|---|---|---|---|
| Base point (zero level in $2^3$ fractional factorial design) | 2.5% | 0.5% | 5.0 | |
| Unit (concentration range of unity level) | 2% | 0.2% | 2.0 | |
| Slope (estimated coefficient from Eq. 1) | 0.484 | 0.131 | –0.184 | |
| Step [unit × slope × ($q$ = 1)] | 0.968% | 0.026% | –0.368 | |
| Trial no. | | | | |
| 1 (base) | 2.5 | 0.5 | 5.0 | 1.85 |
| 2 (base + 1 step) | 3.468 | 0.526 | 4.63 | 2.45 |
| 3 (base + 2 steps) | 4.436 | 0.552 | 4.26 | 2.8 |
| 4 (base + 3 steps) | 5.404 | 0.578 | 3.89 | 2.9 |
| 5 (base + 4 steps) | 6.372 | 0.604 | 3.52 | 2.5 |

7, 11, 12, 13, 16, 17, 18, and 20 were two levels of $2^3$ factorial designs, and experiments 4, 8, 9, 10, 14, and 15 were star points with $\alpha = 1.68$. For compensation, six central points (experiments 1, 2, 3, 5, 6, and 19) were used in this design. The results were then subjected to statistical regression to yield a second-order model equation. The quality of fit of the second-order model equation was expressed by the coefficient of determination, $R^2$, and its

Table 3
Experimental Design and Results of Center Composite Design

| Treatment no. | Coded level of medium composition $X_1$ | $X_2$ | $X_3$ | EPS (g/L) |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 2.78 |
| 2 | 0 | 0 | 0 | 2.82 |
| 3 | 0 | 0 | 0 | 2.99 |
| 4 | 0 | α | 0 | 2.35 |
| 5 | 0 | 0 | 0 | 3.05 |
| 6 | 0 | 0 | 0 | 3 |
| 7 | 1 | 1 | –1 | 2.24 |
| 8 | α | 0 | 0 | 2.75 |
| 9 | 0 | –α | 0 | 2.7 |
| 10 | 0 | 0 | –α | 1.7 |
| 11 | 1 | –1 | 1 | 3 |
| 12 | 1 | –1 | –1 | 2.45 |
| 13 | –1 | 1 | –1 | 2.24 |
| 14 | –α | 0 | 0 | 2.16 |
| 15 | 0 | 0 | α | 3.15 |
| 16 | –1 | –1 | –1 | 1.95 |
| 17 | –1 | 1 | 1 | 2.48 |
| 18 | 1 | 1 | 1 | 2.68 |
| 19 | 0 | 0 | 0 | 2.84 |
| 20 | –1 | –1 | 1 | 2.55 |

| | Coded level of medium composition | | | | |
|---|---|---|---|---|---|
| | –α | –1 | 0 | 1 | α |
| $X_1$ (%) | 3.72 | 4.4 | 5.40 | 6.4 | 7.08 |
| $X_2$ (%) | 0.48 | 0.52 | 0.58 | 0.64 | 0.68 |
| $X_3$ | 3.06 | 3.4 | 3.9 | 4.4 | 4.74 |

statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test. The computer software used was Statistica, version 5.0, by Statsoft (Tulsa, OK).

## Results and Discussion

### *Effect of Nutrients*

Carbohydrates, which play key roles as structural and storage compounds in cells, are distinguished as monosaccharides, disaccharides, and polysaccharides. Mycelia of many fungi will grow to some extent over a wide range of carbon sources. To find the optimal medium, different kinds of carbon sources, including glucose, sucrose, fructose, maltose, and molasses, were used in the medium. The results shown in Fig. 1 reveal the suitability of different carbon sources for the polysaccharide production of

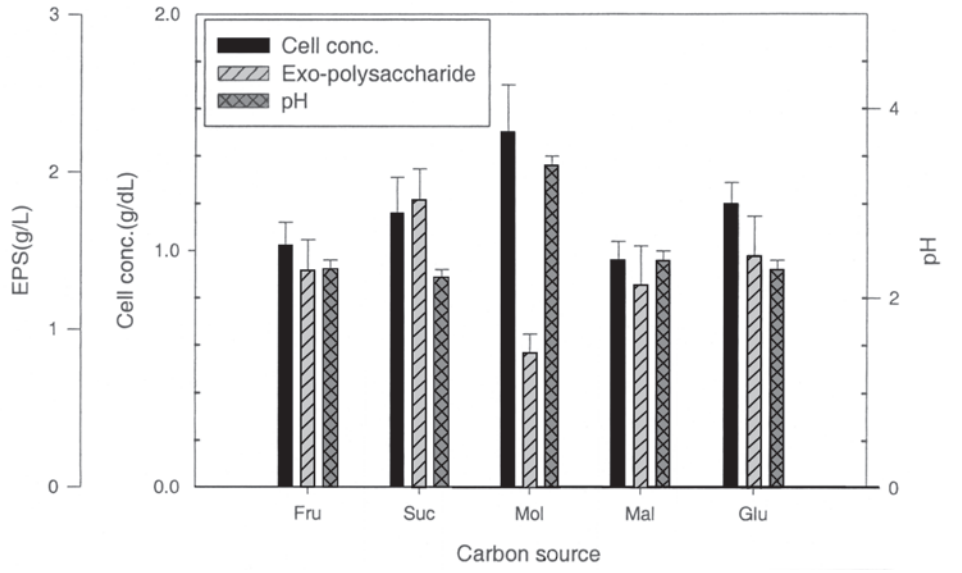


Fig. 1. Effect of carbon source on production of polysaccharides.

*C. sinensis*. Molasses seemed to be the most suitable for mycelial growth, and the concentration of mycelia reached 1.5 g/dL after 7 d of cultivation. However, the production of polysaccharides was very low, and the concentration was only 0.85 g/L. The final pH of the fermentation broth using molasses as the carbon source was higher than that for the other carbon sources. Molasses has been reported to stimulate the growth of many microorganisms, and it contains sugars, nitrogen source, and other nutrients that result in better cell growth. The approximate composition of molasses is 17 to 25% water, 30 to 40% sucrose, 4 to 9% glucose, 4 to 12% fructose, 2 to 5% starch, 7 to 15% ash, 2.5 to 4.5% nitrogen compounds, 0.5 to 4.5% protein, and 1.5 to 6% nonnitrogenous acids with various amounts of vitamins *(14)*. With sucrose as the carbon source, the highest polysaccharide production (1.85 g/L) was found with a cell concentration of 1.1 g/dL. Because sucrose is cheap and suitable for large-scale production, it was chosen as a carbon source in the following tests.

Nitrogen constitutes about 10 to 14% of cell dry weight mainly in the form of proteins and nucleic acids. CSP (Marcor Development, Hackensack, NJ), a widely used nitrogen source, contains 42% protein, 21% lactic acid, 8% sugars, 6% loss on drying (volatile matter and moisture), 16% ash, and 0.21% vitamins. Five different concentrations of CSP were employed in the medium. The concentration of the nitrogen source showed a significant effect on the cell growth and polysaccharide production (Fig. 2). With 0.5% CSP, cell growth increased to 0.96 g/dL; the highest polysaccharide production was found to be 1.75 g/L. When the concentration of CSP increased from 0.75 to 1.25%, the cell concentration continuously increased from 1.18 to 1.36 g/dL; however, the polysaccharide production decreased

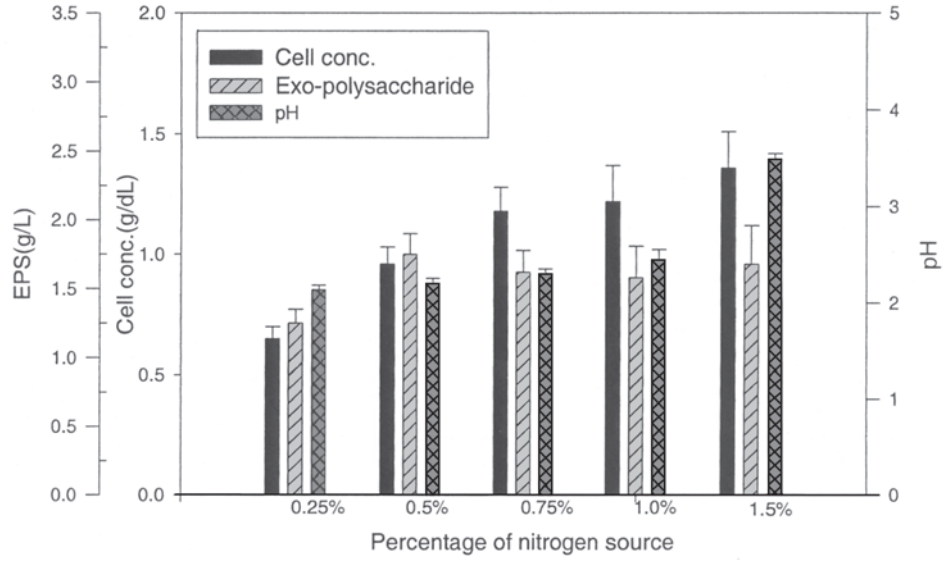


Fig. 2. Effect of nitrogen source on production of polysaccharides.

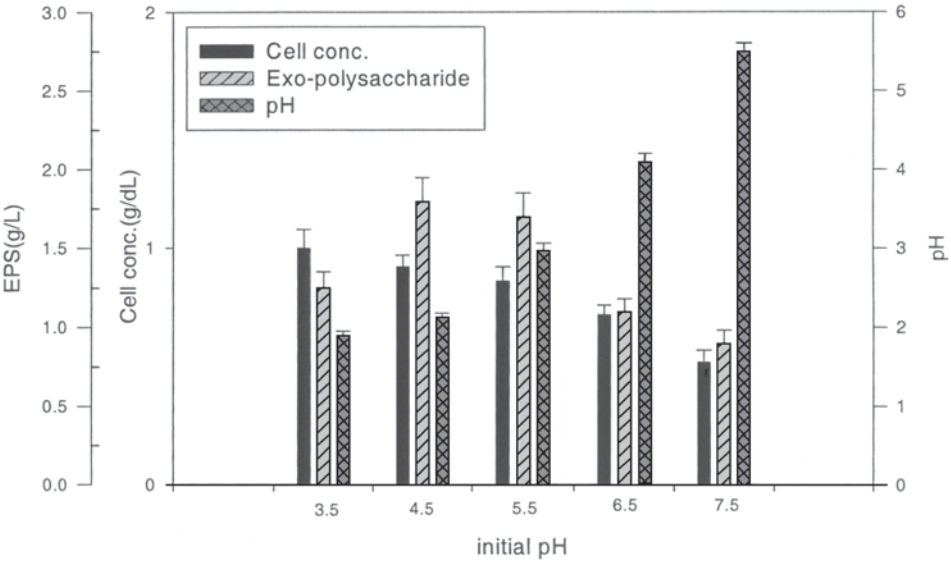


Fig. 3. Effect of pH on production of polysaccharides.

to 1.62 g/L and remained close to this concentration. Regarding cell growth in a high concentration of nitrogen medium, it was observed that cell concentrations were higher as the nitrogen concentration increased. Under these conditions, polysaccharide synthesis showed an opposite pattern to cell growth. Generally, polysaccharide production was higher at a lower nitrogen concentration. Thus, maximum levels were reached in a nitrogen-limiting medium. The low concentration of nitrogen source as well as

0.5% CSP (shown in Fig. 2) was enough to support certain enzyme activity and also allowed suitable growth under these high C/N ratio conditions, which promoted polysaccharide synthesis *(12)*.

The mycelia of various species of fungi will grow in a wide range of pH values. However, for most microorganisms, the most favorable pH range is from 5.0–7.0. The optimal initial pH for growth was determined in the basal medium in a range of 3.5–7.5, incubating for 7 d. The results shown in Fig. 3 indicate that the optimal pH for the highest yield of polysaccharide in the sucrose medium was 4.5–5.5 and that the mycelium concentration reached 0.92–0.86 g/dL at d 7. However, the optimal initial pH for mycelial growth would be dependent on the concentration of carbon source and nitrogen source in the culture medium *(15,16)*. To determine the optimal cultivation conditions by RSM, the initial pH was thus chosen as a factor in the following tests.

## *Optimization of Medium Composition*

### $2^{4-1}$ Fractional Factorial Design and Analysis

A two-level factorial design experiment was used to evaluate the impact of four factors: sucrose ($X_1$), CSP ($X_2$), initial pH ($X_3$), and phosphate ($X_4$). The corresponding polysaccharide production is shown in Table 1. The first-order polynomial equation from the statistical regression is as follows:

$$\text{EPS} = 1.00 + 0.484X_1 + 0.131X_2 - 0.184X_3 + 0.054X_4 \quad (1)$$

From Eq. 1, it was indicated that increasing sucrose ($X_1$), CSP ($X_2$), and phosphate ($X_4$) and also decreasing initial pH ($X_3$) should enhance polysaccharide production. Among the factors, the coefficients of $X_1$, $X_2$, and $X_3$ were larger than $X_4$ on the production of polysaccharides, which meant that the change in concentration of phosphate had less of an effect on polysaccharide production. Thus, the factors were reduced to $X_1$, $X_2$, and $X_3$ for the following experiments.

### Experiment of Steepest Ascent and Analysis

According to Table 2, five experiments were designed to search for the optimum point of the medium by the steepest ascent path. The path began at the zero code level in Table 1. A sequence of equally spaced locations along the path was selected by the regression coefficients of $X_1$, $X_2$, and $X_3$ in Eq. 1 multiplied by the unit in Table 2. In this study, the proportion factor, $q$, was selected as 1. If $q$ were smaller, a more accurate point would be approached but more experiments would be required. On the other hand, a larger $q$ might surpass the optimal region and miss the optimal point. Table 2 shows that trial number 4 reached the highest polysaccharide production (2.9 g/L) at 5.404% sucrose, 0.578% CSP, and pH 3.89. It is worth mentioning that the highest mycelia cell concentration was found at trial number 5 and the lowest at trial number 1 (data not shown); therefore, both

Table 4
Result of Statistical Analysis and Regression Coefficient

| | SE | $t(5)$ | $p$ | Effect | Coefficient |
|---|---|---|---|---|---|
| Mean/intercept[a] | 0.0461 | 63.1785 | 0.000006[a] | 2.9129 | –36.1765 |
| $X_1$[a] | 0.0612 | 5.1262 | 0.0037[a] | 0.3138 | 2.6465 |
| $X_1^2$[a] | 0.0596 | –5.3695 | 0.0030[a] | –0.3203 | –0.1601 |
| $X_2$[b] | 0.0614 | –2.1457 | 0.0847[b] | –0.1318 | 59.2767 |
| $X_2^2$[a] | 0.0604 | –4.5509 | 0.0061[a] | –0.2750 | –38.1897 |
| $X_3$[a] | 0.0612 | 10.2131 | 0.0002[a] | 0.6253 | 6.8868 |
| $X_3^2$[a] | 0.0596 | –5.7259 | 0.0023[a] | –0.3415 | –0.6831 |
| $X_1X_2$[b] | 0.0800 | –2.3450 | 0.0660[b] | –0.1875 | –1.5625 |
| $X_1X_3$ | 0.0800 | 0.4690 | 0.6588 | 0.0375 | 0.0375 |
| $X_2X_3$ | 0.0800 | –1.4695 | 0.2016 | –0.1175 | –1.9583 |

[a]$p < 0.05$.
[b]$0.05 < p < 0.1$.

vegetative growth and poor growth of mycelia did not enhance polysaccharide production.

### Central Composite Design

The results of the steepest ascent path provided an approximated region of optimum medium. Then the experimental design was formulated whose center point was moved in the direction of the condition giving the higher polysaccharide production on the steepest path. The tested range for each factor was 3.72 to 7.08% for sucrose (1% as unity level), 0.48 to 0.68% for CSP (0.06% as unity level), and 3.06–4.74 for pH (0.5 as unity level), as shown in Table 3. Further experiments to optimize chlamydospore production were carried out by using a Box-Wilson central composite design with six star points and three replicates at the center point *(17)*. The extended range of the star point ($\alpha$) from the center point was $N^{1/4}$, in which $N$ was the experiment number of the full factorial design. In this study, $\alpha$ was 1.68 times the unity level. The experimental results are given in Table 3. The second-order polynomial equation from the statistical regression is as follows:

$$\begin{aligned} \text{EPS} = {} & -36.1765 + 2.6465X_1 - 0.1601X_1^2 + 59.2767X_2 - 38.1897X_2^2 \\ & + 6.8868X_3 - 0.6831X_3^2 - 1.5625X_1X_2 + 0.0375X_1X_3 - 1.9583X_2X_3 \end{aligned} \quad (2)$$

The statistical analysis of the $t$-test (see Table 4) indicated that $X_1$, $X_3$, and all the quadratic terms exerted a significant effect on polysaccharide production ($p < 0.05$) and that the terms of $X_2$ and $X_1X_2$ approached a significant effect ($p < 0.1$). The statistical significance of the second-order model equation was checked by an $F$-test (analysis of variance), and the lack of fit was $p = 0.165$ (not shown). The optimal concentration for the three components obtained from the maximum point of the model was calculated to be 6.17%, 0.53%, and 4.44 for sucrose, CSP, and pH, respectively. The model

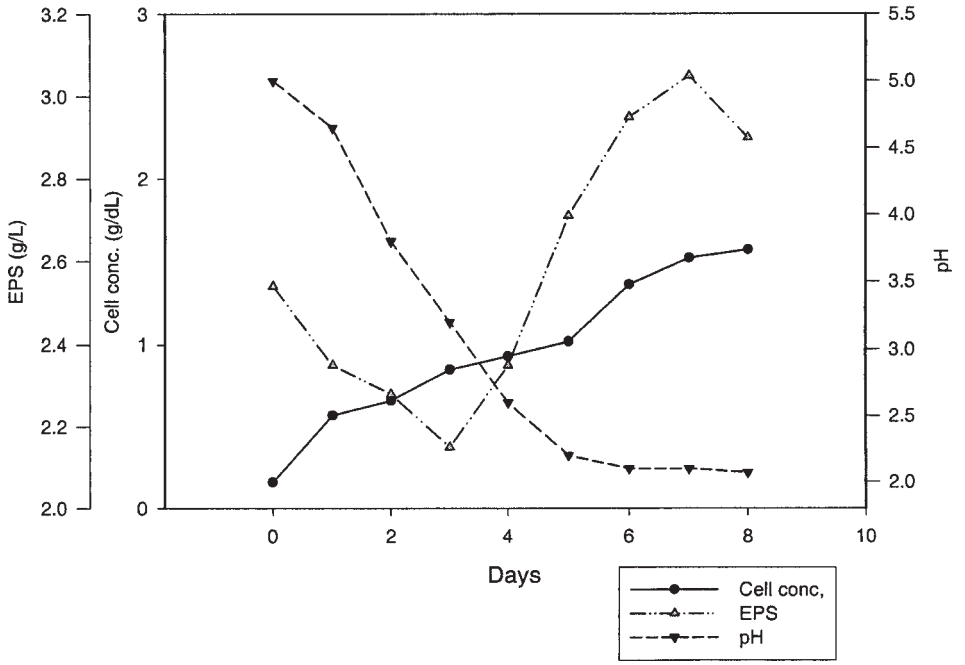


Fig. 4. Time course of cell growth and polysaccharide production using optimal medium in shake flask.

predicted a maximum polysaccharide production of 3.17 g/L, which was within the 95% confidence interval of the predicted maximum polysaccharide.

### *Experiment in Shake Flask and 5-L Jar Fermentor*

*C. sinensis* was cultivated in shake flasks with the optimized medium composition: 6.17% sucrose, 0.53% CPS, 0.5% $(NH_4)_2HPO_4$, 0.15% $KH_2PO_4$, and pH 4.44.

The time course of polysaccharide production is shown in Fig. 4. The maximum production of polysaccharide was 3.05 g/L with a cell concentration of 1.52 g/dL after 7 d of cultivation. This concentration of polysaccharide was lower than the predicted concentration from the results of RSM. The cell concentration showed a continuous increase from the beginning to the end of fermentation, and the maximum cell concentration was 1.57 g/dL with the lowest pH of 2.07. The profile of polysaccharide production first decreased and then increased to the maximum value of 3.05 g/L at 7 d. The decrease in EPSs at the beginning stage of fermentation was possibly owing to the consumption of polysaccharides from CSP in the medium (Fig. 4). Both the polysaccharide consumed from the medium and the polysaccharide produced from *C. sinensis* occurred simultaneously.

A faster rate of polysaccharide production was found in a 5-L jar fermentor (Fig. 5) with the optimal medium. Cells grew fast at the early stage

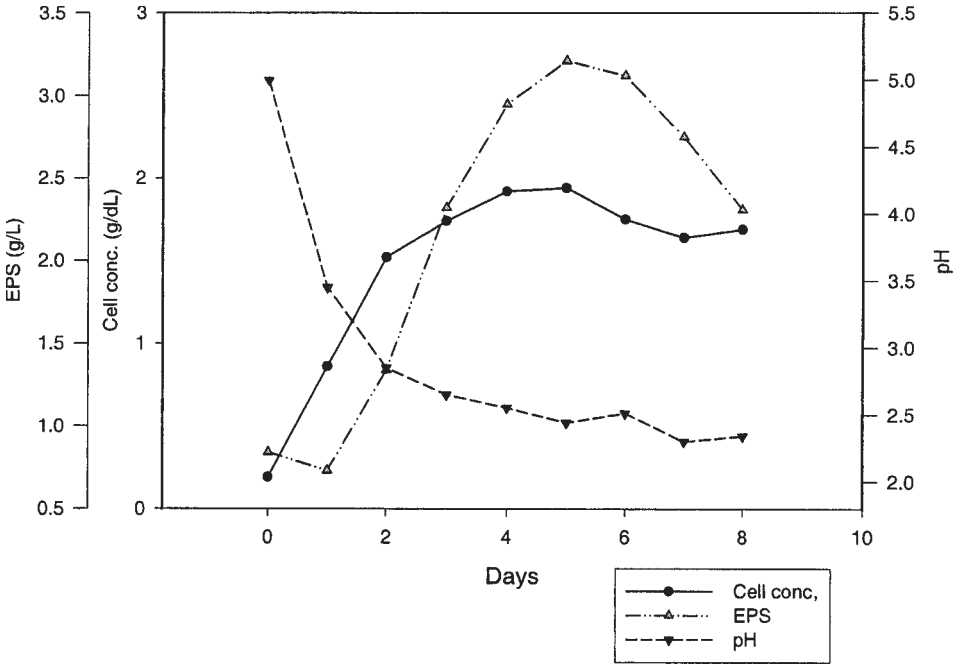


Fig. 5. Time course of cell growth and polysaccharide production using optimal medium in 5-L jar fermentor.

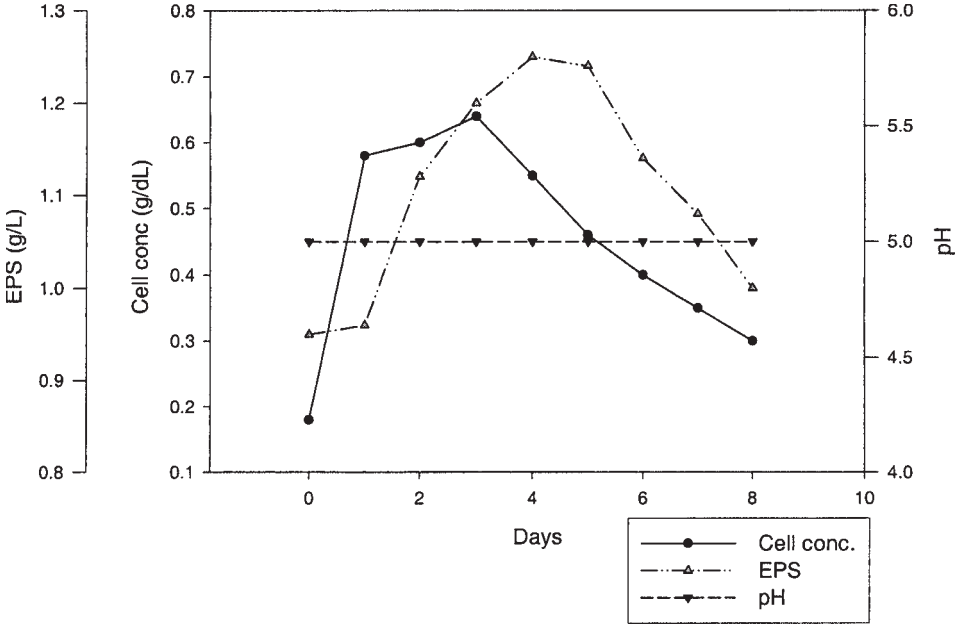


Fig. 6. Time course of cell growth and polysaccharide production using optimal medium with pH control at 5.0 in 5-L jar fermentor.

of fermentation and reached a maximum concentration of 1.85 g/dL at 4 d; meanwhile, the pH decreased to 2.47. After that, the cell concentration decreased to a range of 1.64–1.69 g/dL. The maximum concentration of EPSs was obtained in the fermentor faster than in shake-flask culture. Even a higher value than the predicted value of polysaccharide was found with 3.21 g/L at d 6. This faster production of polysaccharide might be owing to the faster cell growth rate achieved in the jar fermentor. It seemed that the polysaccharide produced was followed by the secondary metabolite and increased only after the cell was not on vegetative growth.

The profile of pH in Fig. 5 shows that the maximum production rate of polysaccharide was relative to the decreasing pH in the broth. The final pH was found as low as 2.3. It seemed that the low pH was necessary for maximal polysaccharide production. To confirm this, an experiment with the pH controlled at 5.0 was carried out in a 5-L jar fermentor. The results of low cell growth and polysaccharide production are shown in Fig. 6. The highest cell concentration was only 0.64 g/dL after 3 d of cultivation, and the polysaccharide concentration reached a maximum of 1.25 g/L after 4 d of cultivation. After that both cell concentration and polysaccharide concentration decreased to 0.3 g/dL and 1.02 g/L, respectively, at the end of fermentation (d 8). Because the high formation rate of polysaccharide was reached only after the pH was below 3.0, it seemed that the related enzyme for polysaccharide production might also have high activity with a better cell growth under this circumstance. Once the pH was controlled at a higher level such as 5.0 both cell growth and polysaccharide were inhibited.

In conclusion, our study shows that the production of polysaccharides of *C. sinensis* could enhance the production of polysaccharides by using an optimal medium composition based on RSM. With this optimal medium, the high formation rate of polysaccharide was reached only after the pH was below 3.0. When the pH was controlled at 5.0, both cell growth and polysaccharide production were inhibited.

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