Optimization of the Medium Composition for Heteropolysaccharide-7 Production by *Beijerinckia indica* L3 Using Response Surface Methodology

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The production of heteropolysaccharide-7 (PS-7) by *Beijerinckia indica* (*B. indica* L3) was evaluated in shaker flask culture. The medium optimization was studied using response surface methodology (RSM). A five-level three-factor central composite design was employed to determine the maximum PS-7 yield at optimum levels for whey lactose, glucose and ammonium nitrate contents. The validity of the model could be determined by the regression coefficient, R^2. The values of R^2 were 0.72, 0.64 and 0.85 in PS-7, DCW and viscosity, respectively. The optimal medium combinations of whey lactose, glucose and ammonium nitrate concentrations on the PS-7 production were whey lactose (2%), glucose (1%) and ammonium nitrate 5 mM, respectively. The result indicated that PS-7 production was affected significantly by the addition of glucose to whey lactose based on medium and C/N ratio.

Key words : *Beijerinckia indica* L3, heteropolysaccharide-7, whey lactose

Introduction

Beijerinckia indica ATCC 21423 is a free-living, strictly aerobic bacterium that can produce copious polysaccharide when growing on carbohydrate-containing medium [15]. The bacterial polysaccharide, heteropolysaccharide-7 (PS-7) produced by *B. indica* has a variety of applications such as stabilizing, viscosifying, emulsifying, thickening and suspending agents [10,11]. The repeating unit of PS-7 composed of D-glucose, L-rhamonse and D-2,6-deoxyglucuronic acid is a tetrasaccharide in the main backbone.

Glucose is somewhat of high price though it is a primary carbon source in synthetic media for polysaccharide production. Thus, there is a growing interest in using renewable natural carbon source of agro-industrial origin because of low cost carbon source for the production of microbial metabolites. In addition, it solves environmental and energy problems related to their disposal. Cheese whey is a nutrient-rich dairy byproduct from cheese production containing lactose, proteins, minerals, trace amount of vitamins and some organic molecules [13]. However, its proper disposal has been a major environmental problem. Recently, the proteins in whey are separated and used as food additives and the remains (mainly lactose) are spray-dried to produce sweet whey powder, which is widely used in the animal feed industry. The whey can be also used as a substrate for the production of valuable products such as polysaccharides and poly-3-hydroxybutyrate (PHB).

For this study, the traditional chemical mutagenesis method was chosen for the mutant screening of *B.indica*. A mutant strain, *B. indica* L3 was obtained for the production of PS-7 from lactose and the polysaccharide production could be enhanced by the addition of glucose in the whey medium or lactose-based MSM medium in previous study [4,15]. Results of previous studies have shown that substrate concentration has a considerable effect on the yield of fermentation products, because the substrate utilization rates could be significantly influenced by the substrate concentration. Consequently, the optimum composition of carbon sources (glucose and lactose from whey) and nitrogen source (ammonium nitrate) was determined for the production of PS-7.

Response surface methodology (RSM) extensively has been applied for the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing processes. RSM is a statistical technique for the evaluation of experimental design, model development, and optimum conditions. RSM can overcome the shortcoming of the classical or empirical methods when interactions between independent factors exist [1,9]. Thus, various carbon and nitrogen concentrations for maximal PS-7

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production were evaluated using RSM to optimize the medium composition using whey-based medium as a main carbon source.

Methods

Bacterial strain and media preparation

B. indica L3, a mutant strain of *B. indica*, was maintained on agar plates of mineral salts medium (MSM, 5.0 g/l KH_2PO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 0.6 g/l NH_4NO_3, 0.4 g/l Bacto yeast extract, 0.2 g/l Bacto peptone, and 20 g/l agar) with 16 g/l lactose and 4 g/l glucose. The seed culture of *B. indica* L3 was prepared in yeast lactose (YL) medium consisted of 3 g/l Bacto yeast extract, 10 g/l lactose and 5 g/l Bacto peptone [15].

Whey medium was prepared by the method outlined by Dlamini and Peiris [2]. Whey medium was prepared with 20 g/l whey lactose with the addition of 5.0 g/l KH_2PO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$ and 1 ml of trace mineral solution containing 1% $FeSO_4 \cdot 7H_20$, 0.1% $MnSO_4 \cdot 5H_2O$, 0.25% $ZnSO_4 \cdot 6H_2O$, 0.2% $CuSO_4 \cdot 6H_2O$ and 0.25% $Na_2MoO_4 \cdot H_2O$.

Analytical methods

The cell growth and PS-7 production were determined by the methods described in previous report [15]. The residual glucose concentration was determined colorimetrically by the phenol sulfuric acid method [3]. Residual sugar was calculated to percentage of total amount of sugar added in the medium. The viscosity measurements of the samples were performed at 30°C using a Brookfield programmable LVD-VIII digital viscometer (Brookfield engineering laboratories, Stoughton, MA, USA) fitted with a small sample adapter using a SC4-34 spindle. Intrinsic viscosities of PS-7 were measured with Ubbelohde (Bukwang Co., Busan, Korea) capillary viscometers in water bath (30.0±0.1°C) and were calculated by the single concentration methods. Intrinsic viscosity were obtained from classical plots according to the Huggins and Kraemer equation [5].

Experimental design and data analysis

A response surface experiments were designed to evaluate the relationships between three independent variables, content of lactose in whey lactose (%, w/v medium, X_1), glucose (%, w/v medium, X_2), NH_4NO_3 (mM, X_3) and three dependent variables, dry cell weight (DCW, g/l), PS-7 (g/l) and viscosity (cP). The specific experimental scheme was designed by a central composite response surface method [14].

The complete design consisted of 17 experimental points in triplicate. Responses of property values under observations were DCW (g/l), PS-7 (g/l) and viscosity (cP). Data were analyzed to fit the following second order equation for each response variables:

$$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2 \quad \text{(Eq. 1)}$$

where Y and b_n values were dependent variables and coefficients, respectively and X_1, X_2, and X_3 were the coded independent variables. All statistical calculations were performed using the SAS/STAT™ statistical package [12].

Results and Discussion

Optimization of medium using RSM

The variances for each of the dependent variables with their corresponding coefficients of multiple determination (R^2) were analyzed and summarized in Table 1.

All dependant factors showed high R-square (R^2) values that the data were adequately explained. Table 1 shows the effect of whey lactose (%), glucose (%), and ammonium nitrate (mM) on DCW, PS-7 and viscosity of medium. The statistical significance of Eq. 1 was checked by F-test, and the analysis of variance (ANOVA) for response surface quadratic model was summarized in Table 2.

The validity of the model could be determined by the R^2. The values of R^2 were 0.72, 0.64, and 0.85 for DCW, PS-7 and viscosity, respectively. The 3-D response surface curves were plotted to explain the interaction of medium components and the optimum concentration of each component required for the PS-7 production.

Fig. 1 showed the effect of glucose addition to whey lactose for the PS-7 production at 5.0 mM, 7.5 mM and 10 mM of ammonium nitrate in the medium. Glucose addition increased the PS-7 production in whey medium at 5.0, 7.5 and 10 mM of ammonium nitrate concentrations. Maximal production was observed at high concentrations of glucose and whey lactose at each of ammonium nitrate concentrations in the medium. However, the yield of PS-7 was decreased with the increase of N source (ammonium nitrate). The maximum yield of PS-7 per g carbon sources was observed at 2% whey lactose, 1% glucose and 5 mM

Table 1. Response surface level combinations of independent variables in the experimental design and responses of dependent variables

Design point	Independent variable[a]			Dependent variable		
	Whey lactose (%, w/v)	Glucose (%, w/v)	Ammonium Nitrate (mM)	PS-7[b] (g/l)	DCW[c] (g/l)	Viscosity (cP)
	X_1	X_2	X_3			
1	-1	-1	-1	2.07	2.73	31733
2	-1	-1	1	1.07	2.51	16616
3	-1	1	-1	3.20	1.63	45590
4	-1	1	1	2.70	3.23	38511
5	1	-1	-1	2.85	1.70	58727
6	1	-1	1	2.98	2.39	46969
7	1	1	-1	3.41	3.01	51708
8	1	1	1	4.00	1.07	38331
9	-α	0	0	3.18	1.56	50449
10	α	0	0	3.00	1.23	53688
11	0	-α	0	2.84	3.26	46790
12	0	α	0	3.24	7.49	43010
13	0	0	-α	3.56	5.77	53088
14	0	0	α	2.50	2.85	32273
15[d]	0	0	0	2.82	3.64	45350
16[d]	0	0	0	2.81	3.55	42460
17[d]	0	0	0	2.77	3.80	47700

Independent variable[a]
X_1: whey lactose -α=0.59%, -1=1%, 0=2%, 1=3%, α=3.41%
X_2: glucose -α=0.59%, -1=1%, 0=2%, 1=3%, α=3.41%
X_3: ammonium nitrate-α=0.59%, -1=1%, 0=2%, 1=3%, α=3.41%
[b]Polysaccharide-7.
[c]Dry cell Weight.
[d]Central points.

ammonium nitrate. The figure indicated that the low concentration of N sources and high concentration of C sources produced high amount of PS-7 due to the high C/N ratio. Jurgen [6] suggested that under the nitrogen-limited condition (high C/N ratio), aerobically growing cells try to store the surplus carbon source to maintain a constant C/N ratio for biomass production. Therefore, the increase of C/N ratio played an important role in the polysaccharide production such as PS-7. From the results in this study, C/N ratio was an important for PS-7 production and showed high yields of PS-7 in low concentration of ammonium nitrate in the medium. This might be explained that low concentration nitrogen content in the medium with high C/N ratio was a starting point for the production of polysaccharide, PS-7.

Viscosity is an important parameter for the quality of polysaccharides. In most cases, higher viscosity is the better for the application of polysaccharide. The viscosity of

Table 2. Regression Coefficients and Analyses of Variance

Coefficient	Polysaccharide-7 production (g/l)	Dry cell weight (g/l)	Viscosity (cP)
b_o	3.426	-4.610	-7253.4
Linear			
b_1	-0.1978	7.725**	14923.0
b_2	0.4558	-0.7388	21329.0*
b_3	-0.3232	0.6423**	5550.2
Quadratic			
b_{11}	-0.00207	-1.632	1171.8
b_{22}	-0.02708	0.3534	-2413.5
b_{33}	-0.004734	-0.02867	-563.8
Two-factor cross			
b_{12}	-0.1487	0.04625	-6426.1***
b_{13}	0.1110	-0.1320	-147.0
b_{23}	0.0480	-0.0405	320.9
R-square	0.7217	0.6369	0.8499
F	2.02	1.36	4.40
Probability of F	0.1836	0.3487	0.0318

Model for analysis of property values used X_1=Whey lactose (%, w/v), X_2=glucose (%, w/v), X_3=Ammonium nitrate (mM) and $Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$
*significant at 10% level.
**significant at 5% level.
***significant at 1% level.

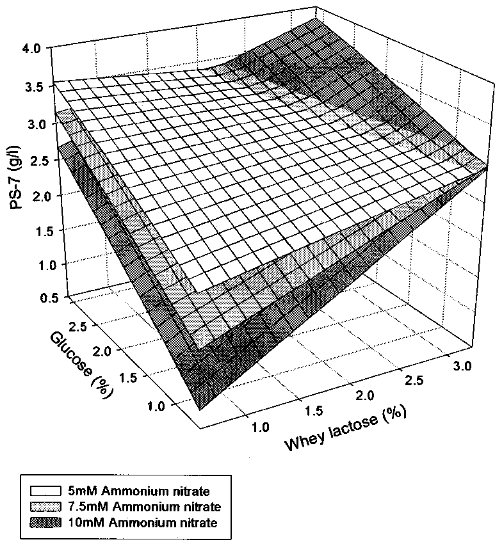

Fig. 1. Effect of glucose, whey and ammonium nitrate concentration on the PS-7 production of *B. indica* L3.

PS-7 was related to the production stage or condition of PS-7 and showed the increase with the increase of PS-7 amount in medium until 3 g/l as shown in Fig 2. The viscosity decreased with further increase of PS-7 concentration over 3 g/l. It might indicate that the molecular

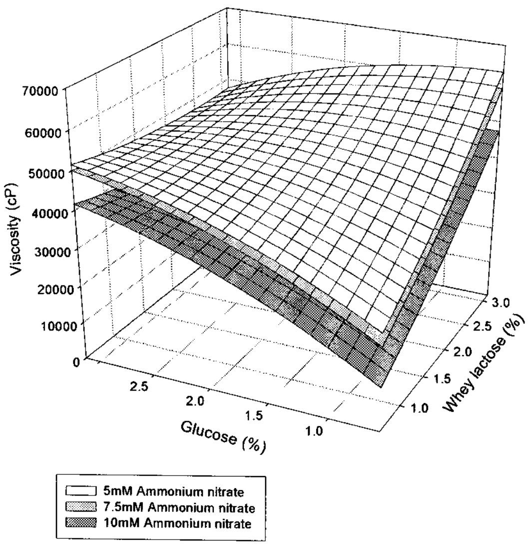

Fig. 2. Effect of glucose, whey and ammonium nitrate concentration on the viscosity of culture after 72 hr cultivation of *B. indica* L3.

weight distribution of PS-7 was decreased. Therefore, the intrinsic viscosities of PS-7 were determined for the samples. The intrinsic viscosity decreased from 0.9 dl/g to 0.2 dl/g, when the concentrations of whey lactose and glucose increased over 2%. This indicates that the molecular size decreased with the high amount of carbon sources in the medium. Lee [7,8] reported that the molecular weight of exopolysaccharide produced by *Aureobasidium pullulan* was changed significantly by the culture condition and various molecular weight patterns of exopolysaccharides were observed from the polysaccharide produced under different culture conditions. Therefore, the optimal condition should be obtained for the production of PS-7 with high yield and viscosity.

In conclusion, PS-7 production was optimized with the addition of glucose (1%) at 5mM of ammonium nitrate in lactose based MSM medium containing 1% whey lactose. PS-7 molecular weight distribution was decreased with further increase of carbon source over 2% carbon content in the medium.

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초록 : 표면반응방법을 이용한 *Beijerinckia indica* L3에 의한 PS-7 생산 최적화

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본 연구는 플라스크 배양을 통해 *B. indica* L3 균주를 가지고 PS-7을 생산할 수 있는 최적 조건을 조사하기 위해서 수행하였다. RSM을 통한 배지의 최적화 실험에서는 whey lactose, glucose, ammonium nitrate 세 가지의 독립변수의 조성에 따라 배양 72시간에 해당하는 DCW, PS-7, 그리고 점도의 변화에 대한 결과를 관찰하였다. 결정 계수(R square)의 값은 PS-7생산량, DCW, 점성에서 각각 0.72, 0.64, 0.85로 신뢰성 있는 값을 획득하였다. Whey lactose와 glucose의 조합에 따른 PS-7 생산에 미치는 효과를 살펴보면 whey lactose (2%), glucose (1%)에서 최적의 PS-7 생산을 나타내었다. 결론적으로 glucose에 whey lactose를 기반으로 하는 배지 첨가와 C/N 비율이 PS-7의 생산에 큰 영향을 미침을 알 수 있었다.