Studies on the Production of Heteropolysaccharide-7 by *Beijerinckia indica*

*Beijerinckia indica*에 의한 Heteropolysaccharide-7의 생산에 관한 연구

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Studies on the Production of Heteropolysaccharide-7 by *Beijerinckia indica*

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ABSTRACT

In this study, the optimal conditions for heteropolysaccharide-7 (PS-7) production by the aerobic bacterium, *Beijerinckia indica*, in a batch culture using shake flask and 5-L bioreactor were investigated. The metabolic flux distribution in *B. indica* under various nitrogen concentrations was analyzed. Furthermore, a mutant strain of *B. indica* was screened and used for PS-7 production with lactose/whey.

The first part of this study was focused on the production of PS-7 under aerobic condition to examine the suitable chemical and physical conditions in respect to quantitative PS-7 formation for this strain. Testing a C/N ratio range of 10~30 g glucose/0.6 g NH_4NO_3, showed that high C/N ratio in the MSM medium improved the productivity of PS-7 with a high viscosity in the culture of *B. indica*. However, the cells produced the highest amount of PS-7 with 20 g/l glucose, and more cell mass was produced at relatively high concentration of ammonium. Therefore, only a small range of ammonium (4~12 mM NH_4) concentration was

favorable for PS-7 production. When KNO_3 was used in the culture of *B. indica*, PS-7 production was not changed significantly. *B. indica* preferred hexoses or glucose-generating sugars for the synthesis of PS-7. Moreover, sucrose was superior carbon source for PS-7 production as compared to glucose and fructose. The cells cultured on fructose produced a few PS-7 with a low viscosity, however, addition of gluconate into fructose medium improved the quantity and viscosity of PS-7.

B. indica metabolized carbon sources through ED and PP pathways. The cell growth and polysaccharide biosynthesis were significantly influenced by environmental nitrogen concentration. Under low nitrogen conditions, a greater amount of PS-7 was converted from glucose rather than glucosamine, glycerol and gluconate. The cell mass in the culture with glucosamine increased, but little polysaccharide was produced. In order to investigate the effects of nitrogen in the culture, a metabolic pathway network under varying concentrations of nitrogen was studied by using metabolic flux analysis. The intracellular flux distributions in *B. indica* showed that the ED pathway was operated dominantly and the PP pathway was maintained to a lesser degree. Triose-3-phosphate cycling was present in the gluconeogenesis pathway, which increased the intracellular pool of hexose-phosphate for polysaccharide synthesis. The culture containing high levels of nitrogen showed a drastic change in metabolic flux distribution. The fluxes in the ED pathway, PP pathway, glycolysis, and TCA cycle were increased. Thus, more carbon source and energy were dissipated and the production of PS-7 polysaccharide decreased. The Malate Dehydrogenase (MDH) activity in the cells cultured at high nitrogen level nearly doubled and confirmed the significant

change in the central metabolism.

Whey, which is a cheap and renewable byproduct from dairy cheese whey production, was used for the production of PS-7. A traditional chemical mutagenesis method was chosen for mutant screening from *B. indica*. One strain, *B. indica* L3, was screened and used for PS-7 production with lactose or whey. The PS-7 production by the mutant strain could be enhanced by the addition of 4 g/l glucose in the whey medium or lactose-based MSM medium. The β-galactosidase was expressed constitutively in the mutant and its activity was higher than that of the parent strain. The mutant cells grown in whey medium produced nearly the same amount of PS-7 with a significantly higher viscosity than that from the parent strain. The compositional analysis of the PS-7 produced by *B. indica* L3 grown in lactose-based MSM medium or whey medium showed that the ratios of Glc/Rha were similar to that from parent strain grown in glucose-based MSM medium. Rheological study showed that the polysaccharide from *B. indica* L3 was twice viscous than that of xanthan and appeared excellent pseudoplasticity. Therefore, the dairy whey can be used as a useful substrate to culture *B. indica* L3 for the production of the PS-7 and the production cost can be reduced.

CHAPTER 1

General Introduction

1-1. Literature review of PS-7

1-1-1. Introduction

Microbial polysaccharides show a wide variety of chemical, physical and biological properties and are applied in food, paint, pharmaceutical and oil mining industry [1,2]. Some of them are commercially used as emulsifiers, stabilizer, gelling agents and suspending agents due to their specific characteristics, such as xanthan and gellan (Table 1.1).

The heteropolysaccharide-7 (S-7 or PS-7) from *Azotobacter indicus var.* myxogenes (ATCC 21423) was identified and examined as a potential candidate for the production of a bacterial polysaccharide with desirable rheological properties by Kang & McNeely [3]. The polysaccharide consists of 73% glucose, 16% of rhamnose and 11% of uronic acid [4]. The fine structure of PS-7 was determined by Gulin et al.[5], which is composed of D-glucose, (D-Glc), L-rhamonse (L-Rha), and D-2, 6-deoxy-glucuronic acid (D-deoxy-GlcA), as shown in Fig. 1.1.

Noticeably, PS-7 contains unusual 2-deoxysugar, 2-deoxy- D-hexauronic acid, similar to the exopolysaccharide from *Sphingomonas paucimobilis* strain I-886 [6]. The backbone of PS-7 has a similarity to that of gellan gum family, which is designated as sphingan by the common genus *Sphingomonas* [7], as shown in Fig. 1.2. PS-7 was reported to be degraded by the sphinganase that cleaves specific gellan- related polysaccharides produced by some species of microorganisms [8].

Table 1.1 Several polysaccharides from microorganisms [1,9].

Origin	Polysaccharide	Components
Xanthomonas campestris	Xanthan	Glucose/mannose/glucuronic acid
Sphingomonas paucimobilis	Gellan	Glucose/rhamnose/glucuronic acid
Alcaligenes faecalis	Curdlan	Glucose
Aurebasidium pullulans	Pullulan	Glucose
Leuconostoc mesenteroides	Dextran	Glucose
Aerobacter levanicum	Levan	Fructose
Rhizobium melilot	Succinoglycan	Glucose/galactose
Zoogloea ramigera	Zooglan	Glucose/galactose
Azotobacer vinelandii	Alginate	Glucuronic acid/ Mannuronic acid
Escherichia coli K5	Hyaluronic acid	Glucuronicacid/ N-acetyl-glucosamine

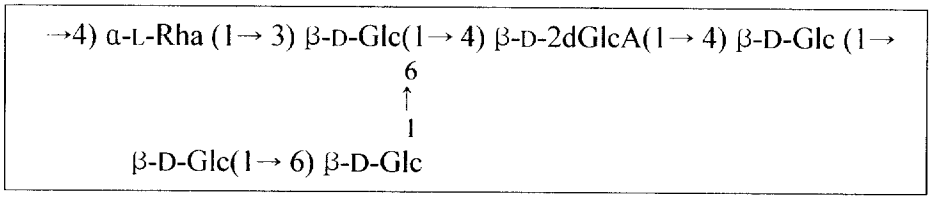

Fig. 1.1 Primary structure of PS-7 from *Azotobacter indicus var.* myxogenes ATCC 21423 (*Beijerinckia indica*). Rha: rhamnose, Glc: glucose, 2dGlcA: 2-deoxy- glucuronic acid.

S60 →4) α-L-RHA (1→3) β-D-GLC- (1→4) β-D-GLCA- (1→4) β-D-GLC- (1→
gellan

NW-11 →4) α-L-MAN (1→3) β-D-GLC- (1→4) β-D-GLCA- (1→4) β-D-GLC- (1→

S-130 →4) α-L-RHA (1→3) β-D-GLC- (1→4) β-D-GLCA- (1→4) β-D-GLC- (1→
wellan
3
|
α-L-RHA or MAN

RHA
S-88 →4) α-L-or (1→3) β-D-GLC- (1→4) β-D-GLCA- (1→4) β-D-GLC- (1→
MAN
3
|
α-L-RHA

S-194 →4) α-L-RHA (1→3) β-D-GLC- (1→4) β-D-GLCA- (1→4) β-D-GLC- (1→
rhamsan
6
|
β-D-GLC- (1→6)α-D-GLC

I-886 →4) α-L-RHA (1→3) β-D-GLC- (1→4) β-D-2dGLCA- (1→4) β-D-GLC- (1→
6 2deoxy
|
β-D-GLC- (1→6) α-D-GLC

PS-7 →4) α-L-RHA (1→3) β-D-GLC- (1→4) β-D-2dGLCA- (1→4) β-D-GLC- (1→
6 2deoxy
|
β-D-GLC- (1→6) β-D-GLC

Fig. 1.2 Structure of repeat units of polysaccharides S-60, NW-11, S-130, S-88, S194, I-886 and PS-7 (after Thorne et al. [7]).

1-1-2. Physical property and application of PS-7

PS-7 is a water-soluble biopolymer and generates high viscosity solution that is about twice as viscous as that of xanthan. The viscosity of PS-7 solutions is stable in a wide range of temperature from 4°C to 93°C and pH from 3.0 to 12.0 [10]. Freezing and thawing of the aqueous PS-7 has only a slight influence on the viscosity and PS-7 shows very good pseudoplasticity, thermostability and excellent suspending ability. PS-7 is incompatible with cationic or polyvalent ions at high pH resulting in gel formation. The properties indicate that PS-7 is a highly useful agent in oil well drilling. Other potential applications, such as dripless water-based latex paint, wall-joint cement adhesive, and textile printing have been reported [11].

1-1-3. Biotechnological production of PS-7

Up to date, only a few groups reported the work related to the production of PS-7. Lee et al. (1997) studied the compositional consistency of PS-7 by *B. indica* utilizing glucose and glucose analogues and found that there was no direct incorporation of these sugars into PS-7 [4]. Jin et al. reported the production of PS-7 using agro-industrial byproducts to reduce the cost of substrate [12]. In another study, chromosomal genes with multiple copies of PS-7 biosynthetic genes were cloned and augmented in *Sphingomonas S7* (ATCC 21423), resulting in increase of yield and viscosity of exopolysaccharide (EPS) [7]. However, a

six-fold increase in phosphoglucomutase activity could not enhance the PS-7 production.

1-2. Bacterial formation of PS-7

1-2-1. Characterization of *Beijerinckia indica*

The bacteria for PS-7 production, *Azotobacter indicus* var. myxogenes (ATCC 21423), was later renamed to *Beijerinckia indica* [4], or *Sphingonomas S7* [7]. *B. indica* is a strictly aerobic, gram-negative bacterium. *B. indica* is straight or slightly curved rods and the size is 0.5-1.2 μm in diameter and 1.6-3.0 μm in length. Lipoid bodies persist in aged cultures. When *B. indica* is grown in a carbohydrate-containing medium, the colonies glisten due to capsular slime exopolysaccharide formation [3,13], as shown in Fig. 1.3.

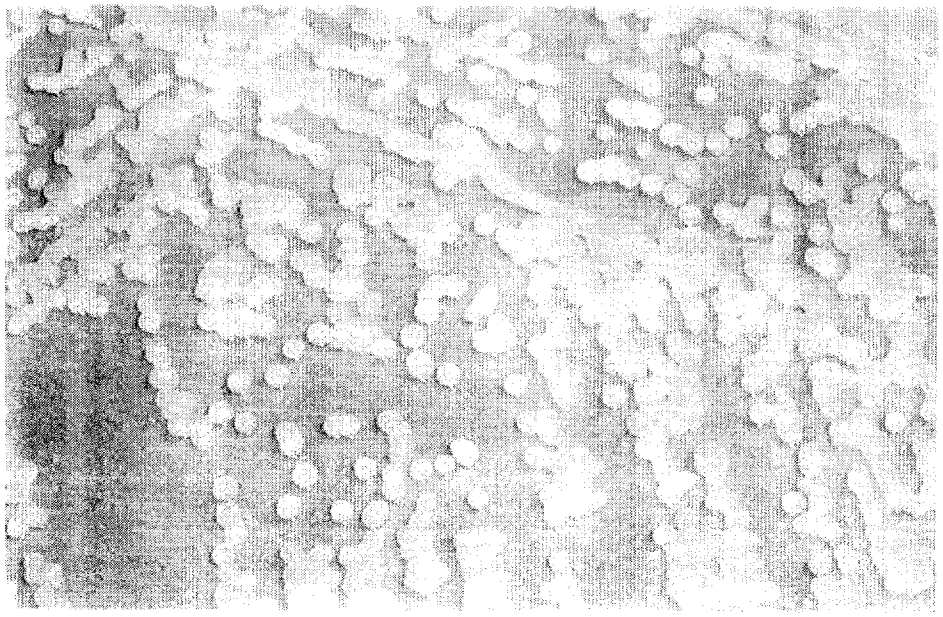

Fig. 1.3 Typical colony of *B. indica* grown in glucose-based MSM medium.

1-2-2. Biological function of PS-7 in *B. indica*

The role of exopolysaccharides (EPS) in nature has not been clearly established and is probably diverse and complex. It has been suggested that they may protect against desiccation, phagocytosis and phage attack, participate in uptake of metal ions, as adhesive agents or ATP sinks or to be involved in interactions between plants and bacteria [14].

Since *Beijerinckia indica* is a soil bacterium, the PS-7 might act as a role for adaptation to drastic change of chemical and physical conditions, such as energy storage, cell adhesion etc. In general, some microbial polysaccharides are produced in carbohydrate-rich media and these organisms employ Entner-Doudoroff (ED) pathway with carbon cycling [15]. Linton proposed that these ED organisms were mostly unable to control their high glycolytic flux. Thus, the extra carbon and energy would be spilled out in the form of EPS by carbon recycling to divert the glycolytic flux towards EPS [16].

1-3. Physiology of PS-7 production

1-3-1. Pathway of the precursors biosynthesis for PS-7 production

B. indica is able to utilize a wide variety of carbohydrates for growth (Table 1.2). Certain types of carbohydrates are, however, utilized more effectively than

others, and the resulting pH of the medium varies depending upon the carbohydrate source employed.

Fig. 1.4 shows the precursors biosynthesis from glucose containing medium. Glucose can be metabolized into glucose-6-phosphate or gluconate, but both intermediates are converted to gluconate-6-phoshpate, and fed into Entner-Doudoroff (ED), Pentose Phosphate pathway (PP) and Tricarboxylic acid cycle (TCA).

1-3-2. Genetics of PS-7 biosynthesis

The gene cluster responsible for PS-7 biosynthesis was identified, cloned and compared with that of *Sphingomonas S88* [17], as shown in Fig. 1.5. With the exception of genes *S*, *R* and *urf31*, all the clustered genes from strain S88 were also located in the *B. indica* DNA and in the same order with no other gap or insertions. The cluster includes four glycosyltransferase genes (*Q*, *K*, *L* and *B*), at least four genes of secretory proteins (*J*, *D*, C and *E*), dTDP-rhamnose biosynthesis operon genes (*rhs ACBD*) and transport genes (*atrD* and *atrB*).

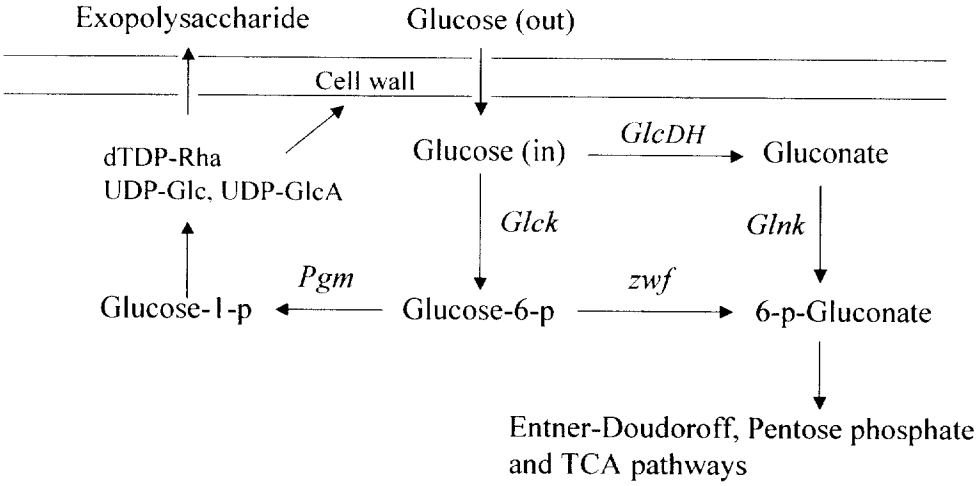

Fig. 1.4 Conversion of glucose into intermediary metabolites, cell walls and exopolysaccharides in *B. indica*. Abbreviation: GlcDH, glucose dehydrogenase; GlnK, gluconate kinase; GlcK, glucose kinase; Rha, rhamnose; Glc, glucose; and GlcA, glucuronic acid (after Thorne et al. [7]).

Table 1.2 Growth of *B. indica* on different carbohydrate.

Carbohydrate	**pH**	Time (days)				
		1	2	3	4	5
D-glucose	A	+++				
D-mannose	A	+++				
D-galactose	A	+++				
D-fructose	A	+++				
D-arabinose	A	+++				
D-xylose	A	+++				
D-ribose	WA					++
D-rhamnose	WA					++
D-sucrose	A	+++				
D-maltose	A	+++				
D-trehalose	A	++				
D-lactose	A			+++		
D-cellobiose	A	+++				
D-melibiose	A				++	
D-raffinose	A			+++		
Dextrin	WA					++
Salicin	A			+++		
Na-Alginate	-		++			
D-mannitol	Alk	+				
Inositol	Alk	+				
Adoitol	Alk	+				

Symbol: A = Acid; Alk = Alkali; WA = Weak acid; - = no change; (+) poor growth; (++) fair growth; (+++) good growth (after Kang & McNeely [3]).

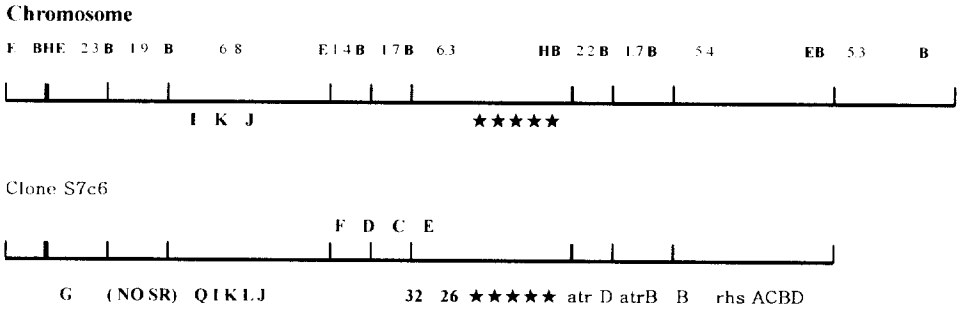

Fig. 1.5 Genetic DNA sequence of the exopolysaccharide synthetase gene cluster of *Sphingomonas* S7 (*Beijerinckia indica*). Abbreviations: B, *Bam*HI restriction endonuclease; E, *Eco*RI; H, *Hind*III; ★★, segment of strain S7 DNA not found in S88 cluster shown (after Thorne et al. [7]).

1-3-3. Nitrogen fixation

Aerobic nitrogen fixers are to be found mainly in the family Azotobacteriaceae that comprises the genera *Azotobacter*, *Azomonas*, *Beijerinckia* and *Derxia*. *Pseudomonas methanitrificans*, a methane-oxidizing organism also fixes N_2 aerobically. *Mycobacterium flavum* and possibly one or two related species are also fixers. The remainders of the aerobic bacteria that can fix N_2 are facultative anaerobes that only fix N_2 anaerobically. Anaerobic N_2 fixation is much more widely distributed [14].

Beijerinckia fixes N_2 under aerobic conditions and also under decreased oxygen pressures (microaerophilic conditions). On agar plates, especially on the conditions of no ammonium or poor nitrogen source, *Beijerinckia* may produce giant colonies containing copious amounts of slime (exopolysaccharide). This slime is often extremely tough, tenacious, or elastic, which makes it difficult to remove part of a colony with a loop. Like other N_2 fixers, *Beijerinckia* sp. requires molybdenum for optimum growth and N_2 fixation. Moreover, unlike *Azotobacter*, the molybdenum requirement cannot be replaced by vanadium [13].

1-3-4. CO_2 as a undesirable product

Under oxygen saturation conditions, respiration and biosynthesis of *B. indica* involves a more important electron sink and most of the carbon is directed to the TCA cycle and produce much CO_2. The biomass yield from substrate is small as

compared to other species and the PS-7 productivity was low as compared to xanthan from *Xanthomonas* [2].

Similar physiology was also found in *Sphingomonas elodea* during the production of gellan gum. In an effort to reduce the CO_2 production and overproduce gellan, a mutant without activity of glucose-6-phosphate dehydrogenase (*zwf*) was constructed, but CO_2 production was the same as the parent strain, thus low production of gellan [18].

1-4. Aims of this work

Several microbial polysaccharides are commercially produced in a large quality to satisfy the need of market. Xanthan and gellan have received approval by FDA and EU. The market of xanthan is estimated 40-50 million tons in 2004 and the price is only about 5~20$/kg depending on grade [19]. In contrast, the price of gellan is about 140 $/kg (Gelrite®, Sigma-Aldrich) and occupies a small market. The xanthan dominates the market due to the high yield from substrate and low price. The total market of polysaccharides is expanded every year. As a competitive gum, PS-7 is a potential alternative due to its novel properties. However, it is an important to reduce the production cost, especially to improve the yield and ability to utilize various carbon sources [1].

The general aim of the presented work is to optimize the process for the high production of PS-7 production. In addition, the biological role of PS-7 in *B. indica* was estimated and that would be of great help for understanding and optimizing

the process.

In order to realize the aim mentioned above, this study has been focused on the following main tasks:

1) Optimizing the culture condition of *B. indica* for PS-7 production in batch culture with flask and 5-L fermenter.

2) Evaluation the effects of various nitrogen and carbon sources in the culture of *B. indica*.

3) Characterization of carbon metabolic pathway of *B. indica*.

4) Evaluation of metabolic flux distribution between high and low C/N ratio in the MSM medium.

5) Obtaining a mutant from *B. indica* that can utilize the whey lactose to produce PS-7 effectively.

6) Optimizing the culture condition for PS-7 production in batch culture of the mutant, *B. indica* L3, using whey as a carbon source.

7) Characterization of the PS-7 produced by *B. indica* L3 using whey.

CHAPTER 2

Heteropolysaccharide-7 Production with Various Carbon and Nitrogen Sources

2-1. Abstract

B. indica was cultured in mineral salts medium (MSM) containing 2.5 mg/l Na_2MoO_4 and 2 mg/l $FeSO_4$ with various carbon and nitrogen sources to improve the production of PS-7. At high C/N ratio, the high concentration of PS-7 was produced until 40 h of the culture, whereas most of the glucose as a carbon source was used for the cell growth at low C/N ratio. However, at high C/N ratio, PS-7 accumulation stopped at 48 h of the culture because the increasing viscosity of the culture broth would inhibit the cell growth and mass transfer. Therefore, the optimal C/N ratio was 14.8 (mol/mol, 20 g/l glucose, 7.5 mM NH_4NO_3). In the culture with various carbon sources, *B. indica* effectively utilized the hexoses or glucose-generating sugars for PS-7 production. Especially, sucrose was the best carbon source for the high production of PS-7 (6.96 g/l). Increase of ammonium concentration could improve cell growth, while less PS-7 was produced. A proper range of ammonium concentration was advantageous to PS-7 production. The additional nitrogen sources of 7.5 mM of NH_4NO_3, glutamate and glutamine after nitrogen source exhaustion showed that nitrogen could regulate the metabolism of carbon sources and thus control the PS-7 synthesis.

2-2. Introduction

Microbial exopolysaccharides (EPS) are produced by various genera of bacteria and yeasts [20], and most of the related studies have focused on the optimization of culture condition for the production of polysaccharides. Noticeably, many

strains produce polysaccharides under nutrient-limited conditions. Among these, nitrogen-limited cultures have been often employed for the production of microbial polysaccharides [21-23].

Accumulation of the exopolysaccharide is controlled by the cell indigenous regulatory mechanism that might respond to the environmental condition, such as availability of carbon source, nitrogen source and so on. The sugars such as glucose can be either converted into exopolysaccharides or into additional cell mass by alternative routes of cell metabolism. Nitrogen source is one of the most important elements required for life, as it is necessary for the production of amino acids, nucleotides, amino sugars, NAD and *p*-aminobenzoate. In the production of commercial xanthan or gellan, the medium contains only a small amount or no nitrogen source [24-26]. In its natural habitat soil, *B. indica* encounters a wide variety of carbon sources and situation of insufficient carbon sources. This bacterium has to adapt to different metabolic patterns under different available carbon sources. Thus, the polysaccharide production by *B. indica* would be affected by various carbon and nitrogen sources significantly.

2-3. Materials and methods

2-3-1. Bacterial strain and medium

Beijerinckia indica HS-2001 is the UV-induced mutant of *Beijerinckia indica* ATCC 21423 and the strain was maintained on agar plates of a mineral salts agar

medium (MSM) consisted of the following components: 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 (Dofico Lab., Detroit, USA), 0.2 g/l Bacto peptone, 20 g/l glucose and 20 g/l agar. The medium was supplemented with 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% $Na_2MoO_4 \cdot H_2O$ and autoclaved. The carbon source (glucose or other carbon sources) was separately autoclaved for 15 min at 121°C and mixed aseptically with the MSM medium before the culture.

Yeast malt (YM) broth (Glucose 10 g/l, peptone 5g/l, yeast extract 2.5 g/l and maltose 2.5 g/l) was also used for inoculation preparation.

2-3-2. Culture methods

2-3-2-1. General culture methods

Starter cultures were incubated in 20 ml MSM medium in a 100 ml shake flask for 24 h at 30°C and 200 rpm in an orbital shake incubator. These cultures were used to inoculate in 50 ml medium in a 250 ml Erlenmeyer flask. The broth (150 ml) cultured for 24 h was used to inoculate in 3 l medium in a 5 l fermenter (KF-5, KFC, Inchon, Korea) installed with DO, pH and temperature probes. One vessel volume per minute (vvm) of air was supplied through a sparger, and the speed of agitation was controlled at 500 rpm. The other conditions were the same unless stated otherwise.

2-3-2-2. Effect of molybdate on cell growth and PS-7 production

Seed cultures were prepared in YM broth. In the MSM medium, different concentrations of sodium molybdate (0, 1, 2.5, 5.0 mg/l) were separately added to the flask culture.

2-3-2-3. Effect of Fe^{2+} on cell growth and PS-7 production

Seed cultures were prepared in YM broth. Different concentrations of iron sulfate (0, 4, 8, 12, 16, 20, 24 mg/l) in the MSM medium were added to the flask culture for 72 h.

2-3-2-4. Effect of nitrogen sources on PS-7 production and cell growth

MSM medium for test of nitrogen effect in the culture of *B. indica*, 0~24 mM of NH_4NO_3, $(NH_4)_2SO_4$ and KNO_3 replaced the 0.6 g/l NH_4NO_3 in MSM medium. The culture was performed in 250 ml flask for 72 h.

2-3-2-5. Effect of glucose/nitrogen ratio on *B. indica* growth and PS-7 production

Initially 10, 20 and 30 g/l of glucose were added in the MSM medium as carbon sources and the culture was performed in 5-l fermenter for 72 h with

intermittent sample at different time.

2-3-2-6. Effect of Nitrogen signal molecule on PS-7 production by *B. indica*

To evaluate the effect of nitrogen source on the production of PS-7, 0.2 ml of the autoclaved NH_4NO_3, glutamate and glutamine were added to the culture to a final concentration of 7.5 mM at the exponential growth.

2-3-2-7. Effect of carbon sources on *B. indica* growth and PS-7 production

The carbon source (20 g/l of fructose, glucose, sucrose) was dissolved in the medium and autoclaved separately for 15 min at 121°C, and then mixed with the MSM medium before the culture for study of the effect of carbon sources. To study the effect of addition of gluconate into glucose, fructose and sucrose medium (15 g/l), 5 g/l of gluconate was supplemented into the culture.

2-3-3. General analytical methods

Culture broths were centrifuged with proper dilutions at 15,000×*g* for 30 min at 4°C to remove the cells. Two volumes of isopropanol was added to the supernatant, and the mixture was kept overnight at 4°C to precipitate the PS-7. PS-7 was obtained as the pellet of precipitation by centrifugation at 10,000×*g* for 30 min. The pellet was washed by isopropanol and dried at 105°C for 4 h. To determine the biomass, the broths were centrifuged and the pellet was washed

with distilled water and dry cell weight was measured after drying the pellet at 105°C for 4 h. The residual glucose concentration was determined colorimetrically by the phenol sulfuric acid method [27]. The broth viscosity was measured at 30°C with a Brookfield DV III viscometer equipped with a 10 ml small-sample adaptor (SC-34) (Brookfield Engineering Laboratories, Stoughton, MA, USA).

2-4. Results and discussion

2-4-1. Optimization of the PS-7 production on batch culture

2.4.1.1. Effect of molybdenum on cell growth and PS-7 production

Molybdenum is required for growth of *B. indica* and cannot be replaced by vanadium. As shown in Fig. 2.1, the cell mass production increased two-fold when sodium molybdate concentration increased from 1 mg/l to 2.5 mg/l in the MSM medium. The PS-7 production was not affected by Molybdenum. Moreover, *B. indica* was cultivated in a relatively nitrogen-limited condition to produce PS-7 and bacteria might have to maintain cell activity with a small portion of nitrogen from N_2 fixation.

2.4.1.2. Effect of Fe^{2+} on cell growth and PS-7 production

Iron is also required for almost all the organisms. *B. indica* is aerobic bacteria and has strong respiratory activity, which requires the Fe-S proteins in the oxidative phosphorylation. As shown in Fig. 2.2, the cell mass production was higher and PS-7 production was poor when no $FeSO_4$ was supplemented into the MSM medium, as compared to other cases with various concentration of $FeSO_4$. There was no significant difference in PS-7 and cell mass production when $FeSO_4$ was higher than 2 mg/l.

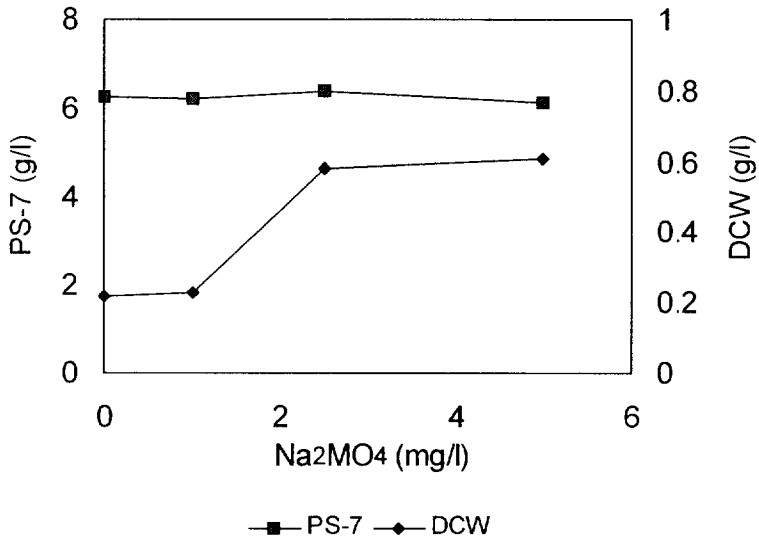

Fig. 2.1 Effect of molybdenum on the growth of *B. indica* and PS-7 production.

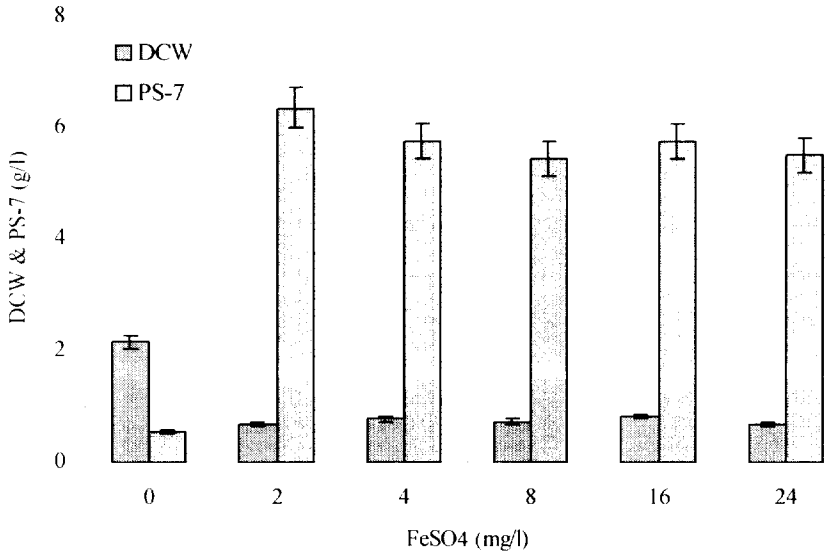

Fig. 2.2 Effect of iron on the growth of *B. indica* and PS-7 production.

2.4.1.3. Effect of agitation on cell growth and PS-7 production

Culture of *B. indica* required sufficient agitation to supply oxygen and enhance mass transfer due to high viscosity of produced polysaccharide. As shown in Fig. 2.3, maximum cell mass (2.9 g/l) was obtained at 600 rpm of agitation, but it decreased rapidly after exponential phase. In the cultivation at 500 rpm, considerable amount of biomass was maintained after reaching maximum (2.2g/l). The cell growth at 300 rpm and 400 rpm showed the same trend. The biomass production continued to increase after exponential phase and decreased at 48 h and 40 h of the culture, for cultivation at 300 rpm and 400 rpm, respectively. The final dry cell weight in the culture at 400 rpm was highest.

PS-7 was continuously accumulated in the culture at 500 rpm and the highest amount of PS-7 was 7.5 g/l at 72 h with a viscosity of 38174 cp and in the culture at 600 rpm, 5 g/l PS-7 was produced and it leveled off after 40 h, but the viscosity was the lower than 13000 cp before 64 h. Final PS-7 production in the culture at 400 rpm was close to that of the culture at 500 rpm. In the culture at 300 rpm, most polysaccharide was produced before 48 h (3.94 g/l).

DO and residual glucose profiles in the cultures at 400, 500 and 600 rpm were similar. DO in the culture at higher agitation gained earlier after dropping to zero. In the case of 300 rpm, the DO dropped and maintained zero due to poor mixing of medium. The final residual glucose at relatively high agitation speeds (400, 500, 600 rpm) was lower (0.7~2.2 g/l) compared to 5.8 g/l in the culture at 300 rpm.

The pH patterns followed decrease, increase and again decrease. pH in the cultures at 500 and 600 rpm was not changed too much. In the cultures at 300 and 400 rpm, pH dropped to a lower 5.85 at 24 h. The final pH in the cultures at 500 and 600 rpm was higher than those in the cultures at 300 and 400 rpm.

From the above results, it seemed that a suitable agitation was required to maintain the cell activity and produce PS-7 with a high viscosity. Cell mass decreased rapidly after exponential growth at higher agitation. At lower agitation (300 rpm), however, cell mass and PS-7 production were the lowest due to poor mixing of medium.

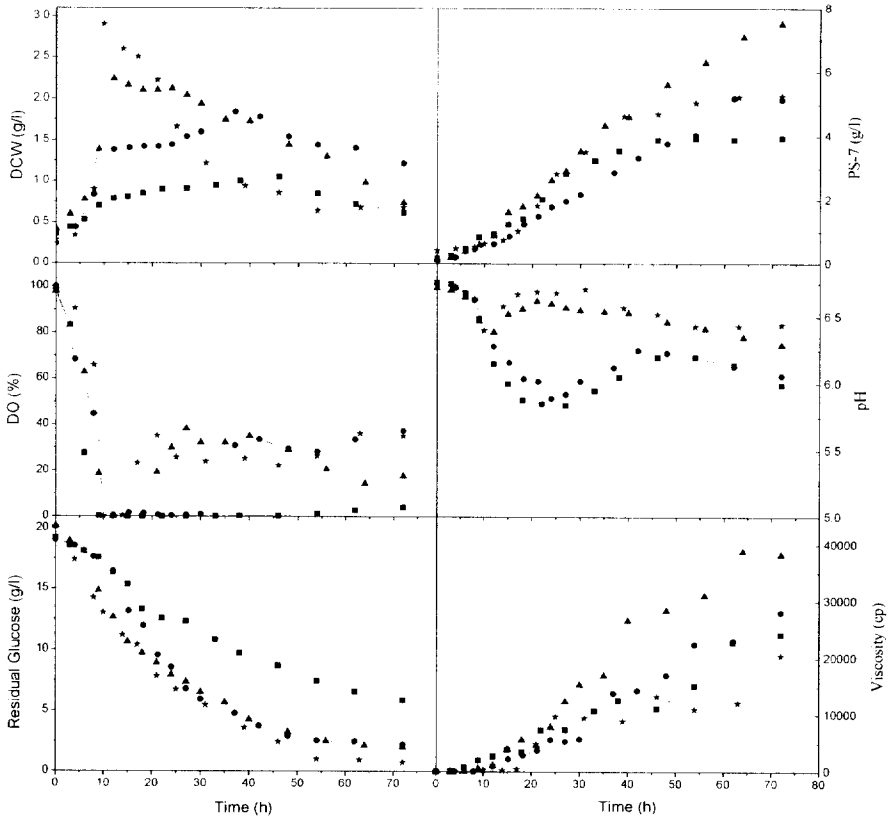

Fig. 2.3 Effect of agitation on the growth of *B. indica* and PS-7 production. Symbols: (■), 300rpm; (●), 400 rpm; (▲), 500rpm; (★), 600rpm.

2-4-2. Effect of nitrogen sources on PS-7 production and cell growth

B. indica is sensitive to the status of available nitrogen and showed metabolic variation in the culture with different inorganic nitrogen sources. As shown in Fig. 2.4, three inorganic nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$ and KNO_3) were used as nitrogen sources to cultivate *B. indica* and produce PS-7. The biomass production was small at low $(NH_4)_2SO_4$ concentration and increased from 0.44 g/l to 2 g/l when $(NH_4)_2SO_4$ increased from 4 mM to 8 mM (Fig. 2.4A). An increase of PS-7 production was also observed when $(NH_4)_2SO_4$ increased from 0 to 4 mM. Thus, the highest polysaccharide production obtained was 6.4 g/l in the culture with 4 mM $(NH_4)_2SO_4$ and then it dropped quickly. In the culture with various concentrations of KNO_3 (Fig. 2.4B), there was no significant difference in PS-7 production, and the highest PS-7 production was 4.1 g/l at 12.5 mM KNO_3. The viscosity of PS-7 from the culture containing KNO_3 was lower than that from the cultures containing $(NH_4)_2SO_4$ and NH_4NO_3. In the culture with NH_4NO_3 (Fig. 2.4C), when NH_4NO_3 increased from 12 mM to 20 mM, the PS-7 production decreased rapidly and the biomass production increased from 0.5 g/l to 1.44 g/l. The narrow range of 4 mM to 12 mM NH_4NO_3 was favorable for PS-7 production. Therefore, the maximal PS-7 production (6.48 g/l) was obtained at 8 mM NH_4NO_3.

Both the nature and concentration of nitrogen affected the cell growth and PS-7 production. Ammonium, nitrate or a combination of ammonium and nitrate, represented totally different nature of nitrogen status in the medium according to their redox state. Ammonium can be assimilated directly by cells for biosynthesis

of cell components. Interestingly, this strain could utilize nitrate though PS-7 production was relatively lower, in contrast to the description in Bergey's Bacteriology Determinative Manual [13]. Increase of ammonium resulted in less PS-7 production. However, the increase of nitrate concentration had no significant effect on polysaccharide production. Reduction of nitrate to ammonia is energy demanding and is not favorable nitrogen source for this strain. Similar phenomena were observed in the study on gellan production that cell growth in the culture with nitrate was slower than that with ammonia, though the presence of nitrate led to high gellan production in comparison with ammonia [24]. *B. indica* produced PS-7 at a low concentration of ammonium nitrate between 4 mM to 12 mM because the ammonium as nitrogen source exerted great influence on the polysaccharide production. In addition, other complex nitrogenous materials could be chosen as alterative to NH_4NO_3, such as soytone [28] and soybean pomace [29] as these natural organic materials have lower or none ammonia and are good for PS-7 production.

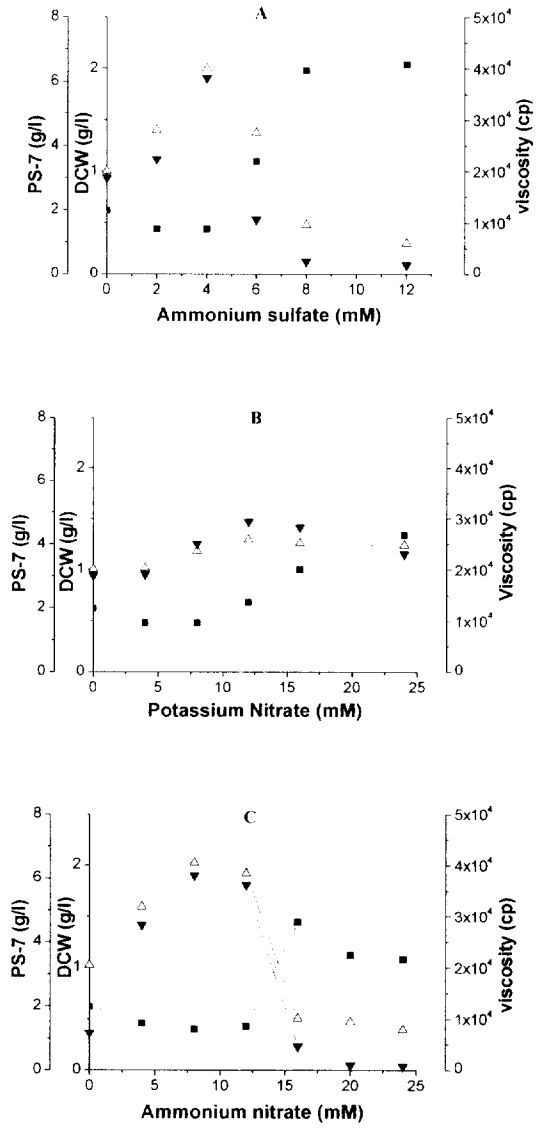

Fig. 2.4. Effect of $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3 on growth of *B. indica* and PS-7 production. symbols: (■) DCW, (△) PS-7, (▼) Viscosity.

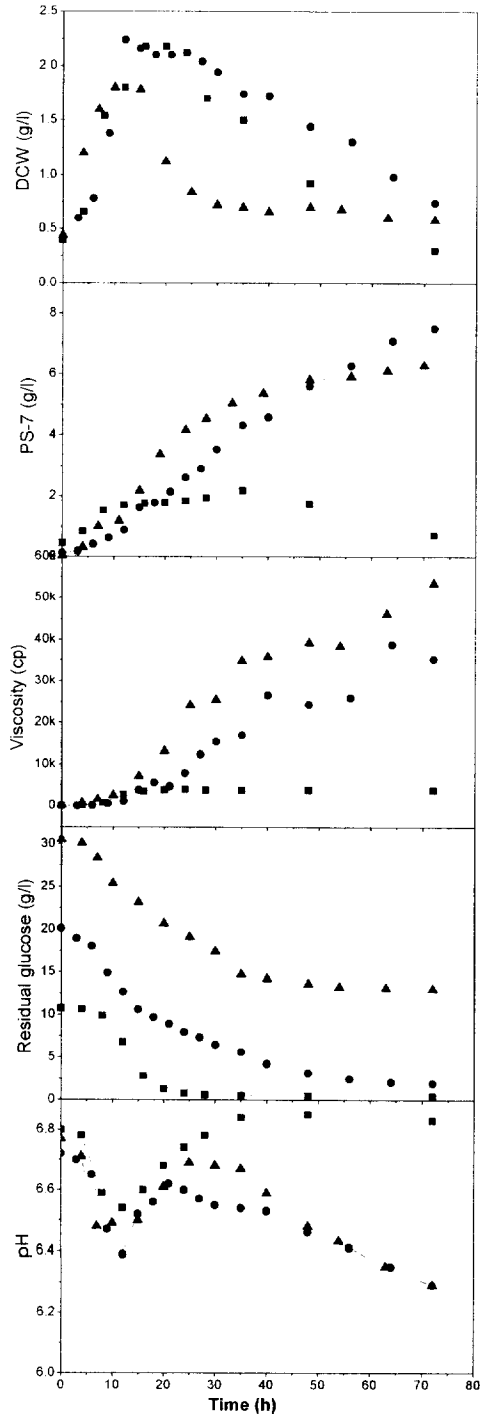

Fig. 2.5 Effect of different concentration of glucose with 0.6 g/l NH_4NO_3: (■), glucose 10 g/l; (●), glucose 20 g/l; (▲), glucose 30 g/l.

2-4-3. Effect of C/N ratio on *B. indica* growth and PS-7 production

The optimal conditions for operation of 5-L fermenter were obtained from the previous experiment [4]. In general, the ratio of carbon and nitrogen sources in the medium affects cell growth pattern. To study the effect of different C/N ratios on the cell growth and PS-7 production, initial glucose concentrations were adjusted to 10 g/l, 20 g/l and 30 g/l in the MSM medium with 0.6 g/l of NH_4NO_3.

As shown in Fig. 2.5, the cell mass reached the maximum at 12 –15 h and then dropped down. The highest cell density in the culture was 2.24 g/l with 20 g/l of glucose, as compared to 2.18 g/l and 1.78 g/l with 10 g/l and 30 g/l glucose, respectively. The final dry cell weight in the culture with 10g/l glucose was lowest compared to other two cases due to the consumption of all glucose. It is interesting to note that the cells began to accumulate PS-7 after the exponential phase when the cell mass decreased. The PS-7 concentration increased continuously in the culture with 20 g/l glucose. In the case of 10 g/l glucose concentration, the PS-7 reached 2.0 g/l at 35 h and then decreased because the substrate (glucose) was exhausted. At the highest glucose concentration (30 g/l), the PS-7 accumulation stopped after 40 h of culture due to low cell activity and mass transfer problem, after which the residual glucose was stable at about 15 g/l. This mass transfer problem seems to exist in the microbial polysaccharides production [24,25].

The apparent viscosity of the culture with 30 g/l glucose was highest compared to that with 10 g/l and 20 g/l glucose, which the highest viscosity (53509 cp) might be due to residual cell activity, though the PS-7 concentration (6.28 g/l) is

even a bit lower than that in the culture with 20 g/l glucose (7.5 g/l). The viscosity of the culture with 10 g/l glucose (3832 cp) was negligible as compared to the other two cases.

The profile of pH in the culture with 20 g/l and 30g/l followed of decrease, increase and again decrease. This is in agreement with the previous report [7]. However, the pattern of pH in the culture with 10 g/l glucose was different from the other two cases. After the pH decreased to 6.4, it increased to over 6.8 after 24 and leveled off.

It seems that appropriate C/N ratio promoted the production of PS-7. In the culture of heterotrophic organism like *Beijerinckia*, the C/N ratio defines the ratio at which the source of organic carbon and reducing equivalents are consumed per source of compound N. While the nitrogen is completely assimilated into biomass, part of the carbon substrate has to be dissimilated or stored in order to provide reducing equivalents as well as energy for substrate assimilation, cell maintenance and so on. Study on alginate production by *Azotobacter vinelandii* showed the C/N ratio that controlled the cellular O_2 consumption, the activity of respiratory system and the polysaccharide production [30]. The author suggested that under the nitrogen-limited condition (high C/N ratio), aerobically growing cells try to dissimilate or store the surplus carbon source to maintain a constant C/N ratio for biomass production. Other reports showed that C/N ratio played an important role in the polysaccharide production [26,31].

2-4-4. Nitrogen signal to regulate PS-7 production

In the regulation of nitrogen metabolism, there are two major assimilation pathways for ammonium, the glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway, which is ubiquitous in bacteria, and the glutamate dehydrogenase pathway, which is an alternative route of assimilation in many bacteria. The GS/GOGAT system is highly regulated in response to the nitrogen availability and the ratio of glutamine to 2-keto-glutarate could affect the expression of this system [32]. The signal of available nitrogen is shown by the intracellular glutamine and the ratio of 2-ketoglutarate to glutamine would regulate the cell metabolism, thus the carbon assimilation and carbon flux to polysaccharide biosynthesis [33].

In the typical culture with 0.6 g/l NH_4NO_3 (7.5 mM), the ammonium nitrate was nearly exhausted after the exponential growth (12 h) in the previous work [34]. To understand the regulation of PS-7 synthesis by nitrogen sources, 7.5 mM of ammonium nitrate, glutamine or glutamate were added separately at 12 h of the culture. As shown in Table 2.1, Addition of NH_4NO_3 had no significant effect on PS-7 production as compared to the control, but the viscosity was lower than the control. Glutamate promoted the consumption of glucose rather than that of the control, showing a lower 1.2 g/l residual glucose. The glutamine addition inhibited the PS-7 synthesis significantly, producing about 5 times more biomass than that of the control (0.24g/l). Thus, the highest cell growth and the lowest PS-7 production were shown in the culture with the addition of glutamine. This indicates that glutamine could directly participate into protein synthesis.

Moreover, the glutamine inhibited further utilization of carbon source, showing a high amount of residual glucose (10.6 g/l).

In general, ammonium is always the preferred as nitrogen source, as it can be assimilated directly into glutamine and glutamate, the key nitrogen donors for biosynthetic reactions. Glutamine can be synthesized by ammonia and glutamate. The additional glutamine led to more biomass production and inhibited PS-7 production. This seems to be that existence of glutamine inhibits the cells to assimilate more extracellular glucose for PS-7 synthesis involved in regulation between PTS dependent sugar transport and nitrogen metabolism [33,35]. Therefore, nitrogen source acts as an indicator of cell metabolic state in *Beijerinckia indica* to regulate the metabolism of carbon sources.

Table 2.1 Effect of nitrogen signal molecular on the cell and PS-7 production [a]

	Dry cell weight (g/l)	PS-7 (g/l)	Viscosity (cp) [b]	pH	Residual glucose (g/l)
Control [c]	0.24	6.64	5732	6.30	2.3
NH_4NO_3	0.28	6.56	4219	6.56	1.2
Glutamate	0.74	5.60	3213	6.36	2.1
Glutamine	1.46	1.92	599	5.08	10.6

[a] Cultures were performed in 250 ml shake flasks for 72 h and additional nitrogen sources were added at 12 hour of culture.

[b] Viscosity was determined using a SC4-34 spindle at 9 rpm.

[c] The culture was performed in MSN without addition of nitrogen source

2-4-5. Effect of carbon sources on *B. indica* growth and PS-7 production

In order to study the efficiency of various carbon sources for the production of PS-7, *B. indica* was grown on MSM media with glucose, fructose, sucrose and gluconate as energy sources. As shown in Table 2.2, maximal PS-7 production (6.96) was obtained when *B. indica* was grown with sucrose. PS-7 production with glucose and fructose came the next, 6.4 g/l and 4.74 g/l, respectively. This is consistent with the results described by Ashtaputre & Shah [36], who also reported that sucrose was the best carbon source for EPS production with high viscosity by the same bacterium.

It seems that *B. indica* prefer the hexoses or glucose-generating sugars for polysaccharide formation. Hexoses were assimilated into cell and metabolized through the ED or Pentose phosphate pathway. The precursor G-6P required for PS-7 synthesis could be produced through gluconeogenesis pathway from triose-3-phosphate from ED pathway or semi-oxidative Pentose Phosphate pathway. The yield of PS-7 in flask batch culture (32%) was smaller as compared to 70% for xanthan production [25]. Even after augmentation of chromosomal genes with multiple copies of biosynthetic gene through recombinant technology in the same strain, the yield of glucose reached about 60% [7]. Overexpression of *pgm* gene (phosphoglucomutase) in *B. indica* [7] or knock-out of glucose-6-phosphate dehydrogenase (*zwf*) of *B. indica* [18] led to no change in PS-7 production. It is assumed that the alternative GlnK (gluconate kinase) route of glucose assimilation (glucose is converted to gluconate, then to

6-phosphogluconate) was dominant over the GlcK (glucose kinase) route (glucose to glucose-6-phosphate). Thus the pool of glucose-6-phosphate would be low or it would cost energy to be formed through triose-3-phosphate cycle [7,18].

The gluconate can be transported and converted to gluconate-6-phosphate by gluconate kinase, and then it can be metabolized and fed into the ED pathway and the PP pathway [18]. Thus, presence of gluconate might affect the assimilation and metabolism of hexoses. Three carbon sources (20 g/l of glucose, sucrose and fructose) mixed with 5g/l gluconate were used as carbon sources for the cultivation of *B. indica* and PS-7 production. As shown in Table 2.2, different metabolic patterns depending on carbon sources were observed. When gluconate was added into the fructose-containing MSM medium, the PS-7 production and its viscosity increased as compared to that with fructose only. The reason might be that degradation of gluconate can provide triose-3-phosphate and pyruvate, and the triose-3-phosate enhances the triose-3-phosphate cycling and thus more fructose can be directed to sugar nucleotides synthesis through the gluconeogenesis [34]. However, the addition of gluconate into sucrose and glucose medium resulted in relatively lower PS-7 productions, corresponding to lower apparent viscosity.

According to Boza [37], the main carbon sources for cellular growth of *Beijerinckia sp.* were glucose, sucrose and fructose. This strain in this work lacks of fructose-6-phosphate kinase [7]. The strains growing in soil such as *B. indica*, where carbon sources of sugar are scare and sugar acids are the most common carbon source, employ the ED pathway dominantly [38]. The intermediate

glucose-6-phosphate can be catalyzed to glucose-1-phosphate and then sugar nucleotides are used for polysaccharide biosynthesis. When cells were grown in glucose or sucrose medium, existence of gluconate might inhibit the phosphoglucoisomerase (*pgi*) activity [15] and thus would hamper the triose-3-phosphate cycling, and then might result in the decrease in PS-7 production.

Table 2.2 Effect of carbon source on growth and polysaccharide production (72 h culture in shake flask)

Carbon source	DCW (g/l)	EPS (g/l)	Viscosity (cp)
Glucose	0.60	6.4	38950
Fructose	0.56	4.72	25615
Sucrose	0.52	6.96	40772
GLC+0.5%Gluconate	0.82	5.28	26694
FRU+0.5%Gluconate	0.54	5.96	34673
SUC+0.5%Gluconate	0.79	5.84	29184

* GLC : Glucose, SUC : Sucrose, FRU : Fructose.

CHAPTER 3

Metabolic Flux Analysis of *Beijerinckia indica* for Heteropolysaccharide-7 Production

3-1. Abstract

In order to investigate central metabolic changes in *Beijerinckia indica*, the cells were grown with different carbon sources and intracellular flux distributions were studied under varying concentrations of nitrogen. Metabolic flux was estimated by combining material balances with extracellular substrate uptake rate, biomass formation rate, and exopolysaccharide (EPS) accumulation rate. Thirty-one metabolic reactions and 30 intracellular metabolites were considered for the flux analysis. The results revealed that most of the carbon sources were directed into the Entner-Doudoroff pathway, followed by the recycling of triose-3-phosphate back to Hexose-6-phosphate. The pentose phosphate pathway was operated at a minimal level to supply the precursors for biomass formation. The different metabolic behaviors under varying nitrogen concentrations were observed with flux analysis.

3-2. Introduction

Metabolic flux analysis (MFA) is a powerful technique used to characterize intercellular fluxes of microorganisms, which aid the identification of potential genetic modifications that may improve the behavior and productivity of microorganisms [39]. The metabolic fluxes distributed within microorganisms are estimated through a combination of biochemical, microbiological, and mathematical approaches, as well as data from experimental measurements. The flux distributions are correlated to the strength of each individual enzyme, which

represents the *in vivo* metabolic state of the cell. The flux information is then used to examine the influence of process parameters or cultivation conditions on the physiological states of microorganisms and to identify rate-limiting steps in pathways. Once such steps are identified, molecular biological techniques can be applied to modify the corresponding gene or enzyme to bring about significant shifts in the yield of desired end products.

In this work, *Beijerinckia indica* was cultivated in media with different carbon sources and a high or low nitrogen content for PS-7 production. Metabolic flux was calculated to investigate the flux distribution in the central metabolism of the strain and metabolic changes under different nitrogen conditions. The Pentose Phosphate pathway (PP), the semi- Embden-Meyerhof-Parnas (EMP) pathway, the Entner Doudoroff (ED) pathways, the Tricarboxylic acid (TCA) cycle, and EPS synthesis were used in the proposed metabolic network for metabolic flux analysis.

3-3. Materials and methods

3-3-1. Culture condition and methods

In order to investigate various nitrogen levels, the cells were grown in MSM media supplemented with 0.6, 1.3 and 1.5 g/l ammonium nitrate (corresponding to 7.5, 16.25 and 18.75 mM). Cultivation was performed in 5-l fermenter as described before.

3-3-2. Analytical methods

The amount of ammonia in the culture was determined by the indophenol blue method [40]. The nitrate was determined by the method by Cataldo et al. [41].

3-3-3. Evaluation of metabolic flux distribution

The biochemical reaction network of *B. indica* was constructed based on previous reports of Klinke et al. [42], Fabien et al. [43] and Vartak et al. [18]. The results of sequence analysis data of the *Pseudomonas* and *Azotobacter* genome were also examined [44,45]. Serial reaction steps were considered as a "lumped" reaction (several reactions combined in one equation). The stoichiometric matrix was formulated according to the biochemical reactions (see Appendix) present in *B. indica*. The metabolite requirements for biomass synthesis were obtained from *Physiology of the Bacterial Cell: A Molecular Approach* [46] with the modifications proposed by Ampe et al. [47]. The principles of stoichiometric flux analysis, based on the metabolite balance, biochemical constraints and pseudo-steady-state assumption for intracellular metabolites, have been extensively described by Valino et al. [48]. Specific metabolite concentration can be balanced by the dynamic flux balance. Mass balance equations were formulated from the biochemical network of *B. indica* (Appendix) by MetaFluxNet® [49]. Boundaries were imposed so that irreversible reactions had nonnegative fluxes. The carbon dioxide and oxygen balance were excluded. The

dimension of the stoichiometric matrix was 31×30 and the-linear equation system was solved by using the *linprog* function from the MATLAB Optimization Toolbox (The Mathworks Inc., MI). The biomass molecular weight was assumed to be 100 g/M with 3% ash. Three fluxes (r_1, r_{29}, r_{30}) were directly calculated from the experimental data and all the fluxes (mM/(g DCW.hr)) were normalized with respect to glucose uptake rate.

3-3-4. Enzyme assays

Enzyme assay of L-malate Dehydrogenase (MDH) in TCA cycle were according to the method described by Labrou and Clonis with some modification. Briefly [50], The cells (5 ml, 12 h of cultivation) were harvested by centrifugation and washed once with 0.9% NaCl (4.5ml) and once with 50mM Potassium phosphate buffer containing 10 μM EDTA (pH 7.5). The cells were suspended in 100 mM Tris-HCl buffer (pH 8.8) and were disrupted by sonicoscillation for 1 min at 4°C. Unbroken cells and debris were removed by centrifugation at 15,000 $\times g$ for 30 min and the supernatant solution was used for the enzyme source. Protein content of the extracts was measured by the method of Bradford et al with bovine serum albumin as a standard using a Bio-Rad kit [51]. Three ml of reaction mixture was prepared by mixing 2.8 ml of 100 mM Tris/HCl buffer (pH 8.8), 0.1 ml of 100 mM NAD^+, 0.1 ml of 1 M L-malate and 0.1 ml of sample. The enzyme assays were performed using a Shimadzu UV-2000 spectrophotometer by measuring the increase in absorbance at 340 nm due to NAD^+ reduction. One unit of enzyme activity, for the oxidation of L-malate, is defined as the amount that

catalyses the conversion of 1 mmol of NAD to NADH, per min at 30° C.

3-4. Results and discussion

3-4-1. Characterization of Carbon Metabolic Pathway in *B. indica*

B. indica was grown in minimal salt media with glucose, glucosamine, gluconate or glycerol as carbon sources. As shown in Fig. 3.1, when *B. indica* was grown with glucose, a significant amount of carbon source had accumulated as exopolysaccharide (6.4 g/l), and final biomass production (0.6g/l) was low due to limited nitrogen content. Glucosamine was a favorable carbon source for cell growth, with a final dry cell weight of 2.66g/l but no production of polysaccharide. In media containing glycerol, the cells grew poorly and produced a small amount of exopolysaccharide with low viscosity. When gluconate was used, a biomass of 1.72 g/l and the PS-7 concentration of 2.1 g/l were produced.

As with the majority of *Pseudomonas sp.* [42], *B. indica* [7] employs the ED pathway, PP pathway, and TCA cycle as central metabolic pathways (Fig. 3.2). This was confirmed by the observation that *B. indica* was able to grow with gluconate. Glycerol was metabolized through triose-3-phosphate [52] and the PP pathway, and gluconeogenesis were active to supply precursors for cell growth and polysaccharide biosynthesis. A likely reason for the increased cell mass production and inhibited polysaccharide production with glucosamine is that glucosamine degrades through the fructose-6-biophosphate pathway [53]. Thus,

the resulting ammonia produced from this pathway was released and the ammonia concentration in the medium increased. The high available ammonia concentration was favorable for cell growth and might inhibit polysaccharide synthesis. This was similar to the result in our previous study in which cell metabolic behavior had changed significantly to inhibit polysaccharide production in a flask culture with high concentrations of nitrogen (18.75 mM) (data not shown). In Fig. 3.2, a central carbon metabolic pathway network and polysaccharide biosynthesis in *B. indica* were proposed as mentioned above and in previous reports [7,18,43].

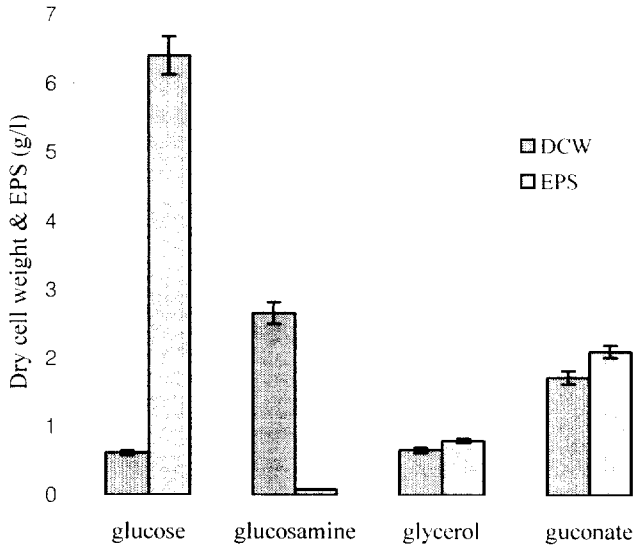

Fig. 3.1 Effect of different carbon sources on cell metabolism.

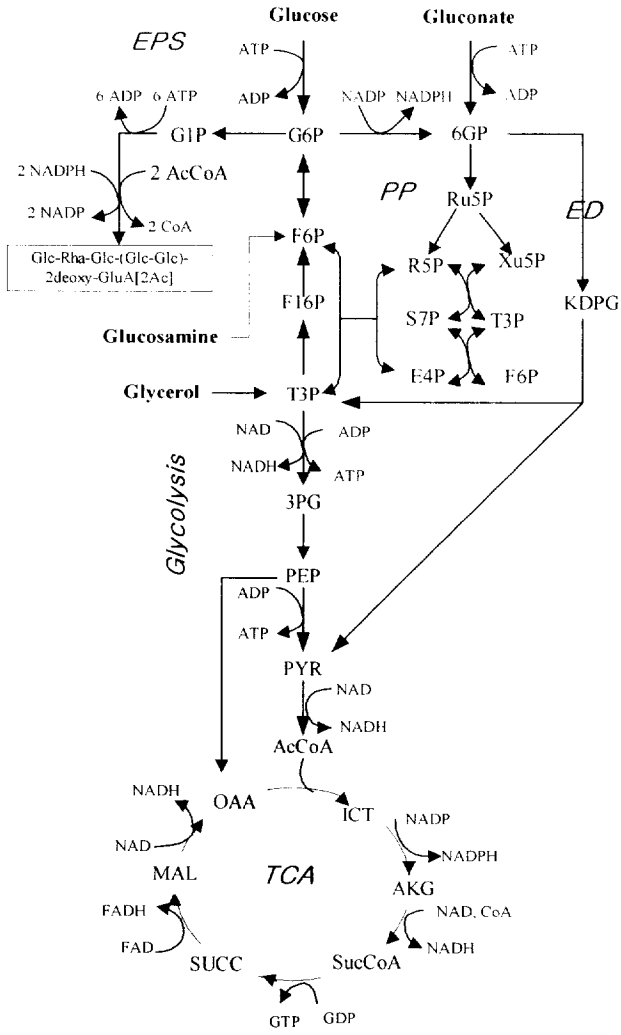

Fig. 3.2 Proposed metabolic pathway network in *B. indica* with different carbon sources.

3-4-2. Growth of *B. indica* under Different Nitrogen Concentrations

As previously mentioned, the findings suggest that ammonia might inhibit polysaccharide synthesis. Thus, the metabolic behaviors of *B. indica* were analyzed with various concentrations of nitrogen sources. Batch cultures of *B. indica* in a 5-L fermenter were carried out aerobically under various ammonium nitrate concentrations (7.5 mM, 16.25 mM, 18.75 mM). As shown in Fig. 3.3, when 7.5 mM NH_4NO_3 was used, the cells reached the exponential phase rapidly, dry cell weight increased slowly from 9 h to 20 h, and then the cell density decreased gradually (Fig. 3.3A). At the cell growth phase at 9 h, approximately 50% of glucose and nearly all ammonia were consumed. Nitrate concentration was constant when ammonia was present in the broth and was only utilized after ammonia was consumed, demonstrating the preference of ammonia to nitrate as a nitrogen source by *B. indica*. The polysaccharide concentration continued to increase and more than 70% of the total polysaccharide was produced after 20 h. The viscosity showed a parallel increase to the PS-7 polysaccharide profile. Glucose was consumed at a moderate rate, corresponding to a gradual accumulation of polysaccharide (Fig. 3.3A).

In the culture with 16.25 mM of NH_4NO_3 (Fig. 3.3B), the profiles of ammonia and nitrate are similar to those in Fig. 3.3A, with the exception that the time for total consumption of ammonia increased. The cell density was increased from 6 h to 16 h. The maximum cell density was 2.2 g/l that was higher than that obtained with 7.5 mM NH_4NO_3 (1.78 g/l). The polysaccharide concentration increased

until 40 h. The maximal PS-7 concentration was 5.9 g/l that was lower as compared to 7.42 g/l obtained with 7.5 mM NH_4NO_3. Glucose consumption in the culture was drastic and nearly 90% glucose was exhausted before 32 h, leading to less PS-7 accumulation and stop of PS-7 production after 40 h.

In Fig. 3.3C (18.75 mM NH_4NO_3), the metabolism profile was significantly different from the former two experiments (Fig. 3.3A and B), even though the NH_4NO_3 concentration was slightly higher than the experiment shown in Fig. 3.3B. Most of the ammonia was consumed at 22 h when the cell density reached stationary phase. However, the nitrate was not utilized. The cells started to produce PS-7 at the cell growth phase. The highest concentration of PS-7 (2.1 g/l) concentration was lower than those of the former two experiments. Viscosity was also low. The residual glucose concentration was constant after 22 h and 60% of glucose was not consumed. Glucose was consumed slowly before 6 h, indicating that cells would have to adapt to the condition of high nitrogen concentration

In Fig. 3.3D, the pH pattern of the culture with a high nitrogen level (18.75 mM) was different from those of low nitrogen levels (7.5 mM, 16.25 mM). The pH decreased to 4.3 due to the consumption of glucose and formation of organic acids. At the relatively low ammonia concentration (7.5 mM, 16.25 mM), pH profiles decreased by the formation of acids at an early stage and was increased due to the utilization of the acids after the depletion of ammonia. The pH of the cultures was also affected when ammonia was consumed. The pH of the culture decreased to 5.0 with 16.25 mM NH_4NO_3, and just 6.2 with the low concentration of ammonia (7.5 mM NH_4NO_3).

Most of the bacteria used for polysaccharide production from glucose or glucose derivatives cannot employ the EMP pathway since the phosphofructokinase activity was absent [18,43,54]. Therefore, glucose was metabolized through the Entner-Doudoroff pathway, as described for *X. campestris* [55], *Sphingomonas* [7] and *Sinorhizobium* [56]. Therefore, less ATP is produced through the ED pathway rather than the EMP pathway. However, triose-3-phosphate can be rerouted to glucose-6-phophate, thus glucose-6-phosphate can be channeled to polysaccharide formation or again into the ED pathway [57].

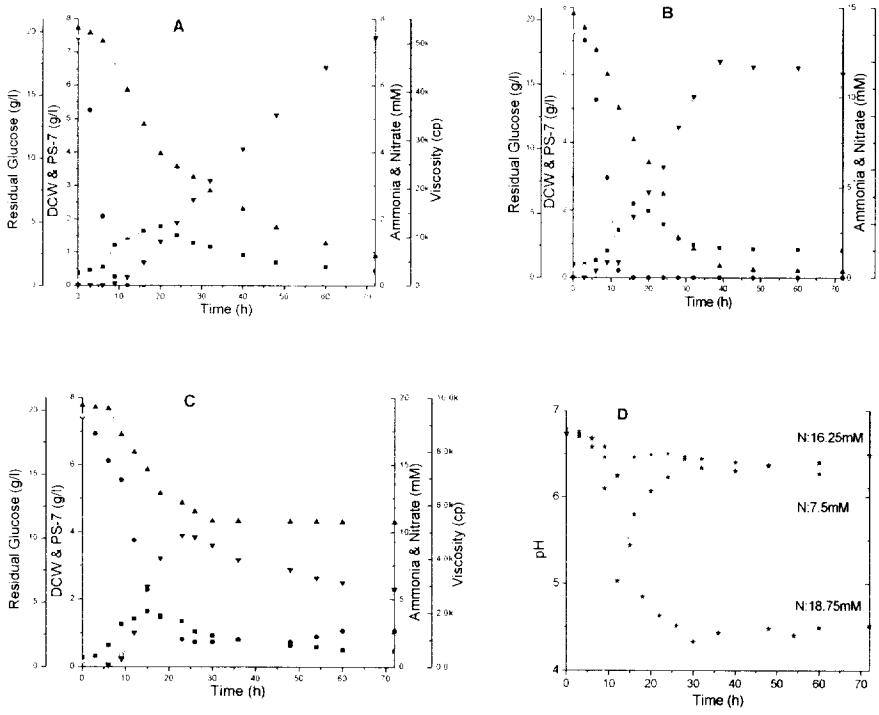

Fig. 3.3 PS-7 productions by *B. indica* under various concentrations of nitrogen source in a 5-L fermenter. A: culture with 7.5 mM NH_4NO_3, B: culture with 16.25 mM NH_4NO_3, C: culture with 18.75 mM NH_4NO_3, D: pH profiles of the cultures with 7.5 mM, 16.25 mM and 18.75mM NH_4NO_3. Symbols: (■), DCW; (□), PS-7; (●), Ammonia; (○), Nitrate; (▲), Glucose; (▼), Viscosity; (★), pH.

3-4-3. Metabolic Flux Analysis between High and Low C/N Ratio

From the findings obtained from the three cultures in Fig. 3.3, it is clear that *B. indica* exhibited different metabolic behavior at different NH_4NO_3 concentrations. Fig. 3.4 shows the calculated metabolite fluxes through the PP pathway, the ED pathway, and the TCA cycle under different nitrogen concentrations. Cells harvested from the cultures with various nitrogen concentrations clearly showed different fluxome pattern.

The ED pathway had operated dominantly while the PP pathway maintained a lesser degree for anabolic precursor synthesis. When nitrogen concentration (NH_4NO_3) was slightly increased in the culture from 16.25 mM to 18.75 mM, the metabolism of bacteria was significantly changed. The gluconate-6-phosphate flux into the pentose phosphate pathway was 5 times higher than that of low nitrogen level conditions, though the flux to biomass was not greater than that of low nitrogen level condition. The PP pathway operated in an oxidative model and the carbon flux was recycled to fructose-6-phosphate and triose-3-phospahte, and approximately 29% increase of r_2 resulted from recycle of fructose-6-phosphate in the PP pathway. The triose-3-phosphate directed to glycolysis (r_5) was also 2 times higher that that in culture with 16.25 mM NH_4NO_3, resulting in more ATP and NADH productions.

The flux into gluconeogenesis pathway (T3P to F6P) was maintained stably at a rate of 1/3 of substrate uptake rate (r_1) and this increased the flux r_{10} (G6P to

6GP) higher than the substrate uptake rate. This was similar to the result (Fig. 3.3) that much more glucose was consumed at the growth phase. The reaction of PEP to PYR was carried out at a slower rate at low nitrogen levels and most of the produced PEP was used for anaplerotic reaction (OAA). At the high nitrogen levels (18.75 mM), this flux was about 16 times higher than those with the low nitrogen levels (7.5 mM NH_4NO_3, 16.25 mM). TCA cycle flux under the high nitrogen concentration was about 1.64 times that at low nitrogen levels. As a result, excessive energy was formed and dissipated. With regard to glycolysis (r_5), the flux from the culture with 16.25 mM NH_4NO_3 was about 70% of that with 7.5 mM NH_4NO_3 condition. This corresponded to low biomass flux and anaplerotic flux r_9 (PEP to OAA).

There was no increase in biomass production or polysaccharide production in the culture with high nitrogen level, and even the produced PS-7 had low molecular weight, which was estimated from the low viscosity. The bacteria utilized the nitrate as a nitrogen source after the consumption of ammonia when NH_4NO_3 was added to the culture. A large amount of residual nitrate was found in the culture with 18.75 mM NH_4NO_3. But the PS-7 with high viscosity could be only accumulated under a high carbon to nitrogen ratio, signifying that the glucose was used for polysaccharide formation according to the flux distribution.

The flux distribution of the cultures with relatively low nitrogen levels (7.5 mM & 16.25 mM) had similar flux distributions. In the culture with 16.25 mM NH_4NO_3, the flux to biomass was less than that cultured with 7.5 mM nitrogen level and more glucose (r_{30}) was channeled to polysaccharide biosynthesis at the

exponential phase, which were similar to the results shown in Fig. 3.3A and B. However, PS-7 accumulated until 72 h in the culture with 7.5 mM NH_4NO_3. In the case of the culture with 16.25 mM NH_4NO_3, the amount of PS-7 reached the maximal level, 5.9 g/l at 40 h. This might be due to that the metabolic activity of the cells was higher and more energy was dissipated (r_{31}) rather than that in the culture with 7.5 mM NH_4NO_3. In addition, the nitrate in the culture with 7.5 mM NH_4NO_3 was depleted early, at 16 h, and this created a high C/N ratio that was favorable for PS-7 biosynthesis. Most of the nitrate in the medium at the level of 16.25 mM NH_4NO_3 was consumed before 40 h after the consumption of ammonium. Utilization of nitrate required more energy compared to ammonium utilization.

In the culture with 18.75 mM NH_4NO_3, the flux in the TCA cycle and the dissipation of energy (r_{31}) had nearly doubled. After cell growth came to a stop, there was still a small quantity of ammonium and much nitrate in the medium. Under a low carbon/nitrogen ratio, the cells could not channel the substrate flux to PS-7 formation. Thus, the amount of PS-7 reached the maximum of 2 g/l at 24 h and then ceased to increase (Fig. 3.3). *Xanthomonas camrestris* can convert 70% of glucose to xanthan. In this study, *B. indica* can only convert about 40% of glucose to PS-7 in batch culture. Whereas, Thorne et al. reported that a recombinant strain with augmentation of EPS biosynthetic gene could convert 60% glucose to PS-7 formation [7]. The reason might be that triose-3-phosphate recycling into glucose-6-phosphate (gluconeogenesis pathway) is energetically more inefficient than glycolysis (T3P to PYR). However, in a ^{13}C-labelled metabolic flux analysis with *Azotobacter vinelanii*, bacteria metabolized glucose

through the Entner-Dodoroff pathway and synthesis of alginate occurred completely from the gluconeogenesis of triose-3-phosphate generated from the ED pathway [54]. In this study, there was approximately 33% triose-3-phosphate recycling to hexose-6-phosphate under all conditions of nitrogen concentrations. This cyclic organization had negative effects on cell metabolism. If no ATP was directly consumed throughout the gluconeogenic part of the cycle, the conversion of two triose-3-phosphates into one glucose-6-phosphate occurs at the expense of a phosphoester bond. Thus, the cyclic organization of carbohydrate metabolism in *Beijerinckia indica* appears to be dissipative at the level of both carbon and energy. The repeat unit of PS-7 includes 2-deoxy-glucuronate and formation of these sugar nucleotides requires 2 M NADPH per repeat unit. Therefore, biosynthesis of PS-7 is more energy demanding rather than xanthan.

3-4-4. Enzyme activity under different nitrogen concentration

To confirm the physiological status of *B. indica* under different nitrogen concentration, one of the important enzymes in TCA cycle, Malate Dehydrogenase was determined. As shown in Table 3.1, Malate Dehydrogenase activity increased with the increase of NH_4NO_3 concentration in the MSM medium. At the high nitrogen concentration (18.75mM), the Malate Dehydrogenase activity nearly doubled as compared to that of the culture with 7.5 mM NH_4NO_3, confirming the drastic metabolic flux change when NH_4NO_3 concentration increased from 7.5 mM to 18.75 mM.

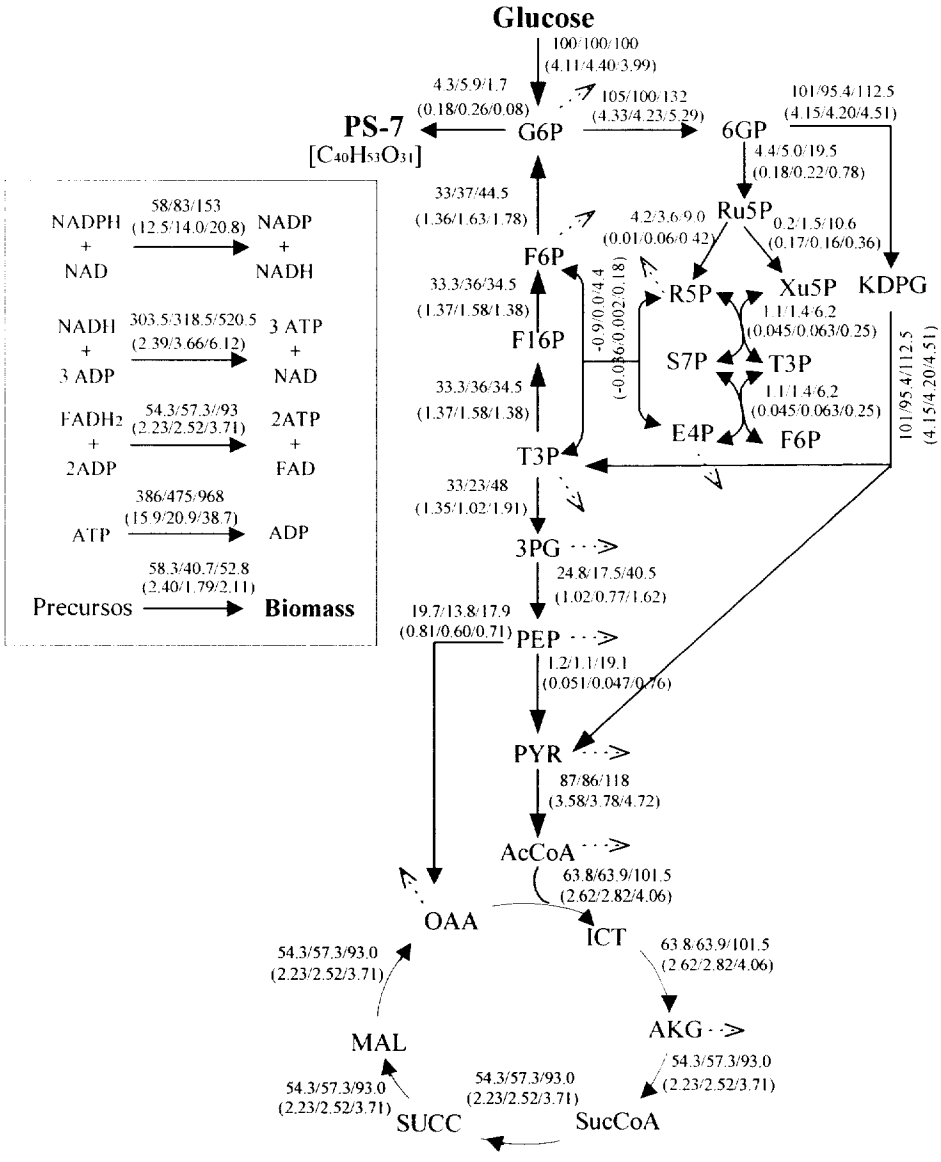

Figure 3.4 Flux distribution under different levels of ammonium nitrate (7.5mM/ 16.25mM/ 18.75mM). The fluxes (mM/(g DCW·hr)) include normalized fluxes and actual fluxes (in parentheses). The symbol, ⇢, indicates the precursors for biomass formation.

Table 3.1 Malate Dehydrogenase activities in *B. indica*

NH_4NO_3 (mM)	Malate Dehydrogenase (U*)
7.5	42
16.25	60
18.75	75

* One unit (U) causes the reduction of one micromole of NAD per minute under the conditions.

CHAPTER 4

Heteropolysaccharide-7 Production from Lactose/whey by *Beijerinckia indica* L3

4-1. Abstract

A mutant strain of *B. indica* L3 screened by a traditional chemical mutagenesis was grown in lactose-based mineral salts medium (MSM) for the production of heteropolysaccharide-7 (PS-7). The beta-galactosidase was expressed constitutively in the mutant and its activity was higher than that in parent strain. The highest amount of PS-7 produced by the mutant was 2.88 g/l with a viscosity of 4530 cp in lactose-based MSM medium. The PS-7 production by the mutant was enhanced by the addition of 4 g/l glucose into lactose-based MSM medium, reaching 5.52 g/l with a viscosity of 39531 cp. PS-7 of 6.18 g/l with a viscosity of 45772 cp was produced by the mutant grown in a whey medium. The PS-7 production by the mutant reached 7.04 g/l when 4.0 g/l glucose was added into the whey medium. Compositional analysis of PS-7 produced by the mutant grown in whey medium or lactose-based MSM medium showed that the ratios of glucose/rhamnose were maintained in the similar range of parent strain, indicating the same compositional structure of PS-7. Optimized conditions for PS-7 production by *B. indica* L3 with whey medium were as follows: 20 g/l whey lactose, 7.5 g/l of $KH_2PO_4 \cdot 7H_2O$, 1 g/l glucose and 1 ml elemental solution. Rheological study showed that the PS-7 produced by *B. indica* L3 was twice viscous than that of xanthan and appeared excellent pseudoplasticity.

4-2. Introduction

Glucose is somewhat of high price though it is a primary carbon source in

synthetic media for polysaccharide production [1]. Thus, there is a growing interest in using renewable natural carbon sources of agro-industrial origin because of low cost feedstock for the production of microbial metabolites. In addition, it solves environmental and energy problems related to their disposal [21,29,58].

Cheese whey is a nutrient-rich dairy byproduct from cheese production process, which contains 4% to 5% lactose, 0.8 to 1% proteins, minerals, trace amount of vitamins and some small organic molecules [59]. 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. Whey can be also used as a substrate for the production of valuable products such as polysaccharides. In this study, the utilization of whey by *B. indica* mutant for the production of PS-7 was evaluated.

4-3. Materials and methods

4-3-1. Bacterial strain and culture conditions

Beijerinckia indica (ATCC 21423) was maintained on agar plates prepared by mineral salts medium (MSM) containing 20 g/l glucose as a carbon source as described in chapter 2. In addition, 20 g/l lactose was added to MSM medium to replace the 20g/l glucose to prepare a lactose-based MSM medium for the

screening of mutants. The mutant strain, *Beijerinckia indica* L3, was grown in MSM medium with 16 g/l lactose and 4 g/l glucose. The seed culture of the mutant was prepared in yeast lactose (YL) medium consisted of 10 g/l lactose, 5 g/l Bacto peptone and 3 g/l Bacto yeast extract.

Whey medium was prepared by the method outlined by Dlamini [60] with some modification as follows: 30 g/l whey powder was dissolved in distilled water and the pH of the mixture was adjusted to 4 by the addition of 1 M HCl. Then the mixture was heated at 100°C for 5 min and cooled to room temperature. The solids were removed by centrifugation at 12,000×*g* for 30 min at 4°C. The supernatant (pH 6.8) was autoclaved and used for the experiments as a whey medium containing about 20 g/l lactose by the addition of 5.0 g/l K_2PO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$ and 1ml of trace mineral solution.

4-3-2. Mutant isolation

Ethyl Methane Sulfonate (EMS) treatment method based on Miller [61] with some modifications was used for the mutation of *B. indica*. The overnight culture of *B. indica* on yeast malt (YM) medium (10 g/l Glucose, 5 g/l Bacto peptone, 3 g/l Bacto yeast extract, 3 g/l Bacto malt extract) was centrifuged at 8,000×*g* for 10 min at 4°C and resuspended in 0.9% NaCl solution. EMS (1%, v/v) was added to cell suspension as a chemical mutagen followed by the incubation at 30°C for 45 min. Fifteen colonies with copious polysaccharide were selected and replated on mineral agar plates in the lactose-based MSM medium. Three colonies with viscous morphology were observed and a colony was chosen for the further study

and designated as *Beijerinckia indica* L3.

4-3-3. Culture methods

One colony of the mutant, *B. indica* L3 was transferred to 20 ml YL medium in a 100 ml Erlenmeyer flask. The culture was incubated for 24 h at 30°C in an orbital shake incubator (200 rpm) and used as a seed culture. The main culture was performed in 50 ml medium with inoculation size of 5% in a 250 ml Erlenmeyer flask for 72 h under the same condition used for the seed culture. The culture in MSM medium containing 16g/l lactose and 4 g/l glucose was used to inoculate the MSM medium or whey medium in a 5 L (working volume 3 l) jar fermenter (KF-5, KFC, Inchon, Korea) installed with DO, pH and temperature sensors. Initial pH of the medium was adjusted to 6.8 with 2 M NaOH. One vessel volume per minute (vvm) of air was supplied through a sparger and the speed of agitation was controlled at 500 rpm. The other conditions were the same to the flask culture unless stated otherwise.

4-3-4. Analytical methods

The cell growth and PS-7 production were determined by the methods described in chapter 2. The residual total sugar concentration was determined colorimetrically by the phenol sulfuric acid method described by Dubois et al. using glucose as standard [27].

4-3-5. Enzyme assays and protein determination

Beta-galactosidase activity was assayed by the method described by Miller [62]. Protein concentration was determined by the method of Bradford et al. with bovine serum albumin (Sigma, MO, USA) as a standard [51]. One unit (U) of enzyme was defined as the amount to produce 1 nmol of O-nitrophenol per gram of protein in 1 min.

4-3-6. Composition analysis

Gas chromatography (GC) analysis was used to determine the composition of sugar repeat units in PS-7 after methanolysis of the oligosaccharide followed by trimethylsilylation [5,63]. Gas chromatographic analysis was performed by a GC-MS system (GC: GC-17A, MS: QP-5050A, Shimadzu) installed with a column (60 m×0.32 mm I.D and 1 mm film thickness, DB-1MS, J&W Scientific). Standard curves were constructed using the GC peak area ratio of the standard monosaccharide of rhamnose and glucose.

4-3-7. Rheological characterization of the PS-7 polysaccharide

The purified PS-7 (from the culture in whey medium) was dissolved in 0.5% NaCl to make a concentration of 5 g/l polysaccharide. Rheological measurements were performed at 30°C using a Brookfield programmable LDV- III viscometer (Brookfield engineering laboratories, Stoughton, MA, USA) fitted with a small sample adapter using SC4-34 spindle. The 0.5% xanthan solution was also

determined for the comparison. The Power Law equation was used a model [36]:

$$\tau = K \bullet D^n \quad \text{.....(1)}$$

in which τ is shear stress (dynes/cm^2), D is shear rate (s^{-1}), K is coefficient index and n is flow behavior index.

4-4. Results and discussion

4-4-1. Comparison of galactosidase activities in the mutant and parent strains

The beta-galactosidase activities of *B. indica* and *B. indica* L3 grown in MSM medium containing lactose and glucose or whey medium were evaluated. As shown in Fig. 4.1, the beta-galactosidase activity of *B. indica* grown in MSM medium with glucose as a single carbon source was negligible, however the activity increased to 31U and 25U in cultures grown in lactose-based MSM medium and whey medium, respectively. However, beta-galactosidase activity of *B. indica* L3 was higher (21U) than that (2U) of *B. indica* grown in MSM medium containing 20 g/l glucose. The highest beta-galactosidase activity (46U) was observed in *B. indica* L3 culture with MSM medium containing 20 g/l lactose. This indicates that beta-galactosidase was expressed constitutively in *B. indica* L3. In addition, the beta-galactosidase activity of *B. indica* L3 was 31U in the culture with whey medium, which was similar to that of *B. indica* grown in lactose-based

MSM medium. The beta-galactosidase activity of *B. indica* L3 dropped when glucose was added to lactose-based MSM medium or whey medium. This indicates that lactose metabolism by *B. indica* L3 was under the catabolite repression by glucose like other bacteria [64-66]. In a lactose-utilizing *Xanthomonas campestris* constructed by expressing high level of beta-galactosidase gene, the xanthan production was poor [65]. In our work, the beta-galactosidase activity of *B. indica* L3 was improved as compared to that in the parent strain. Moreover, the mutant was able to produce a large amount of PS-7 using lactose as carbon source.

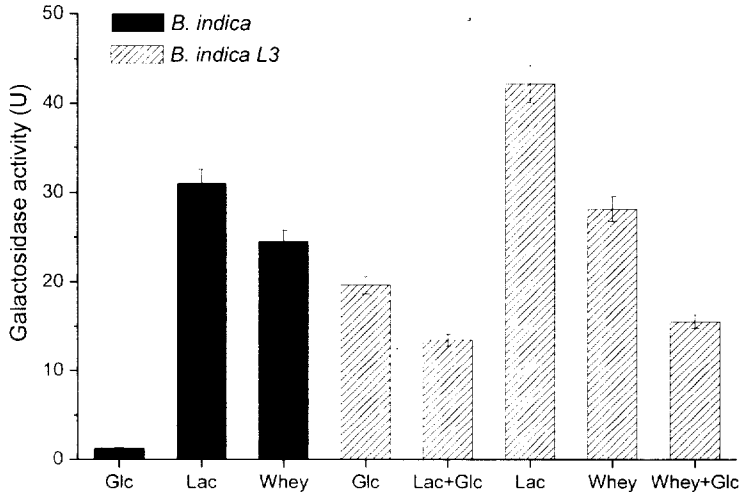

Fig. 4.1 Comparison of the galactosidase activity from *B. indica* and *B. indica* L3 under different carbon sources at 12 h of cultivation. *B. indica* were grown with glucose (Glc, glucose 20 g/l), lactose (Lac, lactose 20 g/l) or whey (whey powder 30 g/l). *B. indica* L3 were grown with glucose (Glc, glucose 20 g/l), lactose mixed with glucose (Lac+Glc, lactose 16 g/l, glucose 4 g/l), lactose (Lac, lactose 20 g/l), whey or whey mixed with glucose (whey+Glc, whey powder 30 g/l, glucose 4g/l).

4-4-2. Effect of glucose in lactose-based MSM medium on *B. indica* L3

The mutant strain, *B. indica* L3, was cultured in the MSM medium with various ratios of glucose to lactose as carbon sources. As shown in Fig. 4.2, *B. indica* L3 grown in lactose-based MSM medium produced a small amount of PS-7 with a low viscosity. However, the PS-7 production was improved by the addition of glucose. The dry cell weight increased from 0.28 g/l to 0.68 g/l and the concentration of PS-7 reached 4.9 g/l in the MSM medium containing 18 g/l lactose and 2 g/l glucose. The PS-7 production further increased to 5.52 g/l and the broth viscosity reached 39531cp by the increase of glucose content to 4 g/l. The PS-7 productions and apparent viscosities in the cultures with addition of more than 4 g/l glucose showed similar results. *B. indica* L3 could utilize glucose as sole carbon source and produced considerable amount of PS-7. The addition of glucose might enhance lactose utilization and provide more hexoses for the central metabolism through Entner-Dodouroff pathway in this aerobic strain [34]. Hsieh et al. also reported that the addition of glucose improved polysaccharide production from thin stillage [67].

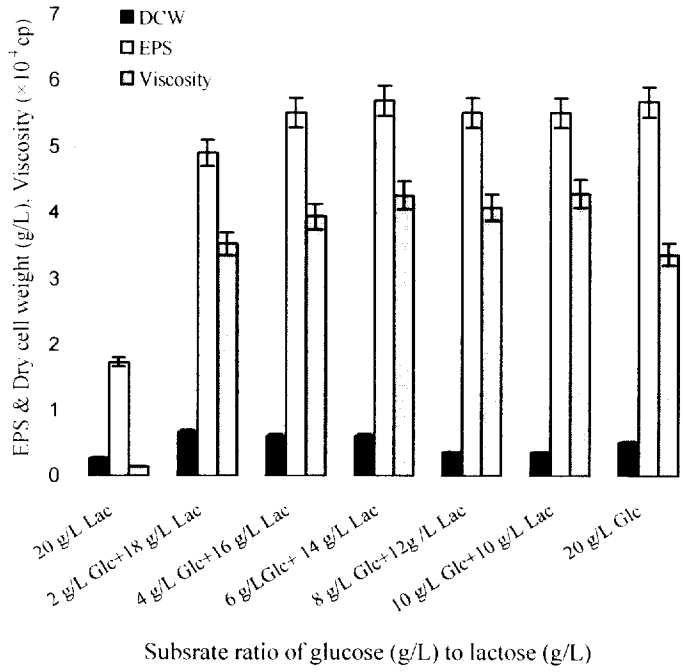

Fig. 4.2 Effect of glucose on PS-7 production in the culture of *B. indica L3* in lactose-based MSM medium in shake flask (total sugar concentration 20 g/l).

4-4-3. Comparison of PS-7 productions by *B. indica* and *B. indica L3*

Table 4.1 shows PS-7 productions from the culture of *B. indica* and *B. indica* L3 in shake flasks with different carbon sources. *B. indica* was cultured in MSM medium with lactose, glucose or whey medium. The amount of PS-7 produced by *B. indica* in MSM medium with glucose (20 g/l) as a single carbon source reached 7.06 g/l. *B. indica* could grow in lactose-based MSM medium or whey medium, however, small amounts of PS-7 (0.82 and 1.08 g/l, respectively) were produced and the viscosities were low accordingly. In the case of *B. indica* L3, relatively lower amount of PS-7 (2.88 g/l) with a viscosity of 4530 cp was produced with lactose as a carbon source. When *B. indica* L3 was grown in the lactose- based MSM medium with 4g/l glucose, it produced considerable amount of PS-7 (5.52 g/l) with a high viscosity (39531 cp). In the culture of the *B. indica* L3 strain in whey medium, the cells produced about 6.18 g/l PS-7 with a high viscosity (45772 cp) and 7.04 g/l of PS-7 was produced in the culture with whey medium containing 4 g/l glucose. Even though most of whey proteins were removed under low pH and high temperature, the residual nutrients in the whey after the treatment might enhance the PS-7 production. Also, the addition of glucose enhanced the carbon channeling to PS-7 production.

To confirm the effect of glucose on lactose (galactose) metabolism, *B. indica* L3 was cultured in MSM medium with galactose as a carbon source or a mixture of galactose and glucose as carbon sources. As shown in Table 4.1, the PS-7 productions in the culture with galactose were similar to that in the culture using

lactose as a carbon source, however, the viscosity was significantly low. Addition of a small amount of glucose could not improve the PS-7 production and viscosity significantly. It seems that glucose in the lactose molecule play an important role for the PS-7 production.

4-4-4. Optimization of whey medium for PS-7 production by *B. indica* L3

The whey has nutrients and elemental gradients, but the phosphate required for cell growth and PS-7 synthesis was low. To optimize the condition for growth of *B. indica* L3 and PS-7 production in whey medium, various concentrations of potassium phosphate were added into the whey medium. As shown in Fig. 4.3, when KH_2PO_4 concentration increased, the PS-7 production increased. Then, PS-7 production leveled off at 7.5 g/l KH_2PO_4, reaching about 6.0 g/l with a viscosity of 49010 cp. The pH in the whey medium increased continuously due to the buffering ability of KH_2PO_4. Therefore, a concentration of 7.5 g/l KH_2PO_4 was chosen for a high production of PS-7.

B. indica L3 was grown on different concentration of whey lactose. As shown in Fig. 4.4, PS-7 production increased with the increase of whey lactose concentration. But when whey lactose was higher than 2%, PS-7 production leveled off and the yield of PS-7 decreased also. The final dry cell weight decreased with high whey lactose concentration probably due to mass transfer limitation. Therefore, 2% whey lactose (30g/l whey powder) was chosen for the production of PS-7 in whey medium.

To find the optimal concentration of glucose for enhancing PS-7 production and viscosity, 1~4 g/l of glucose was added into whey medium. As shown in Fig. 4.5, addition of 1 g/l glucose enhanced PS-7 production and improved the viscosity markedly. When more glucose was added, the effect was not significant.

Table 4.1 Comparison the maximal PS-7 (72 h of cultures).

Strains	Carbon sources [1]	PS-7 (g/l)	Viscosity (cp)
B. indica L3	Lac	2.88±0.22	4530±90
	Lac+Glc	5.52±0.12	39531±1259
	Glc	5.36±0.0.20	36442±750
	Gal	2.90±0.14	60±12
	Gal+Glc	3.56±0.38	2340±60
	Whey	6.18±0.06	45772±167
	Whey+Glc	7.04±0.10	>60000 [2]
B. indica	Lac	0.82±0.02	60±24
	Glc	7.06±0.06	49324±1184
	Whey	1.08±0.08	24±12

[1] *B. indica* L3 were grown in MSM medium containing lactose (Lac, 20 g/l), lactose plus glucose (Lac+Glc, lactose 16 g/l, glucose 4 g/l), glucose (Glc 20 g/l), galactose (Gal, 20 g/l), galactose plus glucose (Gal+Glc, galactose 16 g/l, glucose 4 g/l), or in whey medium (whey, whey powder 30 g/l) or whey medium plus glucose (whey+Glc, whey powder 30 g/l, glucose 4 g/l). *B. indica* were grown in MSM medium containing lactose (Lac, 20 g/l), glucose (Glc, 20 g/l), or in whey medium.

[2] Viscosity was over the limit (60000 cp) of viscometer.

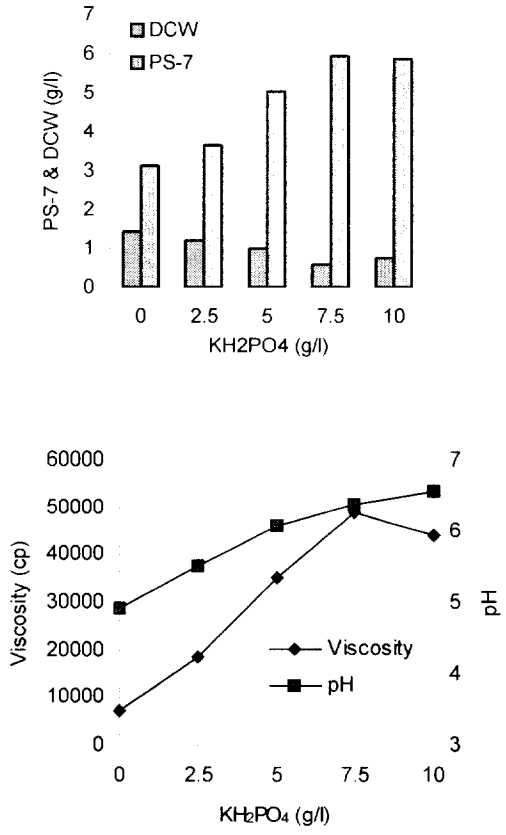

Fig. 4.3 PS-7 production by *B. indica* L3 with various concentrations of KH_2PO_4 in whey medium.

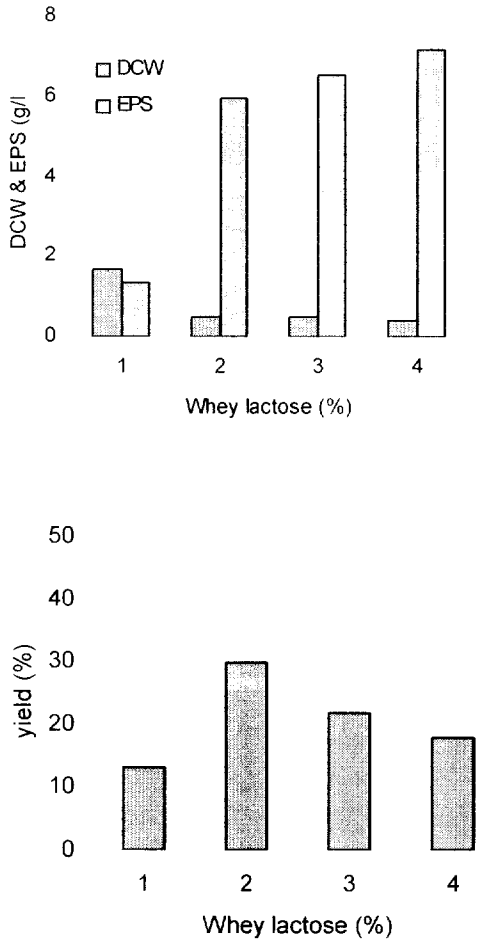

Fig. 4.4 PS-7 production by *B. indica* L3 with various concentrations of whey lactose in whey medium.

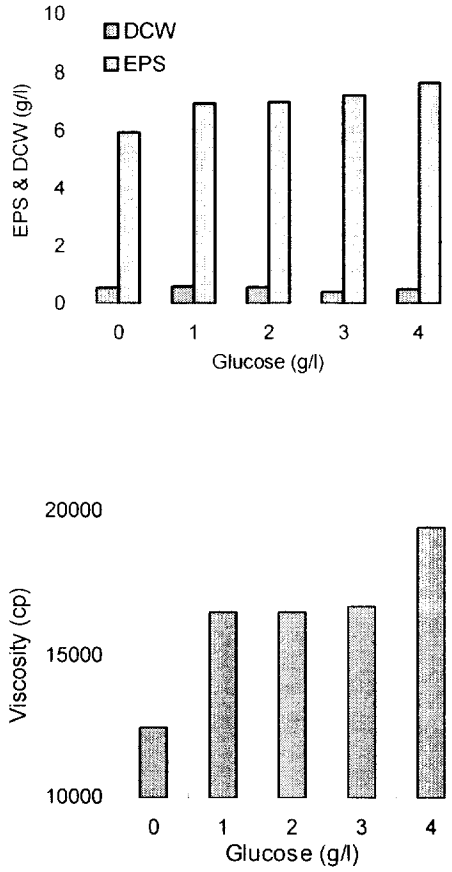

Fig. 4.5 PS-7 production by *B. indica* L3 with the addition of glucose in whey medium.

4-4-5. Production of PS-7 from whey with *B. indica L3*

B. indica L3 was cultured in whey medium or whey medium containing 1g/l glucose and 7.5 g/l KH_2PO_4 in a 5-L fermenter. As shown in Fig. 4.6A and B, the cell density reached a maximum and then decreased rapidly. The final PS-7 concentration reached 6.3 g/l (Fig. 4.6A) and 7.2 g/l (Fig. 4.6B) in the culture in whey medium without or with glucose, respectively. In the culture with whey medium containing 1 g/l glucose, the final residual sugar and biomass were lower than those in the culture with whey medium.

Up to date, several reports indicate that xanthan production with whey was not efficient, although the galactosidase was expressed to some extent in the cells [60, 65,68]. The highest yield of xanthan with whey lactose was 50% using a novel stirrer design in bioreactor, while it was still lower than the average 70% of xanthan yield from glucose-based medium [68]. In this study, *B. indica* L3 could produce significant amount of PS-7 with whey comparable to that of parent strain grown in glucose-based MSM medium.

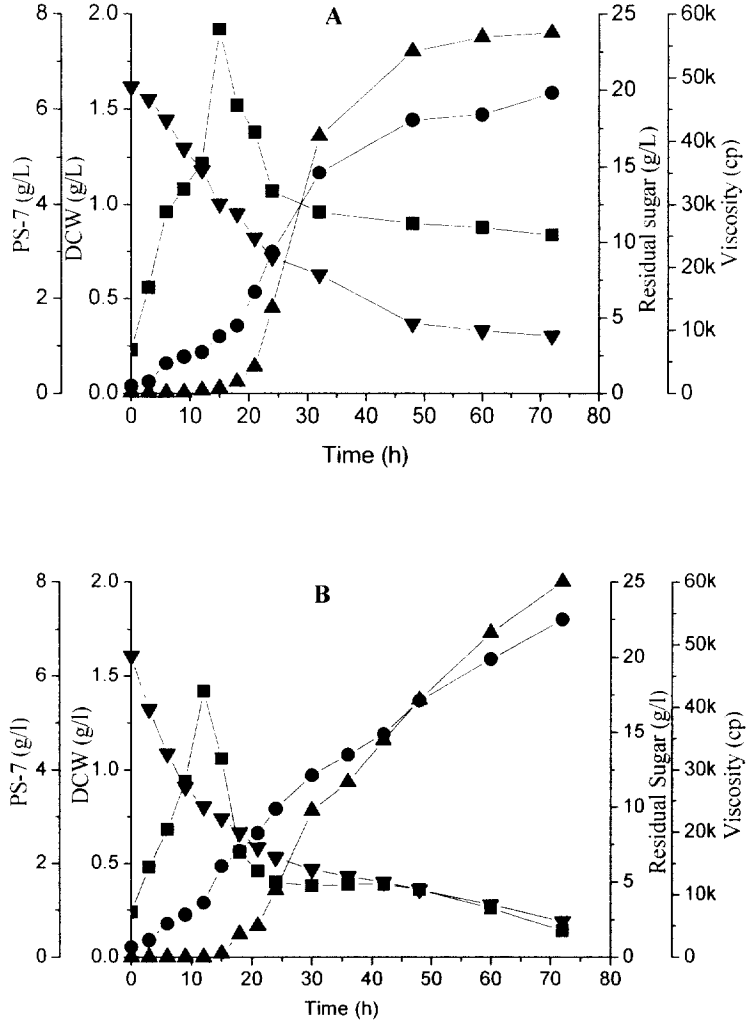

Fig. 4.6 PS-7 production by *B. indica* L3 grown in (A) whey medium (5 g/l KH_2PO_4) and (B) whey medium by the addition of 1 g/l glucose (7.5 g/l KH_2PO_4). Symbols: (■), DCW; (●), PS-7; (▲), Viscosity; (▼),Total sugar.

4-4-6. Compositional analysis of PS-7

The compositional analysis of the PS-7 was carried out by GC-MS. In the previous study, glucose and rhamnose were detected by gas chromatography and the ratio of glucose/rhamnose in the PS-7 was stable between at a range of 4.5~5.0 [4]. Table 2 shows the integration results of Glc/Rha ratios of the PS-7 produced by *B. indica* in glucose-based MSM medium and *B. indica* L3 cultured with lactose-based MSM medium or whey medium. The ratio of glucose/rhamnose of PS-7 produced by *B. indica* L3 cultured in lactose-based MSM medium or whey medium showed similar ratio to that of PS-7 produced by *B. indica* cultured in glucose-based MSM medium.

4-4-7. Rheological characterization of the PS-7 polysaccharide

Rheological study showed that the coefficient index (K) and flow index (n) were 11373 cp and 0.07, respectively, as compared to 5940 cp and 0.2 from xanthan solution (Fig. 4.7). The viscosity of PS-7 produced *B. indica* L3 cultured in whey medium was twice that of xanthan and exhibited high pseudoplasticity (low flow index value).

Table 4.2. Compositional analysis of PS-7 produced by the parent strain grown in MSM medium containing glucose and mutant cultivated with lactose or whey.

Strain	*B. indica* (parent)	*B. indica* L3 (mutant)	
Carbon Source	Glucose	Lactose	Whey
Glc/Rha*	4.67	4.88	4.50

* Glc: glucose, Rha; rhamnose.

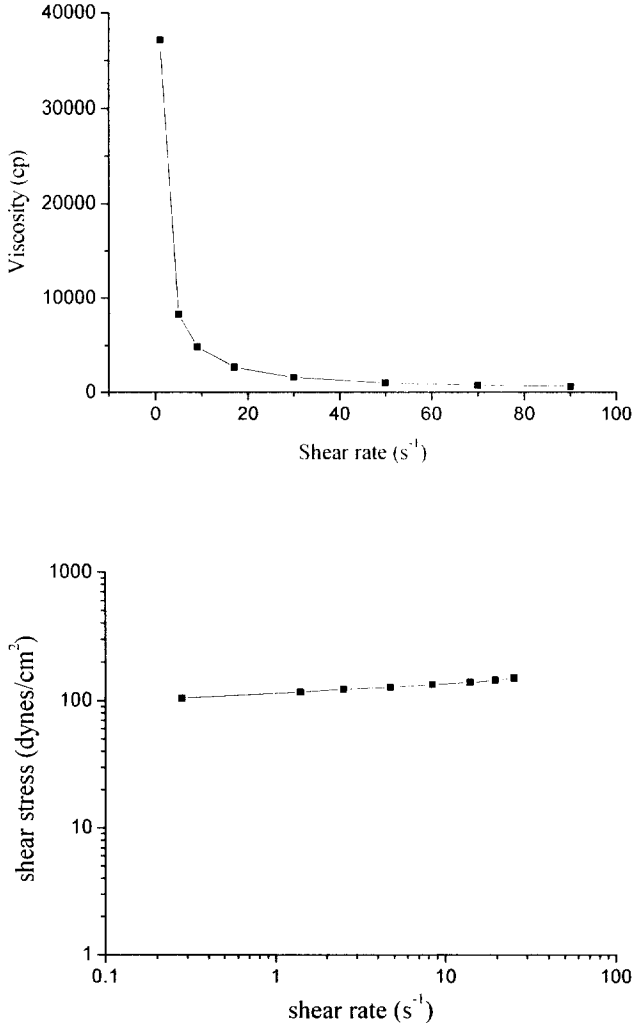

Fig. 4.7 Rheological property of PS-7 produced by *B. indica* L3.

CHAPTER 5

CONCLUSIONS

1. Optimized conditions for the cultivation of *B. indica* fermentation to produce PS-7 are as follows: 2.5 mg/l sodium molybdate and 2 mg/l $FeSO_4$ in MSM medium were required. In batch culture of *B. indica*, an agitation speed of 500 rpm and aeration of 1 vvm were required.

2. At relatively high ammonium concentration, *B. indica* grew well in MSM medium. However, only a small range of ammonium (4mM-12mM) favored PS-7 production. PS-7 production was not changed significantly with variation of KNO_3 concentrations.

3. High C/N ratio in the MSM medium enhanced the productivity of PS-7 by *B. indica* and the apparent viscosity of produced PS-7 increased. The culture with 20 g/l glucose produced the highest amount of PS-7.

4. Supplement of glutamine at exponential phase inhibited PS-7 formation and enhanced the cell growth. Nitrogen source acts as an indicator of cell metabolic state in *B. indica* to regulate the metabolism of carbon sources. Under high C/N ratio, carbon source is to be converted to extracellular polysaccharide as carbon preserve or dissipation.

5. *B. indica* preferred hexoses or glucose-generating sugars for the synthesis of PS-7. Sucrose was the best carbon source for PS-7 production. The cultivation of *B. indica* with fructose resulted in acidic medium and a few PS-7 with a low viscosity was produced. But addition of gluconate into fructose medium improved PS-7 since gluconate affected the metabolic pathway of fructose degradation.

6. Carbon metabolic pathway in *B. indica* was characterized. This strain employs the ED pathway, PP pathway, and TCA cycle as central metabolic pathways.

7. Under low nitrogen condition, a greater amount of polysaccharide was converted from glucose rather than that converted from glucosamine, glycerol and gluconate.

8. The intracellular flux distributions in *B. indica* showed that the ED pathway was operated dominantly and the PP pathway was maintained to a lesser degree. Triose-3-phosphate cycling was present in the gluconeogenesis pathway, which increased the intracellular pool of hexose-phosphate for PS-7 synthesis.

9. Cell growth and PS-7 biosynthesis were significantly influenced by environmental nitrogen concentration. Metabolic flux analysis showed that the culture containing high levels of nitrogen showed a drastic change in metabolic flux. The fluxes in the ED pathway, PP pathway, glycolysis, and TCA cycle were increased. Thus, more carbon source and energy were dissipated and the production of PS-7 polysaccharide decreased.

10. A mutant strain, *B. indica* L3 was obtained to produce PS-7 from whey/lactose. The beta-galactosidase activity of the mutant strain was higher than that of the parent strain.

11. The PS-7 production by *B. indica* L3 could be enhanced by the addition of 4 g/l glucose in the whey medium or MSM medium with lactose as a carbon source. Similar amount of PS-7 with a significantly higher viscosity in whey medium was produced as compared to that from the parent strain.

12. The compositional analysis of the PS-7 showed the mutant strain grown with lactose or whey produced the PS-7 with stable ratio of Glc/Rha that was

similar to the ratio of parent strain. Therefore, this strain can be used for the treatment of dairy and produce valuable polysaccharide.

13. Optimized conditions for *B. indica* L3 cultivation for effective production of PS-7 were as follows: 20 g/l whey lactose, 7.5 g/l of $KH_2PO_4 \cdot 7H_2O$, 1 g/l glucose in the whey medium.

14. The viscosity of PS-7 produced by *B. indica* L3 grown in whey medium was twice that of xanthan and showed excellent pseudoplasticity.

APPENDIX

Nomenclature

T3P	Triose-phosphate
SUCC	Succinate
AcCoA	Acetyl-coenzyme A
OAA	Oxaloacetate
F6P	Fructose-6-phosphate
NADH	Nicotinamide adenine dinucleotide, reduced
E4P	Erythrose-4-phosphate
3PG	3-Phosphoglycerate
G6P	Glucose-6-phosphate
6GP	Gluconate-6-phosphate
F16P	Fructose-1, 6-biphosphate
GLC	Glucose
MAL	Malate
NAD	Nicotinamide adenine dinucleotide
S7P	Sedohepulose-7-phosphate
X5P	xylulose-5-phosphate
PYR	Pyruvate
R5P	Ribose-5-phosphate
BIOMASS	
SucCoA	Succinyl-coenzyme A
PEP	phosphoenolpyruvate

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
ADP	Adenosine 5`-diphosphate
ATP	Adenosine 5`-triphosphate
CIT	Citrate
ICIT	Isocitrate
Ru5P	Ribulose-5-phosphate
PS-7	Exopolysaccharide
FADH	Flavine adenine dinucleotide, reduced
FAD	Flavine adenine dinucleotide
KDPG	2-keto-3-deoxy-6-phosphate-gluconate
AKG	2-keto-glutarate
NADP	Nicotinamide adenine dinucleotide phosphate

Reactions

NO.	Name	Reaction
r_1	Trans1	GLC + [ATP] -> G6P + [ADP]
r_2	Gly01	F6P <-> G6P
r_3	Gly02	F16P -> F6P
r_4	Gly03	2 T3P <-> F16P
r_5	Gly04	T3P + [NAD] + [ADP] <-> [NADH] + [ATP] + 3PG
r_6	Gly05	3PG <-> PEP
r_7	Gly06	PEP + [ADP] -> PYR + [ATP]
r_8	Gly07	PYR + [COA] + [NAD] -> [NADH] + ACCOA
r_9	Gly08	PEP -> OAA
r_{10}	PPP01	G6P + [NADP] -> [NADPH] + 6GP
r_{11}	PPP02	6GP + [NADP] -> [NADPH] + RL5P
r_{12}	PPP03	Ru5P <-> R5P
r_{13}	PPP04	Ru5P <-> X5P
r_{14}	PPP05	R5P + X5P <-> T3P + S7P
r_{15}	PPP06	T3P + S7P <-> E4P + F6P
r_{16}	PPP07	X5P + E4P <-> F6P + T3P
r_{17}	ED01	D6PGC -> KDPG
r_{18}	ED02	KDPG -> T3P + PYR
r_{19}	TCA01	ACCOA + OAA -> [COA] + CIT
r_{20}	TCA02	CIT <-> ICIT
r_{21}	TCA03	ICIT + [NADP] -> [NADPH] + AKG
r_{22}	TCA04	AKG + [NAD] + [COA] -> [NADH] + SUCCOA
r_{23}	TCA05	SUCCOA + [ADP] -> [ATP] + [COA] + SUCC
r_{24}	TCA06	SUCC + [FAD] -> [FADH] + MAL
r_{25}	TCA09	MAL + [NAD] -> [NADH] + OAA
r_{26}	Energy01	[NADH] + 2 [ADP] -> [NAD] + 2 [ATP]
r_{27}	Energy02	[NADPH] + [NAD] <-> [NADP] + [NADH]
r_{28}	Energy03	[FADH] + [ADP] -> [FAD] + [ATP]

NO.	Name	Reaction
r_{29}	Growth	4.11 [ATP] + 0.3547 [NAD] + 1.8225 [NADPH] + 0.0205 [G6P] + 0.0071 [F6P]+ 0.0898 [R5P] + 0.0361 [E4P] + 0.0129 [T3P] + 0.0719 [PEP] + 0.2833 [PYR] + 0.2928 [ACCOA] + 0.1787 [OAA] + 0.1079 [AKG] + 0.1496 [3PG] -> 4.11 [ADP] + 0.3547 [NADH] + 1.8225 [NADP] + [BIOMASS]
r_{30}	EPS synthesis	6 [ATP] + 6 G6P + 2 [NADPH] + 2 [ACCOA] -> PS-7 + 2 [NADP] + 6 [ADP] + 2 [COA]
r_{31}	Energy04	[ATP] -> [ADP]

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SUMMARY (In Korean)

Beijerinckia indica 에 의한 Heteropolysaccharide-7 의 생산에 관한 연구

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본 연구에서는 *Beijerinckia indica* 에 의한 heteropolysaccharide-7 (PS-7)의 최적화 생산조건을 shake-flask 과 5-L bioreactor 를 이용한 배양에서 조사하였다. 또한, 질소원 농도에 따라 *Beijerinckia indica* 의 대사흐름분포를 MetaFluxNet 를 이용 하여 평가하였다. 또한 whey lactose 을 이용할 수 있는 *Beijerinckia indica* mutant 을 얻고 PS-7 생산 최적화 생산조건을 도출하였다.

Beijerinckia indica 를 통한 PS-7 생산 최적화를 위해 다양한 탄소원과 질소원을 첨가하여 각 조성들이 PS-7 생산 에 미치는 영향을 조사하였다. 높은 C/N ratio 에서 PS-7 생산을 배양 40 시간 까지 효과적으로 증가시킬 수 있었으며 낮은 C/N ratio 에선 탄소원으로 첨가한 glucose 가 균체의 생산에 사용되었을 뿐 PS-7 의생산은 낮았다. 하지만, 높은 C/N ratio 에선 배양액의 고점도로 균체의 생산이 정체하는 것으로 보아 본 실험에서는 20g/l glucose 와 NH_4NO_3 7.5 mM 으로 33.3 의 C/N ratio 가 PS-7 생산에 적절한

비율인 것으로 관찰되었다. 다양한 탄소원을 첨가한 결과, *B. indica* 는 PS-7 생산을 위하여 탄소원으로 hexose 또는 glucose-generating 당을 효율적으로 이용하는 것으로 보였다. *Beijerinckia indica* 를 이용한 PS-7 생산을 위해 첨가한 다양한 탄소원 중 sucrose 가 최고의 PS-7 (6.96 g/l)을 생산하였다. 5 l 배양기를 이용한 배양에서 탄소원 20 g/l glucose, 질소원 7.5 mM NH_4NO_3 이 포함된 MSM 배지 내 배양된 *B. indica* 의 세포 농도가 최고 2.5 g/l 이였고, 생산된 최고 PS-7 농도는 7.5 g/l (35174 cp)으로 나타났다. 질소원이 완전히 소모된 12 시간 이후, 7.5 mM 의 NH_4NO_3, glutamine, glutamate 을 첨가한 MSM 배지에서의 *B. indica* 배양 결과, 질소원이 *B. indica* 내에서 탄소원 대사를 조절하는 역할을 하는 것을 관찰할 수 있었으며 최종적으로 PS-7 의 생산을 조절할 수 있었다.

B. indica 는 탄소원 대사를 위해 주로 ED 과 PP pathway 를 이용한다. 즉 균의 생장과 PS-7 생산은 외부의 질소원에 의해 영양을 받는다. 저농도 질소원 조건에서, glucose 첨가시 대량의 PS-7 를 생산하였고, glucosamine, glycerol 과 gluconate를 첨가시 PS-7 생산이 낮았다. Glucosamine를 이용할 때 NH_3 을 많이 방출하여 세포생장을 촉진하였고 PS-7 생산을 억제하였다. 질소의 *B. indica* 에 미친 양향을 알아보기 의해 *B. indica* 의 대사네트워크 를 만들고 질소원 농도에 따라서 대사흐름분포를 조사하였다. 결론적으로, ED Pathway 가 지배적으로 이용되었고, PP Pathway 가 부분적으로 이용되었다. 또한 T-3-P cycling 이 gluconeogenesis pathway 중에 일어났고, 이 cycling 을 통해 PS-7 생산에필요한 hexose-phosphate 의 양이 증가하는 것으로 보였다. 고농도 질소원 조건의 경우, 대사흐름분포가 크게 변하였다. 이때 대사경로 흐름 과 ATP 소비가 증가하였다. 따라서 탄소원이 소모되어 PS-7 생산이 줄었다.

PS-7 생산을 위해 lactose/whey 를 이용할 수 있는 *B. indica* L3 을 화학적인 방법으로 *B. indica* 균주로 부터 얻었다. *B. indica* L3 배양에 있어서 4g/l glucose 를 MSM 배지에 첨가하였을때 PS-7 생산이 향상되었다. 또한 beta-galactosidase activity 조사에서 parent strain 보다 활성이 높게 나타났다. Whey medium 을 이용한 *B. indica* L3 배양에서, *B. indica* 균주가 생산한 PS-7 최고농도에 근접한 최고농도의 PS-7 을 생산했으며 viscosity 도 높았다. PS-7 구성 성분 분석을 통하여 mutant 가 생산하는 PS-7 의 Glu/Rha 비율이 안정된 범위로 parent strain 과 비슷한 수치를 나타내었다. 그리고 *B. indica* L3 가 생산하는 PS-7 의 viscosity 는 xanthan 보다 2 배로 높았고 우수한 pseudoplasticity 을 보였다. 따라서, *B. indica* L3 를 이용하여 PS-7 을 생산시 전체 생산 비용이 감소되고 또한 whey 의 처리문제를 해결할 수 있을 것으로 예상된다.

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Publication List

1. Sung-Mi Lim, **Jian-Rong Wu**, Jin-Woo Lee, Sung-Koo Kim. Optimization of culture condition for the gellan production by Pseudomonas elodea ATCC 31461. *Korea J Life Science*, 2003, 13(5): 705-711

2. **Jian-Rong Wu**, Jeong Hwa Son, Ki-Hong Kim, Yoon-Kwon, Jin-Woo Lee, Sung-Koo Kim. Metabolic Flux Analysis of *Beijerinckia indica* for PS-7 Production. *Biotechnol Bioproc Eng*. 2005, 10:91-98

3. **Jian-Rong Wu**, Jeong Hwa Son, Ki-Hong Kim, Yoon-Kwon, Jin-Woo Lee, Sung-Koo Kim. *Beijerinckia indica L3* fermentation for the effective production of heteropolysaccharide-7 using the dairy byproduct whey as medium. *Process Biochemistry*, In press, 2005

4. **Jian-Rong Wu**, Jeong Hwa Son, Ki-Hong Kim, Yoon-Kwon, Jin-Woo Lee, Sung-Koo Kim. Heteropolysaccharide-7 production by *Beijerinckia indica* with various nitrogen and carbon sources. Korean Journal of Microbiology and Biotechnology, In press, 2005

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Metabolic Flux Analysis of *Beijerinckia indica* for PS-7 Production

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Abstract In order to investigate central metabolic changes in *Beijerinckia indica*, cells were grown on different carbon sources and intracellular flux distributions were studied under varying concentrations of nitrogen. Metabolic fluxes were estimated by combining material balances with extracellular substrate uptake rate, biomass formation rate, and exopolysaccharide (EPS) accumulation rate. Thirty-one metabolic reactions and 30 intracellular metabolites were considered for the flux analysis. The results revealed that most of the carbon source was directed into the Entner-Doudoroff pathway, followed by the recycling of triose-3-phosphate back to Hexose-6-phosphate. The pentose phosphate pathway was operated at a minimal level to supply the precursors for biomass formation. The different metabolic behaviors under varying nitrogen concentrations were observed with flux analysis.

Keywords: metabolic flux analysis, flux distribution, *Beijerinckia indica*, PS-7, exopolysaccharide

INTRODUCTION

The heteropolysaccharide-7 (S-7 or PS-7) from *Azotobacter indicus var.* myxogens was identified and examined as a potential candidate for the production of a bacterial polysaccharide with desirable rheological properties [1]. The bacterial strain was later renamed to *Beijerinckia indica* [2], or *Sphingonomas S7* [3]. A structural analysis of the PS-7 polysaccharide [4] found in *Beijerinckia indica* showed that it has a similar backbone with members of the gellan family of polysaccharides. PS-7 is a water soluble exopolymer that can generate a highly viscous solution with twice the viscosity of xanthan gum. The viscosity of PS-7 solution is stable at a temperature range of 4 to 93°C and at a pH range of 3.0 to 12.0 [5]. PS-7 has good pseudoplasticity and shows excellent suspending ability.

Metabolic flux analysis (MFA) is a powerful technique used to characterize intercellular fluxes of microorganisms, which aid in the identification of potential genetic modifications that may improve the behavior and productivity of microorganisms [6]. The metabolic fluxes distributed within microorganisms are estimated through a combination of biochemical, microbiological, and mathematical approaches, as well as data from experimental measurements. The flux distributions are correlated to the strength of each individual enzyme, which represents the *in vivo* metabolic state of the cell. The flux information is then used to examine the influence of process parameters or cultivation conditions on the physiological states of microorganisms and to identify rate-limiting steps in pathways. Once such steps are identified, molecular biological techniques can be applied to modify the corresponding gene or enzyme to bring about significant shifts in the yield of desired end products.

Beijerinckia indica is a strictly aerobic, gram-negative bacterium. When grown on a carbohydrate-containing medium, the colonies glisten due to capsular slime exopolysaccharide formation [1]. Like other important polysaccharides such as xanthan, gellan, and curdlan, PS-7 production favors high carbon/nitrogen ratio conditions in the substrate [7,8].

In this paper, *Beijerinckia indica* was cultivated in media with different carbon sources and a high or low nitrogen content for EPS fermentation. Metabolic flux was calculated to investigate the flux distribution in the central metabolism of the strain and metabolic changes under different nitrogen conditions. The Pentose Phospate pathway (PP), the semi- Embden-Meyerhof-Parnas (EMP) pathway, the Entner Doudoroff (ED) pathways, the Tricarboxylic acid (TCA) cycle, and EPS synthesis were used in the proposed metabolic network for metabolic flux analysis.

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Short communication

Beijerinckia indica L3 fermentation for the effective production of heteropolysaccharide-7 using the dairy byproduct whey as medium

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Abstract

A mutant strain of *Beijerinckia indica* L3 screened by a traditional chemical mutagenesis was grown in lactose-based mineral salts medium (MSM) for the production of heteropolysaccharide-7 (PS-7). The beta-galactosidase was expressed constitutively in the mutant and its activity was higher than that in parent strain. The highest amount of PS-7 produced by the mutant was 2.88 g/L with a viscosity of 4530 cP in lactose-based MSM medium. The PS-7 production was enhanced by the addition of 4 g/L glucose into lactose-based MSM medium, reaching 5.52 g/L with a viscosity of 39531 cP. PS-7 of 6.18 g/L with a viscosity of 45772 cP was produced from the mutant grown in whey medium. The PS-7 production from the mutant reached 7.04 g/L when 4 g/L glucose was added to the whey medium. Compositional analysis of PS-7 produced by the mutant grown in whey medium or lactose-based mineral salts medium showed that the ratios of glucose/rhamnose were maintained in the range similar to that of parent strain, indicating the same compositional structures of PS-7.

Keywords: *Beijerinckia indica*; Mutant; Heteropolysaccharide-7; Lactose; Whey; Beta-galactosidase

1. Introduction

Beijerinckia indica ATCC 21423 is a free-living, strictly aerobic bacterium that can produce copious polysaccharide when growing on carbohydrate-containing medium [1]. The bacterial polysaccharide, heteropolysaccharide-7 (designated as PS-7) produced by *B. indica* has a variety of applications as stabilizing, viscosifying, emulsifying, thickening, and suspending agents [2,3]. The repeating unit of PS-7 composed of D-glucose (D-Glc), L-rhamonse (L-Rha), and D-2,6-deoxy-glucuronic acid (D-2,6-deoxy-GlcA), is a tetrasaccharide [→4-β-D-Glc*p*-(1→4) -α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-2,6-deoxy-GlcA*p*-(1→] in the main backbone [4]. The structure of PS-7 has the side chain of [β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→] linked at the C-6 of β-D-Glc after rhamnose in the repeating unit.

Glucose is somewhat of high price though it is a primary carbon source in synthetic media for polysaccharide production [5]. 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 [6–8].

Cheese whey is a nutrient-rich dairy byproduct from cheese production containing 4–5% lactose, 0.8–1% proteins, minerals, trace amount of vitamins and some small organic molecules [9]. 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) [10]. In this study, the utilization of whey by *B. indica* mutant for the production of PS-7 was evaluated.

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Optimization of culture condition for the gellan production by *Pseudomonas elodea* ATCC 31461

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Abstract

The gellan was produced by *Pseudomonas elodea* under aerobic condition. In this study, the effects of inoculum size, carbon sources and concentration, nitrogen source, and C/N ratio on the cell growth and the production of gellan were evaluated. The maximum growth of *P. elodea* and gellan production was obtained at 5% (v/v) of inoculum size and glucose showed best results among 9 carbon sources tested. The maximum specific yield of 2.22 and productivity of 0.03 g/ℓ/h were obtained at 1.0% (w/v) of glucose. The maximum gellan production was obtained at medium without ammonium nitrate. This indicates that nitrogen limitation is essential for the production of gellan. The highest cell and gellan production were obtained at 20 of C/N ratio.

Key words – fermentation, gellan, optimum culture condition, *Pseudomonas elodea*

Introduction

Currently, one of the exopolysaccharides with great potential for industrial applications is the gellan gum. The gellan gum (PS-60) is a high molecular mass extracellular anionic heteropolysaccharide produced aerobically from *Pseudomonas elodea* ATCC 31461, renamed as *Sphingomonas paucimobilis* [1,11,21].

The gellan consists of linear repeating tetrasaccharide [→3)-β-D-Glc-(1→4)-β-D-GluA-(1→4)-β-D-Glc(1→4)-α-Rha-(1→)] composed of D-glucose (Glc), D-glucuronic acid (GluA), and L-rhamnose (Rha) residues [4,5]. Gellan contains O-acetyl group that are readily removed by alkali treatment. Acetyl groups in gellan affect the rheology of gels and the deacetylation of native gellan results in a change from soft, elastic and thermoreversible gel to hard and brittle gel. The native state gellan forms a weak gel in water, however when deacetylated by the treatment of alkali, gellan yields a rigid gel much like agar [3,13,18]. From its novel properties of producing a thermoreversible gel when heated and cooled, gellan gains an importance as a potential agar substitute for tissue culture with optical clarity and gel strength at a given concentration. Besides its application as gelling, thickening, suspending, stabilizing, and emulsifying agent in food systems, gellan has been used in enzyme and cell immobilization and gel electrophoresis. Gellan has also potentials for biomedical applications [2,15, 22,24].

The characteristics and the properties of gellan solutions and gels have been extensively studied and a large number of patents have been registered on food and biological applications of gellan, but the knowledge on

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