0273-2289/99/82/0199/$12.50

Biosynthesis of Poly-β-hydroxybutyrate and Exopolysaccharides on *Azotobacter chroococcum* Strain 6B Utilizing Simple and Complex Carbon Sources

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Received February 12, 1998; Accepted October 28, 1999

Abstract

Coproduction of poly-β-hydroxybutyrate (PHB) and exopolysaccharides (EPS) was investigated with *Azotobacter chroococcum* strain 6B isolated from soil samples. The bacterium was cultured using various carbon sources solely or with 0.1 g/L of ammonium sulfate. Ammonium addition resulted in reduced PHB and EPS production with glucose, fructose, and sucrose media, but cellular mass remained constant except for sucrose. Protein was nearly twofold higher in ammonium-grown cultures. Glucose and fructose alone biosynthesized high amounts of EPS (maximum 2.1 and 1.1 g/L, respectively, at 72 h), whereas PHB was accumulated only in glucose-grown cells. Sucrose almost did not produce EPS. Conversely, PHB content was the highest obtained from all experimented conditions (1.1 g/L at 48 h, 40% cell dry wt). When a complex carbon source such as sugar cane molasses was utilized, PHB was accumulated concomitant with EPS production from the initial time to 48 h (0.75 g/L, 37% cell dry wt and 0.6 g/L, respectively), and then PHB decayed at 72 h (0.2 g/L). On the other hand, EPS continued to be biosynthesized (1.1 g/L, 72 h). PHB fractions of total intra- and extracellular biopolymers were calculated. Sucrose-modified Burk's medium without ammonium addition is suggested as a medium capable of diverting the carbon source for the production of intracellular PHB rather than EPS with *A. chroococcum* 6B.

Index Entries: Poly-β-hydroxybutyrate; exopolysaccharides; coproduction; *Azotobacter chroococcum*; complex carbon.

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Introduction

Poly-β-hydroxybutyrate (PHB) is a biopolyester produced in bacteria under unbalanced nutrient conditions, such as nitrogen, oxygen, or phosphorus deficiency *(1)*. PHB is an intracellular carbon and energy storage polymer with industrial applications, because it is biodegradable, thermoplastic, and biocompatible *(2,3)*. Microbial exopolysaccharides (EPS) are commercially useful for their applications as gellifying agents and have the potential to replace alginate polysaccharides from marine algae now used in food and pharmaceutical industries *(4,5)*. *Azotobacter chroococcum* has been reported to produce alginate-like EPS, and enzymes involved in its biosynthesis have been studied *(6)*. *Azotobacter vinelandii* biosynthesized extracellular polysaccharides with galacturonic acid and glucose as the major component of the slime, and EPS components were identical when the energy source provided for the cells was sucrose, glucose, fructose, or ethanol *(7)*.

Within the polyhydroxyalkanoate type of biopolyesters, *Azotobacter* sp. usually biosynthesizes PHB *(8)*. However, the copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate has been produced by a mutant strain of *A. vinelandii (9)*. Extensive biochemical research has been conducted on the regulation of the tricarboxylic acid cycle, glucose metabolism, and PHB biosynthesis in *Azotobacter beijerinckii (10,11)*, and the effect of ammonium addition and oxygen *(12)*. PHB production and molecular weight has been studied in two strains of *A. chroococcum (13,14)*. However, little has been reported for simultaneous production of both types of biopolymers. This coproduction obviously reduces PHB yields. PHB and EPS are competing metabolites for the carbon source utilized by bacteria, and their coproduction has been studied in two strains of *Rhizobium meliloti*. It was concluded that growth in a nitrogen-deprived media was limited and favored EPS production rather than PHB production *(15)*. Because EPS can make up for a large proportion of the biosynthetic product of the bacteria and the *Azotobacter* genus is predominantly mucoid when isolated from its natural soil habitat *(16)*, biochemical and fermentative conditions need to be well established in order to obtain a relative higher PHB content.

The present study examines simultaneous synthesis of EPS and PHB utilizing different simple carbon sources and a complex one, such as sugar cane molasses, combined with the effect of ammonium sulfate addition, to obtain a favorable condition for PHB production. The results indicate that sucrose, without nitrogen addition, is the best condition for diverting the carbon source to PHB biosynthesis with *A. chroococcum* strain 6B.

Materials and Methods

Microorganism and Culture Media

A. chroococcum strain 6B was isolated from rhizospheric soil samples of the Agronomy Faculty Campus, Buenos Aires, Argentina; characterized

and identified by biochemical tests; and deposited as strain 1691 in CCMA WDC 307 Culture Collection. Modified productive Burk's medium was utilized: (10 g/L glucose, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 0.012 g/L $FeSO_4 \cdot 7H_2O$, 0.11 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $Na_2MoO_4 \cdot 12H_2O$, 1.0 g/L K_2HPO_4, and 0.2 g/L NaCl). In sucrose and fructose medium, glucose was replaced by the corresponding carbon sources at the same concentration (10 g/L). In ammonium-containing media, 0.1 g/L of $(NH_4)_2SO_4$ was added, and the pH was adjusted to 7.0. Molasses Burk's medium was identical to modified productive Burk medium with the exception that glucose was replaced with raw molasses with an approximate composition of 1% sucrose, 0.25% glucose, and 0.25% fructose (Tucumán Province sugar cane refineries, Argentina) as the carbon source at 5% (w/v). This medium had an ammonium content of 0.1 g/L. Molasses were autoclaved separately from modified Burk's medium. Stock cultures were maintained at 4°C by periodic transfers on modified productive Burk medium agar slants.

Shake Flask Experiments

The organism was grown aerobically in 250- and 500-mL Erlenmeyer flasks with one-third volume of the culture medium with the carbon sources alone or supplemented with 0.1 g/L of $(NH_4)_2SO_4$. The flasks were incubated in a rotatory shaker at 220 rpm at 30°C for 72 h.

Analytical Methods

Cell growth was monitored by measuring the optical density at 610 nm. Cell dry wt was measured by freeze-drying harvested cells from 2 mL of culture broth. Residual glucose in the broth was determined by an enzymatic method utilizing glucose oxidase/peroxidase (Wiener Laboratory, Rosario, Argentina). Fructose and sucrose consumption were assessed utilizing the phenol-sulfuric method, and measuring absorbance at 488 nm *(17)*. Ammonium was determined using the phenol-nitroprussiate method *(18)*. Protein was determined by the Bradford method *(17)*. PHB content was determined using gas chromatography-flame ionization detection after methanolysis of freeze-drying cells from 4 mL of broth, according to Braunegg et al. *(19)*. A column filled with Reoplex 400 on Chromosorb 80/100 was used. Column temperature was 150°C. EPS were assayed in the culture supernatant (0.5 mL) after trifluoroacetic acid (0.5 mL of 4.25 *N*) hydrolysis in sealed tubes at 100°C for 3.25 h. Samples were freeze-dried and resuspended in distilled water. Carbohydrates were determined utilizing the phenol-sulfuric method and were referred to the initial volume *(15)*.

PHB Extraction and Purification Procedure

Lyophilized cells were extracted for 24 h in a Soxhlet apparatus. Chloroformic extract (150–250 mL) was concentrated by simple distillation to a final volume of 25 mL *(30)*. The concentrated polymer was reprecipitated

twice into 10 vol of cold ethanol into an ice bath. Reprecipitated biopolymer was filtered, acetone washed, and centrifuged (15 min, 4620*g*). Purified PHB was dried in an oven at 60°C until it reached constant weight. Structure was confirmed and purity was assessed by Fourier transform-infrared, ^{1}H, and ^{13}C-nuclear magnetic resonance spectroscopy (spectra not shown).

EPS Extraction and Purification Procedure

Cells from 50 mL of broth were removed by centrifugation (5000*g*, 20 min). To the supernatant was added 0.05 g of NaCl and mixed. This mixture was poured into 200 mL of acetone and stirred. Then it was maintained overnight at 4°C. The material was centrifuged (10,000*g*, 20 min) and then dialyzed overnight at 4°C. EPS was recovered from the freeze-dried pellet *(17)*.

Results

Growth Curves and Specific Growth Rates

Simple and complex carbon sources were utilized in order to assess PHB and EPS biosynthesis on batch cultures of *A. chroococcum* strain 6B. Figure 1 shows growth curves on glucose, sucrose, fructose, and sugar cane molasses. Figure 2 depicts growth curves with the simple carbon sources plus ammonium sulfate (0.1 g/L). The molasses growth curve was not experimented because molasses itself has an ammonium content of 0.1 g/L. Growth on glucose and fructose with ammonium sulfate followed a similar trend with respect to nitrogen-fixing conditions, while sucrose exhibited a higher specific growth rate in the medium containing ammonium sulfate. Molasses had a specific growth rate similar to glucose and was 30 and 35% higher than fructose and sucrose, respectively (Table 1). However, the maximum final absorbances were obtained with fructose and molasses. Growth curves for simple carbon sources with 0.1 g/L of ammonium sulfate had similar specific growth rates with glucose and sucrose, and were nearly 60% higher than with molasses. The final absorbance with the complex carbon source was again significantly higher than with glucose or sucrose alone, and similar to that with fructose and ammonium sulfate.

PHB Production

Figure 3 shows biopolymer profiles for the different carbon sources solely or with added nitrogen. Ammonium sulfate addition resulted in a reduction of PHB accumulation with glucose and sucrose. Also, final cellular mass decreased 30% with sucrose but remained nearly constant with glucose. Protein was twofold higher in ammonium-grown than in non-nitrogen-added cultures, accounting for the similar biomass achieved under both conditions (Table 1). Fructose did not produce PHB with or without added nitrogen and, under our conditions, proved to be an inadequate carbon source for PHB production. PHB from sucrose-grown cells

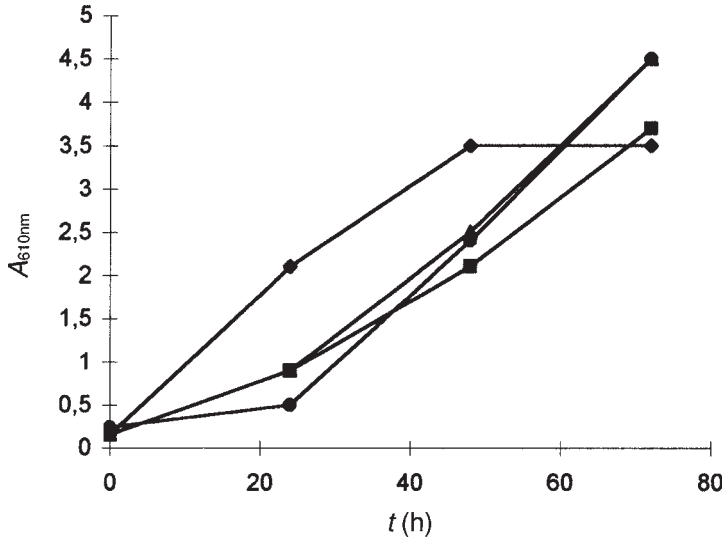

Fig. 1. Absorbance at 610 nm as a function of time for (■) glucose, (▲) fructose, (◆) sucrose, and (•) molasses media for *A. chroococcum* strain 6B.

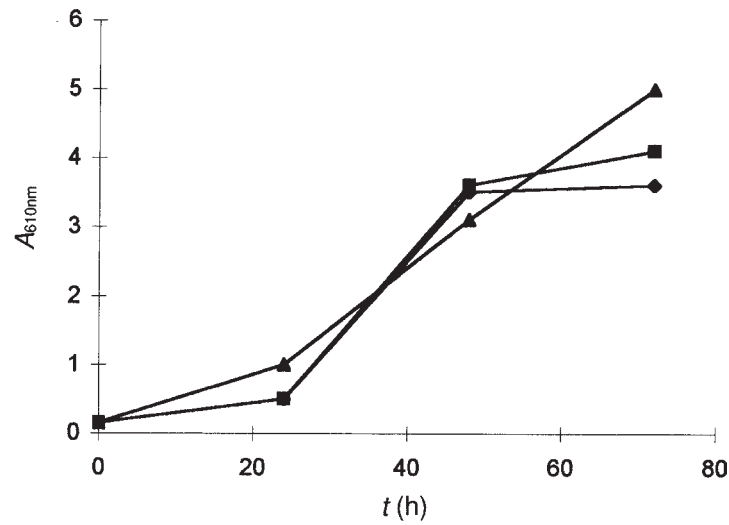

Fig. 2. Absorbance at 610 nm as a function of time for (■) glucose, (▲) fructose, and (◆) sucrose media supplemented with 0.1 g/L of ammonium sulfate for *A. chroococcum* strain 6B.

was the highest obtained from all experimented conditions (1.1 g/L at 48 h, 40% cell dry wt). Cells in molasses medium accumulated high amounts of PHB (0.7 g/L, 37%) comparable to that of glucose-grown cells (0.85 g/L, 48%). In all cases, maximum PHB accumulation was achieved at 48 h of culturing.

Table 1
Effect of Carbon Sources and Ammonium Sulfate Addition on pH, Carbon Source Consumption, and PHB Content by *A. chroococcum* Strain 6B[a]

Carbon source	Final pH	μ (h^{-1})[b]	Carbon consumption (g/L)	Cell dry wt (g/L)[c]	Protein (g/L)	PHB/cell dry wt (g/g)
Glucose	4.7	0.09	8.0	1.65	0.41	0.45
Sucrose	5.8	0.13	7.0	2.75	0.48	0.40
Fructose	5.8	0.06	5.0	1.80	0.45	ND[e]
Molasses	5.6	0.08	4.5	2.00	0.50	0.38
Glucose + NH_4^{+d}	7.2	0.13	9.5	1.80	0.75	0.28
Sucrose + NH_4^{+d}	7.0	0.13	9.5	1.95	0.85	0.16
Fructose + NH_4^{+d}	7.5	0.09	6.0	2.00	0.90	ND[e]

[a]Measurements were taken at 48 h. Simple carbon sources were at 1% (w/v) and molasses at 5% (w/v).
[b]μSpecific growth rate.
[c]Final cell dry wt.
[d]Initial concentration of ammonium sulfate was 0.1 g/L.
[e]PHB not detected.

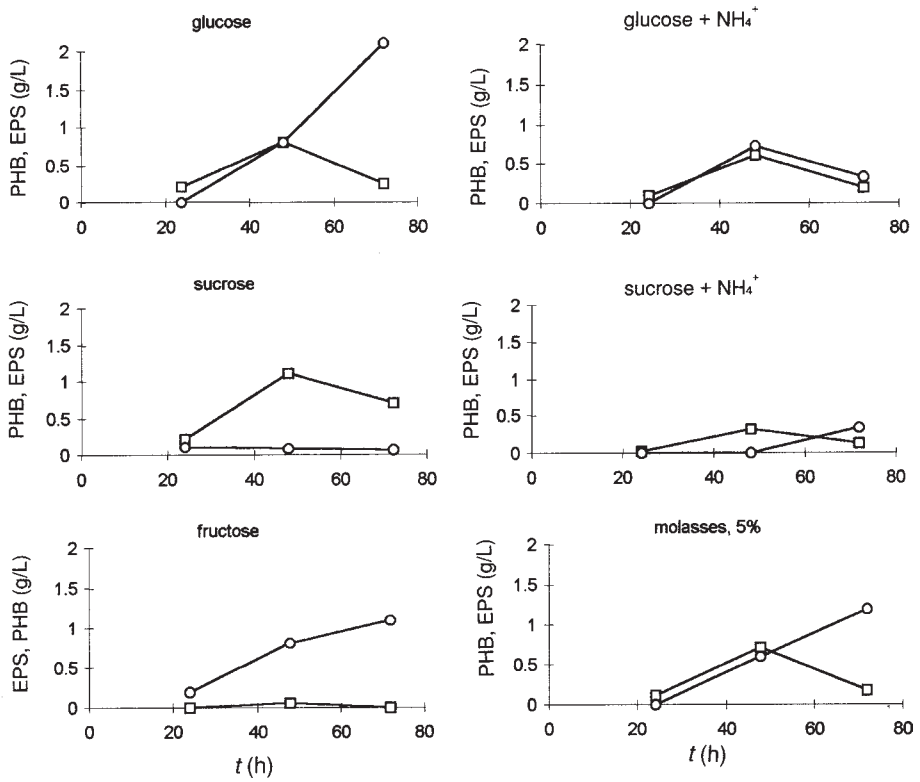

Fig. 3. (□) PHB and (○) EPS production as a function of time for glucose, fructose, sucrose, and molasses media, showing the effect of ammonium addition with glucose and sucrose media.

EPS Production

With glucose and fructose, high amounts of EPS were biosynthesized (maximum 2.1 and 1.1 g/L, respectively, at 72 h) whereas with sucrose there was no EPS production. When a complex carbon source such as sugar cane molasses was utilized, EPS production started at 48 h and continued to be biosynthesized to a maximum value of 1.1 g/L at 72 h. Interestingly, EPS production was reduced when glucose medium was added with 0.1 g/L of ammonium sulfate (2.1–0.3 g/L from 48 to 72 h). Ammonium addition did not affect the EPS profiles of fructose (data not shown), but a small increase was observed with sucrose after 48 h up to 72 h.

In nitrogen-fixing medium with glucose, pH diminished to 4.7, while with fructose and sucrose final pH was 5.8. In ammonium-supplemented media, pH remained near the neutrality. With fructose, a slight alkalinization was observed. Carbon consumption followed growth curves (Table 1). On the condition of maximal EPS production (glucose medium at 72 h), 20% of the initial carbon supplied was converted to EPS.

Calcium, magnesium, and iron (*see* Materials and Methods) consumption did not show significant differences between glucose and sucrose media alone or between glucose and sucrose with fed nitrogen. Iron was completely depleted at 24 h of culturing in both conditions. In glucose and sucrose media, 35% of the initial calcium and 50% of the starting magnesium were consumed after 24 h, and then consumption remained constant until 72 h. With added inorganic nitrogen, calcium and magnesium consumption were 25 and 40% of the initial values at 24 h. Magnesium was assayed between 0.1 and 0.6 g/L and calcium between 0.025 and 0.2 g/L of their corresponding salts. The optimal concentrations for PHB production were 0.4 and 0.11 g/L at 48 h of culturing, respectively (data not shown).

PHB Fraction of Total Biopolymers Produced

The PHB fraction of the total amount of biopolymers produced (defined as the ratio between PHB concentration and PHB plus EPS concentrations) was calculated for the experimental conditions with and without ammonium as a function of time (Fig. 4A,B). Fructose-grown cells showed the same pattern either with or without added ammonium, and the PHB fraction was almost zero. Cultures with glucose decreased their PHB fraction continuously in time, but from 48 to 72 h, in ammonium-containing media, the PHB fraction was markedly increased (from 0.45 to 0.61) (Fig. 4B), reflecting the decrease in EPS production, even when PHB concentration itself was also reduced. Sucrose-grown cells showed the higher values of PHB fraction over the entire culture process. Only in ammonium-added media, from 48 to 72 h, was the PHB fraction significantly reduced (from 1 to 0.28), consistently with an increased EPS biosynthesis (0.33 g/L). This trend was inverse if compared with glucose plus ammonium-grown cells, in which EPS production diminished from 48 to 72 h.

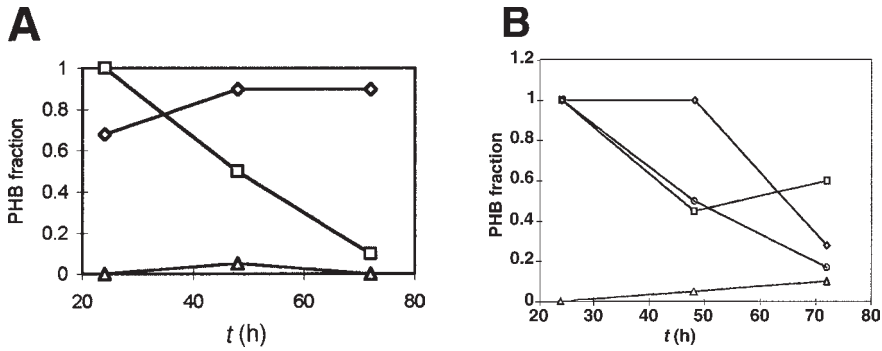

Fig. 4. PHB fraction as a function of time for (□) glucose, (△) fructose, (◇) sucrose, and (○) molasses media for *A. chroococcum* strain 6B. **(A)** Without ammonium salt; **(B)** 0.1 g/L of ammonium sulfate–supplemented media. (The PHB fraction is defined as the ratio between PHB content [g/L] and the sum of PHB plus EPS content [g/L].)

Molasses showed a similar trend on the PHB fraction profile compared to the sucrose plus ammonium medium, but with molasses a continuous decrease in the profile was observed.

Discussion

Concerning growth, sucrose and molasses were the best substrates for final cell mass production under our experimented conditions. Although molasses-grown cells had a relatively low specific growth rate, final absorbances (and cell dry wt) were higher than for cells growing with glucose, and similar for those cells growing in fructose medium.

An extended lag phase observed with molasses can be attributed to an inhibitory effect of molasses components, probably colloid or gum substances (which account for nearly 9 to 10% [w/v] of the composition of raw molasses). However, this effect was overcome, and cells continued to grow at a higher rate than with simple carbon sources. When ammonium was fed to the cultures, final cell mass was slightly higher than in cultures grown under nitrogen-fixing conditions, except for sucrose.

No clear correlation could be obtained between PHB and EPS coproduction and calcium, magnesium, or iron consumption. The reduced calcium consumption observed in ammonium-grown cells was also determined by Jacobson et al. *(21)*, who found that lower amounts of calcium were needed by *A. vinelandii* grown with combined nitrogen.

Fructose-grown cells produced high quantities of EPS (1.1 g/L, 72 h). This is most likely because in the pathway for alginate biosynthesis, the glycolytic intermediate fructose-6-phosphate is the first metabolite converted to mannose 6-phosphate by the action of phosphomannose isomerase *(9)*.

It was shown that ammonium-depleted media favored EPS production rather than PHB accumulation in our strain of *A. chroococcum*, with the

exception of sucrose-grown cells, whose PHB accumulation overcame the EPS biosynthesis. These results agree with those obtained using *Pseudomonas* sp. Higher EPS yields were found in media with a high C/N ratio with glucose and fructose as carbon sources. This was also observed in batch cultures under carbon excess on *Escherichia coli* and *Klebsiella aerogenes (17)*. In addition, nitrogen limitation favored EPS production in continuous cultures of *A. vinelandii (22)*. Acidification was observed in cultures grown under nitrogen fixation and with molasses, and was correlated with EPS excretion, except for sucrose-grown cells. These results agree with the observation that acid production is a common feature of *Azotobacter* and are associated with slime formation *(16)*. Conversions of approx 30% of the initial carbon supplied to EPS production were reported with two strains of *A. vinelandii* after 48 and 110 h of growth *(16,23)*. These values are close to those obtained in our work with *A. chroococcum* strain 6B.

PHB was obtained efficiently utilizing molasses, similarly to sucrose medium. This result can be attributed to the fact that molasses medium contained a high sucrose concentration. However, in molasses medium, the PHB fraction decreased continuously after 24 h (parallel with an increasing EPS). This effect can be attributed to glucose and fructose present in molasses medium, which were proved to produce high amounts of EPS. However, the effect did not appear in sucrose-grown cells, which almost did not produce EPS.

From our results, we can conclude that the sucrose medium was the most suitable of the media experimented for PHB production with *A. chroococcum* strain 6B, because the specific growth rate was the highest obtained in our experimental conditions, PHB productivity was high (0.4 g of PHB/g of cell dry wt at 48 h), and a PHB fraction of total biopolymer close to 1 was produced during the 72 h of culture. However, additional work is necessary to understand the inverse effect of ammonium addition on EPS production for glucose and sucrose-grown cells, and the mechanisms that inhibit EPS production in sucrose-grown cells.

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