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# Effect of Growth Conditions on the Molecular Weight of Poly-3-hydroxybutyrate Produced by *Azotobacter chroococcum* 7B

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**Abstract**—It has been shown that poly-3-hydroxybutyrate (**PHB**) of predetermined molecular weight can be obtained by varying the growth conditions of the producer strain, *Azotobacter chroococcum* 7B: pH, temperature, aeration, presence of sodium acetate as an additional carbon source, or growth on crude complex carbon sources (molasses, vinasse, or starch). High-molecular-weight polymer can be obtained at pH 7.0, optimal for the culture (1485 kDa), temperature 30–37°C (1600–1450 kDa, respectively), and low aeration (2215 kDa). The following factors decrease PHB MW: pH deviation to the acidic (pH 6.0, 476 kDa) or alkaline (pH 8.0, 354 kDa) range or lower temperature (20°C, 897 kDa). Introduction of additional carbon source (sodium acetate) at concentrations in the medium varying from 0 to 5 g/l provides an original method of production of PHB with predetermined MW in a wide range, from 270 to 1515 kDa, with high PHB content in the cell.

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Many bacteria synthesize and accumulate poly-3-hydroxybutyrate (**PHB**) as a carbon storage substance. The content and polymerization degree of this polymer broadly vary depending on bacterial growth conditions. Molecular weight (MW) is an important index, determining physicochemical properties of PHB. It depends on physiological, biochemical, and genetic features of the producer. Different bacteria produce PHB of different MWs. Species of the genus *Azotobacter* produce PHB of MW within 800–2000 kDa; the hydrogen-oxidizing bacterium *Alcaligenes eutrophus*, 600–1000 kDa; the methylotroph *Methylobacterium extorquens*, when grown on methanol, up to 600 kDa, and grown on succinate, to 1700 kDa [1]. The greatest MW of PHB, 20000 kDa, was achieved with a recombinant *Escherichia coli* strain [2].

The degree of PHB polymerization is also affected by producer growth conditions: carbon source, mineral components of the medium, temperature, air flow, etc. [3]. The molecular weight of PHB produced by *M. extorquens* is known to vary from 50 to 800 kDa depending on carbon source, for example, concentration, and methanol : ammonium ratio [4]. In contrast PHB MW is less dependent on pH and is not affected at all by growth temperature.

In experiments with bacteria of the genus *Azotobacter*, PHB MW notably varied with aeration and C : N ratio [5, 6]. With increase in the molar C : N ratio from 34.4 to 137.7, the MW of the polymer decreased eightfold, from 810 to 110 kDa. In *A. vinelandii* UWD, PHB MW decreased from 1500 to 1000 kDa with sugar concentration increase from 2.5 to 5.5%.

This study is dedicated to the effect of growth conditions (pH, temperature, aeration, and presence of additional carbon sources, such as acetate or crude products: molasses, vinasse, or starch) on the molecular weight of PHB produced by *Azotobacter chroococcum* 7B.

## MATERIALS AND METHODS

**Producer strain.** Experiments were performed with *Azotobacter chroococcum* 7B, capable of PHB overproduction. The strain had been isolated from wheat rhizosphere (sod podzol) and maintained in Ashby medium (g/l): $K_2HPO_4 \cdot 3H_2O$, 0.2, $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.006, $CaCO_3$, 5.0; sucrose, 20; agar, 20. All experiments were carried out under laboratory conditions. Overproduction of PHB was achieved by growing the strain with shaking in flasks with 200 ml of Burk medium with an excess of carbon source in the medium (g/l): $MgSO_4 \cdot 7H_2O$, 0.4, $FeSO_4 \cdot 7H_2O$, 0.01, $Na_2MoO_4 \cdot 2H_2O$, 0.006; sodium citrate, 0.5; $CaCl_2$, 0.1, $K_2HPO_4 \cdot 3H_2O$, 1.05, $KH_2PO_4$, 0.2; sucrose, 40 [7].

PHB *isolation and purification.* The protocol of PHB isolation from biomass and purification included extraction with chloroform with shaking at 37°C for 12 h, separation of the PHB solution from cell remains by filtration, precipitation from the chloroform solution with isopropyl alcohol, repeated (3–4 times) dissolution in chloroform and precipitation with isopropyl alcohol, and drying at 60°C.

**Determination of PHB content in cells.** Cell suspension (20–100 mg of dry biomass) was centrifuged at

**Table 1.** Parameters of PHB production by *A. chroococcum* 7B

| Carbon source | Biomass yield, g/l | PHB content, % of dry cell biomass | PHB yield, g/l | Intrinsic viscosity, dl/g | MW, kDa |
|---|---|---|---|---|---|
| Glucose 4% | 4.8 | 77.3 | 3.7 | 9.70 | 1660 |
| Food sugar 4% | 5.2 | 72.4 | 3.8 | 8.88 | 1490 |
| Molasses 6%* | 2.5 | 36.5 | 0.9 | n.d. | n.d. |
| Molasses 4% + food sugar 2% | 2.2 | 47.1 | 1.0 | n.d. | n.d. |
| Molasses 4% + food sugar 2% | 7.3 | 32.0 | 2.3 | n.d. | n.d. |
| Molasses 2 % + food sugar 3% | 6.5 | 59.6 | 3.9 | 4.17 | 590 |
| Vinasse 25% | 5.1 | 73.5 | 3.7 | 5.83 | 890 |
| Starch 20 g/l | 4.0 | 60.2 | 2.4 | 7.68 | 1250 |
| Starch 30 g/l | 5.1 | 63.0 | 3.2 | 7.97 | 1310 |

* Without adding base Burk medium;
n.d., not determined.

5000 *g* for 20 min. Cells were suspended in 10 ml of water and homogenized by 30 s sonication. The resulting cell homogenate had the optical density 0.4–0.5, according to photocolorimeter measurements (cell no. 1, optical path length 1 mm). Two milliliters of the homogenate were mixed with two milliliters of 2 N HCl and heated at 100°C in a water bath for 2 h. The insoluble fraction (PHB grains) was separated by centrifugation at 8000 g for 20 min. The sediment was transferred to test tubes with stopper plugs, and 5 ml of chloroform were added. The tubes were tightly closed and shaken at 28°C overnight. The mixture was centrifuged at 10000 *g* for 15 min, and a 0.1 ml aliquot of the air-dried pure chloroform extract was mixed with 5 ml of concentrated $H_2SO_4$. The mixture was heated at 100°C in a water bath for 10 min. After precipitation, the amount of crotonic acid formed by acidic PHB hydrolysis and dehydrogenation of hydroxybutyric acid was determined with an SF-2000 spectrometer (OKB-Spektr, Russia) at $\lambda = 235$ nm, optical path length 1 cm, against concentrated $H_2SO_4$ [8].

**Determination of PHB MW.** The molecular weight of PHB was determined with a capillary glass Ubbelohde viscometer with suspended level (capillary diameter 0.56 mm) [9]. Viscosity was measured at 30 ± 0.5°C. For the measurement, 10 ml of the solution were poured into the viscometer, which was then placed into a thermostat strictly vertically and incubated there for 15–20 min. The flow time of PHB was determined as the mean of four to six measurements. For determining intrinsic viscosity, measurements were made for four or five PHB concentrations. Various PHB concentrations were obtained by diluting the starting solution of the highest concentration with chloroform. Sample concentrations varied within 50–200 mg PHB per 100 ml chloroform. Flow times varied in the experiments within 40–115 s. Specific viscosity was calculated as

$$\eta_{spec} = (t - t_0)/t_0,$$

where $t_0$ is the flow time of the solvent, s, and $t$ is the flow time of the polymer solution, s.

Molecular weight was calculated from the Mark–Houwink–Coon equation with the following coefficients:

$$[\eta] = 7.7 \times 10^{-5} \times M^{0.82}\ [10].$$

To determine inherent viscosity [η], the experimental intrinsic viscosity values ($\eta_{spec}/C$) were plotted against the polymer solution concentration, $C$. The intrinsic viscosity value [η] was obtained by extrapolating the plot to the Y axis. The accuracy of [η] determination was about 1%. The accuracy of MW calculation from the Mark–Houwink–Coon equation was within 2–5% [9].

The tabulated results were obtained for *A. chroococcum* 7B cultures in Burk medium [7] with modifications according to experimental features.

All experimental variants were performed in triplicate. The tables present the mean values.

## RESULTS AND DISCUSSION

**Biosynthesis of PHB on various carbon sources.** The data presented in Table 1 indicate that the culture poorly grew on 6% molasses (81–84% dry matter, of which 52–54% sucrose, 10–30% amino acids, 9–10% carboxylic acids, and 11–12% inorganic components) or molasses with sugar (4 and 2%, respectively). Use of

**Table 2.** Effect of sodium acetate on PHB production

| Sodium acetate, g/l | Biomass yield, g/l | PHB content, % of dry cell biomass | PHB yield, g/l | Intrinsic viscosity, dl/g | MW, kDa |
|---|---|---|---|---|---|
| 0 | 5.1 | 78.3 | 4.0 | 9.00 | 1515 |
| 2 | 5.1 | 79.5 | 4.1 | 6.93 | 1100 |
| 3 | 4.3 | 72.0 | 3.1 | 3.19 | 427 |
| 5 | 4.0 | 67.5 | 2.7 | 2.19 | 270 |

molasses and sugar at the same concentrations with additions of Burk medium salts allowed greater biomass production than on glucose or sucrose, although the PHB content was lower (32%). Thus, molasses stimulates *A. chroococcum* 7B growth but inhibits PHB production, which is in agreement with data reported in [11]. The optimum ratio was found to be 2% molasses + 3% sugar. It provided intense growth of the culture (6.5 g/l) and satisfactory PHB accumulation (3.9 g/l). Replacement of part of the sugar with molasses makes PHB less expensive, but its MW decreases. Of all carbon sources, the medium with sugar and molasses yielded the lowest PHB MW (590 kDa), which can also happen in cultures grown on pure molasses. Vinasse and malt residue, containing sugars (75 g/l), ethanol (3.3 g/l), volatile fatty acids (1.3 g/l), and polyphenols (1.2 g/l), were good substrates for *A. chroococcum* 7B growth and PHB production. In the culture grown on vinasse, the PHB yield per unit volume was approximately the same as on sugar or glucose (3.7 g/l), although PHB MW (890 kDa) was less than that of the polymer produced on pure sugar.

It is worth noting that *A. chroococcum* 7B is the only species of the genus *Azotobacter* able to consume starch, an inexpensive and readily available carbon source [12]. The yields of PHB with starch as the sole carbon source (20 and 30 g/l) were 2.4 and 3.2 g/l, respectively, and the MW of the resulting PHB was 1310 kDa, close to the value obtained with sugar.

**Effect of sodium acetate on PHB production.** As the lower MW of PHB obtained on the medium with molasses may be due to the admixture of organic acids and additional introduction of organic acids to a sugar-containing medium [7], we performed experiments with different sodium acetate concentrations in the medium. Unlike other PHB producers (e.g., *Ralstonia eutropha*), A. chroococcum is inhibited by high acetate concentrations. Experiments were carried out with three sodium acetate concentrations: 2, 3, and 5 g/l. Food sugar (20 g/l) was used as the main carbon source, and pH was maintained at 7.0. It is apparent from Table 2 that the increase in sodium acetate concentration from 2 to 5 g/l with the presence of sugar as the main carbon source reduced the MW of the PHB produced. At 2 g/l acetate, PHB MW was 1112 kDa; at 3 g/l, 427 kDa; and at 5 g/l, 270 kDa, which is about fourfold less than without acetate (1515 kDa). It is likely that the increase in the intracellular concentration of acetyl groups induces the activity of acetoacetyl-CoA reductase (EC 1.1.1.36). This results in elevation of the concentration of hydroxybutyryl-CoA, a polymerase substrate. High acetate concentrations form more polymerization centers and more initial fragments of polymeric chains, which results in production of PHB with lower MW [3].

The harvest of *A. chroococcum* 7B biomass at 3 and 5 g/l sodium acetate concentrations decreased to 4.3 and 4.0 g/l, respectively, in comparison with 5.1 g/l without acetate. Accumulation of PHB in cells was also reduced by the presence of acetate, although insignificantly. The PHB yield per medium volume unit at 5 g/l acetate was 2.7 g/l, and without acetate, 3.9 g/l. Thus, addition of acetate to the main carbon source (sugar) allows production of PHB of predetermined molecular weight within a wide range from 270 to 1500 kDa.

**Effect of pH on PHB production.** The data on pH effect in Burk medium on the MW of the PHB produced by *A. chroococcum* 7B are shown in Table 3. The polymer of the highest MW (1485 kDa) was obtained at neutral pH, optimal for the culture growth. At pH 6.5, MW decreased to 500 kDa, and at 6.0, to 354 kDa. Growth at pH 8.0 also decreased the degree of PHB polymerization (MW = 476 kDa). Thus, pH variation allows producing PHB of predetermined molecular weight within a wide range. However, this approach to controlling PHB polymerization degree cannot be applied to practice, because acidification of the medium adversely affects the culture growth and reduces the yield of the polymer per volume unit.

**Effect of aeration on PHB production.** Another approach to controlling the MW of the PHB produced by *A. chroococcum* 7B is variation of aeration intensity. As *A. chroococcum* 7B is an obligate aerobe, high aeration conditions were maintained during the first 12 h of the experiments on the effect of aeration on PHB

**Table 3.** Effect of pH on PHB production

| pH | Biomass yield, g/l | PHB content, % of dry cell biomass | PHB yield, g/l | Intrinsic viscosity, dl/g | MW, kDa |
|---|---|---|---|---|---|
| 6.0 | 3.44 | 51.6 | 1.65 | 2.73 | 354 |
| 6.5 | 4.30 | 65.6 | 2.69 | 3.63 | 500 |
| 7.0 | 5.81 | 68.5 | 3.81 | 6.45 | 1485 |
| 8.0 | 5.31 | 57.9 | 2.91 | 3.49 | 476 |

**Table 4.** Effect of aeration on PHB production

| Aeration conditions | Biomass yield, g/l | PHB content, % of dry cell biomass | PHB yield, g/l | Intrinsic viscosity, dl/g | MW, kDa |
|---|---|---|---|---|---|
| Aerobic (shaking at 250 rpm) | 7.66 | 75.3 | 5.8 | 6.44 | 1 480 |
| Aerobic (shaking at 190 rpm) | 3.13 | 74.1 | 2.3 | 7.90 | 1 670 |
| Stationary | 2.76 | 62.5 | 1.7 | 8.65 | 1 820 |
| Anaerobic | 2.87 | 61.3 | 1.7 | 10.90 | 2 215 |

polymerization degree by growing the strain under conventional conditions in cotton-plugged flasks with shaking at 250 rpm. Then aeration conditions were changed. In one series, cells were grown further under the same aeration conditions at 250 rpm; in the second series, at less vigorous shaking, 190 rpm; in the third series, the culture was grown under microaerophilic conditions, without shaking. In the series with anaerobic conditions, flasks were closed with rubber stoppers, air was evacuated with an oil pump, and flasks were filled with nitrogen by blowing nitrogen twice and then shaken until the end of the experiment. The results are shown in Table 4. At reduced aeration, the biomass harvest decreased; correspondingly, PHB yield per medium volume unit decreased, too. Under aerobic conditions (250 rpm), the PHB yield was 5.8 g/l; under microaerophilic or anaerobic conditions, 1.7 g/l.

Decrease of aeration level (series 2, shaking at 190 rpm) at the stationary phase of growth caused an increase in PHB MW from 1480 to 1670 kDa. Culture growth was not observed under microaerophilic conditions, but young (12-h) cells intensely produced the polymer. Its MW (1820 kDa) exceeded the MW of the polymer produced under aerobic conditions by about 30%. The culture did not grow under anaerobic conditions. Young 12-h cells intensely (as under microaerophilic conditions) produced PHB. Its MW (2215 kDa) was twice as high as that of the aerobically produced polymer.

A decrease in the molecular weight of the PHB produced by *A. chroococcum* 7B at higher aeration rates was also observed by other scientists in a semibatch process [5]. At the air flow rate 0.5 vol/(vol/min), the strain produced PHB of 1100 kDa, whereas flow rate elevation to 2.5 vol/(vol/min) reduced MW to 111 kDa. Obviously, the high intracellular NADH/$NAD^+$ ratio at oxygen shortage inhibits enzymes involved in glucose catabolism and the tricarboxylic acid cycle; therefore, acetyl-CoA is channeled to PHB production. Thus, lower aeration at the second stage of *A. chroococcum* 7B growth yields PHB with high MW, 1.5–2.2 MDa.

**Effect of aeration on PHB production.** *Azotobacter chroococcum* 7B was grown at various temperatures: room temperature (20°C), optimum temperature (30°C), and 37°C. The results, presented in Table 5, show that growth temperature had virtually no effect on PHB accumulation or MW within 30–37°C. At room temperature, PHB content in cells was 47.5%, and MW was 879 kDa. This fact appears to be related to growth retardation at room temperature: After 48-h incubation, cells were at an earlier growth stage than those grown at 30°C. Decrease in growth temperature reduces PHB

**Table 5.** Effect of temperature on PHB production

| Growth temperature, °C | Biomass yield, g/l | PHB content, % of dry cell biomass | PHB yield, g/l | Intrinsic viscosity, dl/g | MW, kDa |
|---|---|---|---|---|---|
| 20 | 2.10 | 47.5 | 1.0 | 5.86 | 897 |
| 30 | 7.87 | 73.7 | 5.8 | 9.5 | 1600 |
| 37 | 7.30 | 75.2 | 5.5 | 8.7 | 1450 |

MW, but this approach is not advisable because of poor growth and low PHB content. Temperature elevation to 37°C caused no significant changes of polymerization degree or PHB content.

Thus, variation of pH, aeration rate, and concentration of acetate as an additional carbon source, taken together with use of readily available carbon sources (sugar industry waste) allows production of PHB of predetermined MW within the range from 270 to 2215 kDa, which is important for biotechnological applications. Use of sugar industry waste makes PHB production much cheaper, because the nutrient cost constitutes a significant portion of the polymer cost [13, 14].

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