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POLYHYDROXYBUTYRATE BIOSYNTHESIS BY *Azotobacter chroococcum* 23 FROM RENEWABLE UNREFINED CARBON SOURCES

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Polyhydroxybutyrate (PHB) is a thermoplastic biopolymer posessing unique properties such as biocompatibility and biodegradability. The potential production of PHB from renewable substrates makes this polymer a prospective substitute for thermoplastics chemically synthesised from oil products. As a thermoplastic, PHB can be processed like other synthetic thermoplasts; however, some polymeric properties (thermal stability, short processability window, brittleness, and relatively high cost) limit its feasibility. With the aim of improving the competitiveness of PHB, the various unrefined carbon sources were tested in relation to Azotobacter chroococcum *23 growth, PHB production and polymer properties. Starch syrups from corn and potatoes were found to be the most suitable unrefined carbon sources for PHB production.*

Key words: *biopolymer, poly-β-hydroxybutyrate, unrefined carbon sources, yield.*

INTRODUCTION

The use of biodegradable polymers offers a sound solution for the plastic waste problem. Polyhydroxyalcanoates (PHAs) are naturally biodegradable thermoplastics. For example, poly-β-hydroxybutyrate (PHB) is produced by many bacteria as a carbon and energy reserve (Anderson and Dawes, 1990; Page et al., 1997). The production of PHA from renewable natural resources is ecologically advantageous, compared to thermoplastic and elastomer production from fossil carbon sources (Schlegel, 1992). A major bottleneck in industrial realisation of bioplastics (PHA) is its higher production cost compared to conventional petrochemical plastics. One serious factor determining the economics of PHA production on the industrial scale is the raw material cost (Yamane, 1993). The carbon source still contributes a significant portion (25 %) of the overall production cost (Dale and Linden, 1984). Sugars are one of the most important raw materials of the microbiological industry, as a universal source of energy and carbon for the synthesis of macromolecules. Refined sugars, such as glucose and sucrose, are expensive substrates for industrial use. Presently, the most popular raw materials in the world market are molasses, starches, sucrose, lactose, grains, beets, potatoes and also oils and fats (Schneider and Steinmuller, 1996). In our investigations, the selection of a suitable raw material for the microbiological production of polyhydroxybutyrate was based on the physiological requirements of the producer of *Azotobacter chroococcum* 23 and on the availability of the raw material in the Latvian market.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The *Azotobacter chroococcum* strain 23 (Savenkova et. al., 1993) was used in this study. The basic fermentation medium contained (per litre): 3.0 g NH_4NO_3, 0.64 g K_2HPO_4, 0.2 g KH_2PO_4, 0.41 g $MgSO_4 \times 7H_2O$, 0.1 g $CaCl_2$, 10 mg $FeSO_4 \times 7H_2O$, 0.5 g Na-citrate and 6 mg $Na_2MoO_4 \times 2H_2O$. The fermentation medium (100 ml per 750-ml Erlenmeyer flask) was inoculated with a 4 % (v/v) inoculum that had been pregrown overnight in Burks media with 2 % glucose. Flask experiments were carried out in the 750 ml flasks containing 100 ml culture solution at 30 °C and 190 rpm on a rotary shaker for 48 hours. *A. chroococcum* 23 was cultivated in the fermentation medium supplemented with one of five different carbon sources (4 % w/v of carbohydrates): (1) glucose (reagent-grade chemicals), (2) raw sugar and (3) beet molasses (containing 50–52 % w/w sucrose) (all from Baltic Sugar Company), (4) corn starch syrup (containing 17 % glucose, 14 % maltose, 58 % oligosugars), and (5) potato starch syrup (containing 20 % glucose, 15 % maltose, 52 % oligosugars) (both from Lickeby Aloja, Latvia).

Analytical methods. Cell growth was monitored by measuring the optical density of the culture broth at 540 nm. The cell concentration was also determined by measuring the dry cell weight. Residual (RB), non-PHB biomass was calculated as total dry weight minus PHB dry weight. Glucose concentration was determined using DNS reagent with previous hydrolysis of carbon sources in culture fluid (Miller, 1959). The PHB concentration was determined by gas chromatography (Varian 3700, USA) with benzoic acid as an internal standard (Braunegg et al., 1978). For calibration, sodium-DL-β-hydroxybutyric acid (SIGMA, Deisenhofen, Germany) was used.

PHB isolation and analysis. PHB was extracted from isopropanol-pretreated biomass with hot chloroform and precipitated with isopropanol. PHB intrinsic viscosity [η] measurements were performed in chloroform solution at 30 °C by Ubbelohde dilution viscosimeter. The molecular mass of PHB was determined from intrinsic viscosity, as follows (Akita et al., 1975): $[\eta]=7.7\times10^{-5}\ xM^{0.82}$.

A differential scanning calorimeter (Mettler, Switzerland) was used to characterise time-dependent changes of the degree of crystallinity and thermal properties of samples. The sample size was typically 10–15 mg and they were scanned from -20 °C to +220 °C at 10 °C/min under nitrogen flow to cover glass transition and melting temperature ranges of all components used.

All experiments were repeated at least thrice. Representative results are reported.

RESULTS AND DISCUSSION

The *Azotobacter chroococcum* strain 23 produces PHB during exponential growth. Hence the substrate must simultaneously support both culture growth and accumulation of the polymer in the cells. Of the different raw materials used, *A. chroococcum* 23 growth was highest on glucose and potato starch syrup (both 13.0 $g\cdot l^{-1}$), with PHB concetration in the biomass being higher on glucose than on potato starch syrup (10.2 $g\cdot l^{-1}$ and 8.7 $g\cdot l^{-1}$, respectively) (Table 1). The product (PHB) yield ($Y_{P/S}$) ranged between 0.20 g/g and 0.28 g/g (Table 2). These values were comparable to other PHB producers (Steinbüchel, 1996). The highest substrate conversion to the product $Y_{P/S}$ ratio was 0.28 g/g (Table 2), attained by cultivation of the PHB producer in nutrient medium supplemented with corn starch syrup. The yields of the residual biomass were 0.06–0.19 g/g. The highest yield of residual biomass ($Y_{RB/S}$=0.19 g/g) was obtained using beet molasses as a carbon source, but a comparatively low PHB yield ($Y_{P/S}$ =0.20 g/g) results in insuitability of this cheapest raw material for PHB production. According to our data, the most suitable carbon sources for PHB production by *A. chroococcum* 23 are glucose and potato and corn starch syrups. Previously, it was observed that the best PHB production by another PHB producer *Azotobacter vinelandii* UWD was obtained using beet molasses (Page, 1992b).

Table 1

BIOMASS AND PHB BIOSYNTHESIS BY *Azotobacter chroococcum* 23 GROWN ON PURE AND UNREFINED SUGARS

Carbon source	DCW, $g\cdot l^{-1}$	PHB content, %	PHB concentration, $g\cdot l^{-1}$	Residual biomass, $g\cdot l^{-1}$
Glucose	13.0	78.3	10.2	2.8
Raw sugar	11.3	73.4	8.3	3.0
Beet molasses	10.5	51.8	5.4	5.1
Corn starch syrup	12.6	77.7	9.8	2.6
Potato starch syrup	13.0	67.0	8.7	4.3

Table 2

YIELDS OF BIOMASS, PHB, AND RESIDUAL BIOMASS FROM CONSUMED SUBSTRATES

Substrate	$Y_{X/S}$, g/g	$Y_{P/S}$, g/g	$Y_{RB/S}$, g/g
Glucose	0.34	0.26	0.08
Raw sugar	0.35	0.26	0.11
Beet molasses	0.39	0.20	0.19
Corn starch syrup	0.36	0.28	0.06
Potato starch syrup	0.33	0.22	0.11

Carbon sources affect not only PHB production, but also polymer extractability (Page and Cornish, 1992) and its molecular weight (Casella et al., 1990). The costs of PHB recovery decrease with increasing polymer concentration in biomass. In our experiments, high PHB content was observed in all bacterial biomasses except beet molasses biomass. The highest recoveries of PHB were obtained from biomasses of *A. chroococcum* 23 grown on glucose and starch syrups (about 79 % of the total PHB content) (Table 3). The differences in recovery may be explained by variable composition of cell cultures grown on the substrates. A higher amount of organic solvents would result in a higher recovery, but also increased cost of the end product. The lowest amounts of organic solvents were required for starch syrups (Table 3). The tested substrates resulted in differing physical properties of PHB recovered from the obtained biomasses (Table 4). The intrinsic viscosity for all PHB samples except beet molasses ranged between 11.74 $dl\cdot g^{-1}$ and 5.37 $dl\cdot g^{-1}$, which corresponds approximately to PHB

Table 3

PHB EXTRACTABILITY, IN RELATION TO CARBON SOURCE USED

Carbon source	PHB concentration in biomass, g/g	PHB recovered from total PHB content, %	Chloroform used for full PHB extraction, $ml\cdot g^{-1}$
Glucose	0.77	79.0	150
Raw sugar	0.78	69.3	147
Beet molasses	0.48	67.0	172
Corn starch syrup	0.74	78.6	112
Potato starch syrup	0.71	78.5	116

Table 4

INTRINSIC VISCOSITY AND MOLECULAR MASS OF PHB, IN RELATION TO CARBON SOURCE

Carbon source	Intrinsic viscosity, $dl \cdot g^{-1}$	Molecular mass, kDa
Glucose	11.74	2 094
Raw sugar	8.90	1 494
Beet molasses	3.67	507
Corn starch syrup	5.37	807
Potato starch syrup	6.05	933

molecular weights of 2094–807 kDa. These PHB molecular weight values are required for the polymer treatment (Page, 1992a). The thermal properties and crystalline behaviour of PHB synthesised by *A. chroococcum* 23 from unrefined sugars were similar to those of PHB products synthesised from pure glucose (Table 5 and Figure 1). Similar results have been found by others (Zhang and Wang, 1994). The major expenses in the production of PHA are the costs of substrate and the extraction of polymer from cells (Anderson and Dawes, 1990).

Table 5

THERMAL CHARACTERISTICS OF PHB RECOVERED FROM *AZOTOBACTER CHROOCOCCUM* 23 GROWN ON DIFFERENT CARBON SOURCES

Substrate	T_m, °C	X_C, %	T_g, °C
Glucose	176.0	57.5	3.7
Raw sugar	176.5	50.6	3.6
Beet molasses	175.7	58.2	3.7
Corn starch syrup	175.7	59.9	3.7
Potato starch syrup	176.3	63.5	3.6

T_m, endothermal melting point
X_C, crystallinity
T_g, glass transition temperature

Therefore, our results show starch syrups to be the most cost-effective unrefined carbon source for PHB production.

Further work will be aimed at optimisation of the process of PHB biosynthesis by application of the tested substrates, as well as evaluation of the economical factors, PHB yield, conversion ratios, and PHB properties.

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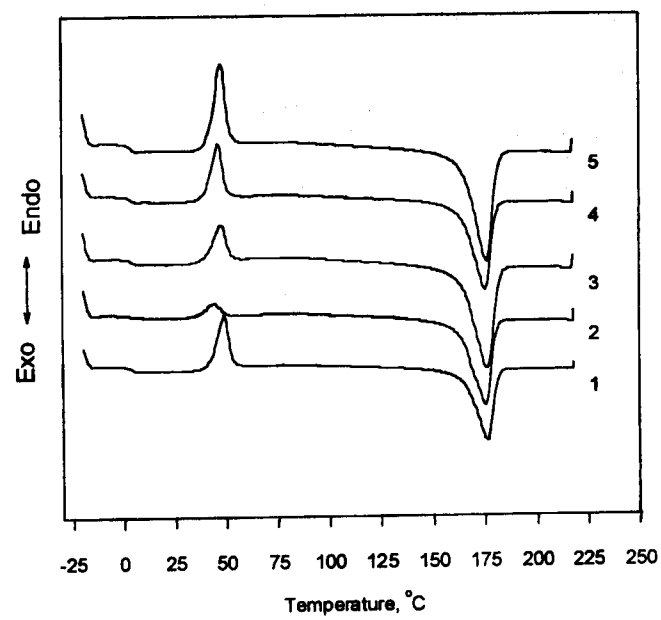

Fig. 1. Thermal curves for PHB samples: 1, PHB from glucose; 2, PHB from raw sugar; 3, PHB from beet molasses; 4, PHB from corn starch syrup; 5, PHB from potato starch syrup. **Endo**, endothermal effect; **Exo**, exothermal effect.

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ATJAUNOJAMO IZEJVIELU IZMANTOŠANA *Azotobacter chroococcum* 23 POLIHIDROKSIBUTIRĀTA BIOSINTĒZEI

Polihidroksibutirāts (PHB) ir termoplastisks polimērs ar unikālām īpašībām: biosavietojamību un biodegradabilitāti. Termoplastisko PHB var pārstrādāt ar parastajām sintētisko termoplastu pārstrādes metodēm, tomēr atsevišķas polimēra īpašības — termiskā stabilitāte, šaurs pārstrādes temperatūru diapazons, trauslums un relatīvi augstās cenas — ir pārstrādi ierobežojoši faktori. Lai veicinātu PHB konkurētspēju, pētīta nerafinētu oglekļa avotu ietekme uz *Azotobacter chroococcum* 23 augšanu, PHB iekššūnu koncentrāciju un iznākumu, kā arī uz polimēra īpašībām. Par piemērotākiem substrātiem PHB biosintēzei atzīti cietes sīrupi.