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THE EFFECT OF SUBSTRATE ON THE MOLECULAR WEIGHT OF POLY-ß-HYDROXYBUTYRATE PRODUCED BY *AZOTOBACTER VINELANDII* UWD

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SUMMARY

Azotobacter vinelandii UWD produced very high molecular weight (MW) (approx. 4 million Daltons) poly-ß-hydroxybutyrate (PHB) when grown in 5% w/v beet molasses medium. The polymer MW decreased as the beet molasses concentration was increased. Similar results were obtained in equivalent concentrations of sucrose (as raw sugar), but the polymer MW was not greater than 1.6 million. This difference was not caused by more severe oxygen-limitation in the beet molasses medium. It appeared that the non-sugar components of beet molasses promoted the formation of higher MW polymer. Fish peptone, a known PHB-yield-promoter in this organism, did not promote the formation of very high MW polymer.

INTRODUCTION

Polyhydroxyalkanoates are reserve polyesters that are accumulated as intracellular inclusions in a variety of bacteria (Anderson and Dawes, 1990). Of these polymers, poly-ß-hydroxybutyrate (PHB) is the most common. There has been a growing interest in the use of these biodegradable microbial polyesters as substitutes for bulk plastics like polyethylene and polypropylene (Byrom, 1987).

Azotobacter vinelandii strain UWD has been shown to be a potentially useful organism for the production of PHB and its copolymers (Page, 1992a; Page *et al.*, 1992). This strain produces relatively large amounts of PHB during growth on unrefined sugars. Beet molasses is an excellent substrate for PHB production and in addition, the non-sugar components of the molasses promote PHB formation (Page, 1992b). Recently this yield-

promoting effect has been duplicated by adding 0.05 to 0.2% w/v fish peptone to the medium, which increases the range of unrefined sugars that can be used for PHB over-production (Page, 1992c). However, in the utilization of these polymers as thermoplastics, the degree of polymerization has to be controlled since it affects mechanical properties and biodegradability (Korsatko *et al.*, 1983). There have been only a few studies to show how growth conditions effect PHB MW (Anderson *et al.*, 1992; Chen *et al.*, 1992; Suzuki *et al.*, 1988). Therefore it was of practical interest to determine if PHB MW from strain UWD was effected by the nature of the growth substrate or other growth conditions.

MATERIALS AND METHODS

A. vinelandii UWD was grown in a beet molasses medium containing salts and 50 μM ferric citrate (Page *et al.*, 1992). The beet molasses contained 50% (w/w) sucrose. Raw sugar medium consisted of the same minimal salts medium containing 2.5 to 5.5% (w/v) raw sugar (Rogers Sugar Co., British Columbia, Canada), 50 μM ferric citrate, 15 mM ammonium acetate and 0.1% (w/v) fish peptone (H0100BT, Protan, Drammen, Norway). Beet molasses fractions: concentrated separator by-product and extract molasses were obtained from The Amalgamated Sugar Co. (Twin Falls, Idaho, USA) and have been described (Page, 1992b). The medium was inoculated with a 4% (v/v) UWD inoculum, from an overnight culture, and incubated (100 ml per 500 ml flask) at 28-30°C for 24 h with rotary shaking at 225 rpm.

The growth and kinetics of PHB production also were studied in batch culture in a 2.5 L Bioflo III fermenter (New Brunswick Scientific Co.) using 5% (w/v) beet molasses medium. The fermenter set-up, process control and growth conditions have been described (Page *et al.*, 1992). Dissolved oxygen concentration (dO_2) was set at 5, 10 or 20% of air saturation and was maintained by an IBM-PC controller. The pH of the culture remained at ≈pH 7.5 during the PHB production phase and incubation temperature was held at 30°C. Whole culture samples were automatically removed at hourly intervals (Page and Rudy, 1992) and the total cell dry weight, PHB dry weight, cell protein and sucrose remaining in solution were determined (Page *et al.*, 1992).

PHB for MW determination was extracted from the biomass from 5 ml of culture using commercial bleach containing 30% Na_2CO_3 (pH 10) at room temperature for 10 min. The dissolved biomass was removed by filtration through a 0.2 μm pore-size glass microfibre filter (Whatmann GF/D) and the PHB remaining on the filter was washed with water, 95% ethanol and air dried. The PHB remaining on the filter was dissolved in 2 ml chloroform at 45°C for 1 h. The PHB solution was filtered through a 0.45 μm pore-size filter (Gelman Nylon Acrodisc, USA) and its weight average molecular weight (MW), number average molecular weight (MN), and dispersity (MW/MN) were determined by gel permeation chromatography in a Shimadzu LC-6A HPLC, relative to polystyrene standards (Budwill *et al.*, 1992).

RESULTS AND DISCUSSION

The MW and MN of PHB decreased with increasing beet molasses concentration (Fig. 1). The MN decreased more rapidly than MW, which resulted in a progressive increase in polymer dispersity at the higher molasses concentrations. Despite these

changes in polymer size, there was no significant inhibition of strain UWD growth (protein g/l) or PHB production (as g/l or % dry weight) over this range of molasses concentrations (data not shown).

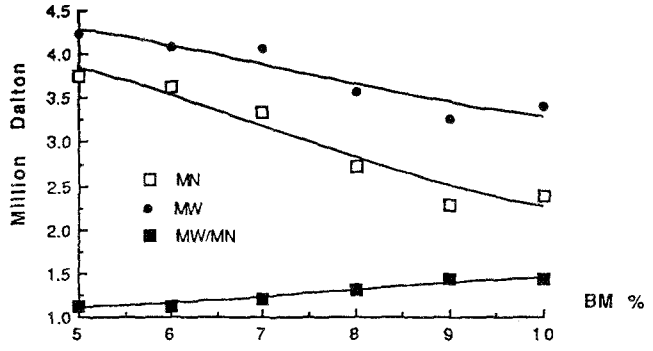

Fig. 1. Effect of beet molasses (BM) concentration on the molecular weight of PHB produced by *A. vinelandii* UWD incubated in shake flask culture for 24 h.

This result was similar to the decrease in PHB MW in response to increased C-source concentration seen in *Methylobacterium extorquens*, *Alcaligenes eutrophus* and *Alcaligenese latus* (Anderson *et al.*, 1992; Chen *et al.*, 1992; Suzuki *et al.*, 1988). To be sure that this decrease in *A. vinelandii* PHB MW was due to increased sucrose concentration and not the other non-sugar components of beet molasses, strain UWD was grown in equivalent concentrations of sucrose (as raw sugar) and PHB MW was determined. There was a definite decrease in polymer MW and MN in response to sucrose concentration (Fig. 2), while PHB yield and cell growth were unaffected (data not shown). However, the polymer MW and MN were significantly lower and dispersity was significantly higher from the raw sugar cultures compared to equivalent molasses cultures (Fig. 1 & 2).

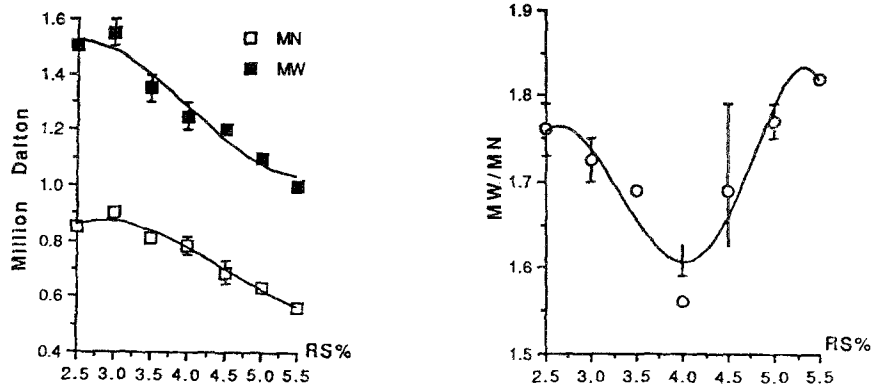

Fig. 2. Effect of raw sugar (RS) concentration on the molecular weight of PHB produced by *A. vinelandii* UWD incubated in shake flask culture for 24 h.

These results suggested that either (a) the increased viscosity of the molasses medium decreased O_2 availability and promoted high MW polymer formation, or (b) the molasses medium contained components that favoured the production of high MW polymer. To test the first possibility, strain UWD was grown in a fermenter with the dO_2 controlled. The MW and MN of PHB were not effected by O_2-limited (5% dO_2) to O_2-sufficient (20% dO_2) aeration conditions (Table 1). Increased aeration increased the consumption of sucrose and the production of cell protein, while it decreased the production of PHB, consistent with respiration and biosynthesis becoming more important electron-sinks than PHB-production (Anderson and Dawes, 1990; Page and Knosp, 1984).

Table 1. The effects of dO_2 on the production and molecular weight of PHB.

dO_2 (% sat)	MW (10^6 Da)	MN (10^6 Da)	PHB (% dry wt)	Protein (g/L)	Sucrose use [a]	PHB prodn [a]	$Y_{P/S}$ (g/g)[b]
5	4.1	3.3	73	0.08	1.0	0.70	0.32
10	4.0	3.1	59	0.14	2.2	0.38	0.23
20	4.0	3.1	48	0.48	2.5	0.20	0.09

[a] Measured as grams per liter per hour.
[b] Yield of g polymer (P) per g substrate (S) used in fermentation.

To test the second possibility, strain UWD was grown in media containing 4% (w/v) raw sugar alone or with: 0.1% fish peptone, 1% beet molasses, 1% concentrated separator byproduct, or 1% extract molasses, as sources of non-sugar compounds. The fish peptone was a source of amino acids and peptides, concentrated separator byproduct contained the majority of the acidic nitrogen-compounds, carboxylic acids and salts of beet molasses, while extract molasses contained the majority of the betaine and amino acids of beet molasses (Page, 1992b). When added at 1% (w/v) these fractions added ≤0.5% sucrose to the culture. Beet and extract molasses had essentially no effect on the MW or dispersity of the polymer formed in raw sugar, but the concentrated separator byproduct caused a definite increase in PHB MW (Fig. 3). Fish peptone, on the other hand, had a definite negative effect on PHB MW and increased polymer dispersity (1.9±0.1 vs 1.5 in the other cultures). This suggests that the acidic nitrogen compounds, the organic acids, or the salts present in beet molasses promote the formation of very high MW PHB in strain UWD.

PHB MW also was affected by the age of the culture. For example, cells grown in 5% beet molasses medium with 5 % dO_2 first produced relatively low molecular weight polymer, which grew to about 4 x 10^6 Daltons by 14 to 16 h (Fig. 4A). By 20 h the MW and MN were similar, giving the lowest dispersity value (1.2). Thereafter, polymer continued to accumulate in the cells (from 65 to 73 % dry wt) and the MW appeared to

increase to a greater extent than MN. This trend was repeated in other fermenter runs containing 5 % beet molasses with 10% (data not shown) or 20 % dO_2 (Fig 4B).

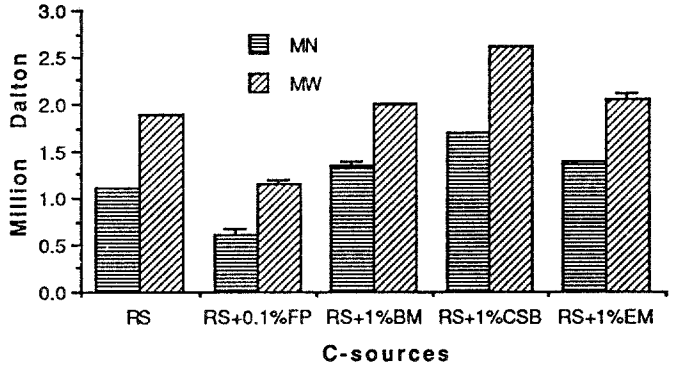

Fig. 3. The influence of fish peptone (FP), beet molasses (BM), concentrated separator byproduct (CSB) and extract molasses (EM) on the molecular weight of PHB produced by strain UWD in 4% raw sugar (RS) medium after 24 h incubation.

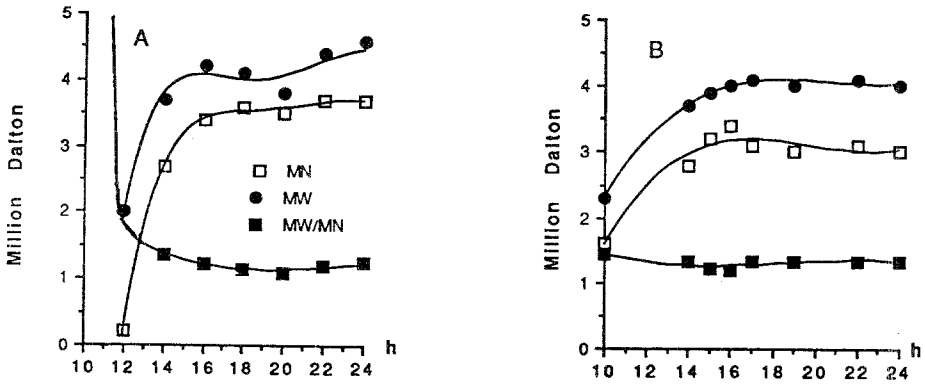

Fig. 4. Changes in PHB molecular weight during growth of strain UWD in 5 % beet molasses medium at (A) 5 % dO_2 and (B) 20 % dO_2.

These data support current models of polymer growth (de Koning and Maxwell, 1993; Gerngross *et al.*, 1993), where the polymer is synthesized first as a water-soluble polymer, then the relatively high MW polymer coalesces to form latex-like inclusions in the cytoplasm. Once an inclusion has been started, it acts as a nucleus for the absorption of other water-soluble polymers. This process determines the number of inclusions per cell, which is usually 9 to 13 in *A. eutrophus* and range from 0.24 to 0.5 µm in diameter (Anderson and Dawes, 1990). However, *A. vinelandii* has about ten-times the volume of *A.eutrophus*, which should permit the formation of either more or larger inclusions. An ongoing electron microscopic study of PHB formation by strain UWD shows that these cells contain about 20 relatively large inclusions (≥0.5 µm diameter) after 24 h in beet

molasses medium, but contain 40 or more inclusions, ranging from 0.10 to 0.44 μm diameter, after 24 h in glucose-fish peptone medium (Page, Sherburne and D'Elia, unpublished data). Thus it would appear that fish peptone-grown cells contain a diverse population of "young" and "old" inclusions which may give the relatively low MW and high polymer dispersity seen in this study.

This study shows that the MW of PHB produced by *A.vinelandii* strain UWD can be altered between one and four million Daltons by the choice of substrate(s) used for polymer formation. Studies are ongoing concerning the enzymology of PHB formation in strain UWD and it will be interesting to determine more accurately the effects of fish peptone and concentrated separator byproduct on PHB synthesis.

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REFERENCES

Anderson, A. J., and Dawes, E. A. (1990). Microbiol. Rev. 54, 450-472.

Anderson, A. J., Williams, D. R., Taidi, B., Dawes, E. A., and Ewing, D. F. (1992). FEMS Microbiol. Rev. 103, 93-102.

Budwill, K., Fedorak, P., and Page, W. J. (1992). Appl. Environ. Microbiol. 58, 1398-1401.

Byrom, D. (1987). Trend Biotechnol. 5, 246-250.

Chen, G. Q., Konig, K. H., and Lafferty, R. M. (1992). Effects of medium conditions on molecular weights of poly-ß-hydroxybutyrate (PHB) and poly-ß-hydroxyvalerate (PHB-co-HV) produced by strains of *Alcaligenese latus*. In: *International Symposium on Bacterial Polyhydroxyalkanoates*, H. G. Schlegel and A. Steinbüchel, ed., pp. 408-409, Goltze-Druck, Göttingen, Germany.

de Koning, G. J. M., and Maxwell, I. A. (1993). J. Environ. Polymer Degrad. 1, 223-226.

Gerngross, T., Reilly, P., Stubble, J. O., Sinskey, A. J., and Peoples, O. P. (1993). J. Bacteriol. 175, 5289-5293.

Korsatko, W., Wabnegg, B., Braunegg, G., Lafferty R. M., and Strempfl, F. (1983). Pharm. Ind. 42, 525-527.

Page, W. J. (1992a). FEMS Microbiol. Rev. 103, 149-158.

Page, W. J. (1992b). Biotechnol. Letters. 14, 385-390.

Page, W. J. (1992c). Appl. Microbiol. Biotechnol. 38, 117-121.

Page, W. J., and Rudy, B. (1993). Biotechniques 14, 228-233.

Page, W. J., and Knosp, O. (1989). Appl. Environ. Microbiol. 55, 1334-1339.

Page, W. J., Manchak, J., and Rudy, B. (1992). Appl. Environ. Microbiol. 58, 2866-2873.

Suzuki, T., Deguchi, H., Yamane, T., Shimizu, S., and Gekko, K. (1988). Appl. Microbiol. Biotechnol. 27, 487-491.