

Use of Poly(ethylene glycol)s To Regulate Poly(3-hydroxybutyrate) Molecular Weight during *Alcaligenes eutrophus* Cultivations

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ABSTRACT: The ability of poly(ethylene glycol)s, PEGs, to control poly(3-hydroxybutyrate), P3HB, molecular weight in a microbial fermentation polymerization process was studied using *Alcaligenes eutrophus* with fructose as the sole carbon source. PEGs varying in molecular weight and end group functionality were added to the cultivation medium subsequent to cell growth, and their effects on polymer formation were evaluated. In general, *A. eutrophus* showed substantial tolerance for PEGs. This was illustrated by similar viable cell concentrations for the medium without PEG, 10% (w/v) PEG-10 000 and 2% PEG-200. Furthermore, detrimental effects on polymer yields were not observed for concentrations of 5% PEG-106 and 10% PEG-10 000. The greatest reductions in molecular weight were obtained when relatively low molecular weight PEG was added to the medium. PEG-106 was most effective in that only 0.25% was required to reduce the number average molecular weight (M_n) by 74%. The largest decrease in P3HB M_n (from 455 000 to 19 400) was observed by adding 10% PEG-106 to the medium. The largest change in P3HB M_n per incremental addition of PEG occurred in the 0–1% PEG concentration range. Supplementing the incubation medium with the monomethoxy ether CH₃O–PEG–OH-350 and PEG-300 resulted in almost identical molecular weight reductions. However, the dimethoxy ether of tetraethylene glycol was not an effective agent for molecular weight reduction. Therefore, interaction between PEG and the PHA production system leading to molecular weight reduction was enhanced for lower molecular weight PEGs and required at least one PEG chain end functionality which may be a hydroxyl group. It is believed that PEG interacts with the *A. eutrophus* synthase in such a way to increase the rate of chain termination by water relative to chain propagation reactions.

Introduction

Poly(hydroxyalkanoate)s (PHAs) are a family of optically active polyesters made by numerous bacteria. PHAs are formed as intracellular inclusion bodies and function as reserve or storage materials. A number of reviews on PHAs that describe biochemical aspects of polymer formation, structural variability, and properties have been published.^{1–8} The process for PHA biosynthesis which involves a fermentation in aqueous media using renewable resources as carbon sources is extremely attractive as an “environmentally friendly” preparative route for plastic production. Also, microbial polyesters are biodegradable in appropriate disposal environments.^{9–14}

Poly(3-hydroxybutyric acid), P3HB, was the first identified member of the PHA family.³ PHAs occur in the cytoplasm of cells in the form of inclusion bodies or granules. Typically, granules have a diameter of 100–800 nm and are believed to be surrounded by an atypical micellar (monolayer) membrane.¹⁵ The PHA synthase and depolymerase system may also be bound to this envelope.^{16–18} The surface components of the granule play an important role in polymer production, assuming they are in close contact with their substrate at all times in order for the microbial cell to synthesize a granule of hydrophobic material in an aqueous environment.¹⁹ It was suggested that the polymerizing unit (synthase), in the form of protein subunits, aggregates into particle form.²³ Thus, upon transition to conditions where PHA accumulation occurs, the PHA synthase becomes granule-

associated. For P3HB biosynthesis, 3-hydroxybutyryl-CoA is the substrate of the PHA synthase.³

Although the biochemistry of PHA (mainly P3HB) biosynthesis has been the subject of much recent work,^{20–23} the mechanism of polymer growth and control of chain molecular weight is not understood. Thus, no rational methods for PHA molecular weight control have been reported. The value of polymerization degree may vary considerably depending on a number of factors including the choice of bacterial production system and fermentation physiological parameters.^{4,24,25} For example, the molecular weight of P3HB produced in a fed-batch culture of *Protomonas extorquens* was affected by the culture temperature, pH, molar ratio of methanol and ammonia, and the concentration of methanol in the medium;²⁴ extraordinarily high molecular weight P3HB having a weight average molecular weight of 3.39×10^6 has been obtained from *Azotobacter vinelandii*.²⁵

Poly(ethylene glycol), PEG, is a neutral water soluble polyether with extraordinary biological properties. PEG is relatively nontoxic to cellular systems,²⁶ large quantities of PEG are absorbed by membranes,^{27,28} and PEG is known to associate with membrane phospholipid head groups.²⁷ Also, alcohols cause changes in membrane lipid composition, leading to increased membrane fluidity.²⁹ Furthermore, it was shown that the osmotic stress imposed by the addition of PEG to media is tolerated by the accumulation of the solutes potassium or calcium ions.³⁰ Interestingly, the nascent PHA in granules remains amorphous at ambient temperature³¹ and PEG is miscible with P3HB.³² Thus, considering the above, it is intriguing to consider that if PEG diffuses from the media into cells, it might accumulate as a miscible component within PHA granules. PEG may also interact with the membrane of the PHA granules, altering the activity/specificity of the PHA synthase. Motivated

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by these ideas, investigations were carried out where PEG ($M_n = 200$, PEG-200) was added to second stage (after cell growth on a non-PHA producing medium) incubations of *A. eutrophus*.³³ The effects of PEG-200 on the conversion by *A. eutrophus* of the carbon source 4-hydroxybutyric acid to polyester were studied. It was found that addition of PEG-200 resulted in the following: (1) changes in the mol % of 3HB, 3-hydroxyvalerate (3HV) and 4-hydroxybutyrate (4HB) repeat units, (2) formation of complex product mixtures which could be separated into high 4HB and high 3HB content fractions, and (3) a method to prepare PHA-PEG diblock copolymers where the carboxylate terminus of 4HB rich PHA chains are covalently linked by an ester bond to PEG chain segments. Thus, the *in-vivo* formation of a natural-synthetic diblock copolymer was demonstrated.

In a preliminary report we described that PEG can be used to modulate the molecular weight of P3HB produced by *A. eutrophus*.³⁴ Specifically, by the addition of PEG-200, substantial molecular weight reductions were achieved that were dependent on PEG-200 media concentration. Thus, the opportunity to control PHA molecular weight by "PEG modulated fermentation"^{33,34} became apparent. Questions arose regarding the effects of PEG molecular weight and chain end structure on PHA molecular weight regulation. In this work, PEGs ranging in molecular weight from 106 (dimer of ethylene glycol) to 10 000 were used in culture media. Furthermore, mono- and dimethoxy derivatives of PEG were also investigated. The effects of PEG structure and concentration on cell yield, viability, polymer yield, accumulation of PEG into cells, and P3HB molecular weight were determined. In this way, PEG structural variables that lead to enhanced interactions with the PHA biosynthetic system for molecular weight regulation were elucidated.

Materials and Methods

Strain Information and Preservation. *A. eutrophus* ATCC 17699 was purchased from the American Type Culture Collection. Methods for bacterial strain preservation and inoculum preparation were described elsewhere.^{33,35-37}

Fermentations for P3HB Production. The method used was described in detail elsewhere^{33,37} and, therefore, is given in summary below. Cells were first grown (30 °C, 24 h) in a nutrient rich medium under aerobic conditions (250 rpm, 100 mL culture volume in 500 mL baffled flasks) and harvested by centrifugation. Typically, the cell dry weight of these first stage cultivations was approximately 3.5 g/L and the extent of polymer formation was limited to only 2.7% of the cell dry weight (~0.1 g/L). For polymer production in a second stage incubation, the washed cells were transferred under aseptic conditions into 100 mL of a filtered sterile nitrogen free minimal medium containing 20.0 g/L fructose as the carbon source, with or without PEG or PEG derivatives. Cultivations were carried out using 500 mL Erlenmeyer flasks at 30 °C, 250 rpm, for 48 h. Viable cell numbers were determined in triplicate from a given flask by the spread plate method at the end of the cultivation period. The cells were harvested by centrifugation (5000*g* force, 25 min), washed with water, lyophilized, and weighed. The total cell dry weight minus the weight of accumulated microbial polyester (see below for isolation procedure) was used to determine the non-PHA residual cell yield (R-CY) in g/L. The PEGs used had number average molecular weight (M_n) values of 106 (ethylene glycol dimer), 194 (tetraethylene glycol), 200, 300, 400, 1000, and 10 000 (PEG-106, TEG, PEG-200, PEG-300, PEG-400, PEG-1000, and PEG-10 000, respectively). In addition, studies were carried out with the monomethoxy ether CH₃O-PEG-350 and the dimethoxy ether of TEG (CH₃O-TEG-OCH₃). All of these PEGs and methoxy derivatives were obtained from Aldrich,

and the molecular weights given were as specified by the manufacturer. Four experimental runs were performed where, within an experimental run, the medium for individual flasks was obtained from a large stock solution and incubations were carried out side-by-side in an incubator shaker. The experimental runs consisted of culture flasks containing the following PEGs or PEG derivatives: (1) a control (no PEG added to the medium), PEG-106, PEG-400, PEG-1000; (2) a control, PEG-200, PEG-10 000; (3) a control, PEG-300, CH₃O-PEG-350; and (4) a control, TEG, and CH₃O-TEG-OCH₃. Standard deviations reported below for values of polymer yield and molecular weight were obtained from three replicate flasks within an experimental run using the medium without PEG.

Polymer Isolation. The intracellular P3HBs formed as well as PEGs accumulated in cells were extracted from cells by stirring a suspension of the lyophilized cells (about 0.5 g) for 48 h in chloroform (80 mL) at room temperature. The insoluble cellular material was removed by filtration and the solvent was evaporated to obtain the "crude product". Purified P3HBs were isolated by redissolving the crude product into a small volume (~4 mL) of chloroform, adding this solution to methanol (30 mL), washing the resulting precipitate with methanol and acetone, and then drying *in vacuo* (50 °C, 24 h, 1.0 mmHg). Unless otherwise specified, the purified products were obtained using one precipitation/washing cycle. PEGs in cells are solubilized during P3HB precipitation and washing. The yield of P3HB in g/L was determined as follows: {cell dry weight (g/L)} × { % P3HB of the cell dry weight } × {0.01}. The % PEG of the cell dry weight was determined from the % P3HB of the cell dry weight by ¹H NMR analysis of the crude product (see below).

Instrumental Procedures. ¹H NMR were recorded with a UNITY-500 spectrometer at 500 MHz using experimental parameters published elsewhere.³³ The molecular weights of P3HB products were determined by gel permeation chromatography (GPC). Polystyrene standards (Aldrich) with low polydispersities were used to generate a calibration curve from which product molecular weights were determined with no further corrections. Details of the GPC method followed a literature procedure.³³

Results and Discussion

When fructose is used as the sole carbon source for polymer synthesis, the PHA formed by *A. eutrophus* is P3HB.³⁸ In this investigation, P3HB accumulation from fructose was carried out in the polymer production medium (second stage incubations) which contained PEG diols of differing molecular weights and end group structures (see Experimental Section). To confirm PEG molecular weights specified by the manufacturer, the M_n values were determined using ¹H NMR spectroscopy by end group analysis (comparison of intensities for terminal CH₂-OH and internal -[O-CH₂-CH₂]- proton resonances at 3.6 and 3.7 ppm, respectively). ¹H NMR determined M_n values were 194, 300, 396, and 1008 for PEG-200, PEG-300, PEG-400, and PEG-1000, respectively. Therefore, there was excellent agreement between the ¹H NMR and manufacturer reported M_n values. Furthermore, the structure of the PEG methoxy derivatives was confirmed by ¹H NMR analysis by comparison of the signal intensities corresponding to terminal O-CH₃ functionalities at 3.38 ppm and internal -[O-CH₂-CH₂]- proton resonances. Relationships between PEG/PEG derivative structure, PEG concentration (% w/v), viable cell concentration, P3HB yield, and molecular weight are considered below.

Viable Cell Concentration and Polymer Yield. The viable cell concentration of *A. eutrophus* in colony-forming units per milliliter (c.f.u./mL) was measured for polymer-producing (second stage) cultivations at the harvesting time of 48 h (see Figure 1). The control experiments for the two experimental runs had almost

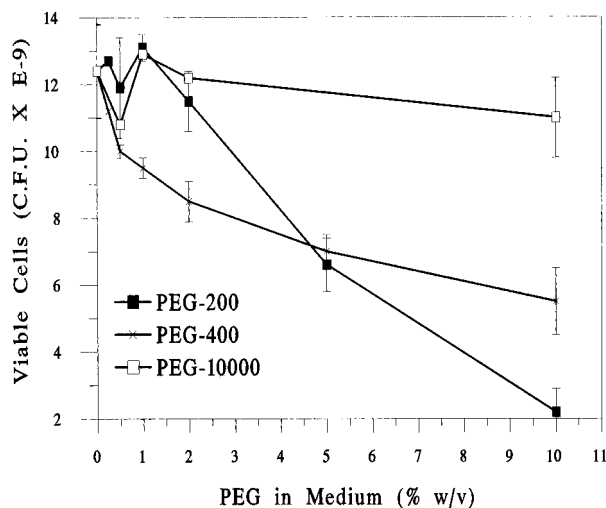


Figure 1. Plot of viable cell concentration as a function of the medium PEG concentration (% w/v).

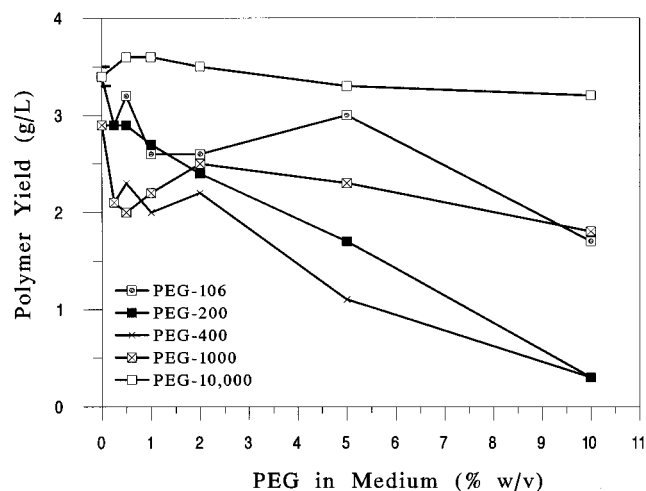


Figure 2. Effect of PEG medium concentration on the P3HB yield in g/L.

identical viable cell concentrations. The addition to the medium of up to 2% PEG-200 and 10% PEG-10 000 had no significant effect on the number of viable cells. In contrast, the addition of from 0.5 to 5% PEG-400 resulted in a regular decrease in c.f.u./mL. Increase in the medium concentration of PEG-200 above 2% to 5 and 10% resulted in a decrease in the number of viable cells by approximately 45 and 85%, respectively. Therefore, PEG-200 can be added at 2% concentrations with negligible effects on the viable cell concentration. In contrast, 10% PEG-10 000 added to the medium did not result in a decrease in the viable cell concentration. A greater tolerance of *A. eutrophus* to relatively large molecular weight PEGs such as PEG-10 000 is likely due to the correspondingly lower osmotic stress on cells. However, we do not have an explanation for the larger negative impact observed for PEG-400 relative to PEG-200 over the concentration range from $\geq 0.5\%$ to $< 5.0\%$.

A plot of polymer yield in g/L versus PEG concentration (% w/v) is shown in Figure 2. For the two experimental runs, shown by solid and dotted lines, the control medium had polymer yields of 2.9 ± 0.1 and 3.4 ± 0.1 g/L, respectively. Polymer yields were not significantly affected by up to 5% PEG-106 and 10% PEG-10 000 medium concentrations. In contrast, addition of 1% PEG-200, PEG-400, and PEG-1000 resulted in decreases in polymer yield of 20, 31, and 26%, respec-

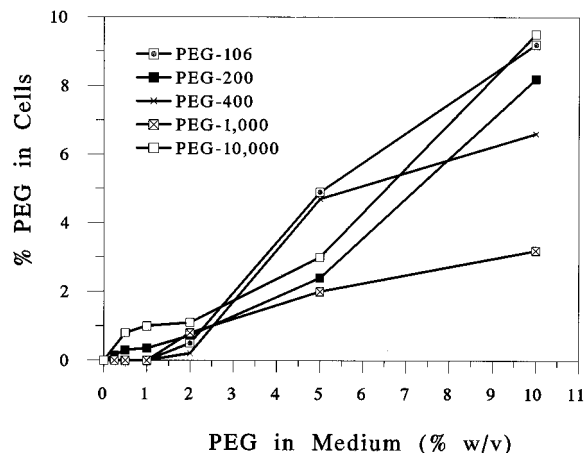


Figure 3. Relationship between the % PEG in the medium and the % PEG accumulated in cells (PEG uptake determined on lyophilized cells).

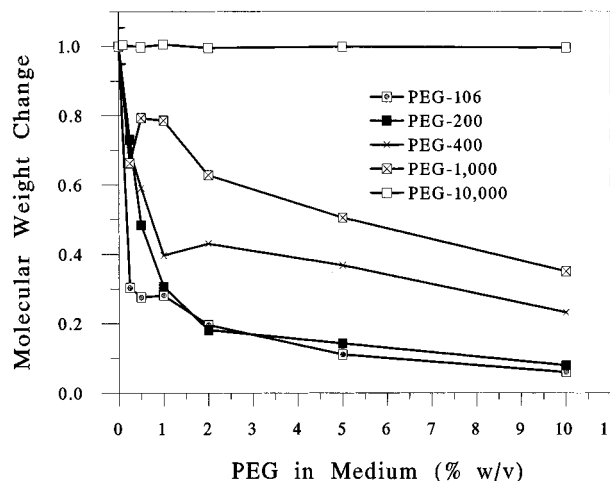


Figure 4. P3HB molecular weight change as a function of the medium PEG concentration. The values of P3HB molecular weight change are given as $M_n/M_{n(0)}$ where M_n and $M_{n(0)}$ are the GPC number average molecular weights of products formed in the medium with and without PEG, respectively.

tively. Similar detrimental effects on polymer yield were observed when the medium concentration of PEG-200 and PEG-400 was increased to 2% and higher (see Figure 2). Thus, the magnitude of changes in polymer yield for a specific PEG concentration varied as a function of PEG molecular weight. Furthermore, the relationship between PEG molecular weight and the resulting viable cell concentration and polymer yield is unclear as regular trends were not observed. It is noteworthy that *A. eutrophus* was able to utilize 2% (w/v) PEG-106 as a sole carbon source and form P3HB in yields of 0.2 g/L. Therefore, relatively high P3HB yields in PEG-106 medium may, in part, be due to the increased availability of utilizable carbon sources.

PEG Uptake and P3HB Molecular Weight. The % PEG of the cell dry weight (see Figure 3) was determined by analysis of the crude product by comparing the ^1H NMR signal intensities of P3HB methyl protons at 1.25 ppm to PEG protons at 3.6–3.8 ppm. At 1% PEG, the dry cell contents of PEG-10 000, PEG-200, PEG-1,000, PEG-400, and PEG-106 were all below 1% and no clear trend was observed between PEG molecular weight and PEG uptake. Increases in PEG uptake from 1 to 2% PEG concentrations were generally small (see Figure 3). However, at PEG concentrations of 5 and 10%, the % PEG in cells reached high

Table 1. Effects of PEG-300 and CH₃O-PEG-OH-350 on Cell Growth, Polymer Production, PEG Uptake, and P3HB Molecular Weight

PEG ^a	PEG added (% w/v)	non-PHA residual cell yield ^b	polymer yield (g/L)	PEG uptake in cells (wt%)	P3HB mol wt change ($M_n/M_{n(0)}$) ^c
PEG-300	0	2.6 ± 0.1	2.6 ± 0.1	0	1 ± 0.05
PEG-300	0.50	2.5	2.5	0.3	0.41
PEG-300	1.00	2.3	3.0	0.3	0.36
PEG-300	2.00	2.1	2.5	1.1	0.27
CH ₃ O-PEG-350	0.25	2.5	3.2	0.2	0.65
CH ₃ O-PEG-350	0.50	2.4	2.8	0.5	0.40
CH ₃ O-PEG-350	1.00	2.3	2.5	0.7	0.35
CH ₃ O-PEG-350	2.00	2.1	2.2	1.0	0.29
CH ₃ O-PEG-350	4.00	1.9	0.6	3.8	0.28

^a PEG or PEG derivative added to the cultivation medium in the second or polymer production stage. ^b The non-PHA residual cell yield (R-CY) is the total cell dry weight minus the weight of accumulated microbial polyester. [R-CY = total biomass (g/L) - polymer yield (g/L)]. ^c The molecular weight of P3HB was measured by GPC. $M_{n(0)}$ is the number average molecular weight of P3HB formed in a medium without PEG [$(4.10 \pm 0.30) \times 10^5$].

levels. For example, at 5% PEG-106 and PEG-200, the % PEG in cells was 5 and 2.2%, respectively. Also, at 10% PEG-106 and PEG-200, the % PEG in cells was 8.7 and 8.2%, respectively. However, once again, trends between PEG molecular weight and the % PEG in cells were unclear at these high concentrations of PEG in the medium.

By introducing PEGs having M_n values ranging from 106 to 1000, the controlled reduction of P3HB molecular weight was achieved (see Figure 4). The extent of molecular weight reduction was dependent on the PEG molecular weight and its concentration in the medium. The molecular weight change in Figure 4 is expressed as $M_n/M_{n(0)}$ (see Figure 4 legend). At 1% concentrations of PEG in the medium, the M_n reduction for PEG-1000, PEG-400, PEG-200, and PEG-106 was 0.80, 0.40, 0.31, and 0.26, respectively. When 10% PEG-10 000 was added to the medium, no significant molecular weight reduction was observed (see Figure 4). This is in spite of the fact that the % PEG-10 000 in cells at the 10% medium concentration was 9.3% (see Figure 3). Therefore, greater molecular weight reductions (lower $M_n/M_{n(0)}$ values) at identical media concentrations were achieved using PEG of relatively lower molecular weight. PEG-106 was particularly effective in that only 0.25% was required to reduce the M_n by 74%. The largest decrease in P3HB molecular weight was observed using 10% PEG-106 (91% to M_n 19 400). The largest change in P3HB M_n per incremental addition of PEG occurred in the 0–1% PEG concentration range. Further addition of PEGs generally caused further P3HB molecular weight reduction but to lesser extents. Since the % PEG in cells is low for 1% PEG medium concentrations (see Figure 3), it appears that relatively low molecular weight PEGs have high specificity for intracellular systems which regulate PHA molecular weight. Therefore, even though large increases in % PEG uptake by cells were observed for media concentrations of 5 and 10% (see Figure 3), a corresponding decrease in P3HB M_n was not observed. In other words, the regulation of P3HB molecular weight does not appear to be a function of the ease by which cells uptake a specific PEG or its corresponding intracellular concentration. Since PEGs of lower molecular weight will have properties which are largely determined by their terminal hydroxyl bearing units, it may be that the extent of P3HB molecular weight reduction is a function of the availability of hydroxyl groups on PEG chains. Alternatively, enhanced molecular weight reduction may require low molecular weight PEG which can interact to a greater extent with the PHA biosynthetic system where terminal hydroxyl functionalities are not required. Another

possibility is that both molecular size and terminal functionality dictate the degree of molecular weight regulation. These models are further considered below by the use of mono- and dimethoxy PEG derivatives.

Methoxy End-Capped PEG Derivatives. Studies were carried out using the monomethoxy ether (CH₃O-PEG-OH-350) and the dimethoxy ether of tetraethylene glycol (CH₃O-TEG-OCH₃). To minimize effects of chain length, P3HB formation using these modified PEGs were compared to results obtained using PEG-300 and TEG, respectively. In Table 1, it is shown that increasing PEG-300 and CH₃O-PEG-OH-350 concentration in the medium results in similar effects on the PHA production system. Specifically, R-CY decreased to similar extents at concentrations $\geq 1\%$. Also, cell uptake values for PEG-300 and CH₃O-PEG-OH-350 as a function of the medium concentration were similar (see Table 1). Most surprisingly, the changes in P3HB molecular weight per incremental addition of PEG-300 and CH₃O-PEG-OH-350 were almost identical (see Table 1). Even at low (0.25%) CH₃O-PEG-OH-350 concentrations, P3HB molecular weight decreased by 35%.

A comparison of the effects of TEG and CH₃O-TEG-OCH₃ on P3HB formation is shown in Table 2. Increasing TEG concentration above 1.0% resulted in decreased R-CY yields. Furthermore, polymer yield decreased at TEG concentrations $\geq 1\%$. TEG accumulation in cells was not detected until media concentrations were 0.5% or greater. Interestingly, the addition to the medium of TEG and its corresponding dimethoxy derivative resulted in dramatically different effects on P3HB formation (see Table 2). Even at 0.25% CH₃O-TEG-OCH₃, substantial reductions in polymer yield were observed. These reductions in P3HB yield were not due to cell death since viable cell counts remained relatively unchanged for concentrations from 0.25 to 5.0% CH₃O-TEG-OCH₃ ($\sim 1.4 \times 10^{11}$ c.f.u./mL, data not shown). Investigation of % P3HB M_n change showed that the incremental addition of CH₃O-TEG-OCH₃ to concentrations of 10% resulted in no significant change in product molecular weight.

From the above we conclude that capping of one hydroxyl group had no apparent effect on the ability of PEG-350 to interact with the P3HB polymer biosynthesis system in *A. eutrophus* to effect molecular weight change. Thus, it may be that interactions between PEG and the PHA biosynthetic system (presumably the synthase) leading to molecular weight reduction are higher for relatively lower molecular weight PEG and only require one PEG chain terminal hydroxyl functionality. In contrast, suitable interactions between

Table 2. Effects of TEG and CH₃O-TEG-OCH₃ on Cell Growth, Polymer Production, PEG Uptake, and P3HB Molecular Weight

TEG ^a	TEG added (% w/v)	non-PHA residual cell yield ^b (R-CY)	polymer yield (g/L)	TEG uptake in cells (wt%)	P3HB mol wt change ($M_n/M_{n(0)}$) ^c
TEG	0	2.7 ± 0.1	2.4 ± 0.1	0	1 ± 0.05
TEG	0.25	2.8	2.6	0	0.34
TEG	0.50	2.7	2.4	0.4	0.24
TEG	1.0	2.5	2.1	0.8	0.23
TEG	2.0	2.3	1.9	3.1	0.13
TEG	5.0	2.2	1.2	2.7	0.80
TEG	10.0	1.7	0.5	9.4	0.88
CH ₃ O-TEG-OCH ₃	0.25	2.5	1.0	0	0.97
CH ₃ O-TEG-OCH ₃	1.0	2.7	1.2	1.5	0.85
CH ₃ O-TEG-OCH ₃	2.0	2.3	0.4	1.5	0.95
CH ₃ O-TEG-OCH ₃	5.0	1.7	0.4	1.4	1.05
CH ₃ O-TEG-OCH ₃	10.0	1.7	0.2	6.9	0.95

^a TEG or TEG derivative added to the cultivation medium in the second or polymer production stage. ^b See Table 1, footnote b. ^c See Table 1, footnote c. $M_{n(0)}$ for this experimental run was $(4.20 \pm 0.28) \times 10^5$.

CH₃O-TEG-OCH₃ and P3HB forming enzymes leading to P3HB molecular weight reduction did not occur. We believe that this result is explained by the nonavailability of terminal hydroxyl groups for CH₃O-TEG-OCH₃. It is interesting to consider that PEG derivatives having chain end functionalities other than hydroxyl may also result in favorable interactions and molecular weight regulation. This is currently under investigation and is supported by our preliminary work.

Conclusions

In general, *A. eutrophus* showed substantial tolerance for PEGs. This was illustrated by similar viable cell counts for the control, 10% PEG-10 000 and 2% PEG-200. Furthermore, detrimental effects on polymer yields were not observed for concentrations of 5% PEG-106 and 10% PEG-10 000. Relationships between PEG molecular weight and resulting effects on cell viability and polymer yield did not follow simple trends. This work showed that PEG is a useful tool to regulate P3HB molecular weight. Larger molecular weight reductions at identical media concentrations were achieved using PEG of relatively lower molecular weight. PEG-106 was most effective in that a media concentration of only 0.25% was required to reduce the M_n by 74%. Studies which compared the abilities of CH₃O-PEG-OH-350 and PEG-300 to regulate P3HB molecular weight showed almost identical molecular weight reductions. Furthermore, CH₃O-TEG-OCH₃ was not an effective agent for molecular weight reduction. Therefore, it was concluded that interactions between PEG and the PHA production system leading to molecular weight reduction are enhanced for lower molecular weight PEGs and require at least one PEG chain end functionality. The chain end functionality found useful in this work was a hydroxyl group. It is important to mention that analysis of all P3HB products by ¹H NMR showed that they did not contain PEG terminal groups. This is in contrast to a previous study where PEG-200 was found covalently linked to the carboxyl chain terminal end of high 4HB containing chains formed by *A. eutrophus*.³³ Thus, for this *A. eutrophus* strain, molecular weight reductions of P3HB by PEG are not due to chain termination reactions by PEG. Instead, PEG likely interacts with the *A. eutrophus* synthase in such a way to increase the rate of chain termination by water relative to propagation reactions. This hypothesis is currently being evaluated by cell free experiments using granules and isolated synthase enzymes.³⁹

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