Chemical Induction of PHB Depolymerases in Bacteria

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Abstract

Petroleum-based plastics have a negative environmental impact based on their accumulation and inability to be broken down. The use of bioplastics is a possible solution to having the convenience of plastics without the environmental harm. Poly-hydroxybutyrate (PHB) is a bioplastic that is synthesized by certain bacteria to store carbon and energy. In order to use PHB as an effective energy source, some bacteria synthesize PHB depolymerases – enzymes that degrade PHB. These enzymes allow PHB to degrade more quickly than petroleum-based plastics. To find a quicker way to degrade PHB, chemical induction of PHB depolymerase activity in *Acidovorax wautersii* strain H8 was observed through turbidimetric assay. These assays were used to measure the cloudiness of solutions, which could be correlated to enzyme activity. PHB itself and its monomer, 3-hydroxybutyrate (3HB), both induced depolymerase activity. Based on PHB assays, 3HB is the more effective chemical inducer for this particular strain of bacteria.

Introduction

Petroleum-based plastics have become increasingly popular and with them have come the concern of their negative environmental impact. As a result of their convenience, plastics are produced at a rate exceeding 300 million tons annually (1) with a projected increase to 810 million tons per year by 2050 (2). Petroleum-based plastics can persist in the environment for thousands of years beyond their intended use, causing problems which include the accumulation of plastics in natural habitats, the potential ingestion and entanglement of plastic by animals, and the leeching of petroleum-based chemicals into environments (1). A potential alternative to using conventional petroleum-based plastics is using bioplastics. Bioplastics are derived from living sources, and some have the potential to be more biodegradable than conventional plastics. Poly-3-hydroxybutyrate (PHB) is one such bioplastic.

Discovered in 1925 by Lemoigne (3), PHB is synthesized by certain bacteria as a form of energy storage similar to fat storage in animals (4). PHB accumulates in storage granules of PHB-producing bacteria, and when these bacteria die, they leave PHB in the environment. PHB-degrading bacteria can then use their extracellular depolymerases to break down and utilize this PHB (3). These depolymerases used by PHB-degraders help to break down PHB into its constituent components, which are non-toxic and naturally found in animals (2). This decrease in environmental harm means that PHB and similar bioplastics can help to alleviate some of the problems caused by conventional plastics.

PHB is in a class of biodegradable polymers known as polyhydroxyalkanoates (PHAs), and is itself considered a short chain length PHA (5). PHAs are promising alternatives to conventional plastics because they can have a wide variety of side chains in the polymer, and so can have similar properties to polyethylene, polypropylene, and other petroleum-based plastics (6). PHB itself has potential as a food containing bioplastic, since its permeability to oxygen is less than that of polyethylene or polypropylene (6). In terms of commercial applications, PHAs have already been used in the creation of shampoo bottles and in waterproof films on the back of diaper sheets (2). PHB also has potential uses in the medical field due to its lack of toxicity and promotion of regeneration. Thus, PHB may be used to construct orthopedic pins, stents, stitches, and nerve guides (7).

Over twenty PHB-degrading bacteria have been isolated from sewage, compost, and water treatment facilities, and they have been further characterized and identified in the laboratory (8). Selection of PHB-degrading bacteria was done by plating bacteria onto plates where PHB was the sole carbon source. The next step was to test whether PHB-degrading enzyme activity was inducible, specifically in *Acidovorax wautersii* strain H8. Work has been done on the characterization of a PHA depolymerase in another *Acidovorax* strain (9), but this study sought to potentially quicken the degradation of PHB through chemical induction of PHB depolymerase.

In order to make PHB and similar bioplastics an efficient alternative to petroleum-based plastics, the degradation of PHB into harmless components needs to be optimized. Currently, PHB degradation occurs

relatively slowly (9), and PHA shampoo bottles were observed to take 5-10 years to degrade in field experiments (2). The optimization of PHB degradation would cause the bioplastic to be rid of more quickly, decreasing its environmental impact. This study sought to answer the question of what chemicals (inducers) could stimulate or inhibit the activity of enzymes responsible for degrading PHB by *Acidovorax wautersii* strain H8, and if activity of these enzymes could be stimulated, which inducers were best at induction. Knowledge of inducers is important because it can provide a solution to bioplastic degradation before the potential problem of PHB-based plastic accumulation occurs.

Methods

Acidovorax wautersii strain H8 was isolated and identified from CSU Chico compost and was tested for PHB depolymerase activity induction with various chemical agents. The chemical agents that were tested were PHB (Sigma Aldrich 363502), 3-hydroxybutyrate (Sigma 166898), polylactic acid (PLA), lactose, and glucose. The bacteria were grown in tryptic soy broth (TSB) overnight, harvested by centrifugation, and diluted to 1:100 in M9 + nutrient broth. This culture was grown to exponential growth, harvested by centrifugation, and resuspended with ¼ original volume M9 salts. The cell suspension was aliquoted into flasks, and then 2 mL of the test inductant was added. Each induction flask was done in duplicate. Samples were taken from each flask at 4, 8, 12, 16, and 24 hours for the first round of experiments (for the second round, samples were not taken at 24 hours). The control sample with no test inductant had an additional sample taken at 0 hours. The samples were centrifuged and the cell-free supernatant containing the exoenzymes was assayed for activity.

The first assay involved mixing the supernatant with a solution of PHB and measuring the absorbance, using a spectrophotometer at 600nm wavelength, as the PHB was broken down by the depolymerase. The A600 of the samples was read at 0, 5, 10, 20, 30, 45, 60, 75, and 90 minutes. The second assay was an Indoxyl acetate assay, which produced a blue product that was measured by a spectrophotometer at a wavelength of 660 nm. In this assay, 0.5 mL of the supernatant was combined with 0.4 mL of phosphate buffer saline (PBS) 10mM, pH 7.4 buffer in a cuvette and equilibrated at 34°C for 10 minutes, then 0.1 mL of indoxyl acetate (10 mg/mL in 100% ethanol) was added to the mixture to start the reaction.

With the assays used, certain outcomes were expected if a chemical was an inducer. In the PHB assay, a decrease in A660 corresponded with a degradation of PHB, so a more substantial decrease indicated induction of enzyme activity. For the Indoxyl acetate assay, an increase in A660 over time indicated induction of enzyme activity because PHB depolymerase oxidizes indoxyl acetate into indigo (10), producing a blue product that increases A660.

Results

PHB assays showed the greatest decrease in A600 for those cultures that were grown in the presence of 3HB and PHB (Fig. 1). Similar results were observed for the indoxyl acetate assays, with A660 increasing most when 3HB and PHB were presented as inducers (Fig. 2). For the PHB assays, greatest enzyme activities for 3HB and PHB induced cultures were from samples taken at 8 hours in the presence of the inducer (Fig. 1). The rest of the inducers (PLA, lactose, glucose) displayed no large difference from the control in terms of enzyme activity for both the PHB and indoxyl acetate assay. For these inducers, the A600 for the PHB assays remained relatively stable with regards to both induction and assay incubation time, while the A660 for the indoxyl acetate assay showed slight increase over assay incubation time in a manner similar to the control (Fig. 2).

Discussion

The results suggest that both 3HB and PHB chemically induce the PHB depolymerase activity of *Acidovorax wautersii* strain H8. According to PHB assays, 3HB appears to be a better inducer for PHB depolymerase activity (Fig. 3). 3HB, being the monomer of PHB, may induce enzyme activity based on its similarity in shape to PHB (11). Chemical induction increases PHB depolymerase activity, but further studies will have to been done to understand the mechanism of induction (i.e. as a cofactor for the enzyme, as an inducer of gene expression, or as a signal for extracellular transport).

Future studies will likely model others that have isolated and characterized PHB depolymerases (3,5,12,13). Similarly, cloning of the gene that encodes PHB depolymerase into other bacteria may be performed. This process would involve isolating the depolymerase, sequencing the N-terminus of the protein, and designing primers to find the gene that encodes the enzyme. Additionally, more induction studies may be performed on other isolates.

Characterizing exoenzyme activity in isolate strains is important because it may be helpful in creating an efficient means toward bioplastic degradation. In order to create environmentally conscious bioplastics, there needs to be a method of degrading them so that their effect on natural habitats and wildlife is reduced. If the degradation of a bioplastics such as PHB can be optimized through the use of inducing chemicals, environmental harm caused by the long-term

persistence and accumulation of petroleum-based plastics may be decreased.

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Figures

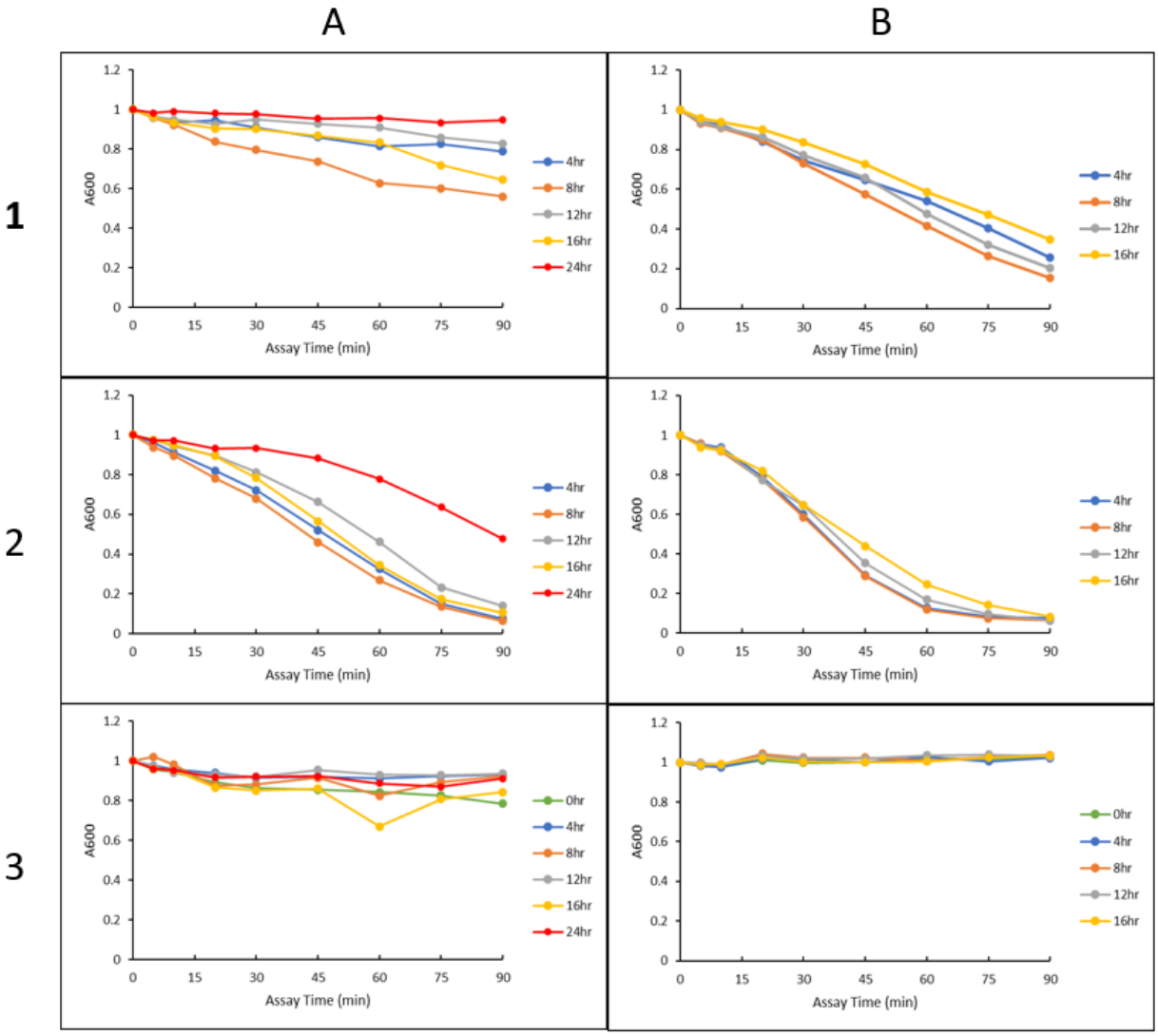

Fig. 1: Normalized absorbances (A600) of PHB assays over time, with results from Spring 2019 (A) and Summer 2020 (B). Each row represents a different inducer tested: PHB (1), 3HB (2), and the control (3).

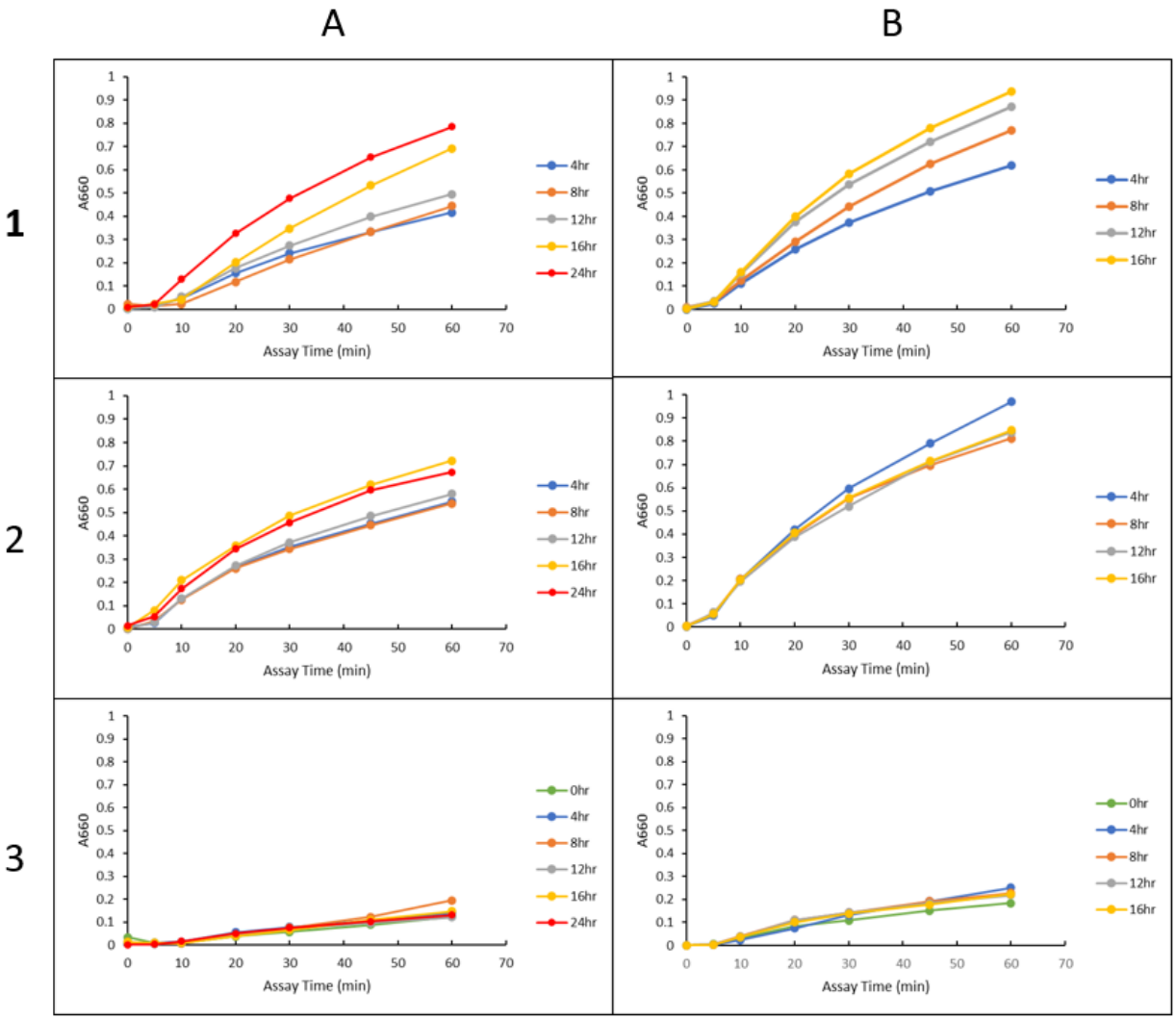

Fig. 2: Absorbances (A660) of indoxyl acetate assays over time, with results from Spring 2019 (A) and Summer 2020 (B). Each row represents a different inducer tested: PHB (1), 3HB (2), and the control (3).

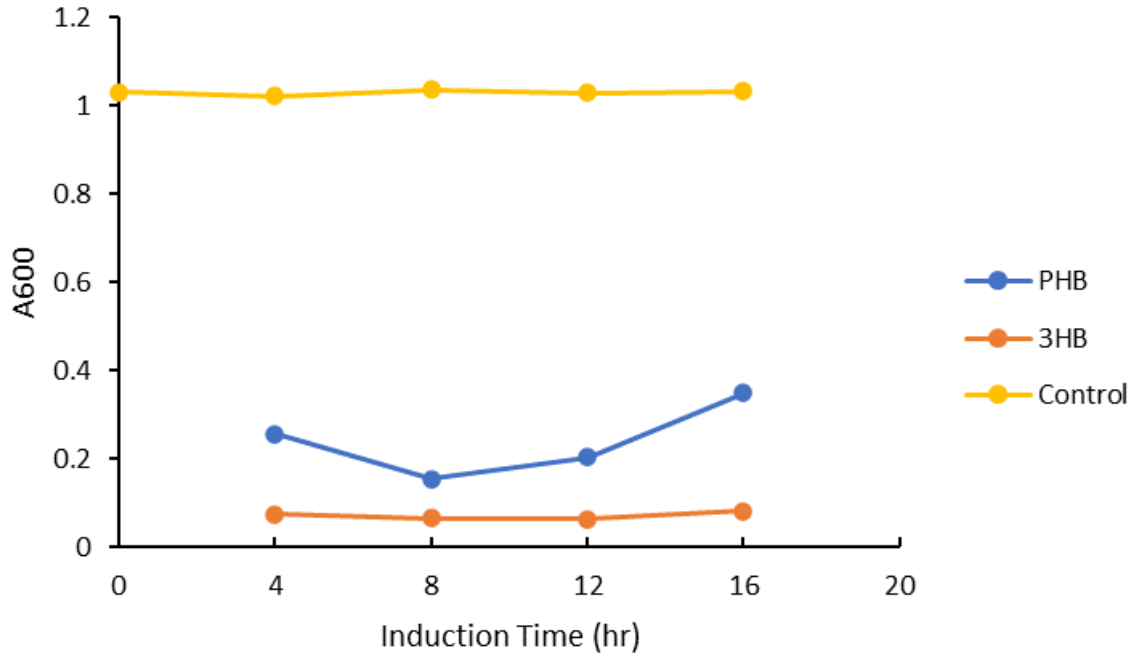

Fig. 3: Normalized absorbance (A600) of Spring 2020 PHB assays at 90min for each induction sampling time.

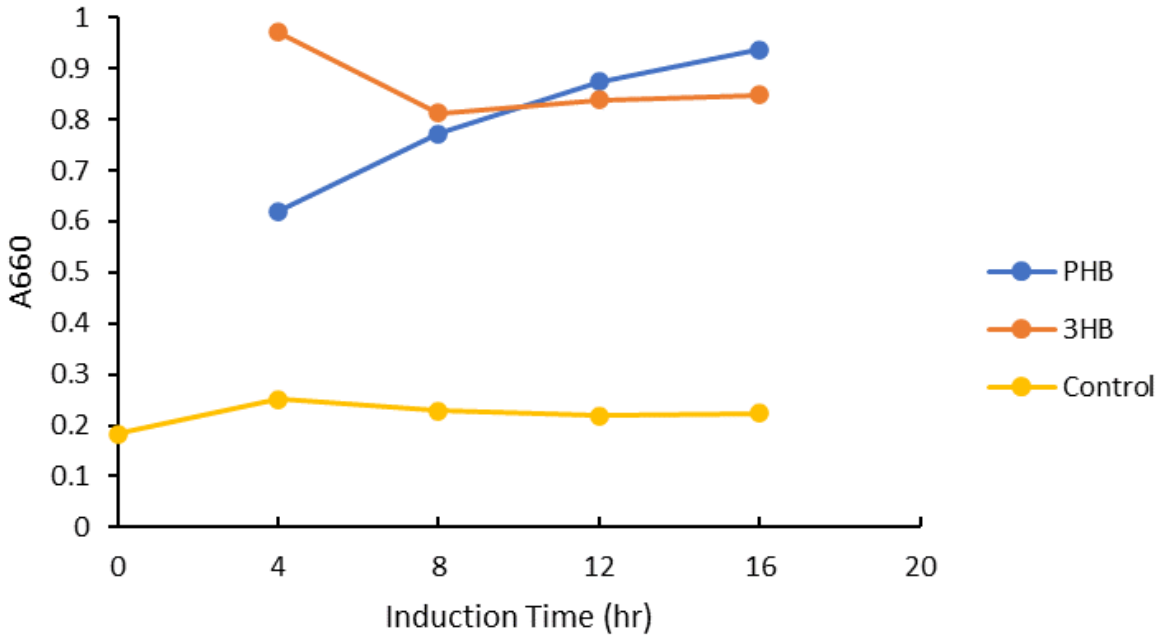

Fig. 4: Absorbance (A660) of Spring 2020 indoxyl acetate assays at 60min for each induction sampling time.