

Isolation and Characterization of Bacteria that Produce Polyhydroxybutyrate Depolymerases[†]

Emily A. Egusa¹, Daniel J. Edwards², MyLo L. Thao³, Larry L. Kirk², and Larry F. Hanne^{1*}

¹Department of Biological Sciences, California State University, Chico, Chico, CA 95929,

²Department of Chemistry and Biochemistry, California State University, Chico, Chico, CA 95929,

³Department of Biological Sciences, California State University, Stanislaus, Turlock, CA 95382

INTRODUCTION

Under conditions of physiological stress, many bacteria convert excess carbon nutrients into storage polyhydroxyalkanoates (PHAs) (1, 2). Poly(3-hydroxybutyrate) (PHB) (Fig. 1A) is one member of this class of compounds comprised of repeating 3-hydroxybutyrate (3HB) subunits (Fig. 1B). Some bacteria accumulate PHB intracellularly in the form of inclusion granules (3). Since PHB and other similar bacterial alkanate polyesters are water insoluble, they can be molded into specific shapes, and many have been used commercially to produce biodegradable thermoplastics for food packaging, disposable utensils, medical devices, and other products (2, 4). Organisms that produce PHB can access these storage granules by producing intracellular depolymerases that hydrolyze PHB into the 3HB monomer that is used as a source of carbon or energy. It is not surprising that, over evolutionary time, other bacteria have evolved extracellular (exoenzyme) PHB depolymerases to scavenge PHB released from dead PHB-producing bacteria (2).

The focus of our undergraduate research group for several years has been on isolating and characterizing bacteria capable of degrading PHB. This article describes three laboratory activities for studying PHB degradation: 1) preparation of a medium that allows visual detection of PHB depolymerase producers, 2) quantification of PHB degraders from environmental samples, and 3) measurement of enzymatic activity in culture supernatants with a simple spectrophotometric assay.

SAFETY GUIDELINES

The safety guidelines are detailed in Appendix 1.

PROCEDURE

1. Formulation of a medium for detection of PHB-degrading bacteria (see Appendix 2 for supplementary instructions)

An essential criterion for successful detection of degraders is the composition of the PHB plate medium. We initially tried to detect PHB-degrading bacteria on PHB plates containing tryptic soy agar (TSA) as a foundation nutrient. Numerous environments were sampled on these PHB/TSA plates, but we were unable to detect any organisms that cleared the PHB. Since depolymerase expression is often repressed in rich media, we began to experiment with PHB plates containing limited nutrients (low concentration nutrient agar). We experimented with various formulations before selecting a growth medium containing 0.5× strength nutrient broth base and final 1 mg/mL sonicated PHB. With this medium, PHB-degrading organisms were readily detected as a clearing of the insoluble PHB (Fig. 2). Note that, although bacterial growth is enhanced by formulations containing high concentrations of nutrients (e.g., normal strength nutrient broth or tryptic soy broth), depolymerase expression is repressed. We found that, for both auxotrophs and prototrophs, a low concentration of nutrients in the presence of PHB will allow for the expression of the PHB depolymerase.

2. Quantification of PHB-degrading bacteria from environmental samples

The PHB/nutrient agar plates can be used to determine the percentage of PHB degraders present in various environments. For example, we have used PHB plates to compare the percentage of degraders from different sewage treatment stages (Fig. 3) and compost stages (data not shown). Samples of sewage and compost were diluted 10⁻², 10⁻⁴, and 10⁻⁶ into sterile water

*Corresponding author. Mailing address: Department of Biological Sciences, California State University, Chico, Chico, CA 95929. Phone: 530-898-6298. Fax: 530-898-5060.

E-mail: lhane@csuchico.edu.

Received: 9 August 2018, Accepted: 22 October 2018, Published: 14 December 2018.

[†]Supplemental materials available at <http://asmscience.org/jmbe>

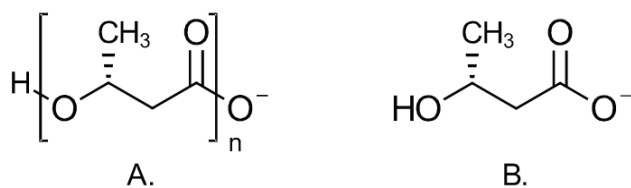


FIGURE 1. A) Structure of PHB polymer (average $n = 400$). B) 3-hydroxybutyrate monomer. PHB = poly(3-hydroxybutyrate).

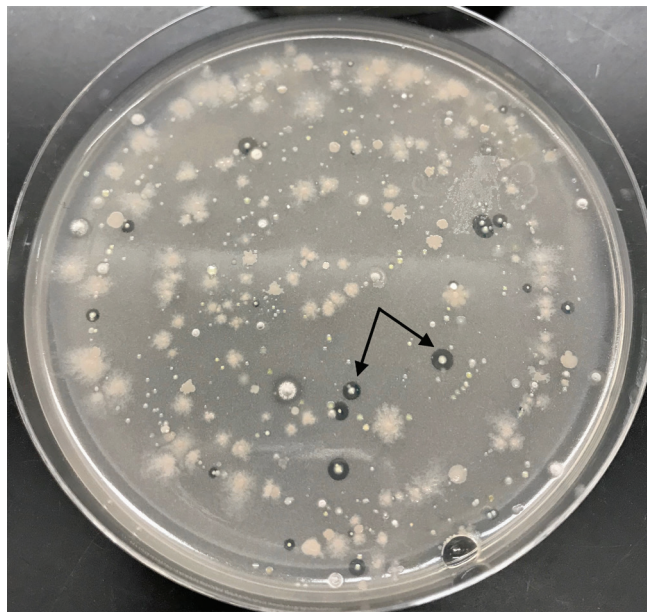


FIGURE 2. Clearing around several colonies on PHB/nutrient agar plates. Polymeric PHB is insoluble (cloudy), but clears upon hydrolysis by PHB depolymerase exoenzyme (example colonies at tips of arrows). PHB = poly(3-hydroxybutyrate).

blanks, 0.1 mL samples of each dilution were spread onto separate PHB plates, and plates were incubated four days at 28°C. The percentage of degraders was determined as a percentage of total colonies that had clear zones surrounding them.

3. Assay of enzyme activity in broth culture supernatant (Appendix 3)

In order to investigate induction specificity, catabolite repression, enzyme stability, or enzyme temperature optimum for isolated PHB degraders, we modified a protocol to assay culture supernatants for enzyme activity (5). Since the PHB polymer is insoluble, a simple spectrophotometric assay can be used to measure enzyme activity (Fig. 4A). Prior exposure of cultures to PHB is required to induce expression of PHB depolymerase activity (Fig. 4B).

CONCLUSION

Environmental microorganisms produce a variety of hydrolytic exoenzymes of commercial value including

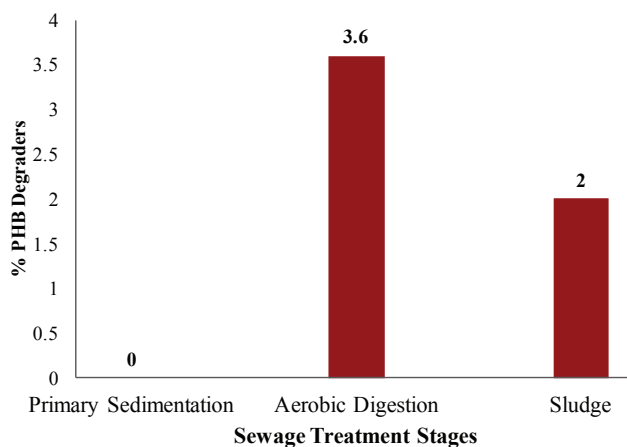


FIGURE 3. Percentage of PHB degraders detected in each of three stages of sewage treatment. Ten-fold dilutions of sample were plated on PHB plates and incubated 4 days at 28°C, and the percentage of colonies with clear zones of PHB degradation was determined. PHB = poly(3-hydroxybutyrate).

proteases, lipases, amylases, and other esterases (6). Organisms that produce these enzymes can be readily detected on appropriate differential media (7–9). For example, amylase producers can be detected on starch plates following flooding with iodine (7), and protease producers can be detected as zones of casein clearing on skim milk plates (9). Over the last four years, we have mentored approximately four students per semester interested in isolating and characterizing organisms that produce depolymerases that degrade the bioplastic PHB. Studies on the PHB depolymerases have included ecological analysis of frequency of degraders in various environments, biochemical and 16S rDNA identification of the organisms, induction studies of PHB depolymerase using inducing agents other than PHB, effect of glucose on induction, extracellular protein analysis of induced and uninduced supernatants on SDS-PAGE gels, and liquid chromatography-mass spectrometry (LC-MS) identification of protein/enzyme isolated from SDS-PAGE bands.

Independent and class projects involving exoenzymes that catalyze biodegradable plastic breakdown offer several advantages. The PHB plate assays allow for easy detection of PHB degraders from environmental samples, and PHB depolymerase enzyme activity from liquid cultures can be monitored by measuring PHB breakdown with a simple spectrophotometer. The strategies presented here can also be readily adapted for the study of other degradable bioplastics, such as PHB-valerate co-polymers (10). Importantly, these projects provide a microbiology-based research platform to explore environmental topics related to plastics. Unlike most molecular biology-based projects we have used in the past to teach research, the depolymerase projects usually yield positive results, even for novice research students. Added benefits of projects based on student-isolated bacteria are a sense of discovery and pride of ownership (11). Student posters and oral presentations on “plastic-degrading” bacteria have been popular due to increased

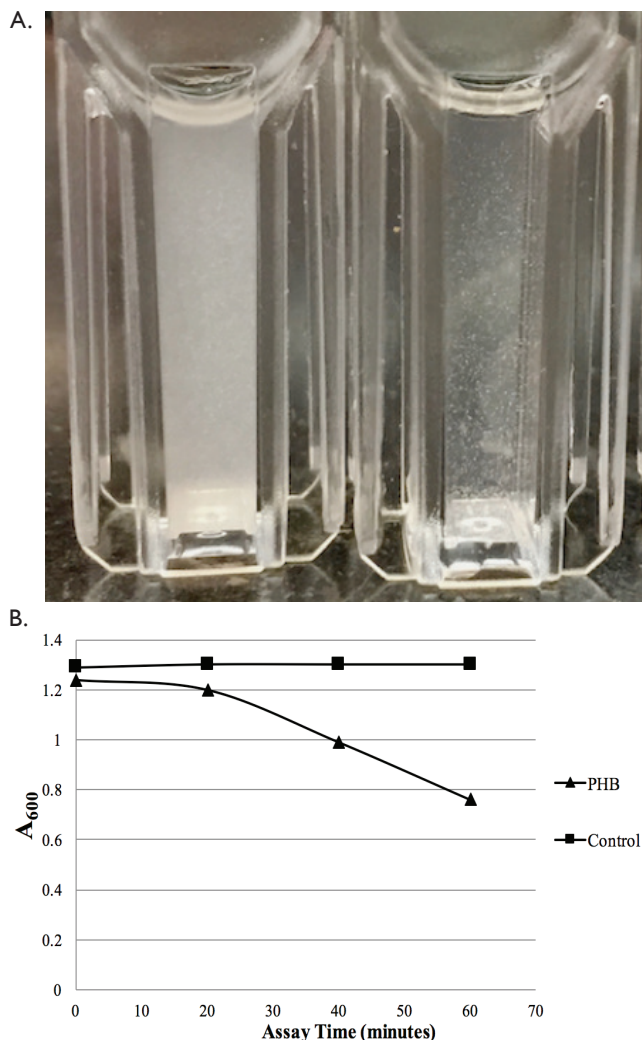


FIGURE 4. A) Photograph of assay cuvettes from supernatants without (left) and with (right) enzyme activity. B) Enzyme activity (decrease in A_{600}) of test supernatant following 8-hour incubation with PHB or no PHB (control) for PHB-degrading isolate *Acidovorax wautersii*. PHB = poly(3-hydroxybutyrate).

public awareness of plastic pollution and the importance of biodegradable plastics.

SUPPLEMENTAL MATERIALS

- Appendix 1: Laboratory safety
- Appendix 2: Biphasic PHB plates for visual detection of PHB-degrading bacteria and fungi
- Appendix 3: Enzyme assay for quantification of depolymerase enzyme activity from broth supernatants

ACKNOWLEDGMENTS

We are grateful to CSUC Facilities Management Services, Sierra Nevada Brewery, Salida Sanitary District, and Hilmar Cheese. The success of the above studies is based on undergraduate researchers who worked hard in the lab and had fun isolating and characterizing a library of PHB-degrading bacteria. The authors declare that there are no conflicts of interest.

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