Supplemental Materials

for

Isolation and Characterization of Bacteria that Produce Polyhydroxybutyrate Depolymerases

Emily A. Egusa1, Daniel J. Edwards2, MyLo L. Thao3, Larry L. Kirk2, and Larry F. Hanne1* 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

Table of Contents
(Total pages 4)

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

*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: lhanne@csuchico.edu.
Received: 9 August 2018, Accepted: 22 October 2018, Published: 14 December 2018.

©2018 Author(s). Published by the American Society for Microbiology. This is an Open Access article distributed under the terms of the Creative Commons Attribution-Noncommercial-NoDerivatives 4.0 International license (https://creativecommons.org/licenses/by-nc-nd/4.0/ and https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode), which grants the public the nonexclusive right to copy, distribute, or display the published work.
Appendix 1: Laboratory Safety

Students who have previously taken a microbiology laboratory course are given a thorough orientation to the laboratory and trained in laboratory protocol according to ASM Guidelines for Biosafety in Teaching Laboratories (http://www.asm.org/index.php/microbelibrary/laboratory-safety-guidelines). BSL2 laboratory safety guidelines from this website were used for environmental sampling since compost or sewage may contain BSL2 organisms. Students are required to wear lab coats, gloves, and safety goggles. Lab benches are disinfected before and after use. Original samples and cultures are autoclaved before disposal. No toxic chemicals or organic solvents were used in any experiments. Although PHB is non-toxic, we recommend minimizing aerosolizing the powder while weighing. The experiments described in this paper could also be safely performed towards the end of an introductory microbiology laboratory course.
Appendix 2: Biphasic PHB plates for visual detection of PHB-degrading bacteria and fungi

This medium is composed of a bottom 12 ml buffered layer and top 4 ml layer that contains a low amount of nutrients and sonicated PHB. A simple formula for making approximately 10 PHB plates is as follows:

A. Bottom agar:
   1. 150 ml M9 salts (see below) + 2.25 grams agar, autoclaved
   2. Cool bottom agar in a water bath to approx. 60 C
   3. Add 1.5 ml 0.01 M CaCl₂ and 0.3 ml 1 M MgSO₄ (autoclaved separately)
   4. Pipette approx. 12 ml bottom agar per petri dish (100 x 15 mm) (gently flame-warm pipette so that the medium doesn’t solidify in the pipette)
   5. Allow plates to solidify at room temperature at least 10 min
   6. Place plates on a warm surface (slide warmer set at approx. 50 C works well)

B. Top agar:
   1. 50 ml M9 salts (no CaCl₂ or MgSO₄), 0.2 gm PHB powdered granules (Aldrich CAS 29435-48-1)
   2. Sonicate to emulsify and disperse clumps without generating significant heat. We typically sonicate with a probe for 10 seconds or in a sonicating waterbath for 1 hour. The sonication probe is disinfected and cleaned with 70 % ethanol.
   3. After sonicating, add 0.75 gm agar, 0.2 gm nutrient broth, autoclave, cool to approx. 60 C
   4. Pipette approx. 4 ml on top of solidified bottom agar, rotate the plate to disperse before it solidifies. Again, a warmed pipette is helpful.

   M9 salts (ref 12)
   Na₂HPO₄  6 gm/liter
   KH₂PO₄  3 gm/liter
   NaCl    0.5 gm/liter
   NH₄Cl   1 gm/liter

Final plates should be slightly hazy (due to insoluble PHB). Plates can be stored for weeks in the refrigerator.
Appendix 3: Enzyme assay for quantification of depolymerase enzyme activity from broth supernatants

In order to measure relative amounts of enzyme production by individual isolated strains, we refined a simple spectrophotometric assay (ref 5) for quantification of enzyme activity. Note that test supernatant, buffer, and substrate (PHB) are equilibrated to the test temperature before mixing to initiate the reaction.

1. Grow test organism overnight, shaking approx. 200 RPM, 28 or 35 C in 80 ml M9 (containing CaCl₂, MgSO₄, and 0.2 % glucose, which had been added to the medium after autoclaving). This is enough cells to set up two inductions. Note that minimal media cannot be used to grow degraders that are auxotrophs.
2. Pellet overnight cultures (approx. 10,000 xg for 10 min at room temperature) and suspend the cell pellet in ¼ volume (20 ml) of M9 salts containing 1 mg/ml sonicated PHB. Dispense 10 ml to two 125 ml flasks. Note that all buffers should be at room temperature, otherwise, cold buffer will shock the cells and delay induction.
3. Allow induction to proceed shaking, 35 C for 4 to 8 hours.
4. Remove 1 ml samples to test cell-free enzyme activity. Cells can be removed from the sample by microcentrifugation at 7,000 RPM for 10 min.
5. Separately equilibrate the following in a water bath at the test temperature for 5 min
   a) Sonicated PHB (10 mg/ml) in M9 buffer
   b) 0.25 ml cell-free supernatant + 0.65 ml M9 buffer in plastic cuvette
6. The reaction begins when you pipette 0.1 ml PHB (#a) into the cuvette (#b).
7. Gently invert to mix, then incubate in a 35 C waterbath. Every 20 minutes invert reaction cuvette to resuspend settled PHB and measure absorbance in a spectrophotometer at 600 nm wavelength (see Fig 4 for typical result).