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Custom fabrication of biomass containment devices using 3-D printing enables bacterial growth analyses with complex insoluble substrates

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ABSTRACT

Physiological studies of recalcitrant polysaccharide degradation are challenging for several reasons, one of which is the difficulty in obtaining a reproducibly accurate real-time measurement of bacterial growth using insoluble substrates. Current methods suffer from several problems including (i) high background noise due to the insoluble material interspersed with cells, (ii) high consumable and reagent cost and (iii) significant time delay between sampling and data acquisition. A customizable substrate and cell separation device would provide an option to study bacterial growth using optical density measurements. To test this hypothesis we used 3-D printing to create biomass containment devices that allow interaction between insoluble substrates and microbial cells but do not interfere with spectrophotometer measurements. Evaluation of materials available for 3-D printing indicated that UV-cured acrylic plastic was the best material, being superior to nylon or stainless steel when examined for heat tolerance, reactivity, and ability to be sterilized. Cost analysis of the 3-D printed devices indicated they are a competitive way to quantitate bacterial growth compared to viable cell counting or protein measurements, and experimental conditions were scalable over a 100-fold range. The presence of the devices did not alter growth phenotypes when using either soluble substrates or insoluble substrates. We applied biomass containment to characterize growth of *Cellvibrio japonicus* on authentic lignocellulose (non-pretreated corn stover), and found physiological evidence that xylan is a significant nutritional source despite an abundance of cellulose present.

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1. Introduction

The degradation of insoluble polysaccharide-based biomass is ubiquitous in nature and essential for the global cycling of carbon and other nutrients (Field et al., 1998; Leschine, 1995). Polysaccharide depolymerization is also an important industrial process for the production of renewable fuels and chemicals (Francesko and Tzanov, 2011; Lynd et al., 2002). In both cases, the carbohydrate active enzymes (CAZymes) of ecologically and industrially relevant bacteria are the primary drivers of biomass turnover, particularly that of insoluble (recalcitrant) polysaccharides such as cellulose or chitin (Beier and Bertilsson, 2013; Mba Medie et al., 2012). Studying microbial polysaccharide degradation requires the ability to measure bacterial growth as these recalcitrant substrates are broken down, however there are challenges making accurate measurements of bacterial growth without the insoluble material interfering. Previous work using plate-based medium showed that acrylamide or silica based solidifying agents were effective in reducing false positives (from bacteria that could degrade agar) when used in environmental screening for polysaccharide degrading bacteria (Bazylinski and Rosenberg, 1980; Gardner et al., 2012). Improved measurements in broth-based media are required to advance the study of insoluble polysaccharide degradation, and while many protocols have been used including optical density, protein measurements, and viable cell counts, these methods have drawbacks (Bradford, 1976; Sieuwerts et al., 2008; Smith et al., 1985). Optical density readings, while able to track bacterial growth in real time, can be erratic upon the mixing of the cells and the insoluble substrate. Protein measurements (*e.g.* BCA or dye-binding assays) are time-delayed, and require that sample be removed from the growing culture. In addition, reagent compatibility and cost need to be taken into account with protein-based assays. For example, if there is plant or chitinous biomass as the growth substrate, the protein found in these heterogeneous substrates will skew the protein measurements. Colony forming unit (CFU) counting is also a time-delayed measurement, even more so than protein-based measures, and also requires the removal of sample. It should be noted that, despite the drawbacks described above, these methods are used successfully with microbes that adhere to insoluble polysaccharides, particularly anaerobes (Alvelo-Maurosa et al., 2016; Yang et al., 2010).

The concept of 3-D printing is nearly 30 years old, but has recently crossed over from exclusively the realm of materials science and moved into engineering, biological, and chemical applications (Chia and Wu, 2015; Kitson et al., 2012; Symes et al., 2012). The core concept

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of 3-D printing (also called additive manufacturing in the trade literature) is a digital file of an object modeled in three dimensions that has been sectioned into thin layers (Conner et al., 2015). A 3-D printer reads the digital file and then constructs the object by the deposition or polymerization of a build material, typically plastic or metal. There are currently multiple methods to create 3-D printed objects, with stereolithography being a common approach for making microfluidic devices (Ho et al., 2015). Briefly, a controlled beam of ultraviolet light strikes a surface of photosensitive plastic material, typically urethane acrylate, and triggers polymerization. A variation of this technique has recently been used to entrap individual bacterial cells and create artificial bacterial communities (Connell et al., 2013). Moreover, the fabrication of replacement parts (*e.g.* no longer carried by the original manufacturer) or custom growth chambers have the potential to both accelerate and broaden the research directions of individual molecular biology laboratories. Both commercial 3-D printing services and 3-D printers themselves are now readily available and increasingly have become a way to rapidly create durable custom components on an individual scale. A robust on-line community for support with the printers and open source sharing of designs, coupled with freely available software, has helped expand the use of 3-D printing to biological research (Baden et al., 2015; Zhang et al., 2013).

Here we report the creation and benchmarking of a set of custom biomass containment devices (BCDs) with 3-D printing that greatly facilitate the optical density measurement of bacterial growth during insoluble substrate degradation. We found that a UV-cured acrylic plastic was superior to nylon or stainless steel for printing small pores in cylinder shapes. The acrylic material was also heat tolerant and able to be autoclaved or alcohol sterilized, making the BCDs reusable. The presence of the BCDs in the growth medium did not interfere with bacterial growth or diminish the observance of growth phenotypes, which allowed for very reproducible experiments. We found that 3-D printed BCDs were cost competitive compared to reagents for protein quantitation or CFU counting, and had the added benefit of allowing growth measurements to be taken in real time. The use of these devices allows for a standardized, rapid, inexpensive, and reproducible way to use optical density measurements in conjunction with insoluble substrates in the growth medium. We used the BCDs to examine, in a physiologically relevant context, lignocellulose degradation in the saprophytic bacterium *Cellvibrio japonicus*. This bacterium secretes a multitude of Carbohydrate Active enZymes (CAZymes) that are proficient at degrading insoluble polysaccharides (DeBoy et al., 2008; Gardner, 2016). Our mutational analysis, coupled with the use of authentic lignocellulose, indicates that *C. japonicus* has a complex nutrient acquisition program, where pentoses represent a significant nutrient input even when there is an abundance of hexoses present for consumption.

2. Materials and methods

2.1. Design, manufacture, and evaluation of biomass containment devices (BCDs)

The stereolithography files (.stl) used in the design of the BCDs were created using a combination of the MakerBot Thingiverse Customizer (http://www.thingiverse.com/apps/customizer) and freely available 3-D computer-aided drafting software (http://www.openSCAD.org). The BCDs were constructed as nested cylinders with an outer diameter of 15 mm and a height of 10 mm. The height dimension was constrained by the height of the light beam coming from the spectrophotometer, which had to pass over the device unobstructed through an 18 mm culture tube. The width dimension was constrained by the inner diameter of an 18 mm tube, and a width of 15 mm prevents the device from moving freely once settled to the bottom of the tube. Three designs were evaluated (Mk 1.0, Mk 2.1, Mk 2.2) for 18 mm culture tubes and one design (Mk 2.3) was evaluated for 1 L shake flasks. The Mk 1.0 BCDs had only pores at the end of the cylinder and were used for material benchmarking. The Mk 2.1 and Mk 2.3 BCDs had large (1 mm) pores, while the Mk 2.2 BCD had small (0.5 mm) pores on the sides of the device. The Mk 2.x designs were used for growth experiments. Using the 3-D printing service ShapeWays (http://www.shapeways.com), we constructed BCD designs in three materials: nylon (Alumide), acrylic (UV cured), and stainless steel (60% steel, 40% bronze).

2.2. Growth media and strains

Strains of *Cellvibrio japonicus* (NCIMB #10462) were grown in defined minimal medium as done previously (Gardner and Keating, 2012). Carbon sources were added sterilely at concentration of 0.5% (w:v). Insoluble carbon sources used were Whatman paper (BioRad) or corn stover (*Zea mays*) obtained from the USDA Sustainable Agriculture Systems Laboratory (Beltsville MD). As was done previously, after insoluble substrates were autoclaved they were extensively rinsed with sterile water to remove trace amounts of oligosaccharides liberated (Gardner and Keating, 2010; Gardner et al., 2012). Soluble substrates tested were glucose (TekNova) or xylan (Megazyme), prepared according to manufacturers instructions. *Escherichia coli* K-12 (CGSC #6300) was grown in lysogeny broth (Bertani, 1951; Bertani, 2004). All experiments using BCDs were grown in a shaking incubator at 30 °C with a high level of aeration (200 RPM) in either 18 mm glass culture tubes or 1 L glass shake flasks as shown in Fig. S1. Growth experiments that did not require biomass containment were performed in a TECAN M200Pro microplate reader (TECAN Trading AG, Switzerland).

2.3. C. japonicus mutant generation

A *C. japonicus* Δ*xylA* in-frame deletion mutant was generated as previously described (Nelson and Gardner, 2015). Confirmation of the correct deletion was performed *via* PCR screening.

2.4. Optical density, colony forming unit, and protein measurement of bacteria growth

Optical density measurements were obtained at 600 nm with a Spectronic 20D+ spectrophotometer (Thermo Fisher). Colony forming unit growth measurements were done as previously described (Gardner and Keating, 2010). For total protein assays, cell lysis was performed with the B-PER Bacterial Protein Extraction Reagent (ThermoFisher) and total protein measurements were performed with a 660 nm Protein Assay Kit (Pierce), both used according to manufacturer's instructions.

2.5. Sterilization procedures

Biomass containment devices were sterilized by autoclaving for a 30 min steam cycle at 121 °C and 16 psi. A different sterilization method tested was exposure ultraviolet germicidal irradiation using a UVG-11 Compact UV Lamp (4 W, 254 nm, 5 cm distance; UVP, Inc., Upland CA). A third alternative sterilization method used was a combination of bleach and ethanol soaking. Briefly, the biomass containment devices were immersed for 20 min in a 10% bleach solution, then rinsed with sterile ddH$_2$O, and then immersed in a 95% ethanol solution for 30 min before being placed in sterile glass culture tubes. The tubes were then dried for 10 min in a 70 °C oven.

3. Results

3.1. Biomass containment devices (BCDs) allow for real-time data acquisition and are a cost competitive choice for bacterial growth analyses

Optical density (OD) readings, colony-forming unit (CFU) counting and protein measurements were compared in terms of temporal delay in data acquisition and cost. The same flasks of *C. japonicus* were used

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