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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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3-D printing, biomass containment, *Cellvibrio japonicus*, lignocellulose, polysaccharide degradation

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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.

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,

91 reagent compatibility and cost need to be taken into account with protein-based assays.
92 For example, if there is plant or chitinous biomass as the growth substrate, the protein
93 found in these heterogeneous substrates will skew the protein measurements. Colony
94 forming unit (CFU) counting is also a time-delayed measurement, even more so than
95 protein-based measures, and also requires the removal of sample. It should be noted
96 that, despite the drawbacks described above, these methods are used successfully with
97 microbes that adhere to insoluble polysaccharides, particularly anaerobes (Alvelo-
98 Maurosa et al., 2016, Yang et al., 2010).

99 The concept of 3-D printing is nearly 30 years old, but has recently crossed over
100 from exclusively the realm of materials science and moved into engineering, biological,
101 and chemical applications (Chia and Wu, 2015, Kitson et al., 2012, Symes et al., 2012).
102 The core concept of 3-D printing (also called additive manufacturing in the trade
103 literature) is a digital file of an object modeled in three dimensions that has been
104 sectioned into thin layers (Conner BP, 2015). A 3-D printer reads the digital file and then
105 constructs the object by the deposition or polymerization of a build material, typically
106 plastic or metal. There are currently multiple methods to create 3-D printed objects, with
107 stereolithography being a common approach for making microfluidic devices (Ho et al.,
108 2015). Briefly, a controlled beam of ultraviolet light strikes a surface of photosensitive
109 plastic material, typically urethane acrylate, and triggers polymerization. A variation of
110 this technique has recently been used to entrap individual bacterial cells and create
111 artificial bacterial communities (Connell et al., 2013). Moreover, the fabrication of
112 replacement parts (e.g. no longer carried by the original manufacturer) or custom
113 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

137 lignocellulose, indicates that *C. japonicus* has a complex nutrient acquisition program,
138 where pentoses represent a significant nutrient input even when there is an abundance
139 of hexoses present for consumption.

140

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 15mm and a height of 10mm. 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 18mm culture tube. The width dimension was constrained by the inner diameter of an 18mm 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 18mm culture tubes and one design (Mk 2.3) was evaluated for 1L 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 (1mm) pores, while the Mk 2.2 BCD had small (0.5mm) 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, Zeitler, Wigstrom, Engel and Keating, 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 18mm glass culture tubes or 1L 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* $\Delta xylA$ 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 600nm 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 660nm 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 (4W, 254 nm, 5cm 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 minutes in a 10% bleach solution, then rinsed with sterile ddH₂O, and then immersed in a 95% ethanol solution for 30 minutes before being placed in sterile glass culture tubes. The tubes were then dried for 10 minutes 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 compare OD and CFU measurements (**Fig 1B**). While the quality of data was good for either approach, the OD data was collected in real time while the CFU data could only be collected after 2 days of incubation due to the requirement of viable cell plating and outgrowth. A second independent experiment using protein measurements had a reduced lag time to data acquisition (~15 min) compared to CFU counting, but still was not in real time. An additional problem with the protein measurement method was that it was not sensitive enough to detect early growth (T_0 - T_4) (**Fig 1C**), which was detectable by both CFU and OD measurements.

We next compared the cost to run these growth analysis experiments. As the tube-based BCDs are small (the interior volume is 1.77 cm^3), the cost of manufacture is primarily from the amount of build material used in its construction. One complete BCD (top and bottom pieces) ranged in price: \$4.76 (nylon; Mk 2.1), \$12.24 (acrylic; Mk 2.1), and \$15.21 (steel; Mk 2.1). While there are a diversity of protein detection kits that are commercially available that range in cost and complexity, it is universal that they all use consumable reagents. CFU counting also incurs a consumable cost in terms of the plasticware required to perform the dilution and plating series required to enumerate the

viable cells in a sample. In contrast the BCDs are re-usable (*vide infra*), therefore giving substantially more value when used with OD measurements (**Table 1**).

3.2. Biomass Containment Devices Are Sterilizable And Reusable. The BCD prototypes (Mk 1.0 design) were evaluated for size relative to the print specifications, ability to be sterilized, and reactivity to bacterial cells. All BCD prototypes were printed to the specified size, with less than 0.1 mm variance (data not shown), indicating that the printing process did not add any additional material from design specifications to make them larger. It was noted, however, that some of the 1 mm x 1mm pores in the steel and nylon BCDs were closed off. For these materials the pore size would need to be increased, but this was not seen as advisable as an increased pore size would allow insoluble polysaccharide substrate to escape the BCD. In regards to the outer perimeter of the BCD prototypes, all three material types were able to fit into 18 mm test tubes (**Fig 1A**).

We assessed the ability of the BCDs to be sterilized by immersing them in an overnight culture of *E. coli* K-12 and then sterilized in one of three ways: UV exposure, surface sterilization with bleach and ethanol, or autoclaving. After a sterilization procedure, the BCDs were then placed in 5 ml of sterile lysogenic broth (LB) (Bertani, 1951) in an 18mm tube and shaken overnight (30°C, 200 RPM) to evaluate the three sterilization methods (**Fig S2**). After 24 hr incubation, 100 µL was spread on an LB plate to confirm that no viable cells were present (data not shown). Exposure to 60 minutes (30 min per inside / outside face of BCD) of ultraviolet germicidal irradiation was not effective in sterilizing any of the BCDs. A combination of bleach followed by ethanol

immersion was an effective means of sterilization for the acrylic and nylon BCDs only. As expected, the steel BCD was able to be autoclave sterilized, but also the plastic BCDs were able to undergo autoclaving multiple times with no adverse effects to shape or function other than a slight yellowing of the acrylic BCD, which was originally translucent. All three BCD materials were able to sustain multiple overnight (12 hr) incubations in a 70°C drying oven with no deformation. While evaluating the BCDs for ability to be sterilized we repeatedly observed that the steel material severely discolored and released a brown precipitate into the medium (**Fig S2**), therefore we discontinued further analysis of this material. Evaluation of the BCD prototypes is summarized in **Table 2**, and based off these benchmarking studies, we continued using the acrylic BCDs to assess growth phenotypes of *C. japonicus* growth using polysaccharide substrates.

3.3. Biomass Containment Devices Do Not Interfere With OD Measurements. The acrylic BCDs were evaluated for reactivity in a defined minimal medium. If the device material inhibited growth, then it would not be suitable. To assess this, we placed autoclaved acrylic BCDs in 5 ml of defined minimal medium with either glucose or xylan as the sole carbon source and inoculated the tubes with *C. japonicus*, a bacterium that does not adhere to recalcitrant polysaccharide substrates and uses a dispersed system of CAZymes for polysaccharide degradation (DeBoy, Mongodin, Fouts, Tailford, Khouri, Emerson, Mohamoud, Watkins, Henrissat, Gilbert and Nelson, 2008, Gardner et al., 2014). We transitioned to evaluating the Mk 2.1 and Mk 2.2 BCD designs for these experiments. These devices have either small (1 mm²) or large (2 mm²) pores on the

sides and ends, which reduced overall cost compared to the Mk 1.0 BCD and also increased the contact area of the insoluble substrate with the medium, which allowed for free exchange between *C. japonicus* cells, the CAZymes secreted, and insoluble substrate encased in the BCD. When compared to control cultures that did not have any BCDs present, we found that both the maximum attained OD and growth rate were similar regardless of the presence of the BCDs in the tube when glucose or xylan was used as a carbon source. In order to further test the utility of the BCDs in soluble polysaccharides, we constructed a $\Delta xylA$ mutant using our previously described method (Nelson and Gardner, 2015). The *xylA* gene encodes a xylose isomerase and is essential for feeding xylose into the pentose phosphate pathway, therefore a $\Delta xylA$ mutant will be unable to use xylan or other xylose-derived oligosaccharides. As shown by **Fig 1C & D**, the presence of the BCD does not exacerbate the growth defects of the $\Delta xylA$ mutant when grown on either soluble polysaccharides or monosaccharides, though as expected is unable to grow in xylan containing media. In addition, the BCDs used with wild type *C. japonicus* did not impact growth under either condition. These data suggest that the acrylic BCDs do not interfere with growth of bacterial cells and perform as well or better than conventional cell growth measurements using glucose (soluble monosaccharide) (**Fig 1B & C**), as well as xylan (soluble polysaccharide) and corn stover (insoluble polysaccharides) (**Fig S3**). Overall, the correlation between OD measurement and cell number for *C. japonicus*, (max OD of 1.1 ± 0.04 equivalent to $1.3 \times 10^9 \pm 3.2 \times 10^8$ CFU for glucose grown cells; max OD of 1.4 ± 0.12 equivalent to $2.3 \times 10^9 \pm 3.8 \times 10^8$ CFU for xylan grown cells; and max OD of 0.298 ± 0.01 equivalent to 1.2

$\times 10^8 \pm 2.3 \times 10^7$ CFU for corn stover grown cells using the Mk 2.2 BCDs), is similar to that previously published for *E. coli* (Sezonov et al., 2007).

3.4. Biomass Containment Devices Are Compatible With Insoluble Substrates

And Scalable To Vessel Size. To probe the utility of the BCDs with insoluble polysaccharides, we re-evaluated two mutant strains under biomass containment. A $\Delta cel5B\Delta cel6A$ mutant and a Δgsp mutant of *C. japonicus* have moderate and severe growth defects when grown in insoluble cellulose, respectively (Nelson and Gardner, 2015), and these strains were tested with cellulose-containing BCDs. As expected the Δgsp mutant was unable to grow in cellulose containing medium, as this mutant is deficient for the entire Type II Secretion System, which is an essential component of carbohydrate active enzyme export (Gardner and Keating, 2010). The $\Delta cel5B\Delta cel6A$ mutant lacks the endoglucanase and cellobiohydrolase enzymes previously been shown to critical for efficient cellulose utilization (Nelson and Gardner, 2015). Wild type *C. japonicus* is unaffected by biomass containment of cellulose and grows well. We chose cellulose as the test case for an insoluble substrate because a previous study examined *C. japonicus* growth on cellulose without biomass containment (Gardner, Crouch, Labourel, Forsberg, Bukhman, Vaaje-Kolstad, Gilbert and Keating, 2014), which allowed us to conclude that BCDs did not adversely effect degradation and consumption of the insoluble polysaccharide. Our analysis indicates that while the maximum growth for wild type and the $\Delta cel5B\Delta cel6A$ mutant was slightly decreased when under biomass containment, the differences in OD were proportional between strains. In addition, we found that there was slightly poorer growth when using the Mk

2.1 (1mm pores) compared to the Mk 2.2 (0.5mm pores) designs (**Fig 2B & C**), although both were able to recapitulate the growth phenotypes observed in the control experiment without biomass containment (**Fig 2A**). This decrease in observed OD with the Mk 2.1 devices was not due to interference by the BCDs, as both designs had negligible background measurements (**Fig S4**).

Large BCDs (Mk 2.3 design) used in 1L shake flasks for growth experiments were equally effective when scaled up to 500 ml volumes of medium (**Fig 2D**). The scalability of the BCDs ensures that large vessels still contain the same percentage of insoluble substrate, typically 0.5% across experiments. While shake flask experiments are not practical for growth analyses (in this case the tube-based BCDs have greater utility), biomass containment in flasks would be useful to facilitate sample removal during an -omics based experiment. Uninoculated flasks containing filter paper without biomass containment started to disperse and create an opaque environment in the flask after 10 hours of shaking (200 RPM), which progressively became more pronounced over the course of the experiment. Conversely, uninoculated BCD control flasks had only minor release of filter paper fragments and like the Mk 2.1 and 2.2 designs, contributed negligibly to background measurements (**Fig S6**).

3.5. Biomass Containment Devices Facilitate Discovery of Complex Phenotypes During Authentic Lignocellulose Degradation. The utility of the BCDs was evident when testing *C. japonicus* mutant strains using a physiologically relevant substrate (corn stover). The heterogeneous and insoluble nature of the authentic corn stover substrate prevented OD measurements without biomass containment (**Fig S3** and **Fig S5**). Shake

flask growth experiments with corn stover were greatly facilitated using the Mk 2.3 BCDs. Without biomass containment, particulate material occluded the shake flask by T_{48} , which was absent in the BCD flask (**Fig S6**). Some fine particles of corn stover did leach out of the BCD over the course of the experiment, however this background contributed negligibly to the overall OD measurements (**Fig 3C**).

We evaluated wild type *C. japonicus*, the newly constructed $\Delta xyIA$ mutant, and a Δgsp mutant in medium with only authentic corn stover as the sole carbon source under biomass containment. The wild type strain of *C. japonicus* was able to grow well on corn stover, although maximum OD attained in experiments using the Mk 2.1 BCDs was lower than experiments using the Mk 2.2 BCDs, despite the Mk 2.1 design having larger pores. As expected the Δgsp mutant was unable to grow using corn stover, which had been shown previously (Gardner and Keating, 2010). Intriguingly, the $\Delta xyIA$ mutant had a modest, but reproducible growth defect (**Fig 3A & B**). The $\Delta xyIA$ growth defect constituted an approximately 33% reduction in maximum growth using Mk 2.1 BCD, which was determined to be significant by a Student's t-test ($p < 0.05$). The $\Delta xyIA$ growth defect was more pronounced using Mk 2.2 BCDs (69% reduction in maximum growth from wild type), and also found to be significant. Similar to what was observed using biomass containment with authentic lignocellulose, the $\Delta xyIA$ mutant has a reproducible growth defect in carbon replete conditions, achieving only 80% of the maximum growth observed for WT *C. japonicus* (**Fig 3D**).

4. DISCUSSION

Bacterial degradation of recalcitrant polysaccharides (e.g. lignocellulose) is currently of great interest for global nutrient cycling studies, as well as biotechnological efforts to cheaply and efficiently produce renewable fuels and chemicals. Previously studied from an almost exclusively biochemical and structural perspective, several reports have stated that *in vivo* studies are required to fully understand lignocellulose degradation, as solely *in vitro* studies will not necessarily find the most efficient enzymes or their true biologically relevant functions (Cartmell et al., 2011, Naas et al., 2014, Zhang et al., 2014). For studies of *in vivo* lignocellulose degradation to be informative, analysis of the complex interactions of lignocellulose-degrading microbes and the insoluble substrate needs to overcome several challenges. Previous work with plate-based media aimed to reduce the number of false positives obtained using agar-based solidified medium, as agar can be used as a carbon source for some polysaccharide degrading bacteria. Both silica and acrylamide based solidifying agents were successful in reducing the number of false positive isolates when re-tested using defined broth-based media (Bazylinski and Rosenberg, 1980, Gardner, Zeitler, Wigstrom, Engel and Keating, 2012). However the transition to broth presented its own challenges, and previous attempts to grow saprophytic bacteria using insoluble biomass required excessive particle reduction, pre-treatment, and settling times to allow for growth measurements. However, for accurate and physiologically relevant analyses to occur, the substrate must be minimally processed. Measuring growth of bacterial strains degrading recalcitrant polysaccharides could improve accuracy with a mechanism to segregate the biomass from the growing cells in a way that does not disrupt the experiment or remove sample from the culture.

Our data evaluating 3-D printing of biomass containment devices (BCDs) suggests a cost-competitive solution that makes bacterial growth measurements fast and very reproducible. Additionally, the flexibility of 3-D printing expedites custom device construction for specific experimental conditions while being both a precise and reproducible method. With the ability to make diverse designs of BCDs, and as the cost of 3-D printing decreases, these devices could be manufactured by a single lab or bought on a fee-for-service basis (Baden, Chagas, Gage, Marzullo, Prieto-Godino and Euler, 2015). In addition, there are a growing number of repositories for 3-D printable models and parts for biomedical and biotechnological purposes.

The described design and implementation of 3-D printed biomass containment devices to facilitate microbial growth measurements highlights their potential as customized, user-generated parts for experiments in fermenters or other vessels where it is advantageous to sequester substrate from bacterial cells. The major utility of the BCDs is the ability to measure growth via optical density readings without removing samples or disrupting the culture, both of which happen with CFU counts and protein measurements. This is of particular importance when performing RNAseq or other transcriptomic experiments, as precise sampling is required to ensure that cells are in a proper growth state (*e.g.* exponentially growing). In addition, it would reduce the amount of plant or animal DNA contaminating the sample. This would prevent some of the filtration steps that are required now for transcriptomic sampling with cells in combination with biomass (Schwalbach et al., 2012). Furthermore, the cost-effectiveness of BCDs becomes apparent with growth experiments that are hundreds of hours long (*e.g.* lignocellulose), which would require proportionally hundreds of agar

plates or protein reactions (**Fig 1B & Table 1**). In these circumstances the cost of the BCD is substantially lower than the consumable costs of the other methods. In addition to reduced sampling cost, the amount of time required to perform sampling is substantially less when using the BCDs compared to viable cell counts, which allows for the inclusion of multiple stains per experiment. In this sense, the use of BCDs allow for higher experimental throughput, something critical when large numbers of genes are being investigated using *in vivo* mutational analysis.

Use of the BCDs allows authentic biologically relevant substrates to be used with the only manipulation being bending the biomass to fit into the device and sterilization. Containment had the benefit of producing fewer small particles that previously were the source of measurement error, and is most evident in shake flask experiments (**Fig S6**). While the Mk 2.3 BCDs move around a great deal more in the flasks than the Mk 2.1 and 2.2 do in the tubes, the background for the flask-based experiments was still low. There was virtually no background in the tube-based BCD experiments because of the restricted movement of the device at the bottom of the tube (**Fig S5**). The Mk 2.2 BCDs did perform better with corn stover substrates, as seen by higher overall growth of the *C. japonicus* strains, which may be a consequence of there being more pores in these devices compared to the Mk 2.1 design because the pore size is smaller.

A $\Delta xylA$ mutant had the expected severe growth defect when xylan was the sole carbon source (**Fig 1E**), but a striking result obtained was that this mutant also has a growth defect when corn stover was the sole carbon source (**Fig 3B**). As the full complement of CAZymes is secreted in the $\Delta xylA$ mutant, both soluble hexoses and pentoses are available for uptake. The growth data suggests that xylose consumption is

an integral part of the *C. japonicus* metabolic program during lignocellulose degradation. These data suggests that *C. japonicus* hexose/pentose metabolism is more similar to *P. aeruginosa* than *E. coli* (Rojo, 2010). Additional studies of the sugar co-utilization in *C. japonicus* will be required to further unravel the regulation and mechanisms of substrate preference in this bacterium, but could prove informative as mixed sugar co-utilization has been intensely studied for the production of renewable fuels. Because it affects product yield, it is currently a barrier for complete utilization of saccharified biomass (Zhang et al., 2015). These studies indicate that novel aspects of *C. japonicus* physiology and metabolism can be discovered using authentic plant biomass in conjunction with the BCDs, which can then be subsequently dissected using defined conditions.

While our results argue for the utility of biomass containment when examining bacterial systems with a dispersed (secreted) enzyme system for polysaccharide degradation, several considerations need to be taken into account. Our results suggest that the type of insoluble substrate placed inside of the BCD influences growth. For example, microcrystalline cellulose powder (Avicel) is not suitable, as this powdered form of cellulose either falls out of the pores or clogs the pores of the BCD (data not shown). While it is possible to make BCDs with very small pores (the accuracy of the 3-D printing method used is ± 0.2 mm), it would come at the cost of allowing less free exchange of medium between the interior of the BCD and the outside. Additionally, BCDs are not appropriate for cells that attach to biomass, such as *Serratia marcescens* binding chitin or *Clostridium thermocellum* adhering to cellulose (Hazlewood et al., 1992, Kawada et al., 2008). The reason for this is that cells bound to the substrate

would be sequestered inside of the BCD, and therefore growth could not to be measured by optical density. In these situations, protein measurements or CFU counting have been used successfully to determine cell growth (Alvelo-Maurosa, Lee, Hazen and Leschine, 2016, Yang, Kataeva, Wiegel, Yin, Dam, Xu, Westpheling and Adams, 2010). Therefore, use of biomass containment can be considered a time and cost saving alternative to generating growth data with environmentally and biotechnologically relevant complex insoluble polysaccharide substrates and ideally suited for saprophytes like *C. japonicus*, which uses a secreted system of cellulases and does not adhere to insoluble polysaccharides substrates (Gardner and Keating, 2010, Hazlewood, Laurie, Ferreira and Gilbert, 1992). As natural biomass has a non-uniform composition the use of the BCDs also helps facilitate and interpretation of complex growth data, and from a biotechnological context, employing BCDs have greater utility in evaluating consolidated bioprocessor microbes (Olson et al., 2012, Zhang and Lynd, 2003), which typically employ secreted CAZyme activities and use non-adhesive bacterial cells. In summary, our proof-of-concept study has shown an effective way to combine 3-D printing and physiological analysis to examine recalcitrant polysaccharide degradation by bacteria. The BCD design was scalable over a 100-fold range (5ml to 500ml), using both purified insoluble polysaccharides and authentic environmentally relevant substrates. Future work will focus on design refinements for tube based BCDs, as well as alternative designs for vessels such as fermenters.

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COMPLIANCE WITH ETHICAL STANDARDS

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FIGURE LEGENDS

Fig 1. Use of biomass containment devices (BCDs) in bacterial growth measurements with soluble carbon sources. (A) BCDs used in this study (from left to right): Mk 1 design in stainless steel (bottom), nylon (middle), and acrylic (top) in an 18mm test tube; Mk 2.1 design, showing separate inner and outer pieces; Mk 2.2 design, showing a complete and closed BCD; and Mk 2.3, showing a large BCD for shake flask experiments. The white bar at the lower right of the image represents a size scale of 15mm. **(B)** Differences in growth analysis measurements of *C. japonicus* using optical density (OD) measurements (closed circles, left Y-axis), colony forming unit (CFU) measurements (open circles, right Y-axis), or **(C)** protein measurement (open circles, left Y-axis). **(D)** Growth analysis of wild type (closed symbols) and a $\Delta xylA$ mutant (open symbols) of *C. japonicus* in minimal defined medium with glucose as the sole carbon source. Growth experiments were performed with no devices (ND, circles), the Mk 2.1 design BCDs (squares), or the Mk 2.2 design (triangles). **(E)** Growth analysis of *C. japonicus* wild type (closed symbols) and a $\Delta xylA$ mutant (open symbols) in xylan medium under conditions with no devices (ND, circles), Mk 2.1 BCDs (squares), or Mk 2.2 BCDs (triangles). All growth analysis experiments were done in biological triplicate at 30°C with high levels of aeration in 18mm test tubes. Error bars indicate standard deviation, but at times are too small to be depicted.

Fig 2. Comparative growth analyses of *C. japonicus* using insoluble cellulose with or without biomass containment. In all panels the growth experiments show wild type *C. japonicus* is represented by closed circles, $\Delta cel5B\Delta cel6A$ as open squares, and Δgsp

as closed inverted triangles. The sole source of carbon used in the growth experiments was filter paper (0.5% (w:v)) in defined minimal medium. All experiments were done in biological triplicate at 30°C with high levels of aeration (200 RPM) in 18mm test tubes. Error bars indicate standard deviation, but at times are too small to be depicted. The three tested strains were evaluated with no device (**A**), the Mk 2.1 devices (**B**), or the Mk 2.2 devices (**C**). Panel **D** represents WT *C. japonicus* grown in shake flasks using the Mk 2.3 BCDs (closed diamonds) with filter paper as the sole carbon source. Uninoculated flasks (open diamonds) were used to track the background generated from insoluble filter paper inside the BCD leaching out. The data for WT growth and the background measurements using Mk 2.3 BCDs were from two independent experiments but are depicted here on the same graph.

Fig 3. Comparative growth analyses of *C. japonicus* using the physiologically relevant carbon source corn stover with or without biomass containment. All three of the panels show growth experiments that were performed in biological triplicate at 30°C with high aeration (200 RPM) in 18mm test tubes. The sole source of carbon was 0.5% (w:v) corn stover in defined minimal medium. The error bars indicate standard deviation and at times are too small to be depicted. Wild type *C. japonicus* is represented in all three panels by a closed circle, $\Delta xyIA$ as closed triangles, and Δgsp as closed squares. The strains were all grown with the Mk 2.1 devices (**A**) or the Mk 2.2 devices (**B**). Growth was unable to be measured with no device because of the amount of dispersed insoluble corn stover. Panel **C** represents WT *C. japonicus* grown in shake flasks using the Mk 2.3 BCDs (closed diamonds) with corn stover as the sole carbon

source. Uninoculated flasks (open diamonds) were used to track the background generated from the insoluble corn stover inside the BCD leaching out. The data for WT growth and the background measurements using Mk 2.3 BCDs were from two independent experiments but are depicted here on the same graph. Panel **D** characterizes growth of WT *C. japonicus* (closed symbols) and a $\Delta xyIA$ mutant (open symbols) either carbon replete or carbon limiting conditions in medium that contains both glucose and xylose. The strains were grown in decreasing amounts of carbon (w:v, 0.25% (circles), 0.1% (squares), or 0.05% (triangles)).

TABLES

Table 1. Cost Analysis of the Biomass Containment Devices Against Colony Forming Unit or Protein Measurement Assays

Method	Cost ^a
Acrylic BCD	\$12.24 per REP; \$36.72 per EXP
Nylon BCD	\$4.76 per REP; \$14.28 per EXP
Steel BCD	\$15.21 per REP; \$45.63 per EXP
Protein Measurement	\$0.71 per REP; \$27.69 per EXP
Viable Cell Count	\$2.28 per REP; \$54.72 per EXP

^a Cost of BCD construction is explained in the text. For other cell growth measurements, the following reagents and conditions were used to calculate cost: Protein: B-PER Complete Bacterial Protein Extraction Reagent (ThermoFisher Cat. # 89821; \$294 for 250ml), 60nm protein assay kit (ThermoFisher Cat. # 22662; \$193 for 450ml), Standard Disposable Polystyrene Cuvettes (Fisher Cat #14955127; \$182 for 500). Per replicate (REP) cost calculated as \$0.71 (660nm protein reagent, B-PER reagent, and cuvette) using micro-assay conditions (150µL of 660 protein reagent and 250µL of B-PER reagent). Per experiment (EXP) cost calculated as \$27.69 using eight time points of data collected (in triplicate; 24 REP) and including a 5-point standard curve (in triplicate, 15 REP). Viable Cell: Petri Dishes with Clear Lid (Fisher Cat. # FB0875712; \$269.84 for 500), Microcentrifuge Tubes (Fisher Cat. # 05408129; \$537.67 for 5000). Per replicate (REP) cost calculated as \$2.28 (using six microcentrifuge tubes and three plates for one dilution series). Per experiment (EXP) cost calculated as \$54.72 using eight time points of data collected (in triplicate; 24 REP).

693 **Table 2. Material Stress Test for Biomass Containment Devices^a**

Material used for BCD	Dry heat tolerance ^b	Autoclave tolerance ^c	Sterile: autoclave	Sterile: EtOH	Sterile: UV	Pore Size Uniformity
Acrylic	++	+	++	++	-	++
Nylon	++	+	++	++	-	+/-
Steel	++	++	++	-	-	-

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695 ^a Key: ++: Excellent; +: Good; +/-: Fair; -: Poor

696 ^b 70°C for 12 hrs in glassware drying oven

697 ^c 121°C and 16psi for 30 min (steam cycle)

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Figure 1. Nelson, Beri, & Gardner

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Figure 2. Nelson, Beri, & Gardner

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Figure 3. Nelson, Beri, & Gardner

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Viable Cell Counting

Time to Data Acquisition
1-2 days

Total Protein Assay

Time to Data Acquisition
15-30 minutes

Biomass Containment Device

Time to Data Acquisition
Real Time

Graphical Abstract