Development of a Microplate Accessory for Improved Bacterial Growth

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Bacterial products are an essential part of many industries that affect people everyday. To make these products, research is conducted using spectrophotometers to quantify the growth patterns of bacteria cultured in 96-well plates. However, limitations to bacterial mixing within these plates and oxygen transfer to the bacteria have reduced the accuracy of this research. Current solutions, such as plate shakers and edited well geometries, do not adequately address these issues, resulting in errors in bacterial growth measurements. This project sought to create a 96-well plate with baffled extrusions entering each well to enhance the dispersion, oxygenation, and growth of cultured bacteria. To ensure usability in a research setting, adjustments to the baffled lid were made until no significant optical interference occurred. Finite element analysis (FEA) also showed the lid could withstand forces reasonably faced during intended use within a laboratory setting. Finally, microbead tests revealed that the baffled lid significantly increased dispersion compared to a plate without a lid through both phase contrast microscopy and fluorescence measurements ($p < 0.0001$). Future work should be done using actual bacteria to demonstrate improved bacterial growth with the baffled lid and finding which baffle design has the best results. If this is accomplished, improvements to bacterial products could be achieved.

Keywords: bacterial growth, 96-well plate, spectrophotometry, optical density

Introduction

Significance and Innovation

The global bacterial products market was valued at $200 billion in 2021 and is expected to nearly double by 2030[1]. Controlled growth of bacteria has become vital for modern food production[2], soil fertilization[3], antibiotic formulation[4,5], and various other applications. To properly execute these processes, an understanding of bacterial proliferation kinetics through development of growth curves is required. Many methods of generating growth curves currently exist, with one of the most common being microplate readers[6]. Microplate readers have utilized the principle of spectrophotometry to measure the concentration of bacteria grown in 96-well plates according to the Beer-Lambert Law for over 70 years[6,7]. During this time, major advances in spectrophotometric technology have increased the sensitivity of microplate readers and allowed for rapid, low volume, and high throughput measurements to be taken[8,9]. However, the lack of innovation in 96-well plate technology has become a critical barrier to the usefulness of this quantification technique[7,10]. Specifically, low oxygen transfer rates (OTR) and inadequate mixing within wells lead to bacterial growth that does not perfectly match actual biological conditions[11]. Inexact data produced by this unrepresentative growth is therefore used to create bacterial products, making them less effective than they could be. Proper growth conditions for bacteria in 96-well plate cultures must be achieved to make this industry as impactful as possible.

Currently, the most prevalent solution for poor oxygenation and mixability in 96-well plates is to use linear or orbital shakers[12]. Many spectrophotometers come equipped with embedded shaking capabilities, and those that do not are operated in tandem with commercially sold shakers[13]. By shaking 96-well plates, a shear force will be created causing the fluid within each well to move. Theoretically, this is meant to agitate the clumps of bacterial cells that form at the bottom of the wells, dispersing them throughout the media[12]. A well-mixed bacterial culture will have a larger surface area over which oxygen can be absorbed, effectively increasing the oxygenation of the culture. Therefore, the idea of using shakers is to have higher oxygen and nutrient uptake in addition to a larger dispersion area, which should result in faster proliferation and better bacterial growth[12,14].

Although shakers have somewhat improved the conditions for bacterial growth in 96-well plates, they have several key drawbacks. Even with the introduction of shaking, OTR_{max} in 96-well plates were lower and much less predictable than those recorded in larger flasks[15,16]. The reported reason for this disparity is an increased force required to overcome surface tension in the wells of microplates in comparison to shaken flasks[10]. Fluid moves in these containers due to inertia generated by the introduction of a centrifugal force via shaking[17]. While inertia increases proportionally with the volume of the container, surface tension remains constant, making fluid in smaller containers like 96-well plates much harder to move. The solution is to create higher shaking frequencies that can overcome the surface tension of microplate wells and move the liquid, but this can result in the bacterial cells being damaged. The optimal shaking frequency needed to thoroughly mix basic media in wells was found to produce cellular shear rates ten times higher

than values reported to damage cells[10,18]. The inability to adequately mix bacterial cells at shear rates low enough to avoid cellular damage makes shaking alone a poor answer to the problems surrounding 96-well plate cultures.

Other previous attempts to improve bacterial growth in 96-well plates involved altering the wells themselves. One group studied how changes in the geometry of the wells would affect the maximum OTR_{max}[19]. They found that as the corners became sharper, the OTR_{max} increased, with conventional cylindrical wells having the lowest OTR_{max} and square wells having the highest. Another paper focused on adding microposts inside of the wells to make the inner surface more jagged[10]. The microposts were meant to disrupt the surface tension of media in the wells without requiring high shaking frequencies. When compared to regular 96-well plates, the peg-filled plates were significantly more effective at distributing contents within the wells over a standard mixing period.

Both of these solutions successfully increased growth conditions for bacteria, but their implementation is infeasible for several reasons. First, there are numerous different types of 96-well plates with different materials and functions. Trying to modify all of these plates would be extremely costly, time-consuming, and require unnecessary updates to the ANSI standards governing well parameters. Also, light scattering due to deflection from the irregular well shapes or microposts reduces the accuracy of OD measurements taken by spectrophotometers. Since 96-well plate bacterial cultures are most frequently applied to growth analysis in spectrophotometers[20], anything that is not compatible with this is unusable.

The proposed research will be innovative by avoiding changes to the standard 96-well plate format. The improvement in conditions will come from an accessory separate from the well plate. This solution will fall under ANSI standard guidelines, will not require any modifications to the current 96-well plate format, and will be compatible with spectrophotometry.

Proposed Solution and Project Aims

Due to issues outlined above, it is clear that a novel solution to the problems facing bacterial growth in 96-well plates is needed. This solution must increase bacterial dispersion within the well plate and allow higher rates of oxygen transfer to the bacteria to occur. It also needs to be compatible with current microplate readers by ensuring proper spectrophotometric measurements can be taken. Finally, it should be economically and structurally feasible to mass produce, strong enough to support clinical needs, and simple for researchers and physicians to use. With these constraints in mind, this project sought to create a device to improve bacterial growth that is durable, easy to use, and does not interfere with OD measurements. To accomplish this goal, two major specific aims were created at the onset of this project.

The first specific aim of this project is to design a 3D printed 96-well plate lid with baffles entering each well that do not disrupt OD measurements. To accomplish this aim, exploration into different baffle geometries and sizes needs to be done to determine characteristics that may be advantageous to bacterial growth. When common themes are found, they can be applied to design different baffles that have a high probability of success. These baffles need to be strong enough to handle any reasonable forces that they may encounter during use in a research setting, meaning finite element analysis will be needed to test their strength. Finally, since the purpose of this lid is to improve bacterial growth as measured by spectrophotometry, the baffles must not cause any light interference when placed in the wells. Baffles that meet all of these requirements can be incorporated into the 3D printed lid and used to explore the second specific aim of this work.

The second major aim will be to test the efficacy of the baffled lid in performing its intended tasks. This aim will be carried out through three different tests. The first will be determining how well the baffles are able to increase dispersion of fluorescent microbeads in the wells in comparison to a plate without baffles. This will be analyzed using both phase contrast microscopy and fluorescence measurements. Netx, the rate of oxygen transfer into the wells will be evaluated through the sulfite oxidation reaction. Finally, *E. coli* will be utilized to quantify whether or not the introduction of the baffled lid actually improves the growth of bacteria in the wells. The baffle design that yields optimal results to all three of these tests and can be reasonably scaled up and manufactured will be chosen for the final lid prototype.

Through the completion of these aims, it is hypothesized that bacterial dispersion within the wells and oxygen transfer to the bacteria will be increased, thereby improving the growth conditions present in 96-well plates. After conducting a review of printable materials and baffle geometries, a lid will be produced that is durable and does not interfere with OD (Aim 1). With this lid, quantification of the bacterial dispersion and OTR_{max} will solidify whether

the addition of baffles promotes bacterial growth (Aim 2). The resulting product will be a patentable lid that can accompany existing microplate accessories in enhancing the usefulness of microplate readers in bacterial analyses. These changes could lead to major advancements in pharmaceuticals, agriculture, and various other technologies that people interact with every day.

Materials and Methods

Materials

3D Printer and Resins

The ELEGOO Saturn Resin 3D printer was used to create the lid prototypes. Two types of resin were used with this printer. The first set of lids was printed using ELEGOO Water Washable 3D Printer Resin LCD UV-Curing Resin 405nm Standard Photopolymer Resin for LCD 3D Printing in the color Ceramic Grey. The second set of lids was printed using ELEGOO ABS-Like 3D Rapid Resin LCD UV-Curing Resin 405nm Standard Photopolymer Resin for LCD 3D Printing in the color Translucent.

Microbeads and Bacteria

FMV - Violet Fluorescent Polymer Microspheres 1.3g/cc - 1-5um were used for microscopy testing. A ratio of 3.75 milliliters of microbeads to 26.25 milliliters of distilled water was used. A highly concentrated *E. coli* live culture (ATCC 25922) was purchased by Cerillo through an online company. This was then diluted by a factor of 100 in Luria-Bertani (LB) broth so that all the bacteria would have enough nutrients to grow and tested in 96-well plates with baffled lids.

Methods

Creation of Baffle Designs and Lid Prototype

Eight different baffle geometries were created using Autodesk Fusion 360 software (Figure 1). Each of the eight baffle designs were created as sketches on a lid base and extruded so that the baffles did not touch either the sides or bottom of the wells on a 96-well plate. Four designs were placed per plate, with each of the quadrants occupying a different design to allow for multiple baffles to be tested at once. This was done to increase efficiency and reduce material waste associated with this project. Lychee Slicer 3D software was used to create printer files with support structures needed during the printing process (Figure 2). These files were then sent to the ELEGOO Saturn 3D printer filled with a printable resin. Two plates with two different sets of baffles were printed: one with designs one through four and one with designs five through eight. Once the print file was completely executed, the lid was scraped off the printing plate and submerged in an isopropanol bath for five minutes to wash off excess resin. All supports were cut and removed using pliers. The top of the lid, with the baffles facing up, and the underside of the lid, with the baffles facing down, were cured using UV light for five minutes per side. The lid was then placed on a 96-well plate to ensure a proper fit.

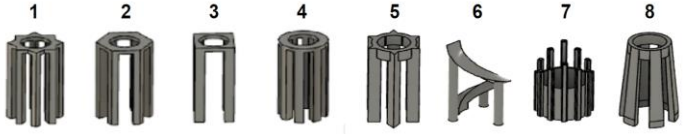

Fig. 1. Individual Baffle Designs in Autodesk Fusion 360. Inspiration for these designs came from mixers used commercially in other applications and the hypothesis that sharp corners would improve mixing within the wells.

Durability Testing

A FEA was completed using Autodesk Fusion 360 software to simulate forces induced from laboratory use. A force of five newtons was simulated and applied to the top corner of each baffle design. Autodesk Fusion 360 software then produced a level of colorimetric visualization of maximum and minimum areas of megapascals each baffle could withstand before failure. This data was exported as an image that included a force scale bar. A physical drop test occurred after the FEA to test structural integrity of the baffles after 3D printing. A range of one to four feet was used to test impact on the face with the baffles, the back side of the lid, and on the four corners of the lid. A team member held the lid in their hand and dropped the lid straight down from each height. The team member then applied pressure by pressing with their hands on multiple points of the baffles to test their

Fig. 2. Baffled Lid Design Process. A.) Autodesk Fusion 360 CAD design. B.) Lychee Slicer 3D printer file with generated supports. C.) ELEGOO Saturn 3D printed lid using Water Washable Ceramic Grey Resin.

overall strength. Qualitative data on the visual appearance of the baffles was collected.

Light Interference Data from Spectrophotometry

A SpectraMax 250 Spectrophotometry was used to test light interference produced by each baffle. Baffle hole diameters 2.5, 3.0, 3.25, 3.50, 3.6, and 3.75 centimeters were tested inside a spectrophotometer to measure the amount of light hitting the extruded lid geometries. The optical density for each hole diameter was compared to a blank non-baffled well.

Microscopy Images and Fluorescence of Microbeads

A LEICA microscope was used to conduct microscopy on microbead solutions. Prior to imaging, a microbead solution was shaken for three hours. The topmost 25 percent of the solution in a well was then pipetted off to avoid sampling from sediment at the bottom of the wells and allow for a representation of microbead dispersion within the media to be made. Ten microliters of solution were placed on a glass slide and examined at 20X magnification using the LEICA phase contrast setting. The same procedure was used to fluorescence readings of the microbeads for each baffle at a 560 nm excitation wavelength.

E. coli Growth Procedure

200 microliters of the *E. coli* diluted in LB broth was pipetted into each of the wells on a 96-well plate. The 3D printed baffled lid was then placed on the 96-well plate. The sides of the plate-lid combo were wrapped with Breathe-Easy Sealing Film to prevent evaporation from incubation. The plate with baffled lid was incubated at 37 degrees Celsius while affixed to a horizontal tilting shaker with tape. After 0, 3, and 24 hours, 50 microliters of solution from one third of the wells were pipetted to a new plate and put into a SpectraMax 250 Spectrophotometer for quantification.

Results

Baffle Evaluation

Table I. Design constraints and considerations for baffled lid prototype ordered by importance.

Need	Design Constraint/ Metric	Unit of Measure	Marginal (acceptable)	Ideal Value
1	Total Height (Plate + Lid)	mm	17.6	17.6*
2	Baffle Volume	mm^3	200 - 240	200*
3	Print time	hours	3 - 6	3*
4	Weight	grams	30 - 40	30*
5	Infill Density	%	15 - 30	15*

* Values less than this would also be ideal, but this is a realistic target

Each baffle was evaluated based on a set of design constraints that needed to be met (Table I). The total lid-plate height must be 17.6 mm or less because of the maximum clearance of commercial spectrophotometers. The volume of the baffles would ideally be less than 200 mm^3 to allow the wells to be filled with at least 200 µl of media, which is the standard amount used to most lab protocols[20]. To maximize efficiency and reduce the complexity of the baffle manufacturing process, print time with the Water Washable Resin would ideally be less than three hours. Finally, lid weight and infill density were factored in to make sure the lid could be easily operated, distributed, and would not waste material. All eight of the baffles met or improved upon the design specifications in Table I, making them suitable candidates for further testing.

Finite Element Analysis and Baffle Durability

A finite element analysis was conducted on all eight baffles as outlined in the methods (Figure 3). A maximum stress of 41.2 MPa was experienced by the baffles when five newtons of force was applied, which is below the tensile strength of the intended final material, polystyrene (46 MPa)[21].

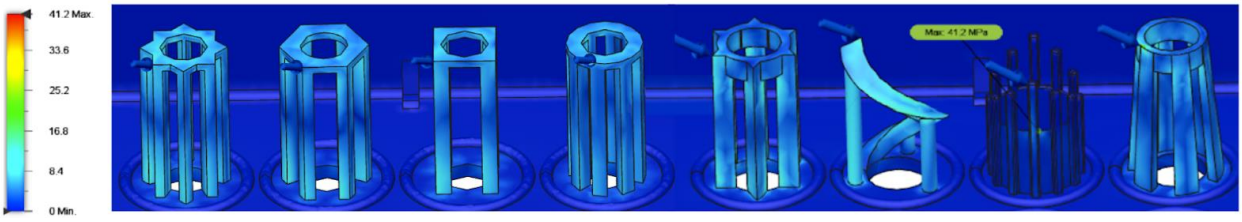

Fig. 3. Finite Element Analysis on eight baffle designs generated using Autodesk Fusion 360. This figure shows the stress profile of each baffle after being subjected to five newtons of force in the area indicated by the blue arrows. The colorimetric scale of the left indicates the relative amount of stress experienced, with blue to green being low stress areas and yellow to red being high stress areas.

Therefore, it was shown that each baffle could withstand the five newtons of force without suffering any permanent deformations. The drop tests resulted in no damage to any of the baffles regardless of drop orientation or tested height. These results show that the baffles are durable and can withstand enough force to be used in a research setting.

Light Interference

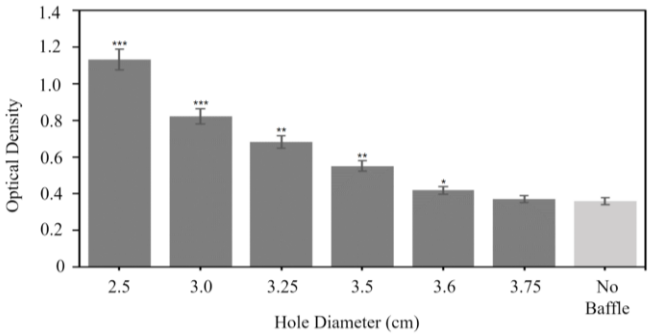

Fig. 4. OD measurements for different baffle hole diameters. 48 samples were taken for each group. Using a post-hoc Tukey's test, significant differences compared to the no lid group were noted with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.0001$).

Optical density measurements were taken of an empty 96-well plate with all six of the baffle hole diameters as well as a control with no lid on (Figure 4). Hole diameters were chosen that could simultaneously be large enough to avoid any light interference while also not being so large as to reduce durability of the baffles by making them too thin. A one factor ANOVA test determined that there was a significant difference between the different groups ($p < 0.0001$), meaning that light interference was occurring due to the addition of the baffles. However, a post-hoc Tukey's test showed that a hole diameter of 3.75 cm had no significant difference to the plate with no lid on, whereas the other hole sizes were significantly different. Therefore, a center hole diameter of 3.75 cm was chosen and implemented into each baffle design.

Microbead Dispersion Microscopy and Analysis

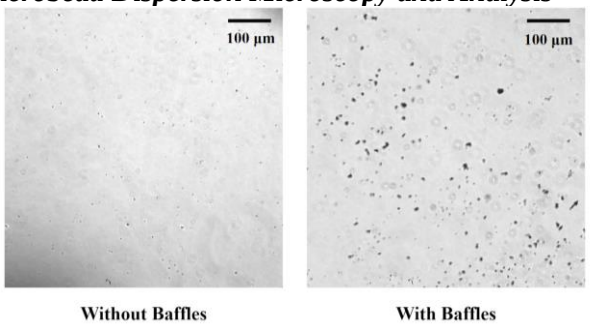

Fig. 5. Phase contrast microscopy images of samples taken from mixed solutions with and without baffles.

After mixing the microbeads within the wells as laid out in the methods section, both qualitative and quantitative measurements of the dispersion were taken. Phase contrast microscopy was used to visualize the top portion of the media when mixed with and without the baffled lid (Figure 5). These images qualitatively displayed a greater concentration of microbeads in the sampled solution when baffles were introduced, as can be seen by the increase in black dots on the image. When evaluated individually, all eight samples mixed with baffles had a significantly higher microbead concentration than the sample mixed without baffles ($p < 0.0001$) (Figure 6). No significant difference in fluorescence was seen between any of the baffled groups when compared to each other ($p > 0.05$).

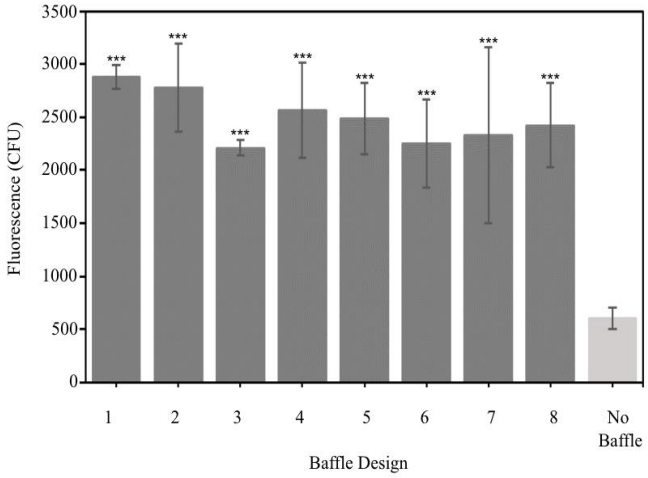

Fig 6. Fluorescence measurements of the eight different baffle designs compared to no baffle. The numbers under each bar correlate the number assigned to each baffle in the methods section. 48 samples were taken for each group. Using a post-hoc Tukey's test, significant differences for each baffle compared to the no baffle group were noted with *** ($p < 0.0001$)

E. coli Growth Analysis

Bacterial concentrations were measured as described in the growth procedure above using both the water-washable and ABS-like resin lids. Through visual examination of the relative turbidity at each time point of the samples with baffles introduced and optical density measurements, it was determined that the baffles were responsible for the stagnation of bacterial growth. This was based on both turbidity and optical density in the baffled wells remaining the same at all three times while increasing with time in the wells without baffles.

Discussion

Interpretation of Baffled Lid Results

Statistical tests confirmed that a baffle hole diameter of 3.75 centimeters produced almost identical optical density

measurements collected when no baffle inside a spectrophotometer. Using this hole diameter, optical density measurements were concluded to experience no light scattering or interference due to the baffles that would create inaccurate measurements. Spectrophotometers shoot a beam of light directly through a monochromator, which isolates a specified wavelength to send through the middle of the wells to the light detector[22]. Therefore, even accounting for light spreading as the beam traverses from the light source to the detector, only the center of the well needs to be unobstructed for optical density to be measured. Since the spread of light within spectrophotometers was not a known variable, systematically increasing the baffle hole diameter until no significant difference between the optical density readings of a well plate with baffles compared to a plate without baffles allowed for any light interference to be avoided.

FEA and drop tests confirmed that each individual baffle could withstand high enough forces to be used in a lab setting. The top corners of the baffles were chosen for application of the force because they are the first place any force would be encountered and carry the highest stress concentration Some of these forces include impacts during transportation of the lids, accidental drops from a shelf to the lab bench, placement of the lid on the well plate, and shear forces experienced while the lid-plate combination is being mixed on a shaker.

Microscopy and optical density were able to show that microbead dispersion increased with the addition of a baffled lid for each baffle design in comparison to unbaffled samples. Because fluorescence and concentration are linearly related, the increase in fluorescence measured for each baffled group is analogous to an increase in concentration of microbeads in that sample. Solid baffle structures were shown to have improved mixing inside the wells while incubating and shaking were occurring. This is due to the baffles creating more turbulent fluid flow in the lids, helping to overcome the surface tension in the wells without increasing shear forces that may kill cells. Disruption of this surface tension led to displacement of media from the bottom of the wells and aided in reducing the sedimentation of the microbeads, which is why increased microbead concentration was observed in the top portion of the fluid.

The *E. coli* growth analysis yielded results that contradicted the hypothesis set at the beginning of this project. Based on previous studies that incorporated physical well modifications, it is not likely that introduction of the solid structure into the wells would directly result in cell death[10,19]. Therefore, the lid material was evaluated as a potential cause of the observed results. Through this search, it was found that photopolymerized resins contain several compounds that are cytotoxic[23]. Since both materials used to create the baffles fall under this category, it is likely what attributed to rapid bacterial death in the well.

Conclusion

The first specific aim of this project was completed by demonstrating that varying the inside diameters of the baffles changed optical density measurements in relation to an unbaffled well. When using a hollow diameter of 3.75 cm, no significant optical interference was detected in relation to the unbaffled condition. FEA and drop testing proved the lid was fit to endure physical forces without breaking, bending, or bowing during intended use. The second specific aim was partially completed by introducing baffles into a solution of microbeads and quantifying an increase in dispersion of microbeads when compared to an unbaffled lid. However, photopolymerized water-washable and ABS-like resins were both discovered to be cytotoxic to bacterial cultures and prevented *E. coli* tests from yielding unbiased results.

Impact

Increasing the amount of oxygen transfer through wells, dispersion of bacteria within the wells, and overall mixability of the wells will allow for bacteria to be grown in a lab more accurately and evenly across the entire plate. This will reduce the error currently present in the microplate bacterial growth format, improve the reproducibility of kinetic growth analyses, and expedite the time required to confidently characterize bacterial growth trends. The baffled lid will therefore help both scientists and engineers artificially grow bacteria in *in vitro* environments to predict how they will perform in clinical or industrial applications. Microbial products, such as vaccines and antibiotics, could then be created using data on bacterial growth that better represents physiological conditions. This could lead to improvements in the efficacy and efficiency of these products and further increase the impact of the $200 billion bacterial products market.

Limitations

This project was conducted through a company external to the University of Virginia, and not all of the materials for lid creation and bacterial testing could be acquired. After finding out the resin was cytotoxic to cells, limits on the material that could be used with the ELEGOO Saturn 3D printer prevented the testing of another material. Access to an extrusion-based printer with the resolution necessary to

print the baffles was also not available, making prototyping with a different material impossible. In addition to this, the acquisition of an anaerobic chamber to test oxygen transfer rates was not available for use.

Based on the data that was collected, the best baffle design of the eight proposed at the onset of the project could not be determined. Each baffle design passed the light interference and FEA durability testing and had a statistically similar dispersion of microbeads within the wells. Since the oxygenation test could not be run and the growth analysis was hindered by the cytotoxicity of the lid, a conclusive decision of the optimal design could not be reached.

Future Work

Successful utilization of a non-cytotoxic material coupled with data supporting the increased bacterial growth of samples using a baffled lid still need to be completed. Dispersion testing, growth analysis, and oxygenation transfer rate tests need to be redone using a non-resin lid and bacterial cultures. ABS plastic or polystyrene would be used in place of ABS-like and water-washable resin and *E. coli* bacteria would be used instead of the microbeads. After conducting these experiments, one of the eight baffle designs needs to be chosen based on the results of these tests as well as the optimization of the design constraints outlined in Table I. Once one baffle geometry and one lid material are chosen, the final lid prototype can be designed, patented, and commercialized for use within a laboratory setting.

End Matter

Author Contributions and Notes

Brooks, N., Mirt, J., and Thomas, J. designed all baffles geometries, printed lid prototypes, created and conducted all experimental protocols, and wrote the final report. Carrier, D., and DeCleene, S. advised on all aspects of the project and assisted with 3D printing. The authors declare no conflict of interest.

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