Bioprinting Using Algae: Effects of Extrusion Pressure and Needle Diameter on Cell Quantity in Printed Samples

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Bioprinting is the fabrication of structures based on layer-by-layer deposition of biomaterials. Applications of bioprinting using plant or algae cells include the production of metabolites for use in pharmaceutical, cosmetic, and food industries. Reported studies regarding effects of extrusion pressure and needle diameter on cell viability in bioprinting have used animal cells. There are no reports regarding effects of extrusion pressure and needle diameter on cell viability using plant or algae cells. This paper fills this knowledge gap by reporting an experimental investigation on effects of extrusion pressure and needle diameter on cell quantity (an indicator of cell viability) in extrusion-based bioprinting of hydrogel-based bioink containing Chlamydomonas reinhardtii algae cells. Extrusion pressure levels used in this study were 3, 5, and 7 bar, and needle diameter levels were 200, 250, and 400 μm. Algae cell quantity in printed samples was measured on the third day and sixth day post bioprinting. Results show that, when extrusion pressure increases or needle diameter decreases, algae cell quantity in printed samples decreases.
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1 Introduction

In bioprinting, biomaterials are deposited layer-by-layer to fabricate structures [1]. In bioprinting, the bioink contains living cells [2] (including animal, plant, and algae cells). Cells in printed structures are immobilized. Immobilization provides a continuous, sustainable cultivation of plant and algae cells [2]. Therefore, immobilized plant and algae cells can grow more in their number (higher cell density) and produce more metabolites per cell than cells suspended in liquid [3]. Metabolites derived from plants [2] and algae [4] are used in products manufactured by the pharmaceutical, cosmetic, and food industries. Algae can also produce vanilla flavoring [4], artificial sweetener [5], and antibiotics [6].

Bioprinting techniques include extrusion-based bioprinting, inkjet-based printing, laser direct writing, and microcontact printing [1]. Extrusion-based bioprinting has many advantages including ease-of-use, availability [7], and compatibility with a wide range of biomaterials [8]. An illustration of pneumatic extrusion-based bioprinting is shown in Fig. 1. In pneumatic extrusion-based bioprinting, bioink is first loaded into the extrusion syringe. To print a structure, the bioink is extruded through the needle using compressed air as the driving force. The printed structure is printed in a cell culture dish, which is placed on the printing platform. Extrusion pressure can be changed during printing by varying the pressure of the compressed air. Additional printing parameters that can be varied are needle diameter, printing temperature, and printing speed. Needle diameter is varied by using extrusion needle of different diameter, printing temperature is varied by changing the temperature of the thermal jacket, and printing speed is varied by changing the speed of the printer head movement.

It has been reported that increasing extrusion pressure, decreasing needle diameter, or doing both at the same time causes cell viability to decrease in bioprinting with animal cells. After bioprinting, cells are considered viable if they are alive and capable of growth and reproduction [9]. It has also been reported [10] that different cell types exhibit different sensitivities to extrusion pressure and needle diameter during bioprinting. In one reported study [10], the survival rate was 90% for printed human tumor cells, and 55% for human stem cells under the same printing conditions.

It is noted that all reported studies regarding effects of extrusion pressure and needle diameter on cell damage in bioprinting used animal cells. For bioprinting using plant or algae cells, there are no reports regarding effects of extrusion pressure or needle diameter on cell viability. Seidel et al. [2] used hydrogel-based bioink containing basil cells in their bioprinting study. They found that both alginate:methylcellulose and alginate:agarose:methylcellulose hydrogels could be successfully used in printing of basil cells. Lode et al. [11] printed *Chlamydomonas reinhardtii* algae cells near human bone cells in printed structures. The *C. reinhardtii* algae provided oxygen required for the survival of the human bone cells in the printed structures. Krujatz et al. [12] investigated algae cell growth in printed structures compared with algae cell growth in liquid suspension and concluded that algae imbedded in printed structures had a higher growth rate.

Because different cells respond differently to changes in bioprinting conditions (including extrusion pressure and needle diameter)

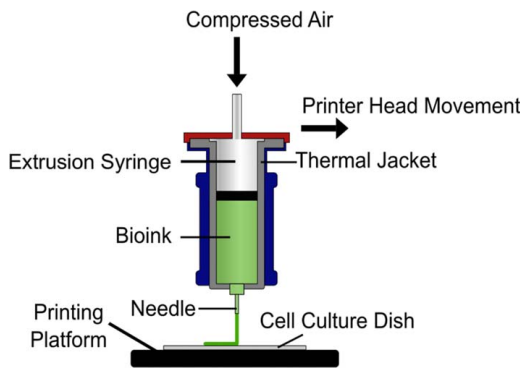

Fig. 1 Illustration of extrusion-based bioprinting process

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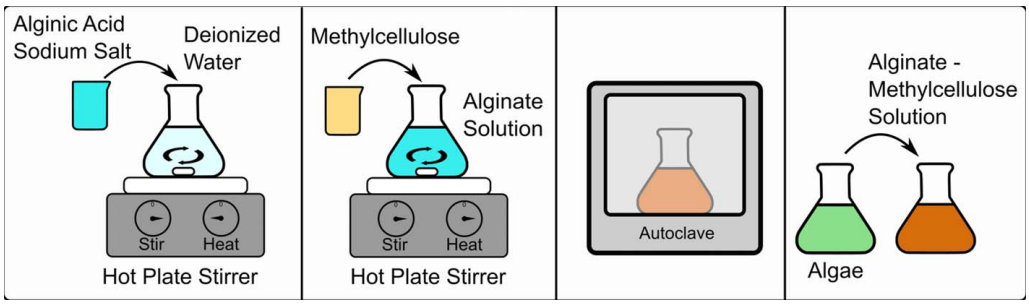

Fig. 2 Bioink preparation procedure

[10], it is unknown how algae cell viability will be affected by changes of extrusion pressure and needle diameter in extrusion-based bioprinting. This paper aims to fill this knowledge gap. It reports an experimental study regarding effects of extrusion pressure and needle diameter on cell quantity (an indicator of cell viability) of *C. reinhardtii* algae in printed samples using an extrusion-based bioprinter.

2 Experimental Procedure and Conditions

2.1 Preparation of *C. reinhardtii* Algae Cells. A 150 ml flask (VWR) was used to prepare 100 ml of tris-acetate-phosphate (TAP) culture media. *C. reinhardtii* algae strain cc125 cells were streaked off from a petri dish and added to the flask. The addition was done in a biosafety cabinet (SterilGARD III Advance, Baker) to maintain a sterile condition. The *C. reinhardtii* algae strain cc125 was selected because it is commonly used in laboratories. The algae cells used in this study were obtained from the *Chlamydomonas* Center (Chlamydomonas Resource Center, University of Minnesota). The flask (containing the TAP-algae solution) was put on a shaker (New Brunswick Scientific Co., Inc., Enfield, CT) for 72 h. The shaker was run at 100 rpm and kept at 22 °C, under lightbulbs to allow growth of the algae cells. Afterward, the algae cell quantity in the TAP-algae solution was measured using Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA) according to the instructions from the cell counter manufacturer.

2.2 Preparation of Bioink. An overview of bioink preparation procedure is shown in Fig. 2. The bioink was based on alginate:methylcellulose hydrogel. Alginate is a natural polymer derived from algae, and methylcellulose is a polymer consisting of numerous linked glucose molecules. Three grams of alginic acid sodium salt (Sigma-Aldrich) were added to 100 milliliters of deionized water in a 500 ml beaker (VWR). This alginate solution was stirred at 900 rpm for five hours on a hot plate stirrer (Fisher Scientific). The alginate solution was then heated on the hot plate stirrer to 90 °C, and 6 g of methylcellulose powder (Sigma-Aldrich) were added to the solution. The alginate:methylcellulose solution was autoclaved in an autoclave (LG 250 Sterilizer, Steris) for 1 min at 121 °C to achieve sterilization. The time and temperature settings for autoclaving were chosen to ensure sterility of the alginate:methylcellulose solution without burning the methylcellulose. After the alginate:methylcellulose solution cooled to room temperature, algae cells in the TAP-algae solution were added to the alginate:methylcellulose solution. The addition of algae cells was performed by pipette (Rainin), an instrument used to deposit precise volumes. The addition was done in a biosafety cabinet (SterilGARD III Advance, Baker). The algae cell concentration in the bioink was 150,000 cells per milliliter of bioink.

2.3 Design of Samples. The samples were designed using Fusion 360 software (Autodesk). Each sample was a disk with diameter of 15 mm and thickness of 1.5 mm. The STL file generated by Fusion 360 software was converted into a G-code file using SLICER software (SLICER.org, USA). This G-code file was then imported into Allevi bioprinter software (Allevi Inc.) via a USB flash drive.

2.4 Printing of Samples. Allevi 2 bioprinter (Allevi Inc.) was used for printing of samples. The printing experiment setup is illustrated in Fig. 3. The prepared bioink was loaded into the extrusion syringe with a needle (Bstean), and printed onto microscope slides (VWR). To maintain sterility, printing was performed inside a biosafety cabinet. After printing, the samples were submerged in 100 millimolar $CaCl_2$ solution for 4 min, a common practice for crosslinking [13]. Crosslinking creates chemical bonds between polymer chains of the printed samples and is necessary for the printed samples to maintain shape fidelity. The microscope slides with the samples were then transferred to cell culture petri dishes (Fisher Scientific) and immersed with TAP solution, as shown in Fig. 4. The culture dishes were placed under lightbulbs to promote algae cell growth.

2.5 Experimental Design. Two sets of one-factor-at-a-time experiments were conducted to investigate the effect of extrusion

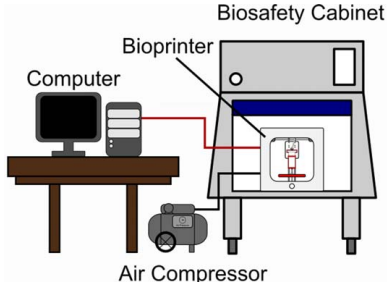

Fig. 3 Illustration of printing experimental setup

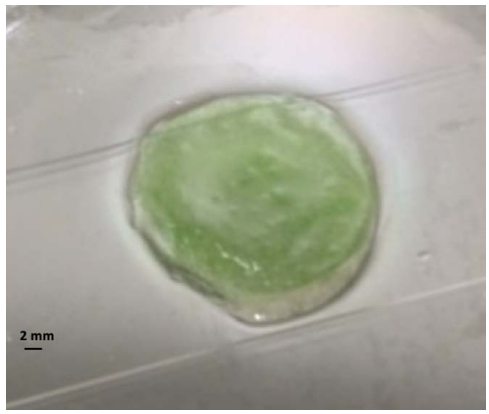

Fig. 4 Image of a printed sample on the third day post printing

pressure and the effect of needle diameter on cell viability. For the first set of experiments, samples were printed by varying extrusion pressure. The three levels of extrusion pressure were 3, 5, and 7 bar. Needle diameter was kept constant at 250 μm. For the second set of experiments, samples were printed by varying needle diameter. The three levels of needle diameter were 200, 250, and 400 μm. Extrusion pressure was kept constant at 4 bar. The constant levels, 4 bar for extrusion pressure and 250 μm for needle diameter, were in the feasible regions of extrusion pressure and needle diameter when printing alginate:methylcellulose bioink with the printer used in this study. These feasible regions would allow continuous strands to be printed and were determined by a systematic experimental investigation. The results of the investigation were reported in a paper published in the Journal of Manufacturing Science and Engineering [14] recently. For both sets of experiments, three samples were printed as replicates for each experimental condition.

2.6 Assessment of Cell Viability. One method to evaluate cell viability in bioprinting is to measure cell quantity after printing, along with other methods such as live/dead staining [9], ATP (adenosine triphosphate) detection from cells [9], and evaluation of cell organelle functions [9]. Because damaged or dead cells do not multiply [15], cell quantity after bioprinting can indirectly measure cell viability. Confocal microscopy, FV1000 microscope (Olympus, Inc.), was used in this study to measure cell quantity in printed samples. Confocal microscopy was also used by other researchers in their bioprinting studies with plant and algae cells [2,11,12]. The measurement procedure is described below.

Step 1: To estimate the average number of algae cells in an algae cell cluster, A. Please note that algae cells grow in clusters instead of being evenly spaced in printed samples. For each printed sample, images of five randomly chosen algae cell clusters on the sample surface were taken. An image of one such algae cell cluster is shown in Fig. 5. The number of cells in each of the five clusters was counted, and the average number of cells per cluster, A, was calculated.

Step 2: To estimate the average number of algae cell clusters in the volume of a z-stack, B. Three z-stacks were captured at three randomly chosen positions for each printed sample. A z-stack is a combination of multiple cross-sectional images. Each image is taken at a fixed depth interval within the sample. Therefore, the z-stack is three-dimensional. The number of clusters in each of the three captured z-stacks were counted, and the average number of clusters per z-stack, B, was calculated.

Step 3: To estimate the average volume of a z-stack, z, by multiplying the depth of each z-stack by the area covered by each image. This area is dependent on the focal length of the optical lens used for imaging. The depth of the z-stacks ranged from 725 to 1008 μm, and the area ranged from 450 μm × 760 μm to 580 μm × 970 μm.

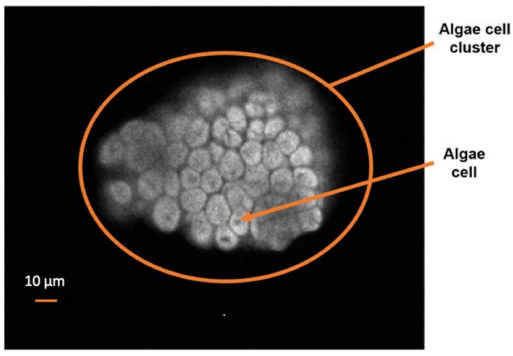

Fig. 5 Image of algae cell cluster on the surface of a sample

Step 4: To calculate the number of cells per cubic millimeter (n) for each sample, Eq. (1) was used

$$n = \frac{A \cdot B}{z} \tag{1}$$

Step 5: To calculate the average number of cells per cubic millimeter of printed samples for each experimental condition, N. For each experimental condition, three samples were printed.

$$N = \frac{n_1 + n_2 + n_3}{3} \tag{2}$$

3 Results and Discussion

Experimental results are presented in Tables 1–4. The effects of extrusion pressure and needle diameter on cell quantity are shown in Figs. 6 and 7, respectively. The error bars in these figures represent 1 standard deviation of cell quantity among the printed samples at a particular level of extrusion pressure or needle diameter.

From Fig. 6, it can be seen that algae cell quantity decreased as extrusion pressure increased. Algae cell quantity was the highest for the samples printed at extrusion pressure of 3 bar, and the lowest for the samples printed at extrusion pressure of 7 bar. These trends were observed in experimental data measured on both day 3 and day 6 post printing. Between day 3 and day 6, the

Table 1 Cell quantity (1000/mm^3) in samples printed at different levels of extrusion pressure and measured on day 3 post printing

Extrusion pressure (bars)	Sample 1	Sample 2	Sample 3	Mean	Standard deviation
3	8.341	7.912	7.880	8.044	0.601
5	5.912	5.414	6.876	6.068	0.922
7	3.984	3.840	3.895	3.907	0.658

Table 2 Cell quantity (1000/mm^3) in samples printed at different levels of extrusion pressure and measured on day 6 post printing

Extrusion pressure (bars)	Sample 1	Sample 2	Sample 3	Mean	Standard deviation
3	28.130	23.383	23.808	25.107	2.984
5	13.217	14.171	15.638	14.342	1.323
7	9.989	9.801	8.384	9.391	0.921

Table 3 Cell quantity (1000/mm^3) in samples printed at different levels of needle diameter and measured on day 3 post printing

Needle diameter (μm)	Sample 1	Sample 2	Sample 3	Mean	Standard deviation
200	5.188	4.923	4.573	4.895	0.308
250	6.024	6.868	6.273	6.388	0.433
400	9.353	8.768	8.828	8.983	0.321

Table 4 Cell quantity (1000/mm^3) in samples printed at different levels of needle diameter and measured on day 6 post printing

Needle diameter (μm)	Sample 1	Sample 2	Sample 3	Mean	Standard deviation
200	9.989	9.982	9.473	9.815	0.295
250	13.194	13.942	13.645	13.594	0.376
400	26.586	22.633	22.247	23.822	2.400

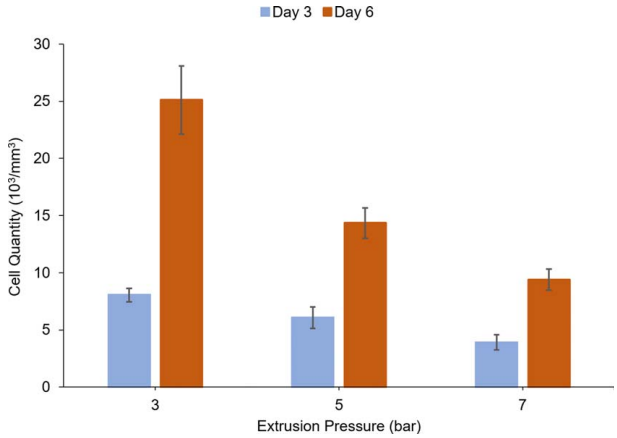

Fig. 6 Effect of extrusion pressure on cell quantity

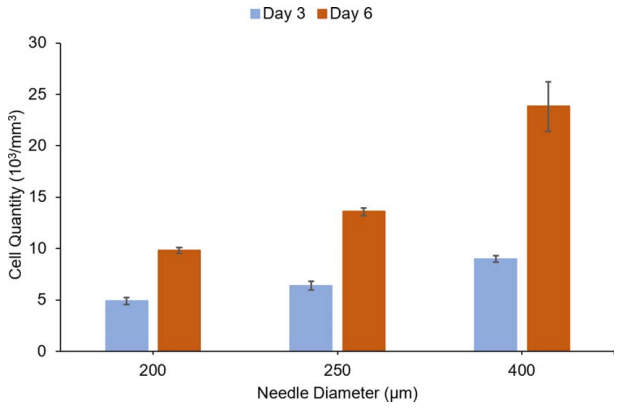

Fig. 7 Effect of needle diameter on cell quantity

samples printed at 3 bar had the largest increase in algae cell quantity, and the samples printed at 7 bar had the smallest increase in cell quantity. Cells that can grow and reproduce are considered viable [9]. Since only viable cells are capable of multiplying, this trend suggests that the samples printed at 3 bar had the highest number of viable cells, while the samples printed at 7 bar had the lowest number of viable cells.

As shown in Fig. 7, algae cell quantity in printed samples increased as needle diameter increased. This trend was observed on both day 3 and day 6 post printing. Between day 3 and day 6, the samples printed with needle diameter of 400 μm had the highest algae cell quantity, and the samples printed with needle diameter of 200 μm had the lowest cell quantity. Because algae cell quantity is an indicator of cell viability, it can be inferred from the data that algae cells in the samples printed with needle diameter of 200 μm and 250 μm were less viable than those in the samples printed with needle diameter of 400 μm.

There are no reported studies on the effects of extrusion pressure or needle diameter on algae cell quantity in printed samples, although the literature contains many reported studies on the effects of extrusion pressure or needle diameter on other aspects of bioprinting (such as compressive modulus of printed samples [16]). However, the trends observed from this study are consistent with reported trends about effects of extrusion pressure and needle diameter on cell viability of animal cells in printed samples.

Table 5 summarizes reported studies regarding effects of extrusion pressure and needle diameter on cell viability in bioprinting using animal cells. Chang et al. [17] reported that cell membranes of liver cells were mechanically damaged by increasing extrusion pressure and/or decreasing needle diameter during printing. Cell viability decreased when extrusion pressure was 20 psi (1.38 bar) or higher, or when needle diameter was 400 μm or smaller. However, some of the damaged liver cells were able to recover over the course of several hours post printing. Nair et al. [18] created a mathematical model to predict percentages of living, injured, and dead rat endothelial cells post printing, based on extrusion pressure and needle diameter used in printing. Extrusion pressure of 20 psi (1.38 bar) or higher resulted in the largest percentage of dead cells. Additionally, the percentage of dead cells increased as needle diameter decreased from 400 μm to 150 μm. Blaeser et al. [19] demonstrated that extrusion pressure of 0.75 psi (0.05 bar) or higher during bioprinting caused immediate mechanical damage to human mesenchymal stem cells. The long-term growth potential of the stem cells that survived the printing process also decreased. Fakhruddin et al. [20] reported that the cell viability of fibroblast cells significantly decreased when extrusion pressure was 4 psi (0.28 bar) or higher during bioprinting. The percentage of dead cells surpassed the percentage of live cells in the printed samples when extrusion pressure was 15 psi (1.03 bar) or higher. Billiet et al. [21] reported that human liver cells were mechanically damaged by extrusion pressures of 14.5 psi (1.0 bar) or higher. Müller et al. [22] found that needle diameter of 260 μm or smaller caused the cell viability of printed cartilage cells to decrease. This trend was observed for both conical and straight needles. Ouyang et al. [10] reported that needle diameter of 260 μm or smaller caused the cell viability of stem cells to decrease. No significant differences in cell viability were reported for needle diameters larger than 260 μm.

Table 5 Reported studies about effects on cell viability in extrusion-based bioprinting using animal cells

Result	Reference	Cell type	Printing condition
As extrusion pressure increased, cell viability decreased	[17–21]	Liver cells, rat endothelial cells, stem cells, fibroblasts	Extrusion pressure ≥ 0.75 psi (about 0.05 bar)
As needle diameter decreased, cell viability decreased	[10,19,21,22]	Liver cells, rat endothelial cells, cartilage, stem cells	Needle diameter ≤ 400 μm

4 Concluding Remarks

In this study, effects of extrusion pressure and needle diameter on algae cell quantity in printed samples were investigated. Major findings are as follows:

- Increasing extrusion pressure caused algae cell quantity in printed samples to decrease. This trend was observed on both day 3 and day 6 post printing.
- Increasing needle diameter caused algae cell quantity in printed samples to increase. This trend was observed on both day 3 and day 6 post printing.

The knowledge about trends of effects of extrusion pressure and needle diameter on algae cell quantity will be beneficial in printing with algae, when selecting extrusion pressure and needle diameter levels. The reason behind these observed trends is, at present, not clear. It is hypothesized that increasing extrusion pressure and decreasing needle diameter will increase shear stress on algae cells being printed, which may cause damage to the algae cells and lead to decreased cell quantity. This hypothesis warrants further investigation. In addition, effects of other printing parameters including printing speed and extrusion temperature on algae cell quantity in printed samples need to be examined.

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Conflict of Interest

There are no conflicts of interest.

Data Availability Statement

The datasets generated and supporting the findings of this article are obtainable from the corresponding author upon reasonable request. The authors attest that all data for this study are included in the paper.

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