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RESEARCH

# Bioprinting of microalgae laden scaffold for tissue regeneration

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**Abstract**
In recent years, microalgae that can be a continuous source of oxygen for cells and rich in bioactive compounds, have been used as an innovative approach to prevent hypoxia after implantation of biomaterials. Oxygen can be provided photosynthetically by the polar microalga (*Chlorella variabilis*) in combination with human cells, even without an external oxygen source. In this study, 3D bioprinting of microalgae laden scaffolds was investigated and characterized before use in wound healing. Prepared bioink consisted of alginate and different ratios of collagen (5, 10 and 15%) and *C. variabilis* cells. Printing was carried out at 10 mm $s^{-1}$ and the pressure was 50 kPa. Each sample had 0.6–0.7 mL volume and $6–7 \times 10^5$ cells. After the 3D bioprinting process, scaffolds were crosslinked with 100 mM calcium chloride for 10 min and incubated. It was observed that the microalgae grew well over 6 days in the scaffold. Also, an oxygen increase from 6 to 12 mg $L^{-1}$ was determined. It was demonstrated that, development of microalgae-laden scaffolds is promising on the delivery of oxygen or bioactive compounds as therapeutic agents.

**Keywords** Bioprinting · Microalgae · Biomaterials · Tissue engineering · 3D-plotted hydrogel · Chlorophyta

## Introduction

Tissue engineering applications provide many opportunities in the field of regenerative medicine, but these applications have some basic problems and the most important of these problems is the inability to provide sufficient oxygen to 3D tissue cultures (Hopfner et al. 2014). In addition, cellular apoptosis and micro-tissue necrosis can be observed after implantation of bioengineered microtissues/cell-loaded biomaterials in in-vivo applications due to oxygen deficiency (hypoxia) (Agarwal et al. 2021).

In order to reduce hypoxia, oxygen releasing materials such as sodium percarbonate, magnesium peroxide, calcium peroxide and hydrogen peroxide have been used as oxygen sources in scaffolds and microspheres (Corrales-Orovio et al. 2023; Ortega et al. 2023; Kang et al. 2024). However, these materials are cytotoxic to cells due to the decomposition of salt by-products and inappropriate release profiles, which adversely affect cell viability (Maharjan et al. 2021).

In order to overcome these challenges, microalgae which can be a continuous source of oxygen for cells, have been used as an innovative approach in recent years (Lode et al. 2015). Oxygen is provided photosynthetically by microalgae when combined with human cells, even without an external oxygen source (Lode et al. 2015). Hopfner et al. (2014) cultured *Chlamydomonas reinhardtii* in scaffold which shows biocompatibility and photosynthetic activity. They then co-cultured the microalgae with fibroblasts which resulted as a decrease in the hypoxic response under low oxygen culture conditions. In another study scaffolds which consisted of *C. reinhardtii* cells were implanted into a mouse. The microalgae survived for at least 5 days and did not elicit a significant immune response. They also achieved a high level of vascularization in the injured area in the presence of microalgae (Schenck et al. 2015).

Additive manufacturing (AM) is a method used in a wide variety of fields for applications ranging from nano to micro and macro scales (Malik et al. 2020). With this production technique, the desired material, such as those containing animal, plant or algae cells, can be created homogeneously layer by layer (Malik et al. 2020; Thakare et al. 2021). Thus, the cells are immobilized in the

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printed structures (Thakare et al. 2021). In recent years, 3D bioprinting of microalgae, which is a layered production technique for the immobilization of microalgae to certain structures for the production of photosynthetic materials, has been used many times (Krujatz et al. 2015; Lode et al. 2015). 3D bioprinting includes many different printing methods where extrusion-based bioprinting is mostly preferred in terms of ease of use, availability and compatibility with various biomaterials (Thakare et al. 2021).

A wide range of biomaterials such as synthetic polymers like poly (ε-caprolactone) (PCL), natural protein-based polymers like gelatin and collagen, and natural polysaccharides like agar and alginate have been used for 3D bioprinting or different cell immobilization techniques (Schütz et al. 2017; Zhao et al. 2019). In recent years, alginate/methylcellulose combinations are the most used (Krujatz et al. 2015; Lode et al. 2015; Malik et al. 2020) including combinations of carboxymethyl cellulose sodium salt (NaCMC)/gelatin/alginate/polyvinyl alcohol (PVA) (Maharjan et al. 2021) and silk-based (Zhao et al. 2019) biomaterials have been evaluated for 3D bioprinting of microalgae-laden structures. Among these polymers, alginate is one of the most widely used biopolymers for 3D bioprinting due to easily crosslinking characteristics to make 3D structures stable and durable after printing. In addition, since the gelation process of alginate occurs very quickly and the process is carried out under mild conditions, it is not harmful to living cells (Schütz et al. 2017). Along with alginate, methylcellulose has been mostly used to increase viscosity (Schütz et al. 2017) and provide 3D printability (Lode et al. 2015). However, methyl cellulose does not have a positive effect on cells other than being a viscosity-increasing agent (Payne et al. 2017).

Collagen is an essential natural polymer for mammalian cells in tissue repair, as it provides cell adhesion and cell differentiation, especially by affecting the cellular activity of fibroblasts. Since it is one of the main components of the extracellular matrix (ECM), it has natural biocompatibility, low cytotoxicity and high water holding capacity (Yang et al. 2018; Xu et al. 2020). For this reason, it has attracted attention as a promising material in 3D bioprinting. Furthermore, Yang et al. (2018) reported that, collagen was more resistant to deformation compared to alginate. Considering the viscosity of collagen at room temperature and changes in viscosity depending on temperature, it is very suitable for 3D bioprinting at room temperature and even highly viscous solutions can be printed thanks to its decreasing viscosity at increasing temperatures (Lai et al. 2008). Due to these properties, collagen can be used instead of methyl cellulose.

In this study, 3D bioprinting of microalgae-laden scaffolds was evaluated by examining the different ratios of polymers to be used as bioink and characterized to be used in wound healing.

As for the selection of microalgae species, the choice of a polar microalgae isolated from Antarctica was driven by its unique biochemical and physiological properties (Inan et al. 2023), which make it highly suitable for wound healing applications. Polar microalgae are known to survive in extreme environments by producing high levels of bioactive compounds such as antioxidants, polyunsaturated fatty acids, and polysaccharides, that have antimicrobial, anti-inflammatory, and regenerative effects. Additionally, these microalgae exhibit exceptional oxygen-producing capabilities due to their adaptation to low-light and low-temperature conditions, making them an excellent natural oxygen source for enhancing cell proliferation and tissue regeneration. By incorporating microalgae into the bioink, this study aims to leverage these beneficial properties to develop a more effective and bioactive scaffold for wound healing, offering an innovative alternative to traditional approaches.

This study presents a significant approach in the field of wound healing by integrating 3D bioprinting with microalgae-laden scaffolds. The novelty lies in the utilization of an Antarctic polar microalgae species, which, to the best of our knowledge, has never been explored in this context. Additionally, by systematically evaluating different polymer ratios in bioink formulation, this research provides valuable insights into optimizing scaffold composition for biomedical applications. The innovative combination of bioprinting technology and bioactive microalgae opens new avenues for the development of advanced wound dressings with enhanced regenerative properties.

## Materials and methods

### Materials

*Chlorella variabilis* YTU.ANTARCTIC.001 was obtained from the Algal Biotechnology and Bioprocess Laboratory in the Bioengineering Department of Yıldız Technical University. Calcium chloride, sodium alginate and acetic acid were supplied from Merck (Germany). Type I collagen from bovine skin was obtained from Kazlıçeşme R&D Centre and Test Laboratory that produces food grade collagen.

### Preparation of bioinks

To prepare a composite hydrogel formulation for 3D printing, different ratios of polymers were examined. First, an alginate solution with the concentration of 3.5% w/v was prepared by dissolving 0.35 g of alginate in 10 mL distilled water, and accordingly varying amounts of collagen (5, 10 and 15% Col) were added into this solution. Each sample was stirred at 400 rpm for 2 h at 50 °C to ensure complete dissolution. Different solutions were evaluated in

the printability test and then the best printed solution was selected for 3D bioprinting. Finally, the selected solution was irradiated with UV light for 15 min to achieve sterilization. After sterilization, microalgae cells were added by pipette to the alginate:collagen solution in a biosafety cabinet. The algae cell concentration in the bioink was 150,000 cells $mL^{-1}$ bioink.

### Preparation of microalgae culture

*Chlorella variabilis* was cultivated in BG-11 nutrient medium (Pandey et al. 2023) in a 250-mL Erlenmeyer flask with a working volume of 150 mL for 10 days. The culture was maintained at a constant temperature of 25 °C under continuous illumination of 108 µmol photons $m^{-2}$ $s^{-1}$, pH 7.2, and a shaking speed of 150 rpm. The growth curve of the culture was determined during cultivation using a UV–Vis spectrophotometer at 680 nm.

### Printability test

In order to find the best printable solution, different alginate:collagen solutions were printed with a 3D bioprinter and analyzed visually. For further detail about the printability, scaffolds with different infill types and layer counts were printed and compared. Honeycomb, triangle and grid infill types and 5, 10, 20 layer counts were used for printing tests of the 10% Col hydrogels. After printing, scaffolds were crosslinked with 100 mM calcium chloride solution for 10 min and these results were investigated and photographed.

### 3D bioprinting of microalgae-laden hydrogel scaffolds

3D bioprinting of the scaffolds was carried out using a Cellink BIOX 3D bioprinter. Prepared bioink was uploaded to a 3 mL cartridge, and a 24G metal needle (or plastic nozzle) was used for printing. 3D printed scaffolds were designed as simple structures of 20 mm × 20 mm × 2 mm dimensions. Printing layer height was determined as 0.2 mm and scaffolds had 10 layer counts. Medium temperature was 25 °C. Printing speed and the pressure were adjusted to 10 mm $s^{-1}$ and 50 kPa, respectively. Each sample had 0.6–0.7 mL volume and had 6–7 × $10^5$ cells. After the 3D bioprinting process, scaffolds were crosslinked with 100 mM calcium chloride for 10 min and incubated.

### Incubation of algae-laden scaffolds

The bioprinted algae-laden scaffolds were incubated in BG-11 medium at a constant temperature of 25 °C under continuous illumination of 108 µmol photons $m^{-2}$ $s^{-1}$ for 16 days. Each sample was incubated in a 50 mL beaker full of BG-11 medium and each beaker was covered with parafilm and the top of the parafilm was drilled for gas transfer. During the incubation period, medium was changed with fresh medium once in three days. Before changing the medium, pH was measured for each sample in order to control.

In addition, growth of the algae was evaluated by measuring the OD at 680 nm at different times. Before measuring absorbance, the algae-laden scaffolds were transferred to a new beaker and 1.5% NaCl solution was added and stirred for scaffold degradation. After degradation of scaffolds, samples were centrifuged at 2655 × *g* for separating microalgae cells from other solved components like alginate, collagen and salt etc. Then, the microalgae pellet was resuspended in 10 mL distilled water and optical density was measured.

### Measurement of dissolved oxygen concentration in water

The dissolved oxygen concentration by microalgae through photosynthesis during the incubation period was measured using a dissolved oxygen probe. Oxygen measurement for the scaffolds incubated in an aqueous environment in a beaker was measured on days 1, 4, 7, 10, 14 and 16. The amount of oxygen was determined as mg $L^{-1}$. After each measurement, the probe was washed with pure water and measurements were performed in triplicate.

### Measurement of chlorophyll-*a* change

To determine the change of the pigment, 5 mL of liquid microalgae suspension was centrifuged 6797 × *g*. Then the supernatant was decanted and 5 mL of methanol was added to the pellet. The tubes were homogenized for 10 min in an ultrasonically assisted water bath at 60 °C. The solution was then mixed for 15 s using a vortex to obtain a homogeneous consistency. Then, it was centrifuged again at 6797 × *g* at 4 °C for 10 min. The absorbance of the supernatant was measured at 666 nm (Zou and Richmond 2000; Macías-Sánchez et al. 2005).

#### Statistical analysis

Experiments were conducted triplicate for the microalgae culture. The data are displayed in the figures as mean ± standard error.

## Results

### Printability test

For the printability test, three different solutions were examined: Col 5%, Col 10% and Col 15%. It was observed

that since the viscosity of the Col 5% solution was very low, the solution was very fluidic and the printed scaffolds did not have shape stability. As seen in Fig. 1, the printed lines were mixed together and the viscosity was not sufficient to form a smooth shape. Since the created scaffolds did not have dimensional accuracy, they were not considered as suitable materials.

Col 10% and Col 15% were easily printed and their scaffolds had shape stability. However, the viscosity of Col 15% was too high, and the 3D printer had to use very high pressure to print. Additionally, Col 15% scaffolds did not have a regular shape compared to Col 10%. The printed structures were able to maintain their shape, but due to high viscosity, there were residues and excesses in the created structures (Fig. 2). Being in such a solid state also reduced the printing speed considerably, and Col 10% had a relatively slower printing speed. Due to these properties, Col 15% material was not preferred as bioink.

The best printability results wer obtained with Col 10% solution because it has appropriate viscosity and can be printed very easily. After printing, the scaffolds achieved shape stability and their surfaces became very smooth. As can be seen in Fig. 3, scaffolds with different filler types and different number of layers were printed and compared for more detailed information about the printability test. In the compression tests of Col 10% hydrogels, honeycomb, triangular and grid filling types and 5, 10 and 20 layers were used. The printed lines were quite smooth in almost all infill types. Even at different layer thicknesses, the porosity of the printed structures was preserved. Additionally, no deterioration in the structures was observed after cross-linking.

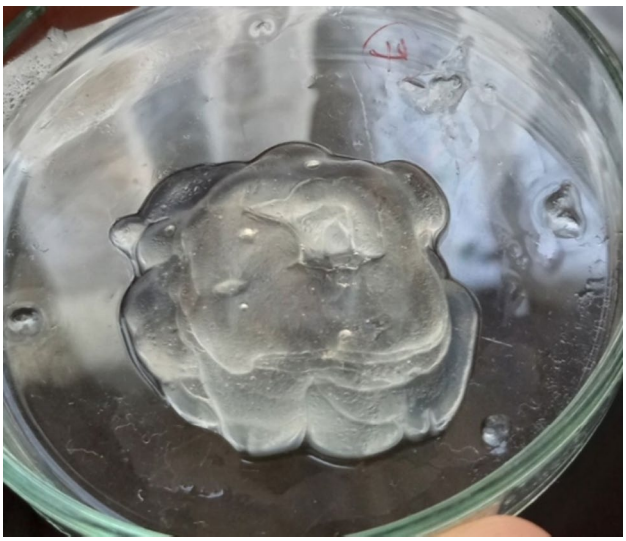


**Fig. 1** Printability test of Col 5% material

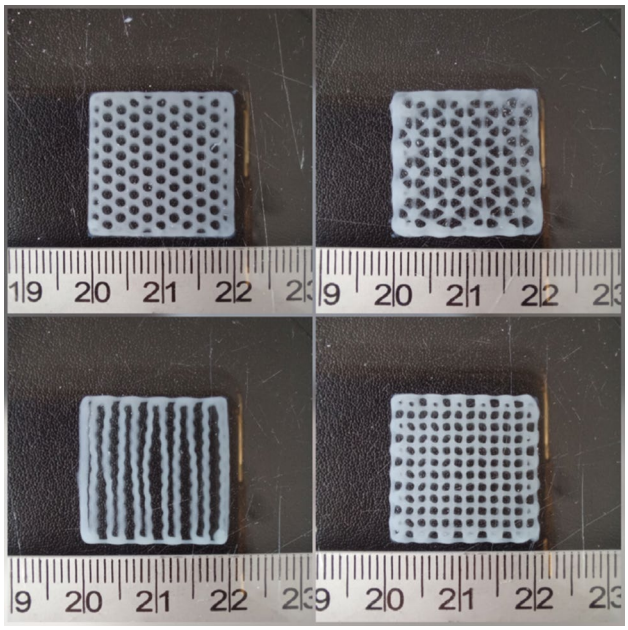


**Fig. 2** Printability test of Col 10% material with different filler type

## Bioprinting of microalgae-laden scaffold

After selecting the Col 10% as the bioink material, it was mixed with *C. variabilis* culture, printed and cross-linked. During the incubation, the pH was measured and growth of microalgae was monitored. While pH was around 7.5 at the beginning, it started to decrease below 6 on the 1st day of incubation, then increased to pH 6.8 and 7 again on the 3rd day of incubation (Fig. 4).

The microalgae growth curve included lag, log, stationary and death phases. It was observed that, the lag phase was in the range of 0–1 days, the log phase in the range of 1–8 days, the stationary phase in the range of 8–14 days and the death phase in the range of 14–16 days (Fig. 5A). Figure 5B shows photographic images and optical microscope images of 3D bioprinted tissue scaffolds on days 1 and 7. Observation of pigment change of microalgae was also performed throughout the incubation. A similar curve to the growth curve of microalgae was observed (Fig. 5C). During the 16-day incubation period, the pigment concentration correlated with absorption at 666 nm increased until the 14 th day. Depending on the death phase, absorption decreased sharply between days 14–16.

The oxygen concentration of the culture first decreased to 5.1 mg $L^{-1}$ on the 1 st day (Fig. 6). Then, it increased again to 8.4 mg $L^{-1}$ by the 7 th day, and to 11 mg $L^{-1}$ until the 14 th day.

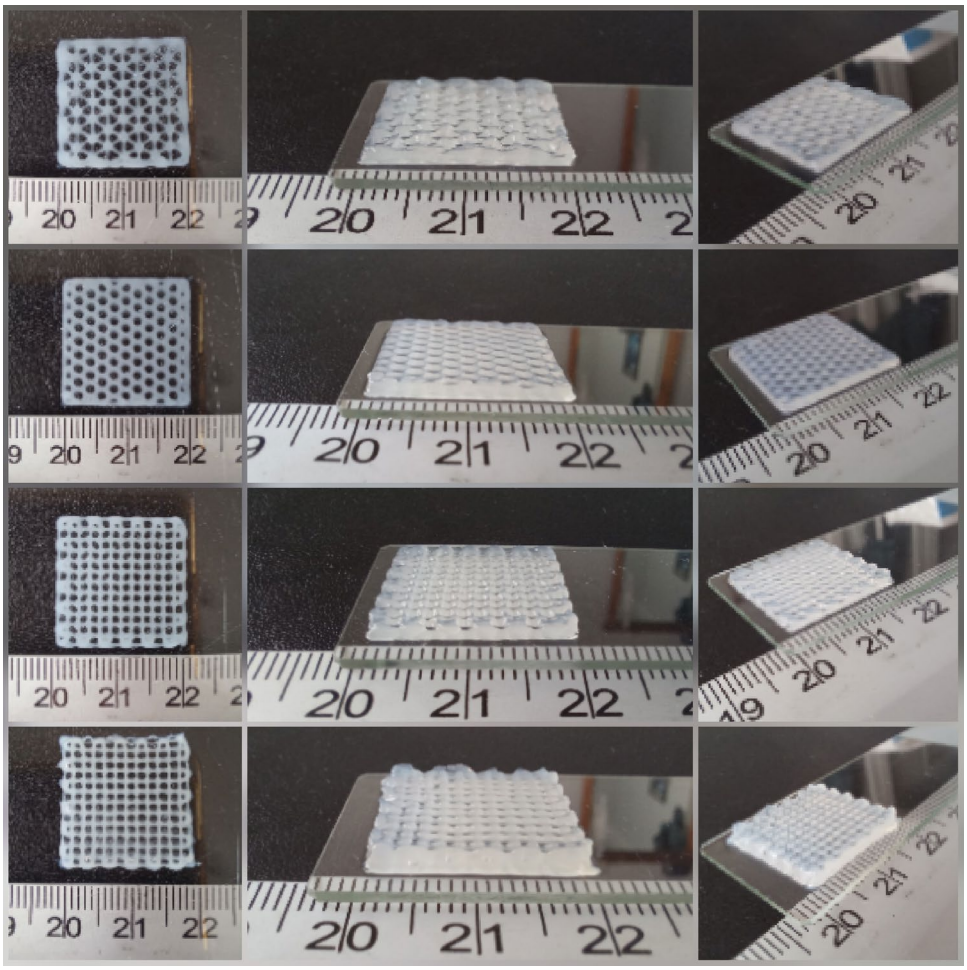


**Fig. 3** Printability test of Col 10% material with different filler type and number of layers

## Discussion

Tissue engineering, together with 3D printing technology, offers solutions to the long-standing challenges of organ transplantation and tissue repair. The basis of this technology is the production of complex structures that mimic natural tissues using biocompatible materials and cell cultures. 3D printing provides precise control over the architecture and composition of structures, allowing researchers to tailor them to specific anatomical requirements and patient needs. This approach holds great promise for producing personalized structures that integrate seamlessly with the body's natural systems, down to bones, tissues and organs (Hopfner et al. 2014; Chung et al. 2020).

Nevertheless, guaranteeing the survival and effectiveness of these constructions after implantation is a crucial challenge in tissue engineering. The availability of oxygen, which is essential for tissue health and cell life, becomes a significant concern. Conventional techniques frequently result in necrosis and graft failure because they are unable to effectively supply oxygen to the deepest parts of designed tissues. Researchers are looking at creative solutions to this problem, like increasing the diffusion of oxygen through the addition of microchannels or porous structures to structures. Furthermore, oxygen-releasing biomaterials are being included into scaffolds through advancements in

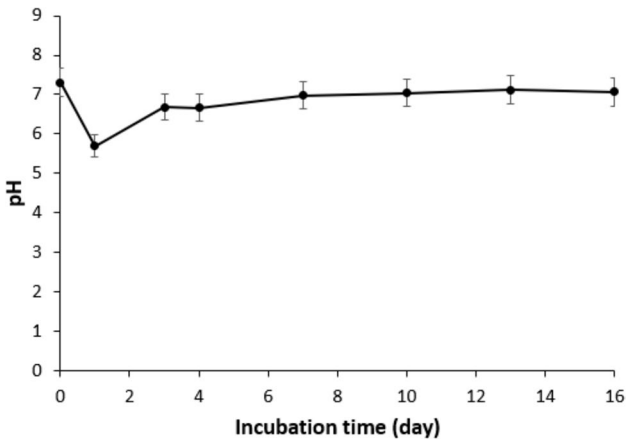



**Fig. 4** Ambient pH values during 16 days of incubation. Measurements were conducted triplicate on the samples. Data are mean ± standard error

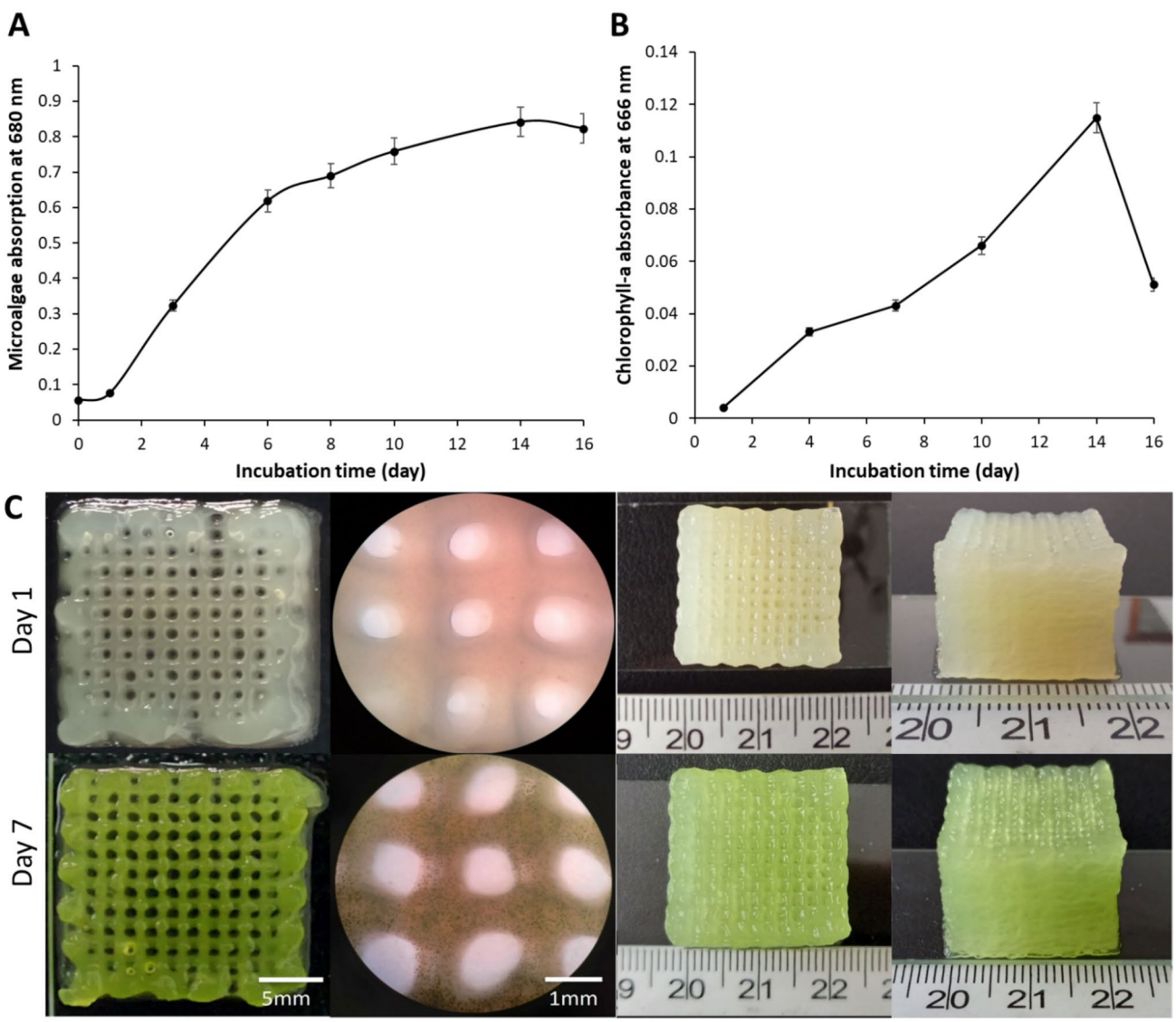



**Fig. 5** **A**-Growth curve of microalgae during 16 days of incubation, **B**-Absorption at 666 nm for the observation of chlorophyll-*a* change during 16 days of incubation **C**-Photographs and optical microscope images of 3D bioprinted tissue scaffolds at day 1 and 7

biofabrication processes in an effort to maintain continuous oxygenation and stimulate tissue regeneration (Gholipourmalekabadi et al. 2016; Agarwal et al. 2021). In order to solve this problem, we aimed to produce microalgae-laden scaffolds produced via 3D printing in this study.

In the microextrusion-based 3D bioprinting process used in this study, one of the main objectives was to extrude cell-loaded hydrogel solutions or bioinks layer by layer through a nozzle and thus create scaffolds with pre-designed porous structures. The created porous scaffolds provide an environment for improving the survival and functions of cells due to the interconnected pores that facilitate the transfer of nutrition and oxygen to the cells, as well as the removal of metabolic wastes of the cells within the scaffold (Malik et al. 2020). To produce such porous scaffolds, a high accuracy in the printing process and the prevention of deformation after printing are required. Visual evaluation of the shape of extruded lines and the quality of layer stacking, as well as measuring the deviation and degree of collapse of printed lines have a great importance (Malik et al. 2020). In this study, scaffolds with varying fillers and number of layers were

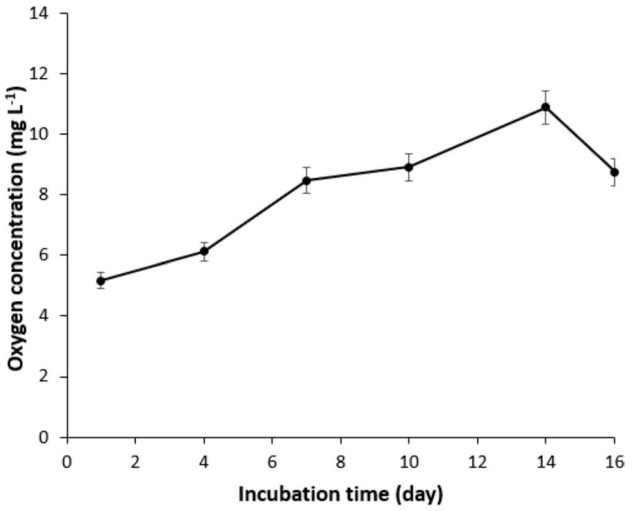



**Fig. 6** Dissolved oxygen values during 16 days of incubation. Measurements were conducted in triplicate on the samples. Data are mean ± standard error

printed and compared with each other for the printability test in accordance with Maharjan et al. (2021) and Cavallo et al. (2023). Col 10% hydrogel printing experiments were conducted using different types of filling such as honeycomb, triangle, and grid. In every form of infill, the printed lines were found extremely smooth and no structural degradation was seen and our results were similar with the literature (Maharjan et al. 2021).

The increase in the amount of oxygen was quite remarkable. Thanks to the ability of microalgae to produce oxygen at this level, their use in tissue scaffolding aims to eliminate the hypoxic effect in tissues. Hopfner et al. (2014) also evaluated oxygen levels and observed a high photosynthetic capacity of the scaffolds and oxygen releasement immediately after light stimulation, reaching peaks of ~ 14% of oxygen. Similarly, Liu et al. (2024) studied 3D polysaccharide-based hydrogel scaffold for microalgae farming without needing liquid media. They stated that the developed product significantly increased dissolved oxygen concentration of 10 mL water from 7.58 ± 0.10 to 22.76 ± 1.94 mg $L^{-1}$.

In summary, the present study has evaluated microalgae in scaffolds for tissue treatment, especially for the treatment of burnt tissues. Although, the use of microalgae-loaded collagen and alginate-based matrices will contribute to the industry in the field of health technologies, to improve the scaffold structure, future studies may be needed to optimize the incubation of microalgae-loaded wound dressings under different conditions. Additionally, more materials need to be researched to develop different bioink formulations. In this way, a potential alternative photosynthetic wound dressing can be produced against hypoxia encountered in wound healing.

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**Author contribution** D.B., B.İ., and D.Y. conceived and designed the experiments. B.İ. and D.Y. performed the experiments and analyzed the data. All authors read and approved the manuscript.

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**Data availability** Data supporting the findings of this study are available within the article. Raw data are available on request from the authors.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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