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Microfiber-flocked surfaces as a novel platform for biofilm and biohydrogen production

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Abstract

For biohydrogen production, immobilized systems offer the potential for improved growth and productivity. In this study, microfiber-flocked surfaces were used for the first time as a strategy to promote photofermentative biofilm formation and enhance biohydrogen production. The surfaces were developed using electrostatic flocking by attaching polyamide microfibers to glass and poly methyl methacrylate (PMMA) surfaces. Parameters such as fiber length, density, and adhesive type were optimized to develop shorter, denser, and vertically oriented microfiber structures, which improved bacterial attachment, light transmittance, and photosynthetic efficiency. For anaerobic cultures, inclined (vertical) photobioreactor provided superior attachment compared to horizontal setups, presumably due to better light exposure and nutrient availability. These enhanced surfaces led to increased cumulative hydrogen production over 21 days during repeated-batch operations with *Rhodobacter capsulatus* in a novel photobioreactor. This resulted in a stable biofilm (Abs_{590} : 0.65 ± 0.04) and a hydrogen productivity of 0.32 ± 0.05 mmol/L.h. Thick biofilms formed on the microfiber flocks, exhibiting interconnected extracellular matrices and nanowire-like structures. In contrast, horizontally oriented surfaces and aerobic conditions were less effective, as sub-optimal light and oxygen atmosphere levels appear to hinder biofilm growth and hydrogen output. Additionally, a combined ultrasonication and Tween surfactant treatment enabled efficient cell removal and biomass recovery, suggesting reusability of the engineered surfaces. These results highlight microfiber-flocked surfaces as a potential platform for enhanced microbial growth and biohydrogen production. The proposed strategy contributes to the development of biofilm-based continuous processes.

Keywords: biofilm photobioreactor, microfiber, electrostatic flocking, *Rhodobacter capsulatus*, biohydrogen

1. Introduction

The world's rising energy demand is fueled by factors such as population increase, the fast industrialization of developing countries, and the high energy consumption of developed nations. The reliance on fossil fuels adversely affects the environment and contributes to climate change. Thus, more sustainable, alternative energy sources are sought to replace fossil fuels in the long term (IEA, 2025).

One alternative energy source that is clean, sustainable, and has the potential to supply energy in the future is hydrogen. Hydrogen can be produced using several widely adopted methods, with steam reforming of methane, coal gasification, and water electrolysis as the primary options for industrial-scale production (Sagir and Alipour 2021). Unlike conventional hydrogen production, which relies on fossil fuels and significant energy inputs, biohydrogen is an attractive alternative for reducing greenhouse gas emissions as it can be produced renewably and sustainably using biological mechanisms, such as those involving photosynthetic bacteria (Fan et al. 2025; Xie et al. 2025).

Purple non-sulfur bacteria (PNSB) are photosynthetic bacteria capable of biological hydrogen production (Sagir et al. 2017). When light and organic compounds are present, PNSB can grow as photoheterotrophs. PNSB can utilize organic acids produced during anaerobic digestion for the conversion to H_2 and CO_2 when organic waste is used as a substrate in H_2 generation (Reungsang et al. 2018). PNSB mostly powers a biological process called photofermentation in which molecular hydrogen is produced in an anaerobic setting in the presence of light and limited nitrogen, using organic substrates as electron donors. Photofermentation is considered an attractive method for utilizing solar energy and other carbon sources over a wide variety of light spectra since it operates under conditions of mild pressure (~ 1 atm) and temperature (25-35 °C), and light with a wide range of wavelengths (350–1000 nm) (Koku et al. 2002; Li et al. 2024). However, low productivity and yields, light availability and nutrient limitations restrict its use. To achieve economic viability, the procedure should be enhanced, particularly in terms of hydrogen productivity (Genc and Koku 2024).

Biomass is a valuable renewable substrate that is derived from industrial and agricultural waste and provides a variety of carbon and nitrogen sources to aid in the production of biohydrogen (Sagir and Alipour 2021). However, in PNSB suspension cultures, low cellular biomass concentration may limit bacteria for photofermentation because bacteria can be

continually washed out of the reactor with effluents, leading to inefficient hydrogen production (Reungsang et al. 2018).

Cell immobilization, which is the process by which bacteria adhere to the surface of a particular support material or matrix to become immobile, can aid in overcoming limitations on biomass concentration in suspension cultures in photofermentative hydrogen generation. Cell immobilization can be categorized into natural and artificial methods to confine living cells to a smaller space and isolate them from their surroundings (Sagir and Alipour 2021; Sagir et al. 2018). For biohydrogen production, cell immobilization is highly effective because biofilm-based immobilization on surfaces including porous glass, activated carbon, and optical fibers greatly improves biomass retention and stability (Tsygankov and Kosourov 2014). By attaching themselves to surfaces or one another, certain microbes can create a unique structure known as a biofilm (Sagir and Alipour 2021). Inspired by natural mechanisms such as biofilm formation, electrostatic flocking offers an engineered approach to create surfaces that promote particle attachment and stability. This method allows for the application of chopped fibers or desired natural or synthetic particles to the surface in the presence of an electric field and an adhesive (McCarthy et al. 2021). Large surfaces can be quickly and affordably covered with electrostatic flocking. Unlike other surface treatment methods such as knitting, weaving, and electrospinning, it provides the ability to change the orientation of the flocked fibers perpendicular to the surface, allowing three-dimensional patterns to be created (Tonndorf et al. 2018). Nonetheless, although biomedical applications have been explored over the years, the application of electrostatic flocking in microbial processes is rare in the literature (Ren et al. 2024; Lu et al. 2024).

The unique microenvironment created by electrostatically flocked surfaces is hypothesized herein to facilitate initial *Rhodobacter capsulatus* attachment, thereby promoting the development of a robust biofilm. In the context of microbial immobilization, biofilm development is crucial. Structured populations of bacteria known as biofilms are enclosed in an extracellular matrix that they produce, which in turn promotes their adhesion to surfaces. Environmental conditions, surface features, and nutrient availability are some variables that affect the ability of microorganisms to produce biofilms (Alotaibi and Bukhari 2021). Increased surface roughness enhances surface area, promoting microbial attachment and biofilm development, which, in this case, may occur on flocked surfaces (Zheng et al. 2021).

Research on capacity of *R. capsulatus* to form biofilms is scarce. The purpose of this work is to illustrate the use of electrostatic flocking, a method that is also understudied in the present literature, to immobilize *R. capsulatus*, a type of PNSB, on polyamide microfiber surfaces. Flock synthesis procedures are adjusted to tune individual characteristics of the microfiber surfaces such as flock area-density and vertical orientation. We also demonstrate the synthesis of biohydrogen in a biofilm photobioreactor (BPBR) developed with these microfiber flocked surfaces. Different growth conditions (aerobic vs anaerobic) were investigated to reveal the growth of bacteria, adherence and formation of biofilm. In addition, different transparent support materials (glass and PMMA) for microfiber flocks were investigated to research their potential use in further scale-up.

We also highlight morphological features of the biofilm structure for *R. capsulatus*, including nano-wire-like structures. This is, to our knowledge, the first study to investigate this process in *R. capsulatus*. Microfiber flock preparation, characterizations, cell harvesting and microscopic analyses are given in the following materials and methods section.

2. Materials and Methods

2.1 Microfiber flocked surface preparation process

An electrostatic power flock machine (Electrosis) was used for microfiber flocking on glass supports. The flocks were spread to the grounded bottom plate and flocked through a grid that features a uniform array of square openings ($14 \times 14 \text{ mm}^2$). The grid was kept at 35 kV positive voltage throughout the whole study. The flocks that align as vertically as the grid imposed would then shoot toward the top plate which is also grounded. The distances between the bottom plate and the grid, and the grid and top plate were fixed at 9 and 12 cm, respectively, based on our previous observations on similar flocks used in similar setups (San 2024).

$1 \times 1 \text{ cm}^2$ square glass surfaces were used to optimize the flocking process and the proceeding UV transmittance studies. $1.25 \times 7.0 \text{ cm}^2$ glass surfaces were used as the flocked surfaces during the growth medium studies. The adhesive-coated glass surfaces were attached to the top plate by two-sided scotch tape. If the desired surface area of the glass surfaces was less than the top plate area, then uncoated glass surfaces were placed beside the coated ones. This was for homogenizing the electric field and thereby keeping the flocks from targeting the bare top plate surfaces.

Two types of adhesives were tested for their performance in flocking and culture studies. 500 μm thick acrylic adhesive films were drawn with a film coater on $1 \times 1 \text{ cm}^2$ glass substrates whereas, 100 μm thick epoxy (Metkon Epocold-R) films were drawn on $1.25 \times 7.0 \text{ cm}^2$ glass.

The microbial system that will form between the bacteria and the support was expected to be a function of the three-dimensional structure formed by the flocks on the glass substrates. The density of the flocks on the surface (i.e., flocks per unit area) and the angles between the flocks and the surface, or the so-called perpendicularity of the fibers, would be the two essential descriptors of this three-dimensional structure. Both were quantified using ImageJ (Schneider et al. 2022). Flocks positioned between 75° and 90° on the glass surface were considered as “vertical” placements.

Polyamide 6,6 (PA 6,6) flocks were supplied by Flokcan Tekstil. Beyond the intrinsic properties of the microfiber flocks, the ultimate structure formed by the flock fibers sticking to the surface will affect how the reactor operates. Several key parameters to consider include the fibers' diameters, lengths, and surface properties. To keep these variables to a minimum, 0.9 dtex (i.e., 10 μm in diameter) flocks, coated with the company's proprietary chemical formulation, were used as received. Nevertheless, flocks with two different lengths, either 0.4 mm or 0.8 mm, were used in this study.

2.2 Microorganism and Media

Rhodobacter capsulatus YO3, a deletion mutant of an uptake hydrogenase (hup-), was used for immobilization and H_2 production (Ozturk et al. 2006). The bacterial culture was cultivated in a modified version of basal medium that contained Acetate (20 mM) and Glutamate (10 mM) (20/10, A/G), as nitrogen and carbon sources, respectively (Biebl and Pfennig 1981). The hydrogen production medium consists of the basal medium containing Acetate (30 mM) and Glutamate (2 mM) (30/2, A/G). The streak plate method was used to isolate colonies and check for contamination after frozen microbes were defrosted and injected onto Mineral-peptone yeast extract (MPYE) agar plates in a laminar flow cabinet for initial activation. The plates were incubated in the dark at 30°C for several days. Following initial activation on MPYE agar plates, the cultures were transferred to 15 mL growth medium with a 10% (v/v) inoculum. For 3-4 days, the cultures were continuously illuminated by a tungsten lamp system at 2500 lux and maintained at 30°C . The cultures were then scaled up to 50 mL of growth medium using the same inoculation ratio. They were then incubated again for 3-4 days (Abs_{660} : 1.5) at 30°C with the same illumination. Finally, reactors for further experiments were then inoculated with

grown cultures (Abs_{660} : 1.5-2.0) with an initial pH of 6.4 under sterile conditions. A Shimadzu UV-1800 UV Spectrophotometer at 660 nm wavelength was used to measure the concentration of bacteria to observe the growth amount.

2.3 Photobioreactors and experimental setup

Borosilicate glass Roux Bottle reactors have been used as photobioreactors (PBR) with approximate outer dimensions of $8.5 \times 4 \times 19.5 \text{ cm}^3$ and a total volume of 350 mL. Five support glasses were placed in the PBRs, sterilized with 10% H_2O_2 , and subsequently rinsed in situ within the photobioreactors using the basal medium. The attachment area of the bacteria is assumed to be the same area as the electrostatically flocculated glass slides with dimensions of $1.25 \times 7.0 \text{ cm}^2$. Fig. 1a shows the experimental setup without bacteria. For each batch round, the reactor was filled to a maximum volume of 350 mL with a 10% v/v inoculation ratio. The illumination rate was kept constant at around 2500 lux at 30 °C. The produced gas was collected via 100 mL syringes, and Fig. 1b shows the operating setup. Fig. 1c and Fig. 1d illustrate the structural characteristics of the PBR setup and glass support slides for attachment.

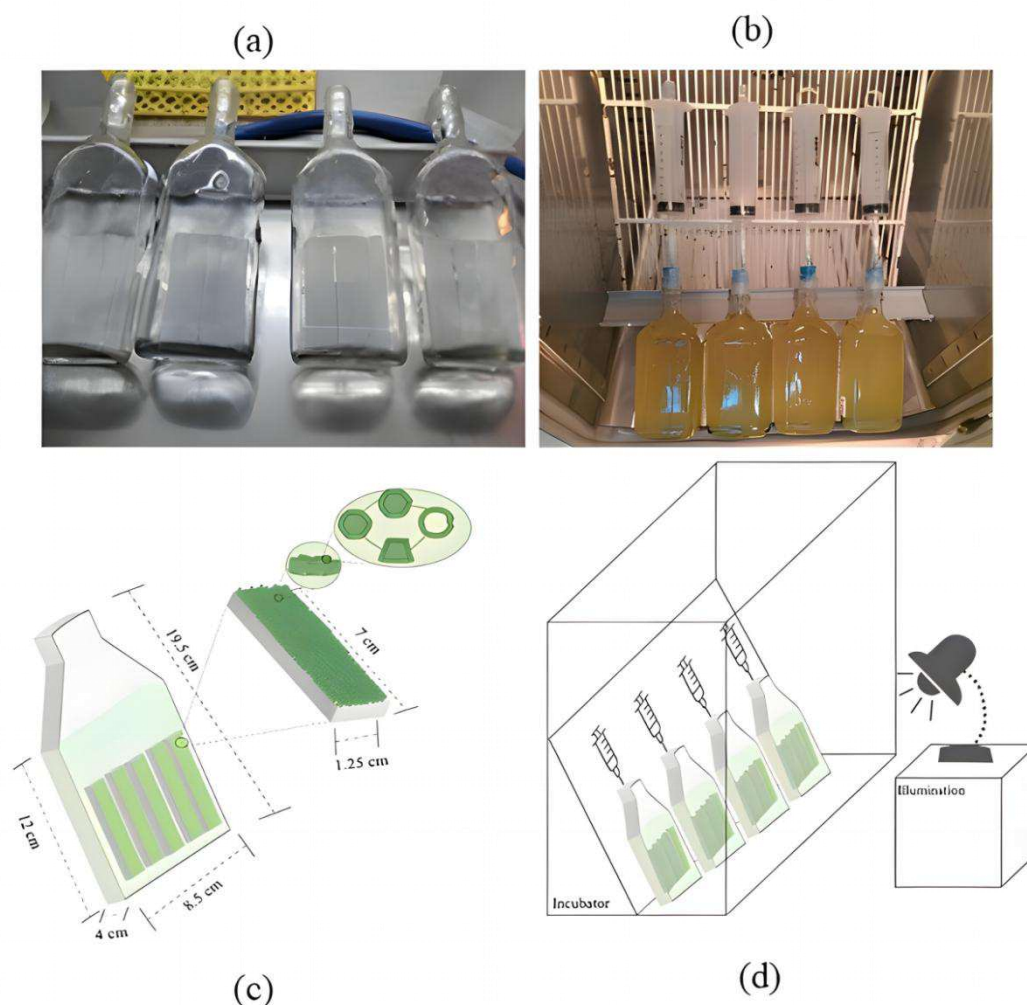


Fig. 1 Experimental setup for biofilm formation and hydrogen production. Photobioreactors (a) before operation and (b) during operation, showing biofilm development. (c) Schematic and dimensions of the microfiber-flocked surfaces used in the photobioreactors. (d) Photobioreactors placed in an incubator under continuous illumination.

2.4 Attachment of bacteria to microfiber flocks

The grown bacteria were inoculated with a 10% v/v ratio into a 350 mL glass Roux flask that contained microfiber flock slides placed facing light. After filling the rest of the working culture volume with the growth medium, the reactors were sealed and flushed with argon for 5 minutes to ensure an anaerobic environment.

The growth and biofilm formation phases were expected to be carried out throughout the first round using 20/10 A/G growth media. A tungsten lamp source that produced approximately 2500 lux of light continuously illuminated the reactors at a 30 °C incubation temperature.

Once the biofilm formation phase was achieved according to phenotypic and microscopic analyses (Round 1), cells in suspension were discarded and fresh hydrogen production medium with 30/2 A/G was added into the photobioreactor for the subsequent batches (Round 2-3) after washing out the unattached bacteria with basal medium. The rest of the process during the experiment remained the same. After being filled with production medium, all reactors were sealed and flushed with argon for 3-5 minutes to ensure an anaerobic environment. Suspension cultures were also run as the control group for the biofilm reactors under the same conditions listed above.

2.5 Analytical methods for microbial, biogas, and surface characterization

The bacteria on the microfiber flocks were examined utilizing a Tecan Vega 3 scanning electron microscope (SEM). The flock slide samples were coated with Au/Pd before the SEM analyses. The bacterial formations around the flock fibers were also investigated using an optical microscope (Motic BA310-Met). SEM images were analyzed by examining at least two flocks from each reactor and at least three distinct regions (two edges and center) within each flock. Syringes (100 mL) were used to continuously measure and record the amount of gas that evolved during the experiment. The produced gas composition was assessed using gas chromatography. The bacterial growth was measured with a Shimadzu UV-1800 spectrophotometer set at 660 nm. To analyze the composition of evolved biogas, a Supelco Carboxen 1010 column (150 x 4.1 mm²) and a Thermal Conductivity Detector were installed

on an Agilent 6890N gas chromatograph. The carrier gas was argon, and the flow rate was 25 mL/min. On average, 90% of the evolved gas was hydrogen.

For analyzing organic acids, 2 mL samples were withdrawn from the PBRs once every 48 hours. A pH meter was used to adjust pH (Mettler Toledo 3311). The Alltech I0A-1000 ion-exchange column was used to measure the samples' organic acid content using High-Performance Liquid Chromatography (Shimadzu 20A series) and a UV detector (Shimadzu FCV-10AT). A mobile phase of 0.085 M H₂SO₄ was used, and the oven was adjusted to 35 °C with a flow rate of 0.6 mL/min.

XPS General Screening Analysis of commercial PA6,6 flock analysis was carried out in METU Central Laboratory in Ankara. A PHI 5000 Versa Probe equipped with a monochromatic Al source was used for the XPS overall scan, spanning 0-1486 eV.

2.6 Live and dead cell fluorescence microscopy

A Confocal Laser Scanning Microscope (CLSM) (Leica SP8) was used to observe distribution and location of live/dead cells bacteria biofilm on microfibers at the National Nanotechnology Research Center (UNAM) in Ankara. Acridine orange (AO) and propidium iodide stains were used for live and dead cell monitoring. Acridine orange (AO) staining solution at a concentration of 10 µg/mL and propidium iodide (PI) staining solution at 20 µg/mL were prepared. A 50 µL of the cell sample was transferred into a tube, and then 50 µL of the premixed AO/PI solution was added. The mixture was gently pipetted to ensure thorough mixing and incubated for 20 minutes at room temperature. After incubation, the stained suspension was gently mixed again, and 15 µL of the sample was loaded onto a glass slide for air-dried fixation and excess stain was washed. For microfiber-flocked samples, 20 µL of a mixed AO/PI solution was applied to a 1 × 1 cm² surface sample. The samples were then washed with basal medium to remove excess dye prior to CLSM analysis.

Fluorescence intensity was estimated by calculating grey values per unit area using Fiji (ImageJ) program. The fluorescently stained different regions of the microfibers were selected and analyzed. Red and green fluorescence intensities were compared at every labeled area to evaluate the overall cell viability.

2.7 Cell harvesting from microfiber-flocked biofilm

A previously refined cell collection approach was used to quantify *R. capsulatus* density (unpublished data). Each flock of microfibers was placed in conical centrifugation tubes

containing 10 mL of 1% (v/v) Tween and maintained for 1 h in this medium. The flocks were positioned vertically in the tube, surface down, to promote cell release. This was followed by 1 h of ultrasonication (Sonorex Bandelin) at 35 kHz to promote further detachment of cells. The resulting pellet was centrifuged (Sigma, 4K15) at 10000 rpm for 10 minutes and following a basal medium wash, was dissolved in 1 mL of basal medium. After that, the cells in the pellet were put into Eppendorf tubes that had been previously weighed and centrifuged for an additional five minutes at 10000 rpm. Following the removal of the supernatant, the leftover pellet was weighed after being in an oven set to 105 °C for a day.

2.8 Biofilm quantification assay

The modified spectrophotometric Crystal violet method was used to measure the biofilm intensity (Kamimura et al. 2022). For the biofilm analysis, flocks with biofilm collected at the end of the rounds were used. Each flock with biofilm received 125 µL of crystal violet diluted with 0.1% distilled water, and they were left to wait for five minutes. Basal medium was used to wash away excess colors that had collected on the flocks' glass surfaces. New falcon tubes were used to hold the samples. The tubes were filled with 4 mL of acetic acid that had been diluted with 50% distilled water. For 15 minutes, it was maintained horizontally with the flocked surface down. After extracting a 1.5 ml sample from the liquid portion, it was diluted with 20% distilled water for measurement. Then, spectrophotometric measurement was performed at 590 nm.

2.9 Data Analysis and Calculations

The consumption rates of acetic acid in photobioreactors were calculated using first order reaction kinetics (Eq. 1). In this context, $[A_0]$ (mM) represents the initial acetic acid concentration, $[A]$ is the consumed substrate at specific time (t: h), and k is the first order rate constant (h^{-1}).

$$[A] = [A]_0 \exp (-k \cdot t) \quad (1)$$

The calculation of hydrogen productivity (mmol/L.h) involved dividing the amount of hydrogen produced (mmol) by the volume (L) of culture placed in the photobioreactor and the duration of illumination (h) (Eq. 2).

$$Q_{H_2} = n_{H_2} / (V_C \cdot t) \quad (2)$$

The experimental groups were analyzed in duplicate/triplicate, and the results are presented as mean $\pm$ standard deviation (SD). Differences between groups were analyzed statistically

using one-way or two-way ANOVA, followed by Tukey's test (Minitab Software). Data with $p < 0.05$ was regarded as significant.

3. Results and Discussion

3.1 Optimization of microfiber flock surfaces

PA6.6 flocked surfaces were produced using 400 and 800 μm fibers. Their vertical alignment and areal density were characterized to optimize light transmittance for PNSB growth and hydrogen production while maintaining sufficient density for biofilm formation.

Flocking parameters such as charging time, plate-grid distance, and initial fiber mass were varied to tune surface properties. At constant voltage and plate-grid distance, charging time controlled areal density and vertical orientation (Table S1). Statistical analysis confirmed that fiber length and charging time significantly influenced areal density ($p < 0.05$).

Surfaces coated with 800 μm PA6.6 flocks for 10 s showed poor vertical alignment (75-90°), whereas other conditions achieved up to $\sim 40\%$ vertical orientation. Reduced alignment at longer flock lengths and charging times is likely due to electrostatic repulsion from pre-deposited fibers (Ji et al. 2018). Using shorter charging times had a positive effect on the verticality of fibers regardless of their length

The amount of microfiber flock on the bottom plate influenced the areal density of the coatings. Using a 1-second charging time as the baseline, flock quantities were adjusted to 100, 50, and 10 mg while keeping all other parameters constant. To assess the effect of flight distance, the grid-top plate spacing was also increased from 12 cm to 24 cm. The corresponding trials were designated in Table S2. SEM micrographs of the side views of the samples are given in Fig. S1.

Microscopy was used to evaluate flock coatings, with areal density measured from uncoated-side images and vertical alignment assessed from top-view coated images using ImageJ. Lower flock amounts (< 50 mg) resulted in poor surface coverage. Vertical alignment was estimated by counting dark spots corresponding to upright fibers. Both areal density and vertical alignment increased as more flocks were applied. Statistical analysis (ANOVA) showed that applied weight and fiber length significantly influenced both properties ($p < 0.05$).

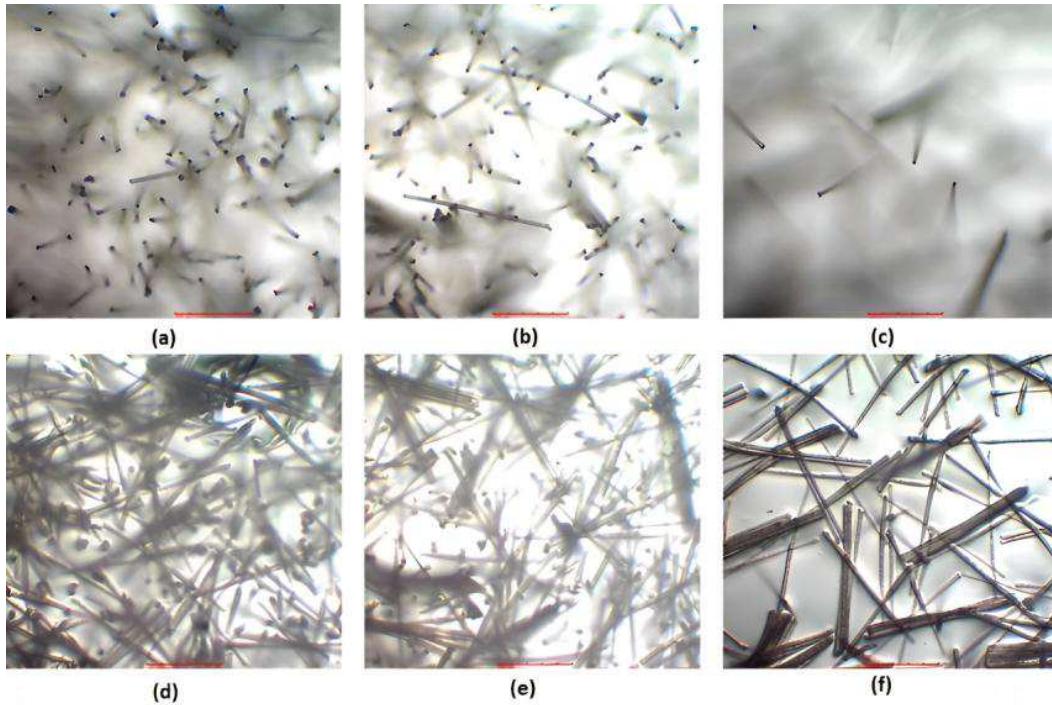


Fig. 2 Optical microscope images of a, d: PA66_100_12, b, e: PA66_50_12, c, f: PA66_10_12. a, b, c: images from the top; d, e, f: images from the bottom. Scale bars represent 200 μm . PA66: Polyamide microfiber. PA66_100_12 corresponds to the glass slides produced by flocking 400 μm long, 100 mg and 12 cm grid-plate distance.

Light transmittance and absorbance of microfiber surfaces and *R. capsulatus* were measured by UV-Vis (Fig. 3). Transmittance increased as fiber density decreased. Samples with 100 mg flocks and a 24 cm gap transmitted $\sim 20\%$ more light than at 12 cm. Considering verticality, density, and transmittance, 100 mg and 12 cm (153 ± 20 fibers/ mm^2) were optimal. A denser layer ($\sim 70^\circ$ fiber angle) improves light use and surface area, enhancing growth and hydrogen production.

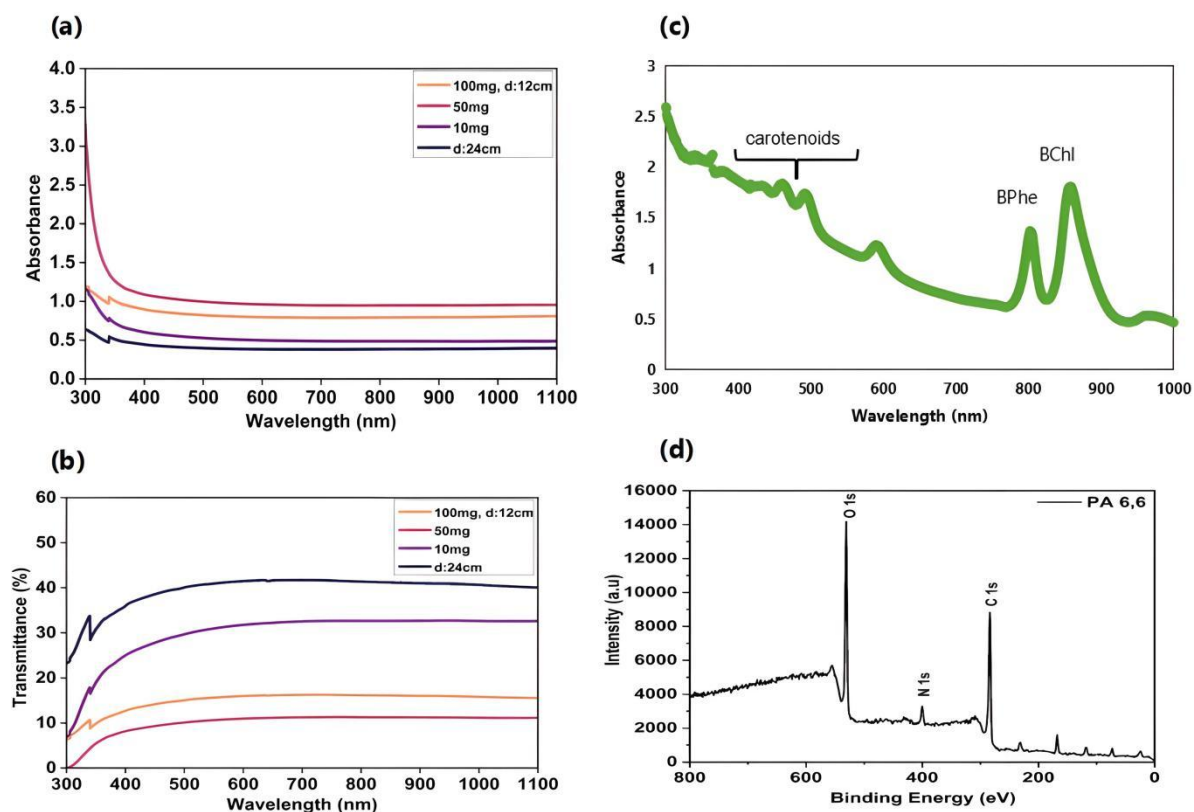


Fig. 3 a) Absorbance, b) % transmission (%T) of spectroscopic values of samples at various amounts (10, 50 and 100 mg) and distances (12 and 24 cm) of flocks. c) *R. capsulatus* spectrum indicating wavelengths of carotenoids, bacteriochlorophyll (BChl) and bacteriopheophytin (BPhe). d) XPS General Screening Analysis of PA6,6 flock.

In addition to carbon (C 1s: 284 eV), nitrogen (N 1s: 400 eV), and oxygen (O 1s: 530 eV) due to the organic matrix, sulfur (S 2s: 231 eV & S 2p: 168 eV) and aluminum (Al 2s: 118 eV & Al 2p: 73 eV) were also detected in XPS analysis, presumably arising from the electrostatic modifier applied by the supplier. The spectrum verifies the chemical integrity and purity of the PA6,6 surface used for flocking (Fig. 3d, Table S3).

3.2 Effects of flock length and adhesive type on bacterial attachment

The synthesized flocks exposed to the basal and growth media were examined by SEM imaging (Fig. S2). The images show that the flocks retain their morphology within these media, with slight precipitation of the salts observed on the surfaces. The data obtained during the growth period demonstrates that short flocks (400 μm) have superior bacterial adherence compared to long flocks (800 μm). In short flocks, the propensity of bacteria to colonize and build biofilms through secretions is evident, in contrast to long flocks, where bacteria are

observed as structures generated by solitary or small populations of bacteria (Fig. S2). On surfaces with short flocks, bacteria were observed to develop network-like structures; however, on surfaces with longer flocks, this tendency was found to be less. These results showed that shorter flocks are more promising, presumably because light penetration plays a significant role, which UV analyses verified to be greater on low-density surfaces. As a result, the studies that followed maintained short flocks with low density, requiring a 1-second flocking time.

After more than 7 days of operation, flock surfaces bonded with acrylic adhesive peeled and detached, likely due to adhesive swelling in water. Therefore, an epoxy-based adhesive was used in subsequent experiments and showed superior long-term durability. The epoxy was heat-cured at 50 °C to form a transparent surface. Control tests confirmed that the epoxy itself supported bacterial growth (Fig. S3), and no separation at the epoxy-glass or epoxy-flock interfaces was observed during hydrogen production. However, due to its low viscosity, thicker epoxy layers (250 and 500 µm) led most flocks to get embedded. Thus, an epoxy thickness of 100 µm was selected as the optimal.

3.3 Effect of photobioreactor configuration on hydrogen production performance and biofilm formation

Following Round 1 (R1), which lasted 7 days during which the bacteria were allowed to colonize and presumably form a biofilm within the flock layer (Fig. 4). Rounds 2 (R2) and 3 (R3) were carried out in the hydrogen production medium to measure the H₂ output of the BPBRs. Hydrogen production was observed within the 14 days of R2 and R3, resulting in a total operation period of 21 days including R1. pH ranged between 6.4 and 7.2 throughout the hydrogen production. The SEM images were obtained on the 7th day and 14th-21st days.

As seen from Fig. 4, the immobilized inclined (30°) design produced the most cumulative hydrogen in Round 2, reaching about 400 mL at the end, followed by the control setup consisting of purely suspension culture ($p < 0.05$). The horizontal photobioreactor configuration consistently produced the least, indicating that it is less efficient in this system for producing hydrogen, due to its inefficient light absorbance onto the flocks (Table 1). The immobilized horizontal reactor was the least productive in Round 3, while the vertical immobilized setup produced the most hydrogen, outperforming the others. This transition suggests that the suspension culture may eventually outperform the horizontal arrangement because of better light availability (Hallenbeck and Liu 2016).

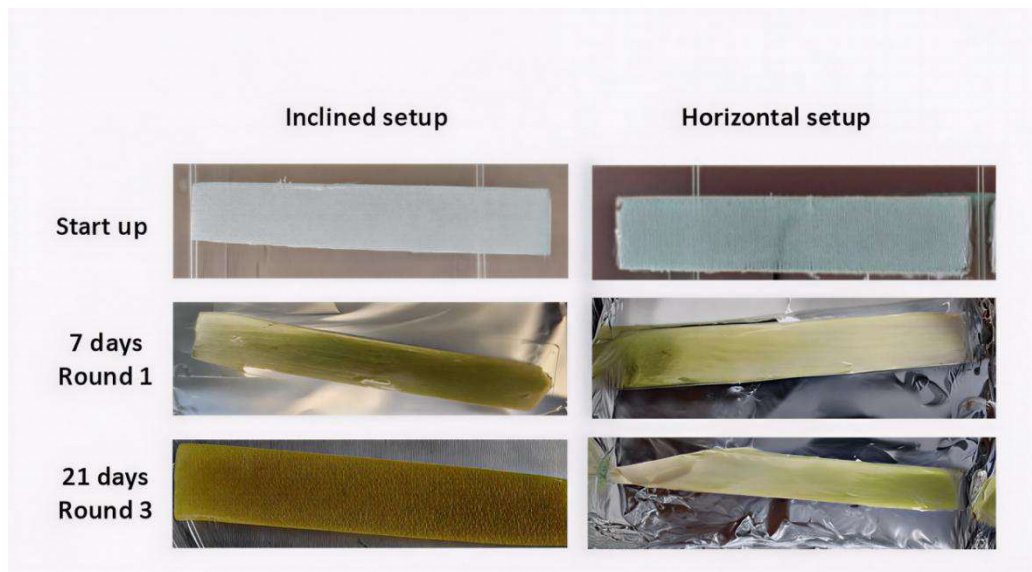


Fig.4 Growth of attached cells on microfiber flocked surfaces at inclined (left) and horizontal setup (right).

The immobilized culture setup maintained consistent productivity over time, whereas the suspension reactor in Round 2 had an initial peak in hydrogen productivity (0.62 ± 0.2 mmol/L/h) that rapidly decreased (Fig. 5b). All setups observed a decrease in productivity in Round 3, but biofilm system maintained a comparatively higher productivity, indicating that it is suitable for a stable hydrogen generation.

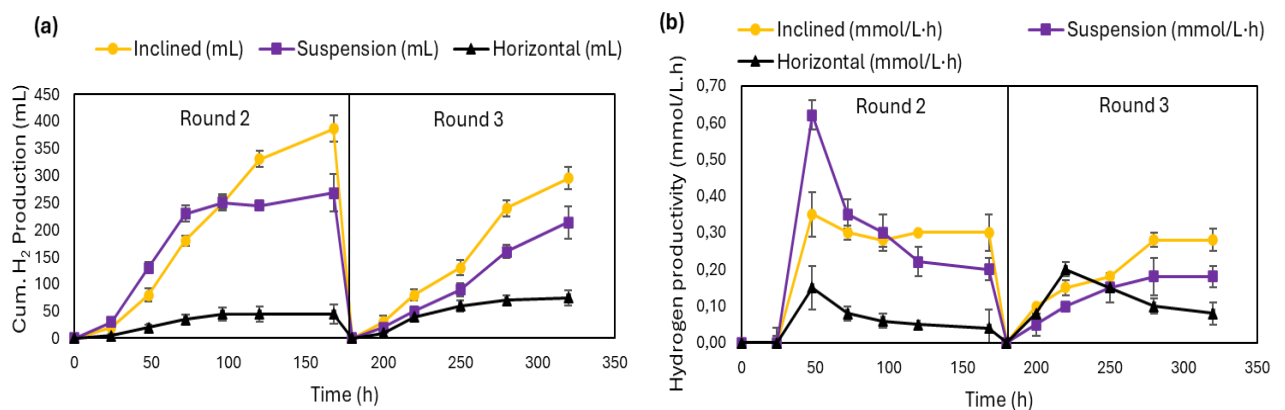


Fig. 5 Cumulative hydrogen gas production (a) and hydrogen productivity (b) of inclined BPBR, horizontal BPBR and suspension culture (Planktonic) of *R. capsulatus* in Round 2 (R2) and Round 3 (R3), 30/2 A/G. The results represent the mean values of two sets of replicates.

According to these findings, biofilm PBRs promote consistent hydrogen production, particularly in the early phases, which makes them suitable for potential use. The fact that the

horizontal design is consistently less successful highlights how crucial the placement of the supporting material is to obtain the illumination properly to maximize hydrogen generation. Fig.6a shows the SEM image of the BPBR inclined setup after the first 7 days, which indicates a dense and uniform biofilm structure with bacteria well-distributed across the surface. Cell clusters appear integrated with the support material, indicating strong adhesion and colonization. By the first 14 days, in Fig. 6b, the inclined setup exhibited a more developed and stable biofilm, with increased bacterial density and clustering. The biofilm assay showed that the inclined flock had an optical density of 0.65 ± 0.04 at 590 nm.

Table 1 Summary of the results of BPBR and suspension photobioreactors. The results represent the mean values $\pm$ SD.

Culture Setup	H ₂ Production (mL)	H ₂ Productivity (mmol/L/h)	Acetic acid Consumption (%)	Dry Cell Weight Suspension (mg)	Dry Cell Weight Flock ² (g/cm ²)	Biofilm assay (Abs:590 nm)
Inclined BPBR	386±24 (R2)	0.36±0.05 (R2)	97.1±2.2 (R1)	300.2±0.1	0.17±0.01	0.65±0.04
	295±20 (R3)	0.28±0.05 (R3)	98.5±1.5 (R3)	157.2±0.2	0.21±0.01	NA
	Mean	341±22	0.32±0.05	97.8 ±1.85	229±0.1	0.19±0.01
Suspension	268±35 (R2)	0.62±0.2 (R2)	98.0±1.3 (R1)	232.3±0.2	0.0021±0.01	-
PBR	214±30 (R3)	0.21±0.2 (R3)	97.2±0.6 (R3)	142.3±0.1	NA	-
Mean	241±32	0.42±0.2	97.6±0.9	187±0.1	0.0021±0.01	
Horizontal	44±18 (R2)	0.14±0.04 (R2)	78.5±1.2 (R1)	NA	0.10±0.01	0.53±0.03
BPBR	72±10 (R3)	0.22±0.04 (R3)	71.7±2.3 (R3)	121.0±0.4	0.14±0.01	NA
Mean	58±14	0.18±0.04	75.1±1.7	121.0±0.4	0.12±0.01	0.53±0.03

R1-R2-R3: Rounds 1,2,3. ¹NA: Not applicable. ²Dry cell weight of harvested cells from a flock.

This trend confirms the inclined arrangement as a potential sustainable configuration for hydrogen generation. On the other hand, horizontal immobilized culture after 7 days of incubation, Fig. 6c, presents a less dense bacterial attachment with minimal biofilm formation. Poor attachment and a non-uniform distribution were observed, which may be attributed to limited light penetration and/or biofilm structure (Fu et al. 2017). These observations agree with

the low productivity and accumulated hydrogen production observed in Fig. 5a and Fig. 5b, further reflecting its inefficiency. The optical density of the horizontal flock was 0.53 ± 0.03 at 590 nm as a result of the biofilm assay. The average dry cell weight of the harvested cells on the horizontal microfiber flock was 0.12 ± 0.01 g. For suspension, there was no flock used, so no SEM image was available.

Harvested microfiber surfaces were obtained and examined under SEM to confirm the detachment (Fig. S4-S5). As a result, inclined systems were utilized in the subsequent experiments after confirming that the immobilization of bacteria provides the greatest advantages for the performance of photobioreactors in the inclined system.

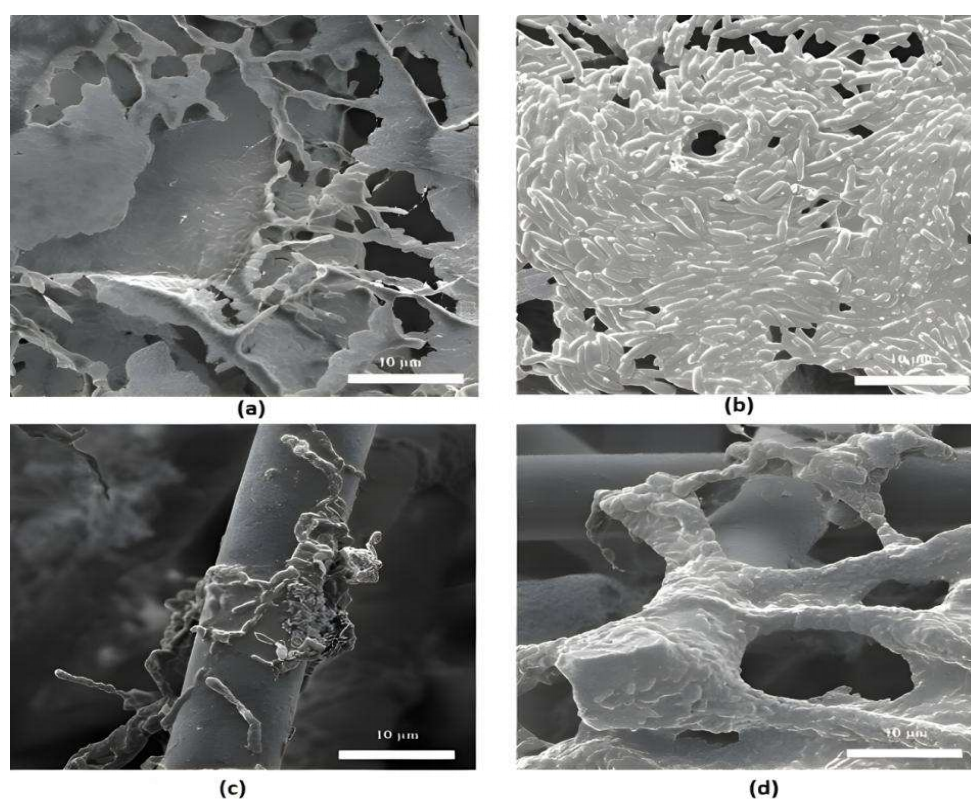


Fig. 6 SEM images of biofilm morphologies under different configurations: (a) inclined BPBR setup after first Round (R1, 20/10 A/G); (b) inclined BPBR setup after second Round (R2, 30/2 A/G); (c) horizontal BPBR after first Round (R1, 20/10 A/G) (d) horizontal BPBR after second Round (R2, 30/2 A/G).

Dry weight analysis has also been performed on the suspension during the experiment. The dry weight analysis of the inclined setup revealed a significantly high biomass production in Round 1. This is a crucial finding, as it indicates a remarkable 152% biomass rise (from 118.7

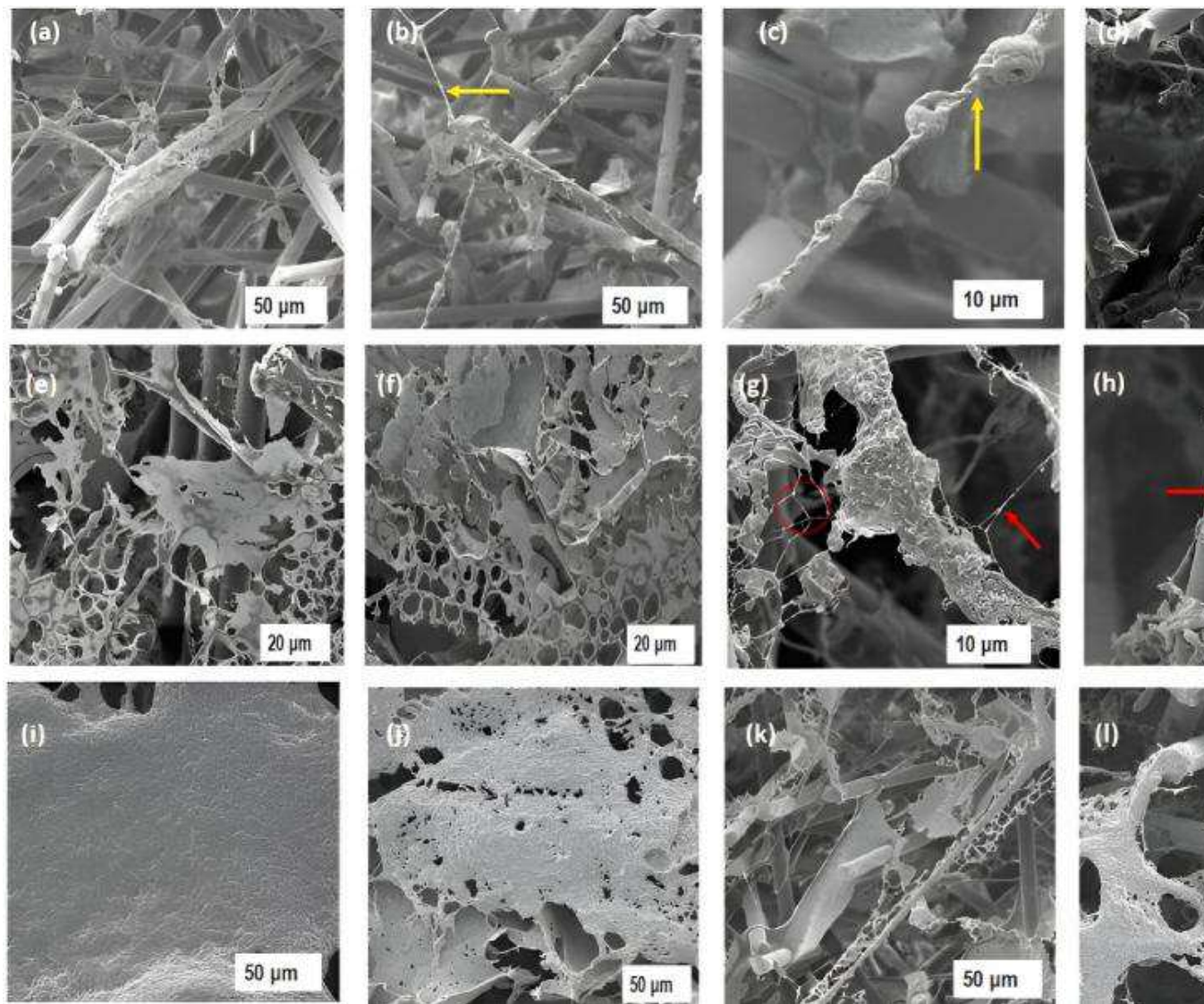
mg to 300.2 mg) on day seven, underscoring the robust biofilm development. In contrast, in subsequent rounds, biomass was 157.2 mg, which was lower than in the first round. This indicates higher cell attachment and biofilm development on the supporting material. The average dry weight in the inclined setup was $0.19 \pm 0.01 \text{ g cm}^{-2}$, whereas the horizontal configuration yielded $0.12 \pm 0.01 \text{ g cm}^{-2}$. In comparison, the cell dry weight in the suspension culture was very low, $0.0021 \pm 0.01 \text{ g cm}^{-2}$. Inclined reactors showed higher cell density, improving activity and efficiency. This confirms that reactor orientation affects biofilm formation and performance, while dry weight drops after medium renewal reflect biomass redistribution between attached and suspended cells.

It was noted that lower hydrogen productivity was obtained compared to earlier studies with the same bacteria using suspension and immobilized systems (Genc and Koku 2024; Sagir and Alipour 2021). However, microfiber-flocked surfaces present several benefits, including easier cell harvesting, the possibility of reusing the same flocked supporting material, and the capability to fine-tune the flocks for various scale applications.

3.4 Morphological and structural characteristics of *R. capsulatus* biofilms

R. capsulatus was observed to form biofilms on microfiber-flocked surfaces during the first 7 days of the process. SEM images (Fig. 7a-f) show the initial attachment of bacteria and the development of early extracellular structures. These structures may represent the initial stages of microbial immobilization and extracellular matrix formation. During this period, the bacterial cell length ranged between 1.5 and 2.5 μm , and biofilm thickness varied among the different flocks. Abiotic factors, including flock density, light intensity, and substrate availability, may influence their variability.

It was observed that the bacteria formed a biofilm across the polyamide microfibers during growth and hydrogen production, exhibiting web-like layers that extended to neighboring regions (Fig. 7e-f). Furthermore, biofilm strands were observed particularly at the top ends of the microfibers that virtually tied together (Fig. 7a-b). These have a striking similarity to the so-called ‘nanowires’, defined as conductive structures that connect intracellular electron transport chains to external electron acceptors, which may function as a crucial path of bioelectrochemical communication in microbial systems (Grattieri et al. 2021). It has also been suggested that such structures could be harnessed for environmental sensing, electricity harvesting, and neuromorphic memory systems at voltages relevant to biology (Guberman-Pfeffer et al. 2024).



458

459 **Fig. 7** SEM images of biofilms showing: (a–f) Biofilm formation during the first round of
 460 process (R1), (g–l) Biofilm maturation during the second round of process (R2), (j–k–l are
 461 from horizontal setup). Red arrows indicate nanowire-like structures. The red circle shows a
 462 network of nanowires (g). Yellow arrows indicate thicker nanowire-like structures.

463 The cone and spherical formations in Fig. 7d–f might indicate localized growth zones or
 464 regions where microbes preferentially attach to specific microfibers influenced by acetate
 465 availability or surface structure. These effects may also be attributed to the acetate-driven
 466 metabolic activity of *R. capsulatus*. Acetate has been shown to have a similarly significant
 467 function in other microbial extracellular electron transfers, as demonstrated in the work on
 468 *Shewanella oneidensis* (Zhang et al. 2021). Although metabolically distinct, *R. capsulatus* and
 469 *S. oneidensis* share the fundamental function of acetate as a substrate and electron donor, which

has been crucial for biofilm formation and electron transport. It may be possible to relate biofilm development to extracellular electron transfer in these species (Liu et al. 2025).

The SEM images for the second round (Fig. 7g-l), however, reveal a more developed biofilm structure, likely due to biofilm maturation following increased carbon substrate availability. The images for this later phase suggest that the wire-like or web-like features evolved into more defined structures, presumably supporting the overall biofilm network. As for Figs. 7j, k, and l, since they belong to the horizontal setup's first round, as mentioned previously, exhibit less adhesion and biofilm formation on the surface, likely due to insufficient illumination in the horizontal orientation. Unlike the inclined setup configuration, horizontal setups exhibit fewer extracellular networks or nanowire-like structures. Moreover, as can be seen from Fig. 7l, the nanowire-like structure exhibits a notable breakout/linkage of nanowire-like structures from microbial clusters. Though less dense than inclined setups, these structures in horizontal setups may represent an adaptive response to the limited light availability, allowing for localized electron transfer. These results are also consistent with lower metabolic activity in these conditions. Further studies are required to reveal the details of the structure.

3.5 Distribution of live and dead cells in biofilms

Live/dead cell fluorescent images of bacteria were obtained at the end of Round 2 (Fig. 8), to verify that the biofilm was indeed an aggregate of mostly viable cells. The cells observed in red are dead bacteria, corresponding to the sum of non-intact, membrane-disrupted and scattered cells, while the live cells were observed as green. It was observed that bacteria attached mostly on polyamide microfibers and the extracellular matrix in biofilm. The cells were mostly alive especially on the edges of microfibers.

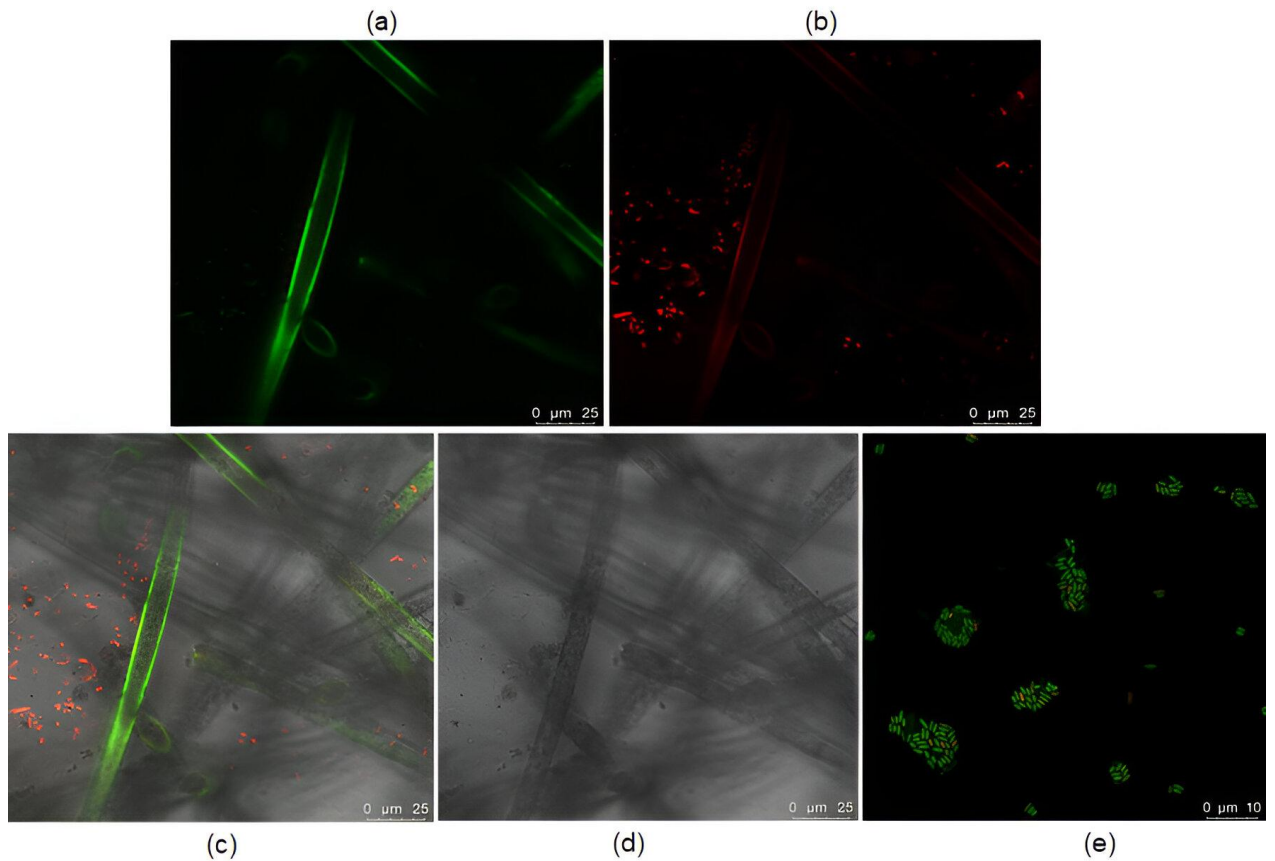


Fig. 8 Confocal Laser Scanning Microscopy images of *R. capsulatus* biofilm on microfibers (a-d). (a), Acridine Orange (AO), (b) Propidium Iodide (PI), (c) Merged (AO and PI), (d) Bright field stained images. (e) Merged (AO and PI) stained images of *R. capsulatus* in suspension culture. AO fluoresce in green and PI exhibits red fluorescence.

Higher live cell densities were obtained on the fiber surfaces of labels 1, 2 and 3 (Fig. 9). The highest live cell density ($>3 \times 10^5$) was obtained on the microfiber (label 2). In contrast, the highest dead cell fluorescence intensity was observed around the biofilm matrix (label 8). The dead cells were observed in red as non intact, membrane-disrupted and scattered. Overall, these results confirm that the biofilm structure consisted mainly of viable bacterial cells attached to polyamide microfibers, surrounded by an extracellular matrix containing a smaller population of dead or lysed cells. The spatial distribution of live and dead cells reveals the heterogeneous nature of the biofilm, showing active growth at the periphery and predominant cell death in the interior, which aligns with typical biofilm physiology.

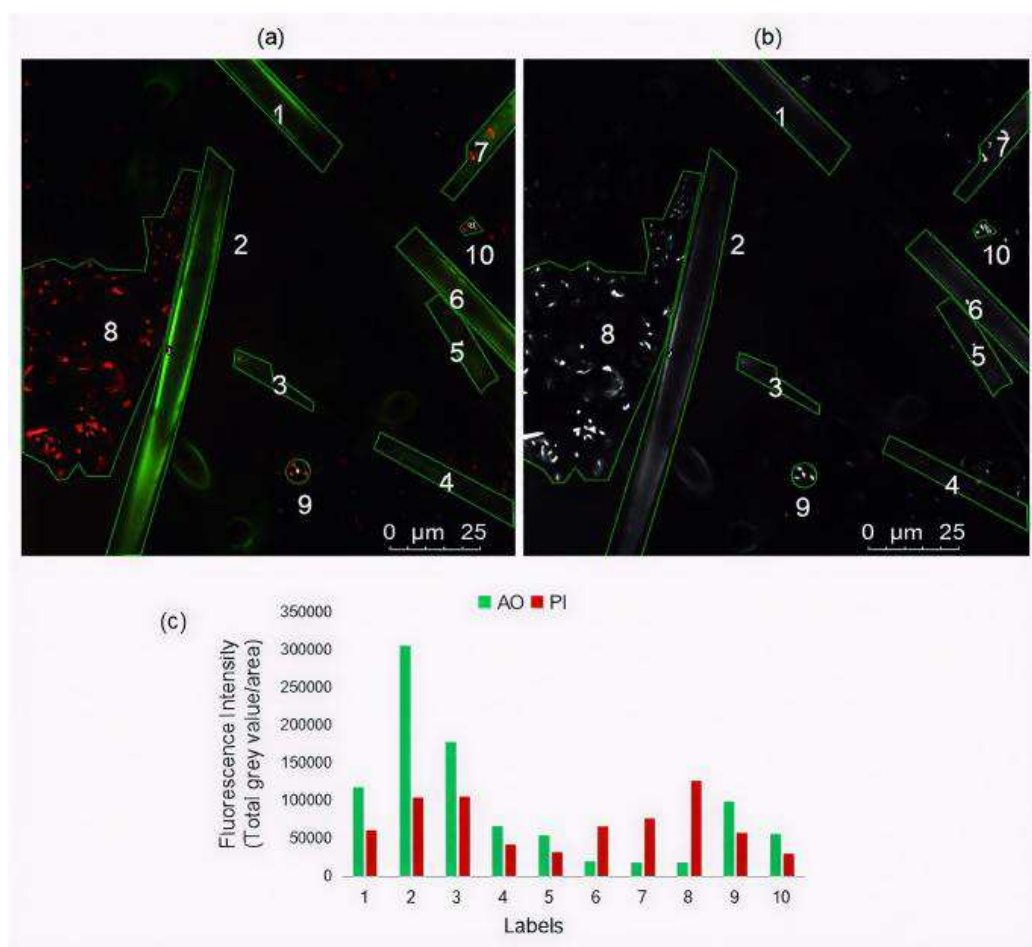


Fig. 9 Live/dead cell distribution of *R. capsulatus* biofilm on microfiber flocks. Confocal Laser Scanning Microscopy images of merged AO-PI (a) and corresponding grey (b) values analyzed. Labels 1-10 are spots analyzed for red and green fluorescence intensity.

3.6 Substrate utilization kinetics in biofilm photobioreactors

In the inclined BPBR configuration, the acetic acid was nearly completely consumed, with 97.1% removal in Round 1 and 98.5% in Round 3 (Table 1). The horizontal configuration showed lower consumption (78.5% and 71.7%), likely due to reduced light availability within the biofilm under horizontal positioning.

First-order consumption kinetics fit well with the concentration data, yielding R^2 values between 0.90 and 0.98 in all the fits. For these fits, immobilized inclined and suspension reactors yielded similar consumption rate constants with 0.028 h^{-1} and 0.030 h^{-1} , respectively (Fig. 10). This means that the transport of acetic acid to the cells on microfibers occurs at a similar rate. Although there is no evidence of significant nutrient mass-transfer limitations for the immobilized cells, it can be noted that the biofilm likely contains a higher cell density. As a

result, the specific nutrient consumption rate may be lower even if the overall consumption rate remains the same. The consumption rate constant of the immobilized inclined setup increased to 0.033 h^{-1} when the initial concentration was increased to 30 mM . However, the horizontal reactor has much slower constant rates ($0.004\text{-}0.007 \text{ h}^{-1}$), meaning substrate persists longer in the reactor.

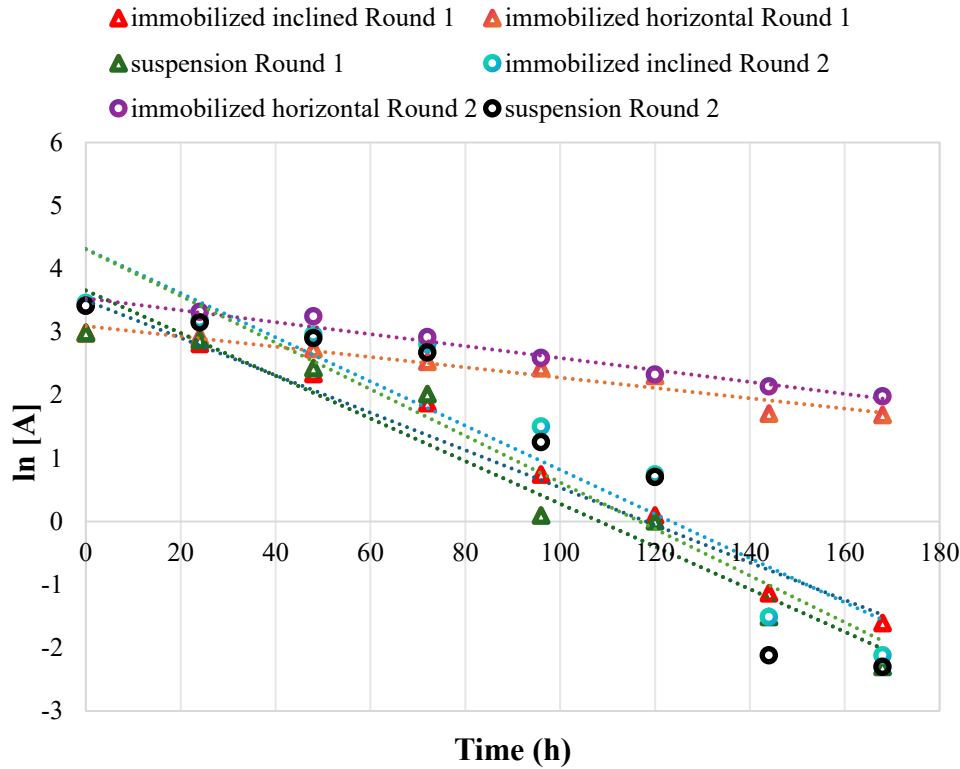


Fig. 10 Acetic acid consumption kinetics of inclined BPBR, horizontal BPBR and suspension PBR in Round 1 (C_0 : 20 mM acetic acid) and Round 2 (C_0 : 30 mM acetic acid).

3.7 Assessment of Glass and PMMA microfiber flocks under aerobic and anaerobic conditions

The feasibility of microfiber flocks were evaluated under different growth modes and support materials to assess its potential for aerobic and anaerobic applications. It was noted that the availability of oxygen (aerobic conditions) significantly affected biofilm characteristics in bioreactors. While both glass and PMMA are transparent and suitable for light-sensitive applications, PMMA allows for simpler construction and flexible design of various support surfaces (Huang et al. 2017).

The behavior of bacteria on fibers flocked to glass and PMMA materials in both aerobic and anaerobic environments was compared to evaluate biofilm formation and hydrogen production. This is important for applications involving bacteria that are attached on microfiber flocks.

The effect of oxygen availability on *R. capsulatus* growth and hydrogen production were tested in a 30° inclined reactor. Biohydrogen evolved only under anaerobic conditions, confirming oxygen's inhibition of nitrogenase; thus, analyses focused on anaerobic data validated by controls. Across two operational cycles (Rounds 2–3), anaerobic systems showed markedly higher hydrogen (Fig. S6). Among glass (GE) and PMMA (PE) supports, the flocked anaerobic photobioreactors (4-GE, 2-PE) achieved the best performance. The quantity of biofilm (Abs₅₉₀) was higher on glass-flocked surfaces (0.70±0.05) than on PMMA (0.58±0.02). Flocked reactors especially glass (4-GE) and PMMA (2-PE) achieved the highest final yields (~170 mL). No biofilm was observed or quantified on non-flocked (control) glass or PMMA supports.

SEM imaging revealed dense, continuous bacterial layers on anaerobic surfaces, matching reduced suspended biomass and elevated hydrogen productivity (Fig. S7). In contrast, aerobic photobioreactors displayed sparse cells, weak biofilms, and negligible hydrogen, underscoring anaerobic conditions as crucial for stable biofilm growth and sustained H₂ production. Overall, flocked glass performed slightly better than PMMA.

Cell dry weight showed an inverse relationship between suspended biomass and surface attachment. High values in Round 1 decreased by Round 3, indicating increased adhesion. Anaerobic conditions were most effective for *R. capsulatus*, with flocked glass performing best.

4. Conclusion

This study investigated biofilm formation and biohydrogen production using tunable microfiber-flocked surfaces for immobilization of *Rhodobacter capsulatus*. A novel biofilm photobioreactor system was developed and operated for 21 days in sequential batch processes.

Optimized microfiber flocks; shorter, denser, and vertically oriented, improved bacterial adherence, light transmittance, and photosynthetic activity, leading to increased biofilm and hydrogen production. Inclined BPBR setups demonstrated higher immobilization efficiency due to improved light exposure and nutrient availability, resulting in the formation of thick

biofilms with interconnected extracellular matrices and nanowire-like structures within 14 days. In contrast, horizontal setups and aerobic conditions performed poorly due to suboptimal light and oxygen levels, whereas anaerobic environments promoted dense biofilms and sustainable hydrogen production. The increased surface area provided by the microfiber-flocked surface enhances biomass attachment, stability, and reusability, making this biofilm system particularly valuable for continuous processes.

The observed nanowire-like structures may support biofilm stability and long-range electron transfer, suggesting potential applications in biohydrogen production, bioelectronics, and environmental remediation. However, further investigation using high-resolution imaging and biochemical techniques is needed to confirm their function in electron transfer.

CRedit authorship contribution statement

E.S.: Supervision, Writing-original draft, Editing, Investigation; **S.C.:** Writing, Investigation, Data curation; **E.M.D.G.:** Writing, Investigation, Methodology; **H.K.:** Writing, Review, Editing; **B.M.:** Writing, Review, Editing; **E.B.:** Methodology; **T.H.E.:** Writing, Review; **M.S.:** Analysis; **M.U.Y.,:** Methodology; **C.S.O.:** Analysis; **B.K.:** Data curation; **B.B.:** Investigation. All authors read and approved the final manuscript.

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