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Understanding *Chlorella vulgaris* acclimation strategies on textile supports can improve the operation of biofilm-based systems

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Abstract

The interest in microalgae biofilm-based systems has been increasing lately due to their high potential for biomass production. However, more studies focusing on the first stages of this bioprocess, such as support selection and inoculum properties, which may finally affect biomass productivity, are required. The aim of this study was therefore to assess the impact of support nature and inoculum properties on microalgae biofilm productivity and physiology. Results suggest that physico-chemical properties of the support (micro-texture, hydrophobicity and chemical functional groups) affect the attachment of *Chlorella vulgaris*. Significant differences in cell-distribution pattern and biofilm structure on polyamide-based (Terrazzo) and cotton-based fabrics were observed. Compared to Cotton, cells grown on Terrazzo showed higher biomass productivity (3.20-fold), photosynthetic capacity (1.32-fold) and carbohydrate pool (1.36-fold), which may be explained by differences in light availability due to support micro-texture. A high inoculum density resulted in a lower biofilm growth, likely due to a lower light/nutrient availability for the cells. Furthermore, when immobilized on fabrics, cells pre-acclimated to 350

μmol photons m^{-2} s^{-1} grew faster than those pre-acclimated to low light (50 μmol photons m^{-2} s^{-1}), demonstrating the influence of light-history of the inoculum cells on biofilm productivity. Therefore, this work confirmed the importance of support and inoculum properties for biofilm-based systems.

Keywords

Chlorella vulgaris, Microalgae biofilms, Physiological properties, Biofilm structure, Textile materials

Declarations

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Competing interests

The authors declare no competing interests.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

S. F. Li, A. Fanesi, T. Martin and F. Lopes designed the experiments, S. F. Li and A. Fanesi

conducted the experiments, S. F. Li, A. Fanesi and F. Lopes analyzed the data, S. F. Li and A. Fanesi wrote the manuscript, F. Lopes revised the article. All authors read and approved the final manuscript.

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1. Introduction

The interest in microalgae biofilm-based systems has been increasing lately due to their advantages, such as lower costs of harvesting, energetic consumption and water demands (Johnson and Wen 2010; Christenson and Sims 2012; Gross et al. 2015), with respect to conventional suspended cultures where cells are in a planktonic state. Studies proposing innovative designs and culture optimization have been thus carried out to fully confirm the feasibility of the biofilm approach for large-scale cultivation (Genin et al. 2014; Li et al. 2017).

The inoculation step is of paramount importance on microalgae bioprocess development. Indeed, it affects biomass productivity and yield (Genin et al. 2014; Moreno Osorio et al. 2020; Li et al. 2021). Biofilm productivity can be clearly improved by increasing the inoculum size (Zhang et al. 2014; Ji et al. 2014; Shen et al. 2017) but eventually light attenuation and/or nutrients limitation may occur thereby decreasing biomass productivity (Huang et al. 2016; Li et al. 2021). Support properties such as hydrophobicity, roughness and surface free energy influence cells colonization efficiency by changing affinity and cell interactions with the substrate (Ozkan and Berberoglu 2011; Cui et al. 2013; Gross et al. 2016; Huang et al. 2018; Tsavatopoulou and Manariotis 2020). Interestingly, Vivier et al. (2021) demonstrated that the microtopography of colonized substrates can also affect biofilm physiology. Rough supports seem to create local microhabitats that may help protect cells from photo-inhibition.

Among supports for microalgae biofilm development, fabrics have been studied due to their flexibility, easy microalgae re-growth after harvesting and low cost compared with other materials (Gross et al. 2013; Gross and Wen 2014; Moreno Osorio et al. 2019; Hart et al. 2021). The majority of these works reported macroscopic data such as biofilm overall productivity (Gross and Wen 2014; De Assis et al. 2019; Brockhagen 2021) and only few described

microscale patterns (e.g. cell distribution on the support/biofilm structure) (Moreno Osorio et al. 2020) or assessed the interplay between fabrics properties and the physiological state of the cells. Supports selection should be therefore carefully considered in biofilm process optimization.

In planktonic cultures, a broad number of studies show that cellular traits such as chlorophyll content or cell size strongly affect growth dynamics of microalgae populations (Post et al. 1984; Sukenik et al. 1990; Urabe and Kagami 2001; MacIntyre et al. 2002). For example, the lower the chlorophyll quota, the more transparent the microalgae and the higher the growth rate or productivity achieved (Sukenik et al. 1990; MacIntyre et al. 2002; Martínez et al. 2018). Some others also reported a decrease in the algal growth rate with the increasing cell size (Urabe and Kagami 2001; Key et al. 2010; López-Sandoval et al. 2014). From these observations, it is clear that the light-history of microalgae may play an important role in biofilm processes, nevertheless at present no information on the subject is available.

In this work, we aim at assessing the impact of fabric nature, inoculum density and its physiology on *C. vulgaris* biofilm initial colonization, productivity and composition (3 days). First, in order to select appropriate fabrics for biofilm development, cells attachment/detachment on five textile materials were investigated. The interplay between surface properties (i.e., relative opening surface area, hydrophobicity, chemical functional groups etc.) and cells retention capability was afterwards discussed. Cotton, considered as an excellent material for biofilm development (Christenson and Sims 2012; Gross et al. 2013), and a polyamide-based fabric with the highest cells retention capability from our study, were then chosen to evaluate the impact of support characteristics on biofilm production, activity and composition. The structure of biofilms formed on the selected fabrics, seldom described in the literature, was characterized using complementary imaging tools (CLSM - Confocal Laser Scanning Microscopy and SEM -

Scanning Electron Microscopy). Finally, in order to test our hypothesis that the physiological status of the inoculum cells affects biofilm growth, cells photo-acclimated to low or high light were immobilized and their growth, composition and photosynthetic activity were measured after 3 days of growth.

2. Materials and Methods

2.1. Planktonic culture maintenance

Chlorella vulgaris SAG 211–11b (Göttingen, Germany) was cultured semi-continuously in 1-L bottles filled with 800 mL 3N-Bristol medium (Bischo and Bold 1963) at 25°C. The cultures were bubbled with filtered air under a continuous illumination of 50 (low light, LL) and 350 µmol photons m^{-2} s^{-1} (high light, HL) (Viugreum 50W LED, Biospherical Instruments, San Diego, CA, USA; the irradiance was measured inside the cultures using a QSL-2100 quantum scalar irradiance sensor). The cultures were kept in exponential phase with a maximum cell concentration of 5.8×10^6 cells mL^{-1} by daily dilution in order to maintain a chlorophyll (Chl) *a* concentration of 0.5 - 1.5 mg Chl *a* L^{-1} to ensure optimal light penetration. The planktonic cultures were pre-acclimated to 50 or 350 µmol photons m^{-2} s^{-1} for at least 8 days before starting any experiments.

2.2. Textile supports characterization

Five textile materials (see table S1_supplementary data for more details) were purchased on https://www.tissusactifs.fr and their micro-texture and physico-chemical properties were characterized with several techniques.

The micro-texture of the materials was observed using a stereomicroscope (Zeiss, Discovery V12, Germany). The images were taken in a size of 2.30 mm × 1.72 mm with 63× magnification (Fig. S1_supplementary data). The area of the pores on the surface of each material was quantified using the Fiji software (Kostajnšek et al. 2021). The relative opening surface area (i.e., the total opening area in 1 cm^2 of textile) was afterwards calculated.

The surface roughness of the textile materials was quantified using a microtopographe (STIL CHR 150, France). At least five positions on each material were randomly selected for roughness determination. The surface roughness (Ra) was analyzed by the Mountains Map Universal software (Digital Surf Sarl 3.0, Besancon, France).

The hydrophobicity of textile materials was determined using the sessile drop test with an automatic drop tensiometer (Tracker Teclis/IT Concept, France). 5 µL of distilled water (as reference liquid) was pipetted onto the surface of the materials, and the images of water drops were analyzed using WDROP 2010 software to characterize the static water contact angle (θ) which reflects surface hydrophobicity ($0° < \theta \leq 90°$, hydrophilic surface; $90° < \theta < 180°$, hydrophobic surface; $\theta = 180°$, ultra-hydrophobic surface, respectively) (Van Oss et al. 1988).

An ATR-FTIR (Fourier Transform Infrared Spectrometer with Attenuated Total Reflectance) PerkinElmer Spectrum-two spectrometer (PerkinElmer, Waltham, MA, USA) was used to analyze the chemical properties of the textile materials. Infrared spectra were recorded in the range of 4000 to 400 cm^{-1} using an accumulation of 32 scans at a spectral resolution of 4 cm^{-1}. Before loading materials, the empty crystal was measured as background.

2.3. Selection of textile supports for biofilm growth

A first screening of supports was done based on the criteria of cells-retention capability (i.e., how many cells were retained on the support) after cell immobilization. The fabric with the highest number of retained cells was then selected for further studies along with cotton (widely described in the literature). Textiles were first cut into squares of 2.4 × 2.4 cm and sterilized in Milli-Q water at 121°C for 15 min. Aliquots of 2 mL concentrated planktonic cultures (1.0×10^7 cells mL^{-1}) were afterwards filtered on the textile materials (with a colonization area of 2.01 cm^{-2}).

After 6-h incubation in 6-well culture plates (Thermo Fisher Scientific, Inc.; each well filled with 8 mL 3N-Bristol medium), the cells on textile materials were harvested by vigorously vortexing the fabrics (for 5 min with 10 mL of growth medium) in 50 mL centrifugation tubes (containing glass beads). Then, the cells in solution (detached fraction), and those on textile materials (successfully immobilized fraction) were quantified using flow cytometry (Guava EasyCyte HT; Millipore, USA).

2.4. Immobilization and growth of C. vulgaris on textile supports

Two initial cell densities corresponding to $(1.5 \pm 0.2) \times 10^6$ cells cm^{-2} (low inoculum cell density, LC, 0.2 ± 0.02 g m^{-2}) and $(7.0 \pm 1.4) \times 10^6$ cells cm^{-2} (high inoculum cell density, HC, 0.8 ± 0.16 g m^{-2}) were obtained by filtrating specific volumes of planktonic culture on Cotton and Terrazzo. More precisely, after a 10-times filtration of the same algal solution, each textile material was washed gently in Petri dishes to remove the loosely attached cells and placed in multi-well culture plates under 100 µmol photons m^{-2} s^{-1}. One coupon was immediately used to verify the cell number just after the inoculation step. Coupons were incubated for three days and the medium was completely renewed every day to avoid nutrient limitation.

2.5. Immobilized growth of C. vulgaris pre-acclimated to two light intensities

In order to test the impact of light-history of the inoculum on biofilm growth, composition and activity, cells pre-acclimated to 50 (LL-acclimated cells) and 350 μmol photons m^{-2} s^{-1} (HL-acclimated cells) were filtrated on Terrazzo with low initial cell density (1.5 ± 0.2) ×10^6 cells cm^{-2} and then exposed to 100 μmol photons m^{-2} s^{-1}. After 3 days, the cells that colonized the fibers were harvested as described above and further measurements such as productivity, photosynthesis and macromolecular composition were carried out (see below for details). Here, biofilms formed from cells photo-acclimated to 50 μmol photons m^{-2} s^{-1} were named as LL biofilms (Biofilms Produced with Low Light acclimated cells) and those formed from cells photo-acclimated to 350 μmol photons m^{-2} s^{-1} as HL biofilms (Biofilms Produced with High Light acclimated cells).

2.6. Relative biomass increase and biomass productivity on textile supports

Although several harvesting steps were performed, no full cell recovery was achieved. Therefore, in order to fully estimate the immobilized biomass, Chl *a* from cells on the coupons was extracted according to the method described in section 2.7b. The number of residual cells was afterwards estimated based on the average cellular Chl *a* content of the mechanically recovered cells.

The relative biomass increase to the initial population on coupon (Rc) was calculated as following according to Eq. (1):

$$R_c = [(\mathrm{C_m} + \mathrm{C_{chl}}) - \mathrm{C_0}]/\mathrm{C_0} \quad (1)$$

where C_m and C_{chl} represent cell numbers obtained by mechanically harvesting and by Chl *a*-content measurements after 3 days, respectively, whereas C_0 stands for the cells on supports at the beginning of the experiment.

Similarly, biomass productivity (P_x, g m^{-2} d^{-1}) was calculated according to Eq. (2):

$$Px = [(X_m + W_t * C_{chl}/S) - X_0]/t \quad (2)$$

where X_m and X_0 represent biomass areal density (g m^{-2}) obtained by mechanically harvesting after 3 days and at the beginning of the experiment, respectively; whereas W_t stands for the average cell dry weight (g $cell^{-1}$), which was estimated by the mechanically harvested biomass after t (here t = 3, d) days; and S is the area (m^2) of the attached culture.

2.7. Physiological traits of sessile cells

Average cell diameter was determined considering a minimum of 300 individual cells using an AxioSkop 2 plus microscope (Carl Zeiss, Oberkochen, Germany) and a 63× magnification lens. Cell volume was calculated using the formula reported by Hillebrand et al. (1999).

Chl *a* was extracted using dimethyl sulfoxide (DMSO), and quantified by measuring the absorption at 649 nm and 665 nm with an Evolution 60S UV–visible spectrophotometer (Thermo Scientific, Madison, WI, USA). The Chl *a* concentration was calculated by the equation reported by Wellburn (1994).

Photosynthetic activity of immobilized *C. vulgaris* was assessed using a Pulse Amplitude Modulation (PAM) fluorometer (AquaPen, AP 110-C, Photon Systems Instruments, Drasov, Czech Republic). After 10 min of dark-adaptation, the relative electron transport rates (rETRs) corresponding to seven increasing actinic lights were used to construct the rapid light curve

(RLC) as described in (Li et al. 2021). The maximum rETR ($rETR_{max}$) was obtained from the RLC fitting function (Webb et al. 1974):

$$rETR = \mathrm{rETR_{max}}\,(1 - \mathrm{e}^{-\alpha \mathrm{I}/\mathrm{rETR_{max}}}) \quad (3)$$

where α represents the initial slope of RLC curve and photo-saturation E_k was computed from $E_k = rETR_{max}/\alpha$. Additionally, the maximum quantum yield F_v/F_m was calculated with the equation:

$$F_v/F_m = (F_m - F_0)/F_m \quad (4)$$

where F_0 and F_m are the minimum and max fluorescence determined after 10 min dark-adaptation, respectively, and F_v indicates the variable fluorescence.

The macromolecular composition of the cells was characterized using ATR-FTIR-spectroscopy (PerkinElmer, Waltham, MA, USA). The FTIR spectra of cells were baselined and maximum absorption values in the spectral ranges corresponding to carbohydrates (C–O–C; 1200–950 cm^{-1}), lipids (C=O; 1750–1700 cm^{-1}) and proteins (Amide I; 1700–1630 cm^{-1}) were used to calculate the relative carbohydrates and lipids contents to proteins (Fanesi et al. 2019; Li et al. 2021).

2.8. Textile biofilms imaging

The overall cell distribution on Cotton and Terrazzo was assessed by stereomicroscopy. Images were taken in a size of 1.72 mm × 1.72 mm with 63 × magnification. Biofilm structure was assessed by CLSM using an inverted Zeiss LSM700 confocal microscope (Carl Zeiss microscopy GmbH, Jena, Germany) equipped with a LD Plan-Neofluar 20×/0.4 Korr M27 objective with a 0.4 N.A. (numerical aperture) (Fanesi et al. 2019). Cells were detected by the

chlorophyll *a* autofluorescence at 639 nm. The images were 640 × 640 µm in size with a z-step of 3.94 µm and a lateral resolution of 1.25 µm.

In order to further investigate the interaction of algal cells with the fabrics, a scanning electron microscope (ESEM, FEI Quanta 200) was used to obtain SEM images with 2000× magnification. A small piece of support (5 mm × 5 mm) was examined at 20 kV accelerating voltage with a working distance of 14 mm in a high vacuum mode. The chamber was precooled to 7 ~ 8 ℃ and the determination of samples was carried out in a solid-liquid phase. Each support observation was performed in at least three random positions.

In addition, biofilm formation on Terrazzo inoculated with a LC-inoculum (Low cell density, ~1.5×10^6 cells cm^{-2}) was observed by Optical Coherence Tomography (OCT; Ganymede 621, Thorlabs GmbH, luebeck, Germany). The field of view was 4 × 4 mm (XY) and the axial depth was 1 mm (Z). The lateral and axial resolution were 8 µm and 1.45 µm, respectively. A refractive index of 1.33 was used for in-situ image acquisitions as the biofilm was aqua-cultured (Wagner and Horn 2017; Fanesi et al. 2022). Images were then analysed using the software ThorImageOCT 5.4.4 (Thorlabs, luebeck, Germany).

2.9. Statistical analysis

All results were presented as mean values ± standard deviations ($n \geq 3$). After the tests of normality (Shapiro-Wilk test) and variance homogeneity (Levene's test), one-way ANOVA analysis of variance followed by Tukey's post hoc test for multiple comparisons in cells, attachment/detachment and physico-chemical properties among different textiles were carried out using IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL). Significant differences in cells growth,

activity, composition and biofilm productivity between different treatments (two supports, two inoculum densities and two inoculum pre-acclimated light intensities) were performed by Students's *t*-tests with pairwise comparisons testing. The statistical significance of the data was shown at the levels of $P < 0.05$, $P < 0.01$ or $P < 0.001$.

3. Results

3.1. Support characterization and selection for C. vulgaris immobilized growth

Characteristic peaks from 2900 to 3500 cm^{-1} were detected on the polyamide-based (Terrazzo) and on the cotton-based supports (Cotton and Nordkap, Fig. 1) suggesting the existence of O-H, N-H and C-H bonds (Shahzadi et al. 2018). Two peaks at 1633 and 1537 cm^{-1} corresponding to C=O stretching in amide-I and N-H bending in amide-II, respectively, were observed only on Terrazzo (Kang et al. 2012; Shahzadi et al. 2018). Also, a peak at 1038 cm^{-1} related to the C-O bonds (Chung et al. 2004) in polysaccharides was found on cotton-based substrata. In the polyester-based supports (Sun silk and Mariella), the C=O bond at 1712 cm^{-1} indicated the presence of ester functional groups (Hoghoghifard et al. 2016).

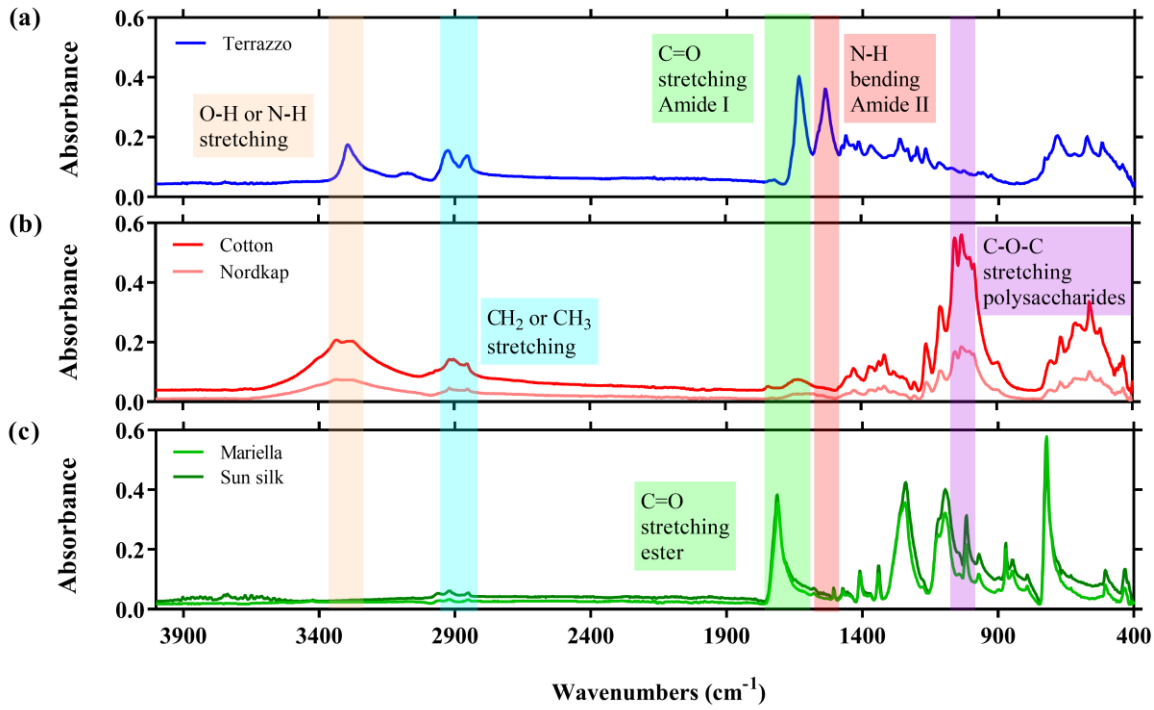

Fig. 1 FTIR-spectra of five fabrics (a, polyamide-based textile; b, cotton-based textile; c, polyester-based textile. Main functional groups in the fabrics are highlighted by different colors) used as a growth support for microalgae biofilm cultivation

Supports were further characterized in terms of hydrophobicity, roughness and relative pore surface opening size (Table S1_supplementary data). All supports but Sun silk and Mariella were hydrophobic (contact angle $\theta > 90°$). In addition, similar roughness values (30 – 50 µm) were detected for all materials except Terrazzo which is smoother (*ca.* 16 µm). Moreover, a broad range of opening sizes, which are larger than *C. vulgaris* cells (2-10 µm), was quantified for each material except for Terrazzo for which no surface opening was detected (Fig. S1_supplementary data).

Fig. 2 shows cells retention and detachment for the different textiles after 6 hours from inoculation. High cell retention capability and low releasing percentages were found for Terrazzo (17.1×10^6 cells cm^{-2}, 36.3%) and Nordkap fabrics (13.1×10^6 cells cm^{-2}, 30.4%). However, similar low values of cell areal densities and high releasing percentages were obtained for Cotton and the polyester-based supports, Mariella and Sun silk (~ 1.8×10^6 cells cm^{-2}, 45 - 80%). Among cotton-based supports, Nordkap presented a higher cell retention capability compared to Cotton though similar physico-chemical properties (surface chemical functional groups, hydrophobicity, roughness) were determined for both supports (Fig. 2, Table S1_supplementary data). Finally, the highest cell areal density and low detachment were obtained for Terrazzo.

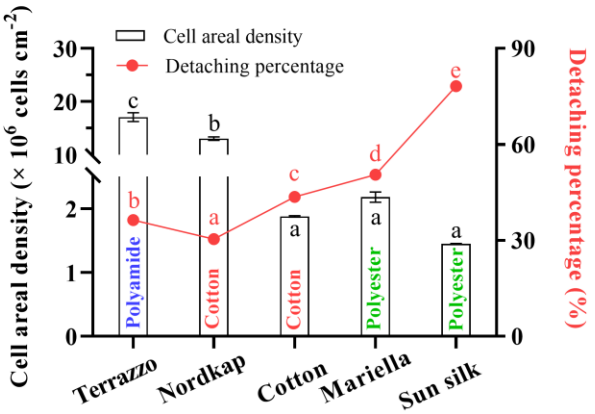

Fig. 2 Cell areal density on different textile materials after 6-h incubation. All the results are shown as mean value ± SD, $n = 3$; Bars and dots with different letters represent the statistical differences among different textiles at a level of $P < 0.05$

3.2. Biofilm structure on Terrazzo and Cotton

The micro-texture of Terrazzo and Cotton and biofilm structure on both textiles were investigated using a stereomicroscope, a CLSM, a SEM (Fig. 3) and OCT (Fig. S2_supplementary data). Terrazzo exhibited tightly-woven fibers with no clearly defined pores (Fig. 3a) while Cotton fibers were loosely knitted resulting in higher porosity (Fig. 3b).

From Fig. 3, it appears that the cells did not cover uniformly the entire fabric at day 3. Cell distribution patterns seemed different for the two supports. Indeed, cells mostly distributed on and in between the tightly-woven fibers, forming cell clusters on the top surface of Terrazzo while it seems that they did attach and grow mainly on the loosely connected fibers all through Cotton's depth.

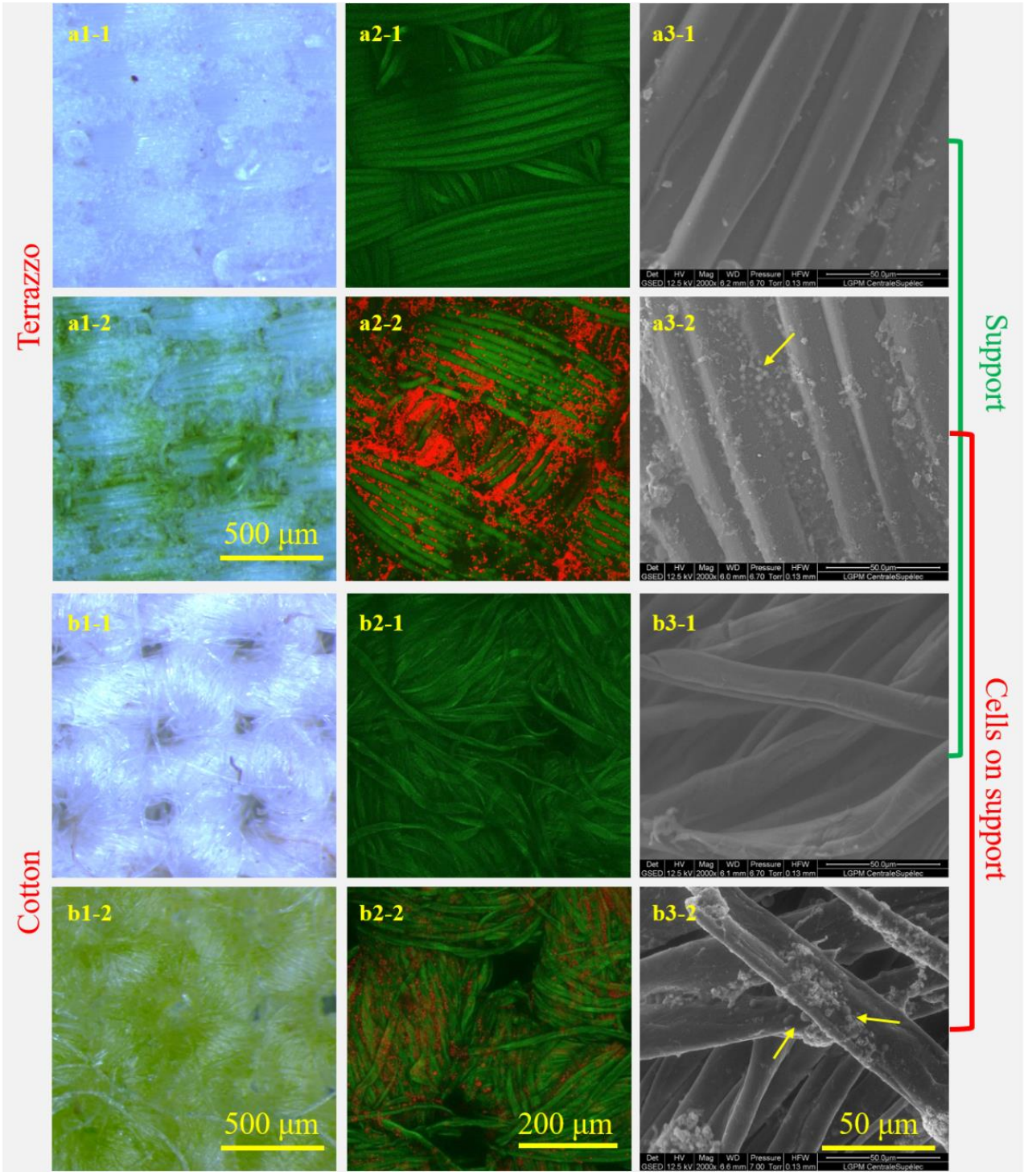

Fig. 3 Microscopic structural observation of supports and cells (pre-acclimated to 50 µmol photons m^{-2} s^{-1}) attached on their fibers at day 3 (A, a-Terrazzo, b-Cotton. a1 and b1 represent stereomicroscope images, a2 and b2 represent CLSM images, a3 and b3 show ESEM images.

Red signal in CLSM images and yellow arrows in SEM images indicate cell or cells clusters on the support fibers)

3.3. Support and inoculum density affect sessile cells growth, activity, composition and biofilm productivity

Relative biomass increase (Rc) for Cotton and Terrazzo inoculated with 50 µmol m^{-2} s^{-1} pre-acclimated cells is illustrated in Fig. 4a. Results show that, regardless of the support material, a higher Rc (6-7 times) was obtained for the LC-inoculated biofilms than those inoculated with HC ($P < 0.001$). On the other hand, productivity was ~ 3.2 times higher for Terrazzo compared to those of Cotton (Fig. 4b, $P < 0.05$) regardless of the inoculum density, suggesting an impact of the support on biofilm development.

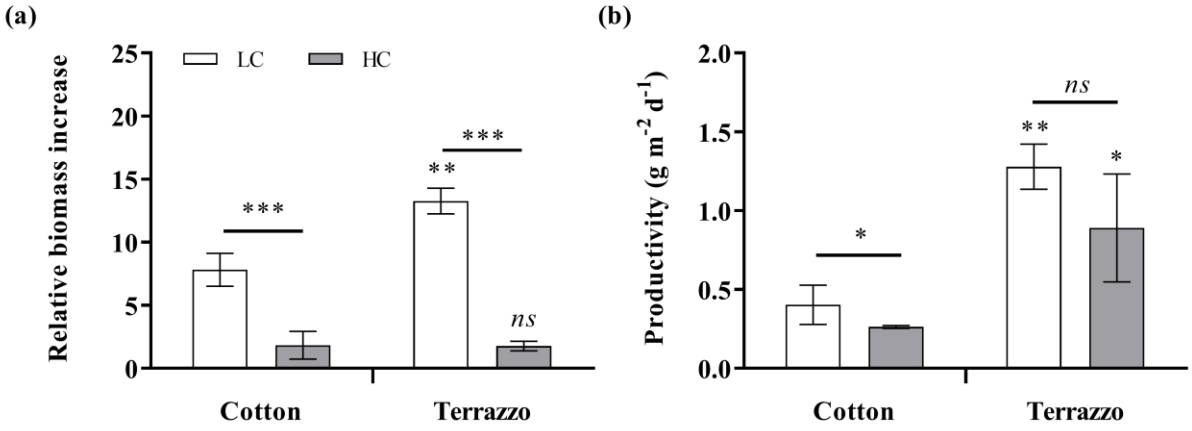

Fig. 4 Effect of support and initial cell density (pre-acclimated to 50 µmol photons m^{-2} s^{-1}) on relative biomass increase (a) and biomass productivity (b) after 3-days cultivation of *C. vulgaris* biofilms. All the results were shown as mean value ± SD, $n = 3$; Bars with ***, ** and *

respectively depict the statistical differences between the immobilized cultures at $P < 0.001$, $P < 0.01$ and $P < 0.05$, and ns represents no difference

In order to get a deeper understanding on the effect of the support material on bioprocess productivity, which is seldom studied, physiological properties of the cells grown on Terrazzo and Cotton in LC condition (i.e., the cell density that improved biomass increase, Fig. 4a) were characterized. Interestingly, differences in photosynthetic activity and composition of cells developed on Terrazzo and Cotton were found (Fig. 5). Similar maximum quantum yield (F_v/F_m around 0.7, Table S2_supplementary data) and cellular Chl *a* ($P > 0.05$, Fig. 5a) were measured for cells on both supports but the cells on Terrazzo presented a higher electron transport capacity (1.32 times) compared to those on Cotton (Fig. 5b, $P < 0.05$). In addition, no significant difference in cell volume and photosynthetic activity parameters other than $rETR_{max}$ (alpha, E_k; Table S2_supplementary data and Fig. 5b) were measured while a higher relative pool of carbohydrates (1.36-fold) was measured for sessile cells on Terrazzo (Fig. 5c, $P < 0.05$).

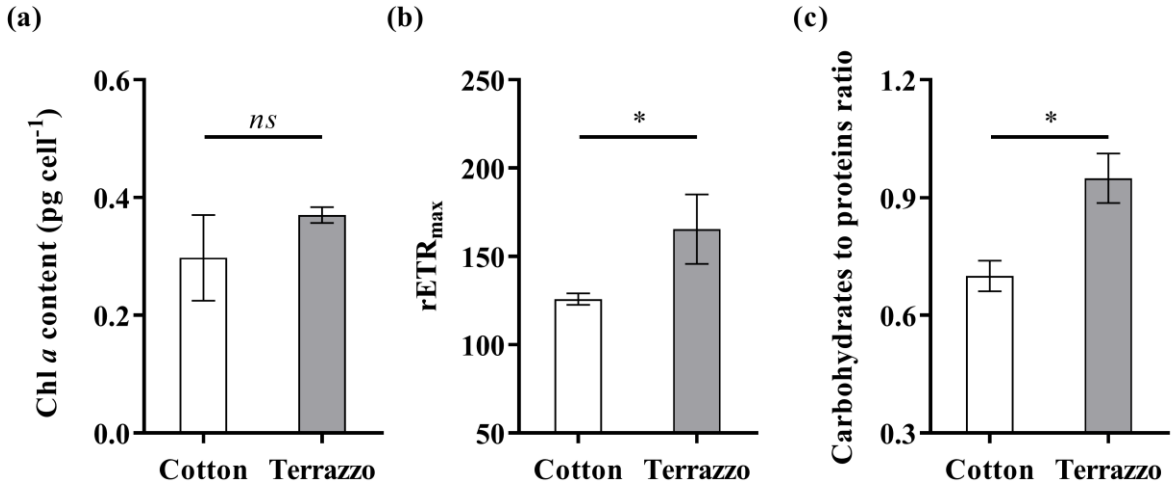

Fig. 5. Chl *a* content (a), relative maximum electron transport rate ($rETR_{max}$, b) and carbohydrates to proteins ratio (c) of *C. vulgaris* biofilms (with the pre-acclimated inoculum at 50 μmol m^{-2} s^{-1}) grown on Cotton and Terrazzo after 3-days cultivation at LC condition. All the results were shown as mean value ± SD, $n = 3$; Bars with * represent the statistical differences between the immobilized cultures on two supports at a level of $P < 0.05$, and *ns* represents no difference

3.4. Light-history of inoculum cells affects biofilm growth and productivity

Table 1 displays the physiological properties of inoculum cells. HC-acclimated cells had higher growth rate (1.38 times, $P < 0.01$), smaller volume (0.69 times, $P < 0.01$), lower cellular Chl *a* quota (0.54 times, $P < 0.001$) and higher electrons transport capacity ($rETR_{max}$, 1.2 times, $P < 0.01$) when compared to their LC-acclimated counterparts. Both populations had no difference in cellular biochemical composition ($P > 0.05$).

Table 1 Physiological traits of inoculum cells pre-acclimated to low (LL, 50 μmol photons m^{-2} s^{-1}) and high light (HL, 350 μmol photons m^{-2} s^{-1})

Cell traits	Pre-acclimation light intensity (μmol photons m^{-2} s^{-1})		
	50 (LL)	350 (HL)	*P*
Growth rate (d^{-1})	1.09 ± 0.20	1.50 ± 0.25	0.0012
Cell volume (μm^3)	56.7 ± 8.1	32.3 ± 3.2	0.0043
Chl *a* (pg $cell^{-1}$)	0.46 ± 0.04	0.26 ± 0.04	0.0007
$rETR_{max}$	181.3 ± 17.2	216.7 ± 24.7	0.0051
F_v/F_m	0.73 ± 0.02	0.60 ± 0.05	< 0.0001
Carbohydrates to proteins ratio	0.79 ± 0.03	0.79 ± 0.06	0.8873
Lipids to proteins ratio	0.15 ± 0.01	0.16 ± 0.00	0.7273

Since lower cell densities seemed to allow immobilized cells to attain higher productivities and faster growth, the effect of light-history of the inoculum was assessed only at low densities (LC).

Fig. 6a presents the relative biomass increase and productivity of biofilms developed on Terrazzo from inoculum cells photo-acclimated to low and high light intensities. An increase in Rc (1.8 times, $P < 0.001$, Fig. 6a) and an improvement in productivity (1.5 times, $P < 0.01$, Fig. 6b) were observed for the HL biofilm (biofilm formed from cells photo-acclimated to High Light; 350 μmol photons m^{-2} s^{-1}) compared to those exposed to low light intensity (LL biofilm, biofilm formed from cells photo-acclimated to Low Light; 50 μmol photons m^{-2} s^{-1}).

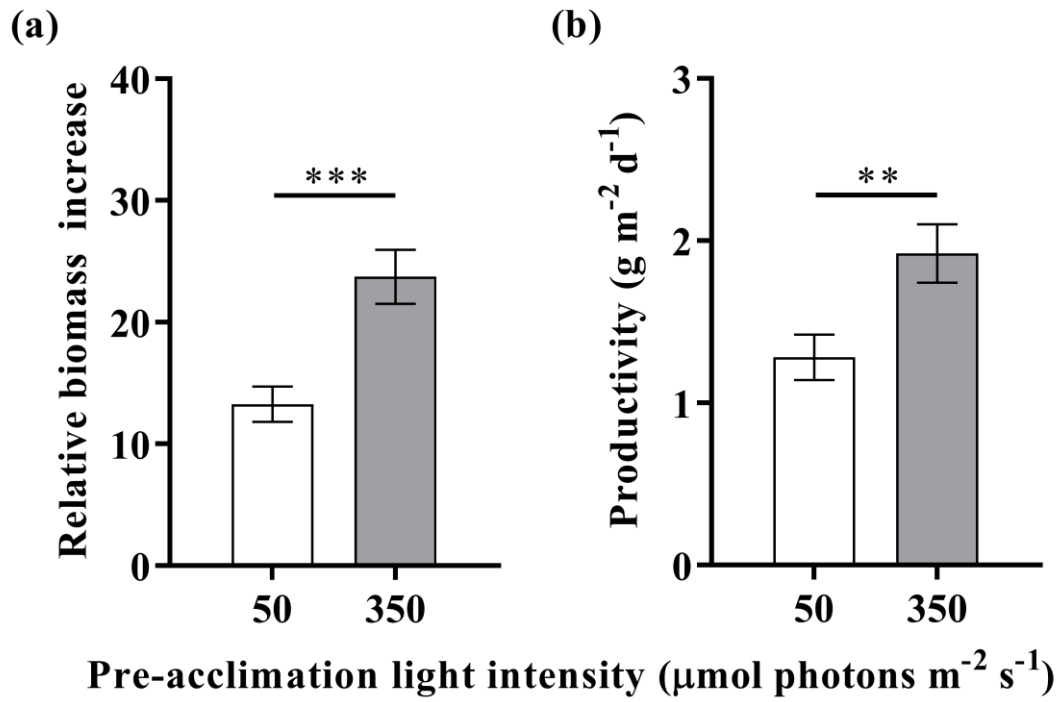

Fig. 6 Relative biomass increase (a) and biomass productivity (b) of 3-day *C. vulgaris* biofilms (inoculated with the pre-acclimated cells to 50 and 350 μmol photons m^{-2} s^{-1}) grown on Terrazzo at LC-conditions. All the results were shown as mean value ± SD, $n = 3$; Bars with *** and ** respectively depict the differences between immobilized cultures at $P < 0.001$ and $P < 0.01$

4. Discussion

With the development of biofilm-based systems for microalgae cultivation, more information and understanding are required to operate them efficiently. Many studies have focused on long-term development (i.e., from weeks to months) of microalgae biofilms as a function of several operational factors such as light intensity, temperature, nutrients and shear stress (Schnurr et al. 2014; Roostaei et al. 2018; Fanesi et al. 2019, 2021). However, only few tried to understand how the first operational steps (e.g. choice of substrate characteristics and light-history of the cells)

affect biofilm growth (Irving and Allen 2011; Genin et al. 2014). In this study, we followed a precise experimental set-up to select promising initial conditions for biofilm development on textile supports.

One of the first steps in biofilm development is represented by the adhesion of cells to a solid substrate (Moreno Osorio et al. 2020). The choice of an optimal support is therefore of paramount importance to promote cell attachment. We tested five fabrics with different characteristics, in terms of chemical functional groups, texture and physical properties in order to target a promising support for biofilm development (Table S1_supplementary data). In accordance with the literature (Cui et al. 2013; Gross et al. 2016), the ability of the supports investigated in this work to retain *C. vulgaris* was strongly dependent on their relative pore opening surface area, chemical properties and the degree of hydrophobicity. The highest cell areal density and the lowest cell detachment were obtained for the fabric "Terrazzo", which is a polyamide-based textile (Fig. 2). The dominant functional groups C=O in amide I and N-H bending in amide II (only detected on this support) together with small mesh openings (Fig. 1, Fig. S1_supplementary data) seemed to be responsible for the higher capacity of this support to retain *C. vulgaris* on its surface. This fabric was therefore selected as a promising support for biofilm growth and compared to a cotton-based fabric (Cotton, in our study) that is typically used in microalgae biofilm studies (Christenson and Sims 2012; Gross et al. 2013; Moreno Osorio et al. 2020; In-na et al. 2022).

In aquatic ecosystems, substrates heterogeneity creates a variety of benthic environmental niches that allows the maintenance of important population and community processes such as primary production (Cardinale et al. 2002; Vivier et al. 2021). Similarly, fabrics with different topographies and physico-chemical properties may create multiple niches that can stimulate specific physiological responses of the immobilized microalgae. Indeed, when *C. vulgaris* was immobilized on polyamide (Terrazzo) or cotton-based (Cotton) fabrics we found that not only the

nature of the substrate alters their productivity, but it also affects their physiological state in terms of activity and macromolecular composition (Fig. 5). It is not surprising that on both fabrics (polyamide and cotton-based) biomass increase was inhibited at high initial cell density, indeed a decrease in light and/or nutrients availability in biofilms due to strong self-shading in densely packed populations may occur as already reported in other works (Roberts et al. 2004; Huang et al. 2016; Roostaei et al. 2018; Li et al. 2021). On the other hand, *C. vulgaris* presented 3.2 times higher productivity on Terrazzo regardless of the initial cell density. This behavior may be related to a different access to light for the immobilized cells on these two supports as a consequence of fabrics micro-topography (Fig. 3, Table 1). Accordingly, cells grown on Terrazzo, mainly distributed on the top of its surface, would have higher access to light than those embedded on the porous and loosely-connected fibers in Cotton. Indeed, cell-shading and loosely connected fibers in Cotton might reduce photons availability for photosynthesis for the cells distributed over the support's depth. The higher light availability on the polyamide-based fabric may thus explain the greater photosynthetic capacity ($rETR_{max}$) (Fig. 5b) and in turn the greater biomass increase, higher productivity and carbohydrates pool observed for this fabric (Fig. 4 and Fig. 5c). Our data is consistent with results of Vivier et al. (2021) who confirmed that support micro-topography provided peculiar micro-habitats impacting biofilm biomass, photosynthetic capacity and efficiency. Also, different cells-distribution patterns due to specific micro-textures of supports have also been documented in previous works (Cui et al. 2013; Huang et al. 2018). Nevertheless, experiments using techniques that can spatially resolve metabolic changes such as imaging PAM and oxygen micro-profiling will be necessary to prove our hypothesis.

The final aim of this study was to test whether the light-history of microalgae could impact early stages of biofilm formation. The inoculum of biofilm-based systems is often represented by a planktonic microalgae population where cells are shifting from a planktonic to a benthic life

style (Moreno Osorio et al. 2020). Choosing cells that could best fit, from a physiological point of view, the new cultivation conditions may shorten the lag-phase or even avoid the collapse of the system. Corcoll et al. (2012) found for example that the light-history of phototrophic biofilms was an essential factor in defining their response to Zn exposure. As expected, light intensity affected the physiological traits of the inoculum with all the parameters indicating a classical light acclimation strategy (Table 1). Interestingly, the light-history of the cells strongly affected the rate of growth and the productivity once the cells were immobilized on the fabrics (Fig. 6). In particular, a high light intensity during the planktonic phase boosted the growth of the cells on the fabrics, which exhibited 1.5 times higher productivity (Fig. 6b). The planktonic cells grown under high-light exhibited a smaller volume, lower Chl *a* content and a higher photosynthetic rate ($rETR_{max}$, Table 1). This is in agreement with studies showing that smaller cells with a higher metabolic rate lead to a greater biomass accumulation (Urabe and Kagami 2001; Key et al. 2010). Our data is also in line with findings described in Martínez et al. (2018) who predicted that, the lower the chlorophyll quota (Chl *a*/C), the higher the maximal productivity achieved in a suspended culture. In our case, it is possible that in biofilms formed by planktonic cells with a lower Chl *a* content light penetrates deeper through the cell layers, influencing positively growth. Similarly, Wang et al. (2015) found that in thick biofilms microalgae cells tend to decrease the amount of chlorophyll to allow more light to penetrate in the deepest layers.

Our data confirm that the inoculation step (i.e. support properties, inoculum density and its physiological status) is of paramount importance in microalgae production when using biofilm-based cultivation approaches. A selection of tightly-woven textiles, low inoculum density of cells pre-acclimated to an appropriate high light could improve the productivity of a biofilm-based system (Cui et al. 2013; Gross et al. 2016).

5. Conclusions

The effects of inoculum density/physiology and support nature on biofilm growth/productivity, activity, and composition were evaluated. Results show a decrease in biofilm growth when using high inoculum density, probably associated to a reduction in light/nutrients availability. Support micro-texture affects cells distribution, consequently impacting biofilm formation and activity. A higher $rETR_{max}$ and carbohydrate/protein ratio were exhibited by cells grown on a polyamide-based fabric (Terrazzo), suggesting a higher light availability to cells than those on Cotton. Our study also suggests that inoculum physiology, poorly considered in literature, affects biofilm productivity. Higher productivities were reported for biofilms inoculated with cells photo-acclimated to high light. Therefore, our data confirm that in the inoculation step, the support selection and inoculum density/physiology must be carefully considered in order to optimize biofilm-based systems productivity.

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