

Evaluating Microalgae Attachment to Surfaces: a first Approach towards a Laboratory Integrated Assessment

Dora Allegra Carbone^a, Immacolata Gargano^b, Gabriele Pinto^a, Antonino De Natale^a, Antonino Pollio^{a*}

^aDipartimento di Biologia, Università degli studi di Napoli "Federico II", Naples, Italy.

^bDipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università degli studi di Napoli "Federico II", Naples, Italy.

anpollio@unina.it)

Recently, the challenge of producing algal biomass at low cost has been faced also with manufacturing systems based on immobilized microalgae. The growth of microalgae as a biofilm reduces the costs of harvesting and also the water demand, allowing at the same time a high biomass productivity. The initial adhesion to a surface is one of the key factors for the formation and maintenance of a stable microbial community, and although the physical properties of the surface have an influence on the adhesion process, the major role is played by species selection. Here we propose a simple procedure to establish on a bench-scale the ability of an algal strain to form a stable biofilm on a surface. As a model organism was selected *Scenedesmus vacuolatus* (ACUF 053) and the progressive adhesion of the microalgae at the surface of two tissues, cotton and jute, was followed with a two-step protocol based on the combination of image analyses and fluorometric measurements. The growth and viability of the algal biofilm were followed through color measurements of inoculated carriers taken at different times using the Trainable Weka Segmentation (a plugin of Fiji) on digital photographs, whereas pulse amplitude modulation (PAM) fluorometry allowed the measurement of algal photochemical activity on two textures. The results indicate that the progressive adhesion of the microalgae reached 80% of the surface of cotton fabrics during the first four days, and that the indicators of cell photosynthetic performance, decreased during the time course of the experiment, suggesting that the reduction of the nutrients concentration in the media could be responsible of progressive decay of the photochemical activity. In conclusion, the method provides reliable data on the extent and metabolic efficiency of the algal attachment to a solid substrate.

Key words – microalgae, *Scenedesmus*, biofilm adhesion, image analysis, PAM fluorometry

1. Introduction

Mass cultures of microalgae are presently at a stage not fully developed, despite years of research. The reason for their reduced exploitation is the too high cost of production, and the efforts of the last years are particularly concentrated on this aspect. The challenge of producing algal biomass at lower costs has been faced also with manufacturing systems based on immobilized microalgae. The growth of microalgae as a biofilm reduces the costs of harvesting and also the water demand, allowing at the same time high biomass productivity (Liu et al., 2013). The initial adhesion of algae to a surface is one of the key factors for the formation of a biofilm, and is ruled by the properties of the surface and the species selection (Irving and Allen, 2011). The production of extracellular polymeric substance (EPS) by unicellular algae is known for decades (Hellebust, 1974), and a wide array of molecules, ranging from carbohydrates to proteins, lipids and vitamins has been detected in the external space that surrounds the cells (Myklestad, 1995), playing a key role in the establishment of the biofilm. The genus *Scenedesmus*, one of the first microalgae tested in suspended-based mass cultivation (Ketchum and Redfield, 1949), has shown a promising attitude to grow on the surface of solid carriers, and different methods of attachment have been proposed (Chen et al., 2014). Recently, we have concentrated our attention on the growth and photosynthetic performances of liquid cultures of *Scenedesmus vacuolatus* in enclosed photobioreactors. The description of photosynthesis behavior of *S. vacuolatus* through

a kinetic model by PAM fluorometry was carried out with the aim of optimizing the biomass growth rate, and to assess the effects of different light operating conditions and photobioreactor design (Gargano et al., 2015). Then, the photosynthetic performances of *S. vacuolatus* under batch, fed-batch, semi-continuous cultivation strategy and under different CO₂ regimes were evaluated with the same model, to estimate the light intensity which maximizes the photosynthesis in liquid mass cultures. Here we propose a simple procedure to establish on a bench-scale the ability of an algal strain to form a stable biofilm on a surface. This two-step protocol is based on a combination of image analyses and fluorometric measurements, providing information on the extent and viability of biofilm formation that can be very useful to assess the attitude of microalgal strains to grow on a solid substrate.

2. Materials and Methods

S. vacuolatus strain ACUF 053 (www.acuf.net) was maintained and grown on Bold's basal medium in 100 mL Erlenmeyer flasks placed in a climatic chamber at $24 \pm 2^\circ\text{C}$, on a shaking apparatus at 60 rpm. Continuous light, at $90 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ was provided by 36 WT12 fluorescent Cool White (Osram light, Munich, Germany). The materials selected for adhesion experiments were cotton and jute, which were cut into square carriers 2,0 cm x 2,0 cm (width 0,3 cm) using a scissor. The carriers were first photographed with a Nikon 5300 digital camera and then, at higher magnifications, with a Leitz Metallurgical Microscope, equipped with a digital camera. Three carriers of each material type, previously sterilized at 120°C for 20 minutes, were placed in a 9 cm diameter glass Petri dish containing 30 mL of sterile BBM. Inocula of *S. vacuolatus* from exponential cultures were poured in each Petri dish, to have a final concentration of 0,5 optical density, assessed with a Specoman 50 spectrophotometer at 600 nm. The Petri dishes were placed on a rotating shaker at 70 rpm and held in the same conditions of light and temperature previously described for the maintenance cultures. Distilled water was added every two days to compensate the evaporation. The nitrogen concentration in the medium was measured as nitrate, by the spectrophotometric method reported by Collos et al. (1999). The adhesion of *S. vacuolatus* to the carriers was measured through a photographic recording of each carrier following the method described by Marasco et al. (2016). The carriers were placed on millimetric paper under controlled light provided by fluorescent lamps. The image were captured with a Nikon D5100. The parameters of the camera were: quality image FINE, image dimension NORMAL, length 3696 pixels, height 2448 pixels, horizontal resolution 300 dpi, vertical resolution 300 dpi, Bit depth 24, focus 5,6, exposition time 1/8, ISO sensibility 320, focal distance 50mm, light source AUTO. The digital images recorded at different times were analyzed with the software Fiji (Schindelin et al., 2012; and also <http://www.fiji.sc>), an open source image processing package based on ImageJ™ and the geometry and radiometry of the images were rectified to allow comparisons. The result of these geometric corrections is a multi-layer file, in which each layer represents a single measurement. The color measurement was analyzed using the Trainable Weka Segmentation (Hall et al., 2009) a plugin of Fiji, that permits to cluster different colours and their tones, distinguishing in this way the substrate from by microalgae. Chlorophyll fluorescence emissions were determined at room temperature by a pulse amplitude modulated fluorometer (Hansatech Fluorescence Monitoring System) elsewhere schematized (Gargano et al., 2015). The Petri dishes were kept in the dark for 30 minutes before starting the tests. Since it is known that the humidity content of carriers is the key factor ruling fluorescence measurements in the lab, each carrier was moisturized for two times at an interval of ten minutes, according the procedure described by Eggert et al. (2006). The protocol used for the PAM analysis was reported by Maxwell and Johnson (2000), with some modification for photosynthetic and non-photosynthetic quenching times (3 minutes). The algae were exposed at three light intensity (39-150-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Fv/Fm were determined with a completely saturating white light pulse (2600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ weak 0,6). The gain of the instrument was 30 and the weak was 1. The light conductor of the fluorometer was always blocked at a distance of 7 mm from the substrate thanks to a special support. The fixed distance was necessary to avoid changes during fluorescence measurement. The measurements were carried out in triplicate for each set of operating strategies and conditions. The experiments were repeated for three times and the results were analyzed with ANOVA test.

3. Results

Cotton and jute fabrics were cut in small squares of 2 cm and were used as carriers for the experiments of *S. vacuolatus* cell immobilization. The experiments lasted 16 days, and the adhesion of the alga to substrates was followed by image analyses and PAM fluorometry. After 2 days, the colonization of the carriers by *S. vacuolatus* attained more than 80% of the surface of each carrier (not shown). Digital image analysis is a low-cost technique that allows the non-destructive recording and quantification of different components of a biofilm (Kaur and Kaur, 2014). Thanks to the colour cluster analysis (Trainable Weka Segmentation), it was possible

to obtain from each image different ranges of hues and colours, which can be observed in terms of visible absorption spectrum. On both cotton and jute, the prevalent colour of the images taken before the inoculation and after the first 24 hours was white, that indicates the absence of algae on the carriers. After 48 hours and until the end of the experiment, the yellow colour prevailed, showing the formation of a subtle layer of microalga, which covered more than 80% of the total surface of both carriers. Finally, the green color occurs in the presence of large biomass of microalgae, indicating the formation of a thick biofilm (Berner et al., 2014), that accomplished to less than 20% of the superficial coverage of both carriers at the end of the experiment (Figure 1a, b).

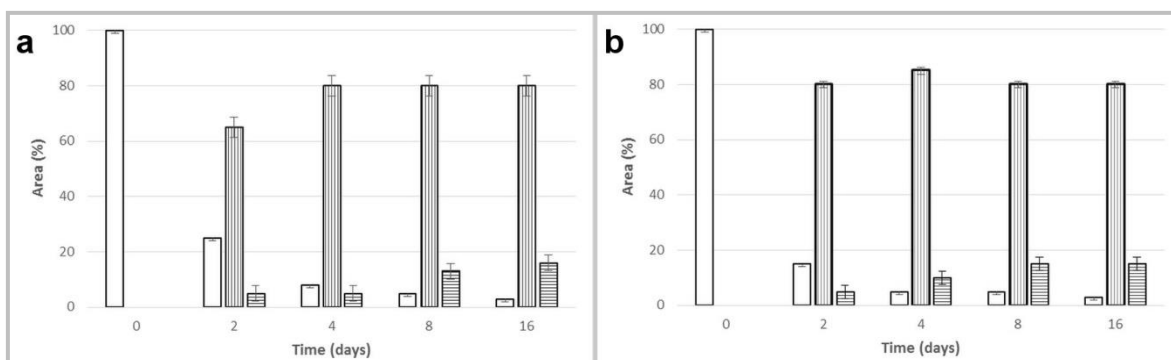


Figure 1. Growth of microalgae on cotton (a) and jute (b) carriers measured with the Trainable Weka Segmentation. Uncolonized area (□), colonized area (▨), biofilm (▩).

The nitrate content of the culture medium in which the carriers were immersed was progressively reduced to less than one tenth of the initial concentration (Figure 2). The depletion of nitrate is generally linked to an increase of the pH (Markau et al., 2014). In this experiment, the initial pH of the medium was 6,5 and achieved values around 9 at the end of the test. The nitrate depletion in the medium in the presence of cotton carriers was initially slower, but the final measurements evidenced similar values of nitrate concentration (4 mg/l) in presence of both carriers (not shown).

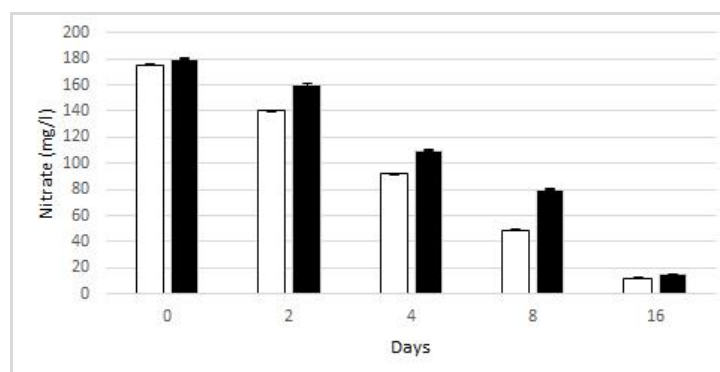


Figure 2. Nitrate content in the culture medium supporting the growth of the carriers. Cotton (■), jute (□).

The adhesion of *S. vacuolatus* to the substrates was also measured in terms of efficiency of the photochemistry of the immobilized algae. Light intensity plays a key role, affecting the photochemical efficiency of attached algae. All the measured parameters varied at different light intensities, resulting particularly sensitive in the range 50-150 PAR. In this interval, we observed the maximum decrease of photochemical efficiency that declined from the initial values of 0,65 for jute and 0,55 for cotton to 0,48 for jute and 0,44 of cotton (Figure 3).

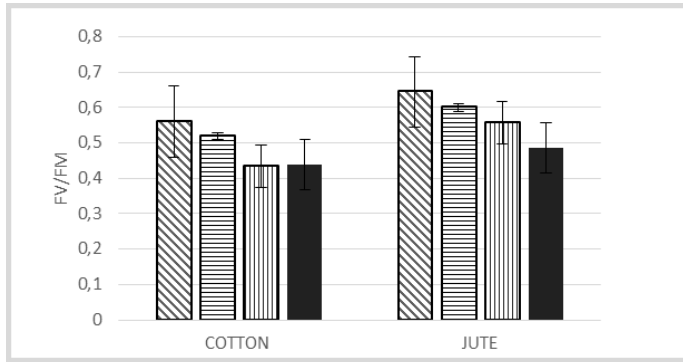


Figure 3. Fv/Fm of *S. vacuolatus* cultures on cotton and jute carriers. 2th day (\ \ \ \), 4th day (≡), 8th day (||||), 16th day (■).

Accordingly, in both type of carriers the microalgae reduced their quantum yield values during the experiment time, with a similar trend, and also in this case higher light intensities showed inhibitory effects on this parameter (Figure 4a, b).

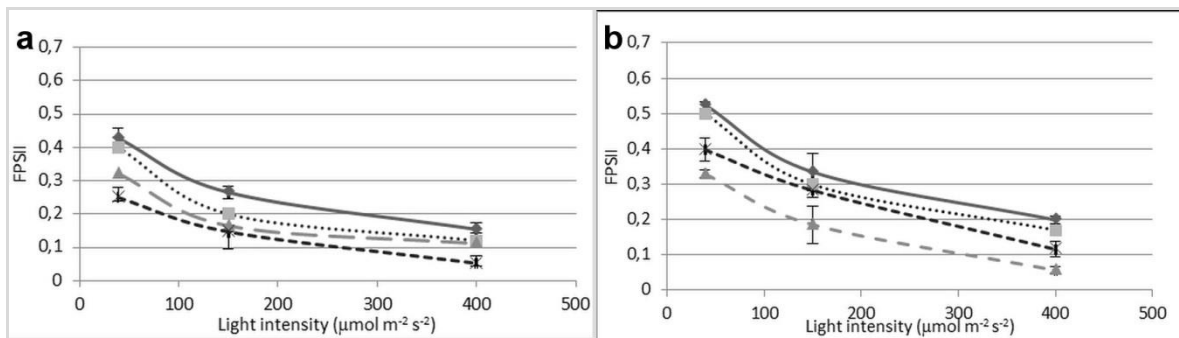


Figure 4. Quantum yield of *S. vacuolatus* cultures grown on cotton (a) or jute (b) carriers. 2th day (—), 4th day (.....), 8th day (- - -), 16th day (- - -).

In our experiments, also NPQ, a parameter related to the photoprotection of PSII (Lavaud et al., 2000), was scarcely influenced by the type of carrier, and depended mainly on light intensity. NPO initially achieved high values already at very low light intensity (0,269 at $39 \mu\text{mol m}^{-2} \text{s}^{-1}$), but in the following days the values decreased to 0,1. At higher light intensities NPQ increased, reaching the maximum values of 0,450 at $480 \mu\text{mol m}^{-2} \text{s}^{-1}$.

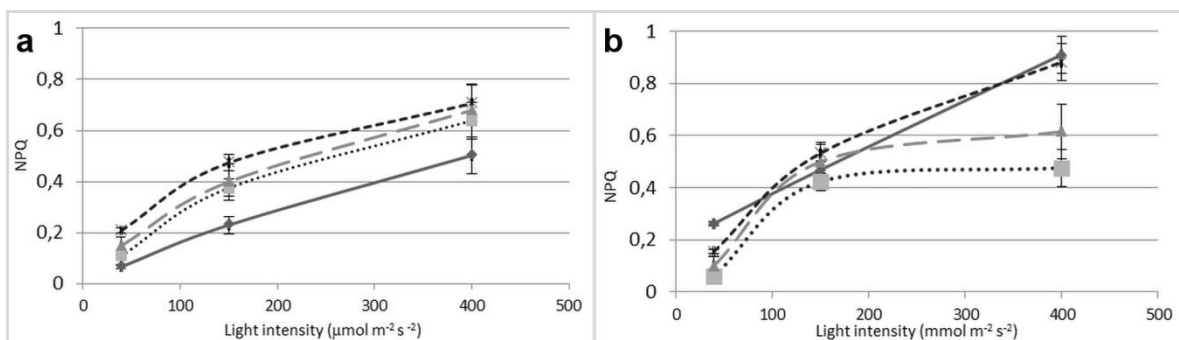


Figure 5. NPQ of *S. vacuolatus* cultures grown on cotton (a) or jute (b) carriers. 2th day (—), 4th day (.....), 8th day (- - -), 16th day (- - -).

4. Conclusion

Immobilized-based systems of microalgal cultivations provide new opportunities for reducing costs and natural resources consumption, but a systematic study on substrate and strain selection is still required. Recent evidence on algal biofilm cultivation system indicates that natural materials like cotton can sustain the algal growth for several months and *Scenedesmus* is showing a very promising attitude in biofilm-based technologies (Gross and Wen, 2014). On the other hand, this genus is characterized by a high number of species (about 800 taxa have been described, according to Hegewald, (1998)); many *Scenedesmus* strains with unknown biotechnological performances are presently held in the algal collections distributed over the world, and a protocol for a high throughput screening is needed. The laboratory approach proposed in this study has shown promising results in terms of reliability, costs and times required; indeed, both the techniques adopted are not expensive and furnish results in short times. Digital image analysis can represent a tool very effective to record the algal growth on attached surfaces, and the development of measuring systems shows that it is possible to furnish a detailed characterization of coloured surfaces (Leon et al., 2006). In a recent study on the colonization of lithic surfaces we have demonstrated that it is possible to investigate the behaviour of different strains of Cyanobacteria and green algae by image analysis and that CLSM microscopy can be a tool to describe in a quantitative way the pioneering attitude of different phototrophic organisms (Marasco et al., 2016). However, this kind of approach requires long times (two months), that represent a limit when it is necessary to gain a quick response on the biotechnological features of a strain. The use of PAM fluorometry coupled with digital image analysis has shown that it is possible provide data also on the physiological status of the attached algal cells, that plays a fundamental importance for the selection of a proper strain. It is possible to evaluate the algal growth through measurements of fluorescence after dark adaptation, as indicated by von Werder and Venzmer (2013); moreover under controlled conditions carbon fixation and PSII are linearly correlated (Maxwell and Johnson, 2000), The Fv/Fm generally has a maximum value of 0,7/0,8 under optimal conditions (Masojidek et al., 2003), but in our test this parameter never achieved these values. A low Fv/Fm ratio can be due to photoinhibition, usually caused by a synergism between high irradiance and other forms of environmental stress, as temperature extremes, or high dissolved oxygen concentration (Bjorkman and Demmig, 1987). However, the test on carriers are made in absence of water, a stress condition frequently experienced by aeroterrestrial microalgae communities (Häubner et al., 2006), that can account for the reduction Fv/Fm ratio. In our tests, we have obtained the better data of photochemical efficiency during the first four days, even though the extension of colonization remained almost constant over the time course of the test. In the following days, the quantum yield decrease was relevant, and it is known that nitrate depletion can leads to a progressive inactivation of PSII reaction centres (Falkowski, 1992; Parkhill et al., 2001). On the other hand, the enhancement of NPQ, which is considered the main protection mechanism against photooxidative damage of photosynthetic machinery (Horton and Hague 1988), points to a stress condition experienced by algal cells attached to the carriers, that needs further experiments to be fully understood. Our results prompt us to develop a protocol based on image analysis and PAM fluorometry tests lasting seven days: it is not necessary to extend them to the second week. In this way, a very high number of strains and carriers could be assayed in a short time. Much work is necessary to fully develop this screening system, particularly more data on the effect of carriers texture and on biochemical mechanisms of adhesion of algal cells to the surfaces, that should be produced by interdisciplinary studies carried out by an integrated team of biologists, biochemists and engineers.

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