Research Article

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Chlorococcum sp. biofilm growth on biochar from olive kernels solid support

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Abstract

BACKGROUND: Biofilm is a complex community of microorganisms including microalgae, bacteria and protozoa, which are adhered to a surface. The purpose of an integrated microalgal processing system is the production of biofuels, biomass and other valuable bioproducts as well as wastewater remediation. Biochar is a carbon-rich and porous substrate, capable of adsorbing nutrients.

RESULTS: In this study, biochar produced from olive kernels pyrolyzed at 400 °C was tested as a solid support candidate for adhering *Chlorococcum* sp. cultures. The substrate used was BG-11 with 1/3 nitrates and BG-11 without nitrates and phosphates. After 15 days of cultivation, the biomass attached to biofilm was measured, while different parameters were determined in the liquid. Among the different concentrations of biochar used, the culture containing 1 g L^{-1} biochar gave the best performance, resulting in 1.28 and 1.24 g microalgae g^{-1} biochar in the presence and absence of nitrates, respectively.

CONCLUSION: The cultures containing BG-11 without nitrogen and phosphorus showed leaching of nutrients in the liquid of the reactor. The present findings provide an alternative use of biochar, enhancing the growth of microalgae, and thus encouraging its use as supporting material in bioreactors.

Keywords: microalgae; biofilm; biochar; olive kernels; nutrient removal

INTRODUCTION

Concerning the huge environmental problems that our planet is facing, the shift from a linear economy to a circular one is required. The main principles concern reuse, recycling and recovery of materials and energy.[1] Recently, the cultivation of microalgae has gained much attention in the scientific community, to exploit it. Their fast growth combined with the ability to be cultivated in a variety of environments (pH, temperature, salinity) in different mediums (freshwater, saline water and wastewater) make microalgae ideal candidates for the production of biomass, lipids and biocompounds (proteins and carbohydrates) and for wastewater remediation.[2,3] Lipids can be converted into renewable biodiesel,[4,5] while proteins and carbohydrates can be used as fertilizers or in aquaculture.[6,7]

Microalgae can be cultivated in suspended and attached cultures. Suspended microalgal cultivation is commonly carried out in open pond systems and closed reactors. Although open pond systems are widely employed due to low construction and operational costs, such systems are sensitive to contamination by microalgal predators, and present low biomass productivity. As far as closed reactors are concerned, they provide better control over pH and temperature, low contamination and evaporation rates and high cell densities; however, the harvesting mode remains a significant challenge.[8] The most usual harvesting techniques include flocculation, membrane filtration and centrifugation.[8-10] In suspended cultures, water must be removed to obtain algal biomass. However, the cost of biomass harvesting is estimated to be at least 20% to 30% of the total cost of biomass production, due to the energy consumption.[8] Additionally, the produced biomass is poor in suspended systems compared to attached ones. *Chlorella vulgaris* showed a 30.4% increase in biomass productivity when cultivated in biofilm form, in contrast to suspended culture.[11] In algal-attached cultures, biomass grows in the form of biofilm on a solid substratum, which can be easily removed by scraping the surface.

Algal biofilms have been proposed as an alternative cultivation strategy in the last decade. An algal biofilm consists of a thin but dense layer of cells combined with bacteria that are connected by an extracellular polymeric matrix (EPS).[12] As the biofilm grows, it becomes thicker, as microalgae near the surface of the biofilm carry out photosynthesis and assimilate nutrients and CO_2 from the medium.[13] Such a structure ensures the coherence of the biofilm on a surface and only uncontrolled turbulence of the liquid surrounding the biofilm can disrupt attachment, as relatively high shear stress is required to enhance nutrient transfer to the biofilm.[12]

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Materials with various chemical properties and textures have been examined and their performance compared, such as metals (e.g. stainless steel, titanium),[14] polymeric materials (e.g. Plexiglass, poly(vinyl chloride)),[15] natural polymers (e.g. cotton, cork),[16] lignocellulosic materials (pine sawdust, rice husk)[17] and some others that have been modified using ultraviolet laser micromachining.[18] There is a trend in the literature that shows that hydrophobic materials perform better without this being the rule and without having given a clear answer about the selectivity of microalgae.[16,19] In addition, a material should be easily available in high quantities, not expensive and environmentally friendly. During olive oil production, four times more wastes are produced than pure product. One of these wastes is olive kernels.[20] Until now, olive kernels have been examined as compost material,[21] catalysts for oil transesterification,[4] direct solid feedstock for biofuel generation for domestic application[22] and superfood.[23]

Biochar is a low-cost material and can be prepared from any biomass. Various raw materials such as malt spent rootlets, coffee grounds, peat and chicken manure have been converted into biochar by thermal decomposition in a furnace using a controlled atmosphere and temperature by physical or chemical activation.[24,25] The calcination atmosphere and temperature determine the composition of biochar and the starting biomass affects the microstructure of the biochar.[26]

There have been very few attempts to use biochar for algal biofilm development, although biochar is an eco-friendly material with many advantages. Indeed, proposing a substratum that is inexpensive, easy to obtain, nontoxic to algal cells and capable of enhancing biomass productivity is crucially important for the scale-up of algal biofilm technology. Walnut shell was tested as substratum in a self-permeating biofilm bioreactor and used for cultivation of *Chlorella vulgaris* and *Scenedesmus obliquus*.[27] The results showed algal productivity of 97.43 and 70.49 g m^{-2} for *C. vulgaris* and *S. obliquus*, respectively, during a cultivation period of 14 days.[27]

The scope of the study presented here was to evaluate the growth of *Chlorococcum* sp., freshwater microalgae, using biochar prepared from olive kernels as a porous carrier. More specifically, it examined (a) the suitability of biochar as a carrier for algal cell attachment, (b) the effect of biochar concentration on the growth of suspended and attached cells and (c) the ability of biochar to provide nutrients for algal growth. Various physicochemical parameters were examined to elucidate the points that help the cells to become attached on such an innovative substratum. Finally, the algal biomass content in terms of proteins, carbohydrates and lipids was determined to investigate the effect of biochar use.

MATERIALS AND METHODS

Algal preculture

Chlorococcum sp. (SAG 5.95) were obtained from the SAG Culture Collection of the University of Göttingen. *Chlorococcum* sp. is a single-celled freshwater green alga, with a capability of potential accumulation and storage of lipids inside the cell. It can be cultivated in synthetic substrate and wastewater and also can afford high biomass yield. Thus, it is an ideal candidate for biofilm production. Algal precultures were prepared with modified 1/3 N BG-11 medium (Blue Green-11 with one third times the nitrate concentration) in 1 L Erlenmeyer flasks. BG-11 medium consists of the following ingredients: Na_2CO_3 (20 mg L^{-1}), $NaNO_3$ (1500 mg L^{-1}), $Na_2MgEDTA$ (1 mg L^{-1}), ferric ammonium citrate (6 mg L^{-1}), citric acid·H_2O (6 mg L^{-1}), $CaCl_2·2H_2O$ (36 mg L^{-1}), $MgSO_4·7H_2O$ (75 mg L^{-1}), K_2HPO_4 (30.5 mg L^{-1}), H_3BO_3 (2.86 mg L^{-1}), $MnCl_2·4H_2O$ (1.81 mg L^{-1}), $ZnSO_4·7H_2O$ (0.222 mg L^{-1}), $CuSO_4·5H_2O$ (0.079 mg L^{-1}), $CoCl_2·6H_2O$ (0.050 mg L^{-1}) and $NaMoO_4·2H_2O$ (0.391 mg L^{-1}). The samples containing BG-11 without N and P did not contain $NaNO_3$ and K_2HPO_4. The flasks were illuminated by fluorescent lights (22 μmol m^{-2} s^{-1}) with constant aeration (3.5 L min^{-1}), and were placed in a walk-in incubator room under controlled environmental conditions at 20 °C.

Biochar preparation

Olive kernels were obtained from a local enterprise in western Greece. The raw materials were placed in an oven at 80 °C, weighed and placed into a ceramic, custom-made vessel (9.9 cm × 9.2 cm height × diameter) sealed with a ceramic cap, thus retaining a limited oxygen atmosphere, about 20% of the required O_2 for full oxidation. The vessel was placed in a gradient temperature furnace (LH 60/12, Nabertherm GmbH, Germany) reaching a temperature of 400 °C and pyrolyzed for 1 h.

Microalgal growth on biochar support

The cultivation of microalgae using biochar as support material was conducted in eight 1 L Erlenmeyer flasks (Table 1). In the first four flasks, 1/3 N BG-11 medium was used (C, CB1, CB5 and CB10), while in another three flasks, BG-11 medium without nitrogen and phosphorus was used (ZNPC, ZNPCB1, ZNPCB5). Both medium cases had a sample without biochar to function as control (C and ZNPC), a flask containing microalgae and 1 g of biochar (CB1 and ZNPCB1) and a flask containing 5 g of biochar (CB5 and ZNPCB5). Additionally, a flask with microalgae cultivated in 1/3 N BG-11 with 10 g of biochar was examined (CB10) and the last sample contained 1/3 N BG-11 with 5 g of biochar without microalgae (B5) (Table 1).

The flasks were kept in a walk-in room and were incubated under controlled environmental conditions: temperature of 21 ± 2 °C and a continuous supply of air at 3 L min^{-1} (air pump, HP-400, Sunsun, Zhejiang, China) filtered by a 0.22 μm syringe filter. The illumination was provided continuously by three 36 W (cool daylight) fluorescent lamps, which were placed next to the cultivation flasks. The light intensity at the surface of the conical flasks was 22 μmol m^{-2} s^{-1}. The duration of the experiment was 15 days.

Samples were taken on days 0, 4 and 15 for the determination of pH, anions, optical density at 650 nm (OD_{650}), cell concentration and chl-a concentration in the liquid, as well as total proteins, total

Table 1. Experimental conditions

Sample	Biochar (g)	Microalgal initial concentration (mg L^{-1})	Substrate
C	0	240	1/3 N BG-11
CB1	1	240	1/3 N BG-11
CB5	5	240	1/3 N BG-11
CB10	10	240	1/3 N BG-11
ZNPC	0	240	Z NP BG-11
ZNPCB1	1	240	Z NP BG-11
ZNPCB5	5	240	Z NP BG-11
B5	5	0	1/3 N BG-11

sugars and lipid content of the suspended biomass. All experiments and determinations were conducted in duplicate. The algal biofilm productivity (g m^{-2}) was calculated as follows:

$$\text{Algal biofilm productivity} = (M_t - M_0)/M_0 \quad (1)$$

where M_t is the dried biomass attached on biochar surface harvested on day t and M_0 is the mass of biochar before cultivation.

The specific growth rate (μ) was determined using the following equation[28]:

$$\mu = \frac{\ln X_t - \ln X_0}{t - t_0} \quad (2)$$

where X_t is the number of cells at time t (days) and X_0 is the initial number of cells at time t_0.

Analytical methods

Microalgal biomass was examined by the determination of total suspended solids (TSS) according to standard methods.[29] The initial concentration of microalgae was 240 mg L^{-1} in the flasks containing microalgae. The pH was measured with a pH meter (pH300/310 waterproof hand-held meter, Oakton Instruments, Singapore). The free algal cell concentration was measured by a Neubauer hemocytometer (0.1 mm, 0.0025 mm^2, Optic Labor, Germany) after algae staining with Lugol's solution to separate the dead from live algae. Anion concentration was determined using ion chromatography (Metrohm 850 Professional IC, Metrohm AG, Switzerland). For the determination of lipids from algal biomass, the procedure of Folch *et al*. was followed.[30] Briefly, the biomass was dried at 90 °C and a measured quantity of dry homogenized biomass was extracted three times with a mixture of chloroform and methanol (2:1). The biomass was removed by filtration through a filter paper and the extracted lipids were transferred quantitatively in a tared Erlenmeyer flask. The procedure was repeated three times to extract all the lipids. The flask was fitted to a rotary evaporator (IKA RV 10, IKA, Germany), until the Folch reagent was removed. The flask was allowed to cool to ambient temperature in a desiccator and then weighed. The weight difference corresponded to intracellular lipids. Total proteins were determined by a modified Lowry method, using bovine serum albumin (BSA) as standard.[31] Briefly, 5 mg of dry microalgal biomass was placed in a tube and 3 mL of 20% (w/w) NaOH and 0.75 mL of 1/15 mol L^{-1} KH_2PO_4 (pH = 4.5) were added. The tube was vortexed and afterward heated at 100 °C for 30 min. The sample was cooled at room temperature, to which was added 0.125 mL of 20% (w/w) $CuSO_4.5H_2O$. The mixture was centrifuged for 20 min at 4000 rpm. The OD of the samples was measured at 540 nm with a UV–visible spectrophotometer. Total carbohydrate concentration was measured by the phenol–sulfuric acid method.[32] A quantity of 5 mg of dry microalgae was placed into a tube and 5 mL of sulfuric acid and 50 µL of phenol (80% w/v) were added. Sulfuric acid hydrolyzes polysaccharides into monosaccharides such as pentose and hexose that dehydrate and rearrange into furfural and hydroxymethylfurfural, respectively. These unstable products condensate with phenol and a yellowish color appears as a result.[33] The OD of the sample was measured after 25 min at 484 nm using a UV–visible spectrophotometer. Total carbohydrate concentration was finally determined using a glucose calibration curve.

Biochar physicochemical characterization

The measurements of specific surface area (SSA), micropore surface area and pore size distribution were performed with N_2 adsorption isotherms at liquid N_2 temperature using a Tristar 3000 porosimeter.

The interaction of *Chlorococcum* sp. biofilm with the surface of the biochars was studied by scanning electron microscopy (SEM) analysis (JEOL 6300, JEOL Ltd). A piece of biochar with attached algae (dehydrated in an oven) was glued a SEM stub with conductive carbon tape. The samples were examined with SEM operating at 20 kV. For each sample, at least four fields were observed at different magnifications of between 250 and 2500. The experiments were conducted in duplicate. All determinations were conducted in triplicate, and the data were recorded as mean ± SD (standard deviation). Data were analyzed and plotted using IGOR Pro (WaveMetrics, Inc., USA). Energy-dispersive X-ray (EDX) analysis of the starting biochar was also performed.

Fourier transform infrared (FTIR) spectrum of the biochar was collected in a 1% (w/w) pellet with KBr in transmittance mode (PerkinElmer Spectrum RX FTIR system). X-ray diffraction (XRD) patterns were recorded using a Bruker D8 Advance diffractometer equipped with a nickel-filtered Cu Kα (1.5418 Å) radiation source. Thermogravimetric analysis (TGA) was performed in with a PerkinElmer TGA system under an air atmosphere, with a heating rate of 10 °C min^{-1}.

RESULTS AND DISCUSSION

Physicochemical characterization of biochar

The biochar from olive kernels pyrolyzed at 400 °C (CB) has a very low SSA of 0.1 m^2 g^{-1}. This is due to the low pyrolysis temperature which is not high enough to decompose significantly the organic phase. Studies showed that only biochars prepared at high pyrolysis temperatures can have a significant amount of micropores.[24,34] On the other hand, this is beneficial for maintaining the shape and the dimensions of kernels and their strength too. Thus, it can provide a significant area for attachment of the microalgae while it makes easier the removal of the microalgae when desired. Interestingly, the EDX analysis shows that there are present high amounts of Na and Cl atoms. The Na (33.4%), Cl (45.3%) and O (15.7%) contents are almost 95% of the heteroatoms (without the C) present in biochar. The rest is P (2.2%) and Ca (3.4%).

The presence of Na and Cl is also evident from the XRD peaks. The XRD pattern of the biochar is presented in Fig. 1. The two main peaks of NaCl are seen, while the broad peak centered at about $2\theta = 22.6^o$ is due to the C(002) amorphous graphitic phase. The NaCl crystallites have a mean diameter of 35 nm.[35-37] The XRD pattern evidences that even at 400 °C the graphitization of the raw biomass can be performed.

The amount of NaCl and other minerals can be estimated with TGA experiments. After a thermal run under air atmosphere, the carbonaceous phase will be burned and released as volatile compounds, mainly CO_2 and CO. The mass left after the full burn of the carbon phase is due to the non-volatile minerals of the biochar. As can be seen for the TGA run (Fig. 2), the decomposition of the carbonaceous phase starts at a low temperature of 300 °C, while the burn is completed at 500 °C.

At higher temperatures the mass is stable and analogous to the minerals, which are almost 7% (w/w) of the biochar, and as evident from EDX analysis and XRD pattern is mainly NaCl.

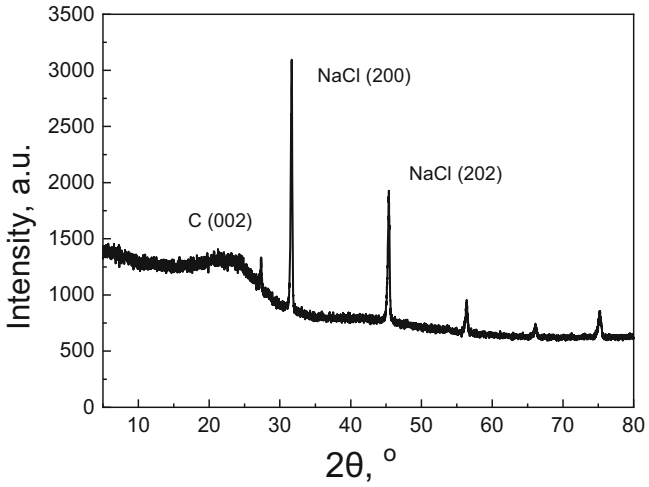

Figure 1. XRD pattern of the biochar used in this study.

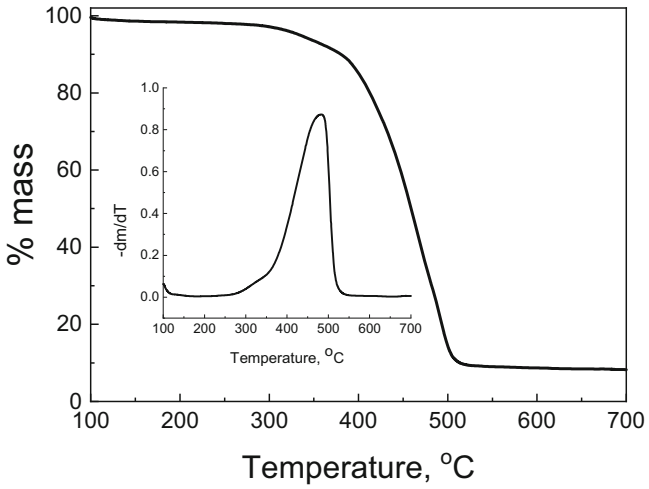

Figure 2. TGA curve of the biochar studied. The run was performed under air atmosphere with a flow of 20 mL min^{-1}. Inset: differential curve of the TGA curve.

The low starting and ending temperatures of the burn imply that the graphitization process was not complete, while the organic phase is almost homogenous. It should be noted that the TGA of raw olive kernels shows that the decomposition starts at a low temperature of 285 °C.[38] Generally, the main components of olive kernels are cellulose, hemicellulose and lignin with typical values of 31.9%, 21.9% and 25.6%, respectively. Also, fat and protein are present in considerable quantities, while acids are also present.[38] The high amounts of cellulose and hemicellulose can be transformed to graphitized carbon during pyrolysis, while the lignin demands higher thermal treatment. This was shown in a previous study[39] where higher pyrolysis temperatures transformed the carbonaceous phase of the biochar to a harder and more difficult-to-burn phase.

FTIR analysis is a helpful technique for the identification of the active groups and the bonds that are present in the biochar. The FTIR spectrum of the biochar can be seen in Fig. 3. The peaks in the FTIR spectrum denote the presence of adsorbed water molecules or –OH groups (3447 and 1037 cm^{-1}), while there is a significant amount of aromatic bonds –C=C– (1588 cm^{-1}).

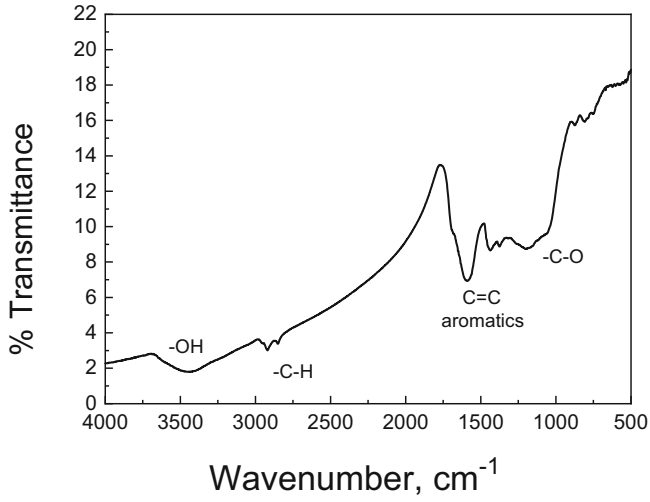

Figure 3. FTIR spectrum of the biochar studied.

This aromatic phase is poor in H, since no bonds are detected above 3000 cm^{-1}, while there are aliphatic –C–H bonds (double peak at about 2900 cm^{-1}).[40,41]

The acidity of the biochar can be estimated from the equilibrium pH of a suspension with a solid-to-liquid (water) ratio of 10% and left to equilibrate for 24 h. The equilibrium pH approaches the point of zero charge of the biochar. This was found to be 6.9, very close to neutral. Generally, this value was found to be significantly higher and in high basic region[4] due to the high amounts of minerals usually found in the form of oxides, and the basic surface groups. In our case, the minerals are mainly NaCl, a compound with no acid–base behavior and the basic surface groups neutralized from the significant fatty acid and phenol concentrations found in the olive kernels. The acidity of the biochar is a dominant factor for the adsorption properties. It was reported and proved with the molecular dynamics approach that the competition of dissolved ions with ionic pollutants for adsorption sites can decrease adsorption. Especially, for soft and polarizable ions such as chloride, it can be concluded that these ions can be adsorbed at the hydrophobic surface of biochar leaving fewer vacant sites available for organic active sites.[42]

Algal cultures

The initial pH for the flasks containing 1/3 N BG-11 was around 8.5 and for the ones without nitrogen and phosphorus was around 7.7. During the algal growth and due to metabolic activities, pH values changed and increased for all flasks containing 1/3 N BG-11 reaching up to 11 on day 4 and around 10.1 on day 15 (average value). For the flasks containing medium without N and P, on day 4 they had pH values around 7.7 and thereafter remained at the same level (Fig. 4(a)), indicating limited photosynthetic activity.

The development of biomass in the liquid was examined through the determination of TSS, cell concentration and OD_{650}. At the beginning of the experiment, the concentration of algal cells was higher in the case where the nutrient contained 1/3 nitrates, compared to the samples that were deprived of nitrogen and phosphorus. On day 0, the cell number for CB1 and ZNPCB1 was equal to 1.4×10^6 cells mL^{-1}. Afterward, during cultivation, the cell population was increased for all flasks and on the day 15, reached a concentration up to 11.2×10^6 cells mL^{-1} for CB5 and 10.4×10^6 cells mL^{-1} for ZNPCB5. The experimental data of

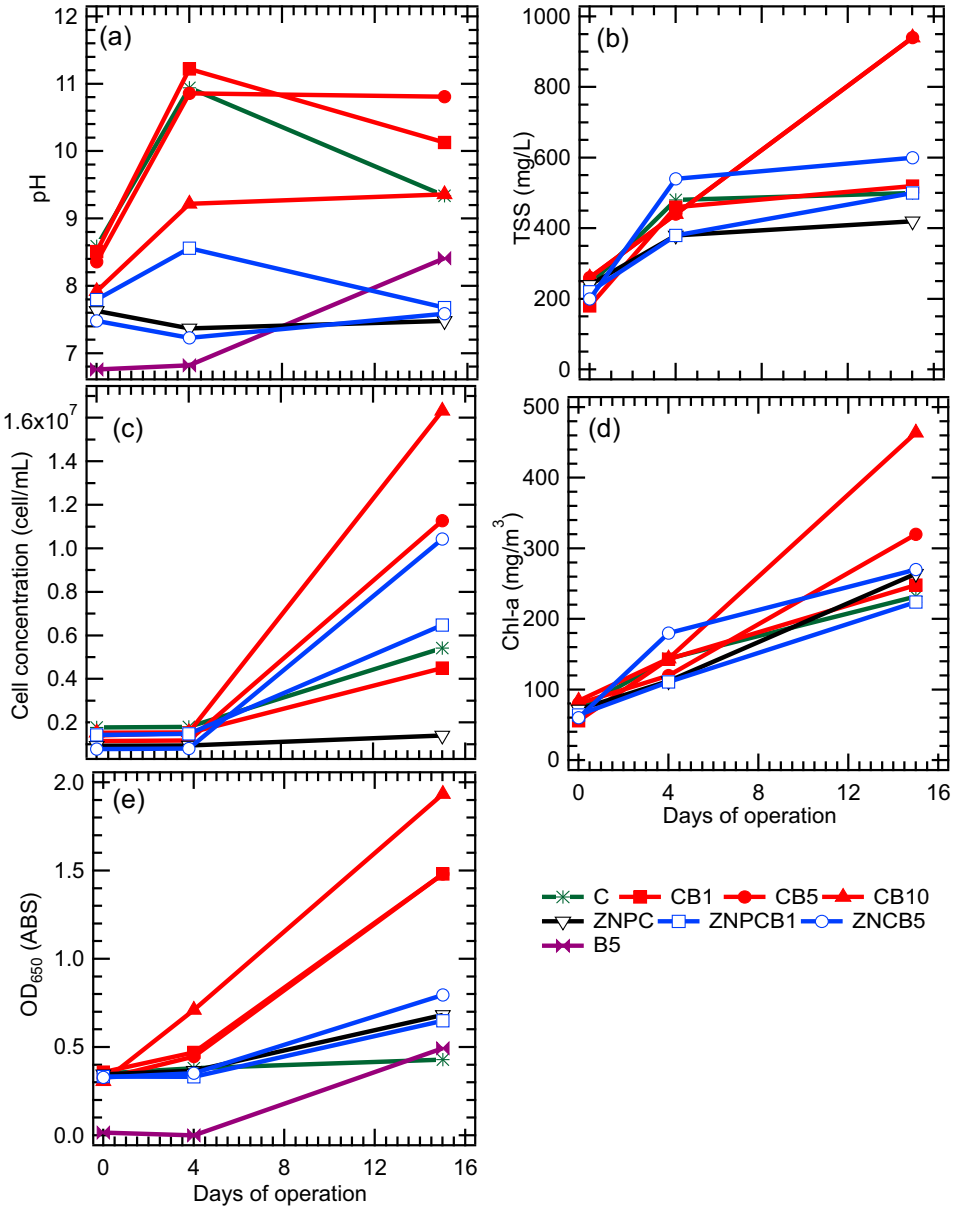

Figure 4. Cultivation of *Chlorococcum* sp. in different mediums (BG-11 enriched with 1/3 N and without N and P). Variation of (a) pH, (b) TSS, (c) cell concentration, (d) chl-a and (e) OD_{650} of *Chlorococcum* sp. in the liquid.

the biomass concentration in the liquid are illustrated in Fig. 4(e), (b), following a similar pattern to cell concentration. The samples at the beginning contained about 0.330 ABS and an initial concentration of 240 mg L^{-1}. On day 15, the culture CB10 contained a TSS concentration of 940 mg L^{-1} and 1.9 ABS, while ZNPCB5 reached up to 600 mg L^{-1} and 0.8 ABS. Tiny pieces that were not visible to the naked eye were detached from the biochar during cultivation, and this is why the concentration of TSS was found to be higher, even though the cell number of CB1 and ZNPCB1 was equal. A higher increase in almost all parameters is observed in the cultures containing 1/3 N BG-11. The chl-a concentration started from 70 mg m^{-3} and reached 464 mg m^{-3} for CB10 and 270 mg m^{-3} for ZNPCB5 cultures (Fig. 4(d)).

Microalgae can uptake a significant amount of nutrients such as nitrogen and phosphorus to synthesize proteins, nucleic acids and phospholipids. At the beginning of cultivation, flasks containing medium with 1/3 nitrogen had a concentration of nitrates of around 360 mgL^{-1}. After 4 days, the nitrates reduced to around 260 mg L^{-1} (27.7%), which were further reduced to zero at the end of cultivation. Nutrients are essential for the development

Table 2. Nutrient content of nitrates on day 0

Sample	Biochar (g)	NO_3^- (mg L^{-1})	Substrate
C	0	359	1/3 N BG-11
CB1	1	369	1/3 N BG-11
CB5	5	378	1/3 N BG-11
CB10	10	364	1/3 N BG-11
ZNPC	0	1.11	Z NP BG-11
ZNPCB1	1	7.07	Z NP BG-11
ZNPCB5	5	1.17	Z NP BG-11
B5	5	396	1/3 N BG-11

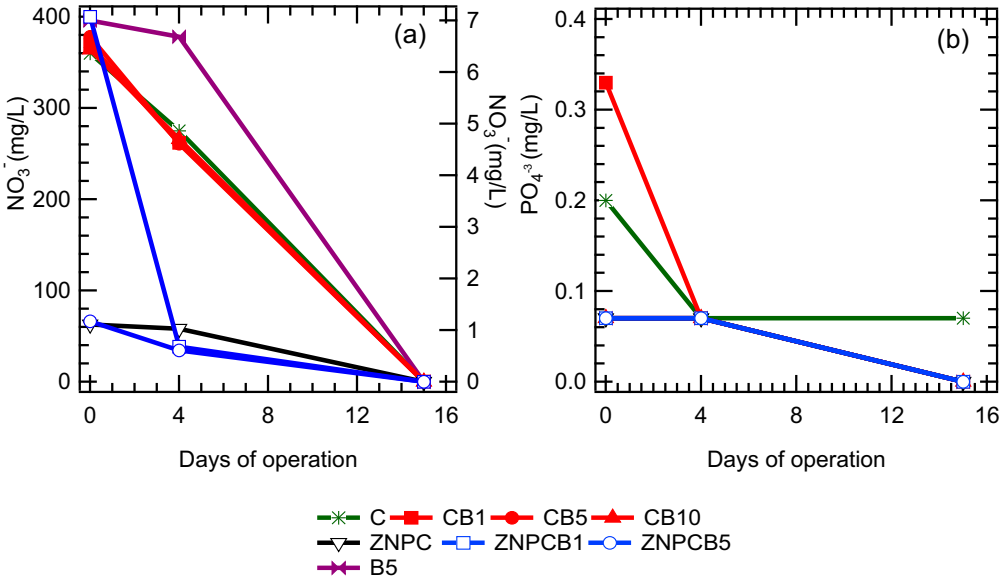

Figure 5. Cultivation of *Chlorococcum* sp. in different mediums (BG-11 enriched with 1/3 N and without N and P). Variation of (a) NO_3^- and (b) PO_4^{3-}. The left-hand *y*-axis shows the NO_3^- consumption of flasks containing 1/3 N BG-11, while the right-hand *y*-axis shows the NO_3^- consumption of flasks containing BG-11 without nitrogen and phosphates.

of microalgal growth. The data of the initial NO_3^- concentration are given in Table 2 and the consumption of nitrogen and phosphorus during cultivation is shown in Fig. 5.

The initial concentration of phosphates ranged from 0.07 to 0.33 mg L^{-1} for the samples containing 1/3 N BG-11, and around 0.07 mg L^{-1} for the sample with BG-11 without nitrogen and phosphorus. The phosphates were completely consumed by day 4, in contrast with nitrates, the determined values for which being significant in the same time interval needing additional time to be further decreased. Of particular interest are the findings for the samples where the medium did not contain nitrates. The concentration of nitrates at the beginning of cultivation was 1.1 mg L^{-1} for ZNPCB and ZNPCB5 and 7 mg L^{-1} for ZNPCB1 culture.

The composition of BG-11 gives the solution a maximum concentration of 364 mg L^{-1}. Beyond the samples which contained BG without N and P, a partially increased concentration of nitrates is also observed in samples CB1, CB5 and B5. This evidence suggests the leaching of nitrates from biochar. Nitrogen is the mineral nutrient most commonly applied in olive cultivation since it is a major nutritional factor affecting plant growth.[34] Nitrogen is stored in the kernels and this is the reason for nitrate appearance. The specific growth rate (μ) at day 15 of cultivation is shown in Fig. 6. The lowest growth rates are found in the cultures CB1 and ZNPCB1 with values of 0.077 and 0.1 d^{-1}, respectively. The latter makes sense since, as indicated above, these are the reactors containing biochar and have the highest concentration of microalgae attached to their surface.

Biomass productivity

On day 15, the biomass productivity was 1.28, 0.13, 0.07, 1.24 and 0.23 g g^{-1} biochar for CB1, CB5, CB10, ZNPCB1 and ZNPCB5 cultures, respectively (Table 3).

In both nutrient cases, samples containing 1 g of biochar performed better, resulting in similar biomass productivity. However, when a higher amount of biochar is present in the culture, it appears not to be beneficial for the attachment of more cells. This

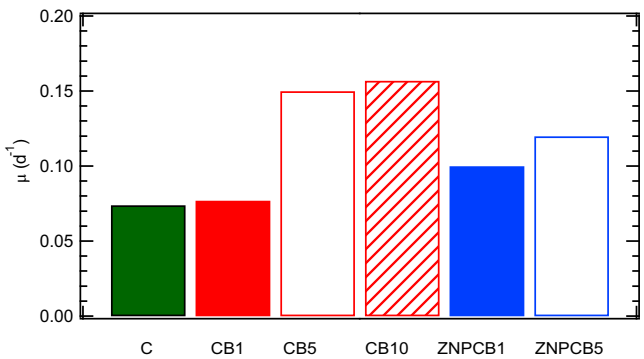

Figure 6. Growth rate of *Chlorococcum* sp. samples in different mediums (BG-11 enriched with 1/3 N and without N and P) on day 15.

evidence needs further investigation. Zhang *et al.* examined pine sawdust as an algal biofilm carrier for wastewater treatment with BG-11 medium, using *Chlorella vulgaris*, biomass production reaching 5.53 g m^{-2} d^{-1}.[17] Three types of lignocellulosic materials (rice husk, pine sawdust and sugarcane bagasse) were applied as bio-carriers for low-cost algal biofilm cultivation of three algal strains (*Chlorella vulgaris*, *Hydrodictyon reticulatum* and *Diplosphaera* sp.). Pine sawdust performed the best (9.61 g·m^{-2} d^{-1}) when cultured with *Diplosphaera* sp. biofilm.[43] Kholssi *et al.* examined *Anabaena cylindrica* using BG-11 culture medium with a biochar as solid support and its growth was enhanced by 80%.[44]

Oil mallee biochar in different concentrations was added to *Chlorella vulgaris* culture in a Tris–acetate–phosphate medium, and the biomass collected was increased from 4.7% to 8.2% in a culture containing 2 g L^{-1} biochar.[45] In our study, the culture liquid at the end of the experiment, including the volume removed to make the measurements and those lost due to evaporation, was finally 800 mL. Using the concentration of suspended solids in the liquid, the microalgal content was estimated. The total biomass (suspended and attached) of each examined sample is given

Table 3. Algal productivity and biomass content on day 15

Sample	Algal productivity (g microalgae g^{-1} biochar	Total proteins (mg L^{-1})	Total carbohydrates (mg L^{-1})	Total lipids (mg L^{-1})	Attached microalgae (mg L^{-1})	Suspended microalgae (mg L^{-1})
C	0.07	59.5	5.07	10.5	-	0.40
CB1	1.28	68.4	3.75	6.3	1.41	0.41
CB5	0.13	65.7	2.7	10.4	0.68	0.75
CB10	0.07	55.1	5.41	8.50	0.71	0.75
ZNPC	0.05	56.5	4.83	17.8	0	0.32
ZNPCB1	1.24	51.4	4.9	19.7	1.41	0.4
ZNPCB5	0.12	46	3.9	3.9	1.16	0.48

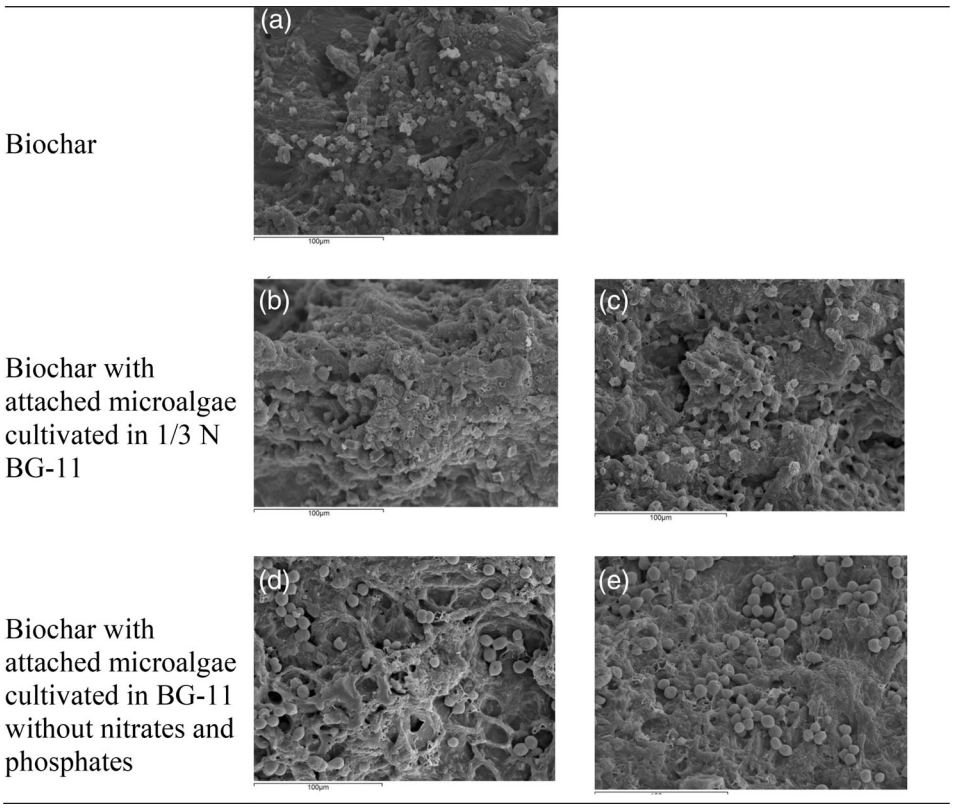

Figure 7. SEM images of *Chlorococcum* sp. attached on biochar surface (×500 magnification).

in Table 3 reaching up to 1.82 g for CB1 and 1.81 g for ZNPCB1. This evidence suggests the suitability of the examined biochar in algal cultivation. Total proteins and carbohydrates were measured in the liquid cultures (Table 3). The attached biomass cultured on biochar from olive kernels could not be fully recovered to quantify the EPS.

EPS is the matrix produced from microalgae attached on surfaces, where the inoculated cells could get adhered with each other and on the substrate.[46] The chemical composition of EPS is affected by various factors such as nutrients, temperature, pH and culture age. The major components of EPS include polysaccharides, proteins (i.e. enzymes and structural proteins), nucleic acid (DNA) and lipids.[47] The same pattern as biomass is followed by total proteins, with an amount of 69% in the case of CB1 and 56% for ZNPCB1. Equally high was the percentage of total carbohydrates for ZNPCB1 (5.4%), while on the contrary, CB10 gave 3.75% (Table 3).

The lipid content results for the case where the BG-11 medium contained 1/3 nitrates showed that the values remained low compared to the zero nitrogen and phosphorus cultures where the lipid content reached up to 20.3% for the ZNPCB5 culture (Table 3). Nitrogen is the most commonly reported nutrient-limiting factor of algal growth.[28]

According to previous research,[48] *Chlorococcum* sp. is a quite hydrophobic strain and the result that the acidity experiment gave about biochar indicated that it was also hydrophobic. Based on the XDLVO model, the reason for the existence of acid–base attraction between two hydrophobic or a hydrophilic and a hydrophobic surface is the hydrogen bonding component of the cohesion energy of water molecules that surround these

surfaces.[49] The formation of biofilm on different substrates has been much studied in the literature and various microalgae have been tested. Hydrophobic species could show the same tendency as *Chlorococcum* sp.; however, several parameters affect the whole process such as pH, temperature, CO_2 availability, light exposure, culture media, physicochemical parameters of the substrate, etc.[15,27] XRD and FTIR analyses gave insight into the surface groups and the recognition of the form of the elements contained in the examined biochar, while TGA indicated its stability. However, it is not yet clear as to the critical factor that is favorable for microalgal attachment. The acidity of the support may play an important role in biofilm formation[50] resulting in higher amounts of biofilm for more polar carriers, although the nature of the biofilm is not altered. Biofilm formation is a complex process that depends not only on the substratum but also on the strain itself.[15,16,48]

Figure 7 presents SEM images (at ×500 magnification), where the surface of the biochar appears to be covered with microalgae. As is seen, a dense algal film is developed on the biochar surface even in cultures cultivated in BG-11 which did contain nitrates and phosphates (Fig. 7(d), (e)).

Despite the large number of published papers dealing with the adhesion of algae and biofilm formation on various surfaces, it is still unclear as to which are the exact parameters affecting biofilm development. The usual concept is to examine the physicochemical properties of each material tested, to have an insight into understanding the adhesion process. The next step is to try to figure out the chemical substances of EPS secreted. To our knowledge, very few studies have examined biochar as a substratum for the growth of algae and especially microalgae. The positive results of the present work showed, however, the ability of biochar to attract microalgae and to be able to produce biofilm, and it certainly is worthy of further investigation.

CONCLUSION

Biochar from pyrolyzed olive kernels at 400 °C was considered as a candidate substratum for algal biofilm development. Due to low pyrolysis temperature the biochar has low SSA, and almost neutral point of zero charge. The minerals present are mainly NaCl. The presence of biochar enhanced algal growth and the biomass attached to the biochar was about three times greater than the biomass grown in the control unit (without biochar carriers). The samples with the better performance were CB1 and ZNPCB1 with similar productivity up to 1.41 mg L^{-1}. Generally, total proteins were more than total carbohydrates and lipids. In the samples with the most biochar included (CB10 and ZNPCB5), the percentage of total protein was reduced, in both cases of media studied, while total carbohydrates do not follow this pattern. Pyrolyzed kernels seem to release nitrates; however, higher quantities of kernels are inhibitory for algal growth and adhesion. Unfortunately, the physicochemical characteristics examined did not give an insight into the biofilm attachment of the biochar. However, the findings of this work show that biochar from olive kernels is capable of attracting algal cells and of promoting algal growth, and is worth applying in the future as an innovative substratum for biofilm development.

AUTHOR CONTRIBUTION

VDT and JV Investigation; IDM Supervision; IDM Conceptualization; VDT and JV Writing – original draft preparation. All authors read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflict of interests, informed consent, or human or animal rights are applicable to this study.

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