Enhancing Biofilm Formation and Microalgae Growth by Preparing Cellulose Substrate With Rough Surface

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Abstract

A series of cellulose films with rough surface were prepared by employing a simple solution casting method and using the waterproof abrasive papers with different grits as the substrate. The cellulose films possessed a rough surface with the maximum height difference (S_z) of 128-217 μm, a macroporous structure with a high porosity of 84.7%-90.5%, and a negative potential between -40.00 and -54.15 mV. Furthermore, the cellulose films exhibited excellent microalgae adhesion properties. After 18 days of attached *Chlorella sp.* cultivation experiments, the average productivities of C-A-120 films (C-A-X, X means the mesh number of the substrate) reached 20.80 g m^{-2} d^{-1}), which is 2.69 times than that of the cellulose film with a smooth surface. The result indicates that the cellulose films with a rough surface and high water adsorption ratio have a huge potential in serving as the substrate of the attached microalgae cultivation to promote microalgae cells growth and biofilm formation.

1. Introduction

Microalgae have been regarded as one of the most promising sources for biofuel (Benemann 2013; Maeda et al. 2018) and other value-added products (Borowitzka 2013; Matos et al. 2017) due to their fast growth rates and high value-added bioproducts production. However, it is still a great challenge to achieve a large-scale commercialized production of microalgae, because one of the key challenges is the high cost of microalgae harvesting and dewatering in conventional suspended culture systems (Bharathiraja 2015; Pierobon 2018). Recently, attached cultivation based on microalgae biofilm has received widespread attention because of the low water demand, the ease of biomass harvesting and its higher concentration (Wang et al. 2017; Gross et al. 2015). For this mode, the cells are fixed to the solid substrate surfaces, which made them separate easily from the culturing medium and significantly reduced the cost of microalgae harvesting and separation.

During the attached cultivation process, the formation of microalgae biofilm depends on various factors including microalgae species, substrate materials, culture conditions, hydrodynamic conditions, et al. (Zhuang et al. 2018) The substrate material is one of the most important factors in the development of an attached culture system, because it influences the initial adhesion and the subsequent growth of the microalgae biofilm (Schnurr and Allen, 2015). Up to now, various materials used in the microalgae attachment have been selected (Rosli et al. 2020). Cellulose material has been regarded as one of the most potential materials due to its high hydrophilicity, non-biotoxicity, low price, and porosity (Zhang et al. 2017). Several kinds of cellulose materials, such as cellulose acetate/nitrate membrane (Ji et al. 2014), newspaper (Naumann et al. 2013), printing paper (Bernstein et al. 2014) and cotton rope (Christenson and Sims 2012; Bernstein et al. 2014), have been evaluated. However, the reported cellulose-based substrate materials are directly obtained from the market, and their structures have not been controlled and designed. Thus, in order to obtain high microalgae productivity and understand the relationship between the structure of cellulose materials and the microalgae growth, it is urgently needed to develop new cellulose materials with novel and defined structures to serve as the substrate of microalgae cells in

In this work, by changing the roughness of the substrate (glass, waterproof abrasive paper with different roughness), a series of cellulose films with different surface roughness were prepared by a solution casting method in ionic liquids. Subsequently, they were used as the supported materials in attached microalgae cultivation (Fig. 1). The structure and properties of cellulose films were characterized by Fourier transform infrared (FTIR) spectra, zeta potential, scanning electron microscopy (SEM), mercury porosimetry and water absorption experiment. The effect of surface roughness on the microalgae adhesion of cellulose films was investigated by attached *Chlorella sp.* cultivation in 24-well tissue culture plate.

2. Experimental

2.1 Materials

The strain *Chlorella sp.* FACHB-1514 was obtained from Institute of Hydrobiology, Chinese Academy of Sciences, which was grown and maintained in sterilized BG 11 medium at 25 ± 2 ^{o}C under a light intensity of 100 μmol m^{-2} s^{-1} in HZQ-QG light incubator (HDL Apparatus) (Yan et al. 2016). The culture with optical density (OD_{680}) about 0.3 was used as the seed for the subsequent attached cultivation experiments. The cellulose (cotton pulp) with a degree of polymerization (DP) of 620 and cellulose content of bigger than 98% was supplied by Hubei Chemical Company Limited (Xiangfan, China), which was dried at 80 ^{o}C under vacuum for 6 h prior to use. The ionic liquid, 1-allyl-3-methylimidazolium chloride (AmimCl), was obtained from Institute of Chemistry, Chinese Academy of Sciences. The water content in the resultant ionic liquid was less than 0.3% as measured by Carl-Fisher method. All the inorganic salts and organic solvents were analytical reagents and used without further purification.

2.2 Preparation of cellulose films

The cellulose films with different surface roughness were prepared in AmimCl using dissolution and regeneration method (Fig. 1) by changing the surface roughness of the substrate. Glass plate and waterproof abrasive papers with different micrometer-sized morphology (40 mesh, 80 mesh, 120 mesh and 300 mesh) were used as the substrate. A typical preparation procedure used is as follows: 4.0 g of cellulose was dispersed into 96.0 g of AmimCl in a flask, and then the mixture was stirred at 80 ^{o}C for 1.5 h to ensure a complete dissolution of cellulose. The solution was degassed in vacuum, then cast onto the abrasive paper (40 mesh) and immediately coagulated in 60 ^{o}C deionized water to make regenerated cellulose films. To remove residual ionic liquid AmimCl in the cellulose films, they were further washed with distilled water at least three times until no Cl^{-} ions were detectable by the $AgNO_3$ test. After solvent exchange with tert-butanol and further freeze drying, the cellulose film (C-A-40) was obtained and kept in a desiccator prior to characterization.

2.3 Microalgae biofilm cultivation

The microalgae adhesion properties of the cellulose films were explored by attached *Chlorella sp.* cultivation procedure used is as follows: the

cellulose films were firstly cut into pieces with the diameter of 1.5 cm, and then were immersed into a beaker with BG 11 media. Then, the cellulose films were placed in the wells of a sterile and transparent 24-well tissue culture plate. The algal culture solutions of 200 μL were slowly added on the cellulose film surface, then the plate was kept in HZQ-QG light incubator (HDL Apparatus) under a light intensity of 100 μmol m^{-2} s^{-1} and at a temperature of 25 ± 2 °C. The sterilized BG 11 medium of 500 μL was added into each well by slowly dripping from the side of the culture-well every 24 h. The fluorescence intensity of attached microalgae cells on the cellulose surface was measured every 3 days. The cellulose film was taken out and rinsed thrice with water to remove planktonic microalgae cells, then the absorbance was measured at 570 nm using a multi well plate reader (Infinite M200, TECAN, Switzerland). Moreover, the surface changes of the cellulose films during the attached microalgae cultivation experiment were observed using photographs and light microscope.

At the end of the attached cultivation experiments (with a total duration of 18 days), the attached microalgae on the surface of cellulose film were quantified by measuring the cell dry weight. The cellulose film was washed ultrasonically three times with deionized water to remove the attached microalgae, then all the washed solution was collected and centrifugated at 8000 rpm to separate the microalgae. The microalgae were dried by freeze drying and then weighed using an analytical scale (XS105DU, Mettler Toledo, Switzerland).

The attached microalgae productivity (g m^{-2} d^{-1}) was calculated as the Eq. (1):

$$\text{Productivity} = (W_1 - W_0) / (A \times t)$$

1

Where W_0 and W_1 are the dry weight of the inoculated and harvested microalgae biomass, respectively. A is the surface area of the cellulose film, which is equal to 1.77×10^{-4} m^2. t is the days of the attached cultivation.

2.4 Measurements

The surface morphology of cellulose films was observed on a Leica DMI4000B inverted fluorescence microscope. The cross section of cellulose films was observed on a JSM-6700F JEOL scanning electron microscope (SEM) at an accelerating voltage of 10 kV. The specimens were coated with platinum before observation. The pore size and its distribution of cellulose films were measured using an AutoPore IV (Micromeritics, USA) instrument.

The surface zeta potential of cellulose films was measured with SurPass zeta potential analyzer (Anton Paar, Austria) at room temperature using BG11 medium (pH = 7.5–7.8) as the electrolyte, and the potential was measured three times to get the average potential for each sample.

The ATR-FTIR spectra of pulp cellulose and regenerated cellulose films were measured with Nicolet™ IS5 [illegible] emperature with a resolution of 4 cm^{-1} and 24

scans per sample.

The water absorption ratio (W_A) of cellulose films was quantified by measuring the dry weight (W_D) and wet weight (W_W) of cellulose films. The cellulose film was dried at 80°C under reduced pressure until constant weight and then was cooled down to room temperature. The sample was taken out and weighed immediately. The weighted cellulose film was submerged in water at 25°C for 48 h, then the water on the surface was removed by filter papers and the cellulose film was weighed. The water absorption ratio (W_A) was calculated using the equations (2) as follows:

$$W_A(\%) = (W_W - W_D) \times 100 / W_D$$

2

The arithmetic mean height (Sa) and the maximum height of the cellulose film surface (Sz) were measured with a confocal laser microscope with a program (OLS4000, Olympus, Massachusetts, USA), which allowed the measurement of surface roughness in a linear manner and in specific areas. The images were captured with a 10 magnification and 0.2 μm registration accuracy. The central area of the cellulose films (500 μm^2) was selected to analyze the Sa and Sz of the cellulose films that was expressed as a numerical value (μm). Three equidistant measurements were performed for each specimen in three different areas.

3. Results And Discussion

3.1 The structure and properties of cellulose films

In order to characterize the morphology of the cellulose films, they were lyophilized to maintain their microstructure. The surface morphology of the prepared cellulose film was studied by visually and optical microscope. The photographs of cellulose films are shown in Fig. 2a. It can be seen that the cellulose films are white and opaque sheets. The photographs also show that the C-G film exhibits smooth surface, while the C-A films exhibit coarse surfaces with irregular shape and a change in the morphology occurs for different C-A films due to the surface roughness of the substrate during the regeneration process. To gain more insights into the surface morphology of those cellulose films, optical microscope observations were further carried out. As shown in Fig. 2b, it can be observed that a large number of concaves cover the surface of the C-A films with diameter of dozens to hundreds μm, and the diameter of the concaves for different C-A films decreases with the increase of the mesh of the substrate, while there is no obvious concave on the surface of C-G films.

Since the microalgae adhesion capacity is highly dependent on the surface properties and structure of the cellulose substrate such as surface hydrophilicity/hydrophobicity, surface charge and surface roughness/micropattern. An attempt was made to measure the water contact angle of the cellulose films, but failed due to the porous structure and water adsorption behavior of the cellulose films. The zeta

 ium at pH 7.5–7.8, which is illustrated in Table.

1. The zeta potential of all the cellulose films exhibited negative values ranging from − 40.00 ± 3.31 mV to -54.15 ± 3.84 mV probably due to the full deprotonation of OH in the cellulose skeleton, which is similar to that of cellulose reported in previous research (Qian et al. 2019). The surface roughness of the cellulose films was measured using confocal laser-scanning microscopy (CLSM), which reports the vertical deviation from the actual surface using the arithmetic mean height (Sa) and the maximum height of the profile (Sz). Sa provides statistically stable and more accurate measurement with optical profilometry. However, it has limitations to differentiate between peaks and valleys on the surface. Sz is more sensitive to peaks and valleys than that of Sa, thus both Sa and Sz were measured. As shown in Table 1, the C-A-120 owned the roughest surface (Sa = 26.17, Sz = 217). This is followed by C-A-80, C-A-40, C-A-300, the smoothest surface of C-G being 15.68 μm (Sa) and 108 μm (Sz). The C-A films show a significant difference between Sa and Sq due to the difference of the substrate, which indicates that it is feasible to design the surface roughness of cellulose film by changing the roughness of the substrate. All the cellulose films were further utilized as the substrate of attached microalgae cultivation and the effect of the surface roughness on the microalgae adhesion was investigated.

Table 1
Preparation and characterization of cellulose films.

Samples	Total intrusion volume* (mL/g)	Average pore diameter (nm)	Porosity (%)	Water absorption ratio (%)	Zeta potential (mV)	RSa (μm)	RSz (μm)
C-A-40	8.57	285.19	90.51	253	-46.47 ± 3.60	21.43	166
C-A-80	5.56	200.68	86.24	306	-49.22 ± 3.19	26.14	211
C-A-120	7.74	258.92	89.13	419	-40.00 ± 3.31	26.17	217
C-A-300	4.93	185.52	84.68	222	-54.15 ± 3.84	18.44	128
C-G	8.68	303.51	89.20	174	-41.12 ± 2.49	15.68	108

* Total intrusion volume was measured at $3*10^4$ psi.

The IR spectra of the cellulose pulp and regenerated C-A-40 film are shown in Fig. 3a. The spectra of C-A-40 is similar to the native cotton pulp, indicating that no chemical reaction occurs during the preparation of the cellulose film. Similar results were also reported in the other reports about the cellulose dissolution and regeneration process in ionic liquids (Zhang et al. 2005). A broad vibration band around 3284 cm^{-1} is assigned to the O-H vibrations of cotton pulp, while the -OH stretching vibration band in the C-A-40 shifts to a higher frequency (3337 cm^{-1}) and becomes sharper and narrower as a result of splitting hydrogen bonds to some extent during the dissolution and regeneration process (De Silva et al. 2015). A [illegible] cm^{-1} is [illegible] in the C-A-40 but not in the cotton pulp, which is assigned

to the C-O stretching vibration in the amorphous region (Zhang et al. 2005). The absorption band at 1426 cm^{-1} is assigned to the CH_2 scissoring motion for the cotton pulp, while it weakens and shifts to a lower wavenumber at 1421 cm^{-1} for C-A-40 due to the destruction of the intramolecular hydrogen bond.

The porous structure of the cellulose films was determined by SEM and mercury porosimeter measurements. The cross-section SEM images of cellulose films are shown in Fig. 2c. The porosity, total intrusion volume, average pore diameter and pore size distribution are shown in Table 1 and Fig. 3b. All the cellulose films possessed high porosities of more than 84.68%, the average pore diameter is in range of 185.52-303.51 nm, which is beneficial to water adsorption. For these cellulose films, the porous structure is formed primarily in the regeneration step. The gelation of the cellulose solution proceeds after immersing the cellulose solution into 60 ^oC deionized water, so the phase separation occurs very quickly due to the high driving force caused by the large concentration gradient and high diffusion coefficient of the ionic liquids in the bath of hot water, resulting in large pores in the cellulose films (Mi et al. 2016). Obviously, there is no relationship between the porosity and average pore diameter and the substrate used in the preparation of cellulose films. The pore volume distributions of the cellulose films are shown in Fig. 3b, the results suggested that the pore size distribution of all the cellulose films is similar and covers the size range between 100 nm and 3 μm, which is less than the size of microalgae and makes the microalgae adhere the surface but not the inner of the cellulose films in attached microalgae cultivation.

The water adsorption ability is of great importance for cellulose substrate in attached microalgae cultivation, which decreases liquid evaporation and maintains moist environment during attached microalgae cultivation. The water adsorption ratio of cellulose films was measured and shown in Table 1. It is found that all the cellulose films display high water-uptake properties with higher than 174% due to their water affinity and porous structure (Jiang et al. 2019). The C-A-120 shows the highest water adsorption ratio (419%), which enables them to absorb a great deal of microalgae cultivation liquid and benefits for the growth of microalgae. The rough surface and good water adsorption ability of cellulose films make them potential in serving as the substrate in the attached microalgae cultivation.

3.4 Microalgae adhesion and growth properties on cellulose films

The surface changes of cellulose films during the attached microalgae cultivation were observed visually by light microscope. The photographs of cellulose films at different time intervals are shown in Fig. 4a. The C-A-120 becomes green at 3th day, while C-G shows green color until 12th day. Moreover, the surface of C-A-120 is much greener and more area is covered with green colour than those of C-G gels until the end of the cultivation. Similar results are also observed by light microscope as shown in Fig. 4b. After 6 days of cultivation, the surface of C-A-120 has been covered completely by microalgae, while the surface of C-G is covered with microalgae partially even after 18 days of cultivation. The growth of microalgae cells on the surface of cellulose films is also conducted by measuring the photosynthetic activity of the cells as an indication of the cellular growth at different time intervals during attached microalgae cultivation. As shown in Fig. 5, the fluorescence intensity on the surface of all C-A films is higher than

that of C-G films. For example, the fluorescence intensity of the C-A-120 is 1.78 times than that of C-G after the 18 days cultivation, indicating that more microalgae cells adhere on the surface of the C-A-120.

The areal productivity of *Chlorella sp.* on the surface of cellulose films were measured and shown in Fig. 5b. After 18 days of cultivation, the area productivity of *Chlorella sp.* biofilm in C-A-40, C-A-80, C-A-120, C-A-300 is 16.28 ± 2.97, 10.44 ± 1.17, 20.8 ± 3.76 and 9.04 ± 2.24 g m^{-2} d^{-1}, respectively, which are 110.6%, 35.06%, 169% and 16.95% higher than 7.73 ± 3.99 g m^{-2} d^{-1} attained in the C-G. The C-A-120 shows the highest area productivity, illustrating a positive correlation between microalgae productivity and the surface roughness of cellulose films. The cellulose films with rougher surface have a relatively larger surface area and deeper grooves, so a bigger microalgae biofilm productivity is obtained (Gross et al. 2016; Huang et al. 2018). Up to now, numerous studies about substrate materials have been witnessed that the substrate materials with an appropriate rough/textured surface provides a “shelter” for the attached microalgae and makes the sloughing of the attached microalgae significantly reduce (Zhang et al. 2020; Guo et al.2019). Moreover, the C-A-120 gains the maximum productivity of 20.8 ± 3.76 g m^{-2} d^{-1}, which is also much higher than that of most cellulose-based substrates in previous reports (Fig. 5c) (Berner et al. 2015), such as 10.92 g m^{-2} d^{-1} for pine sawdust (Zhang et al. 2017), 14 g m^{-2} d^{-1} for cotton rope (Christenson and Sims 2012), 4.29 g m^{-2} d^{-1} for cotton duct (Gross et al. 2013), 9.16 g m^{-2} d^{-1} for cellulose acetate/nitrate filters (Ji et al. 2014). Thus, development of cellulose substrate materials with controllable roughed/textured surface can drastically strengthen algal attachment.

Conclusions

A series of cellulose films have been prepared from an AmimCl solution of cellulose by solution casting using waterproof abrasive paper as the substrate. The cellulose films possess rough surface and macroporous structure together with good water absorption properties. Moreover, the cellulose films exhibit good microalgae adhesion properties. After 18 days of cultivation, the maximum productivity of *Chlorella sp.* biofilm in C-A-40, C-A-80, C-A-120, C-A-300 reaches 16.28 ± 2.97, 10.44 ± 1.17, 20.8 ± 3.76 and 9.04 ± 2.24 g m^{-2} d^{-1}, respectively, which are 110.6%, 35.06%, 169% and 16.95% higher than 7.73 ± 3.99 g m^{-2} d^{-1} attained on the C-G with a smooth surface. These cellulose films with a rough surface and high water adsorption ratio can be considered as the potential candidate for substrate materials in attached microalgae cultivation.

Declarations

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Human and animal rights

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figures

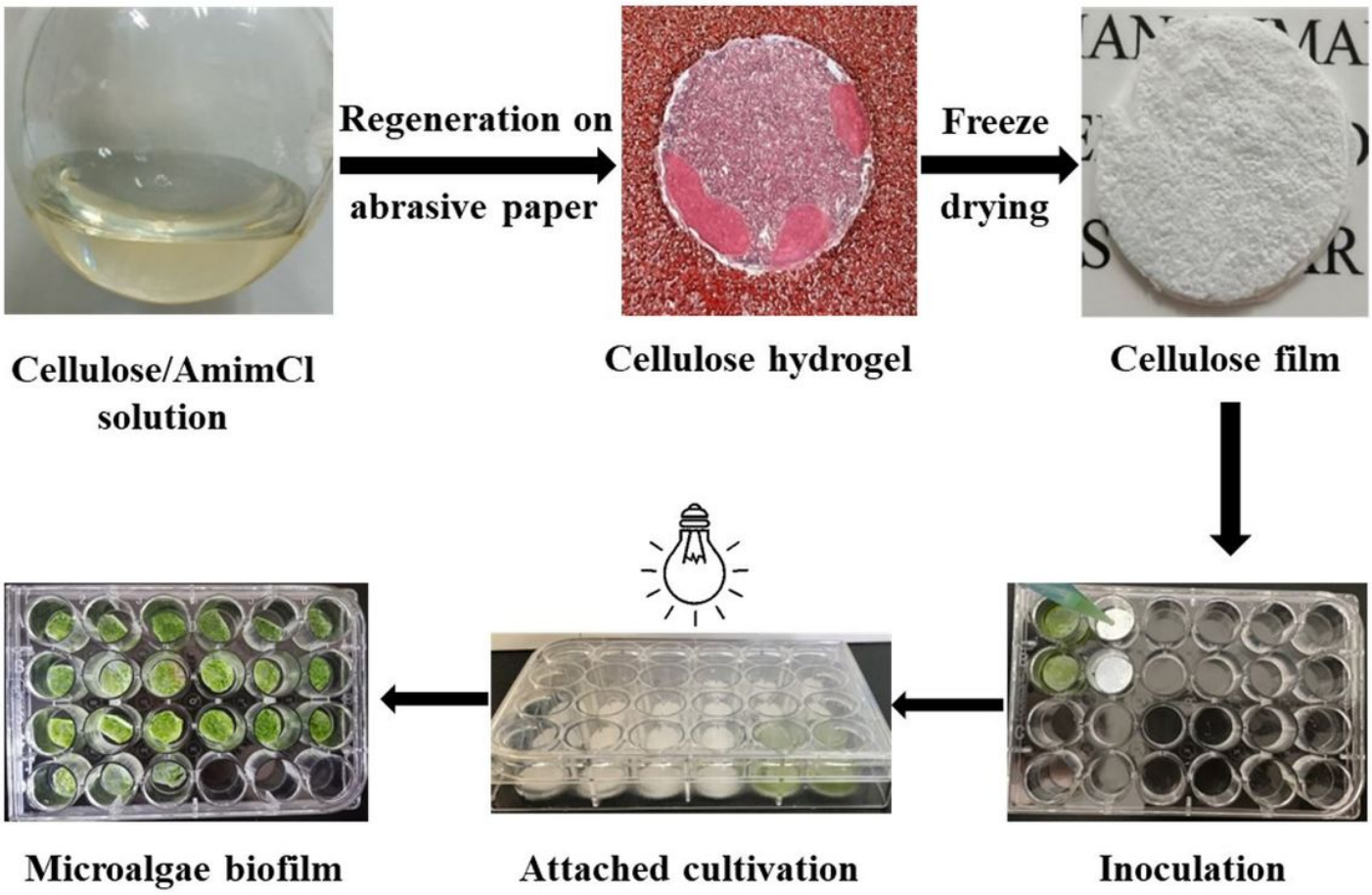

Figure 1

Schematic illustration of the fabrication process of the cellulose films and their application as the substrate materials in attached microalgae cultivation

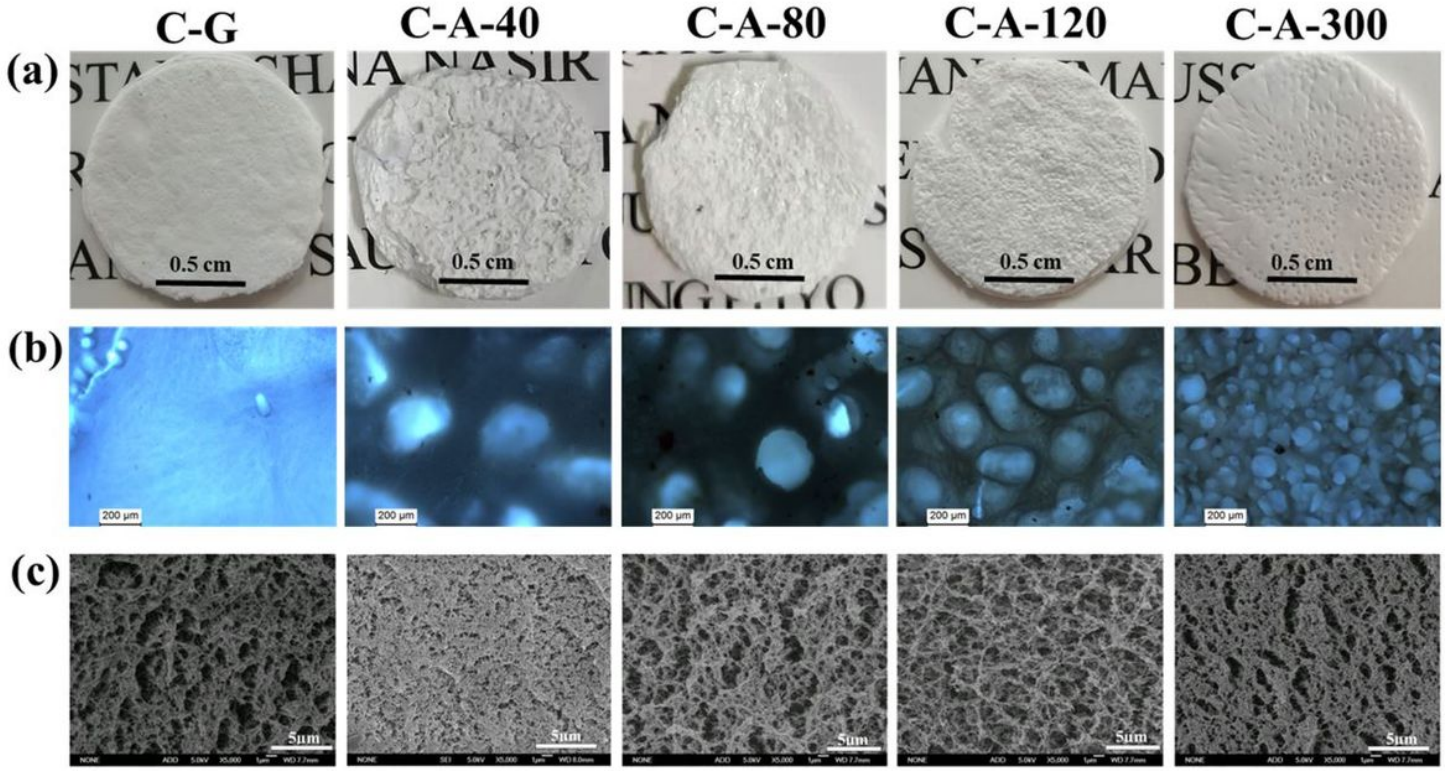

Figure 2

(a) Photographs, (b) light microscope images and (c) SEM cross section images of cellulose films.

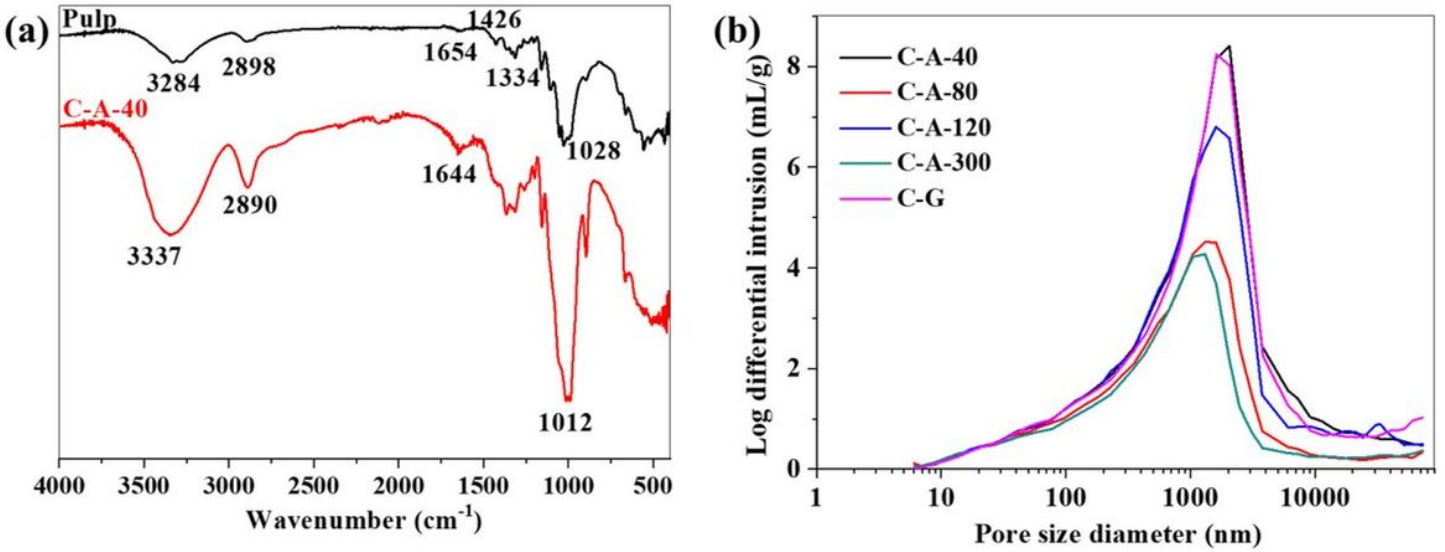

Figure 3

ATR-FTIR spectra and pore size distribution of cellulose films. (a) ATR-FTIR spectra of cellulose films; (b) Pore size distribution of cellulose films according to mercury porosimeter measurements.

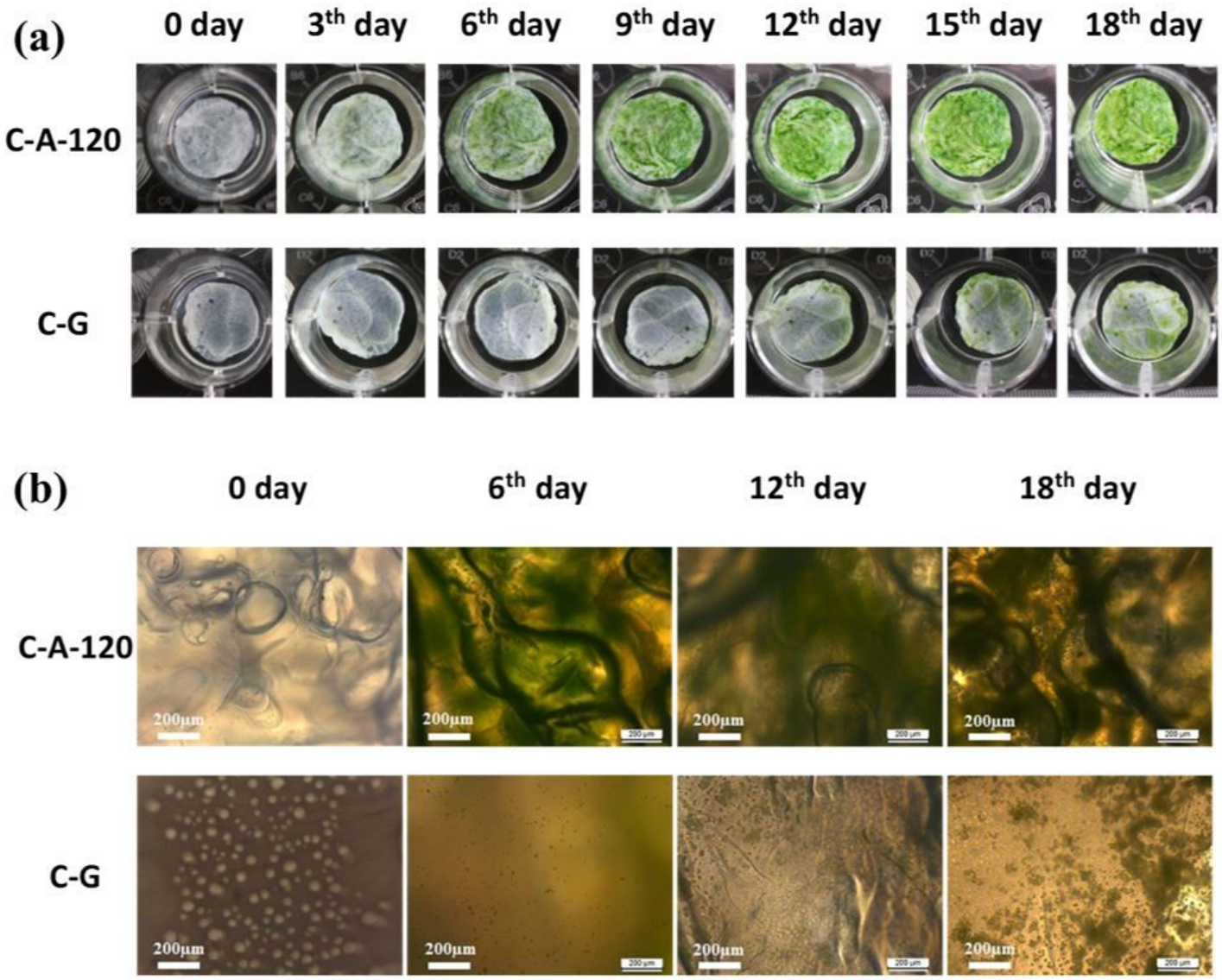

Figure 4

(a) Photographs and (b) light microscope images of cellulose films substrate surface in attached cultivation at different time intervals.

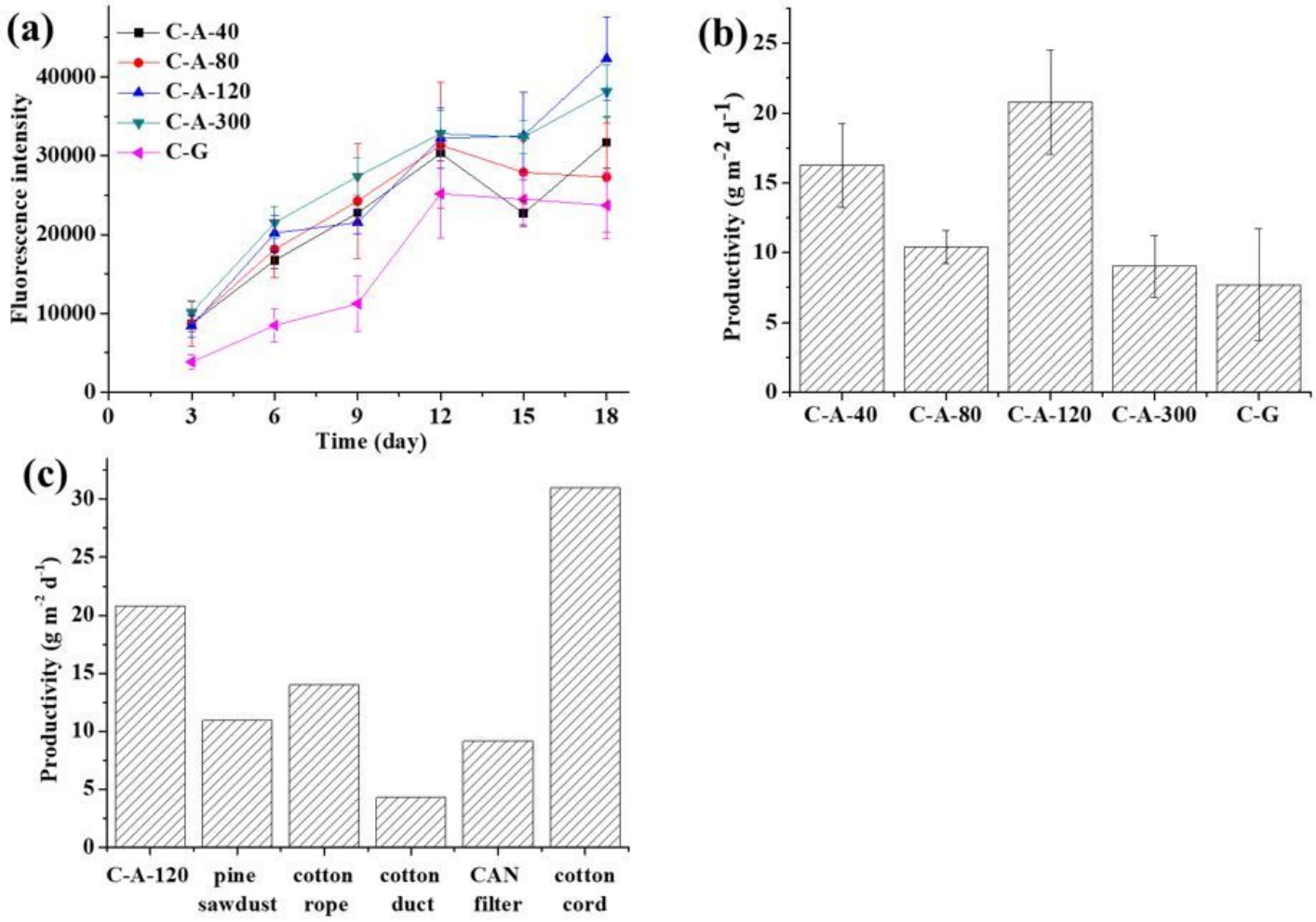

Figure 5

Photosynthetic activities and productivities of Chlorella sp. on the surface of cellulose films. (a) Photosynthetic activities of Chlorella sp. on the surface of cellulose films at different time intervals; (b) Productivities of Chlorella sp. on the surface of cellulose films for 18 days; (c) Productivities of microalgae on the surface of different cellulose substrate materials. Data are shown as mean ± SD, n=3.