Influence of culture conditions on the production of extracellular polymeric substances (EPS) by *Arthrospira platensis*

Mariana Barbalho Silva (mariana_barbalho@ufrj.br)
Universidade Federal do Rio de Janeiro https://orcid.org/0000-0002-5053-409X

Edwin G. Azero
2Departamento de Ciências Naturais, Universidade Federal do Estado do Rio de Janeiro, Avenida Pasteur 458, 22290-240 Rio de Janeiro, Brazil

Cláudia M. L. L. Teixeira
3Laboratório de Biotecnologia de Microalgas,Instituto Nacional de Tecnologia, Avenida Venezuela 82, 20081-312, Rio de Janeiro, RJ, Brazil

Cristina T. Andrade
1Programa de Pós-Graduação em Ciência de Alimentos, Instituto de Química, Universidade Federal do Rio de Janeiro, Avenida Athos da Silveira Ramos 149, 21941-909 Rio de Janeiro, RJ, Brazil. 4Instituto de Macromoléculas Professora Eloisa Mano, Universidade F

Research

Keywords: Arthrospira platensis; Cyanobacteria; Extracellular Polymeric Substances

Posted Date: August 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-21475/v3

Version of Record: A version of this preprint was published at Bioresources and Bioprocessing on August 16th, 2020. See the published version at https://doi.org/10.1186/s40643-020-00337-3.

Abstract

Arthrospira platensis is a cyanobacterium that is of great biotechnological interest, particularly for the food industry, as it possesses a high content of proteins, pigments, lipids and carbohydrates. Cyanobacteria produce extracellular polymeric substances (EPS), which are co-products of secondary metabolism that present thickening or gelling properties. A 3-level factorial design was used to study the combined effect of different nitrate concentrations and photon flux density (PFD) values to evaluate the biomass and EPS production of *A. platensis*. The best result in terms of biomass production was obtained under condition 6 (2 $g.L^{-1}$ $NaNO_3$ and 600 $\mu E.m^{-2}.s^{-1}$) yielding a concentration of 1.292 $g.L^{-1}$. However, condition 1 (0.25 $g.L^{-1}$ $NaNO_3$ and 200 $\mu E.m^{-2}.s^{-1}$) produced the greatest EPS yield (111 $mg.g^{-1}$), followed by condition 9 (2 $g.L^{-1}$ $NaNO_3$ and 1000 $\mu E.m^{-2}.s^{-1}$). FTIR analyses of EPS samples indicated the presence of carboxylate and sulfate functional groups, and rheological studies of the EPS at 5 and 10 $g.L^{-1}$ revealed a dilute solution behavior.

Introduction

The phylum of cyanobacteria comprises a group of diverse prokaryotes. These microorganisms typically use autotrophic metabolism, but can also be grown in heterotrophic and mixotrophic conditions, and are commonly found in brackish or marine environments (Baldev et al. 2015). They are responsible for the incorporation of a large proportion of atmospheric CO_2 and some of them are N_2 fixers (Oliver and Atsumi 2014). Cyanobacteria produce extracellular polymeric substances (EPS), which have a protective function in biofilm formation, a mechanical barrier against desiccation (Decho and Gutierrez 2017), and which also aids in the absorption of heavy metals (Goo et al. 2013). The composition of EPS depends on the species. Generally, they are formed by various substances, such as proteins, polysaccharides, lipids, humic-like substances, DNA, lipopolysaccharides and glycoprotein heteropolymers (Can et al. 2019). EPS may be entirely released into the extracellular environment or may be associated with the cell surface as sheaths, capsules or slime (Pereira et al. 2009).

EPS have attracted industrial interest because of their polyanionic character and that they are a renewable source. They can be used as thickeners, emulsifying agents and biosurfactants, with applications in the food and biomedical industry. Antibacterial (Mundt et al. 2001), antioxidant (Trabelsi et al. 2016) and anti-inflammatory properties (Xiao et al. 2018) have already been reported for EPS of cyanobacteria.

Arthrospira platensis, also called *Spirulina platensis*, is a filamentous blue-green cyanobacterium, belonging to the order *Cyanophycea*, division *Cyanophyta* (Manirafasha et al. 2018). Its biomass is used in human food due to its high protein content (approximately 70% of dry weight). It also produces pigments such as carotenoids and phycocyanin, polyunsaturated fatty acids, several vitamins, minerals and other constituents with antioxidant activity (Gong et al. 2008; Shabana et al. 2017). Sulfated heteropolysaccharides, frequently formed by neutral (xylose, galactose, glucose, fructose, rhamnose,

arabinose and mannose) and uronic acid (galacturonic and glucuronic acids) repeating units, have been identified in the EPS of this species (Trabelsi et al. 2009). Literature reviews suggest that the EPS from *A. platensis* have specific properties, which may contribute to human health. A fraction containing sulfated polysaccharides, at concentrations between 18 and 36 µg/mL, was shown to inhibit the replication of Koi herpes virus (Reichert et al. 2017). Challouf et al. (2011) reported antibacterial activity against *Salmonella typhimurium* and *Staphylococcus epidermis.* Furthermore, antioxidant activity of crude EPS was described by Dejsungkranont et al. (2017).

Although EPS could contribute to numerous industrial areas, the cost of the current extraction and purification procedures, the low yield and the lack of knowledge regarding their structure impede their commercial use at this time. In order to minimize costs and enhance EPS yields, optimization of the culture parameters may be necessary. EPS production by cyanobacteria can be influenced by environmental and nutritional conditions, including temperature (Trabelsi et al. 2009), photonic flux density (PFD) (Villay et al. 2013) and nitrogen starvation (Arad and Levy 2010). The right combination of these parameters could greatly improve EPS production. However, only a few studies have been conducted with the objective to enhance the EPS yield from species of cyanobacteria. For example, the temperature and PFD parameters were optimized for the production of EPS from *Cyanothece* sp., with 140 $mg.L^{-1}$ at 30ºC and 8 $\mu E.m^{-2}.s^{-1}$, and from *Rhodella violacea*, with 600 $mg.L^{-1}$ at 24ºC and 420 $\mu E.m^{-2}.s^{-1}$ (Ohki et al. 2014; Villay et al. 2013). Whilst for *Dunaliella salina* and *Synechocystis* sp., the optimization of the required NaCl concentration was investigated, reaching EPS yields of 944 $mg.L^{-1}$ and 630 $mg.L^{-1}$, respectively (Mishra and Jha 2009; Ozturk and Aslim 2010). Using *A. platensis*, factors of temperature, PFD and the concentrations of NaCl and $NaNO_3$ have been studied individually or in combination by Trabelsi et al. (2009), Chentir et al. (2017) and Dejsungkranont et al. (2016), producing maximum yields of 210 $mg.L^{-1}$, 0.98 $g.g^{-1}$ and 772.1 ±192.9 $mg.g^{-1}$, respectively.

The determination of the best techniques to extract and purify the EPS, as well as the full characterization of the resulting product, could also bring it closer to industrial use (Delattre et al. 2016). In general, the culture medium of the cyanobacteria is centrifuged or filtrated to remove the cells and then the supernatant or filtrate is concentrated to reduce the water content. Some authors have employed the use of a heat bath (Parikh and Madamwar 2006) or a process using a membrane, such as tangential ultrafiltration (Han et al. 2014) or microfiltration (Ahmed et al. 2014), to attain the concentrate. However, membrane processing depends on the viscosity of the culture medium, pore size distribution and transmembrane pressure (Li et al. 2011). The most frequently used technique to recover the EPS from the concentrate is by alcohol precipitation. Under these conditions, some salts present in the culture medium may also co-precipitate. Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have also been used to extract EPS, although to a lesser extent (Budarin et al. 2012). It is worth noting that ultrasound waves may also degrade EPS macromolecules (Du et al. 2011).

As yet, no study in the literature has evaluated the combined effect of nitrate ($NaNO_3$) starvation and PFD on the EPS production from *A. platensis* culture. Nitrogen starvation is known as a stress condition, which

favors the accumulation of some reserve products and the production of EPS from cyanobacteria (Lupi et al. 1994). An increase in PFD has also been related to an increase in EPS yields (Markou et al. 2012). However, the intracellular events that lead to EPS production are still being deciphered. Some authors have proposed two events in diatoms: 1) the combined effect of PFD and nitrate starvation reduce cellular growth and cause an excess of assimilated carbon, which is released as carbohydrates; 2) the electrons accumulated in the photosynthetic electron transport chain induce the production of reactive oxygen species (ROS) that can cause cellular damage, thus EPS are produced to act as a protection barrier against these ROS (Miklestad 1995; Piedras et al. 2010).

In this work, an experimental design methodology was used to investigate the combined effect of nitrogen concentration and PFD on the biomass concentration and EPS content from the cultivation of *A. platensis.* The resulting products were analyzed as for their protein, carbohydrate and metals composition. Furthermore, EPS samples were characterized by infrared spectroscopy, and for their thermogravimetric and dynamic rheological properties.

Materials And Methods

Microorganism

Arthrospira platensis was provided by the Elizabeth Aidar microalgae collection from the Fluminense Federal University (Niterói, RJ/Brazil). The cell suspension of *A. platensis* was first cultivated in 300 mL modified Zarrouk's medium at 32ºC, according to George (1976), to be used as an inoculum. The experiments were carried out at room temperature (23°C) in Erlenmeyer flasks at a PFD of 150 $\mu E.m^{-2}.s^{-1}$ and under constant stirring. All other reagents were purchased from Sigma Aldrich (São Paulo, SP, Brazil). Distilled and deionized water was used in all cases.

Culture media

Prior to experimental conditions, cultures were grown in 5 L bottles with 3700 mL of modified Zarrouk's medium (George 1976) and 300 mL of *A. platensis* inoculum, and incubated in a germination chamber at (32±2)ºC with constant aeration and white LED lighting at a PFD of 1000 $\mu E.m^{-2}.s^{-1}$. Measurements of the PFD were carried out with a QSL 2100 dosimeter from Biospherical Instruments Inc. (San Diego, CA, USA). As LED lamps have unequal values of PFD depending on the region of the bottle surface, through which the light passes, a complete mapping of PFD values was made along 36 points on the surface of the bottle.

Experimental design

Using the Design Expert program, version 10.0.6.0., a 3-level-factorial design (3^2) was applied to plan culture conditions for the production of *A. platensis* biomass and EPS. A total of twelve runs was planned, nine of which for combinations of three levels and a central point in triplicate (Table 1). The independent variables were $NaNO_3$ concentration (0.5, 1.125, and 2 $g.L^{-1}$) and PFD (200, 600 and 1000

$\mu E.m^{-2}.s^{-1}$). The two responses evaluated were biomass concentration ($mg.L^{-1}$) and EPS yield ($mg.g^{-1}$). The effect of each factor was determined by the analysis of variance (ANOVA) for a confidence level of 95%.

Monitoring of growth and determination of the final biomass concentration

A. platensis growth was monitored by daily measurements of the optical density (O.D.) of the cellular suspension at 730 nm for 21 days. The specific growth rate (μ) was determined over the exponential growth phase, and given by Eq. 1:

$$\mu = (\ln x2 - \ln x1)/(t2 - t1) \quad (1)$$

where x_1 and x_2 are the biomass concentrations, and t_1 and t_2 are the times, corresponding to the beginning and the end of the exponential phase, respectively.

To determine the dry biomass weight, aliquots of 20 mL were taken from the culture medium at the end of each experiment and filtered through 0.7 -1.2 μm Sartorius glass fiber membranes that had been previously weighed. After being extensively washed to eliminate excess of salt, the membranes were dried at 105°C in an Ohaus® model MB45 Moisture Analyzer balance for approximately 15 min, and weighed, giving the dry biomass (g) by difference.

EPS production

To recover the EPS from the extracellular medium, the procedure reported by Parikh and Madamwar (2006) was followed. Briefly, the filtered culture medium, which still contained some biomass, was centrifuged at 13000 rpm for 20 min in a Hettich Rotixa centrifuge, model 420R (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The resulting supernatant was heated at 70ºC for 12 h, reducing its volume to ¼. The EPS were then recovered by adding ice-cooled ethyl alcohol. After 12 h at 4ºC, the product was filtered and completely dried in an oven at 60ºC. To eliminate residual salt, the samples were resolubilized in distilled water and then dialyzed against water under constant stirring at room temperature for 24 h. The final product was recovered by adding 95% ethyl alcohol and lyophilized.

Chemical and physicochemical characterization of EPS

The methodology proposed by Dubois et al. (1956) was used to quantify the total carbohydrate content for each recovered EPS sample, by adding H_2SO_4 concentrated at 80% (m/v) and phenol at 5% (v/v). A standard curve was obtained using D-glucose.

Soluble protein was determined by the Folin's method, using bovine serum albumin as the standard (Lowry et al. 1951).

Fourier transform infrared spectroscopy (FTIR) analyses were carried out using a Perkin Elmer spectrometer, Frontier model (Waltham, MA, USA) at room temperature, using KBr disks, in the 4000 –

400 cm^{-1} range, with accumulation of 20 scans and 4 cm^{-1} resolution.

Thermogravimetric analyses were carried out for EPS samples under nitrogen atmosphere on a TGA Q-500 equipment from TA Instruments (New Castle, DE, USA). Approximately, 10 mg of sample were heated from 20 to 700ºC, at a 10ºC/min rate.

The rheological properties of selected EPS samples were investigated at 5 $g.L^{-1}$ and 10 $g.L^{-1}$ at 25ºC with an AR G2 controlled stress rheometer (TA Instruments Inc.), equipped with a coaxial cylinder geometry. Initially, strain sweep tests were conducted as the variation of complex modulus at 6.28 rad/s to determine the linear viscoelastic range. After a period of 10 min, an oscillatory frequency scan was performed, from 10^{-1} to 7 x10^{2} $rad.s^{-1}$ (with a deformation value of 10%), within the region of linear viscoelasticity. Finally, viscosity changes under steady flow regime were investigated as a function of shear rate, from 10 to 10^{2} s^{-1}.

Statistical analysis

The carbohydrate and protein quantifications were carried out in triplicate and the results were expressed as mean values ± standard deviation (SD). One-way ANOVA was applied to the data by using the PAST 3.20 software, available at https:/folk.uio.no/ohammer/past. The mean values were compared by the Tukey's test considering a confidence level of 95% and level of significance ($p < 0.05$).

Results And Discussion

Evaluation of Arthrospira platensis growth under different cultivation conditions

The growth curves of *A. platensis* cultivated in different culture conditions are presented in Fig. 1. As observed, none of the conditions presented an adaptation phase. Condition 6 resulted in the highest growth rate (μ=0.55), in medium with 2 $g.L^{-1}$ of $NaNO_3$ and under 600 $\mu E.m^{-2}.s^{-1}$ of PFD. Condition 2 led to the lowest growth rate, with 1.125 $g.L^{-1}$ of $NaNO_3$ and a PFD of 200 $\mu E.m^{-2}.s^{-1}$, followed closely by number 1 (0.25 $g.L^{-1}$ of $NaNO_3$ and 200 $\mu E.m^{-2}.s^{-1}$ of PFD) and condition 3 (2 $g.L^{-1}$ of $NaNO_3$ and 200 $\mu E.m^{-2}.s^{-1}$ of PFD). All three of these conditions corresponded to the lowest PFD used. The results demonstrate that the *A. platensis* growth rate was practically independent of the $NaNO_3$ concentration, and indicates that the PFD was the limiting factor.

The PFD values may have affected the growth rate, which increased until reaching a point of light saturation and, from this point, there may even have been a decrease in the rate of growth as a consequence of photo inhibition (Carvalho et al. 2011). In cultures grown at 1000 $\mu E.m^{-2}.s^{-1}$ and with $NaNO_3$ at a concentration of 2 $g.L^{-1}$, light saturation may have occurred, which resulted in a lower rate of cell growth. In cultures using a PFD at 600 $\mu E.m^{-2}.s^{-1}$, the $NaNO_3$ concentration was probably the limiting factor for *A. platensis* growth.

Final biomass concentration and EPS yield under different growth conditions

Some researchers reported that different concentrations of $NaNO_3$ and PFD could induce the EPS production from cyanobacteria (Aikawa et al. 2012; Dejsungkranont et al. 2017; Ohki et al. 2014; Villay et al. 2013). Modified Zarrouk's medium has 2.5 $g.L^{-1}$ of $NaNO_3$. Therefore, for the experimental design, a $NaNO_3$ concentration (2 $g.L^{-1}$) close to that for which regular cell growth is already known and a concentration reduced by 75% (0.5 $g.L^{-1}$) were used. For the PFD, the minimum and maximum values that have reportedly been used for *A. platensis* are 57.2 $\mu E.m^{-2}.s^{-1}$ (Chentir et al. 2018), and 700 $\mu E.m^{-2}.s^{-1}$ (Aikawa et al. 2012), respectively.

The values of the final biomass concentration and the EPS yield are given in Table 1. In general, the O.D. values were related to the biomass concentration. However, there was some variation for the intermediate values, although the differences between the expected O.D. and the observed O.D. were not so high, suggesting they could have been caused by small measurements error.

Considering the results, the highest final biomass concentration (1.292 $g.L^{-1}$) was observed under conditions with the highest $NaNO_3$ concentration (2 $g.L^{-1}$). However, for the EPS yield, the best conditions were achieved under the lowest $NaNO_3$ (0.25 $g.L^{-1}$) and PFD (200 $\mu E.m^{-2}.s^{-1}$), which generated 111 $mg.g^{-1}$ of EPS. Although the second highest EPS content (100 $mg.g^{-1}$) was obtained under conditions of the highest values of $NaNO_3$ (2 $g.L^{-1}$) and PFD (1000 $\mu E.m^{-2}.s^{-1}$). In other words, the maximum EPS content (EPS mass/biomass) was attained using both extremes of the experimental design.

The results presented in Fig. 2a and 2b show that the EPS production by *A. platensis* was not directly associated to its growth. As can be observed, the response in which the highest final biomass concentration was obtained differed from that which generated the highest EPS production. In general, metabolic stress may negatively affect cyanobacteria growth, during which cells use their energy to produce reserve compounds such as carbohydrates and EPS (Santos et al. 2019). Condition 9 was an exception, since this condition promoted one of the highest EPS yields and, at the same time, one of the highest final biomass concentrations.

Table 2 shows the results of the coefficients and their interactions, R^2, lack of fit and the p-values for final biomass concentration and EPS yield from *A. platensis*. A 95% confidence level was adopted ($p < 0.05$). The model was significant for final biomass concentration with a p-value equal to 0.0379. It was found that the PFD and its interactions have a significant influence on the model. For the EPS production, the model was significant, with a p-value equal to 0.0164, and the variables that showed a significant influence were the PFD and its interactions.

The regression equation was generated, as shown in Eq. 2 and Eq. 3, according to the values presented in Table 2.

$$\text{Biomass concentration} = 1.07 + 0.0755A + 0.0892B + 0.0138AB + 0.0244\,A^2 - 0.2646\,B^2 \quad (2)$$

EPS yield = 44.54 + 8A – 15.67B + 17.75AB + 12.88 A^2 + 24.88 B^2 (3)

It is noteworthy that the lack of fit values for both biomass and EPS production were significant ($p < 0.05$). In this case, the model does not have a good predictive capacity. Despite the lack of adjustment, it is possible to visualize the best working regions.

In Figure 2a, the response surface graphics show that the conditions which led to the highest final biomass concentration were a PFD of 600 $\mu E.m^{-2}.s^{-1}$ and 2 $g.L^{-1}$ $NaNO_3$. A decrease in biomass production was also observed when the value of PFD was smaller. The PFD can influence cyanobacteria photosynthesis. Excessive or insufficient light may affect the biomass productivity and yield of metabolic products (Carvalho et al. 2011). It is likely that the nitrogen-reduced conditions in this work did not compromise the biomass production and were high enough to sustain the cell growth. However, the lowest PFD negatively affected the biomass production. In this case, it seems that light intensity was insufficient to support the biomass production. Aikawa et al. (2012) also observed that biomass production was related to the PFD, and that *A. platensis* produced the highest final biomass concentration (1.6 $g.L^{-1}$) at 700 $\mu E.m^{-2}.s^{-1}$, the highest PFD value they tested. Under the smallest PFD value, 20 $\mu E.m^{-2}.s^{-1}$, a much smaller final biomass concentration (0.1 $g.L^{-1}$) was attained.

Figure 2b shows the response surface obtained for the EPS content ($mg.g^{-1}$). The highest values were observed at the two extremes, 111 and 100 $mg.g^{-1}$, using conditions 1 and 9, respectively, in addition to condition 3, which also generated an EPS yield of 100 $mg.g^{-1}$. The EPS production from *A. platensis* was also affected by the PFD; however, it was not the only determinant variable. $NaNO_3$ starvation contributes to the increase in the C/N ratio. Consequently, it promotes the incorporation of carbon reserve in the EPS (Otero and Vincenzini 2003). The lowest PFD (200 $\mu E.m^{-2}.s^{-1}$) and $NaNO_3$ concentration (0.5 $g.L^{-1}$) resulted in the highest EPS yield. It is possible that the combined effects led to cells producing more EPS in response to the limited conditions. This result is in accordance with that found by Chentir et al. (2018), who obtained the highest EPS yield, 0.902 $g.g^{-1}$, using 0.5 $g.L^{-1}$ $NaNO_3$ combined with a PFD of 57.2 $\mu E.m^{-2}.s^{-1}$ in *A. platensis*.

Chentir et al. (2017) evaluated the maximization of EPS production from *A. platensis* as a function of variations in NaCl concentration and PFD. Although the PFD did not have any positive effect on EPS production, its interaction with the NaCl concentration provided a 0.98 $g.g^{-1}$ yield of EPS. On the other hand, Dejsungkranont et al. (2017), through studying the effect of PFD on the production of EPS from *A. platensis*, observed that the highest level of PFD (203 $\mu E.m^{-2}.s^{-1}$) favored EPS production (956.4 ± 37.3 $mg.L^{-1}$) as well as biomass (1.5 $g.L^{-1}$). Whereas under the lowest PFD (101 $\mu E.m^{-2}.s^{-1}$), 0.8 $g.L^{-1}$ of biomass and 637.3 ± 41.3 $mg.L^{-1}$ of EPS were obtained.

Trabelsi et al. (2009) carried out a study evaluating the effect of different temperatures and PFD on the final biomass and EPS concentrations by *A. platensis*, and described a possible correlation between the two responses. According to the authors, the production of biomass and EPS are mutually dependent,

and the increase of EPS production may be associated with the kinetics of growth. To achieve a greater EPS content, it is necessary to optimize the PFD while the temperature should be maintained between 30 and 35ºC. In that work, *A. platensis* produced the maximum EPS content at the highest PFD used, 180 $\mu E.m^{-2}.s^{-1}$, with 297.4 ± 11.1 $mg.L^{-1}$. The result for EPS content found by Trabelsi et al. (2009), 210 $mg.L^{-1}$, under 100 $\mu E.m^{-2}.s^{-1}$ and 2.5 $g.L^{-1}$ $NaNO_3$, was twice that of our result, 91 $mg.L^{-1}$.

The results found in the present study demonstrate that the choice of the best culture conditions depended on the response of interest. The results in Figures 2a and 2b showed that the production of EPS by *A. platensis* was not directly associated to growth. Nevertheless, it is possible to choose a condition in which the EPS content as well as biomass concentration could be produced at reasonable values.

Other studies in the literature using different microorganisms have reported the effect of culture conditions on the EPS content and biomass concentration. For *Cyanothece* sp. 113, $NaNO_3$ concentrations between 0 - 200 $mg.L^{-1}$ were used (Su et al. 2007). In this case, the final biomass concentration was observed to increase, reaching 1.2 $g.L^{-1}$ with a $NaNO_3$ concentration of 74.3 $mg.L^{-1}$, but then decreased at values higher than 100 $mg.L^{-1}$. However, a decrease in EPS concentration, from 7 $g.L^{-1}$ to 5$g.L^{-1}$, was reported elsewhere when 200 $mg.L^{-1}$ of $NaNO_3$ was used. In the same work, the PFD effect was evaluated in the 20-100 $\mu E.m^{-2}.s^{-1}$ range and the best condition was found to be 86 $\mu E.m^{-2}.s^{-1}$ for both biomass and EPS production (Su et al. 2007).

FTIR analyses

Figure 3 shows the FTIR spectrum for EPS samples from *A. platensis*. The broad bands observed around 3400 cm^{-1} are attributed to O-H and N-H stretching. The weak absorptions in the region 3000 cm^{-1} to 2840 cm^{-1} are associated to C-H asymmetrical and symmetrical stretching modes of methyl and methylene groups. The absorption at 1650 cm^{-1} is attributed to C=O stretching of carboxylate and amide groups (amide I band). The high-intensity band with maximum at 1442 cm^{-1} is attributed to more complex vibrations, associated to O-H bending (Can et al. 2019; Trabelsi et al. 2009). The most intense absorption of the spectrum, at 1046 cm^{-1}, may be attributed to C-O-C, S-O and P-O-C stretching vibrations. This result evidenced the presence of polysaccharides, proteins/polypeptides, and of sulphate and phosphate groups linked to polymeric substances.

Thermal analysis

Thermogravimetric analysis is an important technique, as it demonstrates the thermal stability of the EPS (Fig. 4). The EPS displayed two stages of thermal degradation. In the first stage, up to 150ºC, 10% weight was lost and this may be attributed to the loss of water and other volatile substances. In the second stage, the degradation of the polymer chain occurred, between 225 and 350°C, with fifty percent of the total mass lost.

The thermal degradation of EPS from microalgae and cyanobacterium was recently reported. Two stages were observed for the EPS from *Dunaliella salina*; in the first, 15% weight was lost at up to 150ºC. In the second, 55% of total EPS weight loss was observed with maximum loss at 240°C (Mirsha et al. 2011). On the other hand, for the EPS from *Nostoc carneum,* three stages of thermal degradation were detected. In the first stage, 15% weight was lost up to 155ºC. In the second stage, with a maximum at 237ºC, 39% weight was lost, attributed to polysaccharide degradation. The third phase occurred up to 378ºC with 32% of weight loss (Hussein et al. 2015).

Carbohydrate and protein quantification

The chemical composition of the EPS depends on environmental conditions and the microorganism studied (Nouha et al. 2018). According to Wingender et al. (1999), the majority of EPS constituents are carbohydrates and proteins. In *A. platensis*, the carbohydrate and protein content were estimated as 55% and 13%, respectively, in a photoautotrophic growth for 25 days (Pignolet et al. 2013). Carbohydrate and protein contents for *A. platensis* EPS determined in the present work are shown in Table 3. The highest contents of carbohydrates, 39.5 ± 2.34 $mg.g^{-1}$ and 38.73 ± 2.55 $mg.g^{-1}$, were observed under conditions 5 (1.125 gL^{-1} $NaNO_3$ and 600 $\mu E.m^{-2}.s^{-1}$) and 9 (2 gL^{-1} $NaNO_3$ and 1000 $\mu E.m^{-2}.s^{-1}$), respectively.

According to Depraetere et al. (2015), when the nitrate source is depleted, protein synthesis is reduced. In the present work, the highest protein concentrations, 7.05 ±0.30 $mg.g^{-1}$ and 6.46 ± 0.20 $mg.g^{-1}$, were observed for intermediate $NaNO_3$ concentrations, under conditions 5 (1.125 gL^{-1} of $NaNO_3$ and 600 $\mu E.m^{-2}.s^{-1}$) and 8 (1.125 gL^{-1} of $NaNO_3$ and 1000 $\mu E.m^{-2}.s^{-1}$), respectively.

Rheological properties

Aqueous solutions of EPS 01 (0.25 gL^{-1} $NaNO_3$ and 200 $\mu E.m^{-2}.s^{-1}$) and EPS 09 (2 gL^{-1} $NaNO_3$ and 1000 $\mu E.m^{-2}.s^{-1}$) were prepared at 5 $g.L^{-1}$ and 10 $g.L^{-1}$, and their rheological properties were investigated. Fig. 5a and 5b show the variation of storage modulus, G', and loss modulus, G", with the oscillatory frequency. Their rheological behaviors were quite similar to that previously observed for other polysaccharides, such as gum algaroba, a galactomannan extracted from *Prosopis juliflora* (Azero and Andrade 2006). At very low frequencies, the viscous character predominated. After the crossing of G' with G", the elastic character was predominant. As expected, the value of the frequency at which the crossing occurs was smaller for the highest concentration. The same behavior was observed for the EPS09 supernatant at 5 and 10 $g.L^{-1}$.

Our results differ from those reported by Chentir et al. (2017), who also assessed the EPS of *A. platensis*. The authors described a gel-like behavior, in which the storage modulus values were higher than the loss modulus values at different concentrations (1%, 2.5% and 5%). Furthermore, Mourhin et al. (1993) found a non-Newtonian character for EPS dispersions of *Spirulina platensis*, which was attributed to its polyanionic nature.

Conclusions

The present work demonstrated that combined values of $NaNO_3$ and PFD were capable of enhancing the EPS yield and biomass concentration of *Arthrospira platensis*. The EPS production was not associated with the growth rate, and under nitrate starvation and the lowest PFD, the highest EPS production was achieved. The FTIR spectra showed that the EPS contained sulphate groups, carbohydrate and proteins, while the rheological studies suggested that the EPS exhibited a dilute solution behavior. In terms of further enhancing the biomass concentration and EPS yield of *A. platensis*, whilst also reducing production costs, alternative culture media could next be evaluated.

Declarations

Acknowledgements

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) for financial support.

Authors' contributions

MBFS contributed to the conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, visualization; **EGAR** contributed to methodology, validation, formal analysis and investigation of rheological experiments; **CMLLT** contributed to the conceptualization, methodology, analysis, writing – review and editing, supervision, project administration, funding acquisition; **CTA** contributed to the analysis, writing – review and editing, supervision, project administration, funding acquisition. All authors read and approved the final manuscript.

Funding

This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES), Finance Code 001.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No potential conflicts of interest were disclosed.

Abbreviations

EPS: Extracellular polymeric substances; NaCl: sodium chloride; $NaNO_3$: sodium nitrate; PFD: Photon flux density; ANOVA: analysis of variance; FTIR: Fourier transform infrared spectroscopy; TGA: thermogravimetric analyses.

References

1. Ahmed M, Poortvliet TCWM., Wijnholds A, Staland LJ, Hasnain S (2014) Isolation, characterization and localization of extracellular polymeric substances from the cyanobacterium *Arthrospira platensis* strain MMG-9. Eur J Phycol 49:143-150 https://doi.org/10.1080/09670262.2014.895048
2. Aikawa S, Izumi Y, Matsuda F, Hasunuma T, Chang JS, Kondo, A (2012) Synergistic enhancement of glycogen production in *Arthrospira platensis* by optimization of light intensity and nitrate supply. Bioresour Technol 108:211–215 https://doi.org/10.1016/j.biortech.2012.01.004
3. Arad S, Levy-Ontman O. (2010) Red microalgae cell-wall polysaccharides: biotechnological aspects. Curr Opin Biotechnol 21:358–364 https://doi.org/10.1016/j.copbio.2010.02.008
4. Azero EG., Andrade CT (2006) Characterization of *Prosopis juliflora* seed gum and the effect of its addition to k-carrageenan systems. J Braz Chem Soc 17:844-850 https://doi.org/10.1590/S0103-50532006000500005.
5. Budarin V, Ross AB, Biller P, Riley R, Clark JH, Jones JM, Gilmour DJ, Zimmerman, W (2012) Microalgae biorefinery concept based on hydrothermal microwave pyrolysis. Green Chem 14:3251–3254 https://doi.org/10.1039/C2GC36202C
6. Can HK., Gurbuz F, Odabas M (2019) Partial characterization of cyanobacterial extracellular polymeric substances for aquatic ecosystems. Aquat Ecol 53:431-440 https://doi.org/10.1007/s10452-019-09699-z
7. Carvalho AP, Silva SO, Baptista JM, Malcata FX (2011) Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. Appl Microbiol Biotechnol 89:1275–1288 https://doi.org/10.1007/s00253-010-3047-8
8. Challouf R, Trabelsi L, Dhieb BR, El Abed O, Yahia A, Ghozzi K, Ammar JB, Omran H, Ouada BH (2011) Evaluation of cytotoxicity and biological activities in extracellular polysaccharides released by Cyanobacterium *Arthrospira platensis*. Braz Arch Biol Technol 54:831-838 https://doi.org/10.1590/S1516-89132011000400024
9. Chentir I, Hamdi M, Doumandji A, HadjSadok A, Ouada HB, Nasri M, Jridi M (2017) Enhancement of extracellular polymeric substances (EPS) production in Spirulina (*Arthrospira* sp.) by two-step cultivation process and partial characterization of their polysaccharidic moiety. Int J Biol Macromol 105:1412-1420 https://doi.org/10.1016/j.ijbiomac.2017.07.009

10. Chentir I, Doumandji A, Ammar J, Zili F, Jridi M, Markou G, Ouada HB (2018) Induced change in *Arthrospira* sp. (Spirulina) intracellular and extracellular metabolites using multifactor stress combination approach. J Appl Phycol 30:1563–1574 https://doi.org/10.1007/s10811-017-1348-3
11. Decho AW, Gutierrez T (2017) Microbial extracellular polymeric substances (EPSs) in ocean systems Front Microbiol 8:1-28 https://doi.org/10.3389/fmicb.2017.00922
12. Dejsungkranont M, Chisti Y, Sirisansaneeyakul S (2017) Simultaneous production of C-phycocyanin and extracellular polymeric substances by photoautotrophic cultures of *Arthrospira platensis*. J Chem Technol Biotechnol 92:2709–2718 https://doi.org/10.1002/jctb.5293
13. Delattre C, Pierre G, Laroche C, Michaud P (2016) Production, extraction and characterization of microalgal and cyanobacterial exopolysaccharides. Biotechnol Adv 34:1159-1179 https://doi.org/10.1016/j.biotechadv.2016.08.001
14. Depraetere O, Deschoenmaeker F, Badri H, Monsieurs P, Foubert I, Leys N, Wattiez R, Muylaert K (2015) Trade-off between growth and carbohydrate accumulation in nutrient-limited *Arthrospira* sp. PCC 8005 studied by integrating transcriptomic and proteomic approaches. PLOS ONE 10:e0132461 https://doi.org/10.1371/journal.pone.0132461
15. Du Z, Li Y, Wanga X, Wana Y, Chen Q, Wang C, Lin X, Liu Y, Chen P, Ruan R (2011) Microwave-assisted pyrolysis of microalgae for biofuel production. Bioresour Technol 102:4890-4896 https://doi.org/10.1016/j.biortech.2011.01.055
16. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356 https://doi.org/10.1021/ac60111a017
17. Ekelhof A, Melkonian M (2017) Enhanced extracellular polysaccharide production and growth by microalga *Netrium digitus* in a porous substrate bioreactor. Algal Res 28:184–191 DOI: 10.1016/j.algal.2017.11.003
18. Goo BG, Baeka G, Choi DJ, Park YI, Synytsyac A, Bleha R, Seong DH, Lee CG, Park JK (2013) Characterization of a renewable extracellular polysaccharide from defatted microalgae *Dunaliella tertiolecta*. Bioresour Technol 129:343-350 https://doi.org/10.1016/j.biortech.2012.11.077
19. Gong H, Tang Y, Wang J, Wen X, Zhang L, Lu C (2008) Characterization of photosystem II in salt-stressed cyanobacterial *Spirulina platensis* cells. Biochim Biophys Acta 1777:488–495 https://doi.org/10.1016/j.bbabio.2008.03.018
20. Han P, Suna Y, Jiaa S, Zhonga C, Tan Z (2014) Effects of light wavelengths on extracellular and capsular polysaccharide production by *Nostoc flagelliforme*. Carbohydr Polym 105:145-151 https://doi.org/10.1016/j.carbpol.2014.01.061.
21. Hussein MH, Abou-elwafa GS, Shaaban-dessuuki SA, Hassan NI (2015) Characterization and antioxidant activity of exopolysaccharide secreted by *Nostoc carneum*. Int J Pharmacol 11:432-439 Doi: 10.3923/ijp.2015.432.439
22. Jesus CS, Assis DJ, Rodriguez MB, Filho JAM, Costa JAV, Ferreira SE, Druzian JI (2019). Pilot-scale isolation and characterization of extracellular polymeric substances (EPS) from cell-free medium of

Spirulina sp. LEB-18 cultures under outdoor conditions. Inter J Biol Macromol 124:1106-1114 https://doi.org/10.1016/j.ijbiomac.2018.12.016

23. Li H, Mao W, Hou Y, Gao Y, Qi X, Zhao C, Chen Y, Chen Y, Li N, Wang C (2012) Preparation, structure and anticoagulant activity of a low molecular weight fraction produced by mild acid hydrolysis of sulfated rhamnan from *Monostrom alatissimum*. Bioresour Technol 114:414–418 https://doi.org/10.1016/j.biortech.2012.03.025
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
25. Lupi FM, Fernandes HML, Tomé MM, Sá-Correia I, Novais JM (1994) Influence of nitrogen source and photoperiod on exopolysaccharide synthesis by the microalga *Botryococcus braunii* UC 58. Enzym Microb Technol 16:546–550 https://doi.org/10.1016/0141-0229(94)90116-3
26. Manirafasha E, Murwanashyaka T, Ndikubwimana T, Ahmed RN, Liu J, Lu Y, Zeng X, Ling X, Jing K (2018) Enhancement of cell growth and phycocyanin production in *Arthrospira* (Spirulina) platensis by metabolic stress and nitrate fed-batch. Bioresour Technol 255:293-301 https://doi.org/10.1016/j.biortech.2017.12.068
27. Markou G, Angelidaki I, Georgakakis D (2012) Microalgae carbohydrates: An overview of the factors influencing carbohydrates production and of main bioconversion technologies for production of biofuels. Appl Microbiol Biotechnol 96:631–645 https://doi.org/10.1007/s00253-012-4398-0
28. Mishra A, Jha B (2009) Isolation and characterization of extracellular polymeric substances from micro-algae *Dunaliella salina* under salt stress. Bioresour Technol 100:3382-3386 doi:10.1016/j.biortech.2009.02.006
29. Mishra A, Kavita K, Jha B (2011) Characterization of extracellular polymeric substances produced by micro-algae *Dunaliella salina*. Carbohydr Polym 83:852–857 https://doi.org/10.1016/j.carbpol.2010.08.067
30. Miklestad SM (1995). Release of extracellular products by phytoplankton with special emphasis on polysaccharides. Sci Total Environ 165:155-164 https://doi.org/10.1016/0048-9697(95)04549-G
31. Mouhim FR, Cornet J, Fontane T, Fournet B, Dubertret G (1993) Production, isolation and preliminary characterization of the exopolysaccharide of the cyanobacterium *Spirulina platensis*. Biotechnol Lett 15:567–572 https://doi.org/10.1007/BF00138541
32. Mundt S, Kreitlow S, Nowotny A, Effmert U. (2001) Biochemical and pharmacological investigations of selected cyanobacteria, International Journal of Hygiene and Environmental Health 203: 327-334 https://doi.org/10.1078/1438-4639-00045.
33. Nouha K, Kumar RS, Balasubramanian S, Tyagi RD (2018) Critical review of EPS production, synthesis and composition for sludge flocculation. J Environ Sci 66:225-245 https://doi.org/10.1016/j.jes.2017.05.020
34. Ohki K, Le N, Yoshikawa S, Kanesaki Y, Okajima M, Kaneko T, Thi T (2014) Exopolysaccharide production by a unicellular freshwater cyanobacterium *Cyanothece* sp. isolated from a rice field in Vietnam. J Appl Phycol 26:265-272 https://doi.org/10.1007/s10811-013-0094-4

35. Oliver JWK, Atsumi S (2014). Metabolic design for cyanobacterial chemical synthesis. Photosynth Res 120:249–261
36. https://doi.org/10.1007/s11120-014-9997-4.
37. Otero A, Vincenzini M (2003) Extracellular polysaccharide synthesis by *Nostoc* strains as affected by N source and light intensity. J Biotechnol 102:143-152 doi: 10.1016/s0168-1656(03)00022-1
38. Ozturk S, Aslim B (2010) Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress. Environ Sci Pollut Res 17:595–602 https://doi.org/10.1007/s11356-009-0233-2
39. Parikh A, Madamwar D (2006) Partial characterization of extracellular polysaccharides from cyanobacteria. Bioresour Technol 97:1822-1827 https://doi.org/10.1016/j.biortech.2005.09.008
40. Pereira S, Zille A, Micheletti E, Moradas-Ferraira P, De Philippis R, Tamagnini P (2009) Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS Microbiol Rev 33:917–941 https://doi.org/10.1111/j.1574-6976.2009.00183.x
41. Piedras FR, Baisch PRM, Machado MICS, Vieira AAH, Giroldo D (2010). Carbohydrate release by a subtropical strain of *Spondylosium pygmaeum* (Zygnematophyceae): influence of nitrate availability and culture aging. J Phicol 46:477-483 https://doi.org/10.1111/j.1529-8817.2010.00823.x
42. Pignolet O, Jubeau S, Vacca Garcia C, Michaud P (2013). Highly valuable microalgae: biochemical and topological aspects. J Ind Microbiol Biotechnol 40:781–796 https://doi.org/10.1007/s10295-013-1281-7
43. Piorreck M, Baasch K, Pohl P (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochem 23:207–216
44. https://doi.org/10.1016/S0031-9422(00)80304-0
45. Reichert M, Bergmann SM, Hwang J, Buchholz R, Lindenberger C (2017) Antiviral activity of exopolysaccharides from *Arthrospira platensis* against koi herpesvirus J Fish Dis https://doi.org/10.1111/jfd.12618
46. Santos RR, Corrêa PS, Dantas FML, Teixeira CMLL (2019). Evaluation of the co-production of total carotenoids, C-phycocyanin and polyhydroxyalkanoates by *Arthrospira platensis*. Bioresour Technol Rep 7:100226 https://doi.org/10.1016/j.biteb.2019.100226
47. Shabana EF, Gabr MA, Moussa HR, El-Shaer EA, Ismaiel MMS (2017) Biochemical composition and antioxidant activities of *Arthrospira* (Spirulina) *platensis* in response to gamma irradiation. Food Chem 214:550– 555 https://doi.org/10.1016/j.foodchem.2016.07.109
48. Soanen N, Da Silva E, Gardarin C, Michaud P, Laroche C (2016) Improvement of exopolysaccharide production by *Porphyridium marinum*. Bioresour Technol 213: 231–238 https://doi.org/10.1016/j.biortech.2016.02.075
49. Su C, Chi Z, Lu W (2007) Optimization of medium and cultivation conditions for enhanced exopolysaccharide yield by marine *Cyanothece*. Chin J Oceanol Limnol 25:411–417

https://doi.org/10.1007/s00343-007-0411-3

50. Trabelsi L, Chaieb O, Mnari A, Abid-Essafi S, Aleya L (2016) Partial characterization and antioxidant and antiproliferative activities of the aqueous extracellular polysaccharides from the thermophilic microalgae *Graesiella*s p. BMC Complement Altern Med 16:2-10 https://doi.org/10.1186/s12906-016-1198-6
51. Trabelsi L, M'sakni NH, Ouada HB, Bacha H, Roudesli S (2009) Partial characterization of extracelular polysaccharides produced by cyanobacterium *Arthrospira platensis*. Biotechnol Biopr Eng 14:27-31 https://doi.org/10.1007/s12257-008-0102-8.
52. Trabelsi L, Ouada HB, Bacha H (2009). Combined effect of temperature and light intensity on growth and extracellular polymeric substance production by the cyanobacterium *Arthrospira platensis.* J Appl Phycol 21:405–412 https://doi.org/10.1007/s10811-008-9383-8
53. Villay A, Laroche C, Roriz C, El Alaoui H, Delbac F, Michaud P (2013) Optimisation of culture parameters for exopolysaccharides production by the microalga *Rhodella violacea.* Bioresour Technol 146:732-735 https://doi.org/10.1016/j.biortech.2013.07.030
54. **Wingender** J, **Neu** RT, **Flemming**HC (1999) Microbial Extracellular Polymeric Substances. Springer-Verlag Berlin Heidelberg 1, 1-19
55. Xiao R, Yang X, Lic M, Lia X, Weib Y, Caod M, Ragauskasc A, Thiesg M, Dingg J, Zhenga Y (2018) Investigation of composition, structure and bioactivity of extracellular polymeric substances from original and stress-induced strains of *Thraustochytrium striatum.* Carbohydr Polym 195:515-254 https://doi.org/10.1016/j.carbpol.2018.04.126
56. Zarrouk C (1966) Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima* Geitler. PhD Thesis, University of Paris.

Tables

Table 1: Experimental matrix for EPS production with the corresponding variables and responses

Experiment number	Factors		Response	
	A - $NaNO_3$ ($mg.L^{-1}$)	B- PFD ($\mu E.m^{-2}.s^{-1}$)	Biomass ($g.L^{-1}$)	EPS ($mg.g^{-1}$)
1	0.25	200	0.78	111
2	1.125	200	0.642	70
3	2	200	0.767	100
4	0.25	600	0.868	47
5	1.125	600	1.07	50
6	2	600	1.292	46
7	0.25	1000	0.871	40
8	1.125	1000	0.94	47
9	2	1000	0.913	100
10	1.125	600	1.073	54
11	1.125	600	1.062	51
12	1.125	600	1.087	45

Table 2: R^2, Lack of fit, p-value, coefficients and effects on quadratic model to responses obtained of biomass and EPS

Source	Biomass yield		EPS	
	Coefficient	p-value	Coefficient	p-value
Model	0.0379	0.0164		
A- $NaNO_3$	0.0755	0.1325	8	0.1846
B- PFD	0.0892	0.0856	-15.67	0.0261
$NaNO_3$ x PFD	0.0138	0.8045	17.75	0.0349
$NaNO_3$ x $NaNO_3$	0.0244	0.7209	12.88	0.1590
PFD x PFD	-0.2646	0.0066	24.88	0.0209
R^2	0.8060		0.8563	
Lack of fit	0.0006		0.0139	

Table 3: Carbohydrates and proteins in different culture conditions.

	Factors		Response	
Experiment number	A - $NaNO_3$ ($mg.L^{-1}$)	B- PFD ($\mu E.m^{-2}.s^{-1}$)	Carbohydrates ($mg.g^{-1}$)	Proteins ($mg.g^{-1}$)
1	0.25	200	23 ± 2.27^{a}	4.67 ± 0.28^{a}
2	1.125	200	23.5 ± 1.19^{a}	$4.2 \pm 1.37^{a,b,c}$
3	2	200	24.43 ± 1.40^{a}	5.46 ± 0.40^{a}
4	0.25	600	27.43 ± 0.37^{b}	3.49 ± 0.10^{b}
5	1.125	600	39.5 ± 2.34^{e}	7.05 ± 0.30^{e}
6	2	600	17.53 ± 0.5^{c}	3.14 ± 0.24^{b}
7	0.25	1000	23.67 ± 1.41^{a}	2.56 ± 0.07^{d}
8	1.125	1000	34 ± 1.49^{d}	6.46 ± 0.20^{e}
9	2	1000	38.73 ± 2.55^{e}	4.80 ± 0.38^{a}
10 (Central Point)	1.125	600	36.63 ± 0.51^{e}	4.18 ± 0.19^{c}

Figures

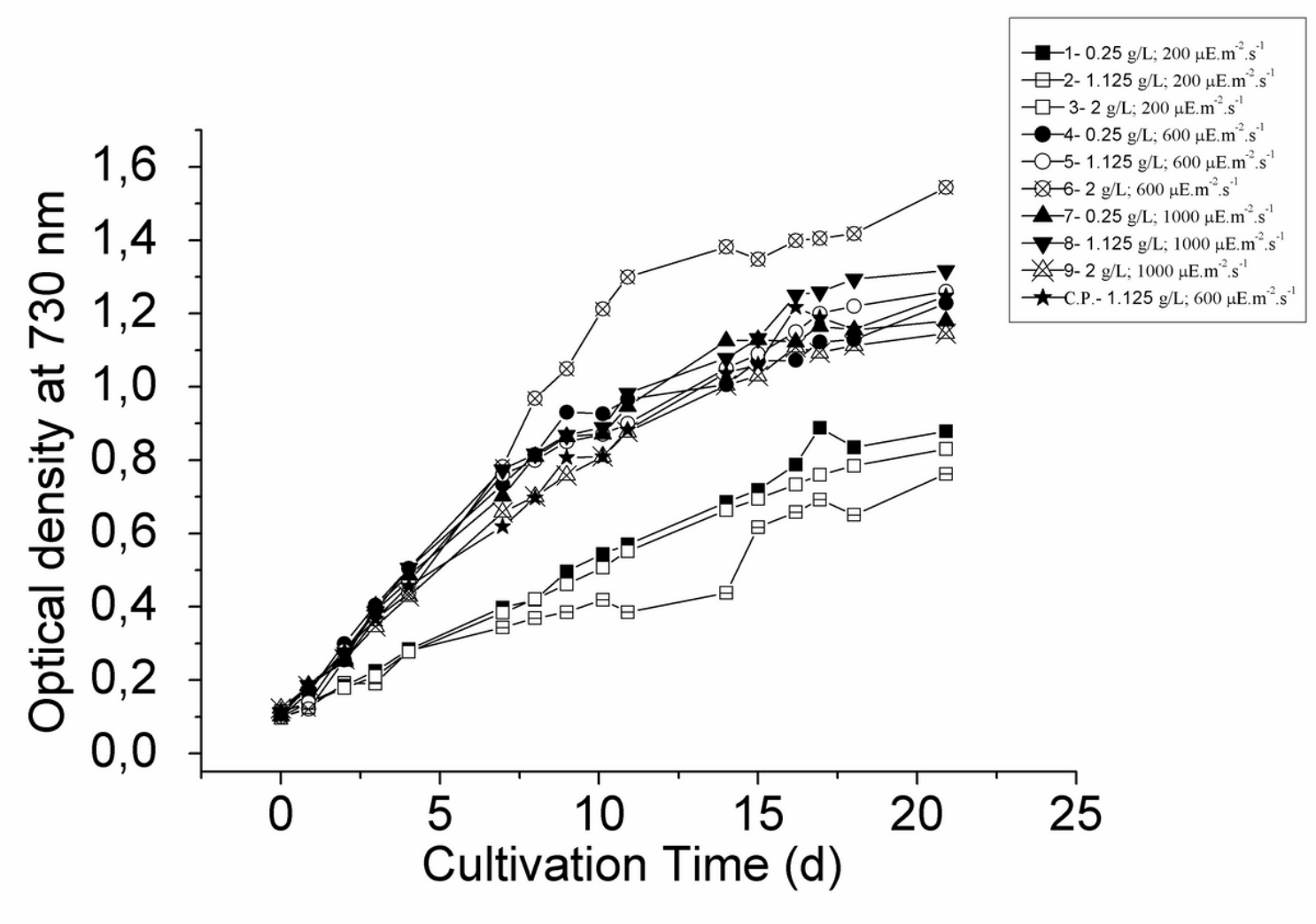
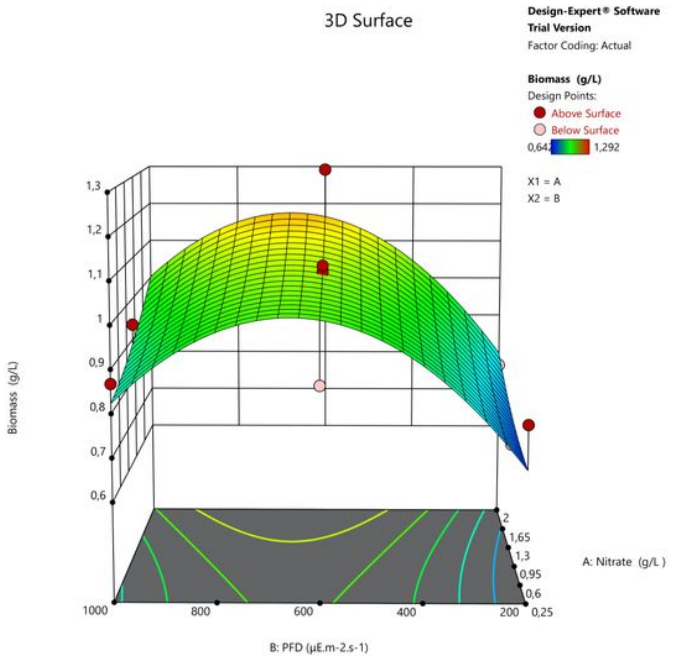


Figure 1

Growth curves of A. platensis at different conditions of NaNO3 and PFD. (C. P. refers to Central Point)



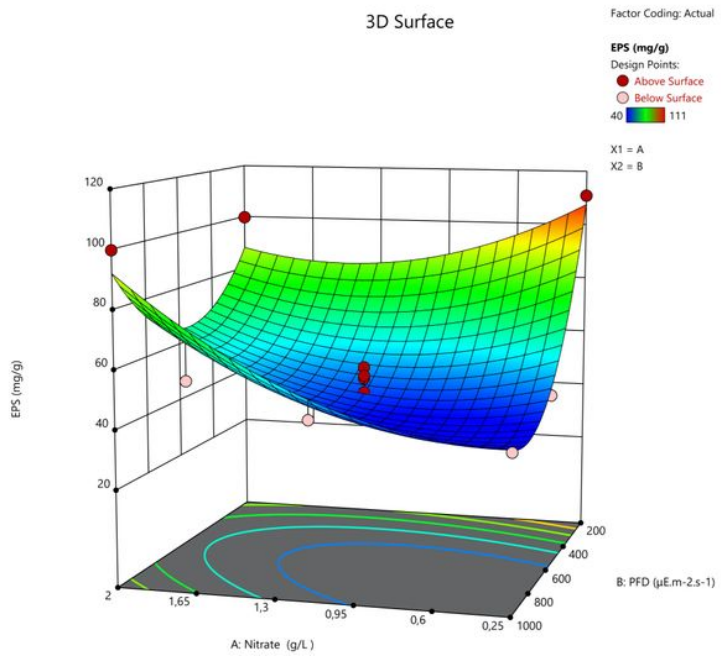
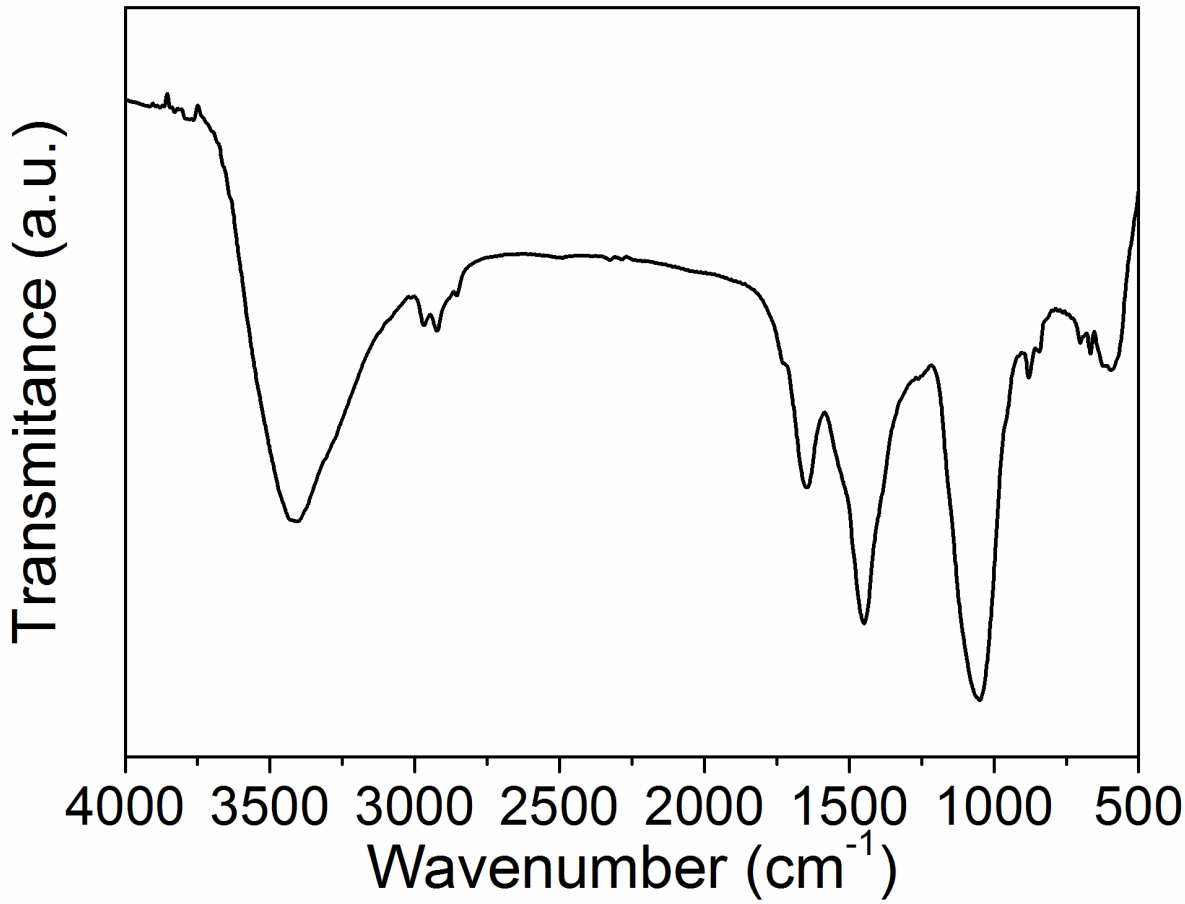


Figure 2

Response surface for biomass (a) and EPS production (b).

Figure 3



Characteristic FTIR spectrum for EPS from A. platensis.

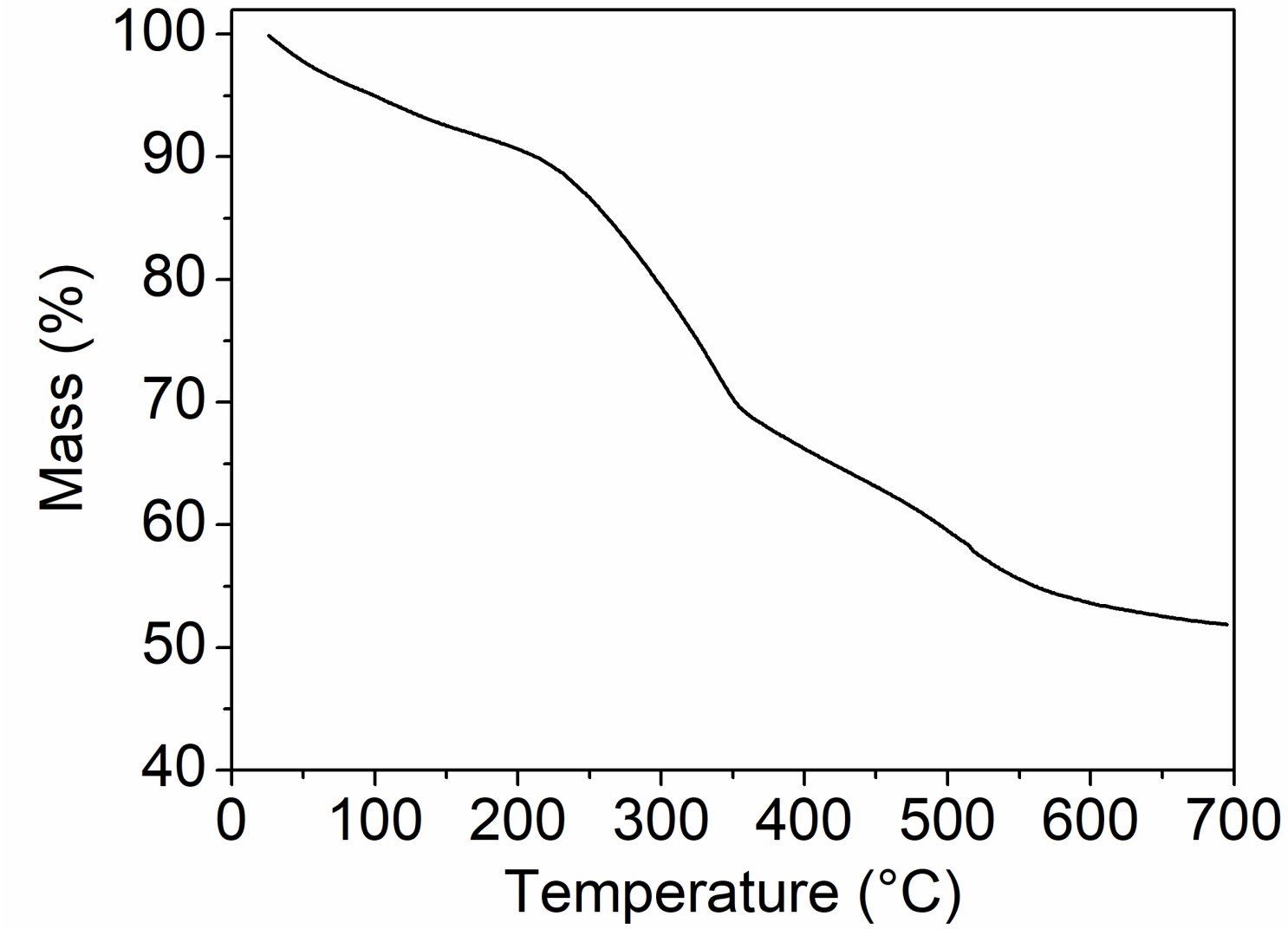


Figure 4

Thermogram for EPS obtained from A. platensis.

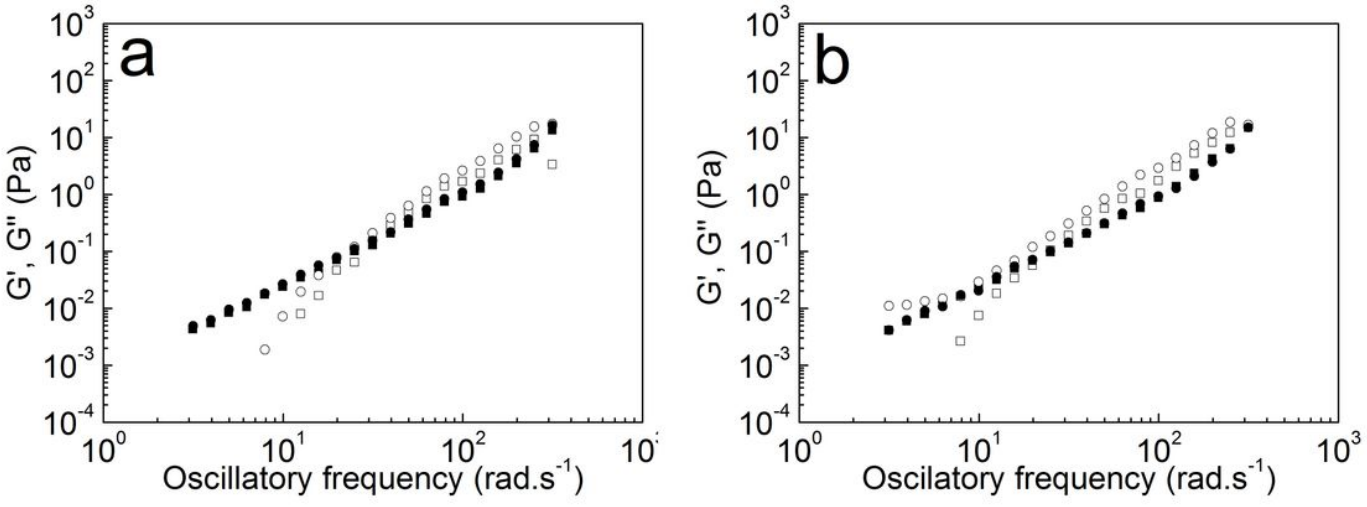


Figure 5

Variation of storage and loss modulus values with oscillatory frequency for the solutions at 5 gL-1 (a) and 10 g.L-1 (b).