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Cultivation of *Euglena gracilis* on Residues from a Food Industry

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***Abstract* – *Euglena gracilis* is a photosynthetic, acidophilic flagellate, also classified as a microalga, growing also in mixotrophic conditions. It can accumulate high-value molecules, but its production is very expensive, especially due to the cost of the required carbon source. The present work was carried out to optimize at lab-scale (4 L photobioreactors, 1.5 L working volume) the batch cultivation of *Euglena* on industrial residues from a firm producing food commodities. The effluent from the anaerobic digestion of the liquid residues was used and mixed with a sugar-rich vinasse. In the first tests different working pH, acidifying agents, stirring methods and vinasse concentrations were tested. After selecting the best operation parameters among them, different kinds and intensity of light were tested, as well as different cultivation times. As the aim of *Euglena* production is to use its biomass in pet feed formulations, it was also important to check contamination, especially by eumycetes, which find favourable growth conditions in the presence of nutrients and sugars and at low pH. The best results were obtained in batch, at pH 5, with the mix of UASB effluent and 2.5 % of vinasse, using purple light, provided by means of LED strips at 50 µmol/m^2/s. The maximum TSS concentration was 1.14 g/L, after 72 h, with a paramylon content total of 28 %. COD removal efficiency, on the contrary, reached the maximum value of 78 % at 144 h and N removal did not vary between 72 and 144 h. The content of pigments also increased between 72 and 144 h. In the selected optimal condition, no significant contamination occurred. The use of UASB effluent enriched with vinasse as substrate proved to be a good, reliable and economic option for *Euglena* cultivation whose performances were comparable or even better than reported for synthetic, sterilized substrates, and occurred when using the lower intensity of purple light.**

***Keywords* – Algal biomass; food commodities; lab-scale testing; paramylon; pigments.**

1. INTRODUCTION

Euglena is a genus of photosynthetic organisms able to grow not only in autotrophy but also in heterotrophy and in mixotrophy. Its economic value lies upon its high protein and carbohydrate content [1]. The proteins in *Euglena* are especially valuable, containing all the 20 essential amino-acids [2] and the carbohydrate component include large amounts of paramylon. Paramylon is a linear β-glucan accumulated by *Euglena* spp. as storage substance, especially in the first stages of its cultivation [3]. According to Barsanti *et al.* [3] the accumulation occurs in the first 48 hours, while later on *Euglena* cells use it and

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paramylon concentration decreases. Some other research attributes paramylon production to environmental stress factors, as salinity peaks [4], causing also some decrease in biomass growth and photosynthetic pigment concentration.

Paramylon use concerns chiefly pharmaceutical and cosmetic applications, due to its antioxidant, antitumoral and antibacterial properties [3], to its vitamin content [5] as well as to its capacity of inhibiting HIV replication [6]. Paramylon is also a dietary fiber and contributes to reduce the levels of cholesterol in blood [7], [8].

Further applications of *Euglena* biomass are in the production of thermoplastics [9] and nanofibers derived from paramylon which can be used to prepare slow-release drugs [10] and in all the fields involving the use of fatty acids, in particular DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) [11].

Unfortunately, *Euglena* cultivation poses a series of challenges. The highest production of biomass and valuable compounds occurs in mixotrophy and this involves high costs for carbon substrate and high risks of contamination. In particular, eumycetes find optimal conditions at low pH and large availability of sugars. The presence of mixed consortia, including bacteria, does not seem necessarily to decrease the production of valuable molecules, as shown by Ouyang *et al*. [12], but certainly affects the reliability of the product quality, and the competition between *Euglena* and other organisms can lead to uncontrolled proliferations.

Our research aimed at verifying the possibility of using residues from a food commodity industry as substrate for growing *Euglena* and to define the best cultivation conditions for biomass, paramylon and pigment production. The origin of such residues would allow to exploit *Euglena* biomass also in sensitive markets, and the possibility of removing at the same time COD and nutrients from the anaerobic effluent would add value to the process. Only few studies are reported in the literature of cultivation of *Euglena* on wastewater or liquid wastes [13]–[16], all carried out in very different conditions, and none using effluents form anaerobic treatment and residues from food commodities industry.

2. Methods and Methodology

2.1. Algal Strain

The *Euglena gracilis* strain used in this study was provided by Istituto Sperimentale Italiano Lazzaro Spallanzani, located in Rivolta D'Adda (CR), Italy. The microalgal inoculum was kept in an indoor glass column photobioreactor, fed on Cramer-Myers Synthetic medium [17], artificially illuminated on a 12-hour light/dark cycle, at room temperature (20.3 °C to 28 °C).

2.2. Photobioreactors

The cultivation experiments were based on a specially designed system made of four Plexiglas column photobioreactors (PBRs) (diameter = 110 mm, height = 40 cm, working volume = 2.5 L) mixed by magnetic stirrers (ASTEROID 40, 2mag AG, Muenchen, Germany) and equipped with artificial lights (50–150 μmol/m^2/s) and sensors to monitor temperature, pH, and dissolved oxygen in continuous (IDEA Bioprocess Technology Srls, Dalmine, BG, Italy) (Fig. 1). Light was provided for 12 hours/day.

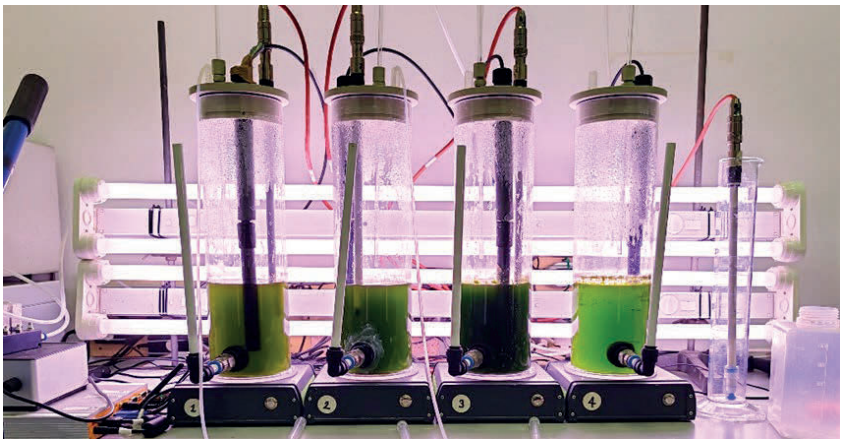


Fig. 1. The bench scale photobioreactors used for the tests.

2.3. Growth Substrates

The Cramer Myers medium [17] was used for reference in order to have a blank. The tested substrate was the effluent from the anaerobic treatment of liquid residues from food commodities (liquids from gas condensation produced in various processes, effluents from the regeneration of resins used for water cleaning), having COD = 230–850 mg/L, TSS = 0.4–0.6 g/L, Total N = 50–190 mg/L, NH_4-N = 50–56 mg/L, and P-PO_4 = 21–32 mg/L, enriched with vinasse (COD = 65 000 mg/L, TSS = 100 g/L, total N = 3000 mg/L, NH_4-N = <20 mg/L, N-NO_3 <10, P-PO_4 = 1000 mg/L). Different samples of the effluent, as such and after nanofiltration, were tested, while only one stock of vinasse was used.

2.4. Analyses

The algal growth was monitored based on the increase of Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS), measured gravimetrically, and on cell counts. An optical microscope at 40× magnification (B 350, Optika, Italy), and a haemocytometer (Marienfeld, Germany) were used for algal cell counts, after treating the sample of algal suspension with 4 M HCl to stop the movement of *Euglena*. The number of cells per milliliter was the average of the counts in 72 square observations (0.04 mm^2).

The analyses of Total Nitrogen, Phosphate (PO_4-P), and soluble Chemical Oxygen Demand (COD) were carried out using spectrophotometric test kits (Hach-Lange, Düsseldorf, Germany, LCK138, LCK238, LCK 338 for total nitrogen, LCK049 for phosphate, and LCK1414 for COD) on filtered samples (0.45 μm). Dissolved oxygen, pH, and temperature were monitored continuously within the PBRs.

The paramylon content of the algal biomass was determined according to Schwartzbach *et al*. [18]: 0.05 L of the microalgal suspension from PBRs was centrifuged to separate the biomass, which was then re-suspended in a solution containing 1 % (w/v) SDS (Sodium dodecyl sulfate) and 5 % (w/v) Na_2EDTA. The biomass was recovered by centrifugation for 10 minutes at 10 000 rpm of the obtained suspension, after 30 minutes incubation at 37 °C. The SDS-Na_2EDTA treatment was repeated, and followed by two washings with hot distilled water (70 °C). After the second wash, paramylon was filtered, collected on glass fiber filters (VWR) and dried overnight at 90 °C to determine its weight.

Pigment concentrations were determined after a two-step extraction in acetone. samples were sonicated for 20 minutes to disrupt the cells and release intracellular contents and pigments into the solvent. According to Pancha *et al.* [19] the concentrations of chlorophyll a, chlorophyll b and carotenoids were calculated basing on the spectrophotometric absorbance at specific wavelength, by the following equations:

Chlorophyll a (μg/mL) = 16.72 OD_{665} – 9.16 OD_{652};

Chlorophyll b (μg/mL) = 34.09 OD_{652} – 15.28 OD_{655};

Carotenoids (μg/mL) = (1000 OD_{470} – 1.63 Chla – 104.9 Chlb)/221, where OD stays for Optical Density.

2.5. Experimental Plan

The selected parameters to be tested to optimize the culture were: pH, concentration of vinasse, time, operation mode (batch, semi-continuous, fed-batch), light wavelength and intensity, aeration, cultivation time. CO_2 and HCl to control pH were also compared. On the whole, 14 batch tests were performed, the first 8 of which are to be intended as preliminary, aimed to define a first selection of the conditions to be further investigated.

The following ones were carried out using the selected pH, vinasse concentration, stirring method and acidification agent and were focused at comparing the effects of different light source (fluorescent lamps and LED), intensity (50 to 150 μmol/m^2/s) and colour (white and purple).

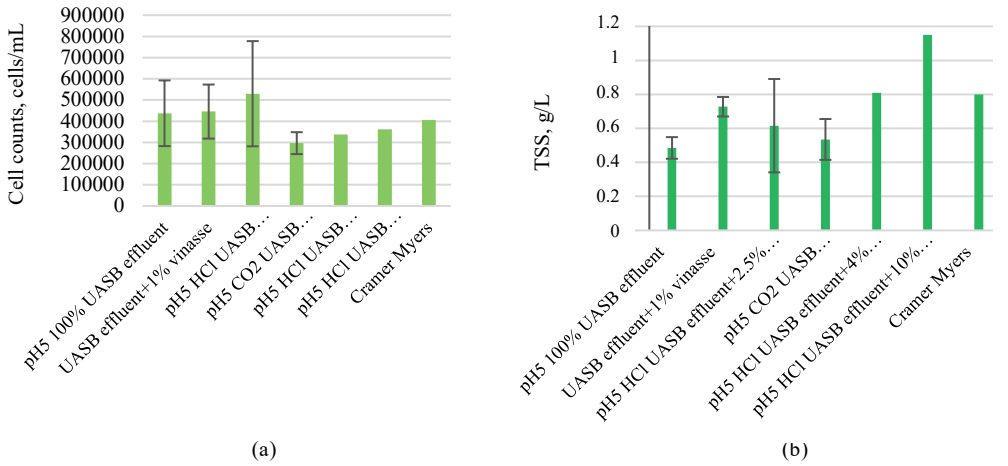
3. RESULTS

The two first tests allowed to select 5 as optimal pH for growing *Euglena gracilis*: at such value the maximum values of VSS and TSS were 0.27 and 0.51 g/L, respectively, and were reached in 48–96 hours. The TSS production rate was 20 mg/L/day and the specific growth rate was 0.23 d^{-1}. At pH 3 all the recorded values were less favourable, while at free pH (without any correction, starting at 8.38 and reaching 8.93 in 96 hours) the removal of nutrients was higher, but contamination by other microorganisms occurred and was probably responsible for such removal.

Test 3 showed clearly that air bubbling had a negative effect both on algal growth and on nutrient and COD removal. Such effect was likely due to CO_2 stripping and to the consequent pH increase, which raised over 9, far from the *Euglena* optimal range and likely to generate high concentrations of free NH_3, toxic for algae. Tests 4 and 5 confirmed that the optimal pH value was 5 and the needed supplementation of vinasse was 2.5 %. Test 6 compared the effect of acidification by CO_2 and HCl addition and showed better algal performance when using HCl.

So, the following tests, aiming at selecting the possible need and optimal dose of vinasse to be added to UASB effluent were carried out at pH 5 without air bubbling, mixing the algal suspension by magnetic stirring.

Fig. 2 shows the averages of the maximum values obtained in the different tested conditions for cell counts and TSS, the most significant indicators for algal growth, mostly obtained between 48 and 96 hours of cultivation.



(a) (b)

Fig. 2. Maximum values of cell counts (a) and TSS (b) in the different test conditions: average and standard deviations (error bars).

It is interesting to observe that the results for TSS and algal cells do not follow the same trend. In fact, even if TSS are generally held as representative of algal growth, in the cases of heterotrophic or mixotrophic their value can be affected by contamination by organisms other than algae. In the case of acidophilic cultures, eumycetes find a particularly suitable condition. Further, when a waste or by-product is used as feed, it may have a starting concentration of TSS whose value sums up to the one due to algal growth. In the specific case, the major influence is related to the amount of vinasse added to the UASB effluent (whose TSS concentration is much lower than the one of vinasse) but also to contamination by bacteria and eumycetes. In fact, it is interesting to observe that the two series of tests with the addition of 2.5 % of vinasse gave exactly the same maximum value for TSS while the maximum algal cell count was higher when pH was regulated by HCl than by CO_2. The synthetic medium Cramer Myers produced maximum cell counts comparable to the ones observed with UASB effluent+vinasse but much lower TSS concentration.

Fig. 3 reports the removal efficiency of nutrients and COD in the different conditions.

A certain variability can be observed, leading to the prevailing absence of significant differences.

Table 1 compares the maximum values for paramylon % in the biomass and the production rate of paramylon in the different conditions, in batch tests, with reference to the cultivation time.

Opposite to some literature data [3], paramylon concentration grew with time, with maximum values between 72 and 96 hours.

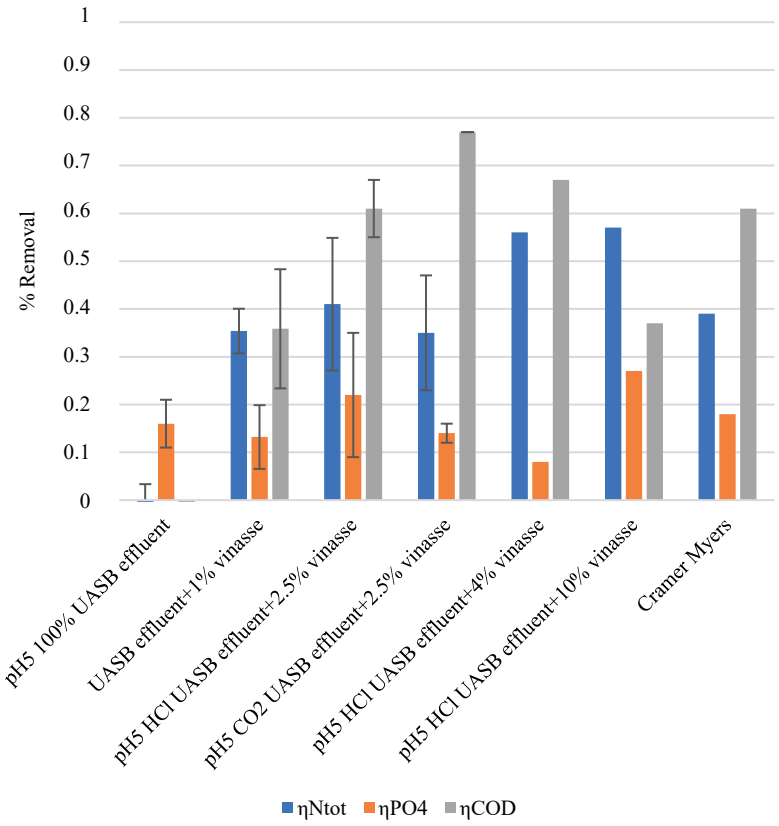


Fig. 3. Percent removal efficiency of nutrients and COD in the different test conditions: averages and standard deviations.

TABLE 1. COMPARISON BETWEEN THE MAXIMUM CONCENTRATION OF PARAMYLON IN THE BIOMASS % AND THE PARAMYLON PRODUCTION RATE

Test condition	**Paramylon % (d.w.)**	**Paramylon Production rate, mg/L/day**	**Hours**
pH5 HCl UASB effluent+2.5 % vinasse	16	30	96
pH5 CO_2 UASB effluent+2.5 % vinasse	4	9	72
pH5 HCl UASB effluent+4 % vinasse	13	37	96
pH5 HCl UASB effluent+10 % vinasse	9	12	72

The concentrations of chlorophyll also increased with time and were higher in the UASB effluent+2.5 % vinasse. For chlorophyll b the increase was observed only at 72 hours and the concentrations were just a little higher for UASB effluent + 2.5 % vinasse and a little lower with 10 % vinasse.

Carotenoids had a different trend, with the highest concentrations at 48 hours with UASB effluent+2.5 % vinasse and lower values for the two other vinasse addition; at 72 hours the value for UASB effluent+2.5 % vinasse slightly decreased while the other two values increased and the maximum was measured in the test with UASB effluent+4 % vinasse where, however, a great amount of eumycetes was detected. Such results are summarized in Fig. 4.

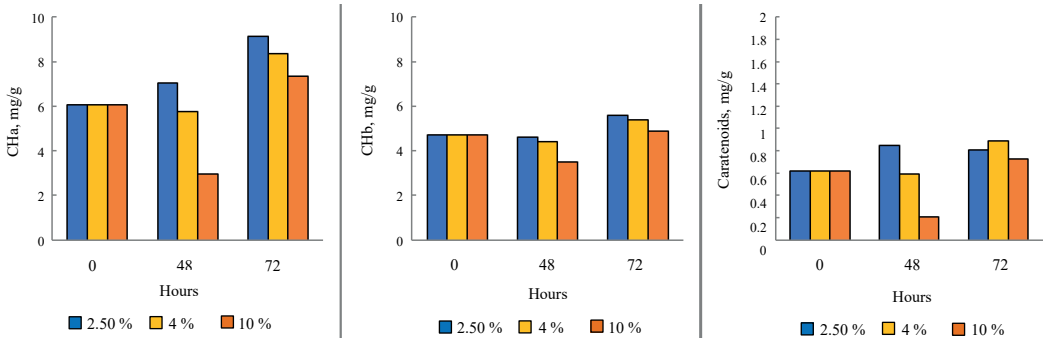
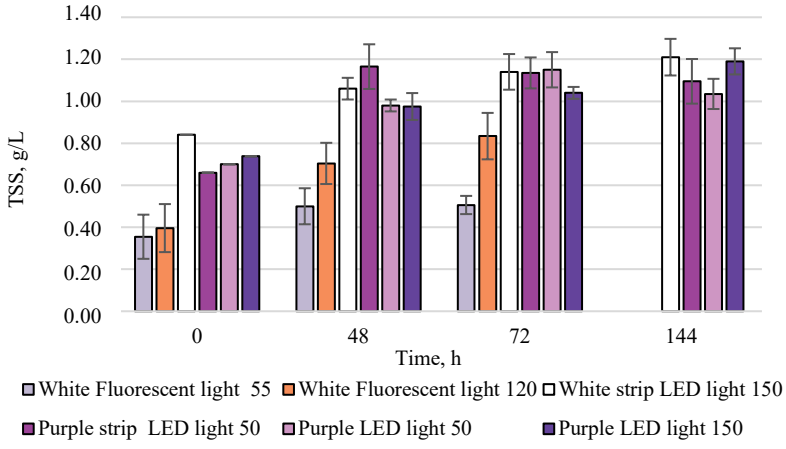
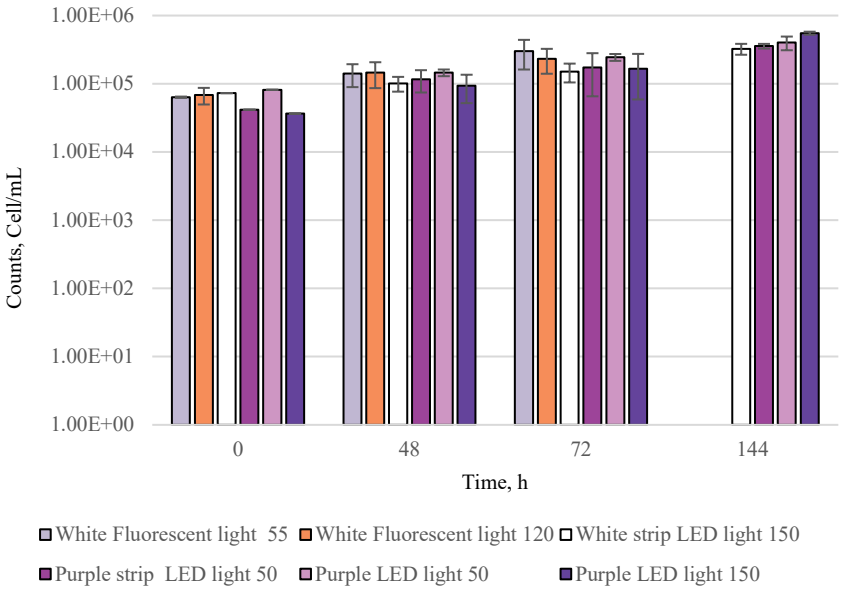


Fig. 4. Trends of pigment concentrations (mg/g) in the biomass in the different test conditions: (a) chlorophyll a, (b) chlorophyll b, (c) carotenoids.

Fig. 5 reports the results of the last 6 tests carried out in different light conditions in terms of cell counts and of TSS concentration. The other operation parameters were the ones selected after the first series of tests. No significant differences were found considering *Euglena* cell counts, however TSS concentration was higher when LED strips had been used.



(a)



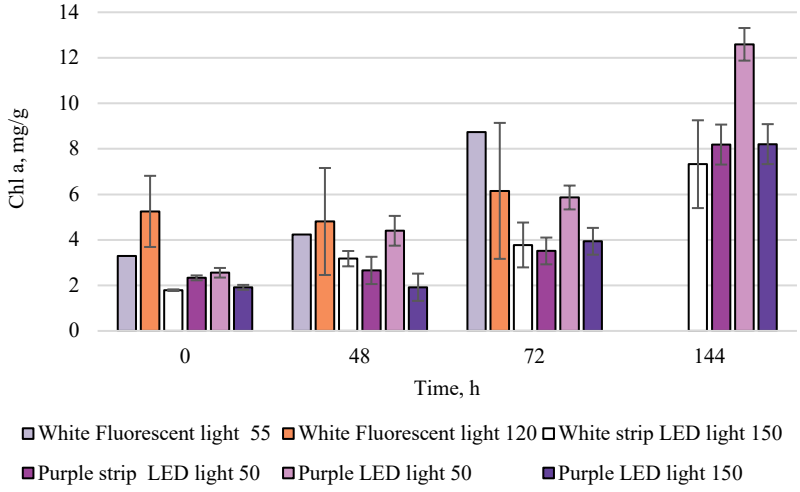
(b)

Fig. 5. Cell counts (a) and TSS (b) at different time intervals in the different test conditions: averages and standard deviations (error bars) for 4 replicates.

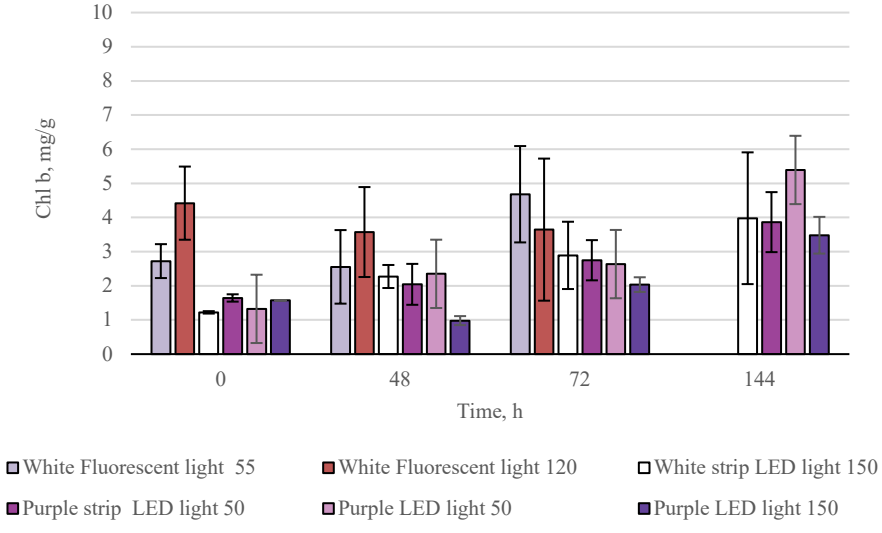
The obtained algal density was good, and similar or higher than reported in other studies [20], [21]. The concentrations of pigments (chlorophyll a and b and carotenoids) and of paramylon in the biomass are reported in Fig. 6, 7 and 8, respectively, while Fig. 9 summarizes the percent removal efficiencies for total nitrogen and COD.

The figures show clearly that the best results were obtained with purple strip LED illumination at 50 µmol/m^2/s. There was no difference in growth performance between 72 and 144 hours; the production of chlorophyll a and b increased significantly from 72 and 144 hours while the accumulation of paramylon decreases in the same time interval. Carotenoid production increased with time, reaching its maximum at 144 h, as well as COD removal efficiency (78 %). No significant differences were observed for nitrogen removal efficiency.

The pigment concentrations were comparable to the ones reported in the literature: chlorophyll a, chlorophyll b and carotenoid average concentrations were 8, 3.9 and 1.3 mg/L, respectively whereas Zhang *et al.* [21] reported 8, 2 and 1.7 mg/L in *Euglena* mixotrophic cultures in synthetic, sterilized medium where, however, the algal biomass density was much lower. Paramylon concentration in the biomass was lower than some of the literature data but higher than obtained by Zhu and Wakisaka [22] in *Euglena* grown in Cramer Myers medium, by Rodriguez-Zavala *et al.* [2], working in heterotrophy in the dark for 5 days, in sterility, using a synthetic medium. Much higher concentrations (60–68 %) are reported by Rubiyatno *et al.* [13] who grew *Euglena* on sewage effluents, sterilized by autoclave, diluted and supplemented with corn steep liquor or waste wine as carbon source and worked at a very small scale (200 mL) for 7 days, producing a very low amount of biomass (maximum value of algal density, at 7 days, below 400 mg TSS/L).



(a)



(b)

Fig. 6. Concentration of chlorophyll a (a) and chlorophyll b (b) in the *Euglena* biomass grown in different light conditions; averages and standard deviations (error bars) for 4 replicates.

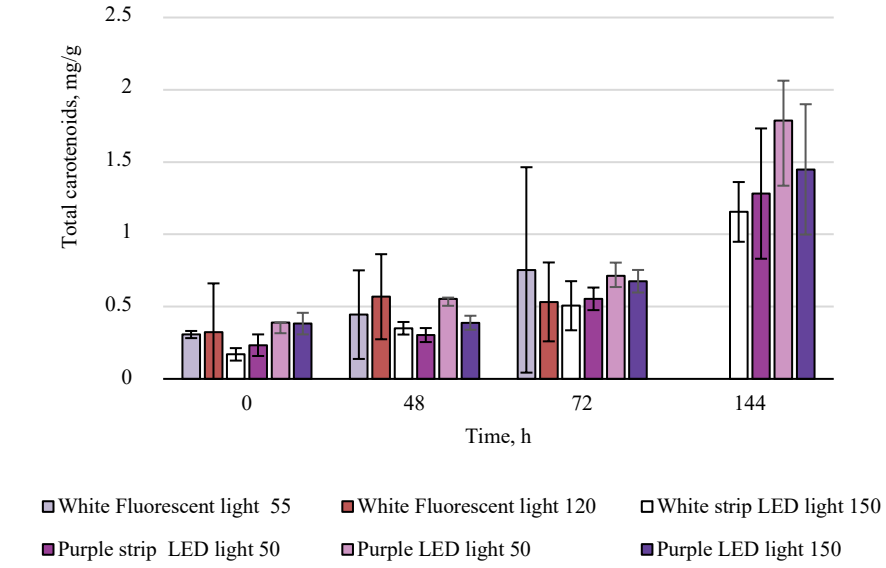


Fig. 7. Concentration of carotenoids in the *Euglena* biomass grown in different light conditions; averages and standard deviations (error bars) for 4 replicates.

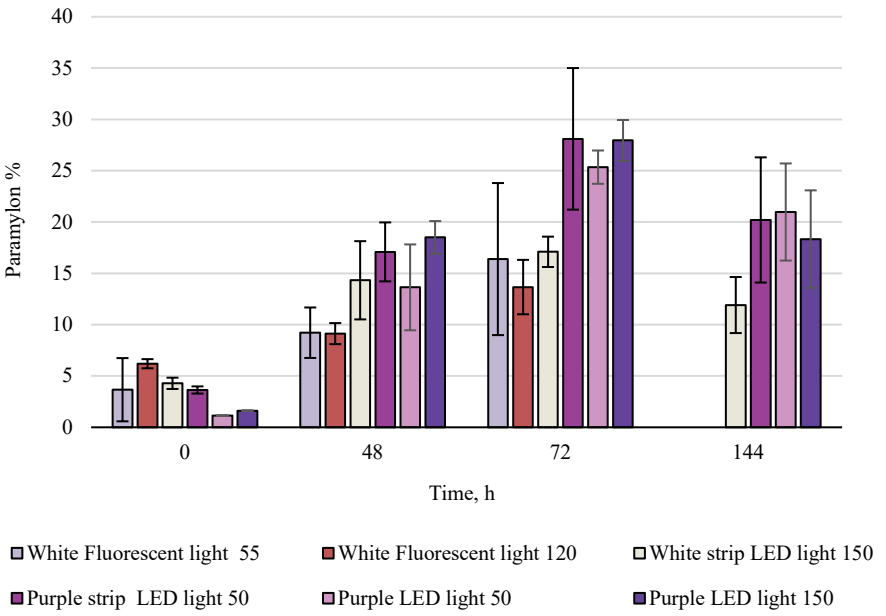
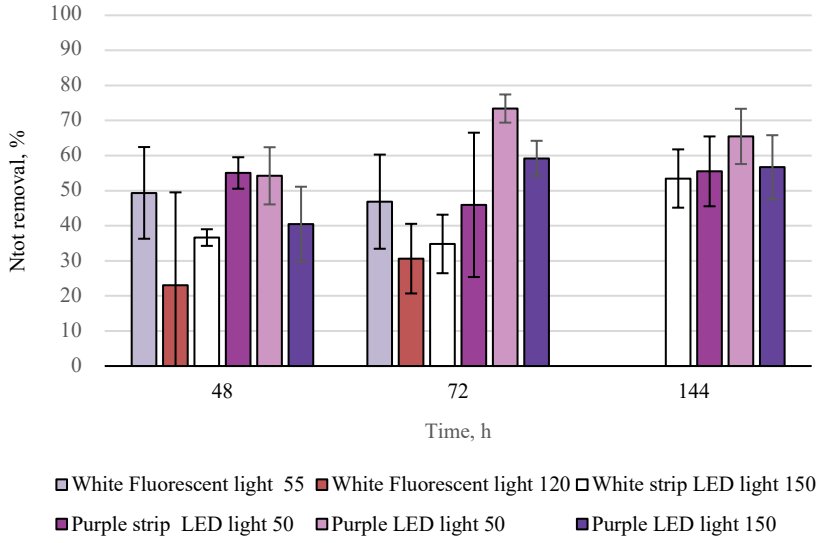
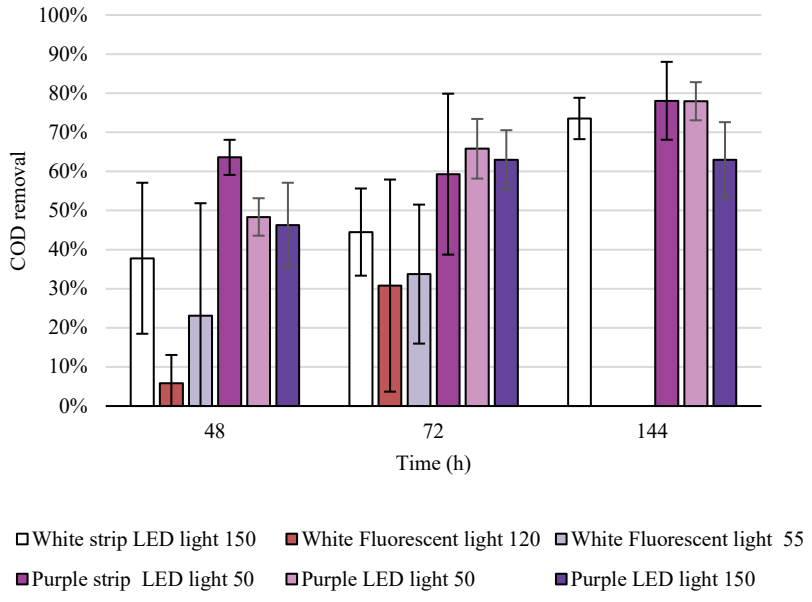


Fig. 8. Concentration of paramylon in the *Euglena* biomass grown in different light conditions; averages and standard deviations (error bars) for 4 replicates.



(a)



(b)

Fig. 9. Percent removal efficiency for total nitrogen (a) and COD (b) in different light conditions; averages and standard deviations (error bars) for 4 replicates.

3. DISCUSSION AND CONCLUSIONS

The experiments led to conclude that *Euglena gracilis* could be cultivated using the UASB effluent deriving from the treatment of liquid residues from a food commodities industry as substrate. As the best growth condition for *Euglena* is mixotrophy and the UASB effluent was rich in nutrients but not in degradable organic matter, the needed carbon was successfully added using vinasse, another residue from the same origin, providing results comparable to the ones obtained using sterilized, synthetic media.

As it is common when growing algae in mixotrophy and heterotrophy, the problem of contamination by other organisms has to be controlled and, in fact, limits the operation mode to batch: both semicontinuous and fed-batch mode would involve too much contamination as demonstrated by some preliminary tests. At the same way the amount of vinasse to be added to UASB effluent has to be limited. This could clearly be seen in the first tests when comparing the algal count and the TSS concentrations: algal counts showed just small increases with increasing vinasse dose, while TSS concentration grows much more and such increase can only be partly explained by the higher TSS input deriving from vinasse. In fact, such hypothesis was regularly confirmed by microscope observations. That is why the addition of 2.5 % vinasse to the UASB effluent was selected as the optimal dose. The last 6 tests were conducted in optimal conditions and this limited the contamination by other organisms.

The overall optimal conditions were defined on the basis of the results obtained in the first set of tests for pH, vinasse concentration, stirring method and acidification agent, and in the last 6 ones for light wavelength, intensity and kind of lamps and were the same for all parameters.

The optimal cultivation time varies according to the prevailing aim of the culture.

If *Euglena* is grown in order to exploit its paramylon content, it is 72 hours. In fact, using purple light, provided by means of LED strips at 50 μmol/m^2/s at 72 h the cell count ($1.7 \cdot 10^5$ cells/mL) and TSS concentration (1.14 g TSS/L) were not different from those recorded at 48 h, but the paramylon content in the biomass concentration reached 28 %, while it was 17 % at 48 h. In terms of cell growth, the increase was around half an order of magnitude in 48 h with no further increase thereafter.

If the prevailing aim is the removal of COD and of nitrogen the cultivation time should be longer: the best results in this perspective were obtained at 144 h. The same time is needed to reach the maximum concentration of all the searched pigments (chlorophyll a and b, and total carotenoids).

It is interesting to remark that the selected kind, intensity and wavelength of light correspond to the lower power consumption and, thus, to the lower carbon footprint and that the use of wastes from food industry is a further advantage in terms of circular economy and involves no sanitary risks in the produced biomass

To summarize, the obtained results are encouraging, especially because little experience is available on this specific topic.

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