Testing *Chlorella vulgaris* strain adapted to winery waste digestate in lab scale semi-continuous system

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Abstract
In this paper anaerobic co-digestion of waste activated sludge and wine lees was integrated with microalgae-based process with the aim of treating digestate and recover lipids as fuel precursors in a circular economy approach. In a one-litre vertical photobioreactor *Chlorella vulgaris* was tested both in batch and semi-continuous mode. Switching the culture from a batch to semi continuous cultivation system, microalgae showed a morphological strain structure change, with a decreased cellular count, a stable dry weight (0.67 ± 0.02 g l^{-1}) and a 17% lipids content (on TS basis). A stable ammonia, calcium and sodium removal of 76.37 ± 8.64 %, 78.25 ± 5.05 % and 29.83 ± 6.24 % respectively was detected.

Keywords: Chlorella vulgaris, digestate, winery, anaerobic digestion, codigestion.

1. Introduction

Nowadays alternative green fuel production is a global challenge to overturn the fossil fuel consume and the environmental-health impact[1]. An important solution can be found in third generation biodiesel from biodegradable, renewable and non-toxic resource as microbial biomass[2]. On this way, microalgae biomass shows the perfect characteristic to be used for biofuel production: i) microalgae has an oil productivity higher than oil crops (136,900 – 58,700 l oil ha^{-1} $year^{-1}$ by microalgae biomass and 5,366 – 172 oil ha^{-1} $year^{-1}$ by plants) [3], ii) can grow on non-arable land with brackish water and on wastewater with a simultaneous phytoremediation effect, iii) can fix CO_2 and/or organic carbon using nitrogen and phosphorous as nutrient, iv) the biochemical biomass composition can be tuned by culture conditions (i.e. temperature, nutrient concentration, salinity etc.)[1].

A sustainable substrate for microalgae biomass production, and therefore lipids storage, is the liquid fraction of digestate from anaerobic digestion (AD) system[4,5]. The supernatant fraction obtained after solid/liquid separation, has usually high ammonia concentration (between 139 and 3,456 mg l^{-1}) [6] that caused most of the inhibition problems; moreover also rotifers, recalcitrant molecules and heavy metals presence can inhibit microalgae growth and decrease the remediation efficiency [7,8]. For these reasons, research studies on microalgae proliferation on digestate, adopts high dilution and sterilization before test [6,9–13]. Microalgae-based processes can potentially close the bioprocess treatment of municipal, agricultural and industrial waste after AD treatment, in a circular economy approach, with secondary high value products recovery and treated water as output [14].

Typically, microalgae cultivated under stress conditions (for example nitrate [15], phosphorous and sulphate starvation [16] and high pH [17]), showed a large lipid storage (30-50 %TS) [18] with a lipids composition that permit its use for biofuel production[2]. As reported by Yang et al. [19], batch cultivation in open pound was not sustainable for its water footprint of 3,726 kg-water kg-$biodiesel^{-1}$ (without water recycling system), where the 81.10% of water was discharged after harvesting. Wang et al. and Bei et al.'s studies [20,21] showed *C. vulgaris* semi-continuous system cultivation on digestate and compost with a biomass and oil production of 1-1.38 g l^{-1} and 25-30 %TS respectively and a promising strain capacity to remove phosphorous, nitrogen and chemical oxygen demand (COD) simultaneously. Semi-continuous process cultivation is the widest applied in large scale open pound cultivation systems and allow to a lower water consumption [21]. Semi-continuous cultivation permit the recirculation of water, where wastewater used as medium could be reutilized instead of fresh water or medium[22]. If wastewater

or digestate [23,24] are used for microalgae cultivation in semi-continuous system, the 90% of freshwater consumption can be reduced [19] with a stable biomass production in long-term cultivation [22].
Wine production is one of the most important agricultural activity in Mediterranean countries, where one hectolitre of wine produced 196 l of wastewater, 1.6 kg of wine lees and 0.1 kg of waste activated sludge [25]. Wine lees is a seasonal waste, with an important environmental footprint considering its composition: organic components (i.e. polyphenols compounds), low pH (between 3 and 4), salinity, heavy metals presence and recalcitrant molecules content that could inhibit microbial land proliferation [26,27]. Only in the middle of 2000s some studies were focused on winery waste and wastewater stabilization using anaerobic digestion process [27] and, on literature basis, only two studies used winery lees in batch microalgae cultivation after sterilization pre-treatment [28,29], with a microalgae biomass production of 1.62 ± 0.11 g l^{-1} [30]and 0.300 g l^{-1} d^{-1} – 0.600 g l^{-1} d^{-1} [31].
In this study *Chlorella vulgaris* was tested in a two steps approach (batch and then semi-continuous) the feasibility of microalgae cultivation on digestate from winery waste treatment (co-digestion of wine lees and activated sludge); ions variation and biomass production was investigated and discussed.

2. Material and methods

2.1 Wine lees, waste activated sludge and digestate characterization

Winery digestate (WL-DIG) was obtained from a lab scale anaerobic digester. AD system was fed with wine lees and waste activated sludge (WAS) collected from a cellar in the North-East of Italy. Digestate, wine lees and WAS chemical-physical characterization was reported in Table 1 and Table 2. AD reactor volume was 4 litres with hydraulic retention time (HRT) of 20 days, and an organic loading rate (OLR) of 2.15 gCOD l^{-1} d^{-1}. The Specific Gas Production (SGP) in the (SSC) state conditions was in a range of 0.35 and 0.40 m^3_{biogas} kg_{VS}^{-1} d^{-1}. Digestate used for microalgae test was previously pre-treated only with centrifugation at 10,161 g for 5 minutes to remove suspended solids. Supernatant digestate was not sterilized before microalgae test. All analysis were carried out following APHA methodology [32].

Table 1: Wine lees, activated sludge and digestate characterization

	WAS	Wine lees	Digestate (WL-DIG)
TS (gTS kg^{-1})	140.53 ± 35.24	179.57 ± 47.38	48.29 ± 5.79 0.24 ± 0.16*
TVS (gTVS kg^{-1})	84.45 ± 64.93	119.22 ± 107.25	12.63 ± 2.23
N ammonia (mgN-NH_4^+ l^{-1})	-	14.10 ± 0.60	478.00 ± 23.00
Phosphates (mgP gTS^{-1})	8.00 ± 2.00	1.50 ± 0.10	-
TKN (mgN gTS^{-1})	168.10	13.92	-
pCOD (mgO_2 gTS^{-1})	669.00 ± 215.00	139.00 ± 58.00	-
sCOD (mgO_2 l^{-1})	-	113.50	252.00 ± 123.00
pH	7.42	3.37	7.58 ± 0.07
Total Alkalinity (mg $CaCO_3$ l^{-1})	-	-	1,962.00 ± 17.00
Partial Alkalinity (mg $CaCO_3$ l^{-1})	-	-	1,456.00 ± 203.00
Na^+ (mg l^{-1})	-	-	388.24 ± 85.42
NH_4^+ (mg l^{-1})	-	-	503.46 ± 59.83
K^+ (mg l^{-1})	-	-	417.55 ± 72.48
Mg^{2+} (mg l^{-1})	-	-	178.09 ± 36.65
Ca^{2+} (mg l^{-1})	-	-	568.97 ± 121.14

**TS value after centrifugation*

2.2 C. vulgaris adaptation and experimental set up

C. vulgaris strain was given by ACUF (Algal Collection of University of Federico II, Naples, Italy). Inoculum was maintained in mixotrophic growth condition with synthetic medium (BG11) in

exponential growth phase with light irradiation (3.9 klux), mechanical agitation (330 rpm) and air bubbling (2.3 vvm) applied in continuous. To adapt *C. vulgaris*, inoculum was cultivated in 300 ml Pyrex flasks and in mixotrophic condition with 1:10 supernatant WL-DIG not sterilized. *C. vulgaris* abundance was confirmed with direct view on optical microscope (40X) and no microbiological predators (e.g. rotifers) were detected. Microalgae growth was monitored with direct cellular count and dry weight to identify exponential growth phase. After consecutive inoculum in microalgae exponential growth phase, supernatant WL-DIG dilution was decreased from 1:10 to 1:5; light irradiation at 3.9 klux, air insufflation at 2.3 vvm and mechanical agitation at 330 rpm, were applied in continuous. In this adaptation phase, inoculum was diluted 1:10 and after 1:5, as for supernatant digestate. When the inoculum was in stationary phase, it was used for two step cultivation test: in the first step was applied a batch culture with WL-DIG 1:5 diluted with BG11, light irradiation (3.9 klux), air bubbling (2.3 vvm) and mechanical agitation (330 rpm) applied in continuous. After 8 days in batch growth conditions the stationary growth phase was achieved and the system was shifted in semi-continuous mode with WL-DIG and applying an hydraulic retention time of 8 days. Inoculum for batch test started with 182.40 ± 14.42 million cell ml^{-1} and 0.06 ± 0.00 g l^{-1}, *C. vulgaris*' cell density and dry weight respectively. Test was performed in one litre tubular vertical PBRs (Pyrex, 465 mm height) with inoculum and WL-DIG 1:5 initial dilution. PBR tests were performed in double.

2.3 Microalgae biomass and effluent analysis

Biomass monitoring was carried out using dry weight and cells count analyses, using acetate cellulose filter with porosity of 0.45 µm (Whatman) in oven at 105 °C and Bürker camber with 10 µl sample on optic microscope (40X, Laika microscope) respectively. Dry weight and cells count analyses were performed two times for day during semi-continuous cultivation, before feeding (24 h) and after supernatant digestate addition.

Dry weight (g l^{-1}) data was obtained with eq. 1:

$$Dry\ weight\ (g\ l^{-1}) = \frac{g_{(filter+dry\ sample)} - g_{(filter\ unladen\ weight)}}{l_{sample}} \qquad \text{Eq. 1}$$

On microalgae biomass, during second test phase, was measured total lipids storage with Folch's gravimetrical methodology [33]. Previously lipids extraction, microalgae frozen biomass was lyophilized using EDWARDS Freeze Dryer Modulyo at -40°C and EDWARDS A653-01-903 vacuum pump. On lyophilized biomass, chloroform and methanol extraction solution (2:1) was applied [34,35]. The mixture was then sonicated for 1 hour and centrifugated at 10,161 g for 5 minutes, to remove cellular remains. Solvent plus lipids solution obtained was cleaned with distilled water and only the organic phase was recovered and transferred in a glass balloon (previously weight) in Rotavapor machinery at 30 °C for solvent removal. Microalgae lipids content was calculated with Eq. 3.

$$Lipids\ total\ storage\ (\%TS) = \frac{g_{oil} - g_{unladen\ weight}}{g_{microalgae\ biomass}} * 100 \qquad \text{Eq. 3}$$

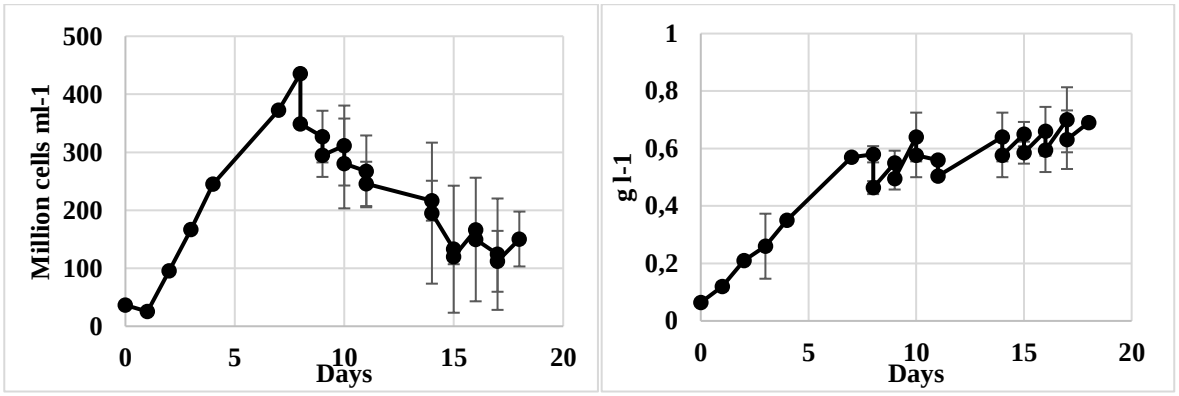
pH and ionic chromatography analyses were performed daily. pH analysis was carried out with Hanna instrument pH probe directly on supernatant filtered at 0.45 µm with acetate cellulose filter (Whatman). Cationic chromatography was applied to identify Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+} variation in supernatant during batch and semi-continuous test. Analyses were performed every 24 h during the batch test and two times per day during semi-continuous system (before and after feeding). Cationic chromatography analysis was performed with Dionex LC25 oven, Dionex GS50 gradient pump, Dionex ED50 detector, Dionex GC12A 2x250 mm precolumn, Dionex IonPack CS12A 2x250 mm column, Dionex CSRS Ultra

II suppressor and N_2SO_4 (Sigma Aldrich) 22mN eluent. Analyses were performed in duplicate for each PBR test.

3. Results and discussion

3.1 Microalgae monitoring and lipids storage

During the batch cultivation with WL-DIG 1:5 diluted (first step), dry weight analysis (Figure 1b) was used to identify stationary growth phase starting (8th day) and thus to switch cultivation system in a semi-continuous mode. This two steps technique, first in batch configuration and after in semi-continuous, was applied by other authors as Ashokkumar [36], Wang [20], Benvenuti [37], Chaiklahan [38], Feng [39], Ruiz-Martinez [40] and Cai [41] in microalgae cultivation on digestate or wastewater. Two step cultivation permits to have the maximum biomass production and after, during the semi-continuous step, the continuous digestate treatment (phytoremediation) with a stable biomass production and secondary high value products storage.



a) b)

Figure 1: Cellular count (a) and dry weight (b) analysis of microalgae biomass on winery digestate (WL-DIG) during batch (8th day) and semi-continuous (18th day) cultivation.

Dry weight and cellular count analyses obtained in this experiment (Figure 1) showed a stable biomass production during semi-continuous feeding with 0.67 ± 0.02 g l^{-1}; on the other hand, cellular count analysis highlighted a cellular number decrease (from 435.00 ± 77.00 million cells ml^{-1} at 8th day to 135.00 ± 38.00 million cells ml^{-1} at the end of the test). Similar cell density decrease during two phase cultivation was detected also by Nwoba et al.'s study [42] with *Chlorella* sp. and *Scenedesmus* sp. Stable biomass production during semi-continuous system was detected by Wang's study [20] (0.76 g l^{-1}) with *C. vulgaris* cultivated on dairy manures digestate, Ashokkumar's study [36] (1.16 g l^{-1}) with *Microcystis aeruginosa* and Ruiz-Martinez's study [40] (0.46 ± 0.65 g l^{-1}) with Chlorophyceae mixed microalgae strain. The stable biomass production and the decrease of cell count obtained by literature data and experimental data obtained could be explained by morphological change in microalgae during semi-continuous system. Switching from batch cultivation to semi-continuous cultivation, it was detected an increase of cells size (data not reported), probably associated with secondary high values storage in microalgae biomass.

Microalgae growth rate was 0.07 ± 0.01 d^{-1} (detected at the beginning and end of test), while the growth rate detected during exponential growth phase was 0.24 ± 0.01 d^{-1}. As reported by Molina Grima's study [43], a low growth rate was required for freshwater microalgae strain to have a lipids storage (from 8% to 57% of lipid storage in a growth rate range between 0.28 d^{-1} and 0.86 d^{-1} during exponential growth phase). The morphological change associated with stable dry weight and growth rate decrease, could be related to lipids storage in microalgae biomass during second step cultivation. For this reason, lipids

gravimetric analysis was performed on microalgae biomass in second step cultivation and it was detected a stable lipids storage of 17.00 ± 4.00 %TS during stationary state condition (SSC). Lipids storage percentage obtained was similar to those obtained by other authors at the end of first HRT in semi-continuous system, (20.00 %TS [39], 18.90 ± 0.47 %TS [36], 16.00 %TS [44] and 16.40 %TS [41]).

3.2 Ions and pH analysis during two-steps cultivation

The behaviour of cationic species in PBRs is reported in Figure 2. During the batch period (days 0-8) no Na^+ and K^+ removal was observed while NH_4^+ and Ca^{2+} concentrations decreased by 100.00 ± 0.00 % and 88.57 ± 2.91 % respectively. Contrary, Mg^{2+} concentration doubled from 31.8 ± 0.00 mg l^{-1} to 63.0 ± 0.24 mg l^{-1} showing a release in the medium. Once reached the stationary growth phase, 125 ml of digestate supernatant was fed daily in order to obtain an HRT of 8 days. Cations removal evaluation was carried out considering average values obtained during days 14-17.

Typically, magnesium removal is liked with biomass increase, such as magnesium is an essential component for photosynthetic system [45], contrary during batch test ammonia and calcium ions were removed constantly. In semi-continuous condition, Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+} removals were 29.82 ± 6.24 %, 76.37 ± 8.64 %, 19.22 ± 5.52 %, 19.22 ± 5.52 % and 78.25 ± 5.05 % respectively. Contrary to removal values obtained in batch cultivation, during semi-continuous cultivation K^+ and Mg^{2+} constantly removed, with a daily biomass production of 0.06 ± 0.01 g l^{-1}. These removal results were in contrast with typical K^+ and Mg^{2+} removal that happened in batch and semi-continuous cultivation, such as monovalent and divalent ions removal is typically associated with microalgae's cell composition (polysaccharides, lipids and protein) and its negatively charged, so cation ions were absorbed with biosorption (80% of cation were absorb in this way), physical adsorption, surface deposition and passive diffusion [46].

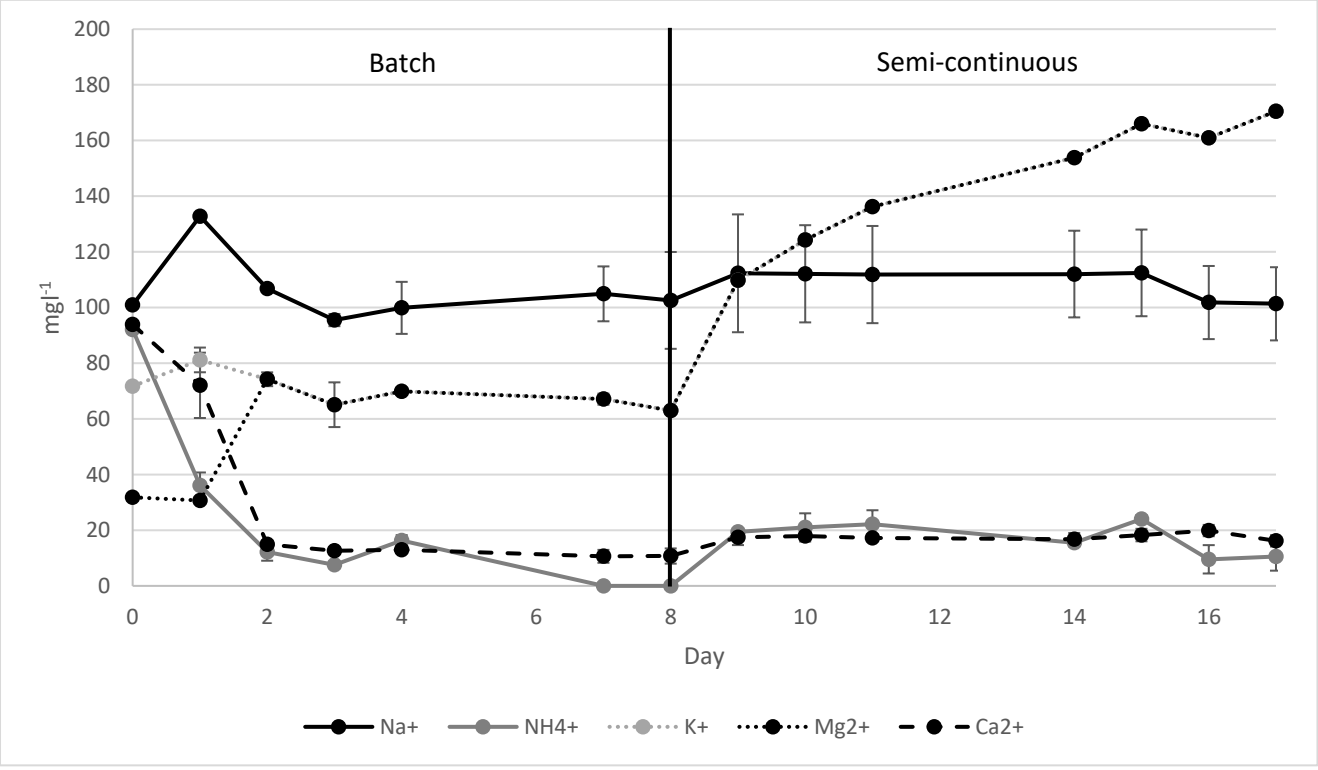


Figure 2: Cationic chromatography analysis for Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+} during batch and semi-continuous test with microalgae cultivation on WL-DIG.

3.2.1 Ion variation's effect on microalgae biomass

Ions consumption during microalgae growth, changed medium condition that influence biomass composition; for example salt stress increase lipids storage [47] and the *C. vulgaris*' Na^+ ion storage was associated with low activity of plasma-membrane Na^+/H^+ anti-porter system [48] and direct bioaccumulation effect [46]. Output's cationic chromatography, during semi-continuous system, showed a stable Na^+ uptake by microalgae. The Na^+ removal from medium gave a salt stress, with a K^+ and Mg^{2+} medium concentration increase during biomass proliferation linked to the K^+ and Mg^{2+} intracellular uptake decrease [46].

Magnesium ion is essential for microalgae growth, inasmuch it is the chlorophyll molecule principal constituent, in fact a semi-continuous system with Mg^{2+} addition showed, for *Scenedesmus acuminate*, a cellular growth increase [49]. So, a Mg^{2+} uptake deficit obtained in semi-continuous system could inhibit microalgae growth associated with low chlorophyll storage [45]. The same problem was detected for K^+ removal, K^+ assimilation is linked with Na^+ intra- and extracellular equilibrium where K^+ uptake is a defence mechanism against Na^+ high concentration's toxic effect [48,50]. Typically, salt stress mechanism defence cellular system is activated when Na^+ concentration increase intracellularly and in medium, so the defence system exchange K^+ with Na^+ that is ejected from cells [46].

Ca^{2+} removal trend detected was similar to K^+ removal reported by Talebi et al. [48], where Ca^{2+} concentration inside cells increase with Na^+ biosorption increase with a bioaccumulation response [46]. Ca^{2+} is essential for microalgae proliferation, it is used in photosynthetic activity, glycerol metabolism, thylakoid membrane integrity, pathogenic resistance, stress-tolerance and signal transduction [47] and its absorption take place thought membrane potential associated with proton pump [50]. The Ca^{2+} removal detected during semi-continuous system with DIG-WL could be associated with the necessity to storage intracellular Ca^{2+} in response to Na^+ uptake in medium to maintain balance ions concentration. The consequent Ca^{2+} starvation decrease biomass yields, but lipids biosynthesis has not been interrupted, in fact cellular size changed during cultivation with size increasing. As reported by Gorain et al.'s study [47], Mg^{2+} and Ca^{2+} were essential nutrient for *C. vulgaris*, where Mg^{2+} increase cellular proliferation and lipids storage, when it was not starved, and Ca^{2+} starvation increase lipids storage (27 %TS and 40 %TS lipids storage respectively). Their study in batch cultivation highlighted the Mg^{2+} starvation negative influence in cellular division, growth yields and chlorophyll synthesis stop, but the lipids storage was close to Ca^{2+} depletion. Semi-continuous applied on microalgae could represent a good solution to have i) good ions medium balance, ii) stress growth condition that increase lipids storage with limit strain death, iii) long term cultivation and a iv) phytoremediation effect.

Ammonia removal detected during batch cultivation was similar that other studies [8,51–56] where ammonia was totally removed at high pH value with air bubbling. pH value was stable during both batch and semi-continuous tests at 9.20 ± 0.20 and this high value allow the ammonia stripping phenomena during cultivation. Semi-continuous feeding showed 76.37 ± 8.64 % of ammonia daily removal, as reported by Park [49] (76.38 %). As reported by Park [49] and Ruiz-Marin [44], high pH value shift ammonia equilibrium from NH_4^+ to NH_3 that increase ammonia stripping, in fact ammonia stripping test without microalgae showed that only the 40% was removed, so the ammonia remaining was direct used by microalgae metabolisms.

4. Conclusion

C. vulgaris cultivated on WL-DIG in two step cultivation showed a preliminarly stable biomass production and lipids storage during the semi-continuous cultivation step. The cellular count decrease (from 435.00 ± 77.00 million cell ml to 135.00 ± 38.00 million cell ml), K^+ and Mg^{2+} increase in medium

did not influence negatively the biomass concentration and lipids storage (0.67 ± 0.02 g l^{-1} and 17.00 ± 4.00 %TS respectively). On the other hand, the necessity of ions implementation to maintain the medium composition stable for microalgae culture could be carried out with digestate semi-continuous feeding. Winery waste digestate could be used as good substrate for microalgae cultivation and further test will be carried out for a long-term validation.

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