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Application of *Scenedesmus obliquus* in the Treatment of a Real Wastewater

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In the present study, the microalga *Scenedesmus obliquus* CCAP 276/38 has been applied in the treatment of a real wastewater (RWW) derived from an anaerobic digestion process of corn silage and livestock wastewater. The liquid phase of the digestate showed a low viscosity value and a high content of ammonia up to 3 g/L. In a preliminary phase, the experimental tests were carried out in Erlenmeyer flasks in order to identify the optimal RWW concentration and, in particular, its influence on biomass growth and productivity. The tests were carried out at 20°C at an artificial dark/light cycles of 12 hours. Three different concentrations were tested: 1, 2 and 3% of RWW in water, in the presence of sodium bicarbonate (NaHCO₃, 50 mM) as inorganic carbon source. The obtained results showed that *S. obliquus* was able to grow in all the tested RWW concentrations even if a higher growth rate and biomass production were observed in the cultures containing 1% RWW. In order to test the influence of N/P ratio on microalgal growth, two different salts, KNO₃ (0,2 g/L) and K₂HPO₄ (0,02 g/L), were added to the medium containing 1% RWW (N/P = 84.4), to correct the N/P value. The biomass growth rate increased in the medium with the lower N/P value (N/P = 27.9). The microalgal production process was scaled-up in a stirred tank photobioreactor (working volume 5 L), in the same temperature and illumination conditions using a medium with the composition optimized in flask tests. The culture was carried out for 124 hours, fed-batch addition of RWW (1%) was done during the fermentation in order to replace the carbon source. The results (growth rate, biomass dry weight and productivity) were compared with those obtained in presence of a synthetic medium with sodium bicarbonate 50 mM as carbon source. The work clearly demonstrated the capability of *S. obliquus* CCAP 276/38 to grow in alkaline wastewater and the possibility to employ this species in the treatment of effluents containing high ammonia concentration.

1. Introduction

Green microalgae are eukaryotic and photoautotrophic unicellular microorganisms able to grow in aqueous media, at different salinity grade, containing carbonates, phosphates, nitrates and sulphates. The wastewaters (municipal, industrial and agricultural) containing inorganic compounds could represent a low-cost medium for the cultivation of different microalgal species; from this point of view, microalgal biomass could be considered a by-product of the wastewater treatment processes (Park et al., 2011). In the last years, the genera *Chlorococcum*, *Chlorella*, *Euglena* and *Scenedesmus* were described as the main carbon sequesters from different authors (Bhola et al., 2014). Among them, different species of the genus *Scenedesmus* have been cultured due to their rapid growth and ability to handle. In particular, *Scenedesmus obliquus* has been described as a versatile organism, characterized by easiness of cultivation and adaptability to different environmental conditions (Raeesossadati et al., 2014). It is known to have a high growth rate and a robust cell wall which makes it resistant to be cultivated in various environmental systems; moreover, it can grow in a wide range of temperatures (15-35°C) and pH values (5 -10) (Singh and Singh, 2014).

The growth of microalgae in culture is influenced by different chemical-physical factors: availability of carbon dioxide, source and type of light (spectral range, light intensity and light/dark cycles), macro and micronutrient concentration in the medium (Koller et al., 2012), inoculum percentage (medium to inoculum ratio) (Alzate et al., 2012), salinity and pH (Cheng and He, 2014).

In literature, several works reported the cultivation of microalgae in wastewaters of different nature to obtain simultaneous biomass production and wastewater treatment (Abreu et al., 2012; Ji et al., 2013; Pereira et al., 2017).

In the present study, the microalgae *S. obliquus* CCAP 276/38 has been applied in the treatment of a real wastewater (RWW) derived from an anaerobic digestion process of corn silage and livestock wastewater. In a preliminary phase, the experimental tests were carried out in Erlenmeyer flasks in order to identify the optimal digestate concentration and its influence on biomass growth and productivity. Afterwards, the microalgal production process was scaled-up in a stirred tank photobioreactor, in controlled conditions of temperature and illumination.

2. Materials and Methods

2.1 Strain and media

The microorganism utilized in the presented work is *Scenedesmus obliquus* CCAP 276/38, a freshwater microalga. *S. obliquus* has been maintained at +4°C on a Proteose-Peptide solid medium (PP agar), containing per litre: MgSO₄*7H₂O, 0.02 g, K₂HPO₄, 0.02 g, KNO₃, 0.2 g, Proteose-Peptide, 1 g, and agar, 20 g. *S. obliquus* has been inoculated on the PP agar and incubated, for 15 days, at room temperature and at the natural dark/light alternation. All the microalgal cultures have been settled in liquid media. A standardized inoculum of 10% v/v has been provided for each tested cultural condition (Pagliolico et al., 2017).

The enrichment cultures of *S. obliquus* have been carried out with the Proteose-Peptide liquid medium (PP), which has the same composition as that of PP agar. The first set of flask tests has been conducted modifying the PP medium by substituting the Proteose-Peptide with NaHCO₃, utilized at three different concentrations, 50, 75 and 100 mM. In the second set of flask tests, *S. obliquus* has been cultivated in a real wastewater (RWW) characterized by the composition reported in Table 1.

Table 1: Composition of the real wastewater (RWW)

Components	Quantities
Suspended solids (total quantity)	0.006 g/L
COD	0.293 g/L
Phosphorous (P)	0.061 g/L
Ammonia Nitrogen (NH ₄ ⁺)	3.730 g/L
Potassium (total quantity)	0.160 x 10 ⁻³ g/L
HCO ₃ ⁻	12.20 g/L
Conductivity at 20°C	16.4 mS/cm

First, the RWW has been utilized at three different concentrations, 1%, 10% and 20%, by means of water dilutions. Then, the RWW was water diluted to a final concentration of 1%, 2% and 3%, and added with NaHCO₃ 50 mM. The 1% RWW has also been added with the salts of the PP medium, K₂HPO₄, 0.02 g/L, and KNO₃, 0.2 g/L. This last medium has been utilized for both flask and photobioreactor tests.

All the media have been thermally sterilized in autoclave (at 120°C and 2 atm for 20 minutes). The NaHCO₃ solutions have been sterilized by filtration (0.22 µm) to avoid precipitation.

2.2 Flasks and photobioreactor tests

The enrichments and the culture tests, as indicated in the "Strain and media" section, have been carried out in 500 mL flasks closed with cotton plugs. The flasks, filled with 200 mL of inoculated media, have been incubated in a photoincubator (Photosynthetic Light bank, Innova 43R/44R) in agitated conditions (125 rpm), at 20°C, with an artificial alternation of dark/light (12 hours of light/12 hours of dark). The photoincubator is equipped with 15 W lamps at a wavelength of 660 nm.

The photoincubator test has been carried out in the R'ALF incubator which has a capacity of 6 litres and is equipped with two radial Rushton turbines. The bioreactor top is equipped by inlets, for the solutions and media, and measurement sensors for pH, temperature and pO₂. The feeding and extraction of the liquids is carried out with peristaltic pumps. The test has been carried out filling the bioreactor with 5 litres of medium at a temperature value of 20°C and at a rotation speed of 125 rpm. The photoperiod (dark/light 12/12) has been guaranteed by six lamps (Osram L18W/12 D Light) controlled by a timer, symmetrically placed all around the bioreactor at a distance of 2 cm from the jacket.

2.3 Analytical determinations

Spectrophotometric determinations at 540 and 620 nm wavelengths have been periodically conducted, together with the pH ones, to control microalgal growth in the cultural medium using a HP 8452A diode array spectrophotometer.

On the basis of the absorbance values at 620 nm has been calculated the growth rate (μ) as follows (Eq. 1):

$$\mu = \frac{LN \left(\frac{OD_t}{OD_0} \right)}{\Delta t} \quad (1)$$

OD_0 is the initial optical density at 620 nm, OD_t is the optical density at 620 nm at the selected time, and Δt is the time interval (hours).

Biomass concentration has been determined after a centrifugation of the culture at 4100 rpm for 10 minutes followed by one washing cycle with distilled water. The precipitated biomass has been dried at 105°C until the achievement of the constant weight. The obtained dry weight value of biomass was utilized to calculate the productivity value (g/L h).

Carbonate (CO_3^{2-}) and bicarbonates (HCO_3^-) concentrations have been determined with an acid/base titration with the method of the double indicator, as reported by Pagliolico et al. (2017). CO_3^{2-} and HCO_3^- concentrations have been calculated as follows (Eq. 2):

$$\begin{aligned} M\text{CO}_3^{2-} \text{ (g)} &= V_b \times \text{NHCl} \times \text{MMCO}_3^{2-} \\ M\text{HCO}_3^- \text{ (g)} &= (V_a - 2V_b) \times \text{NHCl} \times \text{MMHCO}_3^- \end{aligned} \quad (2)$$

V_b is the volume (L) of HCl used in the first titration, V_a is the total HCl volume (L) used in the two titrations, NHCl is the molar concentration of HCl, MMCO_3^{2-} is the molar mass of CO_3^{2-} (g/mol) and MMHCO_3^- is the molar mass of HCO_3^- (g/mol).

3. Results and Discussion

3.1 Flask tests with PP modified medium

In order to evaluate the influence of NaHCO_3 on *S. obliquus* growth behavior, three different concentrations of bicarbonate in the PP medium were tested: 50, 75 and 100 mM, all the tests were triplicated. In Figure 1 the values of biomass concentration (g/L) at the end of the fermentation test (192 hours) are showed, the same behavior was observed for the cell counts (cell number/mL).

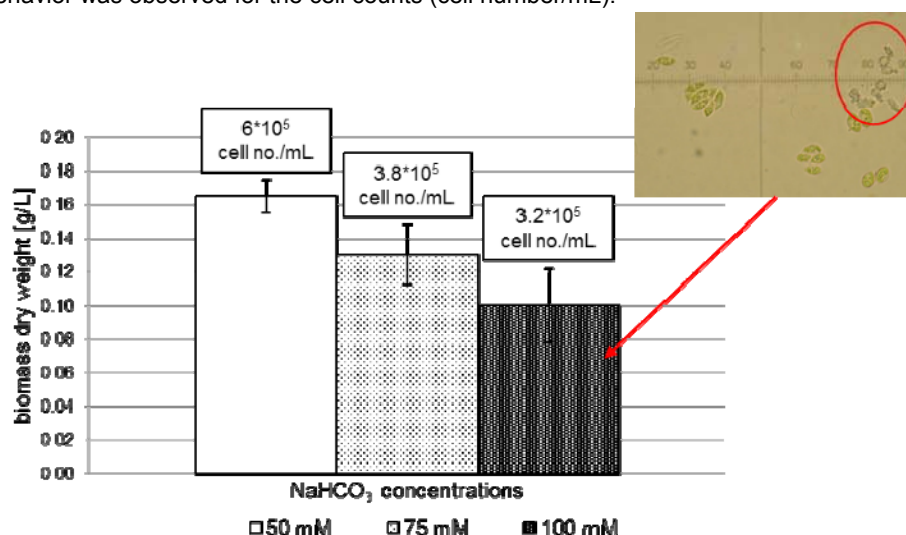


Figure 1. *S. obliquus* biomass dry weight (g/L) and cell counts (cell number/mL), at 192 hours of incubation, at the three different NaHCO_3 concentrations. In the box, the modified morphology at 100 mM is showed.

The obtained values clearly show the inhibition effect of the higher NaHCO_3 concentrations (75 and 100 mM) on *S. obliquus* growth. This result was confirmed by the modified microalgal morphology as showed in the box in Figure 1. The NaHCO_3 concentration corresponding to 50 mM was used in all the following experimental tests.

3.2 Flask tests with real wastewater

The real wastewater composition is reported in Table 1. On the basis of the results obtained in the paragraph "Flask tests with PP modified medium", a 20% RWW in water was used to obtain a final HCO_3^- concentration of about 50 mM. Moreover, in order to identify the optimal dilution of the RWW for microalgal growth, other two lower percentages, 10% and 1%, of RWW in water were tested. In the presence of 20% and 10% RWW no growth was observed; this was probably due to the presence of high concentrations of inhibitory substances in the RWW. On the other hand, the 1% RWW allowed to obtain the microalgal growth even if at this RWW dilution, the bicarbonate concentration is fivefold lower than the optimal concentration reported for the PP modified medium (50 mM). For this reason, the bicarbonate concentration was increased by adding NaHCO_3 50 mM in the 1%, 2% and 3% RWW dilutions in water. As it is shown in Figure 2, the best results were obtained in the 1% RWW medium.

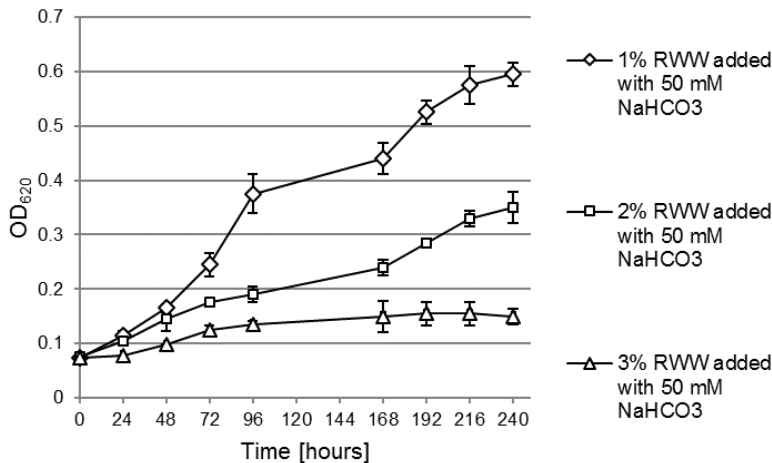


Figure 2. OD_{620} values obtained in the 1%, 2% and 3% RWW medium added with NaHCO_3 50 mM.

Even though this last was the best condition, the corresponding N/P value, 84.4, was not comparable with the 17.2 value of the PP modified medium. For this reason, the 1% RWW medium was added with the PP medium salts (K_2HPO_4 and KNO_3) to obtain an N/P value, 27.9, more comparable with that of the PP modified medium. In Figure 3 these three different cultural conditions for *S. obliquus* are compared: RWW 1% in water, RWW 1% added with salts and PP modified medium.

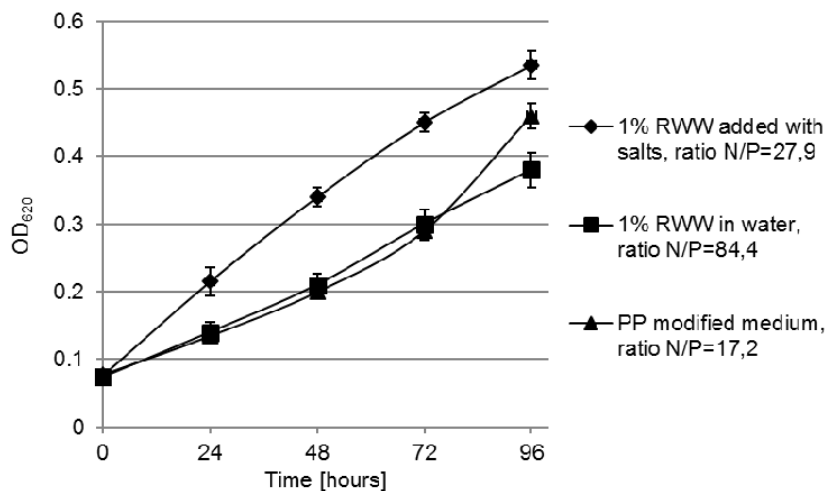


Figure 3. OD_{620} values for *S. obliquus* in 1% RWW, added or not with salts, and in the PP modified medium. The corresponding N/P values are indicated in the legend.

From the reported OD_{620} values it is possible to observe the influence of the N/P ratio on the microalgal growth: the best results were obtained in 1% RWW added with salts where the N/P ratio was more comparable with that of the PP modified medium containing the optimal concentration of $NaHCO_3$ (50 mM).

3.3 Bioreactor test with the 1% RWW medium

On the basis of the information obtained with the flask tests, a process scale-up was performed in a photobioreactor as described in the Material and methods section. *S. obliquus* was cultured in 1% RWW added with salts and the concentration of the bicarbonates was periodically corrected to the initial value by fed batch addition of 1% RWW. In Figure 4, the bicarbonate and ammonia concentrations, and the OD_{620} values are reported together with the photoperiod.

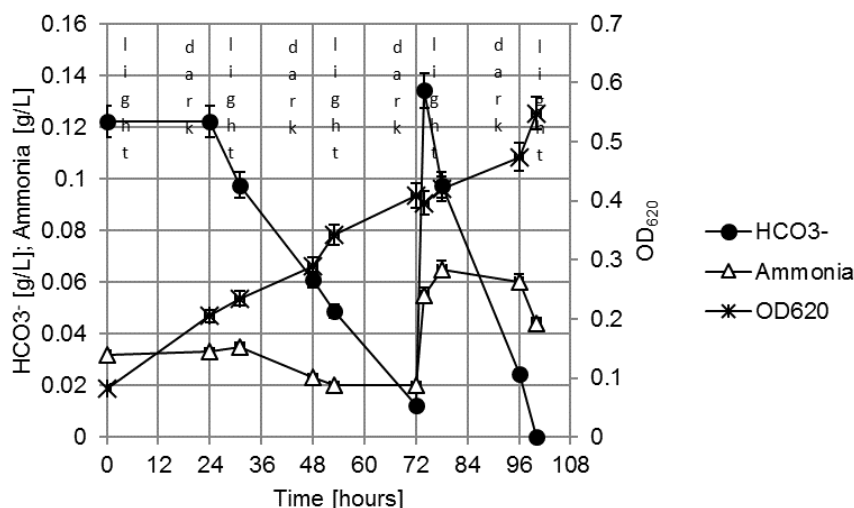


Figure 4. HCO_3^- , ammonia and OD_{620} trends obtained for *S. obliquus* cultured in the bioreactor with 1% RWW added with salts.

It is possible to observe that the bicarbonate concentration in the medium diminishes irrespective of the artificial alternation of dark/light. At 72 hours of incubation, the bicarbonate concentration was corrected to the initial value (about 0.13 g/L) by adding 1% RWW. Afterwards, it is possible to observe that the bicarbonate consumption rate was improved and, about 30 hours later, the concentration was decreased till to zero. At the same time, the ammonia concentration was reduced to 0.02 g/L. According to the OD_{620} values biomass growth rate was almost constant during all the incubation period. The main microalgal growth parameters (i.e. biomass concentration, growth rate and productivity), obtained in the photobioreactor test with 1% RWW added with salts, have been compared with those obtained in a photobioreactor test carried out with PP modified medium (50 mM) in the same cultural conditions (Biolatto, 2013) (Table 2).

Table 2: Microalgal growth parameters in photobioreactor

Parameters	PP modified medium	1% RWW added with salts
Biomass [g/L]	0.248	0.061
OD_{620}	1.1843	0.4743
P [$g L^{-1} h^{-1}$]	0.00358	0.0085
μ_{max} [h^{-1}]	0.026	0.038

The maximum values of growth rate (μ), 0.038 ($hours^{-1}$), and productivity, 0.0085 ($g L^{-1} h^{-1}$), obtained with RWW are higher than those obtained with the PP modified medium (0.026 and 0.00358 respectively). Moreover, even though the biomass concentration at 72 hours was 4 fold lower in RWW than in PP modified medium (0.061 and 0.248 g/L respectively), it has to be considered that the initial concentration of bicarbonates in the 1% RWW medium was about 20 fold lower. The results obtained in the present work has been compared with those reported by Veronesi et al. (2015) related to a digestate derived from the anaerobic treatment of an agro-zootechnical material. The productivity values reported for *Phaeodactylum tricornutum*

(24 mg L⁻¹ d⁻¹) and *Pavlova lutheri* (15 mg L⁻¹ d⁻¹) was lower than that obtained with *S. obliquus* in the RWW medium.

4. Conclusion

The results obtained in the present work showed that the microalga *S. obliquus* CCAP 276/38 was able to grow in a real wastewater derived from an anaerobic digestion process of corn silage and livestock wastewater. This preliminary work clearly demonstrated the capability of the microalga to grow in alkaline wastewater and the possibility to employ this species in the treatment of effluents containing high ammonia concentration.

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