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Production of wild *Chlorella* sp. cultivated in digested and membrane-pretreated swine manure derived from a full-scale operation plant

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ABSTRACT

A wild *Chlorella* sp. was isolated from a manure storage tank and then cultivated under semi-continuous production conditions on four substrates, including digested swine manure (DIG) and digestate-derived liquid fractions obtained by digestate centrifugation (CLF) and ultrafiltration (ULF) in a full-scale digestate treatment plant, in order to evaluate differences in growth and depuration capacities. The microalga was capable of fast growth on some of the substrates, comparable to that obtained with 3N-BBM synthetic medium, productivities being 0.21 g L^{-1} d^{-1} of biomass for ULF and 3N-BBM media, 0.17 g L^{-1} d^{-1} biomass for CLF media and 0.10 g L^{-1} d^{-1} biomass for DIG media.

Algal growth was affected, above all, by the chemical oxygen demand (COD) of the starting culture media which directly affected turbidity and also light availability. Nutrient contents, *i.e.* N and P, did not seem to affect the process. *Chlorella* sp. was capable of reducing about 95%–98% of N-NH_4^+ and 61–73% of COD, while micronutrients were almost completely removed from the cultures. However, most of the ammonia nitrogen was lost to the atmosphere due to the stripping phenomena caused by aeration and high pH during algal growth, so that only 30% of the nitrogen was successfully incorporated in the microalgal biomass.

The isolated *Chlorella* proved to be a strong strain, capable of reducing macro- and micronutrient contents in the digested liquid streams. The full-scale digestate membrane treatment proved to be a suitable process to integrate with microalgae cultivation, improving the growth medium performance in terms of final biomass productivity.

1. Introduction

Livestock farming activities are leading to environmental problems such as the eutrophication of water bodies, air pollution by ammonia volatilization and soil degradation due to over-fertilization [1,2].

Improper land application of animal slurries can lead to a lack in soil nutrient availability [5], thus it is often mandatory to integrate slurry use with chemical fertilizers, thus increasing fertilization and environmental costs [6].

In this context, anaerobic digestion (AD) was suggested as a useful and sustainable technology producing renewable energy (biogas) and nutrient from manure [4,5]. Regarding the recovery of nutrients, the ability of microalgae to take up inorganic N and P is a well-recognized and efficient bioremediation tool for agricultural wastewater treatment. Among these, swine slurry is typically characterized by high N and P concentrations and a good balance of other nutrients which make it suitable for microalgae cultivation [6]. Consequently, the use of microalgae for nutrient removal from wastewater has been considered to be practical, economical and promising [7,8].

In many studies, microalgae have been cultivated using digested swine slurries, secondary effluents or diluted primary piggery wastewater [6,9] above all for the uptake of N, mainly in the ammonia form, and P. An important aspect of microalgae-based treatment is that N is not only removed by cell metabolism (biomass uptake) but, also, a significant portion of N is removed by air stripping and volatilized, above all at alkaline pH and high temperature [10]. Although many studies concluded that microalgae are able to reduce almost 100% of N in agricultural wastewaters, few studies have focused on the volatilized fractions [11,12].

In considering the use of wastewater to cultivate microalgae, another key issue is the negative effect of bacteria on microalgal biomass survival and quality. To solve this problem, slurry needs to be previously sterilized [10], adding costs and energy to the process, and leading to an important constraint in the scale-up of cultivation of microalgae using wastewaters [13].

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This issue could be overcome by using and/or combining different strategies isolating wild microalgae strains that tolerate the utilization of complex substrates, such as livestock slurries and finding suitable technologies.

The aim of this work was to isolate a wild microalgal strain from digested pig slurry and to evaluate differences in growth and remediation performances in sequential digestate liquid fractions sampled from a full-scale digestate treatment plant, based on membrane technology [4]. During microalgal growth a nitrogen balance assessment was carried out to determine the total amount of ammonia lost during the culture process, in order to evaluate the sustainability of the final process.

2. Material and methods

2.1. Substrate sampling and chemical characterization

Digestate (DIG), centrifuged liquid fraction (CLF) and ultrafiltered liquid fraction (ULF) were sampled from a biogas plant equipped with a full-scale digestate treatment unit, already described by Ledda et al. [4]. More specifically, DIG was the effluent of the biogas plant, CLF was the liquid fraction after digestate centrifugation performed by a DODA separator (Mantova, Italy) and ULF was the permeate after ultrafiltration of CLF performed by a 40 kDa grafted Polyacrylonitrile membrane (PAN) (Orelis, France); more details are reported in Ledda et al. [4]. Samples were stored in 1 L bottles at 4 °C overnight and then analyzed.

Ammonia nitrogen ($N\text{-}NH_4^+$) and chemical oxygen demand (COD) were determined using fresh material according to the analytical methods for wastewater sludges [14].

Total Kjeldahl Nitrogen (TKN) on dry biomass was determined using lyophilized pellets according to the analytical methods normally used for wastewater sludges.

Total phosphorus (TP), Na, Mg, K, Ca, Zn, Fe, Mn, Cu, Co, Ni, Cr and As contents were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian, Fort Collins, USA) according to the 3051A and 6020A EPA methods [15,16].

To close the nitrogen balance, both the determination of nitrogen content in the biomass and the set-up of an acid trap to recover stripped ammonia from the algal culture were used. For the latter, 250 mL Erlenmeyer flasks with 10 mL of saturated boric acid solution and 100 mL deionized water were prepared for each trial and connected to the air outlet of the cultures. The amount of ammonia nitrogen in the flask was then determined by acid titration with 0.0005 mol L^{-1} H_2SO_4.

All analyses were performed in triplicate.

2.2. Chlorella sp. isolation and molecular identification

The isolation of the indigenous microalga was performed according to Doria et al. [17]; briefly 25 mL CLF samples were collected from a storage tank at the farm and incubated for 2 weeks in 300 mL Erlenmeyer flasks under 6400 K cool white fluorescent tubes with an average photon flux of 1000 μmol m^{-2} s^{-1}. After that, 100 μL of the supernatant was plated in Petri dishes with 40 mL agarized Bold's Basal Medium [18] and 200 μg mL^{-1} ampicillin.

Plates were incubated for 3 weeks at 25 °C with a 12 h photoperiod at an average irradiance of 50 μmol photons m^{-2} s^{-1}.

Single colonies were transferred to a new Petri dish. After 2 to 3 weeks new single colonies were transferred again to a new Petri dish. After an additional week, each single colony was inoculated in 250 mL liquid 3-fold Nitrogen Bold's Basal Medium (3N-BBM), for a total of 5 isolates. Algal growth was then monitored daily by spectrophotometric optical density measurement at 560 nm (Jenway 7300, Bibby Scientific, UK). Among the isolates, a single culture grown on 3N-BBM showing a high growth rate was further isolated and the strain was identified by microscopic examination as belonging to the *Chlorella* genus.

To confirm microscopic identification, a molecular analysis was performed: culture pellets were subjected to five cycles of freeze-thawing and ground in liquid N_2, then DNA was extracted by using a GeneMATRIX Plant and fungi DNA purification kit (EURx) according to the manufacturer's instructions.

Two primers were used for PCR amplification of regions of the 18S rRNA: the forward GTCAGAGGTGAAATTCTTGGATTTA and the reverse "AGGGCAGGGACGTAATCAACG".

Each PCR reaction was performed in a total volume of 25 μL containing approximately 25 ng of chromosomal DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10× buffer) supplemented to give a final concentration of 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase (Bioline), and 0.5 pmol of each primer.

Amplification was run in a GeneAmp PCR system 2700 (Applied Biosystems) as follows: 1 cycle of 3 min at 94 °C; 37 cycles of 50 s at 92 °C, 50 s at 57 °C and a 50 s extension step at 72 °C plus an additional 10 min cycle at 72 °C. The PCR products were separated by electrophoresis on a 1% (*w*/*v*) agarose gel stained with ethidium bromide. Successful amplifications were purified from the agarose gel, using the Wizard® SV Gel and PCR Clean-up System (Promega Corp., Madison, WI). The PCR products were sequenced on both strands and after that were manually checked obtaining a 650 bp sequence. A comparative analysis was made by comparing the sequence with others from GenBank collected using BLAST giving a perfect match with a *Chlorella* sp. SSU rRNA sequence.

2.3. Microalgae cultivation

According to previous experimental data and to the literature [19] all the substrates were diluted with distilled water to get an initial nitrogen concentration of 124 mg L^{-1} N that is in the range of a common N concentration in synthetic media.

Preliminary trials performed by a batch culture indicated that the DIG substrate diluted to 124 mg L^{-1} N did not support *Chlorella* growth (data not shown). As a consequence of that, this substrate was further diluted until reaching a final N concentration of 60 mg L^{-1}.

The digested liquid streams were neither autoclaved nor filtered, in order to simulate a scaled-up process. Moreover all the substrates were compared to 3-fold nitrogen BBM standard medium (3N-BBM) which contains the same amount of nitrogen, although in the nitric form. Algae growth and biomass productivity on 3N-BBM and the digested liquid streams were measured during semi-continuous cultivation; the dilution rate was kept constant, replacing 65% of the culture every cycle on average, and was determined by a preliminary batch cultivation in all the tested substrates, after which a starting biomass concentration of around 0.15 g L^{-1} was set for the following trials in order to maintain the microalga in the exponential growth phase for two days of culture. For every cycle of cultivation the volume lost by evaporation was corrected with deionized water prior to harvesting.

All the assays were performed in duplicate in 500 mL Erlenmeyer flasks with an operative culture volume of 300 mL for a total of eight trials. Cultures were submitted to constant illumination with two 250 W daylight CFL bulbs with an average irradiance on the flasks of 150 μE m^{-2} s^{-1}.

Air was supplied by a commercial compressor with an average air flux of 0.3 L L^{-1} culture min^{-1} to provide atmospheric CO_2 and to avoid dissolved oxygen accumulation.

Algae growth and dry weight were determined, indirectly, by spectrophotometric measurements at 560 nm. Direct dry weight determinations were also performed during the experiment to confirm data obtained through absorbance measurements. To do this, 10 mL of each culture was filtered through pre-weighed Whatman GF-C filters and subsequently dried at 80 °C for 24 h before being weighed.

The difference between indirect and direct dry weight measurements was, for all cases studied, lower than 5%.