Mixotrophic and heterotrophic cultivation of *Auxenochlorella protothecoides* in chicken manure extract supplemented with sodium acetate: effect of substrate C/N ratio on biochemical composition

I. Langousi, D. Mitrogiannis, G. Markou, D. Arapoglou
Institute of Technology of Agricultural Products, Hellenic Agricultural Organization-Demeter,
L. Sof. Venizelou 1, Lykovrysi, 14123, Greece. E-mail: gmarkou@itap.com.gr

Introduction

Recovery of nutrient and energy from agro-industrial wastes is gaining attention in the context of circular economy. Substantial amounts of chicken manure (CM) are annually generated worldwide due to the increasing livestock production (Yin et al., 2021). Chicken manure is characterized by high content of proteins which can lead to release of high NH_4-N amount during anaerobic digestion (AD) (Singh et al., 2010). One route proposed for CM treatment is the thermophilic acetogenetic fermentation resulting in the bioconversion of organic matter into acetate and inactivation of pathogens. Subsequently, the solid fraction of this process can be used for biogas (bioenergy) production through AD whereas the acetate-rich liquid fraction as substrate for protein-rich microalgae production.

Microalgae are advantageous microorganisms since they can grow in various substrates and wastewater under mixotrophic or heterotrophic conditions (Chalima et al., 2017). Nitrogen source and carbon to nitrogen (C/N) ratio in the culture medium influence the biomass growth and chemical composition (Chalima et al., 2017). Some species are capable to utilize organic N (proteins) of the substrate which are abundant in CM.

In this study, *Auxenochlorella protothecoides* was cultivated in a protein-rich extract of CM supplemented with sodium acetate (as organic C source) in order to simulate a VFA-rich growth medium after CM acetogenetic fermentation. No other nutrients were added in the CM extract. The main objective is to examine the effect of different C/N ratios in the CM substrate along with light conditions (heterotrophy and mixotrophy) on the biomass productivity and biochemical composition as a potential feed supplement.

Materials and methods

Inoculum and substrate

The strain of *A. protothecoides* was obtained from the Culture Collection of Algae and Protozoa SAMS Limited Scottish Marine Institute. The inoculum culture was grown mixotrophically (LED panel illumination and light to dark cycle of 16:8 h) in the following growth medium: 10 mL/L BG-11 medium, 5 mL/L $MgSO_4$, 0.5 g/L K_2HPO_4, 1 mL/L of trace elements solution, 1 g/L peptone (as organic N source) and 6.83 g/L CH_3COONa (as equivalent to 5 g/L acetic acid). The systematic inoculum sub-culturing was performed at 7 d interval, under axenic conditions and magnetic stirring, adding 50 mL inoculum and 200 mL autoclaved substrate in 500 mL Duran flask. The extraction of proteins from CM, collected from a poultry farm in Megara region (Attika), was carried out adding 250 g CM in 1 L of 0.1 M NaOH as an optimum concentration after performing comparative extraction assays with deionized water (DW) and 0.1-0.5 M NaOH. After 24 h, the extract was centrifuged and filtered under vacuum using a Buchner funnel. The properties of CM filtrate were: 6307.1 ± 288.7 mg/L proteins, 5003.1 ± 138.0 mg/L VFAs, 425.3 ± 12.0 mg/L carbohydrates, COD of 27.5 ± 2.6 g O_2/L, 528.1 ± 5.6 mg/L NH_4-N, 7.7 ± 0.2 mg/L NO_3-N, 13.4 ± 0.2 mg/L PO_4-P, 20.6 ± 0.2 g/L total solids, EC = 11.68 mS/cm and pH= 9.55.

Experimental set-up

Biomass growth and biochemical composition was examined at the following C/N ratios (w/w): 10/1, 10/2, 10/4 and 10/6 expressed as total VFAs to extracted CM proteins. To provide the ratios 10/1, 10/2 and 10/4, the CM extract was diluted with DW and supplemented with the appropriate CH_3COONa mass in order to achieve a VFA concentration of 10 g/L (as equivalent acetic acid) in each substrate. Overall, the microalgal cultivation was conducted at four treatments (C/N) under light and dark conditions. The experiment was carried out in 500 mL Duran flasks using 200 mL sterile CM medium and 20 mL inoculum under aseptic conditions, LED panel illumination (5000 lux), photoperiod of 16:8 h, temperature 26 ± 2 °C. The cultures were agitated with filtered-sterilized air at flow of 0.2 L/min.

Analytical methods

Biomass concentration was daily monitored by reading the total optical density (OD_T) of the culture at 750 nm. The OD_{CM} of the supernatant was also measured after centrifugation (5000 rpm, 10 min) and subtracted from OD_T. At the end of each experimental run, the biomass was harvested after centrifugation (5000 rpm, 10 min) and washed twice with DW. Biomass samples rinsed in DW(15 mL) was oven-dried at 60 °C for at least 24 h in order to assess the cell dry weight per L. Concentration of proteins, carbohydrates, lipids and pigments in lyophilized biomass as well as of total VFA in the liquid substrate were determined spectrophotometrically (Cadas 50, Dr.Lange GmBH, Germany) (Markou et al., 2021).

Results and discussion

Each culture was harvested after achieving VFA removal higher than 90%. Specifically, the heterotrophic and mixotrophic cultures at C/N ratio 10/1, 10/2 and 10/4 lasted 6, 7 and 8 d, respectively. The heterotrophic culture at ratio 10/6 was harvested after 10 d and the mixotrophic one after 11 d. These results show that the lower C/N ratio caused slower VFA removal. The net biomass OD (OD_T-OD_{CM}) was observed to increase upon time reaching a plateau at the end of each experimental run. The highest biomass concentration was attained in the mixo- (1.78 g/L) and heterotrophic (1.34 g/L) cultures at ratio 10/6, while the lowest in the mixo- (0.74 g/L) and heterotrophic (0.86 g/L) ones at ratio 10/2 (Fig. 1b). These results indicate that *A. protothecoides* was capable to adapt and proliferate in the CM substrate, which is consistent with a previous study reported an efficient nutrient accumulation under regulated C/N ratio by microalgae (Li et al., 2020).

Regarding the biochemical composition, proteins were found to be the major substance (28.1-44.2% w/w) in the dry biomass followed by carbohydrates (5.9-19.5%) and lipids (4.4-11.9%) (Fig. 1a). The highest protein content was observed at ratios 10/2 (42.9-44.2%) and 10/1 (38.8-40.8%), while the lowest at ratio 10/6 and 10/4 under mixotrophic conditions (28.1% and 30.3%, respectively). At C/N ratio 10/1, carbohydrate and lipid concentrations were higher compared to the other ratios (Fig. 1a). In accordance to that, nitrogen-restricted cultures have been demonstrated to trigger lipid and carbohydrate deposition (Guihéneuf & Stengel, 2015).

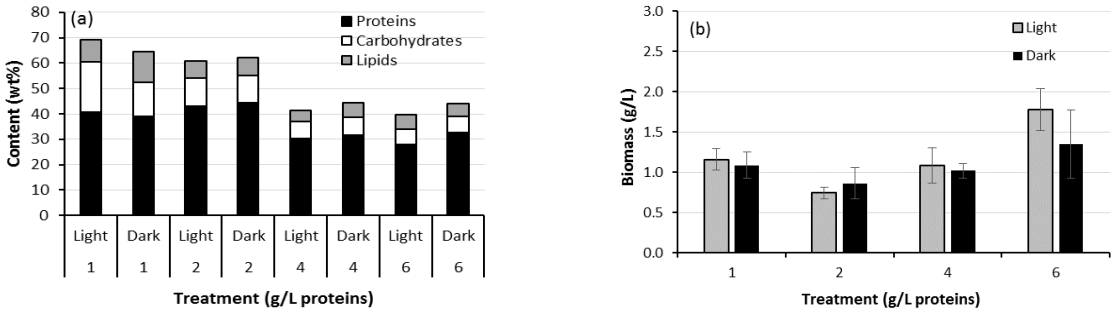

Fig. 1. Biochemical composition (a) and biomass yield (b) of *A. protothecoides* cultivated in CM extract at different C/N ratios under mixotrophic and heterotrophic conditions.

Conclusions

Alkaline extract of CM supplemented with sodium acetate was used as a source of proteins, organic carbon and inorganic nutrients for *A. protothecoides* cultivation. The results indicate that the used medium stimulated the microalgal growth and accumulation of proteins, while at high C/N ratio (10/1) carbohydrates and lipids were promoted. Further research is required to develop and optimize the *A. protothecoides* culture in fermented CM (rich in VFA) supplemented with other C sources such as glycerol and glucose, as well as to examine the effect of these substrates on biomass growth and biochemical composition.

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