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BIOACTIVE COMPOUNDS THROUGH ANAEROBIC DIGESTION OF HETEROTROPHIC MICROALGAE RESIDUES

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ABSTRACT
Several important biomolecules are available into anaerobically digested effluents that were obtained from the biodiesel production process using heterotrophically grown microalga *Chlorella protothecoides.*
Defatted microalgae residues and crude glycerol may undergo anaerobic digestion, separately and in admixture, providing methane/hydrogen and a digestate exploitable for agriculture applications. Furthermore, industrial interesting bioactive compounds such as polyphenols provided with antioxidant activity can be obtained. Anaerobic process offers a promising chance and can be advantageously combined with algae lipid-extraction techniques in order to make it more sustainable.

Keywords: Bioactive compounds, heterotrophically grown microalgae, biodiesel, anaerobic digestion, polyphenols, antioxidant.

INTRODUCTION
Due to the great interest of phenolic compounds as antioxidants this type of molecules find application in several industrial sectors. In addition, the synthetic antioxidants safety has been questioned and the need to replace them has significantly encouraged the research of new sources of natural antioxidant molecules [1]. Solid and liquid residues from anthropic activities, such as those from biomass conversion for bioenergy production, are usually a rich font of bioactive molecules. So, these wastes can be valorised through the recovery of high-value added compounds, contributing to solve problems of disposal and environmental pollution.
Microalgae are a promising alternative for biodiesel production as it can allow a higher yield production of oil per unit area than other source currently used [2]. Microalga *Chlorella protothecoides* has been studied for biodiesel production as it can grow heterotrophically with a high biomass productivity and can accumulate up to 60% of its dry weight in oil [3,4]. The defatted cellular components of microalgae, mainly protein and carbohydrates, can then be further utilized through anaerobic process. On the other hand, the transesterification process, converting oil into biodiesel, generates a glycerol by-product [5].
This work aims to evaluate the potential of the available phenolic molecules in the anaerobically digested effluents, under a biorefinery approach context, valorising the remaining two residues of the biodiesel production process as the crude glycerol and defatted microalga Chlorella protothecoides.

MATERIALS AND METHODS

Chlorella protothecoides was grown in dark heterotrophic conditions, at 28ºC in flasks shaken at 150 rpm, as detailed before [6]. *Chlorella* biomass was recovered by centrifugation, freeze-dried, milled. The defatted microalga residues and the crude glycerol by-product are bio-based materials obtained from the oil extraction process (*n*-hexane) and transesterification steps, respectively. The alga residues, devoid of the residual n-hexane, with a composition of 94.7% total solids, 91.1% volatile solids and the glycerol with 99% mass purity (density = 1.262 g mL^{-1}) were digested in anaerobic conditions (37ºC), separately and in admixture with the anaerobic sludge inoculum, using batch vessels of 80 mL working volume [7]. Four substrates were run in parallel (Table 1): control, with no added substrate (S0), glycerol (S1), microalgae residues (S2) and a mixture of glycerol and microalgae residues (S3). No chemicals for pH control were administered and no other correction or pre-treatments of the substrates were performed. The substrates were digested in triplicate.

Table 1. Composition of the batch anaerobic digestion substrates

Substrates	Concentration [g TS/L]			**S/I**
	inoculum	**microalga residues**	**glycerol**	[VS basis]
S0	58.5	-	-	-
S1	58.5	-	20.0	0.3
S2	58.5	16.7	-	0.4
S3	58.5	16.7	20.0	1.0

TS - total solids, VS - Volatile solids, S0 - control run, S/I – substrate to inoculum ratio

A total of 16 samples were obtained from the anaerobic digestion process, as listed in Table 2. Samples were centrifuged at 4°C for 20 min at 13,200 rpm. The cleared supernatants were separated from the solid residue and assayed for total phenolic content by the Folin-Ciocalteu method. The results were expressed as µg of gallic acid equivalents (GAE) per mL of tested sample. *ortho*-diphenols were also measured in the sample supernatants and the results were expressed as µg of caffeic acid equivalents (CAE) per mL of tested sample [8].

RESULTS AND DISCUSSION

Total phenols and *ortho*-diphenols were detected in all samples of influent and effluent, obtained from the anaerobic digestion process (Table 2).

The greatest quantity of total phenols was measured in the influent S3 mixture (inoculum + alga residues + glycerol), followed by the influents S1 and S2 with a similar amount (63.7-65.2 µg GAE/mL). Concerning the *ortho*-diphenol analyses, the highest concentration was present in the influent S3. The influents S1 and S2 showed the lowest values but within the same range (7.2-8.9 µg CAE/mL). *ortho*-diphenols are an important class of molecules because they are endowed with the highest antioxidant power due to the presence of two hydroxyl groups in ortho position [9]. It is interesting to know that the highest values of the *ortho*-diphenols and total phenols ratio are present in S0 and S3 (0.266 and 0.204, respectively: Table 2), meaning that, in terms of *ortho*-diphenol production, the influent containing both residues (algal and glycerol-S3), was more interesting than the influents holding each residue alone (glycerol-S1 and alga residues-S2).

About effluent samples, a similar trend on total phenols amounts present in influents was noticed. So, the greatest value was recorded in S3 with 57.2 µg GAE/mL (± 3.62), followed by S1 and S2 with 44.3 µg GAE/mL (± 3.62) and 37.4 µg GAE/mL (± 3.62), respectively. Reinforcing these observations were the similar phenols conversion achieved during the anaerobic process. Values from 30% to 40% were registered, having the lowest removal amount obtained in S3.

The presence of *ortho*-diphenol compounds was found in all digested substrates. Nevertheless, the measured concentrations, from about 4 to 8 µg CAE/mL, reveal that the anaerobic process induced a decrease in the amount of this type of compounds, more marked in the digested samples containing glycerol. This behaviour might be explained with a modified microbial flora, induced by the presence of this polyalcohol, with a different metabolism that produces lower amounts of *ortho*-

diphenols. Further analyses are currently in progress in order to establish the qualitative composition (HPLC) and the potential antioxidant power (DPPH) of the whole samples.

Table 2. Phenolic molecules in influent and anaerobically digested effluents

substrates	Description	**Total phenols** [µg GAE/mL sample]	***ortho*-Diphenols** [µg CAE/mL sample]	**o-Dph/Tph**
	Influent			
S0	Inoculum	49.13	13.07	0.266
S1	inoculum + glycerol	65.19	7.22	0.111
S2	inoculum + alga res.	63.72	8.85	0.139
S3	inoculum + alga res. + glycerol	81.42	16.64	0.204
	Effluent			
S0-1	Inoculum	31.16	7.47	0.245*****
S0-2		29.34	6.82	
S0-3		31.34	8.69	
S1-1	inoculum + glycerol	40.02	3.65	0.092*****
S1-2		47.14	4.87	
S1-3		45.66	3.73	
S2-1	inoculum + alga res.	38.54	8.04	0.211*****
S2-2		32.38	7.14	
S2-3		41.41	8.52	
S3-1	inoculum + alga res. + glycerol	54.86	3.90	0.066*****
S3-2		55.38	3.49	
S3-3		61.37	3.98	

*The data shown are the results of the ratio between the average values of *ortho*-diphenols and total phenols

References

[1] N. Balasundram, K. Sundram, S. Samman, Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses, Food Chem. 99 (2006) 191-203.

[2] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25(3), (2007) 294–306.

[3] W. Xiong, X. Li, J. Xiang, Q. Wu, High-density fermentation of microalga Chlorella protothecoides in bioreactor for microbio-diesel production. Appl. Microbiol. Biotechnol. 78 (2008) 29–36.

[4] C.A. Santos, B.P. Nobre, T. Lopes da Silva, H.M. Pinheiro and A. Reis, Dual-mode cultivation of Chlorella protothecoides applying inter-reactors gas transfer improves microalgae biodiesel production, J. of Biotechnology. 184 (2014) 70-83.

[5] R. Halim, M.K. Danquah, P.A Webley, Extraction of oil from microalgae for biodiesel production: A review. Biotechnol. Adv. 30(3), (2012) 709–32.

[6] T. Lopes da Silva, C.A. Santos, A. Reis, Multi-parameter flow cytometry as a tool to monitor heterotrophic microalgal batch fermentations for oil production towards biodiesel. Biotechnol. Bioprocess Eng. 14 (2009) 330–337.

[7] C.M.A. Santos, New Symbiotic approach for sustainable microalgae production: a biorefinery platform, IST, University of Lisboa, Portugal, 2014.

[8] G. del Monaco, A. Officioso, S. D'Angelo, F. La Cara, E. Ionata, L. Marcolongo, G. Squillaci, L. Maurelli, A. Morana, Characterization of extra virgin olive oils produced with typical Italian varieties by their phenolic profile, Food Chem.184 (2015) 220-228.

[9] Z. Sroka, Antioxidative and antiradical properties of plant phenolics, Z. Naturforsch (2005) 833-843.