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Food-industry-effluent-grown microalgal bacterial flocs as a bioresource for high-value phycochemicals and biogas

Sofie Van Den Hende [a,b,*], Jolien Beyls [a], Pieter-Jan De Buyck [a], Diederik P.L. Rousseau [a]

[a] Laboratory of Industrial Water and Ecotechnology (LIWET), Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University, Graaf Karel de Goedelaan 5, B-8500 Kortrijk, Belgium

[b] Centro Nacional de Acuicultura e Investigaciones Marinas, CENAIM, Facultad de Ciencias de la Vida, Escuela Superior Politécnica del Litoral, ESPOL, Km 30.5 Vía Perimetral, Guayaquil, Ecuador

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ABSTRACT

Microalgal bacterial floc (MaB-floc) raceway ponds are a novel sunlight-based technology to grow biomass on food-industry effluent and flue gas (33.9 kg volatile solids (VS) ha_{pond}^{-1} d^{-1}). The MaB-floc biorefinery concept of high-value phycochemicals and biogas was screened to find a suitable valorization strategy for this novel biomass. Freezing and aqueous extraction of MaB-flocs followed by size exclusion chromatography yielded 22.4 g C-phycocyanin (C-PC) kg^{-1} VS with a purity of 1.32 (24.5% recovery) and 9.5 g C-phycoerythrin (C-PE) kg^{-1} VS with a purity of 1.06 (20.9% recovery). Anaerobic digestion of the extracted MaB-flocs resulted in 272 NL CH_4 g^{-1} VS. Moreover, increasing the suspended solid (SS) loading of food industry effluent for one day, significantly reduced the biochemical methane yield by 13.6%, and the C-PC and C-PE yield of total crude extracts by 74.5% and 65.5%, respectively. In contrast, it increased the neophytadiene yield by 45.1%. This study highlights the large potential of these MaB-flocs as a bioresource for production of phycobiliproteins, biogas and neophytadiene. Further research is needed to improve the phycochemical extraction and purification processes, and to confirm a huge economic potential.

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1. Introduction

During the past decades, the interest in microalgae-based chemicals and biofuels has increased because of their potential to reduce the dependence on petroleum-based chemicals and fuels [1]. However, the production of these microalgae-based chemicals is very expensive. To address the current challenges in economic viability of microalgal biomass production, waste streams are increasingly used as a cheap resource of water and nutrients for microalgae cultivation [2].

Food industry produces a large amount of effluent, i.e. 2–73 m^3 wastewater ton^{-1} production [3,4].In Flanders (Belgium), the nutrient limits for this effluent, prior to discharge are 15 mg total nitrogen L^{-1} and 2 mg total phosphorus L^{-1} [5]. Consequently, before it is discharged, this effluent contains sufficient nutrients and water for the production of microalgal biomass. Although this low-strength wastewater can be used as a growth medium for suspended microalgae cultivation, the harvesting cost of these low-density microalgae cultures is high. To avoid this cost, specific reactors types, such as perfusion reactors [6], are required. However, there are also alternatives to the use of these reactor types. In literature, a wide range of microalgae systems based on bioflocculation and biofilm formation have been proposed. These include Rotating Algal Biofilm Reactors [7], algal bristle reactors [8], algal roofs [9], algal turf scrubbers [10], microalgal bacterial flocs (MaB-flocs) in continuous reactors with settling tank [11] or MaB-flocs in a raceway pond operated as sequencing batch reactor (MaB-floc SBR raceway) [12].

It has been shown that MaB-flocs can be produced on low-strength effluent (approximate current discharge norms of 15 mg N L^{-1}; 2 mg P L^{-1}) originating from a wastewater treatment plant of a food-producing company and flue gas in an outdoor SBR raceway (Fig. 1). This experiment yielded on average 5.29 g total solids (TS) m^{-2} d^{-1} and 3.39 g VS m^{-2} d^{-1}, or 19.3 ton TS ha^{-1} y^{-1} and 12.2 ton VS ha^{-1} y^{-1} [5]. This cost highlights the need for a suitable valorization strategy. Nevertheless, as MaB-floc biomass is a novel bioresource, its

Abbreviations: AD, anaerobic digestion; A-PC, allophycocyanin; BMP, biochemical methane potential; BMY, biochemical methane yield; COD, chemical oxygen demand; C-PC, cyanobacterial PC; C-PE, cyanobacterial PE; DM, dry matter; MaB-floc, microalgal bacterial floc; η_{AD}, AD conversion efficiency; NP, neophytadiene; PC, phycocyanin; PE, phycoerythrin; SCOD, soluble COD; TCOD, total COD; TS, total solids; μ_{model}, first order specific methane production rate; VS, volatile solids.

* Corresponding author at: Laboratory of Industrial Water and Ecotechnology (LIWET), Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University, Graaf Karel de Goedelaan 5, B-8500 Kortrijk, Belgium.

E-mail addresses: sofie_vdhende@yahoo.com, shende@espol.edu.ec (S. Van Den Hende), diederik.rousseau@ugent.be (D.P.L. Rousseau).

URL's: http://www.cenaim.espol.edu.ec, http://www.espol.edu.ec (S. Van Den Hende), http://www.enbichem.ugent.be, http://www.enalgae.eu (D.P.L. Rousseau).

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valorization is still a challenging matter because, to the best of our knowledge, no information is available on this till date.

Earlier studies have investigated the potential of paper-industry-effluent-grown MaB-flocs for the production of biogas [13], and of aquaculture-effluent-grown MaB-flocs for the production of biogas [14], shrimp feed [15], and organic fertilizer [16]. However, these studies cannot be used to assess the valorization potential of food-industry-effluent-grown MaB-flocs, because of three reasons: first, MaB-flocs are dominated by microalgae; second, the valorization of microalgae is species-specific [6]; and third, the microalgae species in MaB-flocs differs for each reactor operation and wastewater type. In outdoor-grown MaB-flocs on sewage, the dominating microalgae were *Chlorella* sp. [11]; in on paper-industry effluent, *Chlamydomonas* sp., *Acutodesmus* sp. and *Chlorella* sp. dominated [13], and in aquaculture effluent, *Ulothrix* sp. or *Klebsormidium* sp. were dominant [12]. However, in food-industry-effluent-grown MaB-flocs, cyanobacteria dominated [5].

In an integrated biorefinery approach for algal biomass valorization, typically, a mix of high-value, low-volume products (such as phycochemicals) and low-value, high-volume products (such as bioenergy) is produced. The high-value products enhance profitability, while the low-value products provide scale and energy for the process [17,18].

Cyanobacteria, next to cryptonomads and red algae (Rhodophyta), contain phycobiliproteins [18,19]. Phycobiliproteins are proteins with linear tetrapyrrole prosthethic groups (bilins), and act as photosynthetic accessory pigments [6]. These proteins can be divided into three main classes, depending on their absorption properties: phycoerythrins (PE; with maximum absorption at wavelengths λ_{max} 540–570 nm), phycocyanins (PC; λ_{max} 610–620 nm), and allophycocyanins (A-PC; λ_{max} 650–655 nm) [18]. Phycobiliproteins are high-value compounds and have a spectrum of applications such as in natural dyes in cosmetics and food; phycofluorprobes in immunology, cell biology and flow cytometry; and as therapeutic agent with anticancer, antioxidant, hepatoprotectant, and immunomodulating activity [19]. In this regard, it is hypothized that food industry-effluent-grown MaB-flocs are a potential valuable bioresource of phycobiliproteins.

The remaining extracted MaB-floc biomass needs valorization, for example, by conversion into bioenergy. The energy input: output ratio of bioenergy production from microalgal bacterial biomass from wastewater treatment in raceway ponds is more beneficial for biogas production via anaerobic digestion (AD) than for bio-crude oil, pyrolytic bio-oil, biodiesel, and bioethanol [20].

This article presents a biorefinery concept for phycobiliprotein extraction and biogas production from MaB-flocs grown in an outdoor SBR raceway (28 m^2) in Belgium on food industry effluent (Fig. 1). Moreover, the effect of a day's increase of the suspended solids (SS) loading of the food-industry effluent on this biorefinery concept has been investigated. This is of importance, as a sudden high SS loading due to wash-out of activated sludge is an industrial reality often hard to avoid, for example, in case of bulking or pinpoint activated sludge [4]. The specific objectives of this study are threefold for both (a) MaB-flocs grown on low SS-loaded food-industry effluent, termed low SS-loaded MaB-flocs, and (b) on high SS-loaded food-industry effluent, termed high SS-loaded MaB-flocs. Firstly, aqueous extracts were analyzed for PE and PC quantity and purity, and further purified via size exclusion chromatography. Secondly, the extraction of neophytadiene (NP), a high-value phycochemical, is investigated as an alternative valorization pathway for high SS-loaded MaB-flocs. Thirdly, the biochemical methane yields and conversion efficiencies of extracted MaB-flocs were determined and compared with unextracted MaB-flocs.

2. Material and methods

2.1. MaB-floc origin and characterization

MaB-flocs originated in a pilot-scale outdoor raceway pond (28 m^2; 10 m^3), which was stirred by propeller pumps and treated effluent of a company producing plant-based food (Alpro, Wevelgem, Belgium), as described earlier [5] (Fig. 1). Synthetic flue gas containing 89 ± 2 g CO_2 Nm^{-3} was injected at 5–8 L min^{-1} when the raceway pH was above 8.75. The raceway was operated as a sequencing batch reactor with a hydraulic retention time (HRT) of 2.06 days. To study the effect of SS overloading of raceway influent on the valorization of MaB-floc biomass, aerobic activated sludge of the conventional wastewater treatment plant (Alpro, Wevelgem, Belgium) was added to this influent on 17/11/2014 to increase from <0.01 g Total Suspended Solids (TSS) L^{-1} or <0.01 g Volatile Suspended Solids (VSS) L^{-1} to 1.48 g TSS L^{-1} or 1.16 g VSS L^{-1}. Details on the raceway influent composition are presented elsewhere [5]. MaB-flocs were harvested and dewatered in two steps: (A) concentration by 1 h settling in a settling tank, and (B) dewatering in a filter press (150–250 μm) [5], and stored at −18 °C until further use.

Two samples of dewatered MaB-floc biomass were used in this study. MaB-flocs harvested on 27/10/2014 prior to the SS overloading are referred to as 'low SS-loaded MaB-flocs', while MaB-flocs harvested on 16/12/2014 after the SS overloading are referred to as 'high SS-loaded MaB-flocs'. Dewatered MaB-floc samples were analyzed for TS, VS, total chemical oxygen demand (TCOD) and soluble COD (SCOD), according to Van Den Hende et al. [5]. DNA extraction, PCR and cloning of MaB-flocs were performed based on De Wever et al. [21], with primers P2–P4 for eukaryotic species, and primers 27F-ITS3R for

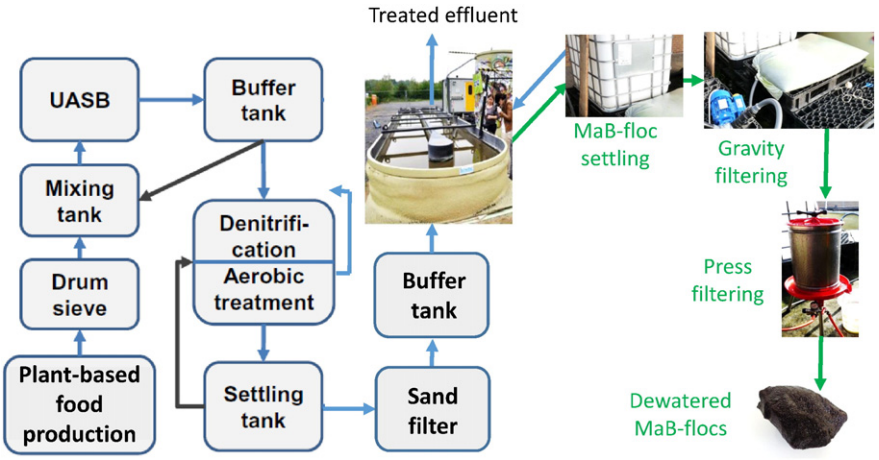

Fig. 1. Origin of MaB-flocs grown on food-industry effluent of Alpro in Belgium. Adjusted from Van Den Hende et al. [5].

prokaryotic species. Sequencing was done by the company Macrogen (The Netherlands).

2.2. MaB-floc extraction

For extraction of C-PE and C-PC, water was used as an extraction solvent according to the total water (g)–MaB-floc TS (g) ratio of 5:1 (total water includes water present in dewatered MaB-flocs). For extraction of NP, methanol was used as an extraction solvent according to a methanol (g)–MaB-floc TS (g) ratio of 7:1. MaB-floc biomass and solvents were stirred in the dark for 1 h at 500 rpm (RER, IKA-Laborthechnik, Germany) at 18–21 °C in 500 mL glass bottles with an effective depth of max. 8 cm, and were centrifuged for 7 min at 5000 *g* at 4 °C (C4236, Analis, Belgium) to separate the extracts from the extracted biomass. For both solvents, the first extraction was followed by a second (1 h) and third extraction (23 h).

2.3. Determination and purification of phycobiliproteins

To determine the C-PE and C-PC contents of MaB-floc aqueous extracts, a sodium phosphate buffer was added to the extracts to reach 0.1 M phosphate buffer (pH 7.0 at 2–4 °C). The C-PE and C-PC contents of these extracts were determined spectrophotometrically (UV-1800, Shimadzu, USA; 0.5 nm) at their maximum absorbance peaks around 568 nm and 618–620 nm [22,23,24], respectively, after subtracting optical density at 730 nm to correct for residual scattering [25], based on the equations given by Sampath-Wiley and Neefus [25]. The recovery of a certain pigment from the crude extract (%) was determined as [(pigment concentration of extract (mg/L) × extract volume (L)) × 100%] / [pigment concentration of crude extract (mg/L) × crude extract volume (L)].

Purity was determined as the ratio of optical absorbance at 568 nm and 280 nm for C-PE [23], and of 620 nm and 280 nm for C-PC [26]. The fluorescence emission spectra were determined using a RF-5301PC spectrofluorometer (Shimadzu, Japan). Size exclusion chromatography was applied as a purification step. The first aqueous extract was passed through a pre-equilibrated Sephadex G-100 column (1 cm × 40 cm–25 cm effective), and was eluted with 0.1 mM phosphate buffer (pH 7.0) at 2–4 °C, adapted from Bhaskar et al. [27]. Eluates were collected in 1 mL fractions and analyzed for C-PC, C-PE content and purity.

2.4. Determination of neophytadiene

The NP concentration of methanol extracts was determined by gas chromatography–mass spectroscopy (GC–MS), with a GC (6890N, Agilent, USA) with a split/splitless injector coupled to a quadrupole MS (5973 N, Agilent, USA) and Agilent MSD Chemstation software, based on Rodríguez-Meizoso et al. [28]. The column used was a 30 m × 0.25 mm fused silica capillary column coated with a 0.25 μm layer of SE-54 (HP-5MS, Agilent, USA). The injection was carried out at 250 °C in a splitless mode. Helium was the carrier gas (1 mL min^{-1}). The oven temperature was programmed from 50 °C (isothermal for 1 min), with an increase of 7 °C min^{-1} to 300 °C, and ending at 300 °C (isothermal for 5 min). The spectra were compared to reference mass spectrometry libraries (Wiley, NIST), and NP standard (Santa Cruz Biotechnology, USA).

2.5. BMP batch tests

The BMY of MaB-floc samples were determined in BMP tests in anaerobic batch reactors of 500 mL at 37 °C with a water displacement system coupled to the headspace as earlier described [14,29], and in accordance with the norm VDI 4630 [30]. Mesophilic anaerobic sludge was collected from a co-digestion plant treating manure and maize silage (Inagro, Roeselare, Belgium). Batch reactors were inoculated with 350 g mesophilic, degassed anaerobic sludge. These reactors were fed with six different dewatered MaB-floc samples as substrate: (1) unextracted, low SS-loaded MaB-flocs; (2) low SS-loaded MaB-flocs after 1 h water extraction; (3) low SS-loaded MaB-flocs after 1 h methanol extraction; (4) unextracted, high SS-loaded MaB-flocs; (5) high SS-loaded MaB-flocs after 1 h water extraction; (6) high SS-loaded MaB-flocs after 1 h methanol extraction. A $VS_{substrate}$ (g):$VS_{inoculum}$ (g) ratio of 0.5 was applied.

All batch assays were performed in quadruplicate, and controls included sole inoculum (blank reactors). BMP assays were ceased when no biogas production was observed for a minimum of four consecutive days, resulting in a total assay duration of 20 days. The batch reactor pH was measured at the start and end of the BMP experiments, and the biogas composition was measured by gas chromatography (GC-TCD, Agilent 6890 Series, USA) and normalized to standard conditions of 273 K and 101.325 kPa, in accordance with Van Den Hende et al. [14].

The cumulative biochemical methane yield, given by BMY_{VS} (NL CH_4 kg^{-1} VS), was modelled using a first-order kinetic model as a function of time t (d), described as $BMY_{VS}(t) = BMY_{0,VS} * (1 - e^{(-\mu t)_{model}})$ in which it was assumed to have an exponential rise to an ultimate biochemical methane yield, given by $BMY_{0,VS}$ (NL CH_4 kg^{-1} VS) and the first order specific methane production rate μ_{model} (d^{-1}) [31]. The model was fitted using Microsoft Excel's solver to minimize the sum of squared of differences between the model and the experimental BMY_{VS} values. The η_{AD} was calculated based on the model $BMY_{0,\,VS,\,model}$ and the TCOD content of MaB-floc samples [14].

2.6. Statistical analyses

Statistical analyses were carried out using the IBM® SPSS® software version 22 (USA). Data is presented as mean ± standard deviation. Data normality and homogeneity of variances were determined using a Shapiro–Wilk test and Levene's test, respectively. In case of normal data distribution and homogeneity of variances, significant differences ($p < 0.05$) were determined by a parametric One-way ANOVA test, and a Tukey post-hoc test. Otherwise, Kruskal–Wallis One-way ANOVA test, and a Tukey post-hoc test were used.

3. Results and discussion

3.1. Phycobiliprotein extraction and purification

In a first step, the phycobiliprotein types present in food-industry-effluent-grown MaB-flocs were analyzed. Phycobiliproteins can be divided into three main classes depending on their absorption spectrum: A-PC, PC and PE. Some cyanobacteria contain phycoerythrocyanin, the fourth type of phycobiliprotein, but phycoerythrocyanin and PE are mutually exclusive [32]. This means that if a cyanobacteria species contains PE, it cannot contain phycoerythrocyanin. Only cyanobacterial PC (C-PC) and cyanobacterial PE (C-PE) were detected in both low and high SS-loaded MaB-flocs. Indeed, as no absorbance peak was observed for A-PC (650 nm) [22,33], the A-PC content in MaB-flocs was negligible in relation to C-PC and C-PE. All MaB-floc extracts showed an absorbance peak of approximately 618 nm, in line with the typical peak for C-PC at 618–622 nm [34,35]. As for C-PE, three types have been described in literature: B-PE (absorption peaks at 545 nm and 565 nm with a 499 nm shoulder), R-PE (absorption peaks at 499 nm and 565 nm with a 545 nm shoulder) and C-PE (absorption peak at 565 nm; typical for cyanobacteria) [18]. None of the aqueous MaB-floc extracts displayed absorption peaks or shoulders at 499 nm or 545 nm, but all showed a peak of around 565 nm. These results suggest that C-PE was present in all MaB-floc extracts.

Fluorescence emission spectra confirmed the presence of C-PC and C-PE in aqueous MaB-floc extracts. Indeed, the emission spectra displayed peaks at 576 and 643 nm, in conformity with the typical

Table 1
Yield, recovery and purity of subsequent phycobiliprotein extracts of low and high SS-loaded MaB-flocs grown on food-industry effluent.

Biomass type	Extract	C-PC			C-PE		
		Yield (mg C-PC g^{-1} $VS_{initial}$)[A]	Recovery of total amount in MaB-flocs (%)	Purity (A_{620}/A_{280})	Yield (mg C-PE g^{-1} $VS_{initial}$)[A]	Recovery of total amount in MaB-flocs (%)	Purity (A_{568}/A_{280})
Low SS-loaded MaB-flocs	First extract (1 h)	61.1	66.8	0.43	30.1	66.1	0.36
	Second extract (1 h)	21.0	22.9	0.32	10.6	23.4	0.29
	Third extract (23 h)	9.3	10.2	0.22	4.8	10.5	0.21
	All extracts	91.4	100.0	–[B]	45.5	100.0	–
High SS-loaded MaB-flocs	First extract (1 h)	5.0	21.6	0.07	4.2	26.5	0.09
	Second extract (1 h)	5.7	24.7	0.13	4.7	29.6	0.16
	Third extract (23 h)	12.5	53.7	0.21	6.9	44.0	0.19
	All extracts	23.3	100.0	–	15.7	100.0	–

[A] $VS_{initial}$ is the VS of the initial dewatered MaB-floc biomass used for extraction.
[B] No data.

emission peaks at 576 nm for C-PE and at 644 nm for C-PC reported by Sobiechowska-Sasim et al. [36].

The second step showed that low SS-loaded MaB-flocs were a valuable source of C-PC. Indeed, these MaB-flocs contained a total of 91.4 g C-PC kg^{-1} VS and 23.3 g C-PE kg^{-1} VS. This C-PC content is similar to those reported for pure cyanobacteria cultures, e.g. 56–194 g C-PC kg^{-1} dry matter (DM) for *Arthrospira* sp. [37,38], but six times higher compared to 16.9 g C-PC kg^{-1} VS for wastewater-fed cyanobacteria-dominated biofilms [7]. The C-PE contents in MaB-flocs are similar to those found in several cyanobacterial species ranging from 10.0 to 26.3 g C-PC kg^{-1} DM [39], and to 16.6 g B-PE kg^{-1} DM obtained for *Porphyridium cruentum* [18].

The major part of C-PC of low-loaded MaB-flocs was recovered during the first 1 h extraction (Table 1). The freezing and thawing of MaB-floc step prior to extraction were key to efficient extraction. Although, on a large (biorefinery) scale, this freezing and thawing step is not the cheapest available method of microbial cell permeabilization, this freezing step has the additional advantage of also being a biomass storage step. This helps to avoid more expensive storage techniques such as freeze-drying. This storage is important as it enables the processing of larger batches of harvested MaB-floc biomass.

During the second and third extraction, another 33.2% of C-PC and 33.9% of C-PE were recovered, but the purity of C-PC, the dominant phycobiliprotein, decreased sharply (Table 1). The purity factor is calculated as the ratio of the absorbance at the pigment peak of the phycobiliprotein and the absorbance at 280 nm, in which the latter absorbance reflects the overall concentration of protein in the extracts [36].

Therefore, only this first extract was used for further purification. While ammonium sulphate precipitation did not result in a significant increase in the C-PC and C-PE purity (data not shown), size exclusion chromatography led to a three time increase in C-PC and C-PE purity (Tables 2, 3). C-PC preparations with a purity factor greater than 0.7 are considered food grade, greater than 3.9 reactive grade, and greater than 4.0 analytical grade, while for PE a purity ratio of 4.0 corresponds to diagnostic and pharmaceutical grade PE [37,40].

This purity is of high importance as it largely determines the market price. For example, C-PC with a purity of 0.5–0.75 is currently (10/09/2015) being sold at 0.13–0.19 € g^{-1}, a purity of 2.5–3.4 at 225.96–1324.40 € g^{-1}, a purity of above 4 at 521.32–5456.00 € g^{-1} (Soley Institute), and a purity of 3.5 at 88,528–110,880.00 € g^{-1} (Sigma Aldrich). R-PE with a purity of 4.6. is currently (10/09/2015) being sold at 93,000.00–135,000 € g^{-1} (Sigma Aldrich), a purity of 5.3 at 4400–17,600.00 € g^{-1} (Chromaprobe), analytical grade at 2860–12,320.00 € g^{-1} [41]. B-PE is currently (10/09/2015) being sold at 44,000.00 € g^{-1} [18]. The purity of C-PC and C-PE of the eluate with the highest purity is 1.32 and 1.06 respectively, and, thus, theoretically food grade (Tables 2, 3). Nevertheless, since grown in wastewater, the extracted pigment might not be accepted as food grade regardless of the extract's protein purity, and, therefore, its further purification to reactive or analytical grade is recommended. Furthermore, it will improve the economic potential of the presented biorefinery concept. As shown in Fig. 2, 758 g C-PC and 322 g C-PE can be produced from the daily amount of MaB-floc biomass produced in a 1 ha raceway pond in Northwest Europe based on pilot-scale results of biomass productivity [5] and the results of this study. These obtained results highlight the potential of low SS-loaded MaB-flocs as a bioresource of C-PC and C-PE, and warrant a detailed economic analysis and further research to improve the purification process.

The third step investigates the effect of increasing during a day the suspended solids (SS) loading of the food-industry effluent on this biorefinery concept. This is important because a sudden surge in loading

Table 2
Yield, concentration, recovery and purity of extraction (1 h) and purification (Sephadex G-100) of C-PC of low SS-loaded MaB-floc biomass grow on food-industry effluent.

Process	Sample	Yield (mg C-PC g^{-1} $VS_{initial}$)[A]	Concentration (mg C-PC mL^{-1} extract or eluate)	Volume of extract or eluate (mL)	Amount in extract or eluate (mg C-PC)	Recovery of total amount in MaB-flocs (%)	Recovery of total amount in crude extract (%)	Purity (A_{620}/A_{280})
Extraction	Crude extract	61.1	12.9	2.0	25.8	66.8	100.0	0.43
Purification	Highest purity eluate	22.4	3.9	2.4	9.5	24.5	36.7	1.32
	Second highest purity eluate	36.7	4.5	3.5	15.5	40.1	60.0	1.02
	Total of above eluates	59.1	4.2	5.9	24.9	64.6	96.7	1.15

[A] $VS_{initial}$ is the VS of the initial dewatered MaB-floc biomass used for extraction.

Table 3
Yield, concentration, recovery and purity of extraction (1 h) and purification (Sephadex G-100) of C-PE of low SS-loaded MaB-floc biomass grow on food-industry effluent.

Process	Sample	Yield (mg C-PE g^{-1} $VS_{initial}$)[A]	Concentration (mg C-PE mL^{-1} extract or eluate)	Volume of extract or eluate (mL)	Amount in extract or eluate (mg C-PE)	Recovery of total amount in MaB-flocs (%)	Recovery of total amount in crude extract (%)	Purity (A_{568}/A_{280})
Extraction	Crude extract	30.1	6.4	2.0	12.7	66.1	100.0	0.36
Purification	Highest purity eluate	9.5	1.4	2.8	4.0	20.9	31.6	1.06
	Second highest purity eluate	2.7	0.8	1.3	1.1	5.8	8.8	0.94
	Total of above eluates	12.2	1.3	5.1	5.1	26.7	40.2	0.99

[A] $VS_{initial}$ is the VS of the initial dewatered MaB-floc biomass used for extraction.

of suspended solids (SS) due to flushing of activated sludge is an industrial reality often harder to avoid, for example, in case of bulking or pinpoint activated sludge [4].

Microbial community analyses confirmed the presence of cyanobacteria in MaB-flocs. The cyanobacteria species present in low SS-loaded MaB-flocs are closely related to the *Geminocystis* sp. strain NIES-3708 (97.3% similarity) [42], and also to an uncultured cyanobacteria species (97.7% similarity) [43]. Coccal blue-green cyanobacteria, which dominated the low SS-loaded MaB-flocs, formed large colonies of over 1000 μm. *Geminocystis herdmanii* never forms large colonies, and may only form clusters of 2 to 4 cells during a brief period of a couple of hours [44]. In contrast, the formation of small clusters has been reported for *Geminocystis papuanica* [44]. Diatomea species were found which are closely related to *Nanofrustulum* cf. *shiloi* (99.8% similarity) [45] and to *Staurosira elliptica* (99.4% similarity) [46], were also found in high SS-loaded MaB-flocs.

High SS-loaded MaB-flocs contained 23.3 g C-PC kg^{-1} VS and 15.7 g C-PE kg^{-1} VS (Table 1). Compared to low SS-loaded MaB-flocs, this is 74.5% lower for C-PC and 65.5% lower for C-PE. In contrast to low SS-loaded MaB-flocs, the major part of C-PC of low-loaded MaB-flocs was not recovered during the first 1 h extraction (Table 1). During the second and third extraction, another 58.4% of C-PC and 73.6% of C-PE were recovered (Table 1). Moreover, the purity factors for both phycobiliproteins were much lower when the SS loading was increased (Table 1). Hence, the purification of these crude extracts was not included in this study. This decrease in C-PC and C-PE yields highlights the need for the extraction of an alternative high-value phycochemical for high SS-loaded MaB-flocs.

3.2. Neophytadiene extraction

To find an alternative high-value phycochemical, especially for high SS-loaded MaB-flocs that showed a decreased phycobiliprotein

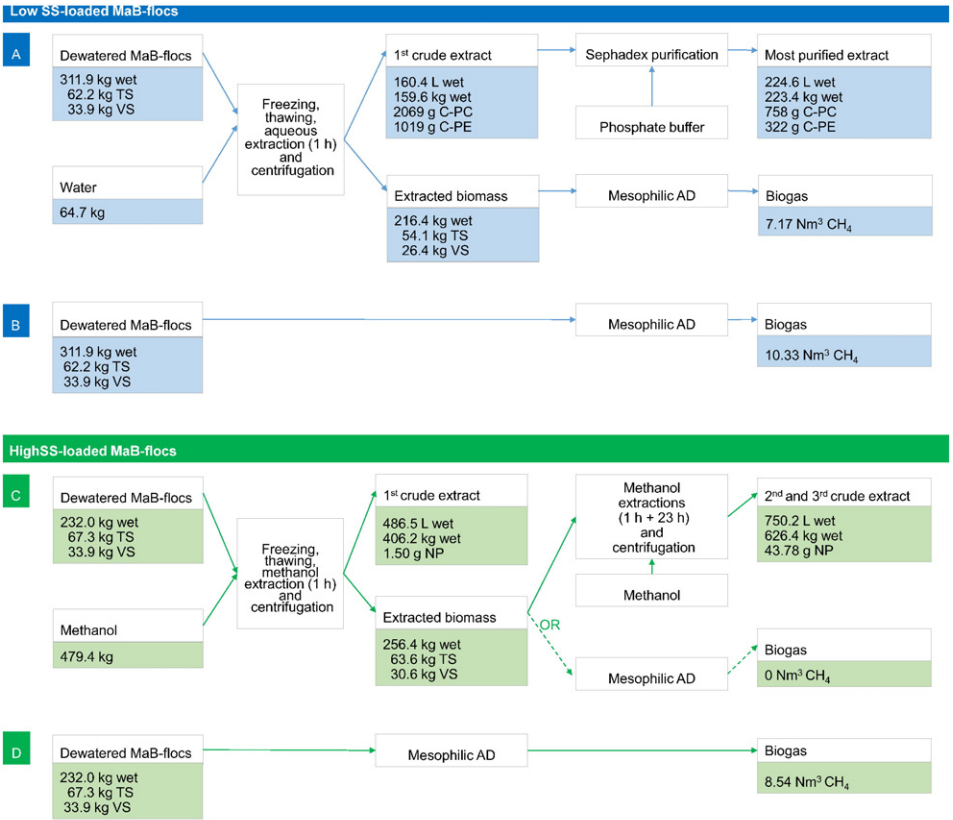

Fig. 2. Mass flow diagram of the proposed MaB-floc biomass valorization scenarios: (A) C-PC, C-PE and biogas production from low SS-loaded MaB-flocs, (B) biogas production from low SS-loaded MaB-flocs without extraction, (C) NP production and biogas from high SS-loaded MaB-flocs, and (D) biogas production from high SS-loaded MaB-flocs without extraction. Data was normalized to 33.9 kg VS, as 33.9 kg VS $ha^{-1}_{pond\ area}$ d^{-1} was produced in an outdoor pilot-scale raceway [5].

Table 4
Yield, recovery and purity of subsequent NP extracts of low and high SS-loaded MaB-flocs grown on food-industry effluent.

Biomass type	Extract	Yield (μg NP g^{-1} $VS_{initial}$)[A]	Recovery of total amount in MaB-flocs (%)	Concentration (μg NP mL^{-1} extract)
Low SS-loaded MaB-flocs	First extract (1 h)	b.d.[B]	0.0	b.d.
	Second extract (1 h)	495	53.8	39.0
	Third extract (23 h)	426	46.2	42.2
	All extracts	921	100.0	27.1
High SS-loaded MaB-flocs	First extract (1 h)	44	3.3	3.1
	Second extract (1 h)	706	52.8	58.2
	Third extract (23 h)	587	43.9	58.6
	All extracts	1337	100.0	39.9

[A] $VS_{initial}$ is the VS of the initial dewatered MaB-floc biomass used for extraction.
[B] Below detection level (no peak observed).

content, MaB-flocs were extracted with organic solvents and screened for the presence of several phycochemicals via GC–MS (data not shown). High SS-loaded MaB-flocs proved to be a valuable source of the diterpene NP (neophytadiene; $C_{20}H_{38}$; 7,11,15-trimethyl-3-methylidenehexadec-1-ene). The total NP yield of all extracts and the NP concentration of the pooled extracts were 1.5 times higher in high SS-loaded MaB-flocs compared to low SS-loaded MaB-flocs (Table 4). For both MaB-floc types, the third extract (23 h) still yielded around 45% of the total NP (Table 4). This demonstrates that NP extraction is a much slower process compared to aqueous phycobiliprotein extraction (Table 1), highlighting the importance of a long extraction period. In this study, methanol was chosen as an extraction solvent because of its greater effectiveness compared to diethylether, ethanol and hexane (data not shown). Alternative extraction solvents used for the extraction of NP, such as ethyl acetate [47] and cell permeabilization techniques remain to be screened for their cost-effectiveness and impact on the total biorefinery concept. This should be part of future research.

Compared to other NP contents of algae found in literature, the NP contents in both high and low SS-loaded MaB-flocs are high, i.e. 673 mg NP kg^{-1} TS and 502 mg NP kg^{-1} TS, respectively. Santos et al. [48] detected only 98.2, 11.3 and 6.8 mg NP kg^{-1} TS for the macroalgae *Codium tomentosum*, *Ulva lactica*, and *Gracilaria vermiculophylla*, but with a short extraction period of 0.5 h. NP has also been found in microalgae such as *Phormidium* sp. [28] and *Synechocystis* sp. [49], but quantitative data is lacking.

NP is a phycochemical found in cyanobacteria, macroalgae and plants [50,51,52,53]. This phycochemical can be formed via dehydration of phytol, a metabolite from chlorophyll hydrolysis. Indeed, Changi et al. [54] demonstrated the dehydration of phytol to NP in high temperature waters, and Blumer et al. [55] showed acid dehydration in zooplankton gut. In tobacco leaves, the concentration of NP significantly increases upon curing and aging [56]. In this regard, a possible explanation for increased NP concentration in high SS-loaded MaB-flocs might be due to an increased conversion of phytol into NP due to an increased presence of zooplankton and/or acidification during the night phase in the MaB-floc reactor. However, the latter hypothesis is yet to be confirmed.

NP has been found to have antimicrobial, antifungal, antipyretic, analgesic, antioxidant, and anti-inflammatory functions [47,48,57]. NP has been suggested to be a tobacco flavour enhancer and may as such act as

Table 5
Biomass and AD characteristics of MaB-flocs grown on food-industry effluent with different SS loadings: comparison of unextracted, water-extracted and methanol-extracted MaB-flocs.

Parameter	Unit	Low SS-loaded MaB-flocs			High SS-loaded MaB-flocs		
		Unextracted	Water-extracted	Methanol-extracted	Unextracted	Water-extracted	Methanol-extracted
MaB-floc biomass before AD[A]							
TS	% of dewatered biomass	19.95	25.56	26.72	29.00	22.72	26.52
VS	% of dewatered biomass	10.86	12.19	13.35	14.60	10.72	11.94
VS TS^{-1}	%	54.45	47.70	49.97	50.35	47.20	45.01
TCOD VS^{-1}	mg TCOD g^{-1} $VS_{substrate}$	1167	1873	4531	1488	1378	6681
SCOD VS^{-1}	mg SCOD g^{-1} $VS_{substrate}$	430	329	3214	352	213	5685
SCOD $TCOD^{-1}$	%	36.86	17.59	70.94	23.67	15.49	85.10
AD in batch reactors[B]							
pH_{start}		7.91 ± 0.02	7.95 ± 0.08	7.88 ± 0.01	7.87 ± 0.01	7.90 ± 0.07	7.86 ± 0.03
pH_{end}		7.96 ± 0.01[a]	7.94 ± 0.03[a]	7.63 ± 0.02[c]	7.94 ± 0.01[a]	7.89 ± 0.02[b]	7.57 ± 0.00[d]
Content CH_4	%-biogas	72.1 ± 2.8[a]	70.2 ± 2.2[a]	46.2 ± 1.7[b]	69.7 ± 3.0[a]	67.4 ± 2.0[a]	42.9 ± 1.5[b]
$BMY_{0,TCOD,exp}$	NL CH_4 kg^{-1} TCOD	264 ± 4[a]	145 ± 3[c]	−2 ± 0[d]	170 ± 1[b]	174 ± 9[b]	−4 ± 1[d]
$BMY_{0,TS,exp}$	NL CH_4 kg^{-1} $TS_{substrate}$	168 ± 3[a]	129 ± 2[b]	−5 ± 1[d]	128 ± 1[b]	113 ± 6[c]	−12 ± 2[e]
$BMY_{0,VS,exp}$	NL CH_4 kg^{-1} $VS_{substrate}$	308 ± 5[a]	271 ± 5[b]	−9 ± 1[d]	254 ± 1[c]	240 ± 12[c]	−27 ± 4[e]
$BMY_{0,VS,model\ C}$	NL CH_4 kg^{-1} $VS_{substrate}$	305 ± 4[c]	272 ± 3[b]	n.d.[C]	252 ± 1[c]	235 ± 9[d]	n.d.
μ_{model}[C,D]	d^{-1}	0.287 ± 0.007[c]	0.296 ± 0.027[c]	n.d.	0.456 ± 0.009[a]	0.397 ± 0.045[b]	n.d.
$R_{correlation,\ model}$[C]		98.6 ± 0.2	98.3 ± 0.1	n.d.	98.7 ± 0.2	98.3 ± 0.1	n.d.
$\eta_{AD,\ model}$[E]	%	74.7 ± 1.1[a]	41.5 ± 0.5[c]	n.d.	48.4 ± 0.2[b]	48.7 ± 1.9[b]	n.d.
Maximum revenues and avoided costs[F]							
Electricity	€ kg^{-1} $VS_{substrate}$	0.33	0.28	0	0.30	0.26	0
Thermal energy	€ kg^{-1} $VS_{substrate}$	0.13	0.11	0	0.12	0.10	0
Total	€ kg^{-1} $VS_{substrate}$	0.47	0.39	0	0.42	0.36	0
$VS_{substrate}/VS_{unextracted}$	%	100.0	77.9	83.9	100.0	87.0	90.4
Total	€ kg^{-1} $VS_{unextracted}$	0.47	0.30	0	0.42	0.31	0

[A] Averages of 2 analyses per sample.
[B] Averages and standard deviations of 4 AD batch reactors, values with different labels are significantly different according to a parametric One-way ANOVA or non-parametric Kruskal–Wallis One-way ANOVA and a Tukey post hoc test ($p < 0.05$).
[C] Parameters of applied first order kinetic model [30], no data presented for methanol-extracted biomasses due to negative values of $BMY_{0,VS,exp}$.
[D] Production rate constant.
[E] AD conversion efficiency.
[F] Max revenues and avoided costs are calculated according to methodology presented in Van Den Hende et al. [14] and are based on $BMY_{0,VS,model}$.

a flavour carrier by entrapping volatiles in the tobacco smoke aerocol [57]. Recently, the use of NP as an additive for liquid cigarettes to improve the aroma and evaporation rate has been patented [58]. Similar to phycobiliproteins, NP is a high-value chemical with market prices for its analytical grade in the same range as that of C-PC and C-PE. For example, analytical grade NP is currently (2015) being sold at 26,620–10,650 € mg^{-1} (Santa Cruz Biotechnology, TRC). These results show that NP extraction can be an interesting alternative to the MaB-floc valorization pathway. Further optimization is needed to increase the NP recovery, find a suitable purification process, and to confirm the large economic potential.

3.3. Biogas production

The BMY of unextracted and extracted MaB-flocs was determined by BMP tests to evaluate their potential for anaerobic digestion to biogas. The $BMY_{0,VS,model}$ of unextracted MaB-flocs (Table 5) are in the mid-range compared to microalgae, i.e. 50–510 NL CH_4 kg^{-1} VS [20,59,60,61], and are higher than those of microalgal biomass cultivated outdoors in a sewage-treating raceway pond, i.e. 170 NL CH_4 kg^{-1} VS [60]. Moreover, they are similar to paper-industry wastewater MaB-flocs grown indoors, i.e. 208–305 NL CH_4 kg^{-1} VS, and higher compared to aquaculture wastewater-fed MaB-flocs cultivated outdoors in a raceway pond, i.e. 133–227 NL CH_4 kg^{-1} algae VS [14]. The maximum total revenue and the avoidable costs of by electricity and heat production in a combined heat-and-power system from the biogas (Table 5) are nearly double compared to aquaculture wastewater-grown MaB-flocs, i.e. 0.23 € kg^{-1} MaB-floc VS. [14]. But these values are very low compared to the valorisation potential via extraction of phycochemicals (Sections 3.1, 3.2), and, therefore, AD of food-industry-effluent-grown MaB-flocs cannot be recommended as the sole valorization pathway.

Aquaeous extraction of the MaB-floc biomass significantly decreased the $BMY_{0,VS,model}$ by 10.8% for low SS-loaded MaB-flocs (Table 5). Aqueous extraction of low SS-loaded MaB-flocs led to a decrease in the solubility of TCOD, and resulted in a η_{AD},which is significantly lower compared to unextracted MaB-flocs (Table 5) but is still in the mid range of the η_{AD} of wastewater-grown activated sludge, i.e. 23–61% [62,63,64]. Increasing this η_{AD} by energy-consuming pretreatment methods is not warranted, but potential lies in an enzymatic pretreatment of MaB-flocs and the optimization of the AD inoculums [13]. However, from an economic point of view, it could be more cost-effective to improve the purification of MaB-floc extracts rather than the η_{AD}, as the conversion of aqueous extracted MaB-flocs into biogas would only add 0.30 € kg^{-1} $VS_{unextracted}$ to the total maximum revenues and avoidable costs (Table 5). This value is low compared to the potential economic revenues of phycochemicals. Nevertheless, AD of extracted low SS-loaded MaB-flocs is recommended, as the disposal of wastewater-grown biomass as waste would be an additional cost [4].

Compared to low SS-loaded MaB-flocs, the μ_{model} of high SS-loaded MaB-flocs was significantly higher, indicating a faster hydrolysis (Table 5). But the $BMY_{0,VS,model}$ of high SS-loaded MaB-flocs was significantly lower compared to low SS-loaded MaB-flocs (Table 5). Aquaeous extraction of these high SS-loaded MaB-flocs significantly decreased the $BMY_{0,VS,model}$ by 6.7% (Table 5).

Based on the results of phycobiliprotein and NP extraction from high SS-loaded MaB-flocs (Sections 3.1, 3.2), not an aqueous extraction for phycobiliprotein recovery but a methanol extraction for NP recovery is preferred. The BMY of methanol-extracted MaB-flocs was lower compared to the blank reactors only containing inoculum. Methanol extraction resulted in a significant lower AD reactor pH (Table 5). This could be due to the toxicity of methanol felt by AD microorganisms. In this study, methanol was chosen as extraction solvent, as it was more effective for NP extraction compared to diethylether, ethanol, and hexane (data not shown). Despite the fact that other researchers have reported the efficient AD of methanol-containing waste into biogas [65], future research should include the screening of less toxic but more effective solvents for NP extraction. Moreover, due to the limited amount of extracted MaB-floc biomass that can be produced per food company (23–27 kg MaB-floc $VS_{extracted}$ for 0.88 ha pond area per daily 1500 m^3 effluent of the food company Alpro), co-digestion with other biomasses, preferable with a high C: N ratio to avoid ammonia inhibition [52], would be needed.

4. Conclusions

The biorefinery concept of high-value phycochemical extraction and biogas production from food-industry-effluent-grown MaB-flocs was assessed. Freezing and aqueous extraction of MaB-flocs followed by size exclusion chromatography yielded 22.4 g C-PC kg^{-1} VS with a purity of 1.32 (24.5% recovery) and 9.5 g C-PE kg^{-1} VS with a purity of 1.06 (20.9% recovery). Anaerobic digestion of the extracted MaB-flocs resulted in 272 NL CH_4 g^{-1} VS. Moreover, increasing the suspended solids (SS) loading of food-industry effluent for one day, significantly reduced the biochemical methane yield by 13.6%, and the C-PC and C-PE yield of total crude extracts by 74.5% and 65.5%, respectively. In contrast, it increased the NP yield by 45.1%. This study highlights the large potential of these MaB-flocs as a bioresource for the combined production of phycobiliproteins and biogas in case of low SS loading, and for NP extraction in case of high SS loading of the food industry effluent. Further research is needed to improve the extraction and purification of these phycochemicals, and to confirm the economic potential.

Contributions

SVDH designed the experimental set-up, contributed in lab work, data analyses and statistics, and wrote the manuscript. JB approved the experimental setup, contributed in lab work and data analyses of extractions and anaerobic digestion, and revised the manuscript. P-JDB approved the experimental setup, contributed in lab work, material and method-writing and data analyses of anaerobic digestion, and revised the manuscript. DNPR approved the experimental set-up, and revised the manuscript.

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