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Anaerobic co-digestion of pig manure and algae: Impact of intracellular algal products recovery on co-digestion performance

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KEYWORDS

Anaerobic digestion; codigestion; microalgae; biorefinery; pre-treatment

ABSTRACT

This paper investigates anaerobic co-digestion of pig manure and algae (*Scenedesmus* sp.) with and without extraction of intracellular algal co-products, with views towards the development of a biorefinery concept for lipid, protein and/or biogas production. Protein and/or lipids were extracted from *Scenedesmus* sp. using free nitrous acid pre-treatments and solvent-based Soxhlet extraction, respectively. Processing increased algae methane yield between 29% and 37% compared to raw algae (VS basis), but reduced the amount of algae available for digestion. Co-digestion experiments showed a synergy between pig manure and raw algae that increased raw algae methane yield from 0.163 to 0.245 m^3 CH_4 kg^{-1} VS. No such synergy was observed when algal residues were co-digested with pig manure. Finally, experimental results were used to develop a high-level concept for an integrated biorefinery processing pig manure and onsite cultivated algae, evaluating methane production and co-product recovery per mass of pig manure entering the refinery.

1 INTRODUCTION

Algae are an interesting feedstock for the production of biofuels, chemicals, cosmetics and animal feed (Milledge & Heaven, 2014; Passos et al., 2013). Advantages of algae include: (i) the capacity to grow on fresh, brackish, saline and wastewater streams; (ii) tolerance to a wide variety of environmental conditions; (iii) an ability to be cultivated on land not suitable for food production; and (iv) be produced all year round (Uggetti et al., 2014; Ward et al., 2014). Currently, most approaches for algae-based biorefineries (i.e. facilities to convert algae into multiple valuable products, including biofuels) are not economically viable, due to high costs of algae cultivation and valorisation (Milledge & Heaven, 2014). Consequently, strong research efforts aiming to improve biofuels and/or biochemical production yields from algae are being made (Milledge & Heaven, 2014; Uggetti et al., 2014). One particular opportunity, which is the focus of this work, is value-adding to algae residue by using it as a feedstock for anaerobic (co-)digestion.

Anaerobic digestion (AD), which converts organic matter into biogas and a stabilised digestate, is a proven technology for the management of organic-rich streams (Mata-Alvarez et al., 2014). AD has been identified as a key process to make algae biorefineries commercially feasible (Milledge & Heaven, 2014; Uggetti et al., 2014), and can be used to treat either raw algal biomass or algal residue after extraction of valuable intracellular products (Keymer et al., 2013; Passos et al., 2013; Ramos-Suárez & Carreras, 2014; Sialve et al., 2009). The viability of algae AD is highly dependent on: (i) the organic concentration of the feedstock, since harvesting and concentrating algae biomass is a major cost; and (ii) the biochemical methane potential (B_0) of the algae (Uggetti et al., 2014). The latter depends on the algae culture strain and its cultivation conditions, which impact their composition (carbohydrate, protein and lipid content) as well as cell wall structure (Alzate et al., 2014;

Sialve et al., 2009; Uggetti et al., 2014). Reported algae B_0, mostly mono-cultured, are highly variable ranging from 0.130 to 0.600 m^3 CH_4 kg^{-1} VS (Mussgnug et al., 2010; Ward et al., 2014). Unfortunately, the methane yield from natural mixed algae cultures grown in less controlled systems (real world application) are found in the lower range, rarely exceeding 0.300 m^3 CH_4 kg^{-1} VS (González-Fernández et al., 2011; Keymer et al., 2013; Passos et al., 2013). This fact has raised the interest on algae pre-treatment techniques, with and without co-products recovery, aiming to improve algae biodegradability through cell wall disruption (Milledge & Heaven, 2014; Ramos-Suárez & Carreras, 2014). Under this rationale, the feasibility of an algae-based biorefinery is mainly linked to: (i) the co-products economic value; (ii) biogas value as electricity and/or heat energy; (iii) algae harvesting and concentration, where harvested algae may not only be thickened, but also dewatered or even dried before processing for co-products extraction (Alzate et al., 2014; Sialve et al., 2009; Ward et al., 2014).

Anaerobic co-digestion (AcoD), the simultaneous anaerobic digestion of two or more substrates, improves economic viability of AD plants due to the potential for higher methane production than through digestion of single substrates (Mata-Alvarez et al., 2014). The increase in methane production from AcoD is mainly a result of increased organic loading rate; however, synergism (i.e. a complementary relationship between substrates that improves digestion performance) can further enhance methane production (Astals et al., 2014; Mata-Alvarez et al., 2014; Ramos-Suárez & Carreras, 2014). Beyond the implementation and operation expenses, onsite cultivation of algae presents some advantages over the use of other co-substrates (Mata-Alvarez et al., 2014). Such advantages include: (i) reduced or nullified co-substrate transport cost, which is one of the most important co-substrate selection criteria; (ii) minimising the effect of seasonality of some agro-industrial co-substrates, where supply

can be variable or cease; and (iii) providing a co-substrate in regional areas where co-substrates are otherwise utilised or are not available. Taking into account these facts, algal biomass appears as a potential co-substrate for animal manure digester located in rural/remote areas.

The potential of using algae as a co-substrate has recently been reported in several publications; however, these studies focus on sewage sludge or carbon-rich waste as the main substrate (Cecchi et al., 1996; Mata-Alvarez et al., 2014; Ramos-Suárez & Carreras, 2014; Zhong et al., 2012), while few studies have evaluated AcoD of animal manure and algae (González-Fernández et al., 2011; Miao et al., 2014; Sarker et al., 2014). From a nutrient balancing perspective, AcoD of algae and manure does not seem obviously attractive, because both substrates are characterised by a relatively low carbon-to-nitrogen (C/N) ratio (< 10) (Mata-Alvarez et al., 2011). However, Gonzalez-Fernandez et al. (2011) and Ramos-Suarez and Carreras (2014) observed that synergism is not always linked to the C/N ratio of the mixture when using algae as co-substrate and therefore AcoD of algae and pig manure warrants further investigation. Furthermore, previous algae AcoD studies have only tested raw algae in co-digestion mixtures and have not considered AcoD of algae residues after extraction of valuable co-products. There is a present need for a study on AcoD of algal residues to provide critical insights into AD plants aiming to process algae grown on anaerobic digestion supernatant.

The primary goal of this study is to evaluate anaerobic co-digestion of pig manure and algae (*Scenedesmus* sp.) with and without extraction of intracellular algal co-products. Algae processing targeted the extraction of lipids (solvent-based Soxhlet extraction) and/or protein (free nitrous acid pre-treatment) as high-value co-products. Biomethane potential tests were

used to assess the effect of pre-treatment and co-product extraction on substrate biodegradability and degradation rate. Finally, the results of the study were used to evaluate a high-level concept for an integrated biorefinery treating pig manure and onsite cultivated algae.

2 MATERIALS AND METHODS

2.1 Raw algae, manure and inoculum origin

Pig manure was collected as a representative composite sample of an entire direct-flush from a grown-out pig shed near Perth (WA, Australia). The sample was shipped immediately (chilled on ice-bricks) to The University of Queensland, and was received cold and stored at 277 K until use. Dry algal biomass was obtained from a pilot-scale open algal cultivation raceway (Pinjarra Hills, Australia). The raceway had a total volume of 30 m^3 with 1.5m wide channels and 0.2 m depth. The green algae *Scenedesmus* sp. was cultured in an open pond and algal biomass was collected by filtration and dried in a solar collector and drying tunnel assembly. Microscope observation showed that the majority of the biomass was *Scenedesmus* sp. with small amounts of sand, grit and salt crystals.

Anaerobic inoculum was collected from the bottom (~2 m depth) of a partially covered anaerobic lagoon, which treats flush manure from a specialised breeder piggery located near Grantham (QLD, Australia). After collection, inoculum was stored at 277 K. Prior to commencement of the biomethane potential (BMP) tests, the inoculum was degassed at 310 K for one week. The specific methanogenic activity of the inoculum at 310 K was 0.09 kg COD-CH_4 kg^{-1} VS day^{-1}.

2.2 Algae high-value products extraction

Processed algal residues were prepared for the digestion/co-digestion testing. The processing steps extracted protein with free nitrous acid (FNA) (Section 2.2.1), and/or lipids via a solvent-based Soxhlet extraction (Section 2.2.2).

2.2.1 Protein extraction

FNA pre-treatment was carried out to release protein from algal cells. Dry algal biomass was re-suspended in deionized water at 47 g L^{-1}, and pH was adjusted to 5.5 using 0.1 M HCl. Sodium nitrite stock solution 30 g NO_2^--N L^{-1} was then added to the suspension resulting in an initial concentration of 0.3 g NO_2^--N L^{-1}. The FNA dose was selected from previous experiments (Bai et al., 2014), where 0.3 g NO_2^--N L^{-1} led to moderate cell disruption with algal biomass releasing most protein. The algal suspension was treated in a well-mixed (550 rpm) reactor for 48 h, with pH maintained constant at 5.5 ± 0.2 through periodic manual addition of 0.1 M HCl. During the pre-treatment, FNA concentration was monitored by nitrite and pH measurements, and calculated as in Eq. 1 (Bai et al., 2014), where S_{NO2} is the dissolved nitrite concentration (g NO_2^--N L^{-1}), pH is the suspension pH, and T is the operational temperature (298 K).

$$\text{FNA}\left(\text{g HNO}_2 - \text{N L}^{-1}\right) = \frac{S_{NO2}}{e^{\frac{-2300}{(T)}} \cdot 10^{pH}} \quad (1)$$

After FNA pre-treatment, algae biomass residues were recovered by centrifugation (2,500 *g* for 5 min) and decanting the supernatant, where released protein was contained.

2.2.2 Lipid extraction

Lipid extraction was done on raw and FNA pre-treated algal biomass using a serial Soxhlet

159 extraction apparatus and n-hexane:ethanol (3:1, v/v) as extraction solvent (Bai et al., 2014).
160 Lipid extraction yields were quantified in duplicates after 6 h of extraction. After lipid
161 extraction, the algal biomass residues were dried to constant weight in a vacuum desiccator to
162 remove residual organic solvent.
163

164 **2.3 Chemical analytical methods**

165 Analyses of the total fraction were performed directly on the raw samples. For analyses of the
166 soluble fraction, the samples were centrifuged at 2,500 g for 5 min and the supernatant was
167 filtered through a 0.45 µm PES Millipore® filter. The content external to the cells and cell
168 debris was quantified by analysing the supernatant of the centrifuged samples.
169 Total solids (TS) and volatile solids (VS) were measured according to Standard Method
170 2540G (Eaton et al., 2005). Total chemical oxygen demand (tCOD) and soluble chemical
171 oxygen demand (sCOD) were measured using a Merck COD Spectroquant® test kit (range
172 0.5-10 g L^{-1}) and a Move 100 colorimeter (Merck, Germany). Volatile fatty acids (i.e. acetic,
173 propionic, butyric and valeric) were analysed with an Agilent 7890A gas chromatograph
174 equipped with an Agilent DB-FFAP column. NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P, total
175 Kjeldahl nitrogen (TKN) and phosphorous (TKP) were determined with a Lachat Quik-Chem
176 8500 flow injection analyser. Total protein was measured using the bicinchoninic acid
177 method with bovine serum albumin as calibration standard (Smith et al., 1985).
178 Polysaccharide (carbohydrate) concentration was determined using the anthrone method with
179 glucose as standard (Raunkjaer et al., 1994). Lipid content was determined using a Wilks
180 Enterprise Inc. InfraCal TOG/TPH analyser, with S-316 as the extraction solvent. Biogas
181 composition (CH_4, CO_2 and H_2) was determined using a Shimadzu GC-2014 gas
182 chromatograph equipped with a Valco GC valve (1 mL sample loop), a HAYESEP Q 80/100
183 packed column (2.4 m length; 1/8" outside diameter, 2 mm inner diameter) and a thermal

conductivity detector (TCD). The chromatograph injector, oven and detector temperatures were set at 75, 45 and 100 °C, respectively, and 28 mL min^{-1} of Argon at 135.7 kPa was used as a carrier gas.

2.4 Biomethane potential tests

Biomethane potential (BMP) tests were carried out according to Angelidaki et al. (2009) in 160 mL glass serum bottles at mesophilic conditions. All tests contained 35 mL inoculum and an amount of substrate that provided an inoculum to substrate ratio of 2 (VS-basis). Bottles were flushed with 99.99% N_2 gas for 1 min (4 L min^{-1}), sealed with a rubber stopper retained with an aluminium crimp seal and stored in temperature-controlled incubators (310 ± 1 K). Tests were mixed by swirling once per day. A blank test containing inoculum and no substrate was used to correct for background methane potential of the added inoculum. All tests and blank were done in triplicates, and all error bars indicate 95% confidence limit on the average of the triplicates. Biogas volume was measured using a manometer at the start of each sampling event. Accumulated volumetric gas production was calculated from the pressure increase in the headspace volume and expressed under standard conditions (273.15 K, 100.00 kPa). At each sample event, the biogas composition was determined by gas chromatography using the GC configuration described in Section 2.3.

Pig manure was co-digested with four different algae co-substrates: (i) raw algae; (ii) lipid extracted algae residue (after Soxhlet extraction); (iii) protein extracted algae residue (after FNA extraction); and (iv) protein & lipid extracted algae residue (after FNA extraction followed by Soxhlet extraction). Specifically, three mixtures were tested between pig manure and raw algae (15, 30 and 50 % in co-substrate on a VS-basis), and two mixtures were tested between pig manure and each algae residue (15 and 30% in co-substrate on a VS-basis). In

addition, BMP assays of each individual substrate (i.e. pig manure, raw algae and the three algal residues) were carried out to establish reference degradation parameters (Fig. SI of supplementary data summarises BMP set-up). The chemical characterisation of the substrates under study is provided in Table 1.

2.5 Model implementation and data analysis

Mathematical analysis of the BMPs was based on the IWA Anaerobic Digestion Model No. 1 (ADM1). Process kinetics and substrate biodegradability were the two targeted parameters to compare mono- and co-digestion experiments (Astals et al., 2014). As hydrolysis was assumed to be the rate-limiting step during AD of manure and algae (Costa et al., 2012; Ramos-Suárez & Carreras, 2014), the BMPs were modelled using first order kinetics (Astals et al., 2014). In contrast to the conventional one-substrate model, in this study all substrates (manure and algae) were modelled through a two-substrate model, where substrates are split into a rapidly biodegradable and a slowly biodegradable fraction (Eq. 2) (Wang et al., 2013). This approach improved fitting of the algae (raw and residual) mono-digestion BMP profiles and exploration of the effect of the algae pre-treatment over the rapidly and slowly biodegradable fractions of the substrates (Wang et al., 2013).

$$r = \sum_i \left(f_{fast,i} \cdot k_{hyd,fast,i} \cdot X_i \cdot C_i\right) + \sum_i \left(f_{slow,i} \cdot k_{hyd,slow,i} \cdot X_i \cdot C_i\right) \quad (2)$$

where r is the process rate (g COD L^{-1} day^{-1}), f_i is the substrate biodegradability (-), $k_{hyd,i}$ is the first-order hydrolysis rate coefficient of the substrate (day^{-1}), X_i is the substrate concentration (g VS L^{-1}) and C_i is the tCOD-to-VS (COD/VS) ratio of the substrate. Biodegradability (f_i) is used for model-based analysis in order to normalise analysis between substrates. The f_i can be converted to B_0 using the conversion factors provided in Table 1,

234 with material with a COD/VS ratio of 1 having a conversion factor of 0.350 m^3 CH_4 kg^{-1} VS
235 (B_0/f) (Astals et al., 2014).
236
237 The degradation model was implemented in Aquasim 2.1d. Parameter estimation and
238 uncertainty analysis were simultaneously estimated with a 95% confidence limit as per
239 Batstone et al. (2009). Parameter uncertainty was estimated using a two-tailed t-test on
240 parameter standard error around the optimum and non-linear confidence regions were also
241 tested to confirm the linear estimate was representative of true confidence. The objective
242 function used was the sum of squared errors (χ^2), where average data from triplicate
243 experiments were used as the model target.
244
245
246 **3 RESULTS AND DISCUSSION**
247 **3.1 Extraction of high-value products from algal biomass**
248 The increase in polysaccharide and protein concentrations in the supernatant of the algal
249 suspension after 48 h FNA pre-treatment reflects lysis of the algal cell wall and the
250 subsequent release of intracellular organic compounds (Fig. 1). *Scenedesmus* sp. is known to
251 have a rigid cell wall composed of poorly biodegradable carbohydrates (Ramos-Suárez &
252 Carreras, 2014; Ward et al., 2014). Hence, the increase of polysaccharides concentration in
253 the liquor, especially insoluble (particulate) compounds, shows that FNA pre-treatment was
254 able to break apart the algal cell wall but could not solubilise it. The cell wall disruption
255 produced by the FNA pre-treatment caused release of large amounts of protein into the liquor
256 leading to a protein concentration increase from 0.5 g L^{-1} to 5.0 g L^{-1} (i.e. release of 0.25 kg
257 of protein per kg VS of algae) (Fig. 1). The protein release yield obtained in this study is in
258 agreement with those reported in previous studies using other pre-treatment techniques such

as sonication, high-pressure homogenization and enzyme hydrolysis (Keris-Sen et al., 2014; Safi et al., 2014), indicating that FNA pre-treatment is an effective technology to facilitate protein recovery.

Lipid extraction yield for *Scenedesmus* sp. was 0.14 kg lipids kg^{-1} VS, which is in good agreement with previously reported values (Keymer et al., 2013; Ramos-Suárez & Carreras, 2014). Moreover, the efficiency of lipid extraction increased up to 0.19 kg lipids kg^{-1} VS after the FNA pre-treatment (Fig. SII of supplementary data), indicating that the disruption of algal cell wall caused by the FNA pre-treatment allowed to improve the contact between the solvent and intracellular lipids. The improvement of green algae lipid extraction yield after pre-treatment has been previously reported by Bai et al. (2014), who used different FNA concentrations with *Tetraselmis striata* M8, and Lee et al. (2010), who evaluated five pre-treatment techniques on *Scenedesmus* sp. Comparing the results with those reported by Lee et al. (2010), it can be observed that the lipid yield after the FNA pre-treatment was similar to that reached by their optimal reported pre-treatments (i.e. bead-beating and microwaves). However, the increase of the lipid extraction yield (1.5-fold) recorded in the present study was lower than that reported by Lee et al. (2010) (up to 5.5-fold). This difference may be related to differences in pre-processing (i.e. drying and grinding) of the raw algae as well as the different lipid extraction method.

3.2. Influence of product recovery on algal biomass anaerobic digestion

Fig. 2 displays the experimental and modelled methane production profiles of the four algae mono-digestion experiments, while Table 2 shows the model outputs for biodegradability (f_{fast}, f_{slow}, f_{total}) and degradation kinetics ($k_{hyd,fast}$, $k_{hyd,slow}$). The low methane yield of the *Scenedesmus* sp. (0.163 ± 0.010 m^3 CH_4 kg^{-1} VS), linked to the resistance of the cell wall to

bacterial degradation, is in good agreement with values reported elsewhere (González-Fernández et al., 2011; Keymer et al., 2013; Mendez et al., 2014; Ramos-Suárez & Carreras, 2014). Model outputs clearly illustrated that pre-treatment could improve algal biodegradability (f_{total}), even when co-products had been extracted. All algal residues (i.e. after co-product extraction) had an improved biodegradability as compared to raw algae, with lipid extracted algae showing the greatest increase (f_{total} from 0.31 to 0.48). This improvement in biodegradability with extraction is believed to be a result of cell wall disruption, which made intracellular organic matter more bioavailable.

Model outputs also indicated that the improvement in biodegradability following lipid extraction was mainly related to an increase of the rapidly biodegradable fraction (f_{fast}), whilst protein, and protein & lipid extracted algae was due to an increase of the slowly biodegradable faction (f_{slow}). The latter phenomenon could be explained by the conjunction of two factors: (i) the solvent-based extraction may be more severe disrupting the algal cell wall than the applied FNA dose; and (ii) the fact that the algae particles released during lipid extraction remained inside the Soxhlet thimble and were subsequently digested, whereas the particles (soluble and insoluble) released during the FNA pre-treatment were removed after the centrifugation of the pre-treatment suspension.

Processing of algae for co-product extraction did not appear to have a significant impact on the degradation kinetics. A finding which was consistent with results reported by Ramos-Suarez and Carreras (2014) who digested residual *Scenedesmus* sp. biomass after protein and lipid extraction and Keymer et al. (2013) when digesting solvent-based lipid extracted *Scenedesmus* sp.

309 The present results indicate that algae pre-treatment is an effective strategy to enhance of
310 algae B_0. Actually, in the present study, algal biomass B_0 improvement was achieved even
311 after removing lipids and/or FNA solubilised organic matter (carbohydrates and protein),
312 which is not a common practice in studies devoted to algae pre-treatment prior to AD
313 (Uggetti et al., 2014; Ward et al., 2014). However, the co-production of high-value products
314 and biofuels has been identified as more economically viable for algal biorefineries than the
315 production of bioenergy alone (González-Fernández et al., 2011; Milledge & Heaven, 2014).
316 Under this biorefinery concept (where algal valuable products are removed from the system
317 prior to AD), two extreme scenarios could occur: (i) a biorefinery with a fixed algae
318 production capacity, where co-products recovery reduces the amount of algae available for
319 AD; and (ii) a biorefinery with a flexible algae production capacity, where an increase on
320 algae production compensates the organic matter lost during co-products recovery. Pre-
321 treated algal B_0 can only be used as a comparative parameter if the biorefinery is able to
322 produce extra algae and keep the anaerobic digester organic loading rate stable. On the
323 contrary (when the biorefinery algal production is the stable parameter), the overall methane
324 yield (B'), defined as the methane yield per gram VS of algae before the pre-treatment, is a
325 more appropriate parameter to compare the performance of an anaerobic digester; because
326 although pre-treatment increased algae B_0, co-products extraction reduces the amount of
327 organic matter going into the digester. Table 3 shows B_0, B' and co-product extraction yield
328 of each of the evaluated pre-treatments. Results indicated that only lipid extraction was able
329 to enhance the digester methane production under both scenarios, while protein and protein &
330 lipid extracted algae had a significant reduction of B' due to the extraction of large amounts
331 of organic matter during FNA pre-treatment.
332
333

3.3. Anaerobic co-digestion of pig manure and raw or processed algae

Assessment of the interaction mechanisms (i.e. synergistic and antagonistic) during co-digestion of pig manure and algae (raw or processed) was carried out by comparing the experimental profiles with the theoretical ones (generated by the combination mono-digestion profiles over time and proportioned to the amount of substrate present). As illustrated in Fig. 3, actual and theoretical curves overlap in most experimental trials, indicating no strong interaction between substrates. Thus, co-digestion performance (kinetics and extent) could have been assessed by combining the results from mono-digestion experiments. In all cases, the introduction of algae into the manure led to a reduction of the B_0 since the B_0 of algae is lower than the B_0 of pig manure; such reduction was approximately proportional to the amount of algae and manure in the mixture. However, two raw algae mixtures (70% manure + 30% raw algae; 50% manure + 50% raw algae) showed a methane yield significantly higher than the theoretical methane yield (Fig. 3A). The fact that the methane yield improvement was only observed in the mixtures with a higher algal concentration does not necessarily imply that the synergy did not occur at the low-level mixture (85% manure + 15% raw algae). Indeed, it may suggest that raw algae rather than manure was the substrate further degraded (increased biodegradability). Under this rationale, the increased algae biodegradability in the lowest mixture would have been masked by the low proportion of algae, which in the mixture only accounted for about 10% of the methane production.

The comparison between the actual and modelled methane curves, when the mixtures were simulated using the set of parameters from the mono-digestion BMP modelling (Table 2), show that curves overlap in most of the tests; however, small differences could be observed in B_0 values (see Fig. SIV of supplementary data). To better understand the interaction between the substrates, AcoD profiles were modelled giving freedom to kinetic (k_i) and/or

359 extent (f_i) parameters. Model outputs from different scenarios (data not shown) indicated that
360 (i) substrate degradation kinetics were not influenced by co-digestion; and (ii) B_0
361 discrepancies were mainly linked to algae biodegradability rather than manure
362 biodegradability. Consequently, AcoD profiles were modelled using fixed hydrolysis rates
363 ($k_{hyd,fast,i}$ and $k_{hyd,slow,i}$) and fixed biodegradability for pig manure ($f_{fast,manure}$ and $f_{slow,manure}$)
364 (values shown in Table 2), with algae biodegradability as the fitted variables for each mixture
365 ($f_{fast,algae}$ and $f_{slow,algae}$). With the exception of the 85% manure + 15% protein & lipid extracted
366 algae mixture, model outputs confirmed that pig manure and residual algae co-digestion
367 could be modelled using a single set of parameters. The observed minor differences between
368 the measured and modelled profiles would likely be attributed to experimental/analytical
369 error rather than to synergic mechanisms (Figure SV of supplementary data). Likewise,
370 model outputs confirmed the increase of raw algae biodegradability when co-digested with
371 pig manure. As an average, raw algae f_{fast} increased from 0.20 to 0.26 while f_{slow} increased
372 from 0.11 to 0.20, which represents approximately 0.030 and 0.050 m^3 CH_4 kg^{-1} VS
373 additional methane potential respectively. Therefore, it can be concluded that raw algae B_0
374 increased from 0.163 to 0.245 m^3 CH_4 kg^{-1} VS due to synergistic mechanisms. Supporting the
375 hypothesis made by Gonzalez-Fernandez et al. (2011) about improved algal biodegradability
376 when co-digesting algae (a mixture of *Chlorella vulgaris* and *Scenedesmus obliquus*) and pig
377 manure.
378
379 Previous algae AcoD studies mainly linked the synergistic improvement in methane yield to
380 the nutrient balance (Mata-Alvarez et al., 2014). However, in BMP assays the composition of
381 the digestion mixture is primarily controlled by the inoculum properties rather than by the
382 properties of the added substrates. Moreover, if the improvement was related to an optimised
383 C/N ratio, similar behaviour should have been observed when co-digesting manure and algal

384 residues, even though some organics were extracted. However, no synergies were observed
385 for co-digestion of pig manure and algal residues. Based on this outcome, it is theorised that
386 the enhancement of the raw algae biodegradability in the presence of pig manure was related
387 to other factors, such as the addition of specific microbes within the pig manure able to
388 disrupt algal cell wall. Furthermore, this statement would also explain why no significant
389 improvement in methane yield was observed during manure and algae residues co-digestion
390 trials where the algal cell wall had been disrupted by the pre-treatment technique.
391 In this regard, *Scenedesmus* sp. cell wall has been described as a rigid wall of cellulose and
392 hemicellulose, which together with the sporopollenin-like biopolymer provides great
393 resistance to enzymatic degradation (Mendez et al., 2014; Mussgnug et al., 2010). Lu et al.
394 (2013) reported the occurrence of *Lactobacillus* and *Clostridia* in fresh pig manure,
395 independent of pig age and diet, while other studies have shown that *Lactobacillus* and
396 *Clostridia* are very effective at degrading cellulosic organic matter (Calderon Santoyo et al.,
397 2003; Li & Liu, 2012; Mussatto et al., 2008; Sethi & Scharf, 2001).
398
399 **3.4. An integrated biorefinery approach to treatment of pig manure and algae**
400 Fig. 4A presents a high-level flow diagram for an integrated biorefinery, which combines
401 manure treatment and algae cultivated onsite using supernatant from digested pig manure.
402 Cultivated algae may be directed for biogas production or processed to extract valuable co-
403 products with the algae residues recycled for biogas production through anaerobic digestion.
404 Biorefinery final configuration will be influenced by two main factors: (i) the cost (capital
405 investment and operating expenses) of the extraction process and the revenue obtained from
406 the sale or use of co-products; and (ii) the biogas production. To assess the potential for
407 methane production, B* (defined as the maximum methane production per gram VS of pig
408 manure entering the system) was used to compare the different scenarios considered in this

409 study (Fig. 4). Using B* is more suitable than B_0, since the introduction of algae into the
410 manure digester will reduce B_0 of the mixture, as the B_0 of algae is lower than the B_0 of pig
411 manure, although it will increase the digester methane production due to the additional VS
412 (organic loading rate increase). The system's calculation base was pig manure (q_{manure}) with
413 characteristics similar to that of the manure used in this study, i.e. a VS concentration of 25 g
414 VS L^{-1} and an ammonium concentration in the AD effluent of 1.0 g NH_4^+-N L^{-1}. The typical
415 growth yield of the algae used in the present study is estimated to be 12.5 kg VS kg^{-1} NH_4^+-
416 $N_{removed}$ (Rusten & Sahu, 2011; Uggetti et al., 2014), while other parameters used to develop
417 Fig. 4B were obtained from results in the present study. Considering the aforementioned
418 values, a reasonable maximum algae production capacity ($q_{algae,max}$) was estimated at 0.5 kg
419 VS kg^{-1} VS of manure entering the system. Nonetheless, the amount of algae recovered
420 (q_{algae}) would be influenced by the efficiency of both the algae cultivation system and the
421 harvesting system.
422
423 Fig. 4B shows the progressive improvement of B* in relation to algae recovery efficiency
424 ($q_{algae}/q_{algae,max}$); illustrating the extra methane production from adding raw and processed
425 algae into the pig manure digester (0.350 m^3 CH_4 kg^{-1} VS represents methane yield for pig
426 manure mono-digestion). Fig. 4B also demonstrates the improvement on AcoD performance
427 due to the recorded synergy between manure and raw algae. In fact, the synergy between
428 manure and raw algae shifts the optimal scenario for methane production from co-digestion
429 of lipid extracted algae to co-digestion of raw algae. However, this scenario does not
430 consider value from co-product extraction nor the processing cost. Finally, the results in Fig.
431 4B can be used together with product value calculations (energy, lipids and/or protein),
432 processing costs, market analysis and sensitivity testing to determine the highest-value
433 approach for such a biorefinery.

CONCLUSIONS

A biorefinery concept is presented for co-treatment of pig manure and algae (*Scenedesmus* sp.). Free nitrous acid pre-treatment was used on algal biomass to recover protein (0.25 $kg_{protein}$ kg^{-1} VS_{algae}) and to improve the algal lipid extraction yield from 0.14 to 0.19 kg_{lipids} kg^{-1} VS_{algae}. Co-product extraction enhanced algal methane yields from 0.163 to 0.223 m^3 CH_4 kg^{-1} VS, but reduced the mass of algae available for digestion. Synergy between manure and raw algae increased the methane yield of algae from 0.163 to 0.245 m^3 CH_4 kg^{-1} VS. Conversely, no synergies were observed between manure and processed algae co-digestion trials.

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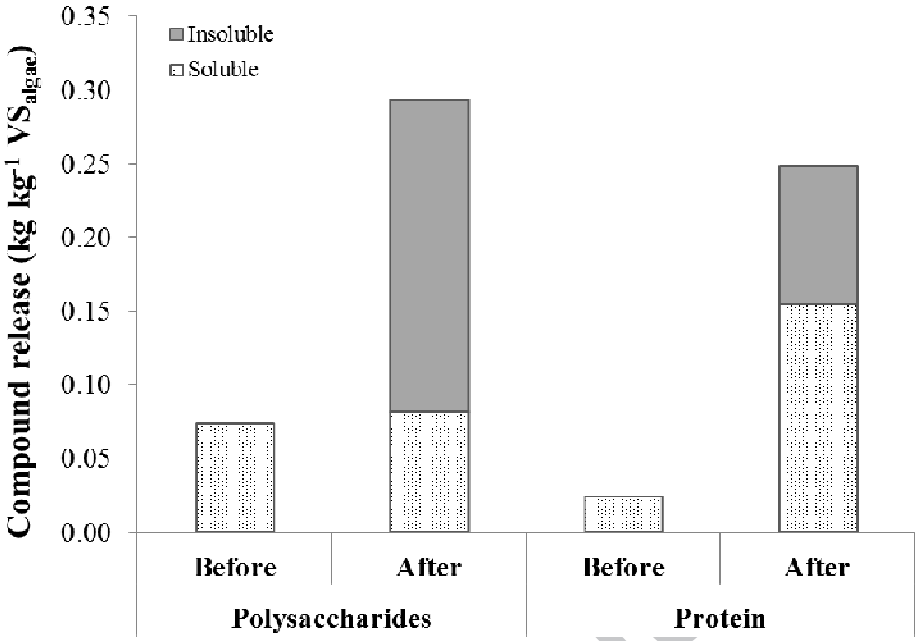

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458 **Fig. 1** Protein and polysaccharides release, on VS-basis, in the supernatant of the algal

459 suspension before and after raw algal biomass FNA pre-treatment. Results in g L^{-1} are shown

460 in Fig. SII of supplementary data.

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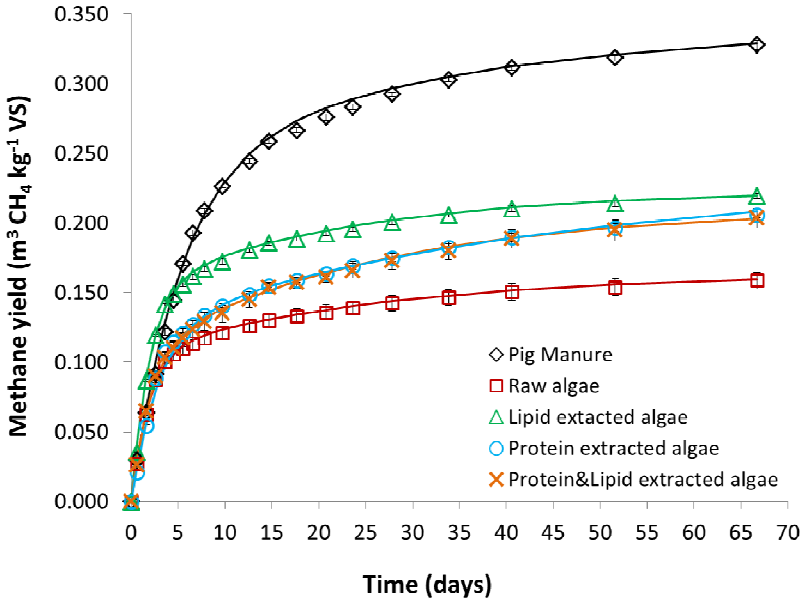

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466 **Fig. 2** Cumulative specific methane production in time of mono-digestion BMPs (symbols)

467 and their corresponding modelled profile (solid lines).

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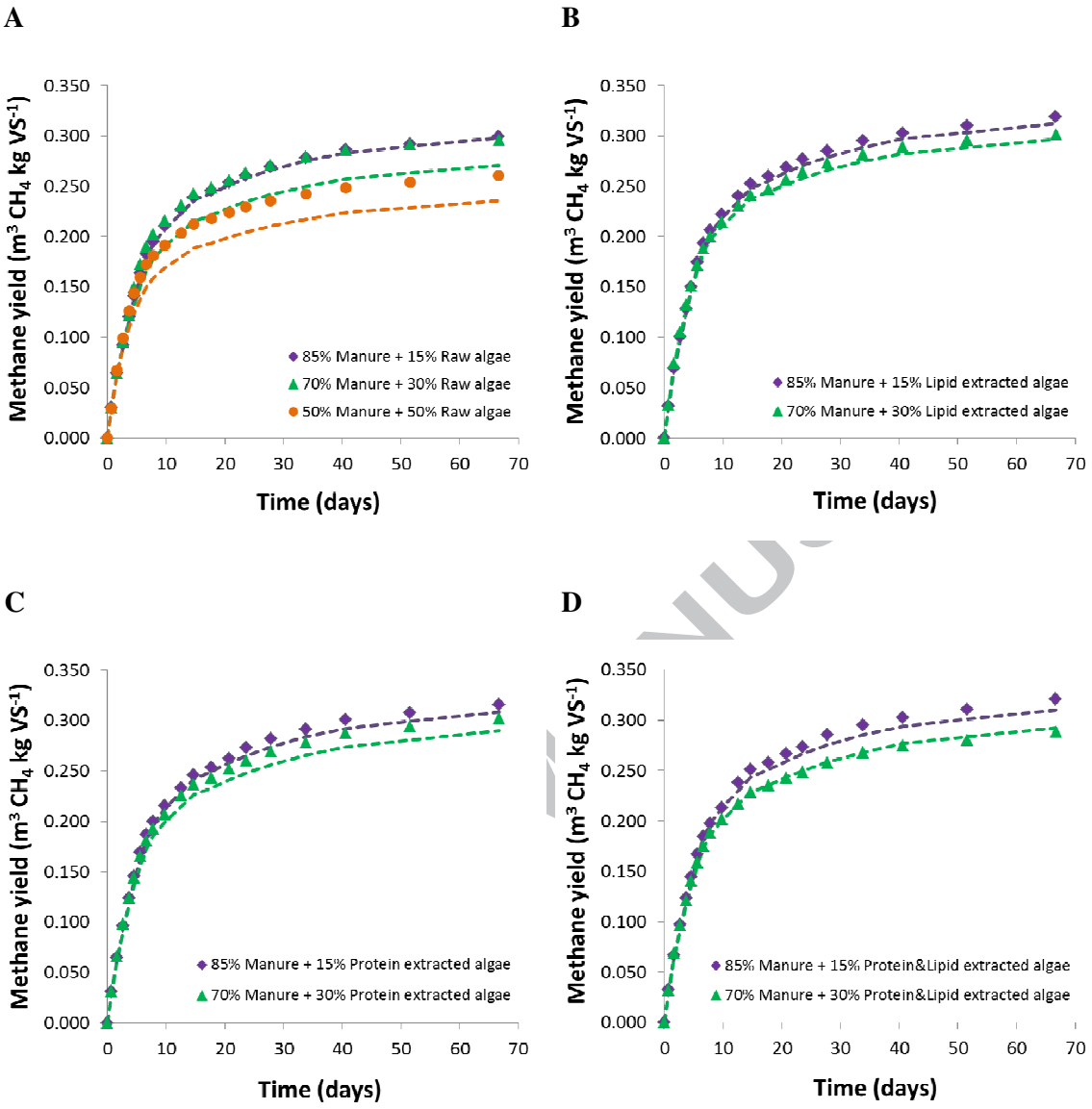

472 **Fig. 3** Experimental and theoretical methane production over time for co-digestion of (A) pig
473 manure and raw algae, (B) pig manure and lipid extracted algae, (C) pig manure and protein
474 extracted algae, and (D) pig manure and protein & lipid extracted algae. Symbols (♦,▲,●)
475 show experimental measurements and dashed lines the theoretical profile.

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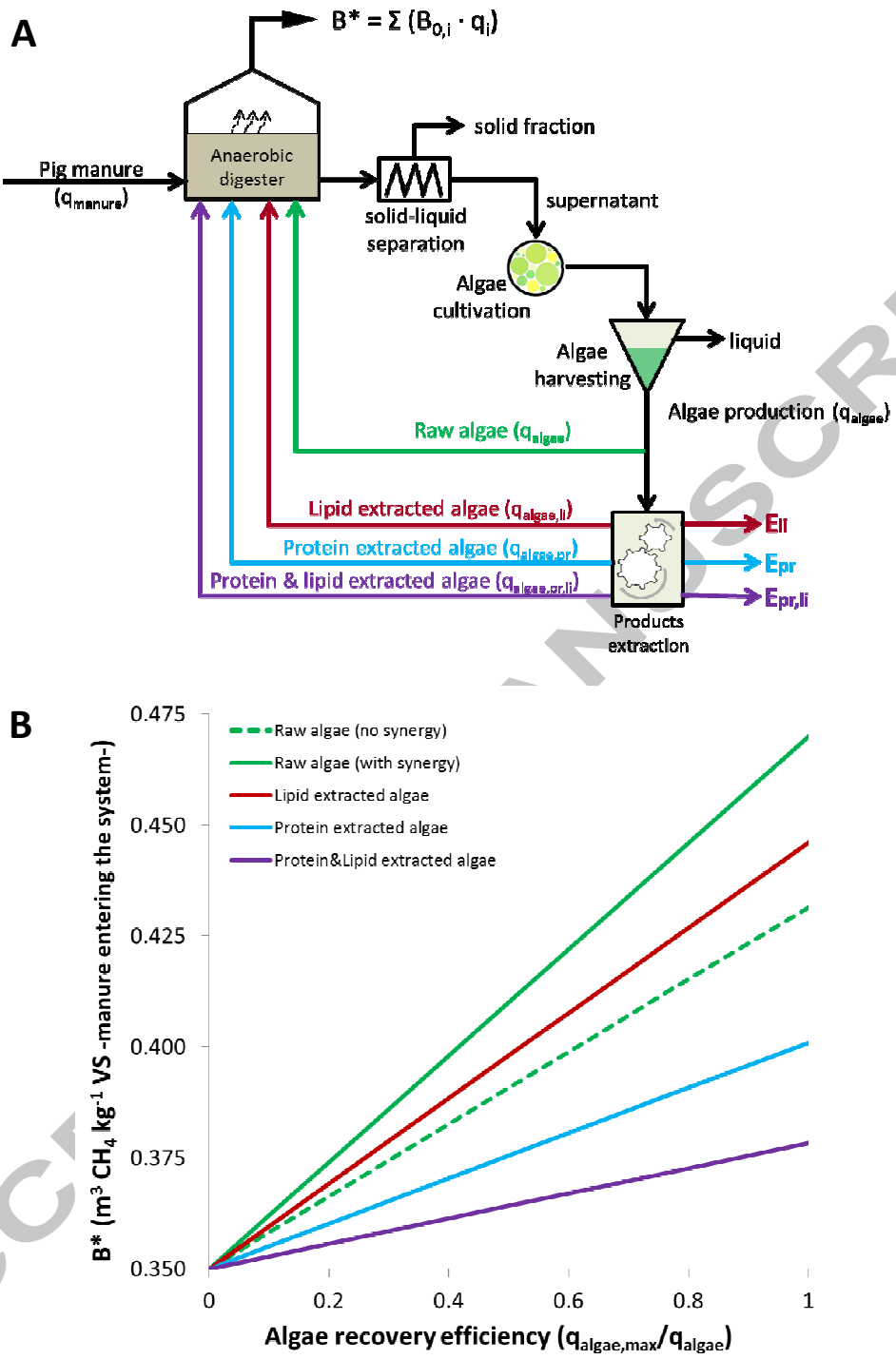

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481 **Fig. 4** (A) Flow diagram of the integrated manure and algae biorefinery considered in this

482 study, (B) evolution of B* in relation to algae recovery efficiency in the biorefinery displayed

483 in Fig. 4A

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485 **Table 1.** Chemical characterisation of the substrates under study

Parameter	Units	Pig manure	Raw Algae	Lipid extracted algae	Protein extracted algae	Protein & lipid extracted algae
TS	g kg^{-1}	34.6 ± 1.9	68.6 ± 1.5	56.0 ± 2.2	56.8 ± 2.4	54.9 ± 2.9
VS	g kg^{-1}	24.5 ± 2.1	30.0 ± 1.3	23.8 ± 2.0	25.9 ± 2.2	22.7 ± 2.6
tCOD	g O_2 kg^{-1}	33.7 ± 0.5	45.4 ± 1.3	32.0 ± 0.6	44.6 ± 0.7	36.1 ± 0.5
sCOD	g O_2 kg^{-1}	4.8 ± 0.4	5.9 ± 0.3	4.9 ± 0.1	1.7 ± 0.3	2.2 ± 0.4
VFA	g L^{-1}	1.9 ± 0.1	2.3 ± 0.1	1.7 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
Nitrite	mg N L^{-1}	-	-	-	28.9 ± 0.1	2.3 ± 0.1
Ammonium	mg N L^{-1}	624 ± 44	52.5 ± 1.8	16.0 ± 0.3	15.1 ± 0.2	4.1 ± 0.5
Phosphate	mg P L^{-1}	49.6 ± 3.4	18.4 ± 0.5	10.5 ± 0.1	37.8 ± 0.6	20.9 ± 0.5
B_0/f	m^3 CH_4 kg^{-1} VS	0.480	0.532	0.469	0.602	0.557

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490 **Table 2** Model parameter outputs for the mono-digestion BMP tests.

	$k_{hyd,fast}$ (d^{-1})	$k_{hyd,slow}$ (d^{-1})	f_{fast} (-)	f_{slow} (-)	f_{total}* (-)
Manure	0.19 ± 0.01	0.025 ± 0.005	0.50 ± 0.01	0.23 ± 0.03	0.74 ± 0.04
Raw algae	0.54 ± 0.01	0.037 ± 0.010	0.20 ± 0.01	0.11 ± 0.01	0.31 ± 0.02
Lipid extracted algae	0.50 ± 0.02	0.043 ± 0.009	0.32 ± 0.01	0.15 ± 0.01	0.48 ± 0.02
Protein extracted algae	0.39 ± 0.02	0.028 ± 0.009	0.20 ± 0.02	0.17 ± 0.02	0.37 ± 0.04
Protein + lipid extracted algae	0.50 ± 0.05	0.038 ± 0.005	0.19 ± 0.01	0.19 ± 0.01	0.38 ± 0.01

491 * $f_{total} = f_{fast} + f_{slow}$

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496 **Table 3** Comparison between the algae methane yield (B_0) and the algae methane yield per

497 gram VS of original raw algae feed (B').

	Algae methane yield (B_0)		Overall methane yield (B')		Co-product extraction
	Yield (m^3 CH_4 kg^{-1} VS)	Increase (%)	Yield (m^3 CH_4 kg^{-1} VS)	Increase (%)	Yield (kg kg^{-1} VS algae)
Raw algae	0.163	-	0.163	-	-
Lipid extracted algae	0.223	37%	0.192	18%	0.14 (lipids)
Protein extracted algae	0.222	36%	0.102	-38%	0.29 (carbohydrates) 0.25 (protein)
Protein & lipid extracted algae	0.211	29%	0.057	-65%	0.29 (carbohydrates) 0.25 (protein) 0.19 (lipids)

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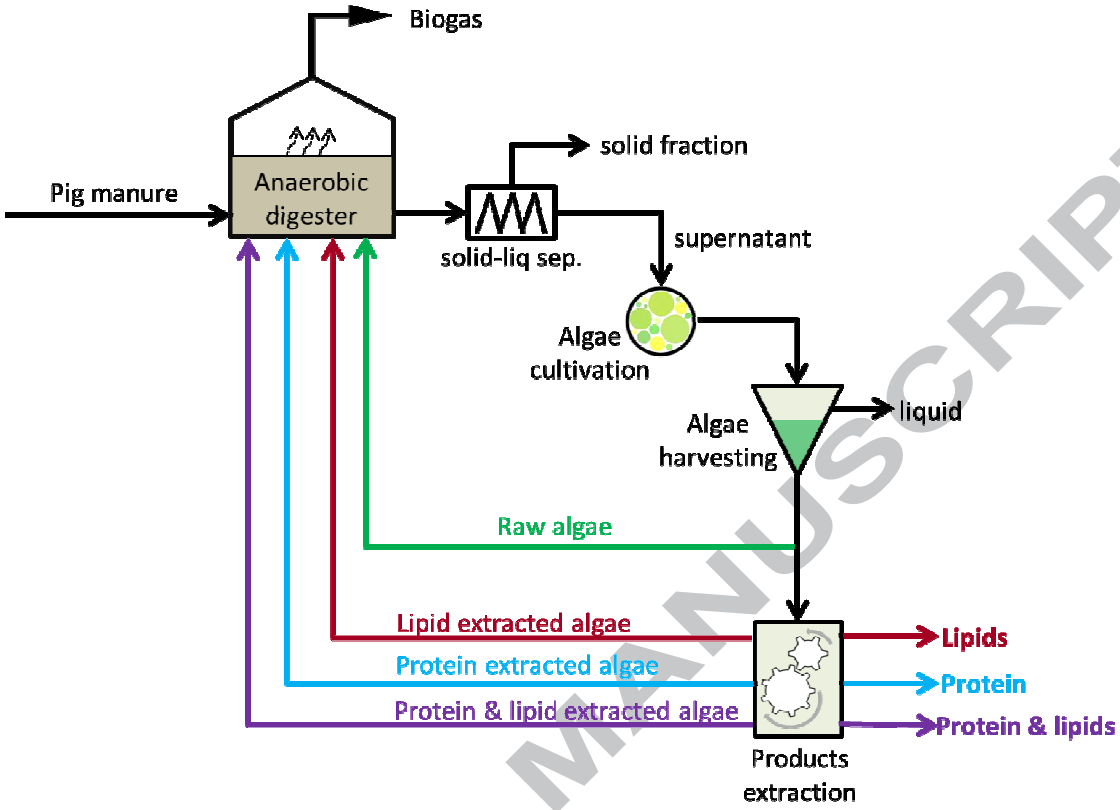
502 **GRAPHICAL ABSTRACT**



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* Pig manure was anaerobically co-digested with raw and processed algae.

* Processing increased algae biodegradability but not its degradation rate.

* Synergy between raw algae and pig manure increased methane yield of the mixture.

* There was no significant synergy between processed algae and pig manure.

* Concept was presented for a combined biorefinery processing pig manure and algae.