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Co-digestion of microalga-bacteria biomass with papaya waste for methane production

Glenda Cea-Barcia, Jaime Pérez and Germán Buitrón

ABSTRACT

The anaerobic co-digestion of microalga-bacteria biomass and papaya waste (MAB/PW) was evaluated under semi-continuous conditions. Microalgae-bacteria biomass was obtained from a high rate algal pond fed with municipal wastewater and artificially illuminated. The co-digestion of MAB/PW was evaluated using a 1:1 (w/w) ratio and an organic loading rate of 1.1 ± 0.1 g COD/L/d. Enzymatic activity assays of papain were performed in the feeding to determine the activity of this enzyme in the substrate mixture. A methane yield of 0.55 L CH_4/gVS and 68% of total volatile solid removal were observed. The volumetric productivity was 0.30 ± 0.03 L CH_4/L/d with a methane content of 71%. It was observed that papaya waste was a suitable co-substrate because it maintained a low ammonium concentration, decreasing the risk of inhibition due to ammonia and then increasing the methane yield of the microalgae-bacteria biomass compared to the biomass alone. The pretreatment effect by the addition of papaya waste on the microalgae-bacteria biomass was supported by the papain activity remaining in the substrate.

Key words | co-digestion, methane, microalgae, papain, pretreatment

Glenda Cea-Barcia
Jaime Pérez
Germán Buitrón (corresponding author)
Laboratory for Research on Advanced Processes for Water Treatment, Academic Unit Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México,
Blvd. Juriquilla 3001, Querétaro 76230,
México
E-mail: *gbuitronm@iingen.unam.mx*

INTRODUCTION

Microalgae feedstock appears as a promising alternative to CH_4 production because of the high lipids and carbohydrates accumulation during starvation as well as the high biomass productivity (Bahadar & Bilal Khan 2013). Currently, the use of microalgae-bacteria systems for wastewater treatment is particularly attractive because of their ability to produce inexpensive O_2, to remove nutrients, pathogens, and heavy metals, and to fix CO_2 during a photoautotrophic process (Arcila & Buitrón 2016). That represents a considerable gain in the carbon available for CH_4 production compared to classic aerobic processes and an inexpensive alternative to biomass production (Zamalloa *et al.* 2011; Chisti 2013).

The theoretical methane yield for different microalgae species varies between 0.47–0.80 L CH_4/g VS depending on the composition of the microalgal biomass (Sialve *et al.* 2009). However, experimental studies have shown lower methane yields (MY) ranging from 0.09 to 0.45 L CH_4/g VS, depending on the species and culture conditions (Golueke & Oswald 1959; Sialve *et al.* 2009; Alzate *et al.* 2012). Because of the cellulose cell wall of microalgae, the hydrolysis of the macromolecules present in the microalgae structure can be difficult, lowering the MY. The experimental MY can be increased by a pretreatment step to disrupt the microalgae cell wall before anaerobic digestion. However, the excessive energy input to maximize the methane conversion might negatively impact the feasibility of this technology.

Co-digestion of organic substrates has been described as an attractive strategy to optimize methane production (Mata-Alvarez *et al.* 2014). The addition of highly biodegradable substrates increases the organic load and supplies additional nutrients, which can attenuate the inhibition that would occur during digestion of the individual substrate. For example, Yen & Brune (2007) reported that the addition of waste paper to a mixture of *Scenedesmus* and *Chlorella* resulted in an improved MY due to an increased cellulase activity. Park & Li (2012) achieved an increase in the organic loading rate (OLR) up to 3 g VS/L d with a specific MY of 0.54 L CH_4/g VS in the anaerobic co-digestion of algae biomass with lipid waste. In contrast, combining swine manure and microalgal biomass, both with high nitrogen contents, did not result in significant MY improvement (González-Fernández *et al.* 2011). The best co-substrate for microalgae anaerobic co-digestion should have a high carbon-to-nitrogen ratio to minimize the inhibitory effects

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of the ammonia released. Reducing ammonia inhibition is a key factor in the anaerobic digestion of microalgae (Vargas *et al.* 2016). Fruit wastes can be an appropriate co-substrate for microalgae because of the high biodegradability and carbon-to-nitrogen ratio (Ge *et al.* 2014). Among different fruit wastes, papaya waste is an interesting alternative due to the presence of the papain enzyme. Previously, papaya waste was proven to have high biodegradability and to be suitable to be used even in the composting process (Lim *et al.* 2011). Raw papain consists of a mixture of carbohydrates and enzymes, of which the major enzyme is the cysteine protease papain. Papain digests most protein substrates more extensively than the pancreatic proteases. Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides (Asbóth & Polgár 1977; Azarkan *et al.* 2003). Horst *et al.* (2012) reported that the treatment of diatom *Phaeodactylum tricornutum* cells with papain facilitates lipid extraction and Kose & Oncel (2015) reported an increase in the digestibility of *Chlorella vulgaris* treated with pancreatin enzyme.

In this study, the anaerobic co-digestion of microalga-bacteria biomass, generated during municipal wastewater treatment, and papaya waste was evaluated under semi-continuous conditions. The performance of the reactor regarding the methane yield and the methane production rate were evaluated, assessing the effect of papain on microalgae-bacteria biomass.

METHODOLOGY

Microalga-bacteria biomass

Microalgae-bacteria aggregates were generated in a 50 L high rate algal pond (HRAP) operated with 10 days of hydraulic residence time (HRT) and artificially illuminated as described by Arcila & Buitrón (2016). Primary municipal wastewater from a wastewater treatment plant was used as the substrate. Under these conditions, the growth of agglomerates formed by Chlorophyta (mainly *Stigeoclonium* and *Scenedesmus*), diatoms and bacteria were developed (Figure 1). The effluent of the HRAP was settled. The microalgae-bacteria biomass was harvested from the settler tank and further concentrated to feed the anaerobic reactor.

Papaya waste

Papaya waste (includes skin, pulp and seed) was collected from the fruit and vegetable market. The waste was sliced (into pieces of about 0.5 cm of diameter) in a crusher equipped with stainless steel rotating blades. The waste was mixed thoroughly and then stored at −20 °C. Papaya waste batches were brought to room temperature and then mixed with microalgae-bacteria biomass to feed the reactor.

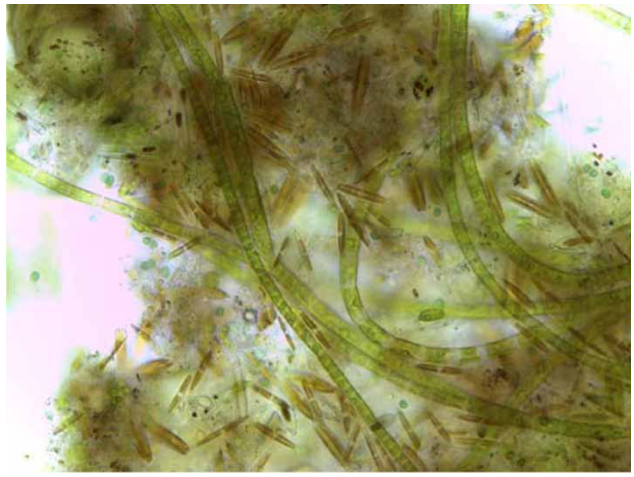

Figure 1 | Microscopic image of the microalgae-bacteria biomass cultivated in wastewater (magnification: 400X).

Anaerobic reactor and inoculum

The anaerobic reactor was fed with two microalga-bacteria to papaya waste (w/w) ratios (MAB/PW): 1:1 and 1:4, reaching a mean concentration for the mixture of 23 ± 1.7 gTS/L and 18.7 ± 1.5 gTVS/L. The 1:4 ratio was used at the start-up stage (first 20 days of operation) and the 1:1 ratio at the operational stage. Co-digestion was performed in a stirred lab-scale reactor of 5 L. The entire reactor was covered to reduce light penetration and hence prevent microalgae growth. The temperature was regulated at 35 °C and pH was monitored but not controlled. The produced biogas volume was continuously quantified (Ritter Drum-type Gas Meter TG-05). The reactor was fed twelve times per day just after the extraction of the digested sludge and was operated with 31 days of HRT and an OLR of 1.1 ± 0.1 gCOD/L/d. Once a week, the total and soluble phase of the feed and the digestate were characterized. The reactor was inoculated with granular anaerobic sludge from a brewery wastewater treatment plant operating at 35 °C. The initial solid content was 27 gTS/L and 19 gVS/L. The maximum specific methane activity of the sludge was 17 mLCH_4/gVS/d. Before the co-digestion assay, the inoculum was kept under endogenous conditions for 14 days to reduce non-specific biogas generation.

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Enzymatic assay of papain

Enzymatic assays of papain (EC 3.4.22.2) were performed to determine the presence of papain in the feed mixture and its possible effect on microalgae-bacteria biomass. Assays were conducted according to the titrimetric rate determination method proposed by the Sigma-Aldrich Quality Control test procedure. The titrimetric analysis consists of determining the number of moles of reagent (titrant), required to react quantitatively with the substance being determined. In this case, the reaction is according to Equation (1):

$$\mathrm{BAEE} + \mathrm{H_2O} \xrightarrow{\text{Papain}} \mathrm{N}\alpha\text{–Benzoyl} - \mathrm{L} - \text{Arginine} + \text{Ethanol} \quad (1)$$

where one unit of papain hydrolyze 1.0 μmole of BAEE (Nα-Benzoyl-L-Arginine Ethyl Ester) per minute at pH 6.2 at 25 °C and the titrant (NaOH) reacts with the hydrolysis product (Nα-Benzoyl-L-Arginine). The calculation was made following Equation (2):

$$\frac{Units}{mL\ enzyme} = \frac{0.05N1000}{TV} \quad (2)$$

where 0.05 is the volume in milliliters of NaOH used to maintain the pH at 6.2., N is the normality of NaOH, 1,000 is the conversion factor from millimoles to micromoles, T is the time in minutes required to maintain the pH at 6.2 of a 50 μl aliquot sample and V is the volume in milliliter of enzyme used.

One mL of either papaya waste, feed mixture or microalgae-bacteria suspension were used as enzyme preparation. The results are expressed in Units/mL enzyme or Units/mg TVS. Additionally, solubilization kinetics of COD, carbohydrates and proteins of the feed mixture were analyzed by measuring the respective concentrations in the soluble phase during 7 days of storage at 4 °C to determine the effect of the papain on the solid phase (microalgae-bacteria biomass).

Analytical methods

Total solids (TS), total volatile solids (TVS) and total Kjeldahl nitrogen (TKN) were determined according to standard methods (APHA 2005). Total chemical oxygen demand (COD_T), soluble chemical oxygen demand (COD_S), total ammonium ($N\text{-}NH_4^+$) and total phosphate ($P\text{-}PO_4^{3-}$) were measured by a colorimetric method using Hach vials. Samples were centrifuged (600 × g, 10 min), followed by filtration at 1 μm (Whatman GF/A filter), to separate the particles from the soluble phase. The carbon dioxide and methane produced were periodically analyzed using a gas chromatograph (Agilent 6890 N) equipped with a thermal conductivity detector and a 30 m long (0.53 mm id) Carboxen 1010 Plot column. The temperature of the injection port, column and detector, were 200, 100 and 230 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 4 mL/min. Liquid samples were taken at the outlet for the analysis of volatile fatty acids (VFAs). 1 mL of sample was centrifuged at 600 g for 5 min. VFAs concentrations were determined using a chromatograph (Varian 3300) fitted with an FID detector and a 15 m long (0.53 mm id) Zebron ZB-FFAP column. Injector and detector temperatures were maintained at 190 and 210 °C, respectively. The temperature of the column was maintained at 45 °C for 1.5 min; then, it was increased to 135 °C at a rate of 8 °C/min. The carrier gas was nitrogen at 9.5 ml/min. Carbohydrate concentration was determined by the phenol-sulfuric acid method, using glucose as a standard according to Dubois *et al.* (1956), and the protein concentration was analyzed according to the Lowry *et al.* (1951) method using seroalbumin as standard.

RESULTS AND DISCUSSION

Methane production and content

The start-up of the bioreactor was carried out during the first 20 days of operation where the reactor was fed with a MAB/PW ratio of 1:4. This ratio was applied to promote the activity of methanogenic inoculum under co-digestion conditions. Subsequently, the reactor was fed with a MAB/PW ratio of 1:1. Figure 2 shows the reactor performance

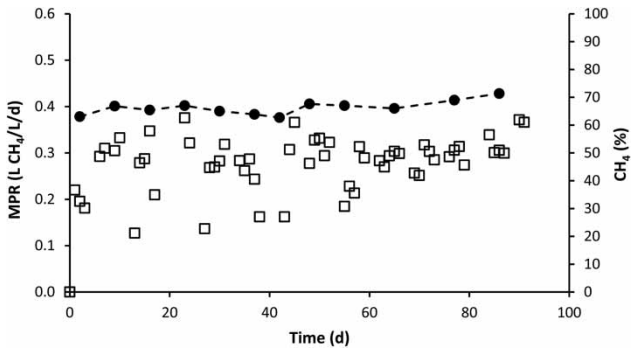

Figure 2 | MPR (squares) and methane content (circles) during the start-up of the reactor (0 to 20 days) and co-digestion period (20 to 95 days).

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during the start-up and the co-digestion operation time. The MPR, expressed in L CH_4/L·d, indicates the quantity of methane that can be produced per unit reactor working volume. The improvement of this parameter is important in maximizing the efficiency of the anaerobic digestion process. Moreover, the methane content in the biogas is related to the process efficiency. A low methane content implies some form of inhibition that decreases the methanogenic activity within the microbial consortium.

A relatively elevated coefficient of variation of 34.3% for the MPR was observed (0.27 ± 0.09 L CH_4/L/d) during the first 58 days; however, the methane content was maintained around 63%. From day 59 forward, the coefficient of variation of the MPR decreased to 10.1%, indicating the stabilization of the reactor (0.30 ± 0.03 L CH_4/L/d). For this period, the methane content in the biogas increased up to 71% (Figure 2). Those values agree with the results obtained by Xie *et al.* (2012) who reported MPR values around 0.27 L CH_4/L/d using an OLR of 1 g TSV/L/d similar to the OLR used in the present study (0.6 g TSV/L/d). MPRs as high as 0.8 L CH_4/L/d were obtained at a higher OLR (2 g TSV/L/d) and using lipids as co-digestate (Park & Li 2012).

Effect of co-digestion on methane yield and digestate characteristics

MY (L CH_4/g VS_{fed}), expresses the substrate conversion efficiency into methane. Figure 3 shows the MY during the reactor operation. When the reactor was operated with a MAB/PW ratio of 1:1, an average MY of 0.55 ± 0.07 L CH_4/g VS_{feed} was obtained. Other studies (Olsson *et al.* 2014; Caporgno *et al.* 2015) have reported MY values of

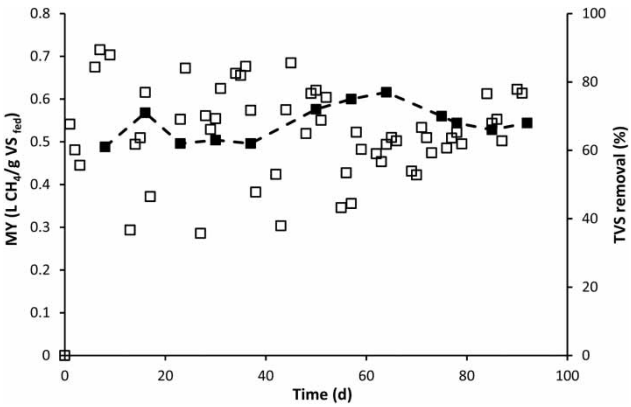

Figure 3 | MY (open squares) and TVS removal (filled squares) during the start-up of the reactor (0 to 20 days) and co-digestion period (20 to 95 days).

0.4 L CH_4/g VS_{feed} for the co-digestion of saline microalgae and lipid waste under an OLR of 2 g TSV/L/d. When the OLR was increased to 3 g TSV/L/d, the MY was also increased (0.54 L CH_4/g VS_{feed}). Moreover, co-digestion studies of microalgae and sewage sludge in batch reactors report MY between 0.3 and 0.4 L CH_4/g VS.

Figure 2 also presents the TVS removal. The TVS, COD_T and COD_S removals achieve 68%, 72% and 91%, respectively, evidencing the good reactor performance (Table 1). The results showed that about 8% of organic nitrogen has been mineralized (Table 1), which was evidenced by a decrease of TKN values in the digestate. The released of $N\text{-}NH_4^+$ in the digestate reached concentrations of 311 ± 50 mg/L. Important levels of $N\text{-}NH_4^+$ have been observed in the microalga digestate, ranging between 800 and 1,100 mg $N\text{-}NH_4^+$/L (Ras *et al.* 2011; Alzate *et al.* 2012). High levels of $N\text{-}NH_4^+$ increase the $N\text{-}NH_3$ toxicity. That behavior is related to the high protein content of microalgae, which can vary between 20% and 65% of the dry weight (Becker 2007; Alzate *et al.* 2012). The co-digestion of microalgae with papaya waste improved the $N\text{-}NH_4^+$ profile, maintaining the reactor at a non-inhibitory level of $N\text{-}NH_4^+$.

On the other hand, the digestion of fruit wastes at a high OLR becomes difficult because of the rapid acidification, resulting in the inhibition of methanogenic activity (Mata-Alvarez *et al.* 1992). The co-digestion of microalgae with papaya waste shows a synergistic effect, decreasing the ammonium to carbon ratio due to the papaya organic matter addition and the pH control levels due to the microalgal biomass. That resulted in an improvement in the performance of the fruit wastes digestion.

Evaluation of co-digestion performance

After 95 days of operation, the reactor was only fed with papaya waste at the same operation conditions (HRT and OLR) to determine the MY of this waste (data not shown). Table 2 summarizes the MY obtained in the present study

Table 1 | Feed and digestate physicochemical parameters for the co-digestion

	Feed	Digestate
COD_T (g O_2/L)	35 ± 3.2	9.8 ± 2
COD_S (g O_2/L)	–	0.92 ± 0.1
$N\text{-}NH_4^+$ (mg/L)	13 ± 9	311 ± 50
PO_4^{3-} (mg/L)	283 ± 110	162 ± 37
TKN (mg $N\text{-}NH_4^+$/L)	1,015 ± 10	938 ± 39
pH	7.0	7.15 ± 0.3

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Table 2 | Comparison between the different MY and biodegradability percentage obtained for each substrate

Substrate	MY (L CH_4/gCOD_{fed})	Biodegradability (%)
Papaya waste	0.312	89.1
Microalga-bacteria biomass[a]	0.144	41.1
Theoretical mixture of biomasses[b]	0.205	58.6
Co-digested MAB/PW[c]	0.230	65.7

[a]Arcila and Buitrón (2016).
[b]Calculated by using the data of papaya waste and microalga-bacteria biomasses alone under the condition applied to the reactor.
[c]Experimentally obtained during co-digestion.

and compared with the MY of microalga-bacteria biomass obtained in a previous study.

The MY (regarding COD) during the reactor operation was calculated by using Equation (3):

$$MY = \sum_{0}^{n} \frac{CH_{4TP}}{COD_{TF}} \quad (3)$$

where MY is methane yield in L/gCOD_{fed}, CH_{4TP} is the total production of methane during the operation time in L, COD_{TF} is the COD_T fed in g O_2, and n is reactor operation days.

The MY values were calculated for the co-digestion period (from day 20 to day 95) and the papaya waste feeding, after day 95 (Table 2). The waste conversion efficiency or biodegradability, expressed in percentage, was calculated considering the theoretical production yield of 0.35 L CH_4/$COD_{degraded}$ at 1 atm and 0 °C. Results indicate an increase in the biodegradability of the microalga-bacteria biomass under co-digestion from 41.1 to 65.7%. That represents an increment on the MY of 59.8% (0.230 versus 0.144 L CH_4/gCOD_{fed}). Similar co-digestion improvements have already been reported when microalgae and sewage sludge were co-digested (Olsson *et al.* 2014; Caporgno *et al.* 2015). It is interesting to note the synergistic effect of co-digestion: a higher biodegradability was observed in the experimental reactor than the one expected from theoretical calculations of the mixture of microalga-bacteria biomass with the papaya waste. That indicates that papaya waste improves the biodegradability.

The evidence that the co-digestion improves the MY of the microalgae-bacteria biomass, increasing its biodegradability, suggested a possible effect of pre-treatment by the papaya waste on the microalgae-bacteria biomass. On the other hand, the co-digestion improved the performance of the system due to a decrease in the concentration of VFAs of the digestate (101 mg acetate/L papaya waste in digestate against 5 mg acetate/L microalgae-bacteria/papaya waste in digestate), becoming an alternative to operating a higher OLR without an acidification of the system.

Effect of papaya waste on the feed mixture

The presence of papain activity in the feed mixture was determined to evaluate the effect of papaya waste on microalga-bacteria biomass degradability. Figure 4 shows the enzyme activity measured in the raw papaya waste, in the feed mixture at time 0 and after 3 days of storage at 4 °C. As a control sample, the activity was measured in the microalgae-biomass suspension to determine potential interferences of other substrates with the methodology. When the feed mixture is prepared, 80% of the initial enzyme activity was lost. Nevertheless, after three days of storage at 4 °C, the papain activity of the feed mixture remains (loss of 29% of activity). These results evidence the presence of active papain in the feed mixture and suggest a possible hydrolysis of the enzyme on the microalgae-bacteria biomass. The hydrolysis kinetics of the feed mixture was performed by measuring the COD_S, carbohydrates and proteins of the soluble phase over time (Figure 5). Proteins concentrations measured by Lowry protein assay increase during the incubation period. The Lowry protein assay can measure protein during enzyme fractionations, small peptides and free amino acids such as tyrosine and tryptophan (Lowry *et al.* 1951). However, the COD_S and carbohydrates kinetics are inconclusive. The carbohydrates consumption kinetics could be the result of the solubilization and consumption of carbohydrates present in the solid and liquid phase respectively, and COD_S kinetics could be the result of the solubilization and consumption of different macromolecules.

The main structures of the biomass that might be hydrolyzed by papain are microalgae cell walls and bacteria aggregates. Rupture of cell walls can result in the release

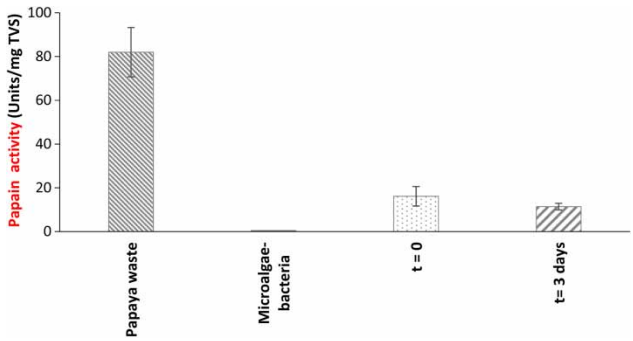

Figure 4 | Papain activities of papaya waste and feed mixture measured by titrimetric rate determination method using BAEE as substrate.

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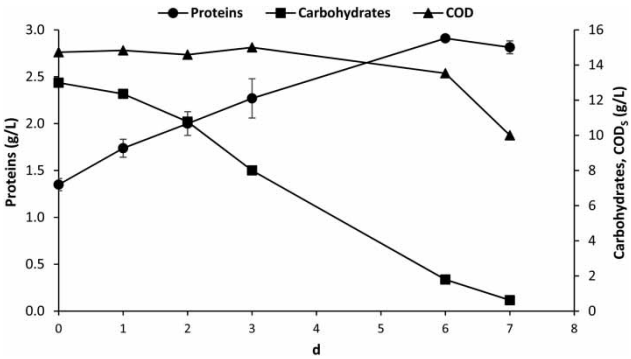

Figure 5 | COD_S, carbohydrates and proteins concentrations in the soluble phase of the feed mixture over seven days of storage at 4 °C.

into the medium of other cellular components, such as intracellular proteins, carbohydrates, chlorophyll and DNA. The microalgae from the Chlorophyceae class possess cell walls with assemblages of polymers containing hyp-rich glycoproteins and arabinogalactan proteins (Domozych *et al.* 2012). Papain can interact with the proteins contained in these structures.

CONCLUSIONS

Anaerobic co-digestion of microalgae-bacteria biomass and papaya waste was evaluated. Co-digestion improved the biodegradability of the microalga-bacteria biomass and overall performance of the system indicated by the increment of the MY of the microalgae-bacteria biomass from 0.144 to 0.230 L CH_4/gCOD_{fed} (0.55 ± 0.07 L CH_4/g VS_{feed}). The co-digestion poised the ammonium concentration of the microalga-bacteria biomass and decreased the risk of acidification of papaya waste. Furthermore, it was possible to determine the presence of papain activity in the feed mixture and its effect on the solid phase, showing solubilization of proteins. The results demonstrate that the co-digestion of microalga-bacteria biomass with a residue containing enzymatic activity, such as the papaya waste, is an effective strategy for an efficient methane production system from microalgae. However, more research and development is required to make this technology an economically viable process.

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