Anaerobic co-digestion of microalgae *Chlorella vulgaris* and potato processing waste: effect of mixing ratio, waste type and substrate to inoculum ratio

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Abstract:

This investigation aimed to evaluate the feasibility of potato processing waste (PPW) as a co-substrate for *Chlorella vulgaris* in batch biochemical methane potential (BMP) tests. Three parameters were examined: *C. vulgaris* and PPW mixing ratios (100:0, 75:25, 50:50, 25:75 and 0:100), PPW type (potato discarded parts (PPW_{dp}) and potato peel (PPW_{p})), and substrate to inoculum ratio (0.5 and 1.0 SIRs). The mixing ratio was a significant factor with higher methane yields obtained with 25:75 *C. vulgaris*: PPW. The type of PPW also affected methane yield, as *C. vulgaris* co-digested with PPW_{dp} increased methane yield by 22 – 47%, versus a 12 – 32% enhancement with PPW_{p}. Moreover, an SIR of 1.0 led to an accumulation of soluble COD, resulting in decreased methane yield relative to an SIR of 0.5. Overall, the current study showed that PPW are suitable feedstocks for co-digestion with microalgae, with the enhanced methane yields attributed to a balance C/N ratio. However, the extra nitrogen in the seed inoculum of the BMP test may have obscured the full benefits and hidden some synergistic effects. Therefore, follow-up studies should be carried out in continuous anaerobic digesters to verify the full potential of PPW as a co-digestion substrate for microalgae.

Keywords: Anaerobic co-digestion, microalgae, *Chlorella vulgaris*, potato processing waste, C/N ratio, substrate to inoculum ratio.

Abbreviation

AD	Anaerobic Digestion
ANOVA	Analysis of Variance
BBM	Bold's Basal Medium
BCA	Bicinchoninic Acid
BMP	Biochemical Methane Potential
BSA	Bovine Serum Albumin
C/N	Carbon to Nitrogen
COD_t	Total Chemical Oxygen Demand
COD_s	Soluble Chemical Oxygen Demand
DW	Dry Weight
FAN	Free Ammonia Nitrogen
HRT	Hydraulic Retention Time
OLR	Organic Loading Rate
PPW	Potato Processing Waste
PPW_{dp}	Potato Discarded Parts
PPW_p	Potato Peel
SIR	Substrate to Inoculum ratio
STP	Standard Temperature and Pressure
TS	Total Solids
VFA	Volatile Fatty Acids
VS	Volatile Solids

1. Introduction

Fossil fuels, as unsustainable and polluting sources of energy, will become depleted, therefore, alternative renewable energy sources are attracting close attention. Anaerobic digestion (AD) is a well-established biological process in which anaerobic microorganisms convert organic materials to methane rich biogas.

Microalgae are considered to be promising sustainable sources of biomass for bioenergy production, including biodiesel, bioethanol and biogas [1, 2, 3]. Further, microalgae are also being investigated as biological nutrient scrubbers in wastewater treatment systems [4, 5], presenting opportunities to utilise the resultant biomass in numerous downstream processes. Microalgae contain a range of organic macromolecules such as carbohydrates, proteins and lipids, most of which can be fermented to generate biogas via AD. Moreover, the cultivation of microalgal biomass in photobioreactors generates a biomass with a relatively consistent nitrogen composition [6]. Therefore, microalgal biomass used as a feedstock would have less variation in nutrient content between batches, and help stabilise the AD process. However, an unbalanced carbon to nitrogen (C/N) ratio can cause low methane yields and an unstable AD process when using microalgae as a mono-digestion feedstock. The optimum C/N ratios for AD range from 20/1 to 30/1, but values for microalgae have been reported from 4.65/1 to 17/1 [7, 8, 9, 10]. Co-digestion of microalgae with other carbon-rich substrates, such as waste paper and maize can balance the C/N ratios and increase methane yields [6, 8, 11]. Animal manures have also been introduced for co-digestion with microalgae, but these types of feedstock are unpopular either due to their relatively low C/N ratios or because ultimate methane yields were not significantly improved [9, 10]. Besides the above co-substrates, food waste (e.g. kitchen waste) can also be an optimised co-substrate due to its high biodegradability [12]. However, kitchen waste is a mixture of different waste types [12]. The use of a specific food waste type has not been fully investigated.

The potato is among the top five global food crops, with production levels reaching 377 million tonnes in 2016 [13], with around 60% of harvested potatoes being processed into industries [14]. Potato processing waste (PPW) consists of discarded parts (PPW_{dp}) (whole or cut potatoes discarded due to size, blemishes or failing to meet the standard quality for human food) and potato peel (PPW_p) [15, 16]. The typical manufacturing losses are approximately 8% of the total potato weight [14], which accounts for around 18.10 million tonnes of waste generated in 2016. There is growing interest in strategies to treat these waste streams, particularly as they represent zero value waste from the manufacturing process with associated removal and disposal costs [17]. Further, potatoes contain high levels of nutrients and decomposing potato waste has the potential to contaminate both ground and surface water [18]. Given these factors, there is a need for an integrated, environmentally-friendly solution for PPW treatment. The carbohydrate content of PPW is typically around 55.6 – 68.7% of dry weight, primarily as starch [15, 17, 19], which is easily broken down into monomers or simple sugars [20]. The C/N ratios of potato waste ranges from 12.1/1 to 30.0/1 [15, 21], and therefore it is a promising feedstock for anaerobic co-digestion with other low carbon substrates [22, 23]. Since mono-digestion of microalgae does not show good digestion performance, co-digestion with PPW could be a promising way to enhance methane yields. However, there is little information available about the anaerobic co-digestion of microalgae with PPW.

Biochemical methane potential (BMP) tests have been widely used in previous studies to quantify the production rates and yields of biogas, and also to characterise the biodegradability of various substrates [24, 25, 26]. The substrate to inoculum ratio (SIR) is crucial during BMP tests as it ensures a balance of the bacteria and archaea that carry out the acidification and methanogenic processes [27, 28, 29].

Therefore, the aim of this study was to investigate the feasibility of PPW as a co-substrate for co-digestion with the microalga *Chlorella vulgaris* in batch BMP tests. The effect of mixing ratios between *C. vulgaris* and PPW on methane yield was investigated. In addition, the influence of PPW type (discarded parts (PPW_{dp}) and peel (PPW_{p})) and SIR were also evaluated. Moreover, since PPW is a new co-substrate for co-digestion with microalgae, apart from the investigation of above operating parameters, further efforts are needed to describe the kinetics of the co-digestion process as well as the synergistic effects caused by microalgae co-digestion with PPW. Lastly, the new knowledge yielded from this work could potentially provide useful information for the development of an economically viable microalgae co-digestion process.

2. Materials and methods

2.1 Experimental design

Since the aim of this work was to investigate the potential of PPW as a co-digestion substrate with microalgae, microalgae was mixed with PPW in different ratios to determine the optimum mixtures for successful co-digestion as well as to improve the chemical properties of microalgae as an AD feedstock. Therefore, the current study utilised a 5×2×2 mixed factorial design, including one within-independent variable, i.e. the mixing ratios between *C. vulgaris* and PPW, with five levels: 100:0 (i.e. mono-digestion of microalgae), 75:25, 50:50, 25:75 and 0:100 (i.e. mono-digestion of PPW) on the basis of volatile solids (VS). The proportions were selected based on previous microalgae co-digestion studies [9, 11]. The first between-independent variable was PPW type, either PPW_{dp} or PPW_{p}. The second between-independent variable was the substrate to inoculum ratio (SIR), where substrate and anaerobic inoculum were mixed to achieve a ratio of 0.5:1 or 1:1 on the basis of VS [30, 31]. A summary of the experimental design is shown in Table 1. The dependent variables were biomethane potential (experimental final methane yield and BMP kinetic results), and process stability measured as concentrations of soluble COD (COD_s) and free ammonia nitrogen (FAN).

Table 1. Summary and coding of the experimental design for microalgae co-digestion trials, VS = volatile solids. Treatment coding: D = potato discarded parts (PPW_{dp}), P = potato peel (PPW_p), 1-5 corresponds to the mixing ratio, A = SIR of 1.0, B = SIR of 0.5.

***C. vulgaris* : PPW (based on VS)**									
100:0		**75:25**		**50:50**		**25:75**		**0:100**	
D1A	P1A	D2A	P2A	D3A	P3A	D4A	P4A	D5A	P5A
D1B	P1B	D2B	P2B	D3B	P3B	D4B	P4B	D5B	P5B

2.2 Microalgae and potato processing waste

The *C. vulgaris* strain (CCAP 211/63) was obtained from the Culture Collection of Algae and Protozoa, UK. *C. vulgaris* was cultured in Bold's Basal Medium (BBM) in 10 L Nalgene carboys [32, 33] at 19 ℃ under artificial light (a mean luminance of 2500 Lux) with a 16:8 light dark photoperiod. The cells were harvested during stationary phase (after 35 days of culture) and concentrated by sedimentation and washed by centrifugation (3392×g for 10 minutes) and re-suspension in 2 L of distilled water to remove the culture medium.

The simulated PPW was made in two groups: PPW_{dp} and PPW_p. The waste, with 2 L of distilled water, was homogenized using a kitchen blender.

The feedstocks were characterised by their total solids (TS), volatile solids (VS), total chemical oxygen demand (COD_t), carbohydrate and proteins content, as well as carbon (C) and nitrogen (N) content are summarised in Table 2.

C. vulgaris had a C/N ratio of 6.43/1 (Table 2). The addition of PPW_{dp} enhanced the C/N ratio to within a range of 8.03/1 to 22.77/1. The C/N ratios were also increased by adding

PPW_p (ranging from 7.99/1 to 19.86/1). For these co-digestion mixtures, the carbohydrates content increased whereas the protein content decreased.

Table 2. Characterisation of *Chlorella vulgaris*, potato discarded parts (PPW_{dp}) and peel (PPW_p), and co-digestion mixtures. TS = total solids, VS = volatile solids, COD = chemical oxygen demand, C/N = carbon to nitrogen ratio.

	TS (g/L)	**VS (% TS)**	**COD/VS**	**Proteins (% VS)**	**Carbohydrates (% VS)**	**C/N**
Chlorella	3.6±0.6[b]	96.96±1.20	1.6±0.1	35.94±3.09	20.01±3.32	6.43
PPWdp	11.5±8.2[a]	90.07±5.82	1.8±0.5	16.41±2.28	76.99±1.34	40.78
PPWp	8.1±5.9	88.21±5.30	1.6±0.2	17.82±1.32	63.32±10.62	28.59
25% PPWdp	4.6±1.3	94.95±0.99	1.5±0.1	27.14±3.30	44.43±2.52	8.03
50% PPWdp	5.4±1.8	92.67±2.62	1.5±0.0	25.71±3.12	51.67±3.44	11.24
75% PPWdp	8.8±5.1	91.48±3.67	1.5±0.2	21.03±1.23	67.15±2.31	22.77
25% PPWp	3.9±1.1	93.86±0.91	1.5±0.1	25.16±2.83	42.38±4.33	7.99
50% PPWp	4.1±1.2	90.02±3.86	1.4±0.2	23.61±1.50	51.78±5.22	11.19
75% PPWp	6.3±3.5	88.68±5.28	1.3±0.2	21.02±0.05	53.96±8.98	19.86

[a] the large variations in TS content was due to different amounts of raw substrate having been diluted with distilled water

[b] Mean ± standard deviation, n = 4.

The anaerobic seed inoculum was collected from a manure-based farm anaerobic digester located at Cockle Park Farm, Northumberland, UK (lat: 55.215024, long: -1.6846638). The seed inoculum had a TS of 15.2 ± 0.1 g/L, a VS/TS of 63.00 ± 1.00%, a pH of 7.96 and high concentrations of NH_4^+-N (4100 ±141 mg/L) and FAN 433 ± 15 mg/L.

2.3 Biochemical methane potential (BMP) test

Batch BMP tests were performed based on the guidelines recommended by [34], using glass bottles with a capacity of 160 mL and closed with butyl rubber seals and aluminium caps. An addition of 10% v/v (9 mL) of $NaHCO_3$ (5g/L) solution was made to each test bottle to maintain the pH value [35]. Quantities were calculated to obtain 90 mL of final liquid volume and to allow 43.75% of headspace. The biogas production was measured volumetrically, and on each measurement day, a 10 mL syringe was connected to the top of the BMP bottle to measure the daily biogas production before measuring the methane percentage, and also to make sure that internal pressure was equal to atmospheric pressure [36]. Each BMP assay was performed in triplicate for each individual substrate in order to identify the biogas production level and percentage of methane, and a blank test containing only inoculum was also performed and subtracted from the treatment bottles. The volume of methane was calculated under STP conditions (0 ℃, 1atm), and detailed methods can be found in Supplementary Information.

2.4 Analytical methods

TS and VS were determined according to the APHA standard methods [37]. The total chemical oxygen demand (COD_t) of all feedstocks and soluble chemical oxygen demand (COD_s) at the end of digestion were measured using Merck Millipore COD cell test kits (VWR, UK). Concentrations of ammonium nitrogen (NH_4^+-N) were measured using Merck Ammonium cell test kits (VWR, UK). To obtain the soluble phase, samples were centrifuged at 3392×g for 10 mins and then filtered using a 0.2 μm nylon filter (VWR, UK). Carbohydrate content was measured via the phenol-sulfuric acid method, using D-glucose as a standard [38]. Protein content was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Sciencitific Pierce, 23227), with bovine serum albumin as the standard. FAN was calculated based on Equation (1) [39].

$$\text{FAN} = \frac{NH_4^+ - N \times 10^{pH}}{e^{6344/(273+T)} + 10^{pH}} \quad (1)$$

For elemental analysis, *C. vulgaris*, PPW_{dp}, PPW_p and all mixed feedstocks were oven dried at 60 ℃ until the weight was constant, and analysed for carbon (C) and nitrogen (N) using an Elementar VarioMAX CNS analyzer. The methane composition of the biogas was determined by a GC-FID instrument (Carlo-Erba 5160 GC) with hydrogen as the carrier gas and the injector at 150 ℃ and FID at 300 ℃. Methane standards (10% or 80% CH_4 balanced with CO_2; Scientific and Technical Gases Ltd., UK) were used in triplicate injections of 50, 40, 30, 20 and 10 µL of standard gas to make a standard curve. Triplicate injections of a 50 µL sample of biogas, taken from the headspace of the BMP bottles using a 100 µL gastight syringe (SGE, 100R-V-GT), were qualified by reference to the standard curve.

2.5 Kinetics of anaerobic digestion

The modified Gompertz equation has been used in many previous studies, and also assumes that methane production is proportional to the microbial activity which indicates the growth of microorganisms [26, 40, 41]. The kinetic data obtained from all digesters were checked for the fitness of the modified Gompertz by Equation (2):

$$M = P \times \exp\left\{-\exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

where M is the cumulative methane production (ml/gVS) at time t, P is the methane yield potential (ml/gVS), R_m is the maximum methane production rate (ml/gVS/d), λ is the duration of lag phase (d), t is the digestion time (d).

2.6 Synergistic effect

The synergistic effect is the inner reaction produced by the co-digestion of different feedstocks [26]. This effect can be calculated as Equation (3):

$$\alpha = \frac{Experimental\ Yield}{Theoretical\ Yield} \quad (3)$$

where "Experimental Yield" is the methane yield obtained from BMP test for each co-digestion mixture, "Theoretical Yield" was calculated from the sum of experimental yields of the individual substrates (mono-digestion) taking into account the mixing ratio (on the basis of VS) of each substrate contained in the final mixture. "Theoretical Yield" can be calculated as Equation (4) [42]:

$$Theoretical\ Yield = MY_{CV}\ R_{CV} + MY_{PPW} R_{PPW} \quad (4)$$

where MY_{CV} and MY_{PPW} are the experimental methane yields of mono-digestion of *C. vulgaris* or PPW (PPW_{dp} or PPW_{p}). R_{CV} and R_{PPW} refer to the mixing ratio for *C. vulgaris* and PPW (PPW_{dp} or PPW_{p}) in the co-digestion mixture.

2.7 Statistical analysis

Experimental data (final methane yield, kinetic data, COD_s, and FAN) were analysed by a 3-way mixed analysis of variance (ANOVA) with the Bonferroni post hoc test [43] using IBM SPSS statistics, version 23. The 95% confidence interval of differences ($p<0.05$) was chosen to define the statistical significance. MATLAB, R2015a was used to calculate P, R_m and λ for each digester.

3. Results

3.1 Biomenthane potential of mono- and co-digestion

3.1.1 Experimental BMP

Figure 1 shows the cumulative methane produced by mono-digestion of *C. vulgaris*, and co-digestion with PPW_{dp} (Figure 1A) or PPW_p (Figure 1B) for 1.0 SIR. The methane yields were inhibited during the first 7 days for all experimental conditions, but after 9 days the methane yields increased linearly with time. Mono-digestion of *C. vulgaris* produced 158 mL CH_4/g VS, compared with the yields of 232 and 340 mL CH_4/g VS by mono-digestion of PPW_p and PPW_{dp}, respectively.

Figure 2 shows the cumulative methane produced by mono-digestion of *C. vulgaris*, and co-digestion with PPW_{dp} (Figure 2A) or PPW_p (Figure 2B) for 0.5 SIR. After 3 days, the methane yields increased linearly with time for all treatments. The lowest yield was 176 mL CH_4/g VS by mono-digestion of *C. vulgaris*, while the high methane yields were 439 and 348 mL CH_4/g VS by mono-digestion of PPW_{dp} and PPW_p, respectively.

For both SIRs, co-digestion of the mixtures showed methane yields between those of the two mono-substrates, and the mixing ratios between *C. vulgaris* and PPW had a significant effect on the final methane yields ($F(4,32)=100.68$, $p<0.001$). A greater PPW introduction relative to *C. vulgaris* led to an improvement of the methane yields, and post hoc testing with Bonferroni correction showed that the methane yields achieved by co-digestion of 25:75 *C. vulgaris* and PPW (mean of 266 mL CH_4/g VS) were significantly higher than the mixing ratios of 100:0 (mean of 167 mL CH_4/g VS, $p<0.001$), 75:25 (mean of 205 mL CH_4/g VS, $p<0.001$) and 50:50 (mean of 236 mL CH_4/g VS, $p=0.031$).

Moreover, the type of PPW also had a significant effect on yields ($F(1,8)=52.94$, $p<0.001$), with PPW_{dp} giving significantly higher methane values (mean of 263 mL CH_4/g VS) than both mono-digestion or co-digestion with PPW_p (mean of 222 mL CH_4/g VS).

To assess the impact of SIR on methane yields, it was found that the overall methane yields were significantly affected by SIR ($F(1,8)=54.82$, $p<0.001$). Methane yields produced at 0.5 SIR (mean of 264 mL CH_4/g VS) were significantly higher than at 1.0 SIR (mean of 222 mL CH_4/g VS).

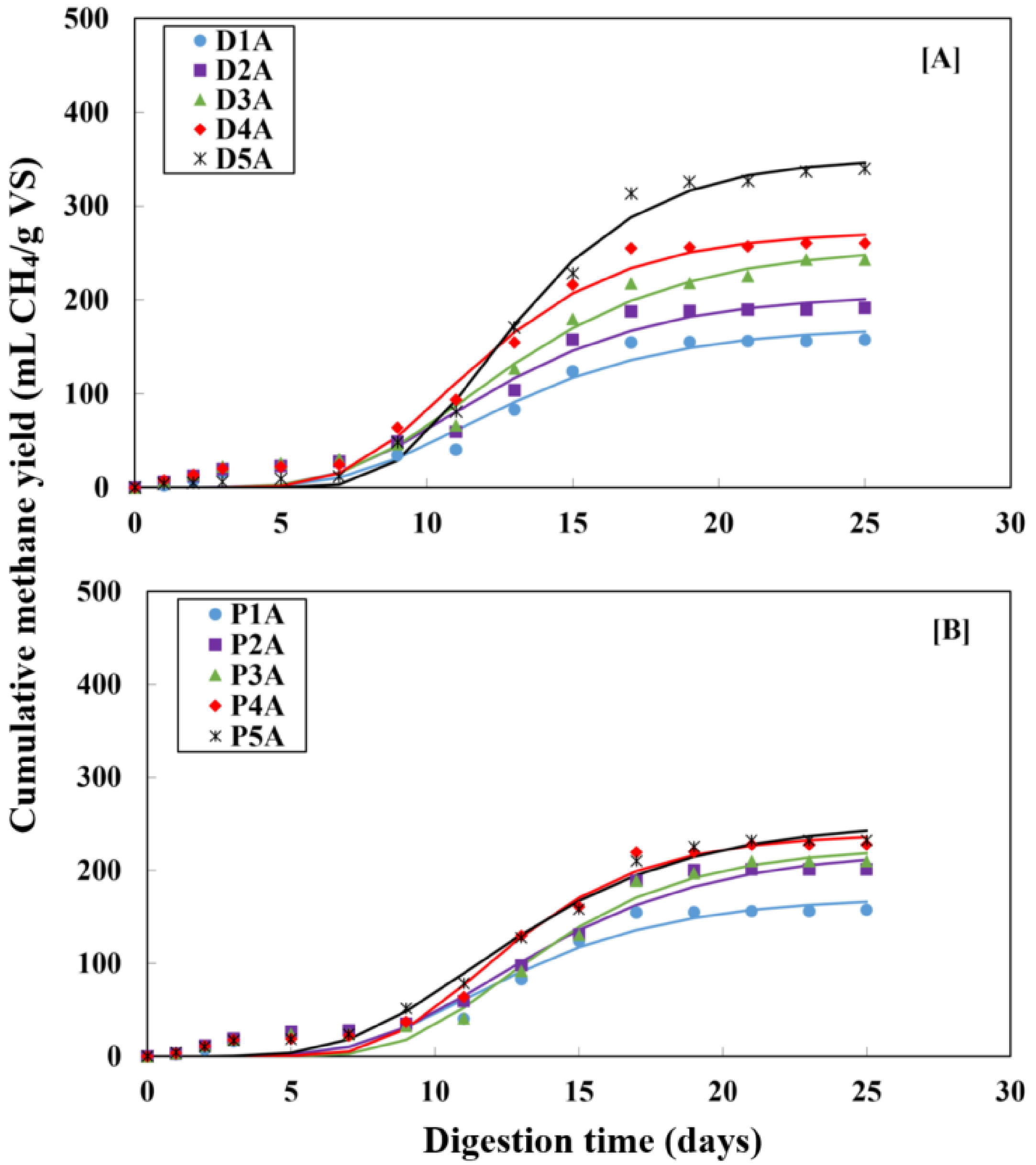

Figure 1: Cumulative methane yield of *Chlorella vulgaris* co-digested with PPW for 1.0 SIR with [A] potato discarded parts (PPW_{dp}) and [B] potato peel (PPW_p). The solid line represents the Gompertz model fit data. Co-digestion with PPW_{dp} at 1.0 SIR (D1A-D5A), co-digestion with PPW_p at 1.0 SIR (P1A-P5A).

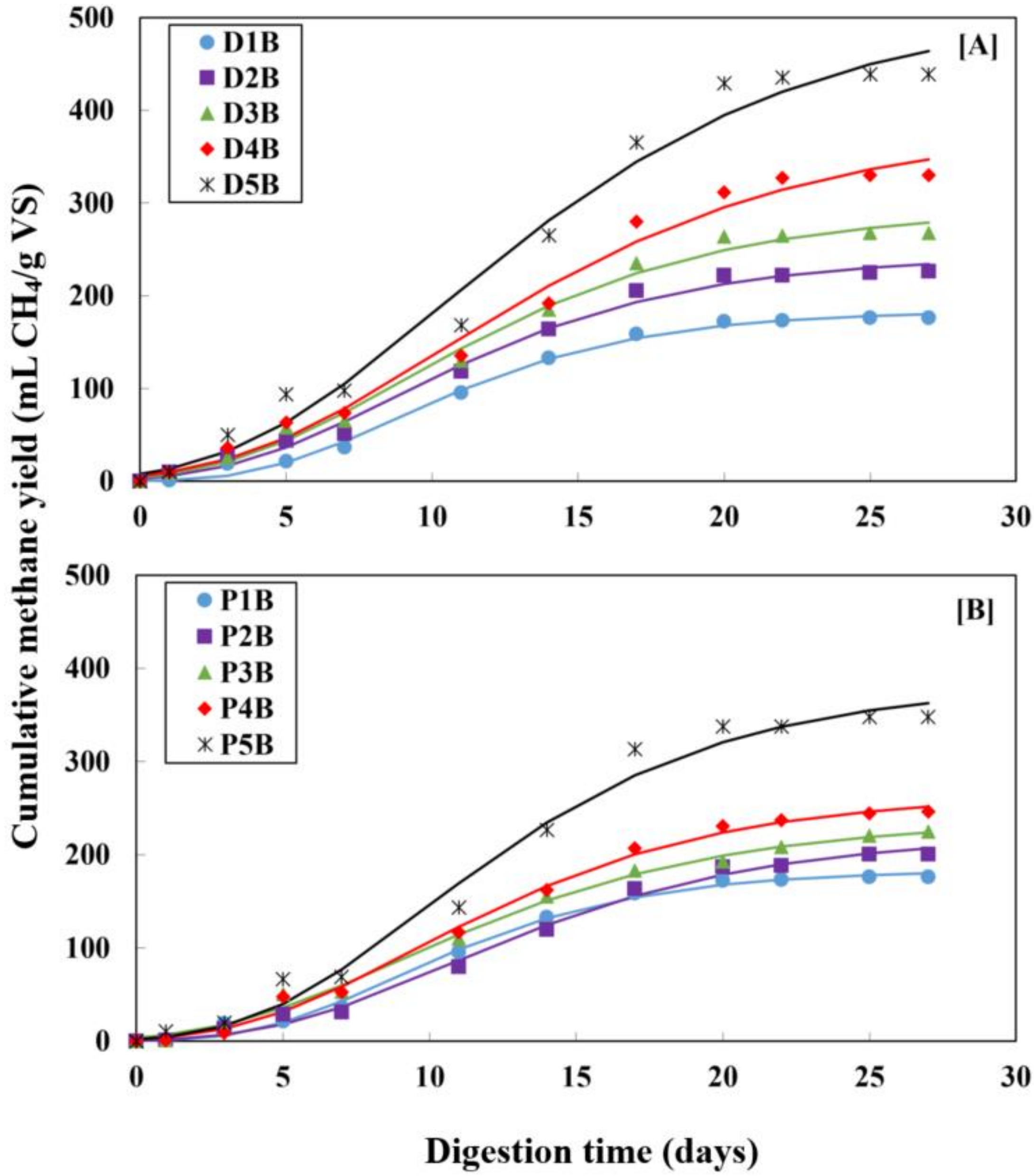

Figure 2: Cumulative methane yield of *Chlorella vulgaris* co-digested with PPW for 0.5 SIR with [A] potato discarded parts (PPW_{dp}) and [B] potato peel (PPW_p). The solid line represents the Gompertz model fit data. Co-digestion with PPW_{dp} at 0.5 SIR (D1B-D5B); co-digestion with PPW_p at 0.5 SIR (P1B-P5B).

3.1.2 BMP kinetic model

The modified Gompertz model was applied to the experimental BMP data and used to determine the maximum methane production rate (R_m) and lag phase (λ) for each substrate (Table 3). The values of R_m obtained by co-digestion with PPW were gradually improved as the proportions of PPW_{dp} or PPW_p were increased, and the mixing ratios between *C. vulgaris* and PPW had a significant effect on R_m ($F(1.736,32)=18.52$, $p<0.001$). Post hoc testing with Bonferroni correction indicated that the highest R_m achieved by mono-digestion of PPW (mean of 29.53 mL CH_4/g VS/d) were significantly higher than the mono-digestion of *C. vulgaris* (mean of 15.50 mL CH_4/g VS/d, $p=0.001$), co-digestion of 75:25 *C. vulgaris* with PPW (mean of 17.05 mL CH_4/g VS/d, $p=0.002$) and co-digestion of 50:50 *C. vulgaris* with PPW (mean of 19.64 mL CH_4/g VS/d, $p=0.005$). The PPW type also had a significant effect on R_m ($F(1,8)=17.89$, $p=0.003$). Co-digestion or mono-digestion with PPW_{dp} gave higher production rates (mean of 22.90 mL CH_4/g VS/d) than with PPW_p (mean of 19.19 mL CH_4/g VS/d). The values of R_m were also significantly affected by SIRs ($F(1,8)=58.28$, $p<0.001$), and R_m produced by 0.5 SIR (mean of 17.70 mL CH_4/g VS/d) were significantly lower than those produced by 1.0 SIR (mean of 24.40 mL CH_4/g VS/d).

The SIR had a significant effect on time values of λ ($F(1,8)=177.59$, $p<0.001$), with values of λ at 0.5 SIR (mean of 3.52 d) significantly shorter than at 1.0 SIR (mean of 7.58 d). The effect of mixing ratios on time values of λ was not significant ($F(4,32)=0.54$, $p=0.711$). Also, the values of λ did not significantly differ between PPW_{dp} and PPW_p (means of 5.35-5.75 d, $F(1,8)=1.76$, $p=0.221$). However, there was a significant interaction effect between the mixing ratios, type of PPW and SIR ($F(4,32)=3.58$, $p=0.016$). This interaction effect can be seen as at 0.5 SIR, when the type of PPW is PPW_{dp}, the values of λ being reduced as increasing the proportions of PPW.

Table 3. Summary of modified Gomerptz kinetics data for *Chlorella vulgaris* co-digestion with PPW_{dp} or PPW_p at two substrate to inoculum ratios (SIR). Co-digestion with PPW_{dp} at 1.0 SIR (D1A-D5A) or 0.5 SIR (D1B-D5B); co-digestion with PPW_p at 1.0 SIR (P1A-P5A) or 0.5 SIR (P1B-P5B). R_m = maximum methane production rate, λ = duration of lag phase.

1.0 SIR	R_m ($mLCH_4/gVS/d$)	λ (d)	R^2	0.5 SIR	R_m ($mLCH_4/gVS/d$)	λ (d)	R^2
D1A	15.75	7.14	0.9659	**D1B**	14.33	4.06	0.9957
D2A	21.45	6.85	0.9899	**D2B**	23.91	3.90	0.9879
D3A	18.97	6.76	0.9730	**D3B**	15.74	2.95	0.9923
D4A	22.77	7.17	0.9817	**D4B**	17.60	2.82	0.9924
D5A	40.90	8.73	0.9942	**D5B**	26.15	3.11	0.9845
P1A	15.75	7.14	0.9659	**P1B**	14.33	4.06	0.9957
P2A	29.41	7.22	0.9865	**P2B**	19.67	3.15	0.9882
P3A	18.77	7.57	0.9739	**P3B**	13.32	4.48	0.9945
P4A	22.94	8.76	0.9775	**P4B**	13.85	2.70	0.9953
P5A	21.45	6.85	0.9899	**P5B**	23.91	3.90	0.9879

3.1.3 Synergistic effects of co-digestion

To study the synergistic effects produced by co-digestion of *C. vulgaris* with PPW_{dp} or PPW_p, the theoretical yields of co-substrates were calculated based on Equation (4). Figure 3 shows that the synergistic effects (experimental yields higher than theoretical yields) were only found in co-digestion of *C. vulgaris* with PPW_p for 1.0 SIR.

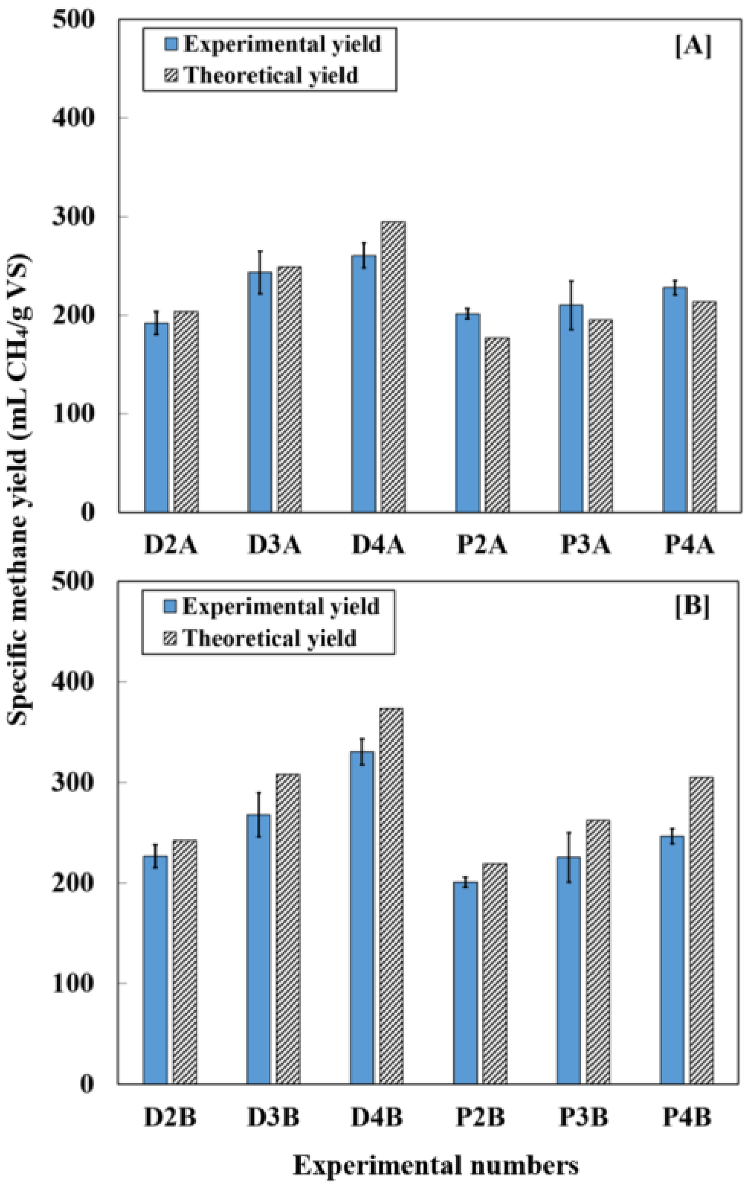

Figure 3: Experimental and theoretical methane yields for the co-digestion of *Chlorella vulgaris* with PPW at [A] 1.0 SIR and [B] 0.5 SIR. Potato discarded parts (PPW_{dp}) at 1.0 SIR (D2A-D4A) and 0.5 SIR (D2B-D4B); or with potato peel (PPW_p) at 1.0 SIR (P2A-P4A) and 0.5 SIR (P2B- P4B). Error bars = mean ± SD, n=2.

3.2 Process stability

In the current study, concentrations of COD_s, NH^+_4-N and pH were measured at the end of the BMP tests. The concentrations of FAN were calculated based on the values of pH and NH^+_4-N. Figure 4 shows that the highest COD_s present at the end of the BMP tests was 3210 mg/L and was produced by mono-digestion of PPW_{dp} at 1.0 SIR. The mixing ratios had a significant effect on COD_s ($F(4,16)=38.26$, $p<0.001$). The overall COD_s obtained by digestion with PPW_{dp} were significantly higher than with PPW_p ($F(1,4)=122.51$, $p<0.001$). The concentrations of COD_s improved with increasing the SIR ratio, and the values obtained by 0.5 SIR were significantly lower than by 1.0 SIR ($F(1,4)=1473.68$, $p<0.001$). The significant effect on COD_s was also qualified by an interaction effect between mixing ratios, type of PPW and SIR ($F(4,16)=33.49$, $p<0.001$). This interaction effect can be seen as the similar amounts of COD_s being produced by co-digestion of *C. vulgaris* with PPW_p for both SIRs. During co-digestion of *C. vulgaris* with PPW_{dp}, similar amounts of COD_s were obtained at 0.5 SIR; however, for 1.0 SIR the concentrations of COD_s increased significantly with increasing proportions of PPW_{dp}. Specifically, mono-digestion of *C. vulgaris*, and co-digestion with 25% and 50% PPW_{dp} produced similar amounts of COD_s. However, the concentrations of COD_s increased significantly when the proportions of PPW_{dp} were at 75% and 100%.

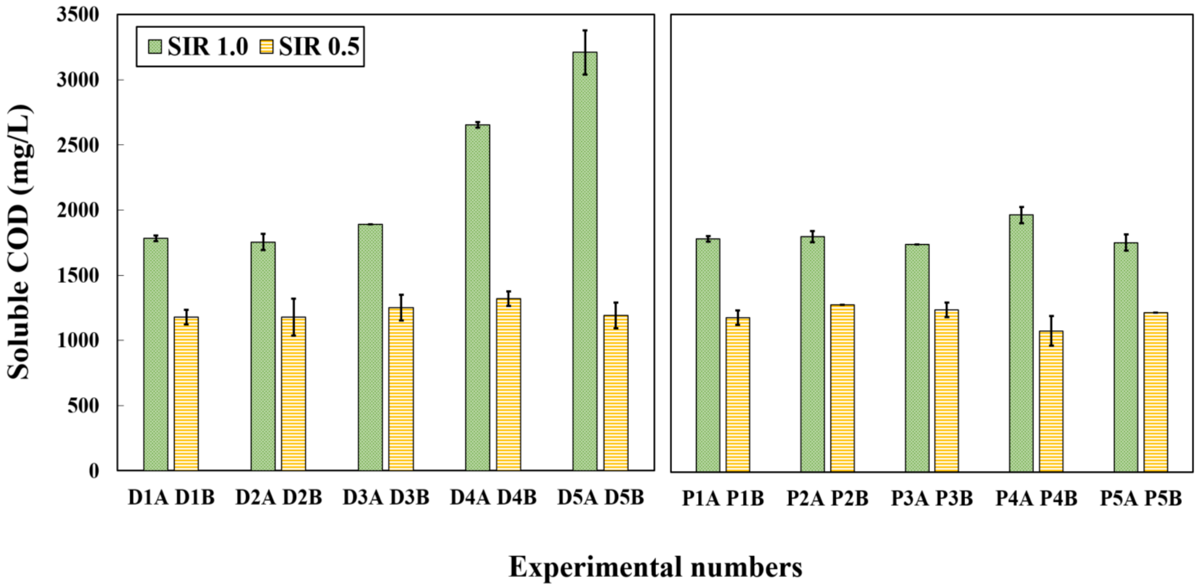

Figure 4: Concentrations of soluble COD obtained at the end of co-digestion of *Chlorella vulgaris* with potato discarded parts (PPW_{dp}) at 1.0 SIR (D1A-D5A) and 0.5 SIR (D1B-D5B); or with potato peel (PPW_p) at 1.0 SIR (P1A-P5A) and 0.5 SIR (P1B- P5B). Error bars = mean ± SD, n=2.

Figure 5 shows that the highest FAN of 123 mg/L was obtained by mono-digestion of *C. vulgaris* at 0.5 SIR, and the lowest value of 20 mg/L was obtained by mono-digestion of PPW_{dp} at 1.0 SIR. The mixing ratios of PPW had a significant effect on FAN ($F(4,16)=20.70$, $p<0.001$). Also, the concentrations of FAN were significantly increased at lower SIR ($F(1,4)=626.42$, $p<0.001$). The main effects of mixing ratios of PPW and SIR on concentrations of FAN can also qualified by a significant interaction effect between these two factors ($F(4,16)=15.44$, $p<0.001$). This interaction effect can be seen as, for 0.5 SIR, the concentrations of FAN showed a clear decreasing trend as increasing the proportions of PPW_{dp} or PPW_p. The type of PPW also had a significant effect on concentrations of FAN ($F(1,4)=13.86$, $p=0.020$), with PPW_{dp} giving significantly lower FAN concentrations (mean of 63 mg/L) than both mono-digestion or co-digestion with PPW_p (mean of 72 mg/L).

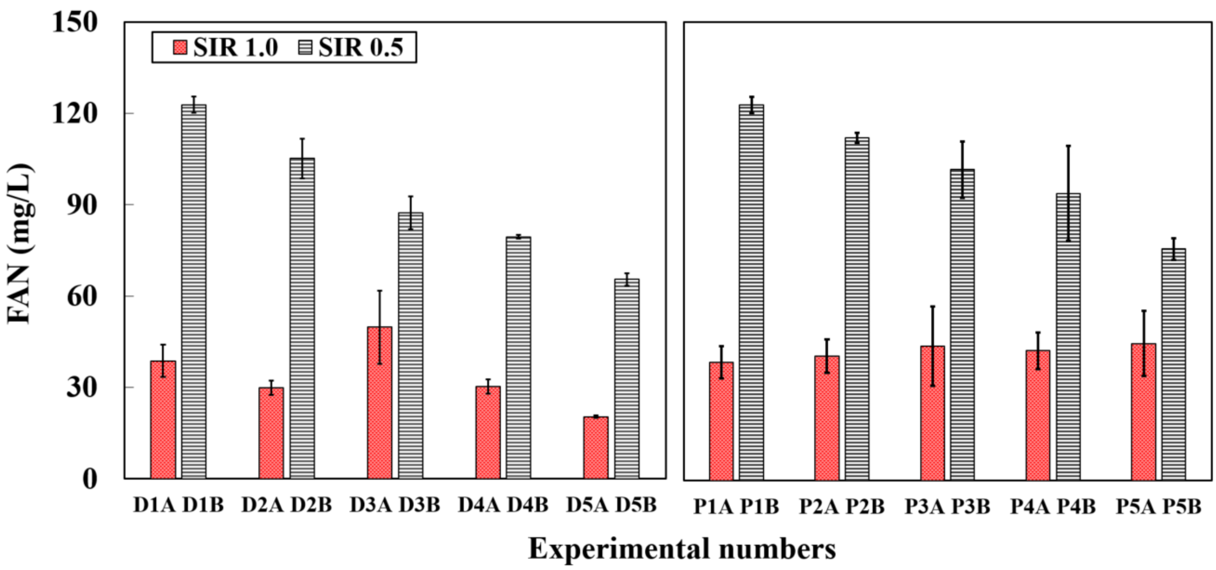

Figure 5: Concentrations of free ammonia nitrogen (FAN) obtained at the end of co-digestion of *Chlorella vulgaris* with potato discarded parts (PPW_{dp}) at 1.0 SIR (D1A-D5A) and 0.5 SIR (D1B-D5B); or with potato peel (PPW_p) at 1.0 SIR (P1A-P5A) and 0.5 SIR (P1B- P5B). Error bars = mean ± SD, n=2.

4. Discussion

4.1 Effect of co-digestion on biomethane potential and process stability

In the current study, 158 and 176 mL CH_4/g VS were produced by mono-digestion of *C. vulgaris*, which is lower than previously reported by [9]. Their results showed that 250 mL CH_4/g VS was produced by mono-digestion of *Chlorella sp.* and a possible reason for this is that the growth media they applied to cultivate the microalgae was a mixed media containing synthetic and real AD swine effluent. Consequently, the mature *Chlorella sp.* was harvested with a C/N ratio at 17/1, which is much higher than in the current study (6.43/1).

The benefits of co-digestion of microalgae with carbon-rich feedstocks are to rebalance the C/N ratio, reduce the concentration of inhibitory compounds affecting methanogens, and provide a stable AD process [11]. In the current study, the addition of PPW_{dp} or PPW_p to *C. vulgaris* both resulted in an increase in the C/N ratio, and the results indicate that PPW has the potential to be an effective co-substrate for microalgae co-digestion in terms of generating more balanced C/N ratios. Consequently, the co-digestion of *C. vulgaris* with PPW_{dp} increased methane yields by 22 – 47% above that of *C. vulgaris* mono-digestion, while co-digestion with PPW_p increase it by 12 – 32%. The co-digestion of wheat straw with mixed microalgae in batch BMP tests were investigated by [44]. Their results showed that the final methane yield increased by only 5 – 9% compared to microalgae mono-digestion, which is lower than the current study (Table S1 in Supplementary Information). Wheat straw is a lignocellulosic biomass consisting of 40.8 – 49.8% of cellulose, 26.4% of hemicellulos and 19.6 – 22.9% of lignin [45, 46, 47]. Lignocellulosic biomass comprises a strong structural matrix formed by the digestible polymers (cellulose and hemicellulose) being embedded within the relatively recalcitrant lignin component, and therefore requires additional treatment to be broken down completely into simple sugars. However, 65.0 – 85.0 % of the carbohydrate in potato waste is present as starch [17, 48], and unlike lignocellulosic biomass, it is easily broken down into sugars [20]. Therefore, this suggests that PPW could be more efficient than lignocellulose biomass as a co-substrate with microalgae.

When mixed microalgae was co-digested with food waste in batch BMP tests, and the methane yields were enhanced by 4.99 fold as in comparison with mono-digestion of microalgae as reported by [12] (Table S1 in Supplementary Information). In their study, the seed inoculum was taken from a continuously stirred lab-scale AD digester treating food waste, and the increased microbial diversity in the seed inoculum could digest food waste directly and consequently enhanced methane productivity. Therefore, this likely explains why their enhancement is much higher than the current study.

Improved kinetics data is another benefits of co-digestion of two feedstocks [44, 49]. In the current study, the addition of PPW to *C. vulgaris* significantly increased the values of R_m, while a significant reduction the values of λ was seen for the co-digestion with PPW_{dp} at 0.5 SIR. The improved kinetics may also suggest that PPW could be a useful co-substrate in co-digestion with microalgae. Moreover, the concentrations of FAN were significantly reduced by co-digestion with PPW, and FAN is regarded as the active component leading to ammonia inhibition for AD process [50]. Therefore, in the current study, the results indicating that co-digestion of microalgae with PPW brings further benefits by reducing the risk of ammonia toxicity.

The synergistic effects would be an additional benefits provided by co-digestion of different feedstocks [26]. However, in the current study, synergistic effects in final methane yields were only found for the co-digestion of *C. vulgaris* with PPW_p at 1.0 SIR. Research to identify the possible mechanisms leading to the improvement of co-digestion performance has not focused entirely on the balancing of C/N ratios in feedstock. Some studies reported that the synergistic effect of co-digestion of microalgae with other co-substrates was attributed to certain micronutrients and essential trace elements provided to the microorganisms, and this may hiding the true benefits of the co-digestion [6, 51]. However, in the current study, additional nutrients were not supplied in BMP bottles, and therefore the presence of potentially toxicity components during digestion was thought to influence the synergistic effects seen in co-digestion studies [42]. In the current study, the seed inoculum was collected from a manure-based anaerobic digester with high concentrations of ammonia nitrogen, especially FAN which was measured at over 400 mg/L. This inoculum was used without diluting and washing, and could have provided extra nitrogen in BMP bottles, masking the true benefits of the co-digestion mixtures in batch BMP tests. Consequently, for 0.5 SIR, the concentrations of FAN were significantly higher than for 1.0 SIR because of the relatively higher proportion of inoculum that was added into the BMP bottles. Therefore, this likely explains why the synergistic effects of the co-digestion substrates were only found for 1.0 SIR.

4.2 Effect of mixing ratios on biomethane potential and digester stability

The co-digestion of *C. vulgaris* with PPW, the methane yields also affected by the mixing ratios between *C. vulgaris* and PPW proportions. In the current study, the best performance was found at ratio of 25:75 *C. vulgaris* and PPW compared to ratios of 75:25 and 50:50. Wang et al. [9] found that the C/N ratio of swine manure was 35/1, and compared to mono-digestion with *Chlorella sp.*, the methane yields improved by around 13 – 28% after co-digestion with these two substrates (Table S1 in Supplementary Information). This improvement is similar to that achieved by co-digestion of PPW_p with *C. vulgaris*, however, in the previous study the highest yield of 348 mL CH_4/g VS was obtained from co-digestion with 6% *Chlorella sp.* and 94% swine manure on the basis of VS. Since the 25:75 ratio was the highest co-digestion ratio investigated in the current study that contained *C. vulgaris*, it is possible that higher methane yields might have been found at ratios containing greater proportions of PPW (e.g a 10:90 ratio). However, in the current study, the results showed that the concentrations of COD_s increased with increasing proportions of PPW to microalgae, and the highest COD_s observed by mono-digestion of PPW. According to [52], the COD_s produced in an anaerobic process corresponds mainly to oxidation produced volatile fatty acids (VFAs). Moreover, potato waste has high degree of soluble components and high biodegradability resulting in rapid and strong acidification, and consequently accumulated more VFAs which may have inhibited the activity of methanogens [15, 53]. Therefore, the results may indicate that adding higher proportions (> 75%) of PPW to microalgae increased the possibility of generating high VFAs concentrations that might inhibit the AD process. Similarly, the mono-digestion of PPW creates a possibility that the AD process might inhibited due to VFAs accumulation, although it obtained highest methane yields in the current batch BMP tests. In the current study, the C/N ratios in the mixtures of *C. vulgaris* with PPW_{dp} or PPW_p at a mixing ratio of 25:75 were 22.77/1 and 19.86/1, respectively, both within the optimal range quoted for AD process [11, 54]. Therefore, the current study suggests that a mixing ratio of 25:75 might provide more optimal conditions for the co-digestion of microalgae and PPW.

4.3 Effect of type of PPW on biomethane potential and digester stability

The addition of PPW_{dp} or PPW_p to *C. vulgaris* increased the C/N ratios and the final methane yields. Therefore, both of these sources of PPW could be used as co-digestion substrates with microalgae. For PPW_{dp}, a higher VS/TS ratio was determined than for PPW_p. Li et al. [55] indicated that a substrate with a higher VS/TS ratio contains higher concentrations of organic content, which is more appropriate for methane production. PPW_{dp} consisting of 54.3 – 76.8 % of dry matter as starch [48], while [14, 17] observed for PPW_p that between 34.3 – 52.1 % of dry matter is starch. In the present study, the total carbohydrates content for PPW_{dp}

was 77.0 % of dry weight, and 63.3 % of dry weight for PPW_p, suggesting that PPW_{dp} contained higher amounts of starch (around 85% of total carbohydrates) than the values (65% of total carbohydrates) for PPW_p, although the starch analysis was not determined directly. Spets et al. [56] reported that starch is easily broken down into monosaccharides, and the higher starch contents in anaerobic feedstocks may improve their anaerobic biodegradability [53]. Therefore, in the current study, the higher methane yields achieved by mono- or co-digestion with PPW_{dp} was probably a result of their higher starch content with respect to PPW_p. Moreover, increased methane production rates (R_m) and shorter lag phase (λ) were seen during mono- or co-digestion with PPW_{dp} compared to PPW_p. These results suggest that the PPW_{dp} contained greater concentrations of soluble components than PPW_p. Furthermore, mono- or co-digestion with PPW_{dp} resulted in the lower concentrations of FAN than mono- or co-digestion with PPW_p. Since FAN has been reported as the major inhibitor of an AD process [50], therefore, this result indicates that digestion with PPW_{dp} could have more chance to avoid ammonia toxicity for methanogens than with PPW_p.

4.4 Effect of SIR on biomethane potential and digester stability

In the current study, the results showed that methane yields were increased at lower SIR, and agree with previous studies using different substrates [27, 28, 29]. An optimum SIR in the digester is considered to contain the balanced amount of anaerobic microorganisms for digestion of both primary and intermediate products [27, 28]. However, the lag phase (λ) in the present study significant reduced at lower SIR, indicating that the activity of methanogens was limited for 1.0 SIR compared to 0.5 SIR; supported by the findings of [29, 55].

The concentrations of COD_s decreased at the lower SIR, which agree with a previous study reported by [29]. And higher COD_s concentrations were measured at the end of digestion for 1.0 SIR, compared to 0.5 SIR, and hence it is likely that much of this was from VFAs. In the current study, a significant interaction effect was observed between the mixing ratios, type of PPW and SIR, and may indicate that the co-digestion of *C. vulg*aris with higher proportions (> 75% VS) of PPW has the potential to accumulate more VFAs in terms of COD_s under higher SIR. In the present study, the concentrations of FAN were significantly increased at lower SIR, contrasting the results obtained by [52]. In their study, it was found that the final concentrations of FAN was reduced with a decreasing SIR. The high concentrations of FAN present in the seed inoculum could have been responsible for the observed effects in the current study.

4.5 Benefits from co-digestion with PPW

In the current study, the results show that PPW could be a promising co-substrate for co-digestion with microalgae to enhance methane production. Currently, the most fundamental challenge that exists in proving the economic viability of converting microalgal biomass to methane, or alternative biofuels, is the relatively low concentration of harvested microalgal biomass generated from cultures [58]. This means that without the implementation of costly biomass concentration technologies, low organic loading rate (OLR), and short hydraulic retention time (HRT) or both would be experienced when using microalgal cultures directly as a substrate for a large-scale AD plant. The results in this study show that the concentrated *C. vulgaris* contained relatively low levels of TS and VS, and the addition of PPW could increase the load of biodegradable organic matter in terms of TS and VS. Therefore, if the co-digestion of microalgae and PPW can be applied in large-scale AD, the OLRs may potentially be increased compared to the mono-digestion of microalgae. Moreover, increasing the OLRs can also reduce the size of the digester, and subsequently reduce capital costs [59]. Besides the low biomass concentration, high production costs are another challenge when using microalgae for biofuel production [60]. Production costs are reported to be between

$100,000–1,000,000/ha depending on the cultivation system used [61]. The results in the current study also show that the best co-digestion performance was achieved at a mixing ratio of 25:75 of microalgae and PPW compared to ratios of 75:25 and 50:50. Therefore, when running a large-scale co-digestion digester, less input of microalgal biomass may be required, which may consequently improve the economic feasibility of using microalgae for methane production.

Lastly, the results show that the highest methane yields were obtained by the mono-digestion of PPW, but this increased the possibility of generating high VFA concentrations. Therefore, methanogenesis was the rate-limiting step for the easily biodegradable PPW. However, hydrolysis was the rate-limiting step for microalgae due to their resilient cell wall. Therefore, based on these findings, a two-stage anaerobic co-digestion process could be proposed to further increase methane production from microalgae.

Overall, co-digestion of microalgae with PPW could be a possible step to enhance the feasibility of biogas production from microalgae. In addition, since PPW are generated in larger amounts, co-digestion with microalgae could also be an environmentally-friendly and economical solution for PPW disposal.

5. Conclusion

This investigation has demonstrated the possibility that potato processing waste (PPW) could be used effectively as a feedstock co-digestion with microalgae. In batch BMP tests the methane production rates and final methane yields were all increased significantly as the proportion of PPW in the mixed waste was increased. Addition of relatively high proportions of PPW could decrease the concentrations of FAN, and improve digestion performance and stability by reducing the likelihood of ammonia toxicity. The PPW_{dp} and PPW_{p} co-digestion feedstocks both show good potential for co-digestion with microalgae. Co-digestion of *C.*

vulgaris with PPW_{dp} increased the methane yields the most, by 22 – 47%, whilst co-digestion of *C. vulgaris* with PPW_p enhanced the methane yields by 12 – 32%. Methane yields and duration of lag phase were both affected significantly by the variation of the SIRs. The residual level of COD_s present at the end of BMP tests was greater at the higher SIR, and may limit observed methane yields.

Overall, the investigation suggests that PPW_{dp} and PPW_p are both promising feedstocks for co-digestion with microalgae. The enhanced methane yields resulting from co-digestion can be attributed mainly to the balanced C/N ratios. However, the presence of relatively high concentrations of ammonia in seed inoculum could have hidden the true benefits of the co-digestion. Therefore, follow-up studies should carried out using continuously fed anaerobic digesters to verify the potential of PPW as a feedstock for co-digestion with microalgae. Moreover, changes in microbial communities during the AD process are also linked to the performance of anaerobic digesters. However, microbial community information during microalgae AD is still limited. Therefore, further investigation should involve the microbial community analysis that may provide insights at a molecular level to assist the digestion of microalgae. In addition, as microalgae with a robust cell wall this may also lower methane production, therefore, further studies should also investigate potential pre-treatments and extend the current co-digestion work by feeding pre-treated microalgae and PPW.

Declaration of interest

The authors report no conflicts of interest.

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Supplementary Information

Anaerobic co-digestion of microalgae *Chlorella vulgaris* and potato processing waste: effect of mixing ratio, waste type and substrate to inoculum ratio

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The volume of dry biogas under STP conditions obtained during the process was calculated based on equation 1 [1].

$$V_0 = \frac{V*(P-P_W)*T_0}{P_0*T} \tag{1}$$

where V_0 is the volume of dry biogas under STP conditions (0 ^{0}C, 1atm) (mL), V is the volume of biogas produced (mL), P is the pressure of the gas phase at the time of reading (hPa), P_W is the vapour pressure of water as a function of the temperature of the ambient space (hPa), T_0 is the normal temperature, T_0=273K, P_0 is the normal pressure, P_0=1013 hPa and T is the temperature of the fermentation gas, T=37 ^{0}C (310K).

The methane content of dry gas was calculated based on equation 2:

$$CH_4^D = CH_4^H * \frac{P}{P-P_W} \tag{2}$$

where CH_4^D is the methane content of dry biogas in % by volume, CH_4^H is the methane percentage in humid gas by volume, P is the pressure of the gas phase at the time of reading (hPa), and P_W is the vapour pressure of water as a function of temperature of the ambient space (hPa).

The volume of methane produced under STP conditions was calculated based on equation 3 [2].

$$V_{CH_4} = V_H + V_S - V_{H_0} \tag{3}$$

where V_H is the calculated daily methane production in the headspace (mL), V_S is the daily measured methane in the syringe (mL), and V_{H_0} is the volume of methane produced from the headspace on the previous day (mL).

Table S1. Comparison of current study with previous microalgae co-digestion studies

Microalgae Strains	Co-substrates	Reactor Type	Methane enhancement	Microalgae: Co-substrate Mixing ratio [a] (%)	References
Mixed microalgae (C/N=7.40)	Wheat straw (C/N=95.40)	BMP	5 – 9%	20:80	[3]
Nannochloropsis salina (C/N=5.36)	Sewage sludge (C/N=13.88)	BMP	Approx. 8 – 25%	25:75	[4]
Scenedesmus sp. + *Chlorella* sp. (C/N=NA[b])	Food waste (C/N=NA[b])	BMP	1.67 – 4.99 fold	20:80	[5]
Chlorella sp. (C/N=17.00)	Swine manure (C/N=35.00)	BMP	13 – 28%	6:94	[6]
Chlorella sp. (C/N=4.86)	Chicken manure (C/N=12.28)	BMP	29 – 77%	20:80	[7]
Chlorella vulgaris (C/N=6.48)	PPW_{dp} (C/N=40.78)	BMP	22 – 47%	25:75	This study
Chlorella vulgaris (C/N=6.48)	PPW_p (C/N=28.59)	BMP	12 – 32%	25:75	This study

[a] Mixing ratio based on VS ratio for best co-digestion performance;

[b] NA- not identified;

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