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Short Communication

Sequential ethanol fermentation and anaerobic digestion increases bioenergy yields from duckweed

O. Calicioglu, R.A. Brennan*

The Pennsylvania State University, Department of Civil and Environmental Engineering, University Park, PA 16801, USA

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ABSTRACT

The potential for improving bioenergy yields from duckweed, a fast-growing, simple, floating aquatic plant, was evaluated by subjecting the dried biomass directly to anaerobic digestion, or sequentially to ethanol fermentation and then anaerobic digestion, after evaporating ethanol from the fermentation broth. Bioethanol yields of 0.41 ± 0.03 g/g and 0.50 ± 0.01 g/g (glucose) were achieved for duckweed harvested from the Penn State Living-Filter (*Lemna obscura*) and Eco-Machine™ (*Lemna minor/japonica* and *Wolffia columbiana*), respectively. The highest biomethane yield, 390 ± 0.1 ml CH_4/g volatile solids added, was achieved in a reactor containing fermented duckweed from the Living-Filter at a substrate-to-inoculum (S/I) ratio (i.e., duckweed to microorganism ratio) of 1.0. This value was 51.2% higher than the biomethane yield of a replicate reactor with raw (non-fermented) duckweed. The combined bioethanol-biomethane process yielded 70.4% more bioenergy from duckweed, than if anaerobic digestion had been run alone.

1. Introduction

The economic and environmental disadvantages of fossil fuel consumption have increased the search for alternative resources to fulfill world's growing energy and chemical needs (Jung et al., 2016). At the same time, conventional bioenergy crops have also been posing social, economic, and environmental challenges. Duckweed (*Lemnaceae*), a family of fast-growing, simple, floating aquatic plants, consisting of 38 species in five genera (Les et al., 2002), has been demonstrated to be a technically feasible alternative feedstock for bioethanol production due to several advantages: it can accumulate high amounts of starch (up to 46% of dry mass) under nutrient starvation (Zhao et al., 2015); has relatively little lignin content (1–3%); its small size (0.1–1 cm) eliminates the need for milling; and, because it floats, the harvesting process is relatively simple (Cui and Cheng, 2015). Duckweeds are resilient to a broad range of nutrient concentrations; therefore, they can be grown on wastewater steams (Cheng and Stomp, 2009).

Due to its high and manipulatable starch content, duckweed is regarded as a promising bioethanol feedstock in the current literature. The studies conducted to date have focused on the utilization of the starch component only (Xu et al., 2011; Yu et al., 2014), or the fermentation of cell wall carbohydrates as well (Ge et al., 2012; Zhao et al., 2014). The high level of variability in wastewater compositions, however, may cause uncertainties in starch and bioethanol potentials from wastewater-derived duckweed biomass. By comparison, a more resilient pathway for duckweed valorization could be anaerobic digestion, since this process converts not only sugars, but also proteins and lipids into biomethane. In addition, anaerobic digestion can be used to stabilize residual organics in the ethanol fermentation broth, and thereby help to compensate for the costs of ethanol production and distillation (Wu et al., 2015). Indeed, the sequential process of ethanol fermentation and anaerobic digestion has been shown to increase the overall bioenergy yield of several other substrates such as food waste (Wu et al., 2015), oat straw (Dererie et al., 2011), and corn stalks (Vintilă et al., 2013). This combined approach may improve the sustainability of large-scale biorefineries.

Although some work has focused on ethanol production from duckweed, reports on its anaerobic digestibility are limited to a very few studies. An early study on anaerobic digestion of manganese-contaminated duckweed produced a maximum biogas yield of 176 ml/g with a methane content of 60% (Jain et al., 1992). Other work conducted on duckweed has focused on its co-digestion with other substrates, such as dairy manure (Triscari et al., 2009), to help balance the C/N ratio.

To ensure that neither limitations nor inhibition will occur during anaerobic digestion due to substrate loading, the substrate-to-inoculum ratio (S/I) should be optimized (Chynoweth et al., 1993). The S/I not only affects total methane yield, but also its production rate (Alzate et al., 2012). In the current study, the potential of increasing bioenergy yields obtained from duckweed grown in an ecological wastewater

* Corresponding author.
E-mail address: rab44@psu.edu (R.A. Brennan).

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treatment system for nutrient removal was investigated using a sequential process: fermentation of duckweed and distillation of the resulting bioethanol, followed by anaerobic digestion of the residual fermented duckweed. In addition, the effects of S/I ratio on anaerobic digestion performance were evaluated through biochemical methane potential (BMP) assays.

2. Materials and methods

2.1. Analytical methods

Total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), and volatile dissolved solids (VDS) were determined according to Standard Methods No. 2540 (APHA/AWWA/WEF, 2012). The suspended portion of samples was separated on glass fiber filters (AP40; Millipore, Billerica, MA, USA) using a vacuum filtration apparatus. Chemical Oxygen Demand (COD) was measured according to the closed reflux colorimetric method as described in Standard Methods, No. 5220 (APHA/AWWA/WEF, 2012).

Glucose and ethanol quantification were performed using a Waters high performance liquid chromatograph (HPLC) equipped with a refractive index detector (Waters, Milford, MA) and a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as the mobile phase. The detector and column temperatures were constant at 35 °C and 65 °C, respectively. Prior to HPLC analysis, samples were centrifuged at 4 °C for 20 min at 5200 × *g* and the supernatant filtered through 0.2 µm nylon syringe filters. Theoretical maximum glucose concentration was calculated according to Gulati et al. (1996).

Headspace gas volumes of anaerobic reactors were measured at 25 °C using a water displacement device filled with 0.01 M hydrochloric acid to prevent microbial growth. Volume readings were reported at standard temperature and pressure. Volumetric methane concentrations were determined by withdrawing headspace from the reactors using a 250 µL airtight syringe (Hamilton, Reno, NV, USA) and injecting into a gas chromatograph (model SRI310C, SRI Instruments, Torrance, CA, USA) equipped with a 6 foot molecular sieve column (Altech, 5605PC, MD) held at 80 ∘C.

2.2. Plant material and cultivation

Duckweed used in this study was obtained on May 27, 2015, from two sources: 1) an open tank dedicated for growing duckweed in the Penn State Eco-Machine™ (EM), which is a pilot-scale ecological wastewater treatment system receiving on average (n = 4) 3.6 ± 1.1 mg/L phosphate, 0.1 ± 0.0 mg/L ammonia, and 11.1 ± 3.0 mg/L nitrate; and 2) an open pond within the effluent spray fields of the Penn State Wastewater Treatment Plant, a.k.a. the "Living-Filter" (LF), receiving on average (n = 3) 2.2 ± 0.4 mg/L phosphate, 2.3 ± 0.9 mg/L ammonia, and 7.8 ± 0.8 mg/L nitrate. In both sources, duckweed was naturally present and had not been subjected to a frequent harvesting regime.

To identify the duckweed species present in each source, total DNA was extracted from duckweed tissue using a PowerPlant® Pro DNA isolation kit (QIAGEN, Hilden, Germany), and then amplified using a two-barcode PCR protocol (Borisjuk et al., 2014). After amplification, the DNA fragments were purified using a GeneJET PCR purification kit (ThermoFisher, Waltham, MA), and sent to the Genomics Core Facility (The Pennsylvania State University) for processing. Following a BLAST-based protocol for duckweed species identification (Borisjuk et al., 2015), the EM duckweed was identified as a co-culture of *Lemna japonica/minor* (100% sequence identity to accession numbers KJ9211760.1 and DQ400350.1, respectively, in the NCBI database) and *Wolffia columbiana* (99.6% sequence identity to accession number GU454371.1); whereas the LF duckweed was identified as a monoculture of *Lemna obscura* (100% sequence identity to accession number GU454331.1).

For use in these experiments, harvested duckweed was rinsed with tap water and dried at 50 ± 2 °C to a constant weight over two days. The composition of the dried duckweed was determined by first grinding and sieving through mesh No. 20 (850 mm opening size), and then sending to Dairy One Wet Chemistry Laboratory (Ithaca, NY). The composition of EM duckweed was reported as 16.9% cellulose, 23.9% hemicellulose, 4.3% starch, 2.0% lignin, 26.0% crude protein, and 0.73 g VS per g TS. The composition of LF duckweed was reported as 17.0% cellulose, 18.1% hemicellulose, 15.9% starch, 1.1% lignin, 17.0% crude protein, and 0.81 g VS per g TS.

2.3. Inocula

2.3.1. Yeast strain

For fermentation of duckweed, *Saccharomyces cerevisiae* (ATCC 24859) was enriched in culture medium with the following constituents (concentrations in parentheses are g/L): glucose (20); yeast extract (Difco, Sparks, MD) (6); $CaCl_2{\cdot}2H_2O$ (0.3); $(NH_4)_2SO_2$ (4); $MgSO_4{\cdot}7H_2O$ (1); and KH_2PO_4 (1.5). The culture was grown at 30 °C for 24 h before being transferred to fermentation flasks as the inoculum.

2.3.2. Anaerobic seed

Anaerobic seed was obtained from the Penn State Wastewater Treatment Plant secondary anaerobic digester. The inoculum was starved for two days prior to use in the BMP assays. The TS of the starved seed was 23.9 ± 0.5 g/L, and the VS was 15.7 ± 0.7 g/L, which is 65.8 ± 5.1% of the TS.

2.4. Fermentation experiments

Enzymatic saccharification of the duckweed was performed in 500 ml flasks with 200 ml distilled water and 10 g duckweed (dry weight). The pH was adjusted to 7.0 ± 0.1 with 2 M hydrochloric acid prior to liquefaction by autoclaving at 95 °C under 103 kPa for 1 h. Flasks with EM and LF duckweed received 0.6 ml and 1.98 ml of α–amylase (Sigma Aldrich, A3403, USA) respectively, based on the starch content of each duckweed type, to achieve an amylase loading of 5000 units/g starch. Following liquefaction, the pH was adjusted to 4.8 ± 0.1 with glacial acetic acid. After pH adjustment, 60 mg and 198 mg glucoamylase (Sigma Aldrich 10115, USA) were added to each flask containing EM and LF duckweed, respectively. In addition, all flasks received 2 ml cellulase (60 filter paper unit/g cellulose). Saccharification was then performed at 50 °C, while mixing at 120 rpm for 24 h in flasks sealed with cotton stoppers and parafilm. All experiments were conducted in triplicate under sterile conditions.

Following saccharification, the pH of each flask was increased to 7.0 ± 0.1 by dosing with 2 M sodium hydroxide, and then 2 ml yeast culture was added. Flasks were incubated at 30 °C while mixing at 120 rpm for 48 h. Glucose and ethanol concentrations before and after fermentation were quantified. Fermented ethanol was then evaporated by vacuum extraction after the pH was increased to 7.8 ± 0.1 by 2 M sodium hydroxide addition, in order to avoid escape of volatile fatty acids (VFAs) from the slurry. The triplicates for each duckweed type were then combined and subjected to BMP assays.

2.5. Biochemical methane potential (BMP) assays

The BMP assays with duckweed were carried out based on the protocol proposed for bioenergy crops and organic wastes (Angelidaki et al., 2009) with slight modifications. Batch reactors (160 ml total volume, 120 ml working volume) were filled with 24 ml inoculum, and substrate (either raw EM or LF duckweed, or residual fermentation slurries, FEM or FLF), to provide an S/I of 0.5 or 1.0. To account for the effect of endogenous gas production by the anaerobic inoculum, control bottles were prepared with the same amount of anaerobic seed, but without substrate. Blank bottles were prepared with duckweed, but

Table 1
Bioethanol, biomethane, and bioenergy yields from Eco-Machine (EM) and Living-Filter (LF) duckweed biomass through separate and coupled ethanol fermentation and anaerobic digestion processes.

		Eco-Machine™ (EM)	Living-Filter (LF)
1	*Bioethanol production*		
a	Theoretical maximum glucose (g)	11.8 ± 0.7	18.3 ± 0.9
b	Glucose recovery (g/L)	6.5 ± 0.8	17.9 ± 0.6
c	Glucose recovery (%)	55.5 ± 6.7	97.6 ± 3.4
d	Ethanol produced (g/L)	3.2 ± 0.3	7.3 ± 0.3
e	Ethanol yield (g ethanol / g glucose)	0.50 ± 0.01	0.41 ± 0.03
f	Ethanol yield (g ethanol / g TS)	0.07 ± 0.01	0.15 ± 0.01
2	*Biomethane production*		
a	Raw duckweed methane yield (ml CH_4/g VS)	258 ± 0.0	259 ± 0.3
b	Raw duckweed methane yield (ml CH_4/g TS)	183 ± 0.0	192 ± 0.2
c	Fermented duckweed methane yield (ml CH_4/g VS)	328 ± 0.1	390 ± 0.1
d	Fermented duckweed methane yield (ml CH_4/g TS)	261 ± 0.0	289 ± 0.0
3	*Bioenergy production*		
a	Ethanol from raw duckweed (kJ/g TS)	1.9 ± 0.2	4.3 ± 0.2
b	Methane from raw duckweed (kJ/g TS)	6.8 ± 0.0	7.5 ± 0.0
c	Net ethanol recovered after distillation (kJ/g TS)	1.4 ± 0.2	3.7 ± 0.2
d	Methane from fermentation residue (kJ/g TS)	8.9 ± 0.0	9.1 ± 0.0
e	Total energy yield of coupled process (kJ/g TS)	10.3 ± 0.2	12.8 ± 0.2
f	*Energy gain of coupled over separate processes (kJ/g TS)	3.5 ± 0.2	5.3 ± 0.2

* 3f = 3e 3b (Energy gain of coupled over separate processes has been compared to the maximum energy gain potential of the separated process).

without inoculum addition. To determine if the duckweed reactors were lacking in alkalinity or other nutrients for microbial growth, the effect of basal medium addition (Vanderbilt Medium, VM) (Uludag-Demirer et al., 2008) was also tested. After the initial pH was adjusted to 7.2 ± 0.3 by adding 2 M solutions of hydrochloric acid and sodium hydroxide, the bottles were purged with a 80/20 (by volume) mixture of N_2/CO_2 gas for 3 min prior to sealing with butyl rubber septa and aluminum crimp tops. Reactors were incubated at 35 ± 0.5 °C for 45 days. Gas volumes and contents were quantified periodically, until the weekly gas production was less than 5% of the cumulative value. Test and control reactors were run in triplicate, whereas blank reactors were run in duplicate. Biogas volumes in control bottles were subtracted from those of tests before reporting.

2.6. Overall bioenergy yields

The overall bioenergy yields of ethanol fermentation, anaerobic digestion, and the two processes coupled together were calculated for both duckweed sources, using lower heating values of ethanol and methane of 29.7 MJ/kg and 35.8 MJ/kg, respectively (Wu et al., 2015). For these calculations, the yields of ethanol (Table 1) and biomethane (Fig. 1) were considered on a TS basis. The energy input and output associated with enzyme, yeast, and pH adjustment were assumed to be negligible.

3. Results and discussion

3.1. Fermentation experiments

Ethanol fermentation potentials of duckweed obtained from the EM and LF were quantified in terms of glucose recovery, glucose recovery efficiency, ethanol concentration in the fermentation broth, fermentation efficiency, and ethanol yield (Table 1). The results revealed that only 55.5% of the glucose could be recovered from EM duckweed after enzymatic saccharification. Since α-amylase was added in proportion with the starch content, EM duckweed received lower quantities of the enzyme. Therefore, the poor glucose yield for EM duckweed can be attributed to a slower rate of liquefaction due to lower α-amylase availability.

Despite relatively low glucose recoveries, the ethanol concentration observed in the EM duckweed fermentation broth was 3.2 g/L, which corresponds to an ethanol yield of 0.50 g/g glucose recovered. This relatively high conversion efficiency might be a result of ongoing enzymatic activity, which may have increased glucose availability during the fermentation process and consequently boosted its simultaneous conversion into ethanol. By comparison, the glucose recovery for the LF duckweed was 17.9 g/L, corresponding to 97.6% of the theoretical value. This value is similar to the sugar recovery reported by Xu et al. (2011), as 96.8% of the theoretical glucose saccharification of *S. polyrrhiza* starch using the enzymes α-amylase, pullulanase, and amyloglucosidase for hydrolysis. The ethanol concentration in the LF duckweed fermentation broth after 48 h was 7.3 g/L, which corresponds to a yield value of 0.41 g ethanol/ g glucose recovered. This result is slightly lower than the average value reported by Yu et al. (2014) as 0.44 g/g (as glucose) for duckweed grown on Schenk & Hildebrandt medium and sewage wastewater, following sugar recoveries of 94%.

3.2. Biochemical methane potential (BMP) assays

Approximately 90% of the total biogas production was observed in the first 20 days in all reactors. The biogas production was proportional to the VS concentration of substrate provided. The biomethane yields of the reactors varied between 141 and 390 ml CH_4/g VS_{added} (Fig. 1a–d), which is comparable to that reported by Jain et al. (1992) as 176 ml CH_4/g VS_{added}. No methane production was observed in blank reactors (data not shown).

The raw EM duckweed yielded slightly lower biomethane (234 ml CH_4/g VS_{added}), compared to that of LF duckweed (260 ml CH_4/g VS_{added}) at an S/I value of 0.5. However, for an S/I of 1.0, EM and LF duckweed yielded similar biomethane (258 and 259 ml CH_4/g VS_{added} respectively). Compared to BMP assays conducted on other raw bioenergy crops, these values are consistent with the literature. For instance, lignocellulosic feedstock such as straw, yielded a methane potential between 180 and 320 ml CH_4/g VS, whereas starch crops showed higher, yet comparable, methane yields of 250–406 ml CH_4/g VS_{added} for corn, and 310–430 ml CH_4/g VS_{added} for potatoes. In general, both raw and fermented EM duckweed reactors yielded less biomethane than their LF duckweed counterparts. This could be explained by the lower readily biodegradable (i.e., starch) content and higher recalcitrance (i.e., lignin) of EM duckweed.

Interestingly, basal medium (VM) addition had a negative effect on biomethane yields. This result may be related to the higher buffering capacity and higher pH values in reactors supplemented with VM, compared to reactors with no VM supplementation. Indeed, final pH measurements revealed pH values from 7.2 to 7.6 for VM-supplemented reactors, compared to pH values from 6.5 to 7.0 for reactors with no VM addition (data not shown). High pH conditions may have resulted in an "inhibited steady state", during which the ammonia concentrations may have risen to levels high enough to cause process instability and temporary VFA accumulation (Montingelli et al., 2015).

In general, higher biomethane yields were observed in reactors with a larger S/I of 1.0 (Fig. 1A–D). The highest biomethane yield among all reactors was 390 ± 0.1 ml CH_4/g VS_{added}, in the reactor with fermented LF duckweed (FLF) without VM addition, at an S/I of 1.0. This value was 51.2% higher than the corresponding raw duckweed reactor with no VM addition at an S/I of 1.0 (LF 1.0). The superior biomethane production in reactors fed with fermented duckweed indicates that upstream ethanol fermentation had a positive impact on methanogenic activity. This has previously been attributed to direct interspecies electron transfer pathways triggered by the presence of ethanol in methanogenic digesters (Zhao et al., 2017), which enhance the synthrophic metabolism of VFAs such as propionate and butyrate (Zhao et al., 2016). Biomethane produced with both fermented duckweed types was higher than that reported for the anaerobic digestion of food waste fermentation residues of 248 ml CH_4/g VS_{added} (Wu et al., 2015).

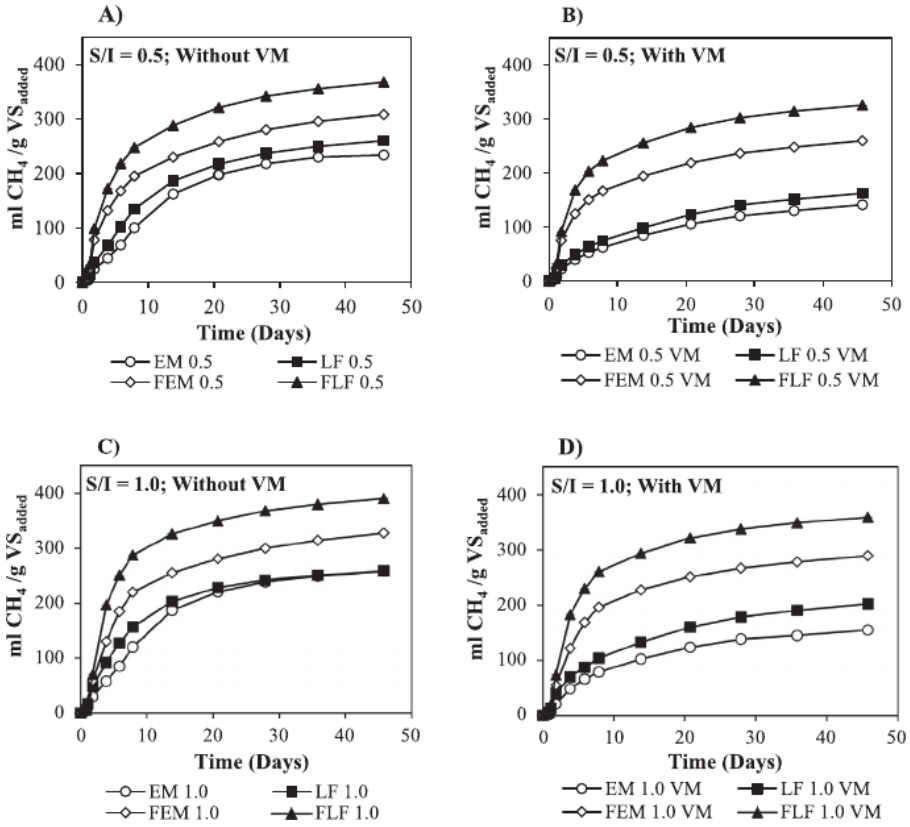

Fig. 1. Cumulative methane production (ml CH4/g volatile solids added) in batch reactors fed with raw Eco-Machine™ duckweed (EM), raw Living-Filter duckweed (LF), fermented Eco-Machine™ duckweed (FEM), fermented Living-Filter duckweed (FLF) at different substrate-to-inoculum (S/I) ratios and with and without the addition of Vanderbilt Medium (VM): A) S/I = 0.5, without VM; B) S/I = 0.5, with VM; C) S/I = 1.0, without VM; D) S/I = 1.0, with VM.

3.3. Overall bioenergy yields

Overall bioenergy yields of EM and LF duckweeds by separate and sequential processes of ethanol fermentation and anaerobic digestion are summarized in Table 1. Comparison of the separate processes revealed that biomethane production from duckweed provides higher energy gain. Therefore, 100% of the duckweed biomass allocated to biomethane production was used as the basis of comparison for the energy yield performance of the coupled process. However, it is important to note that relative market values of bioethanol and biomethane may lead to a difference in the allocation of duckweed end products. The highest bioenergy yield in this study was obtained from LF duckweed subjected to the coupled sequential bioethanol and biomethane process, which provided 70.4% higher overall energy yield compared to sole biomethane production. This value is comparable to the literature. For example, thermochemically pretreated oat straw recovered 85–87% higher heating value from the biomass in the coupled process, which is 28–34% higher than direct anaerobic digestion (Rabelo et al., 2011). Based on these results, the coupled process seems more attractive for enhancing bioenergy gain. Techno-economics of the coupled process must still be taken into account to arrive at a definitive conclusion.

4. Conclusion

In this study, it was demonstrated that significant methane production from duckweed is possible. Contrary to the current literature, from an energy yield standpoint, anaerobic digestion of duckweed seems to be a more reasonable approach than its fermentation into ethanol. Nevertheless, upstream ethanol fermentation results in even higher (51.2%) biomethane yields when compared to anaerobic digestion of raw duckweed, increasing the overall energy gain by 70.4%. To further demonstrate the technical feasibility of a coupled system, mass and energy balances, as well as a techno-economic analysis of the coupled system, must be performed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.02.053.

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