

Process Development and Optimization of Wastewater Derived Integrated Biorefinery for Production of Bio-Based Energy Carriers Employing *Spirodela polyrhiza*

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Abstract

The excessive use of agrochemicals to fulfill the high demand of agricultural products has been imposing numerous negative impacts on the environment for decades worldwide. Untreated wastewater with high chemical load and unutilized nutrients of fertilizers and pesticides released from agricultural farms results in contamination and intoxication of clean water bodies. Giant duckweed specifically *Spirodela polyrhiza* specie has shown the potential to efficiently uptake nutrients from nutrient rich waters thus, it can be employed cost-effective approach for phytoremediation of agricultural runoff. *S. polyrhiza* biomass also serves as an alternative feedstock for the production of biofuels which is another precedence of using this method. In this study, the potential of *S. polyrhiza* for removing nitrogen and phosphates from the agricultural runoff was determined by growing it in the agricultural runoff under lab conditions. Upto 70% and 78% removal of nitrogen and phosphates respectively was achieved during the treatment of agricultural runoff by *Spirodela polyrhiza*. The starch content of *Spirodela polyrhiza* was elevated 58% during 10–12 days of nutrient starvation at the end of the agricultural runoff treatment process. The biomass obtained was pretreated with dilute acid that liberated 96.4% glucose from the starch-rich biomass. This glucose was used as a substrate for the fermentation process carried out by *Saccharomyces cerevisiae* QG1 MK788210 yeast strain which yielded 99% volumetric conversion of glucose into bioethanol. The remaining fermentation vinnasse was anaerobically digested and 589 Nml/g VS biogas yield was obtained. To the best of our knowledge this is the first study that develops a lab-scale bioprocess in terms of integrated wastewater derived *S. polyrhiza* biorefinery.

1. Introduction

Intensive use of fertilizers and pesticides in the agriculture sector to fulfill global food production demand has adversely affected and contaminated terrestrial, aquatic as well as the coastal marine environment by adding a wide range of hazardous chemicals and loads of nitrogen and phosphorus fertilizers in agricultural wastewater (Carvalho and Security, 2017). Agricultural wastewater is one of the leading cause of water pollution which not only intoxicates water but also pose serious threats to human as well as aquatic life. It also contaminates water reserves causing eutrophication that makes water unavailable for public use (Shrestha et al., 2016). Higher population growth rates along with an alarming increase in the toxic pollutants have tremendously increased the demand for clean water globally. To subside water scarcity, multiple physical and chemical purification techniques and treatments like membrane filtration, adsorption, chemical precipitation, oxidation, photocatalysis, as well as certain biological methods have been introduced and adopted to clean up wastewater and make it reusable by eliminating organic, inorganic and biological pollutants (Bolisetty et al., 2019). With the advent of various modern technologies designed for water purification, there is still room for modification or finding new cost-effective, and reliable wastewater treatment methods that may remove pollutants from wastewaters.

Duckweed is a tiny plant that floats on the water surface and multiply through the vegetative budding of fronds (leaf like thallus). Duckweeds are monocotyledonous plants distributed in five different genera (Lemna, Wolffia, Landoltia, Wolffiella and Spirodela) and 37 diverse species. Over the past few decades,

duckweed has been investigated as a potential source of cleaning up nutrient rich wastewater due to its ability of up taking excessive nutrients and tolerance to high nitrogen and phosphorus load. A few of these small floating hydrophyte duckweeds that belong to family *Lemnaceae* like *Spirodela oligorhiza*, *Spirodela punctate*, *Lemna japonica* have been previously explored for biological nutrients removal from wastewater under different conditions generating high protein biomass (Cheng and Stomp, 2009; Xu and Shen, 2011; Zhao et al., 2015).

In addition to duckweed's phytoremediation abilities, it is also considered as a potent source of starch-rich biomass based on its faster growth rates along with high dry weight starch content of On dry basis 3–75% increase in starch content can be obtained from different species on applying different growing conditions. Variation in plant growing conditions for example nutrient concentration in the growth medium, phosphate concentration and pH can also increase the total starch accumulation in the plant. Due to these capabilities, duckweed is a promising feedstock for the biofuels production (Cui and Cheng, 2015).

By doubling its mass every 16 to 24 hours under suitable environmental conditions, its growth rate is higher than many other plants. Cultivation of duckweed plants could reach to the maximum level of $29 \text{ g m}^{-2} \cdot \text{day}^{-1}$ (on dry basis) and on annual basis it can reach to the maximum level of $106 \text{ t ha}^{-1} \cdot \text{year}^{-1}$ (dry basis). On comparing this annual yield with other crops like wheat $3.15 \text{ t ha}^{-1} \cdot \text{y}^{-1}$, corn $7.48 \text{ t ha}^{-1} \cdot \text{y}^{-1}$ and barley $3.70 \text{ t ha}^{-1} \cdot \text{y}^{-1}$, duckweed is a potential starch crop for the bioethanol production (Cui and Cheng, 2015).

Spirodela polyrhiza, also known as giant duckweed has been studied previously under nutrient starved conditions and the acid pretreated biomass was utilized for the production of bioethanol (ul ain Rana et al., 2019). In this study, *Spirodela polyrhiza* was grown in agricultural runoff that was synthesized at the lab by mixing NPK fertilizer in distilled water to test the capability of the plant to uptake nitrogen and phosphorus from the agricultural runoff. The biomass synthesized by the plants during their growth in agricultural runoff was used further for the production of bioethanol and biomethane via biorefinery approach.

2. Materials And Methods

2.1 Collection and Culturing of Duckweed (*Spirodela polyrhiza*):

Plant sampling was done from local garden ponds in Islamabad, Pakistan. The plant was then identified to be *Spirodela polyrhiza*, which is commonly known as giant duckweed. The plant was submitted to the National Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, and its accession number was acquired. *Spirodela polyrhiza* being an aquatic plant needs a stable environment, adequate light, optimum pH, and temperature range for proper growth. Hoagland Media (KNO_3 1.515g/L, KH_2PO_4

0.68g/L, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 1.18 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.492g/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.00022g/L, H_3BO_3 0.00285g/L, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.00012g/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.00008 g/L, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.00362g/L, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.0054 g/L and tartaric acid 0.003g/L) was used to grow plants. Hoagland Media contains all the vital micro and macronutrients needed by the plant to grow including. Healthy fronds of *Spirodela polyrhiza* were selected and washed carefully with 30% sodium hypochlorite solution to remove algal and bacterial contamination and were afterwards suspended in hoagland solution present in 10" × 10" sized clear plastic containers. The pH of Hoagland solution was maintained at 5.8 and plants were placed in the growth chamber under fluorescent light (continuous illumination with light intensity of 120–150 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 25°C (Singh et al., 2018).

2.2 Preparation and Culturing of *Spirodela polyrhiza* in Agricultural Runoff:

Agricultural runoff was prepared by dissolving NPK fertilizer in distilled water. About 9.8 grams of solid NPK was weighed and dissolved in 1000 ml of distilled water to form a solution similar to agricultural runoff. pH of this solution was adjusted at 5.8. A solution was then prepared by mixing 66% lab prepared agricultural runoff and 34% Hoagland solution and was poured into a 12" × 6" glass box and *S. polyrhiza* was cultured in it. The mixing of NPK and Hoagland solution was done to ensure the availability of all necessary nutrients because NPK solution lacks the other essential micronutrients required for plant growth. Instead of plastic boxes, glass trays were used in the experiment to avoid the absorption of nitrates and phosphates in plastic. Before culturing the plants in agricultural runoff, the initial nitrogen and phosphorous content present in the solution was determined. Afterward, a few healthy plants from the cultured stock and were allowed to grow in the prepared agricultural runoff. pH of the final solution i.e agricultural runoff was adjusted at 5.8 and the bioremediation was carried out in the afore mentioned growth chamber. The bioremediation was carried out for 30 days till the decline in nitrogen and phosphate concentration became constant. Nitrogen and phosphate concentration was determined every 3rd day. The plants were harvested once the bioremediation reaction was halted for further study.

2.3 Biochemical Analysis:

For the determination of nitrogen and phosphorous content present in the agricultural runoff, Total Nitrogen Determination Kit and Phosphate Determination Kit (Merck, NY, USA) were used. The analysis was carried out using Spectroquant® Prove UV/VIS spectrophotometer (Merck, NY, USA). For determination of starch content in plant biomass, Appenroth method was used (Appenroth et al., 2010). For this purpose, about 200 mg of fresh plant was ground thoroughly and homogenized in 4 ml of 18% HCL. The mixture was then stirred for 1 hour at 5 °C. The completely homogenized mixture was collected and centrifuged at 12000 RPM for 20 minutes. Later on, the supernatant was collected and mixed with Lugol's solution (KI 0.5% w/v and I_2 0.25% w/v in distilled water) in 1:1 ratio. Optical density of this solution was taken at 530 nm and 605 nm via spectrophotometer. The starch content was then calculated through following equations:

$$\text{S\%} = [\text{Cs} \times \text{Vol (extr)} \times 100] / \text{FW}$$

Where, Vol (extr) was the plant extract volume after homogenization with HCl (mL), FW was fresh weight (mg), $C_s = A_{605}/(0.07757 - P + 4.463)$ and A_{605} and A_{530} were the absorbances at 605 nm and 530 nm.

P was calculated by using the formula:

$$P = [(7.295 \times A_{605}/A_{530} - 4.463)/(7.757 - 0.729 \times A_{605}/A_{530})] \times 100$$

2.4 Dilute Acid Pretreatment of Plant Biomass

The plant biomass was pretreated with dilute acid at previously optimized conditions in order to convert plant starch to glucose. For this purpose, the biomass was treated with 0.2% sulfuric acid for 1 hour at 121 °C and the feedstock loading rate was kept to be 100g/L. Pretreated plant biomass suspension was centrifuged at 14000 RPM for 20 minutes and supernatant (acid hydrolysate) was collected (ul ain Rana et al., 2019). DNS method was used for determination of the glucose concentration present in the solution after pretreatment (Miller, 1959).

2.5 Fermentation of Glucose obtained from *Spirodela polyrhiza* Biomass by *Saccharomyces cerevisiae* QG1 MK788210.

Saccharomyces cerevisiae QG1 MK788210 was indigenously isolated and previously optimized to give highest ethanol yield by fermenting pretreated hydrolysate of *S. polyrhiza* (ul ain Rana et al., 2019). The pH of acid hydrolysate was adjusted to 4.8. Fermentation was carried out by adding 15% v/v of 72 hours old yeast suspension, 70% v/v acid hydrolysate containing released glucose and 15% v/v yeast fermentation media (yeast extract 9 g/L, peptone 7 g/L, KH_2PO_4 6 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L and $(NH_4)_2SO_4$ 1 g/L) to fermentation reactor. Nitrogen sparging of the fermentation reactor was carried out to develop anaerobic conditions and reactor was air sealed. Fermentation was carried out at 30 °C and 150 RPM and incubation time was 48 hours.

2.6 Analysis:

For determination of ethanol titer after fermentation, enzymatic ethanol detection was carried out using Megazymes Ethanol Assay Kit (Bray, Ireland) according to manufacturer's instructions.

Inoculum for anaerobic digesters was obtained from an already running reactor and was degassed for 3 weeks at 37°C. TS and VS determination in order to calculate inoculum to substrate ratio for anaerobic digestion was carried out by the method proposed by Sluiter et al., (Sluiter et al., 2008).

2.7 Anaerobic Digestion Reactor Setup

For the purpose of anaerobic digestion 100 ml glass reactors with air tightening rubber stoppers having outlet for gas collection were used. The reactors were attached to 60 ml gas collection vials. The working volume of the reactors was kept to be 70 ml. The inoculum to substrate ratio was kept to be 1:4 (Holliger et al., 2016). Cellulose was used as positive control whereas inoculum alone was used as negative

control. All the reactions were carried out in triplicates. In order to calculate the amount of inoculum and substrate to be added in reactor according to 1:4 ISR equations 2 and 3 were used:

$$\text{Amount of Substrate} = \frac{\text{Working Volume} \times \text{VS of Inoculum}}{\text{VS of Inoculum} + 4 \times \text{VS of Substrate}} \dots\dots (\text{Eq. 2})$$

$$\text{Amount of Inoculum} = \text{Working Volume} - \text{Amount of Substrate} \dots\dots (\text{Eq. 3})$$

The reactor was operated for 27 days. In order to determine amount of biomethane produced, biogas was passed through reactor containing scrubbing solution (3M NaOH) which effectively absorbed CO₂ and released methane (Abdeen et al., 2016). The biogas and biomethane values obtained were converted and reported in normalized liters (at standard temperature and pressure).

3. Results And Discussion

3.1. Phytoremediation of Agricultural Runoff by *Spirodela polyrhiza*

Agricultural runoff was prepared in lab by dissolving commercially available NPK fertilizer in distilled water in such a way to mimic the nitrogen and phosphate concentration that is present in naturally occurring agricultural runoff. Nitrate and phosphate concentration were kept higher as compared to reported concentration to evaluate the plant's ability to survive in higher NPK load. Table 1 shows the chemical composition of NPK fertilizer used to prepare agricultural run-off. Numerous studies have suggested and reported the utilization of duckweed species for phytoremediation of wastewaters rich in nutrients like nitrates and phosphates which includes municipal wastewater and agricultural runoff (El-Kheir et al., 2007; Landesman et al., 2010; Ceschin et al., 2019; Kreider et al., 2019). Agricultural runoff was selected in this study for analyzing the potential of *Spirodela polyrhiza* to remove nitrates and phosphates from wastewater. Plants were cultured in mixture containing 66% lab prepared agricultural runoff and 34% Hoagland media to supplement plant growth with micronutrients. The experiment was conducted for 30 days and total nitrogen and total phosphates in the agricultural runoff were measured after every 3rd day. The concentration of nitrates and phosphates in the runoff declined rapidly in the first 22 days. However, after 22nd days the removal of nitrogen and phosphate by the plant slowed down and gradually stopped by day 30th. The results indicated 70% removal of nitrogen and 78% removal of phosphates from the wastewater by *Spirodela*, which indicates that this plant can efficiently and effectively phytoremediate agricultural runoff. Table 2 shows the obtained Nitrogen and phosphate contents removed by *Spirodela polyrhiza* from Agricultural Runoff during 30 days of treatment. Previously Mohedano et al., 2012 has reported 98% and 98.8% total N and total P recovery from swine waste water using *L.punctata* in full scale duckweed ponds (Mohedano et al., 2012) whereas Xu and Shen reported removal efficiencies of 83.7% and 89.4% for total N and total P respectively for *Spirodela oligorhiza* in 8 weeks (Xu and Shen, 2011). The lower removal efficiency of duckweed in this study is due to the fact that the agricultural

runoff prepared in this study had far more initial nitrogen and phosphate concentration than the previously reported studies.

Table 1
Composition of NPK Fertilizer used in the lab as synthetic agriculture runoff.

Chemical	w/w percentage
Total Nitrogen (N)	20%
Potassium (K ₂ O)	14%
Phosphorus Pentoxide (P ₂ O ₅)	8%
Sulphur Soluble in water	9.7%
Magnesium (Mg)	2%
Copper, Zinc, Iron, Boron, Molybdenum	Traces

Table 2
Nitrogen and phosphate contents removed by *Spirodela polyrhiza* from agricultural Runoff during Treatment Days.

Treatment Days	Total Phosphate Content (mg/L)	Total Nitrogen Content (mg/L)
1st	20	161
4th	18	138
7th	16	122
10th	13	97
13th	10	77
16th	9	51
19th	5	48
22nd	5	47
25th	4	47
28th	4	47
30th	4	46

3.2 Starch Accumulation by *S. polyrhiza*:

Various studies have reported that plants of duckweed specie has the ability to accumulate starch in their biomass at varying growth conditions (Xu et al., 2011;Xiao et al., 2013). This high starch plant biomass is then a potential feedstock for third generation bioethanol production (Ge et al., 2012). A decreased bioavailability of nitrates and phosphates in the plant growth media is one of the factors that increase starch concentration in plant biomass (Ekperusi et al., 2019). The starch content of *Spirodela polyrhiza* was continuously monitored during the phytoremediation experiment. A continuous elevation of the starch levels in the plant biomass was observed as the nitrogen and phosphate concentration in the agricultural runoff declined. By the day 30th a 58% (Fresh Weight) of total starch content increase in plant biomass was observed (Fig. 1).

3.3 Dilute Acid Pretreatment of *Spirodela* Biomass Harvested after Phytoremediation:

The plant biomass harvested after phytoremediation of agricultural runoff had elevated levels of starch which can effectively be utilized for production of bioethanol. As the yeast used in process of fermentation for production of ethanol can only uptake glucose, thus the plant biomass with starch was pretreated with dilute acid at conditions previously optimized specifically for high starch *Spirodela* biomass, for conversion of plant starch to glucose. About 96.4% conversion rate of starch to glucose was obtained after the pretreatment.

3.4 Fermentation of Glucose Obtained After Pretreatment with *Saccharomyces cerevisiae* QG1 MK788210 for Bioethanol Production:

Batch fermentation of plant derived glucose was carried out using yeast strain *Saccharomyces cerevisiae* QG1 MK788210 which was previously optimized to give 93% theoretical yield of ethanol while using 150 g/L of glucose. The pretreated plant hydrolysate was fermented using afore mentioned yeast strain at previously optimized conditions and 99.8% ethanol theoretical yield was achieved.

3.5 Determination of Total and Volatile Solids of Inoculum and Feedstock

The TS and VS of substrate and inoculum for anaerobic digestion to produce biogas was carried out. Table 3 shows the obtained TS and VS of fermentation vinasse.

Table 3
Total Solids and Volatile Solids Content of Substrates and Inoculum
Used for Anaerobic Digestion.

Substrate	Total Solids (%)	Volatile Solids (%)
Fermentation Vinnasse	5.48 ± 0.34	4.65 ± 0.2
Inoculum for Anaerobic Digestion	1.79 ± 0.058	1.23 ± 0.03

3.6 Anaerobic Digestion of Fermentation Vinnasse:

Anaerobic Digestion in batch mode for fermentation vinnasse, cellulose as positive control and inoculum as negative control was carried out for 27 days. The biogas and biomethane yield of 0.6 NL/g VS and 0.5 NL/g VS was obtained, respectively (Figs. 2 & 3). Use of fermentation vinnasse for production of biogas in addition to providing a biorefinery approach for treatment of agricultural runoff via *S. polyrhiza* can also help in management of distillery waste and can also be used by distillery itself instead of natural gas. According to an estimate carried out by O'shea et al., (2020) 64% of the distillery's natural gas consumption can be reduced if anaerobic digestion of distillery byproducts is carried out and resultantly produced biogas is utilized by distillery instead of natural gas (O'Shea et al., 2020). This will also lead to decrease in green house gas emission due to natural gas burning in distilleries.

4. Conclusion

This study concludes that the duckweed species *Spirodela polyrhiza* is a potent option to resolve two major issues simultaneously. First, it can remediate agricultural runoff by removing excessive nutrients out of it that reduces water pollution. Second, the plant biomass obtained as by-product could be used as a potential feedstock for the production of bioethanol and biomethane that addresses the problem of the energy crisis. *S. polyrhiza* was able to achieve 70% and 88% removal of nitrates and phosphates, respectively with 58% enhancement in its starch content. Dilute acid pretreatment converted 96.4% of this starch into glucose which was further fermented to bioethanol by indigenously isolated and optimized yeast strain *Saccharomyces cerevisiae* QG1 MK788210. Starchless biomass and fermentation vinnasse was then anaerobically digested for biomethane production with the yield of 0.6 NL/gVS. All the results obtained indicate that *Spirodela polyrhiza* has the capability of finding promising applications within the fields of bioremediation and bioenergy and an integrated agricultural runoff derived *S. polyrhiza* biorefinery is a perfect approach to achieve sustainability in terms of both environment and energy.

Declarations

Declaration of Interest:

The authors declare that they have no competing interest.

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CRedit authorship contribution statement

Qurrat ul ain Rana: Conceptualization, Data Curation, Formal Analysis, Methodology, Writing Original Draft. Fatima Riaz: Investigation, Methodology, Data analysis, Writing. Saleha Perveen: Methodology, Data Curation, Writing – Review and Editing. Muhammad Irfan: data Curation, Formal Analysis, Writing – Review and editing. Sidra Ali – Data Curation, Writing – Review and Editing. Malik Badshah: Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing.

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Figures

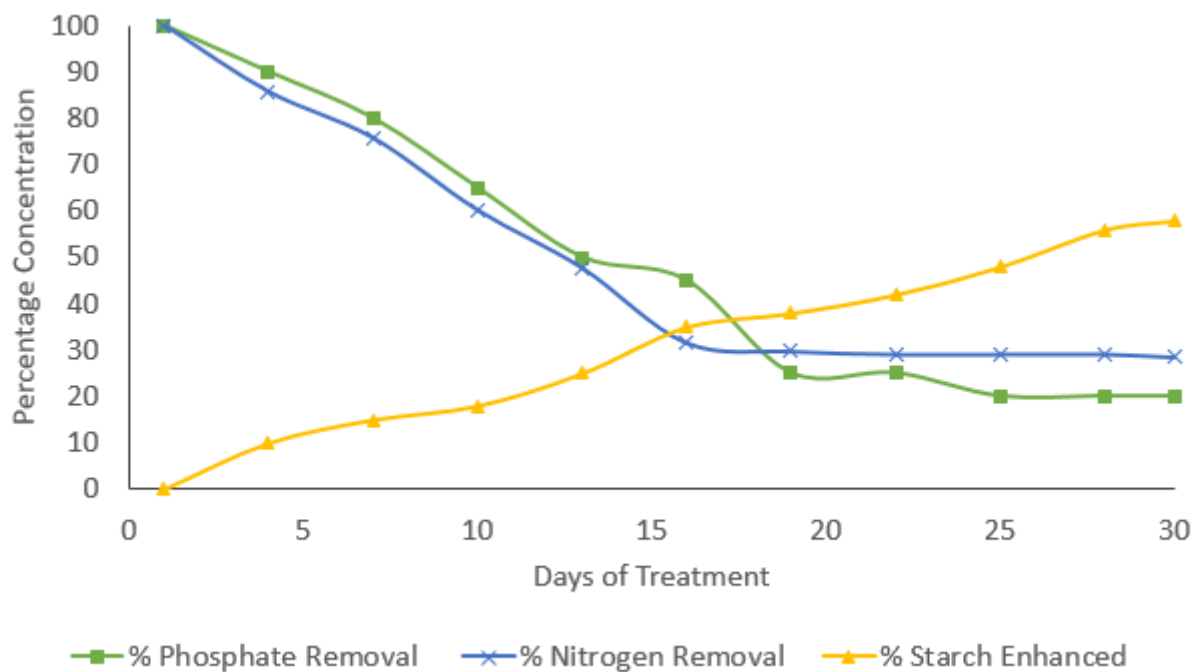


Figure 1

Percentage Reduction of Nitrogen and Phosphate Concentration in Agricultural Runoff with Percentage Elevation of Starch Levels in *Spirodela polyrhiza* Biomass.

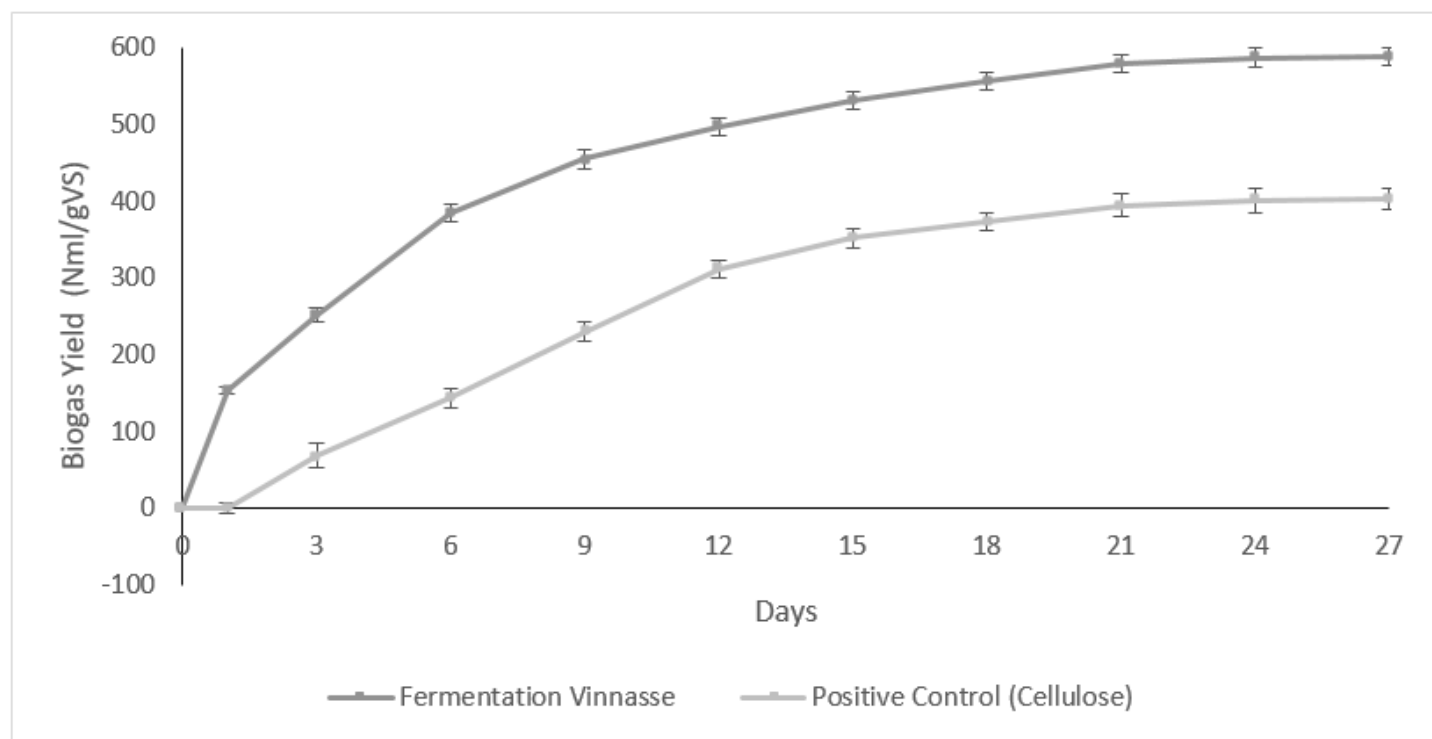


Figure 2

Biogas yield using plant biomass as substrate.

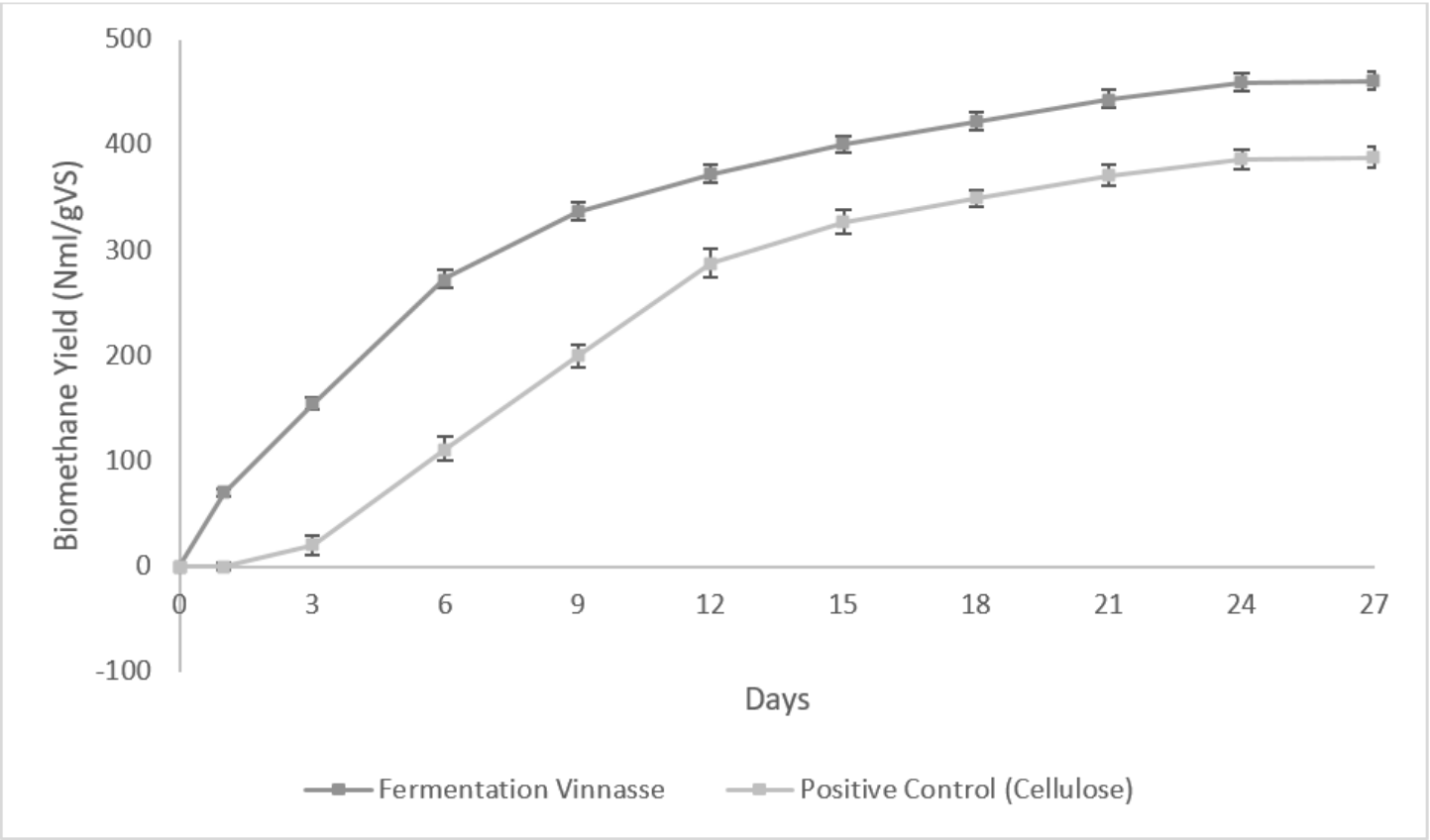


Figure 3

Biomethane yield using plant biomass as substrate.