

Article

Effect of Bioaugmentation with Anaerobic Fungi Isolated from Ruminants on the Hydrolysis of Corn Silage and *Phragmites australis*

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Abstract: Anaerobic fungi produce extracellular hydrolytic enzymes that facilitate degradation of cellulose and hemicellulose in ruminants. The purpose of this work was to study the impact of three different anaerobic fungal species (*Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27, and *Piromyces rhizinflatus* YM600) on hydrolysis of two different lignocellulosic substrates, corn (*Zea mays* L.) silage and reed (*Phragmites australis* (Cav.) Trin. ex Steud.). Biomass from each plant species was incubated anaerobically for 11 days either in the presence of live fungal inoculum or with heat-inactivated (control) inoculum. Headspace gas composition, dry matter loss, soluble chemical oxygen demand, concentration of volatile fatty acids, and chemical composition were measured before and after hydrolysis. While some microbial activity was observed, inoculation with anaerobic fungi did not result in any significant difference in the degradation of either type of plant biomass tested, likely due to low fungal activity or survival under the experimental conditions tested. While the premise of utilizing the unique biological activities of anaerobic fungi for biotechnology applications remains promising, further research on optimizing culturing and process conditions is necessary.

Keywords: *Anaeromyces mucronatus*; lignocellulose; *Neocallimastix frontalis*; *Piromyces rhizinflatus*; pretreatment; hydrogen; biomass



Citation: Ravi, B.; Nkongndem Nkemka, V.; Hao, X.; Yanke, J.; McAllister, T.A.; Lee, H.; Veluchamy, C.; Gilroyed, B.H. Effect of Bioaugmentation with Anaerobic Fungi Isolated from Ruminants on the Hydrolysis of Corn Silage and *Phragmites australis*. *Appl. Sci.* **2021**, *11*, 9123. <https://doi.org/10.3390/app11199123>

Academic Editor: Ramaraj Boopathy

Received: 9 August 2021

Accepted: 24 September 2021

Published: 30 September 2021

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1. Introduction

In Europe, more than 13,638 biogas plants (72%) utilize the agricultural feedstocks out of 18,943 biogas plants [1], among which corn (*Zea mays* L.) silage is the major feedstock [2]. While corn silage is the most widely used energy crop for biogas production, there is interest in using other sources of plant biomass for environmental, economic, and societal reasons [3]. For example, *Phragmites australis* (Cav.) Trin. ex Steud. (common reed) is a perennial invasive wetland plant species in North America that produces substantial quantities of biomass of up to 30 t ha⁻¹ y⁻¹ [4]. While all parts of the common reed can be used for both biogas and biofuel production [5], the estimated biogas yields reported in the literature are only 150 L kg⁻¹ volatile solids (VS) of fresh material compared to grass and pig manure that yield more than 280 and 340 L kg⁻¹ VS, respectively [4,6]. The issues of low degradability and poor conversion to biogas are also applicable to other potential energy crops, such as *Miscanthus* and *Arundo donax* L. [7,8].

In nature, one of the most efficient systems for unlocking the energy found in lignocellulosic substrates is the rumen animals such as cattle and sheep. While the stepwise fermentation process (hydrolysis, acidification, acetogenesis, methanogenesis) that occurs during anaerobic digestion (AD) is crudely similar to the digestive process in the rumen, it

is far less efficient [9]. One reason for the reduced efficiency of AD compared to the rumen likely lies in differences in the microbial populations between these two environments. The anaerobic digestive system of the rumen has been extensively studied, and anaerobic fungi (AF) are known to be involved in the digestion of the most recalcitrant lignocellulose within the rumen [10]. Anaerobic fungi use rhizoids to physically penetrate and disrupt the lignin layer of lignocellulose, while also enzymatically degrading plant cell walls using a diverse suite of extracellular hydrolytic enzymes, including cellulases, hemicellulases, pectinases, and phenolic acid esterases [11]. Some of the extracellular hydrolytic enzymes produced by these organisms are freely released into the milieu; others are bound to the cellular surface as components of multi-enzyme cellulosomes [12]. Using feruloyl esterase activity, AF cleaves the bonds between hemicellulose and lignin, increasing the access of microbial enzyme to hemicelluloses. Although AF are known to degrade lignin, they do not utilize the lignin themselves [13]. While AF are known to play an essential role within the rumen, their presence, abundance, and activity level in AD is not well understood.

Bioaugmentation involves adding specific microorganisms into a system or process in order to improve its efficiency [14]. Several studies have been conducted using bioaugmentation with bacteria or fungi as a pretreatment for the hydrolysis of lignocellulosic substrates prior to AD [12]. In one study, the addition of thermophilic *Geobacillus* sp. strain AT1 to a biogas reactor using sewage sludge as substrate resulted in a 210% increase in biogas production due to the protease activity of the microbe [15]. In another study, 22 isolates of white rot fungi were used individually to pretreat wheat straw, with the greatest lignin degradation and subsequent increase in biogas yield (from 0.293 L g⁻¹ to 0.343 L g⁻¹) obtained from an isolate of *Pleurotus florida* [16].

Recently, studies utilizing AF to improve biogas production and speed up substrate degradation have been reported [17–19]. To date, isolates of the genera *Anaeromyces*, *Neocallimastix*, and *Piromyces* have been added to AD systems in an effort to improve lignocellulose degradation and ultimately improve methane yield [18,19]. A previous study [18] demonstrated increased biogas yields from different substrates, such as maize silage, anaerobic sludge, and microcrystalline cellulose, with bioaugmentation of AF in fed batch semicontinuous digesters. In that study, addition of 8 mg dry mycelium of *Anaeromyces* sp. (strains KF8 or JF1) or mixed cultures of 1.9 mg dry mycelium of *Anaeromyces* sp. KF8 and *Piromyces* sp. KF9 increased biogas yield by up to 22%. Although the study demonstrated an increase in biogas yield with AF, the researchers did not determine if the increase in biogas occurred as a result of the addition of AF or the anaerobic microbes that were already present in the sludge. Another study [18] explored bioaugmentation of a two-stage reactor with *Piromyces rhizinflata*, using corn silage and cattail as substrates, which resulted in an initial increase of H₂ and CH₄ production but with no overall increase in biogas production. They proposed that this response occurred as a result of rapid wash out of AF from the anaerobic digester systems. There may also have been additional challenges with integration of AF into the microbial populations within the AD. A recent study [20] surveyed 10 agricultural biogas plants for the presence and transcriptional activity of AF, concluding that survival and activity were impeded by the process conditions prevalent in commercial scale biogas systems.

Based on the seeming lack of activity from AF in commercial biogas systems [20] and poor survival of AF bioaugmented into lab-scale AD systems [18], this study was designed to evaluate the efficacy of AF as a hydrolytic pretreatment for lignocellulosic biomass. We evaluated the effect of three different fungal species (*Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27, and *Piromyces rhizinflatus* YM600), which were previously isolated from ruminants and known to possess hydrolytic activity against lignocellulosic substrates, on microbial hydrolysis of corn silage and common reed.

2. Materials and Methods

2.1. Feedstock

Corn silage (*Zea mays* L.) and common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) were used as substrates for fungal hydrolysis. Corn silage was obtained from a commercial beef cattle feedlot in Lethbridge County, Alberta, Canada. Common reed, harvested in July, was obtained from Ridgetown, ON, Canada.

2.2. Anaerobic Fungal Strains, Media, and Culturing Conditions

Pure cultures of three AF were obtained from the microbial collection lab at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre: *Anaeromyces mucronatus* YE505 (elk isolate), *Neocallimastix frontalis* 27 (cow isolate), and *Piromyces rhizinflatus* YM600 (moose isolate). Inocula of the fungal cultures were maintained anaerobically at 39 °C in modified semi-defined Lowe's medium B [21] with barley straw (ground <1 mm) as the sole carbon source. The ground barley comprised 5% of the mass (0.05 g) of the anaerobic media (about 5 mL) in the test tube and was then autoclaved for 20 min at 120 °C with 103.4 kPa pressure. After autoclaving, the media was cooled down and fungal cultivation was carried out using the Hungate technique [22]; tubes were inoculated by transferring fungal biomass from already existing culture tubes using a Pasteur pipet under anaerobic conditions. After inoculation, tubes were incubated at 39 °C in an incubator for 4 days to allow for fungal growth, and then the AF with spent medium was transferred to Erlenmeyer flasks at the start of the hydrolysis experiment.

2.3. Hydrolysis Experiment

Hydrolysis of plant biomass was evaluated in 0.5 L Erlenmeyer flasks. The total solids (TS) content of all flasks was set at 7.9% (*w/w*). A single lot of anaerobic sludge was obtained from a commercial scale biogas facility (Lethbridge Biogas LP) that co-digests livestock manures with industrial food processing waste. Anaerobic sludge was autoclaved for 20 min at 120 °C with 103.4 kPa pressure to inactivate background microbial activity and then used as a buffering solution in each flask. Triplicate samples of autoclaved sludge were analyzed and used to determine the chemical and physical properties. The sludge had a pH of 7.88, total bicarbonate alkalinity of 16.66 g L⁻¹ and TS of 1.66%.

A total of 36 flasks were used for this hydrolysis experiment. Flasks containing either corn silage or common reed were individually inoculated with each of the three AF in triplicate. Each corn silage flask contained 200 mL of anaerobic sludge, 80 mL fungal inoculum (comprising 20% of the total working volume), 92.8 g of corn silage, and 100 mL of distilled water. Each common reed flask contained 200 mL of anaerobic sludge, 80 mL fungal inoculum, 57.6 g common reed, and 140 mL of distilled water. Control flasks were also set up in triplicate in a manner identical to those described above, except that the fungal inocula were first killed by autoclaving prior to addition to the flasks. Inoculated flasks were then flushed with nitrogen for 1–2 min to ensure anaerobic conditions and sealed with butyl rubber stoppers connected to aluminum gas tight bags (Multilayer Transofoil, Flextrus AD, Sweden) as described in [23]. Flasks were equipped with sampling ports for gas and liquid sample extraction. The experiment was conducted under mesophilic conditions (40 ± 1 °C) by placing flasks in a water bath (2870; Thermo Fisher Scientific, Waltham, MA, USA) and manually agitated at least three times a day.

2.4. Analytical Methods

2.4.1. Gas Analysis

Gas samples (10 mL) were taken daily from the headspace of each flask and transferred to 5.9 mL evacuated glass vials (Exetainer; Labco Limited, Lampeter, UK) prior to analysis using gas chromatography (GC). Gas samples were analyzed for CO₂ and CH₄ concentrations using a two-channel micro-GC (Varian 4900, Palo Alto, CA, USA) equipped with a thermal conductivity detector [24]. Operational parameters of the GC were as follows: channel A (H₂ analysis) injector 110 °C, column oven 40 °C, argon carrier gas at

150 kPa; channel B (CH₄, CO₂ analysis) injector 80 °C, column oven 40 °C, helium carrier gas at 100 kPa. Total gas volume from each flask was captured in individual gas-tight bags and quantified using a 0.1 L glass syringe (Perfektum™ Jumbo Glass Syringes, Cadence Science™, Cranston, RI, USA). Gas volumes reported were normalized to 0 °C and 1 atm.

2.4.2. Liquid Analysis

Liquid samples were extracted from a sampling port on each flask every 48 h using a 10 mL syringe and divided into aliquots for further analysis as described below. The TS and VS of liquid samples were measured following a standard protocol [25]. To estimate the extent of lignocellulose hydrolysis and the amount of remaining dissolved organic matter, soluble chemical oxygen demand (COD) was determined according to the manufacturer's protocol (Dr. Lange test kit HR mercury free, 20–1500 mg L⁻¹, Mississauga, ON, Canada). Samples used for COD analysis were first syringe filtered through 0.45 µm nylon filter (Chromatographic Specialties Inc., Brockville, ON, Canada) and then digested using a digital reactor block (HACH DRB200, Loveland, CO, USA) at 150 °C for 2 h. After digestion, absorbance of the sample was measured using a spectrophotometer (DR900, HACH, Mississauga, ON, Canada).

The pH and total bicarbonate alkalinity were measured using a BIOGAS titration Manager (R41T114, HACH, Vézenaz, Switzerland). Liquid samples were also analyzed for volatile fatty acids (VFA; acetate acid, n-butyrate, iso-butyrate, propionate, n-valerate, iso-valerate, and caproate) by GC (Agilent 6890 N, Agilent, Mississauga, ON, Canada). The samples were prepared by first filtering using 0.45 µm nylon filter (Chromatographic Specialties Inc., Brockville, ON, Canada), then 25% meta phosphoric acid was added to the filtered sample in the ratio of 5:1 sample to acid. The gas chromatograph was equipped with a flame ionization detector maintained at 250 °C, and a fused silica capillary column (ZB-FFAP, 30 m × 0.32 mm × 1.0 µm: Phenomenex, Torrance, LA, USA). The equipment was set at split mode and the split injection ratio was 5:1. Helium was used as the carrier gas and the analytical steps were performed according to the procedures outlined in Gilroyed et al. [26].

Concentration of soluble ions (NH₄⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺) were determined after filtration through 0.45 µm filter paper using ion chromatography (ICS-1000 and DX-600, Dionex, Sunnyvale, CA, USA). The concentration of free (unionized) NH₃ was calculated as previously reported [27]. To determine the ratio of total carbon to total nitrogen in samples, a subsample (5 mg) was freeze dried for 1 week and ground to a size < 0.15 mm using a Cyclone sample mill (UDY Corporation, Fort Collins, CO, USA), and then analyzed using a CNS analyzer (NA-1500, Carlo Erba, Rodano, Italy) linked via a continuous flow interface to an Optima isotope ratio mass spectrometer (Micromass, Manchester, UK).

2.4.3. Fiber Analysis and C:N

Fiber analysis was performed to characterize the composition (cellulose, hemicellulose, and lignin content) of corn silage and common reed before hydrolysis. Before taking samples for analysis, corn silage and common reed were thoroughly mixed in the containers that they stored to obtain a uniform and unbiased sample for analysis. Triplicate samples of each feedstock were air dried for 1 week and then ground through a screen of 1 mm mesh size in a tabletop mill grinder (Wiley mill standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA). The contents of lignin, hemicellulose, and cellulose were analyzed according to a modified method of [28] with thermal stable amylase (Termamyl® 120, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) and sodium sulfite (S430-3 sodium sulfite anhydrous, Fisher Scientific Int., Inc., Pittsburgh, PA, USA) included in the NDF procedure [29]. Total carbon and total nitrogen concentrations were determined from freeze-dried, finely ground samples using a Model 1500 Nitrogen/Carbon analyzer (Carlo Erba Instruments, Milan, Italy).

2.5. Statistical Analysis

One-way ANOVA and repeated measures ANOVA tests were performed for statistical analysis using IBM SPSS version 24.0. The different treatments (*Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27, and *Piromyces rhizinflatu*s YM600) were kept as independent variables, and the different analytical tests, such as average cumulative hydrogen and CO₂ gas production, and changes in COD, pH, and VFA, were considered as dependent variables.

3. Results and Discussion

3.1. Feedstock Characteristics

The TS of corn silage and common reed were 33.5% ± 0.6 and 54.7% ± 0.8, of which 96.3% ± 0.6 and 94.9% ± 0.4 were VS, respectively (Table 1). The C:N ratio of corn silage and common reed was 31.2% ± 0.1 and 26.3% ± 0.7, respectively; both values were almost within the optimum range of 20 to 30 for AD [30]. In terms of fiber composition, corn silage had about two times lower ($p < 0.05$) concentrations of cellulose, hemicellulose, and lignin than common reed (Table 1).

Table 1. The physicochemical characteristics of corn silage and common reed.

Parameters	Corn Silage	Common Reed
TS ¹ (%)	33.5 ± 0.6	54.7 ± 0.8
VS ² (% TS)	96.3 ± 0.6	94.9 ± 0.4
VS added (g)	30	30
Moisture content (%)	66 ± 0.6	44.3 ± 0.8
Total carbon: Total nitrogen ratio	31.2 ± 0.1	26.3 ± 0.7
Hemicellulose (% of TS)	12 ± 4.3	28.7 ± 0.4
Cellulose (% of TS)	17.2 ± 1.8	38.7 ± 0.4
ADL ³ (% TS)	2.7 ± 0.0	7.9 ± 0.3

¹ Total solids. ² Volatile solid. ³ Acid detergent lignin.

3.2. Gas Production and Composition

Cumulative methane production was <1 mL g⁻¹ VS in all treatments for both corn silage and common reed substrates. Since only hydrolysis was conducted in this study, minimal methane volume was expected. Anaerobic fungi are known to produce H₂ and CO₂ during substrate hydrolysis [31]. Nkemka and Gilroyed [18] demonstrated that bioaugmentation with anaerobic AF into a two-stage AD can increase H₂ production within the system in the days following inoculation. In our study, an initial increase in hydrogen (Figure 1) and CO₂ (Figure 2) production were observed with all fungal species that were added to digesters containing corn silage. Over the course of the 11-day hydrolysis experiment, all three fungal treatments produced similar cumulative volumes of H₂, in the range of 46–60 mL g⁻¹ VS ($p > 0.05$) (Figure 1a). Similar trends were observed for CO₂ gas production, with cumulative CO₂ production for all three treatments of corn silage in the range of 78–93 mL g⁻¹ VS ($p > 0.05$) (Figure 2a). The initial increase in gas production observed for corn silage may have been due to the fact that the material had already undergone ensiling. Additionally, background microbes such as H₂-producing *Clostridia* are known to be present in corn silage and were likely actively contributing to the gas production observed [32].

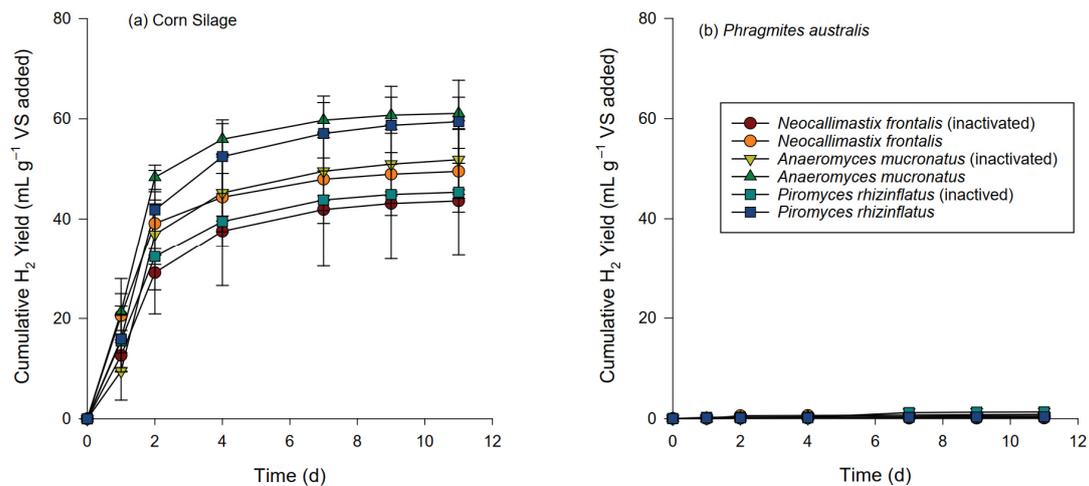


Figure 1. Cumulative hydrogen yield in the bioaugmentation of three different anaerobic fungal species: (a) corn silage (*Zea mays* L.); (b) common reed (*Phragmites australis*). Error bars show standard deviation.

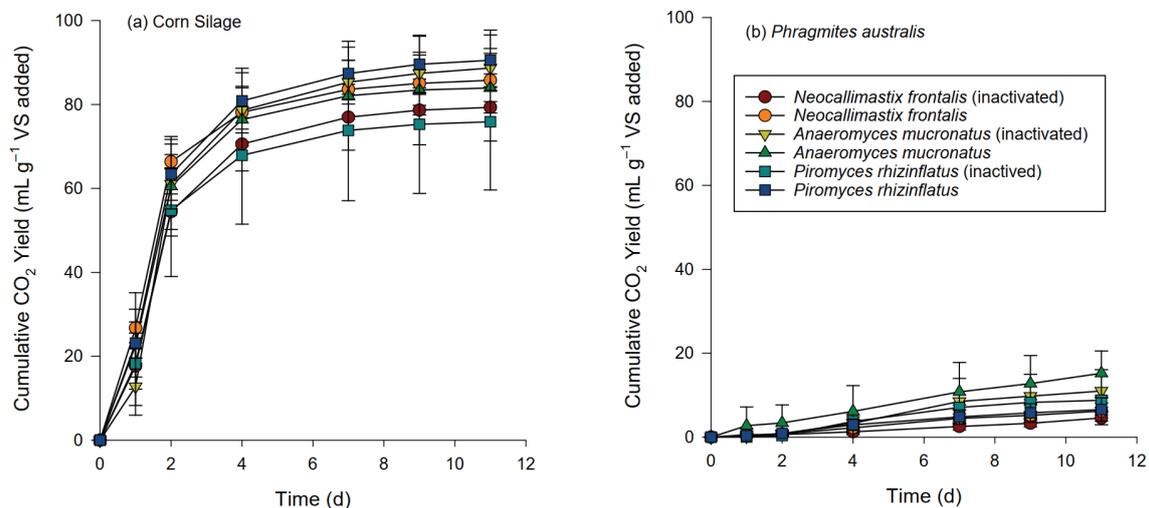


Figure 2. Cumulative carbon dioxide yield in the bioaugmentation of three different anaerobic fungal species: (a) corn silage (*Zea mays* L.); (b) common reed (*Phragmites australis*). Error bars show standard deviation.

During hydrolysis of common reed, smaller volumes of H_2 ($<1 \text{ mL g}^{-1} \text{ VS}$) (Figure 1b) and CO_2 ($<15.5 \text{ mL g}^{-1} \text{ VS}$) (Figure 2b) gas were evolved from all three treatments than was observed with corn silage ($p > 0.05$). Further studies are required to either eliminate the contribution of background microflora present on the substrate, or to account for the magnitude of their activity within the overall microbial consortia present during substrate hydrolysis.

3.3. Chemical Changes during Hydrolysis

Chemical oxygen demand was measured over the course of the hydrolysis experiment to examine the amount of soluble COD released due to hydrolysis and for further AD (Figure 3). Soluble COD concentrations for corn silage treatments trended upwards over time but did not significantly differ pre- and post-hydrolysis (Figure 3a). Similarly, soluble COD concentration did not increase in common reed (Figure 3b), regardless of treatment ($p > 0.05$), and was approximately half the value compared to corn silage. The higher initial COD concentration in corn silage compared to common reed was likely due to conversion of some corn biomass to soluble fermentation products during the ensiling process.

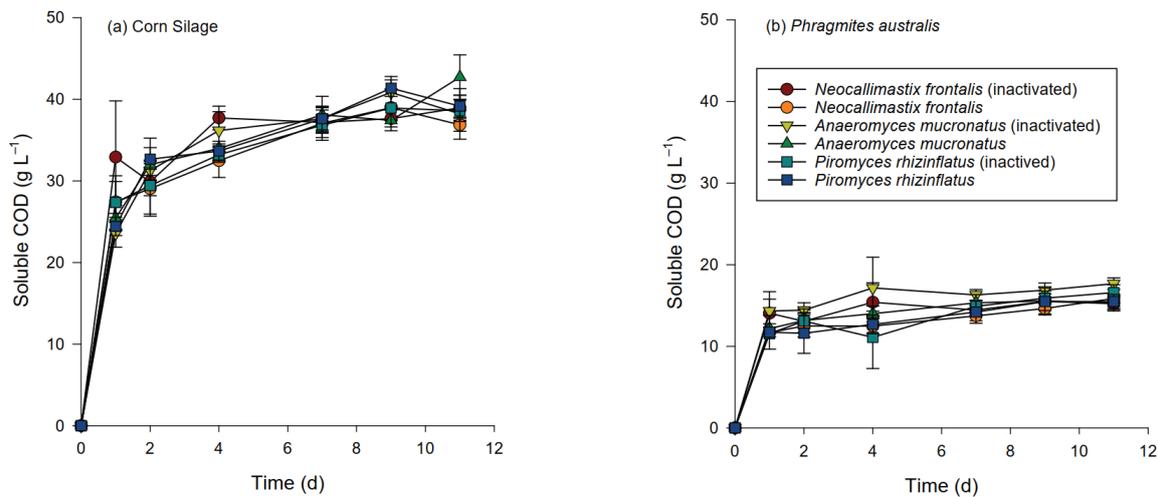


Figure 3. Soluble COD production in the bioaugmentation of three different anaerobic fungal species: (a) corn silage (*Zea mays* L.); (b) common reed (*Phragmites australis*). Error bars show standard deviation.

The main VFA produced during hydrolysis of corn silage were acetic, propionic, and butyric acids. Total VFA concentration trended upward over the course of the hydrolysis experiment for corn silage in all treatments; however, these increases were not statistically significant (Figure 4). In comparison, VFA production during hydrolysis of common reed was limited, with no significant difference in concentration observed over the course of the experiment for any treatments. It is unlikely that the lack of VFA accumulation could have been attributed to microbial conversion, as minimal gas volume was produced, suggesting an overall lack of microbial activity. The absence of a functional methanogenic phase in the experimental system could have been inhibitory. The inhibitory concentration of VFA for the specific fungal species investigated here is unknown, but it is possible that the concentrations observed were detrimental to continued fungal growth.

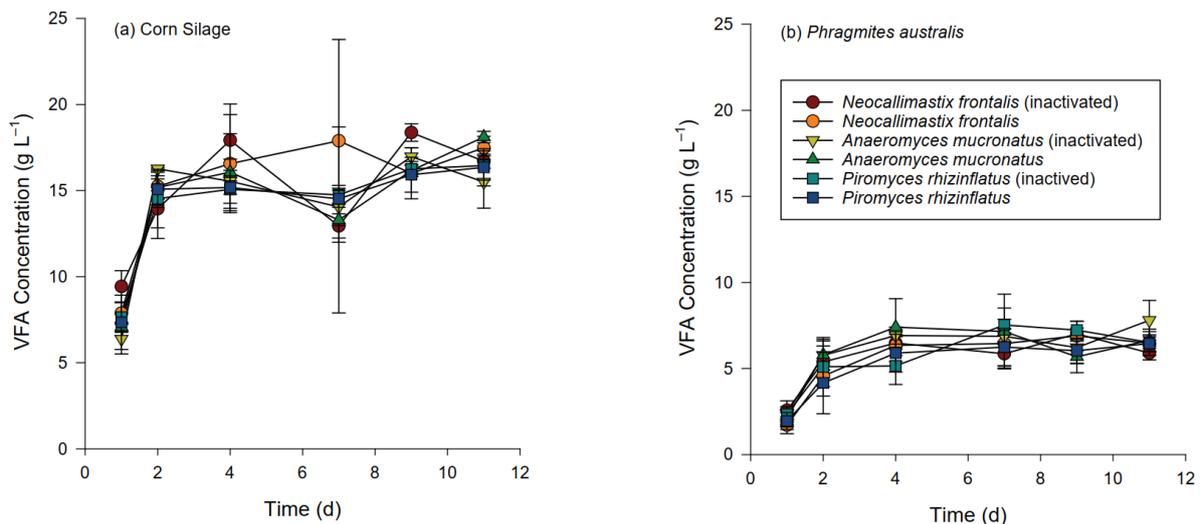


Figure 4. Volatile fatty acids (VFA) production in the bioaugmentation of three different anaerobic fungal species with and without activation: (a) corn silage (*Zea mays* L.); (b) common reed (*Phragmites australis*). Error bars show standard deviation.

The pH of the digestate in corn silage flasks decreased from approximately 7.0 on day 1 to 5.6–5.8 on day 11 for all treatments (Figure 5a). The pH of digestate in common reed flasks ranged between 7.2–8.0 on day 1 (Figure 5b), which was not statistically different from corn silage ($p > 0.05$). At the end of hydrolysis, the pH values of common reed decreased to 6.1–6.9, which again was not statistically different from corn silage ($p > 0.05$).

The optimum pH range for the growth of AF is between 6.0 and 7.0 [33], so reduction of pH to <6.5 due to VFA accumulation may have contributed to conditions unfavorable for anaerobic fungal activity. In the rumen, there is both a constant supply of buffering capacity as well as organic acid removal through the production of saliva and the symbiotic activities of the host animal and microbial consortium, respectively. The complexity of the rumen system is difficult to mimic in vitro in the laboratory, but a better approximation of the conditions which are favorable for AF to survive and be active will be essential for future success in this area of research.

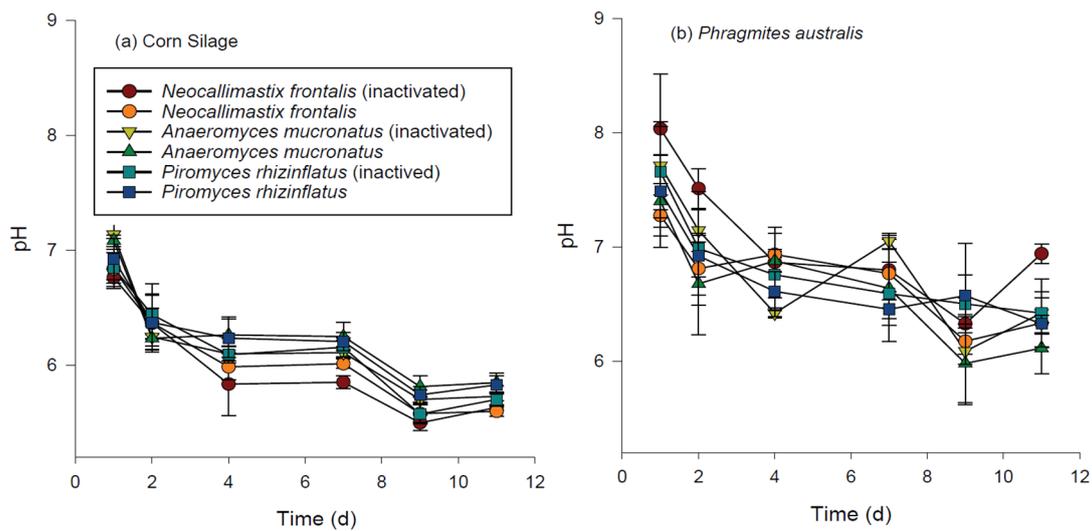


Figure 5. Changes in pH in the bioaugmentation of three different anaerobic fungal species with and without activation: (a) corn silage (*Zea mays* L.); (b) common reed (*Phragmites australis*). Error bars show standard deviation.

Ammonia is known to inhibit hydrolysis during AD at >200 mg L⁻¹ [34], but that threshold was not exceeded in our study (Table 2). Similarly, metals can inhibit biological hydrolysis processes when present in sufficient concentration. Alkaline metals, such as Na⁺, Mg²⁺, K⁺, and Ca²⁺, up to a range of 400 mg L⁻¹ help maintain alkalinity and pH in AD. However, higher concentrations would cause toxicity and inhibit AD processes [34]. In our study, Na⁺ and Mg²⁺ concentrations were very low (<1 mg L⁻¹) in all treatments and well below reported inhibitory levels, i.e., <750 mg L⁻¹ for Mg²⁺ [35] and 3500–5500 mg L⁻¹ for Na⁺ [36]. Concentration of K⁺ was 1–3 mg L⁻¹ for all treatments, again below the inhibitory concentration of 400 mg L⁻¹ [34]. Similarly, for Ca²⁺ the values were <1 mg L⁻¹ for all treatments and below inhibitory concentrations (>7000 mg L⁻¹). Based on this, the low degree of hydrolysis observed in all treatments was not likely caused by inhibition from ammonia or metals.

When considering our overall results, we can conclude (1) that there was limited hydrolytic activity in any of the reactors, regardless of fungal species or substrate type, and (2) the activity that was present was likely due to background microflora, including the bacteria that are present on the feedstock and not the AF. The most likely explanation for these results is that the AF were unable to survive, or at least be active, in the environment provided in this study. Low activity and survival of anaerobic fungi when applied to non-rumen environments has been reported by others [17,18,20].

Table 2. Effect of ammonia and alkaline metals in the bioaugmentation of three different anaerobic fungal species.

Substrate	Fungal Species	Active/Inactivated	NH ₃ mg L ⁻¹	Na ⁺ mg L ⁻¹	K ⁺ mg L ⁻¹	Mg ²⁺ mg L ⁻¹	Ca ²⁺ mg L ⁻¹
Corn Silage	<i>Neocallimastix frontalis</i>	Inactivated	N/A	0.87 ± 0.18	2.14 ± 0.13	0.14 ± 0.00	0.17 ± 0.00
	<i>Neocallimastix frontalis</i>	Active	N/A	0.80 ± 0.08	2.20 ± 0.08	0.17 ± 0.02	0.23 ± 0.02
	<i>Anaeromyces mucronatus</i>	Inactivated	N/A	0.79 ± 0.08	2.19 ± 0.17	0.15 ± 0.02	0.21 ± 0.03
	<i>Anaeromyces mucronatus</i>	Active	N/A	0.75 ± 0.02	2.07 ± 0.02	0.14 ± 0.01	0.20 ± 0.02
	<i>Piromyces rhizinflata</i>	Inactivated	N/A	0.72 ± 0.03	2.00 ± 0.11	0.12 ± 0.00	0.18 ± 0.01
	<i>Piromyces rhizinflata</i>	Active	N/A	0.72 ± 0.03	2.08 ± 0.09	0.13 ± 0.01	0.20 ± 0.01
<i>Phragmites australis</i>	<i>Neocallimastix frontalis</i>	Inactivated	78.45 ± 5.62	0.57 ± 0.03	1.63 ± 0.02	0.12 ± 0.01	0.19 ± 0.02
	<i>Neocallimastix frontalis</i>	Active	14.71 ± 0.09	0.61 ± 0.02	1.64 ± 0.03	0.12 ± 0.00	0.22 ± 0.01
	<i>Anaeromyces mucronatus</i>	Inactivated	41.10 ± 0.42	0.53 ± 0.04	1.59 ± 0.02	0.10 ± 0.00	0.21 ± 0.00
	<i>Anaeromyces mucronatus</i>	Active	37.66 ± 14.56	0.64 ± 0.02	1.58 ± 0.03	0.08 ± 0.00	0.16 ± 0.01
	<i>Piromyces rhizinflata</i>	Inactivated	44.11 ± 2.05	0.59 ± 0.03	1.63 ± 0.07	0.08 ± 0.00	0.16 ± 0.00
	<i>Piromyces rhizinflata</i>	Active	25.81 ± 0.36	0.58 ± 0.03	1.58 ± 0.08	0.10 ± 0.00	0.21 ± 0.01

Anaerobic fungi are known for having a close symbiotic activity and interspecies H₂ transfer with other microbes in the rumen [37,38]. The absence of these relationships, or the lack of time for such relationships to develop using the experimental design of this study, may also account for the poor hydrolytic activity observed. Coculturing AF with other rumen hydrolytic bacteria, such as *Fibrobacter succinogenes*, could be a potential approach to take in the future to increase viability [39]. Joblin et al. [40] inoculated *F. succinogenes* together with methanogenic cocultures of *Caecomyces/M. smithii* grown on rye grass. They found that there was an increase in stem degradation and attributed this to complementary fibrolytic activities between the two species. By comparison, our study utilized only AF to help degrade the substrate. The heat sterilization used to eliminate background microflora from anaerobic digestate may have limited potential for symbiotic relationships to develop between AF and bacteria. Conversely, antibiosis has been reported between ruminal bacteria and AF in laboratory studies [41], which highlights our current poor understanding of AF ecology.

Yildirim et al. [42] recently reported up to a 60% increase in biogas yield from animal manures bioaugmented anaerobic fungi. In that study, the authors used an undefined mixture of AF isolated from a cow's rumen, resulting in an AF community composed of >6 groups (including *Anaeromyces* spp., *Neocallimastix* spp., and *Piromyces* spp. used in our study) [43]. It is possible that the mixed AF culture approach is a better strategy for ensuring AF activity and survival when used in bioaugmentation compared to single species inoculations, as we have described here. The benefit of a mixed AF culture could be due simply to higher diversity increasing the chances for survival under artificial conditions, and/or could be due to interactions between the different community members. The combination of our results and those by [42] strongly suggest that successful outcomes from the addition of AF to hydrolysis and/or anaerobic digestion may require use of mixed complex communities.

4. Conclusions

Hydrolysis of corn silage and common reed were not improved by bioaugmentation with three different species of AF, as evidenced by a lack of significant H₂ production or substrate degradation compared to controls. The most likely explanation for these results is that AF had low activity and/or survival in the anaerobic fermentation systems used in this study. More research is required to better understand survival of AF in anaerobic digestion processes to determine the feasibility of exploiting these organisms for lignocellulosic degradation.

Author Contributions: Conceptualization, B.R. and B.H.G.; methodology, B.R., V.N.N. and J.Y.; software, B.R. and B.H.G.; validation, B.R., V.N.N. and B.H.G.; formal analysis, B.R.; investigation, B.R. and V.N.N.; resources, X.H., J.Y., T.A.M. and B.H.G.; data curation, B.R. and V.N.N.; writing—original draft preparation, B.R.; writing—review and editing, V.N.N., C.V., X.H., J.Y., T.A.M., H.L. and B.H.G.; visualization, B.R. and B.H.G.; supervision, X.H., H.L. and B.H.G.; project administration,

B.H.G.; funding acquisition, X.H., T.A.M. and B.H.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Natural Resources Canada Program of Energy Research and Development (grant number Inc001), Agriculture and Agri-Food Canada (grant number T.2002.36), and the Natural Sciences and Engineering Research Council of Canada (grant number 400806).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors wish to thank Kurtis Baute, Kimberley Van Overloop, Greg Travis, Douglas Marchbank, Stephanie Montienth, Winnie Lao, and Raene Barber for their technical assistance on this research project.

Conflicts of Interest: The authors declare no conflict of interest.

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