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Original Research Paper

# Enhancing waste degradation and biogas production by pre-digestion with a hyperthermophilic anaerobic bacterium

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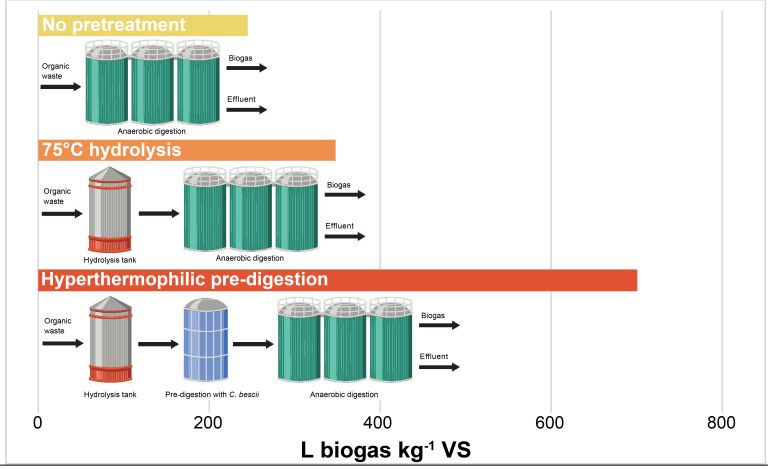
## HIGHLIGHTS

- Several organic wastes successfully pre-digested with *Caldicellulosiruptor bescii*.
- Waste destruction and biogas production increased in all cases.
- Pre-digestion doubled biogas production from dairy manure and waste activated sludge.
- Optimization requires optimizing both pre-digestion and digestion retention times.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The hyperthermophilic anaerobic bacterium, *Caldicellulosiruptor bescii*, is effective in degrading and solubilizing lignocellulosic materials. Laboratory studies have characterized the chemistry of the process for crystalline cellulose and switchgrass, but the data are insufficient for engineering commercial plants to use *C. bescii* for pre-digestion of waste streams. The purpose of this study is three-fold: 1) to identify any potential toxicities in *C. bescii* pre-digestion and biogas production from several wastes; 2) to determine the potential enhancement of biogas production by anaerobic digestion of pre-digested dairy manure and waste activated sludge; and 3) to identify variables that must be quantified and controlled for engineering commercial, continuous-flow systems for waste disposal and biogas production incorporating *C. bescii* pre-digestion. Tests were run at lab-, bench- and pilot plant-scale with *C.bescii* pre-digestion and controls run at 75°C and pH 7-8 followed by mesophilic anaerobic digestion at 37-41°C. The lab- and bench-scale tests demonstrate that *C. bescii* is capable of growing on several organic wastes and pre-digestion with *C. bescii* increases conversion of waste into biogas, typically by a factor of 2 or more. Incorporation of *C. bescii* pre-digestion in an optimized commercial system is predicted to provide 75-85% volatile solids conversion to biogas with 75% methane when digesting dairy manure and sewage sludge. Achieving these results at a commercial scale requires further work to quantify *C. bescii* growth and enzyme production rates, as well as rates of base- and enzyme-catalyzed hydrolysis of the polymeric materials, e.g., lignocellulose, in the waste in order to optimize retention times.

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**Abbreviations**

| | |
|---|---|
| AD | Anaerobic digestion |
| ASB | Anaerobic secretome bioreactor |
| COD | Chemical oxygen demand |
| CSTR | Continuously stirred tank reactor |
| DWAS | (anaerobically) Digested waste activated sludge |
| IBR | Induced bed reactor |
| LTHP | Low temperature hydrolysis process |
| TFS | Total Fixed Solids, defined as solids remaining after heating in air at 550°C |
| THP | Thermal hydrolysis process |
| TS | Total Solids, defined as mass remaining after drying at 105°C |
| TSS | Total Suspended Solids, defined as mass captured by a filter and dried at 105°C |
| VS | Volatile Solids, determined as weight loss from heating in air at 550°C |
| VSS | Volatile Suspended Solids, defined as dry solids captured on a filter and lost by heating in air at 550°C |
| WAS | Waste activated sludge |
| ΔG | Change in Gibbs energy |

## 1. Introduction

Transformative advancement in renewable energy production by anaerobic digestion (AD) of waste streams requires an inexpensive, simple, and scalable pretreatment to increase organic waste conversion into biogas (Atelge et al., 2020; Zamri et al., 2021). Production of biogas by AD offers a proven, readily-scalable, and well-understood mechanism for energy production and disposal of organic wastes. However, inefficient conversion of waste into biogas, typically 30-40% in mesophilic digesters without pretreatment (Rico et al., 2011; Nasir et al., 2012; Atelge et al., 2020; Tabatabaei et al., 2020; Liu et al., 2021), makes it difficult for AD to be an economically viable source of renewable energy. Improving the economic viability of AD in the renewable energy market, therefore, requires a low-cost, efficient pretreatment that consistently and significantly increases the fraction of biomass converted into biogas (Carrere et al., 2016; Anukam and Berghel, 2020; Atelge et al., 2020; Cheah et al., 2020; Sevillano et al., 2021).

Pretreatment of organic wastes prior to AD by physical (e.g., mechanical pulverization, cavitation, and limited pyrolysis), physicochemical (e.g., steam explosion and ammonia fiber explosion), chemical (e.g., acid hydrolysis, alkaline hydrolysis, high-temperature organic solvent pretreatment, and oxidative delignification), biological (e.g., lignin degradation by white- and soft-rot fungi), and electrical methods, and various combinations thereof, have existed for several decades, but are energy inefficient and are often not economically viable (Lee et al., 2016; Kumar and Sharma, 2017; Vyas et al., 2018; Anukam and Berghel, 2020; Atelge et al., 2020). To date, the only economically successful pretreatment method for increasing degradation and biogas production is the thermal hydrolysis process (THP), in which the influent is heated to 130-180°C for 30-60 min. THP of sewage sludges increases biogas yield by 50%, decreases viscosity, allowing higher loading rates, decreases effluent chemical oxygen demand (COD) by 50%, improves dewatering, and provides sterilized, odor-free compost (Liao et al., 2014).

The optimum system for waste pretreatment depends on the physical and chemical characteristics of the waste being treated, and for some wastes, a pretreatment that uses a thermophilic biological component may provide many of the same advantages as THP at less cost. A biological pre-digestion process is more energy-efficient than THP because it operates at lower temperatures and pressure. However, for some wastes, the optimum pretreatment may be to add thermophilic biology post-THP, which could be done with no additional energy cost because the influent is already heated. Such a combination of compatible pretreatments may provide a significant increase in performance over THP or biological pre-digestion alone for some wastes.

Many wastes are recalcitrant for AD because the organic solids are large, polymeric molecules, e.g., lignocellulose, that are not directly accessible to methanogens (Sayara and Sanchez, 2019; Atelge et al., 2020). Hydrolysis of these polymeric materials into small, soluble molecules or ions makes them readily accessible for methanogenesis and improves the rate and efficiency of conversion of the substrate into biogas by AD. Laboratory studies of enzyme-catalyzed hydrolysis and metabolism of the products of hydrolysis of switchgrass with *Caldicellulosiruptor bescii* (Kateava et al., 2013; Basen et al., 2014; Straub et al., 2019) have demonstrated a potential for substantially improving biogas yields and for increasing processing rates of lignocellulosic wastes by AD. *C. bescii* is a hyperthermophilic obligate anaerobe that grows at pH 7-8 and 70-80°C (Yang et al., 2010; Kataeva et al., 2013; Basen et al., 2014), and produces more than 50 different exozymes (Lochner et al., 2011), mostly hydrolases. The wealth of exozymes produced by *C. bescii* suggests pre-digestion is capable of catalyzing the hydrolysis of many other polymers such as polysaccharides, esters, and amides found in waste streams. Although *C. bescii* may not be capable of metabolizing some of the molecules from hydrolysis of some wastes, methanogenesis may still occur. The temperature and processing time of *C. bescii* pre-digestion also provide a side benefit of pasteurizing influent materials, which makes bio-solid byproducts more valuable as soil amendments.

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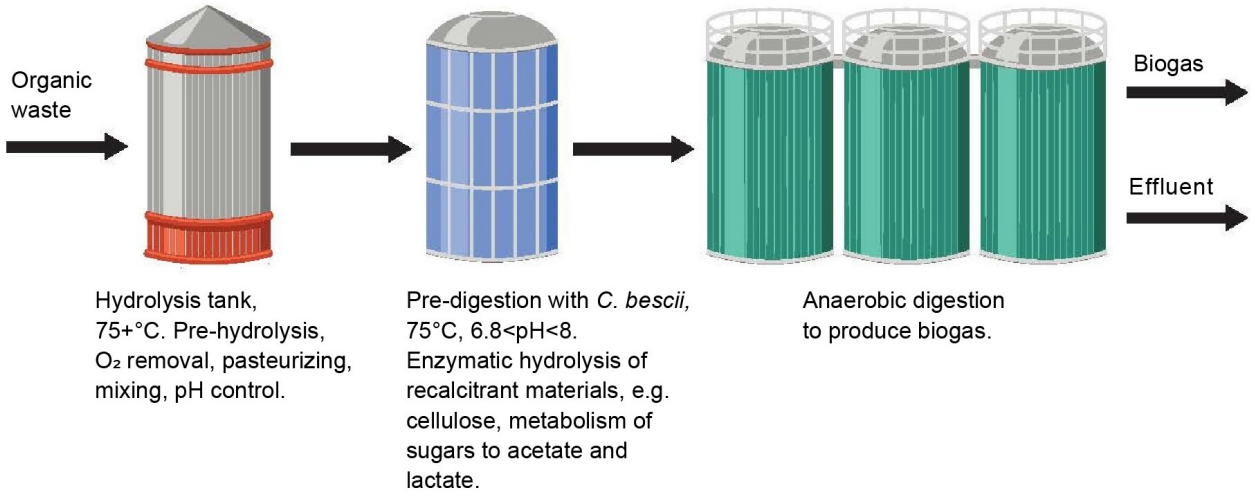


**Fig. 1.** The proposed commercial process occurs in three steps. First, the feedstock is mixed and heated in a hydrolysis tank to drive off $O_2$ and reach the requisite temperature and pH for the growth of *C. bescii*. Second, the feedstock is pre-digested in an anaerobic secretome bioreactor (ASB). Third, the predigested feedstock is anaerobically digested to produce biogas in a conventional anaerobic digestion vessel.

**Figure 1** shows a schematic diagram of how *C. bescii* pre-digestion would be implemented in a commercial plant for producing biogas from an organic waste stream. In the first stage or tank, an organic waste containing polymeric organic materials is suspended in water in a mixing-hydrolysis tank at 75°C or higher where partial hydrolysis of the substrate occurs, $O_2$ is removed by decreased solubility and reaction with the organic material, and the suspension is pasteurized. Note that this hydrolysis tank could be a THP tank which may be advantageous for some wastes. Pre-digestion by *C. bescii* takes place in a second stage or tank (termed an anaerobic secretome bioreactor, ASB) at 75°C and pH 7-8. *C. bescii* is a free-living organism, and at least some of the exozymes are free in the solution (Lochner et al., 2011), so the ASB tank must be well mixed to optimize contact with the substrate.

The temperature in the ASB is high enough to provide relatively fast reactions and short retention times, but not so high as to require special materials or designs for tanks, pumps, and fittings or to incur excessive heating costs. In the last phase, AD takes place in a third vessel that could be thermophilic or mesophilic. Although it was not examined in this study, thermophilic digestion may be advantageous since the energy cost of heating the influent has already been incurred during the pre-digestion phase. The hypothesis tested in this work is that pre-digestion of wastes with *C. bescii* will significantly increase the amount of volatile solid (VS) destroyed and therefore increase the yield of biogas and methane.

Engineering a commercial plant requires quantification of the chemistry of the processes for both pre-digestion and AD. Test-tube experiments described by Kataeva et al. (2013) and Basen et al. (2014) found that *C. bescii* rapidly depolymerizes and solubilizes switchgrass lignocellulose, producing sugars, acetate, lactate, and lignin fibers. Based on this information, the chemistry of the pre-digestion process with cellulose as the substrate is as follows (**Eq. 1**):

cellulose + $H_2O$ → glucose, represented as $C(H_2O)$ Eq. 1

After the cellulose is hydrolyzed, some of the glucose is taken up and metabolized to acetic and lactic acids as presented in **Equation 2**:

$C(H_2O)$ → acetic acid, $CH_3COOH$, & lactic acid, $CH_3CH(OH)COOH$ Eq. 2

These acids are immediately neutralized by bases in the media (Straub et al., 2019), represented here as bicarbonate (**Eqs. 3** and **4**):

$CH_3COOH + HCO_3^- \rightarrow CH_3COO^- + H_2O + CO_2(g)$ Eq. 3

$CH_3CH(OH)COOH + HCO_3^- \rightarrow CH_3CH(OH)COO^- + H_2O + CO_2(g)$ Eq. 4

The solution containing acetate, lactate, and remaining sugars is then transferred to the anaerobic digester, where the reactions are as follows (**Eqs. 5-7**):

$CH_3COO^- + H_2O \rightarrow CH_4(g) + HCO_3^-(aq)$ Eq. 5

$2CH_3CH(OH)COO^- + 2H_2O \rightarrow 3CH_4(g) + 2HCO_3^-(aq) + CO_2(g)$ Eq. 6

$2C(H_2O) \rightarrow CH_4(g) + CO_2(g)$ Eq. 7

Because bicarbonate ion is not volatile, the reaction presented by **Equation 5** produces 100% $CH_4$ gas, the reaction presented by **Equation 6** produces biogas with 75% $CH_4$ and 25% $CO_2$, and the reaction shown by **Equation 7** produces biogas with 50% $CH_4$ and 50% $CO_2$. Thus, because of the production of acetate and lactate from the metabolism of sugars, pre-digestion with *C. bescii* has the potential to increase methane concentration in the biogas. **Figure 2** shows how the biogas composition from methanogenesis of the products of *C. bescii* pre-digestion of cellulose depends on the mix of substrates.

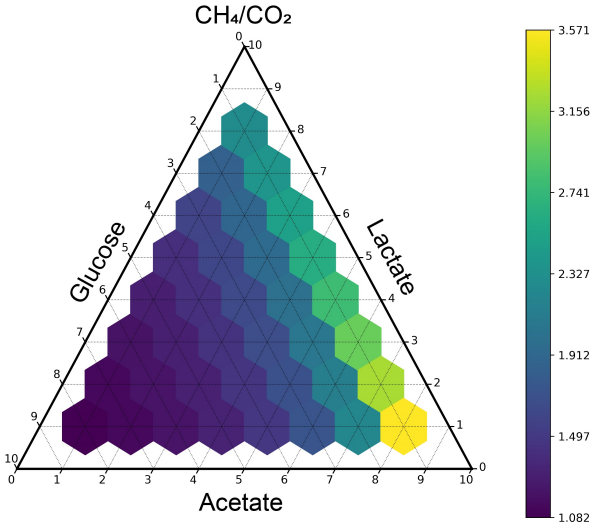


**Fig. 2.** Biogas composition as a function of the relative concentrations of acetate, lactate, and glucose in the anaerobic digestion feedstock. The colored bar shows the ratio of $CH_4$ to $CO_2$ from 3.6 (78% $CH_4$) to 1 (50% $CH_4$).

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The thermodynamics of the pre-digestion process is very favorable if there is a sufficient supply of base (alkalinity) in the pre-digestion vessel. The Gibbs energy change (ΔG) for hydrolysis of cellulose to glucose at the optimum temperature for growth of *C. bescii*, 75°C, is negative and large (Popovic et al., 2019), but the uncatalyzed reaction is extremely slow under most conditions. The exozymes from *C. bescii* catalyze rapid hydrolysis of cellulose and hemicellulose to glucose and other sugars. The *C. bescii* cells metabolize the sugars to acetic and lactic acids, but ΔG for the reaction is approximately zero, so the bacteria do not gain any energy for growth and other activities until the reaction with base occurs. Neutralization of acetic and lactic acids by bicarbonate to produce $CO_2$ gas provides a negative ΔG for the metabolism of sugars by *C. bescii* and thus provides the energy for growth and activities of *C. bescii*. The ΔG° values for the reaction of acetic and lactic acid with the bases commonly found in waste treatment systems are given in **Table 1**. The relatively large, negative ΔG° values show the reactions will go to completion even at very low concentrations of the acids.

**Table 1.**
ΔG° (kJ $mol^{-1}$) for the reaction of acetic and lactic acids with bicarbonate ion, hydroxide ion, and aqueous ammonia at 25°C. Note that these values are more negative at higher temperatures.

| Base | $HCO_3^-$ | $OH^-$ | $NH_3$ |
|---|---|---|---|
| Acetic acid | -17.5 | -52.8 | -25.8 |
| Lactic acid | -22 | -58 | -31 |

The three specific purposes of this work are: 1) to identify any potential toxicities associated with *C. bescii* pre-digestion and AD of pre-digested substrates from several different wastes, 2) to determine the potential enhancement of biogas production by the AD of pre-digested dairy manure and waste activated sludge (WAS), and 3) to identify variables that must be quantified for engineering commercial, continuous-flow systems for waste disposal and biogas production by *C. bescii* pre-digestion followed by AD. Potential toxicities in pre-digestion and methanogenesis were tested by determining the enhancement of biogas production in a bench-scale batch process. Pre-digestion and AD of WAS was further characterized in a lab-scale semi-continuous flow system, and dairy manure was further characterized in a bench-scale batch system. The commercial feasibility of *C. bescii* pre-digestion of dairy manure followed by AD for methane production was further examined in a semi-continuous flow pilot plant. In this study, *C. bescii* pre-digestion was compared with the low temperature hydrolysis process (75°C) (LTHP) (Lu et al., 2008; Ferrer et al., 2008) that was used as a control.

## 2. Materials and Methods

### *2.1. Caldicellulosiruptor bescii cultivation*

*C. bescii* was obtained as lyophilized inactivated culture from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, accession #6725. A culture was first grown on cellobiose in media 516 according to the instructions from the DSMZ (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium516.pdf) and used to inoculate the ASB vessels for pre-digestion, regardless of feedstock type.

### *2.2. Feedstock descriptions*

Diverse feedstocks containing various amounts of cellulose and other polymers were evaluated in the initial part of the study. Giant king grass (GKG, African *Pennisetum purpureum* Schumach [Poaceae]) was grown at the BYU Life Sciences Greenhouse from corms obtained from an experimental agricultural plot in California. GKG leaves were separated from stems, after which the material was chipped, shredded, and screened to 0.65 cm. Kentucky Bluegrass (*Poa pratensis* L. [Poaceae]) clippings were obtained from a local resident's lawn in Provo, UT. Mixed municipal green waste was obtained from the compost yard of the Springville, UT Wastewater Treatment Facility. The mixed green waste consisted of a chipped mixture of lawn clippings, leaves, branches, other plant materials, dirt, and pebbles. The raw material was sifted through a 0.65 cm screen and the fine material used in tests. Corn mash was obtained from a brewery in Colorado. Grass silage was obtained from Roeslein Alternative Energy. Dairy manure was obtained from the Bateman Dairy in Mosida, UT. Waste activated sludge (WAS) was collected from the DAF unit, and anaerobically digested waste activated sludge (DWAS) was collected from the digesters at the Orem, UT Wastewater Treatment Facility. The feedstock for the wastewater digesters was 50% primary sludge with 4-5% total solids (TS), and 50% WAS from the DAF containing 4% TS. The first digester was thermophilic at 55.6°C, and the second was mesophilic at 37.8°C with a total retention time of 21 d. All feedstocks were dried at 105°C and weighed to determine water content, and dried samples were analyzed for C, N, and P by the Brigham Young University Environmental Analytical Laboratory (https://pws.byu.edu/eal).

### *2.3. Experimental systems for pre-digestion and anaerobic digestion*

Three systems were used in the experiments. One system was a flow-through lab-scale system, with two 1-L pre-digestion anaerobic secretome bioreactor (ASB) vessels connected in series and connected to a 10-L anaerobic digestor. Another system was a bench-scale system with a 30-L pre-digestion (ASB) vessel and a 60-L digester that were not connected, so this system could only be used for batch operations. Both of these systems were twinned, with one inoculated with *C. bescii* and the other operated at the same conditions serving as a control. The third system was a pilot-scale, semi-continuous feed, 7500-L total volume system with a mixing tank, pre-digestion in an ASB, and two AD tanks. This system differed from the system detailed in **Figure 1** because the mixing tank was not heated. Details of the construction of these systems are given in the **Supplementary Data**; only relevant operational information is provided here.

#### *2.3.1. Lab-scale system and experiments with WAS and anaerobically digested WAS (DWAS)*

The lab-scale system was comprised of twin systems with two 1-L ASB vessels connected in series for pre-digestion and a 10-L vessel for AD. All three tanks were connected with 2.5 cm iron tubing from the top of one tank to the bottom of the next. The 1-L vessels were stirred at 100 rpm with paint stirrers and maintained at 75°C. The AD vessel was maintained at 37°C. Biogas was measured with Whisper series integrating flow meters calibrated with NIST traceable Mesa Labs FlexCal series flow meters. WAS and DWAS were evaluated in the lab-scale system. Waste was fed into the system twice a day with 200 mL portions from a stock that was kept in a refrigerator at 4°C to prevent reaction outside the system. The actual volumes used in the ASB tanks were 800 mL each and 5 L in the AD tank. Retention time in the ASB was 4 d and retention time in the AD was 12 d. *C. bescii* was acclimated for at least two days after the inoculation before data collection began. Pre-digestion of WAS with *C. bescii* noticeably reduced the viscosity but not sufficiently to allow complete transfer of solids so only total suspended solids (TSS), volatile suspended solids (VSS), and COD were determined in the input and AD effluent. Although complete transfer occurred with DWAS, for comparison, TSS, VSS, and COD were also determined in the input and AD effluent.

#### *2.3.2. Bench-scale system and experiments with dairy manure, WAS, and various other wastes*

The bench-scale system was comprised of twin systems with 30-L vessels for pre-digestion and 60-L vessels for AD. The pre-digestion vessels were stirred continuously at 40 rpm by paddle stirrers that extended to the bottom of the vessels. The AD vessels were maintained at 37°C and were not stirred. Biogas was measured with Whisper series integrating flow meters calibrated with NIST traceable Mesa Labs FlexCal series flow meters. Because the ASB and AD vessels were not connected, experiments were performed as batches. Feedstocks were mixed with sufficient deionized water to make a 29-L suspension of 3-5% solids and poured into the top of the 30-L vessels, and after the temperature reached 75°C, one liter of *C. bescii* culture was added to one of the 30-L vessels and one liter of distilled water was added to the other 30-L vessel. The control system was operated at the same temperature as the *C. bescii* vessel. The pH in the vessel with *C. bescii* was measured with a Cole-Palmer double junction electrode and pH meter calibrated with standard pH 4 and 7 buffers. The

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pH was maintained between 7 and 8 by periodic addition of KOH pellets as needed. The 30-L vessels were washed with distilled water between trials.

In the initial series of experiments on WAS, dairy manure, stems and leaves of giant king grass, grass clippings, green waste, grass silage, and corn mash, pre-digestion was conducted for 3-5 d after which the vessels were drained through a 0.16-cm screen, and the screened liquid was pumped into the anaerobic digesters after it had cooled to <40°C. Processing times, screening, and transfer were the same for the control and *C. bescii* systems. Two L of sludge was harvested from the bottom of the digester and added back into the digester along with the pre-digested material to maintain the methanogen culture in the digesters. Gas production was monitored until the rate dropped to <200 mL/h, at which point the digestion was considered complete.

The second set of experiments in the bench-scale system evaluated dairy manure in more detail with a 2-d retention time in the ASB. TS, total volatile solids (VS), and COD were determined in the suspensions before loading into the 30-L vessels. In these experiments, dairy manure from pre-digestion was not screened, and a 5-L representative sample from the 30-L vessels was pumped into the digesters. Because the AD tanks were not stirred, TS, VS, and COD values for digester effluent could not be obtained.

*2.3.3. Pilot plant and experiments with dairy manure*

**Figure S1** in the **Supplementary Data** is a design schematic diagram of the pilot plant. The system has two 938-L storage tanks, an 1875-L ASB pre-digestion vessel, and two 1875-L AD vessels operated in parallel. One AD vessel is a continuously stirred tank reactor (CSTR), and the other is an induced bed reactor (IBR). The ASB was operated at 75°C. The IBR was operated at 37°C, and the CSTR was operated at 41°C. The pH in the ASB was stable at 7.2-7.8. To inoculate the ASB tank, one liter of *C. bescii* was first grown on cellobiose, transferred to 30 liters of 4.5% manure at 75°C, and twenty-five liters was used to inoculate the ASB.

The feedstock for the pilot plant was obtained from the Bateman Dairy in Mosida, UT. Fresh manure was obtained after passage over a slope screen to remove sand and excess water, hauled to the pilot plant in storage tanks, and used within 72 h. The manure suspension in the feed tank was continually mixed using a paddle-type mixer rotating at 60 rpm. Biogas was collected in Tedlar (Cel Scientific) and Mylar (SKC Quality) sampling bags for transport to the lab for analysis for $CH_4$ and $CO_2$.

Analysis of samples taken from the feedstock, the ASB effluent, and both digester effluents included COD, TS, VS, and alkalinity.

Experiments were done at two different retention times in the ASB and AD tanks, 2 and 4 d and 4 and 8 d, respectively. The feedstock was pumped into the ASB at 5 gallons/h. The volume in the ASB was 500 gallons during the 4-d trials and 250 gallons during the 2-d trials. The volume of the ASB tank was maintained with a capacitance level controller (Mercoid Continuous Level Transmitter, Dwyer Instruments, Inc., Michigan City, IN) operating with a Labview program. The solution in the ASB tank was continuously mixed using a macerating pump (AMT) that pulled solution from the bottom of the tank and introduced it into the middle of the 12' tall ASB at a rate of 0.8 $m^3$/min.

*2.4. Analytical methods*

TS and VS were determined by adding a 15-mL sample to a 25-mL ceramic crucible. To determine TS, the crucible and sample were heated to 105°C for 24 h, cooled in a desiccator, and weighed. To determine VS, the dried sample and crucible were placed in a muffle furnace at 550°C for 15 min, cooled in a desiccator, and weighed to determine total fixed solids (TFS). VS was calculated from the recorded masses as (TS – TFS). TS, VS, and alkalinity measurements were done with adherence to the Standard Methods for the Examination of Water and Wastewater (Baird et al., 2017). Determinations of COD in suspensions were done with Hach COD kits TNT 823 and TNT 824, Loveland, CO (Baird et al., 2017). P and N in suspensions were done with Hach kits TNT 845 and 828, respectively. The presence of live *C. bescii* was determined by microscopy, but feedstock particles interfered with the enumeration of live cells.

*2.4.1. Analysis of biogas for $CH_4$ and $CO_2$ concentrations*

For all three systems, biogas was collected in Tedlar (Sigma-Aldrich, St. Louis, MO) or Mylar (SKC Quality) gas sampling bags for analysis for $CH_4$ and $CO_2$ with Edinburgh Sensors gas analyzers (Livingston, United Kingdom) calibrated with Airgas (Radnor, PA) standard mixtures of $CH_4$ and $CO_2$.

*2.5. Stoichiometric relations between biogas yield or methane yield and VS destruction*

The following stoichiometric relations relate the fraction of carbon in VS to the amount of biogas and methane that can be obtained from the conversion of VS to biogas. The biogas yield is directly proportional to the %C in the VS (**Eq. 8**).

$$m^3 \text{ biogas } kg^{-1} \text{ VS} = 0.0187(\%C \text{ in VS}) \quad \text{Eq. 8}$$

Typical values of %C are 45-50%C in vegetative plant material and 75-85%C in fats and oils.

The methane yield is also proportional to the percentage of carbon in the VS (**Eq. 9**):

$$m^3 \ CH_4 \ kg^{-1} \text{ VS} = 0.030(\%C \text{ in VS}) - 0.79 \quad \text{Eq. 9}$$

As a reference point, the calculated biogas yield from cellulose is 0.747 $m^3$ biogas/kg VS destroyed, and the biogas has 50% methane, so the estimated amount of methane produced is 0.272 $m^3$ $CH_4$/kg VS destroyed. These relations were used to estimate VS destruction, biogas yield, and methane yield when VS destruction or biogas and methane yield could not be measured.

## 3. Results and Discussion

*3.1. Results of preliminary experiments on various wastes*

The properties of the feedstocks, increases in biogas methane content, methane produced, and biogas produced by *C. bescii* during pre-digestion relative to the controls for the preliminary experiments done in the bench-scale system are provided in **Table 2**. The feedstocks tested produced on average 87% more biogas and 95% more methane than the controls, with a concomitant increase in VS destruction. Feedstocks with higher

**Table 2.**
Properties of feedstocks and ratios of methane content, total methane, and total biogas from *C. bescii* pre-digested feedstock to a 75°C hydrolyzed control.

| Feedstock | %C in TS | %N in TS | %P in TS | %CH4 in biogas, pre-digest/control[a] | Ratio $CH_4$, pre-digest/control[a] | Ratio biogas, pre-digest/control[a] |
|---|---|---|---|---|---|---|
| WAS | 39.5 | 6.7 | 2.0 | 1.22 | 1.71 | 1.39 |
| Cow manure | 39 | 1.9 | 0.05 | 1.03 | 1.60 | 1.37 |
| Stems GKG | 42 | 2.2 | 1.1 | 1.05 | 1.47 | 1.41 |
| Leaves GKG | 41 | 1.1 | 0.5 | 1.09 | 2.84 | 2.62 |
| Grass clippings | 43 | 3.1 | 0.6 | 1.11 | 1.25 | 1.14 |
| Green waste 1 | 22 | 2.1 | 0.02 | 0.95 | 1.71 | 1.77 |
| Green waste 2 | 22 | 2.1 | 0.02 | 1.15 | 1.55 | 1.34 |
| Grass silage | 48 | 0.8 | 0.09 | 1.25 | 4.24 | 3.21 |
| Corn mash | 47 | 5.0 | 0.9 | 1.02 | 2.04 | 2.01 |

[a] Both pre-digestion and control experiments were run until the rate of biogas production dropped to <200 mL/h; ratios are calculated from total biogas and $CH_4$ production during a run.

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cellulose/lignin ratios (i.e., GKG leaves, grass silage, and corn mash) produced more biogas from pre-digested materials compared with controls than feedstocks with lower cellulose/lignin ratios (i.e., dairy manure and GKG stems). The methane content (%) in the biogas also increased for all the tested wastes except green waste 1. No toxicities to either *C. bescii* or methanogens were apparent, as demonstrated by the increase in biogas production and observations of live *C. bescii* in the effluent after pre-digestion.

### *3.2. Results of experiments on WAS and DWAS*

VSS from WAS was reduced by 52% in the ASB tank in the lab-scale system by solubilizing solids. Also, as a consequence of *C. bescii* pre-digestion, the viscosity of WAS was noticeably reduced, probably because of enzyme-catalyzed hydrolysis of the polyhydroxyalkanoates (biodegradable polyesters) synthesized by filamentous bacteria. Experiments on WAS diluted to 4% TS in the bench-scale system with a 2-d retention in the ASB and 17 d in the AD showed 85% conversion into biogas with 70% methane. The control, hydrolyzed at 75°C for 2 d, showed 49% conversion into biogas with 69% methane. These data show *C. bescii* could be used to double biogas production by pre-digesting the influent to a WAS digester. Table 3 shows in the lab-scale system, AD of DWAS after *C. bescii* pre-digestion gave approximately 69% reduction in VSS and 81% more biogas than the control. Although the total amount is relatively small, i.e., 33 L biogas $kg^{-1}$ VSS, the data on DWAS in Table 3 show that even after lengthy thermophilic AD, there is still some material that can be hydrolyzed by *C. bescii* and made available for AD.

**Table 3.**
Properties of DWAS used in the lab-scale system, biogas produced, biogas composition, and waste destruction.

| Analysis | *C. bescii* DWAS | Control DWAS |
|---|---|---|
| %C in TS | 32.8 | 32.8 |
| %N in TS | 4.53 | 4.53 |
| %P in TS | 3.92 | 3.92 |
| TSS, g $L^{-1}$ | 12.9 | 12.9 |
| VSS, g $L^{-1}$ | 8.26 | 8.26 |
| COD, $gO_2$ $L^{-1}$ | 14.5 | 14.5 |
| Biogas, L $kg^{-1}$ VSS | 32.9 | 13.3 |
| $CH_4/CO_2$ in biogas | 2.65 (73% $CH_4$) | 2.48 (71% $CH_4$) |
| %VSS destruction | 8.80 | 3.56 |

**Table 4.**
Properties of dairy manure used in the bench-scale system, biogas produced, biogas composition, and waste destruction.

| Analysis | *C. bescii* COW | Control COW |
|---|---|---|
| %C in TS | 36.3 | 36.2 |
| %N in TS | 3.03 | 3.02 |
| %P in TS | 0.78 | 0.78 |
| TS, g $L^{-1}$ | 40.7±10.7[a] | 37.7±4.7[a] |
| VS, g $L^{-1}$ | 25.3±6.3[a] (58% C) | 23.0±2.7[a] (59% C) |
| COD, $gO_2$ $L^{-1}$ | 52.2 | 44.7 |
| Biogas, L $kg^{-1}$ VS | 476 | 265 |
| Methane, L $kg^{-1}$ VS | 362 | 182 |
| $CH_4/CO_2$ in biogas | 3.18 (76% $CH_4$) | 2.22 (69% $CH_4$) |
| %VS destruction | 44[b] | 24[b] |

[a] Note the ±values represent the difficulty of obtaining a representative sample of the suspension, not the uncertainty in the measurement method.
[b] Calculated from the measured biogas volume with Equation 8.

### *3.3. Results of experiments on dairy manure*

Table 4 provides the properties of dairy manure, as well as biogas produced, biogas composition, and waste destruction in the more detailed experiments on dairy manure run in the bench-scale system. Figure 3 shows biogas production, the rate of biogas production, and the methane content of biogas from dairy manure as functions of time. *C. bescii* pre-digestion of dairy manure nearly doubled the amount of biogas per kg VS, and on average, methane content of

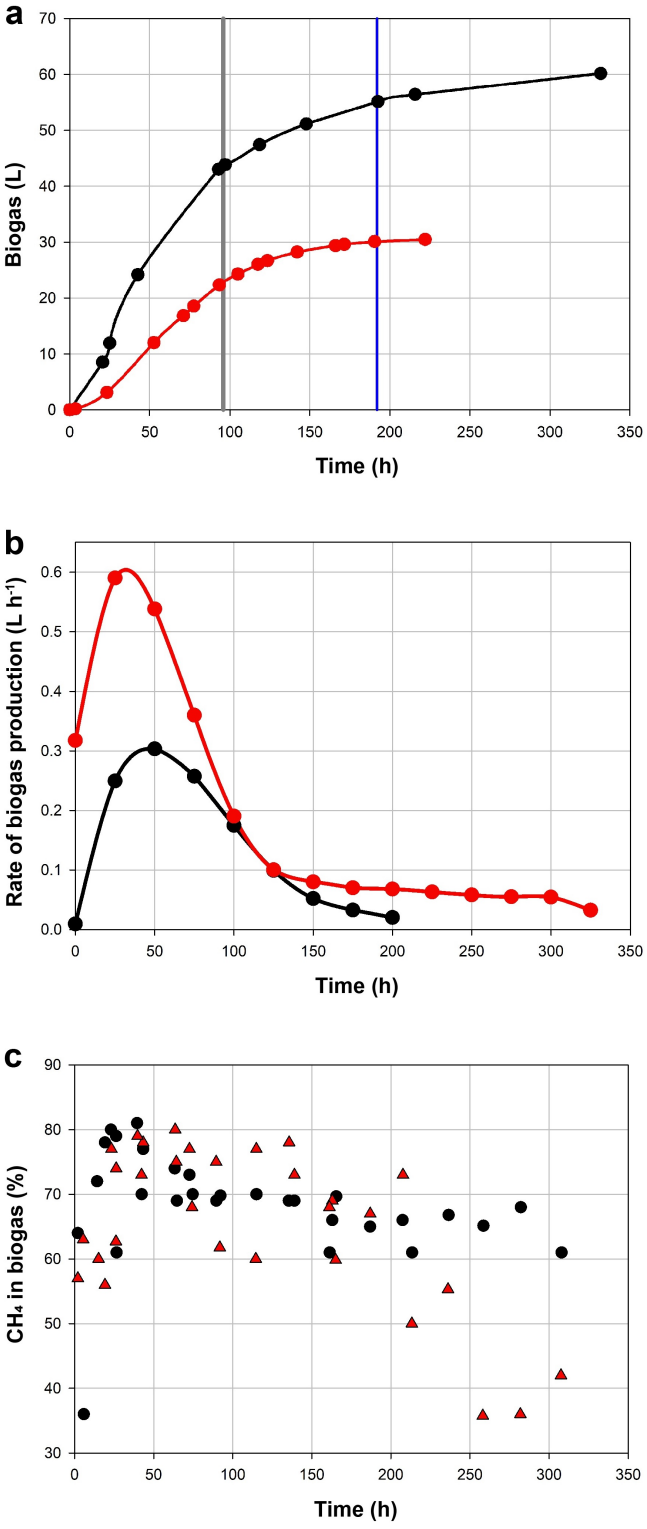


**Fig. 3. (a)** Representative plot of biogas production as a function of time from dairy manure in the bench-scale, batch system. Black circle and line are from *C. bescii* pre-treated substrate, red circle and line are from the control. The grey vertical line is 4-d retention time, and the blue vertical line is 8-d retention time; **(b)** Rates of biogas production in **a**; **(c)** Methane content of biogas as a function of time from four runs in the bench-scale, batch system. Black circles are *C. bescii* pre-treated substrate, and red triangles are from the control.

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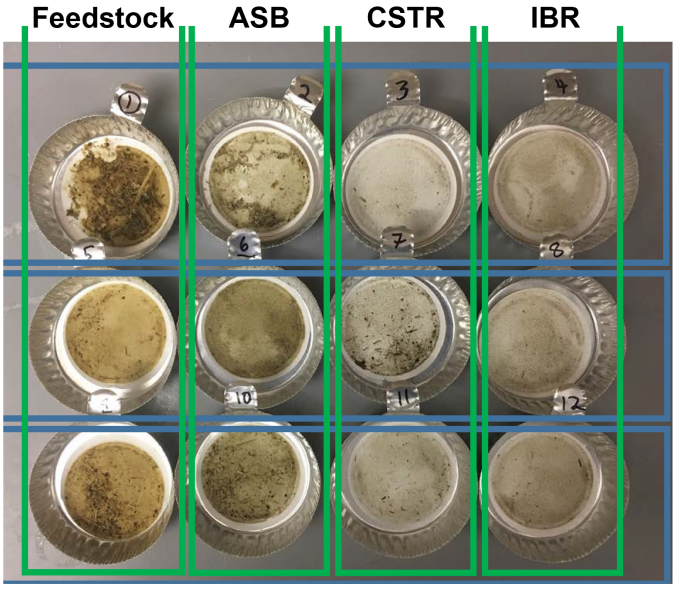


**Fig. 4.** TSS from 1.5% dairy manure after *C. bescii* pre-digestion for 38 h followed by 77 h of mesophilic anaerobic digestion.

the biogas was about 10% greater than in the control hydrolyzed at 75°C for the same time. The higher methane content in biogas from pre-digested dairy manure was primarily caused by very high methane, 85%, at the beginning of digestion (Fig. 3c), which could only be produced from a significant concentration of acetate in the AD feedstock (see Fig. 2). Also, note that the highest rate of biogas production (Fig. 3b), occurs at the same time as the high methane content indicating digestion of acetate by acetoclastic methanogens.

An initial test run in the pilot plant with *C. bescii* pre-digestion of dairy manure demonstrated a major decrease in TSS (Fig. 4) and an 89% reduction in COD from the feedstock to the AD effluents. The C/N ratio of dairy manure in the ASB feedstock was approximately 12. Total N and total P were 1160 and 300 mg $L^{-1}$ in the feedstock and in the effluents, respectively. After the initial test runs, the four sets of data shown in Table 5 were collected during continuous, steady-state operation for 2-3 weeks each. Table 5 gives the average results for runs with *C. bescii* pre-digestion, control runs, and literature data for mesophilic digestion with no pretreatment in a very similar AD system (Rico et al., 2011). *C. bescii* pre-digestion increased VS reduction compared with 75°C hydrolysis control runs by 19% with a 2-d ASB retention time and by 55% with a 4-d retention time. *C. bescii* pre-digestion gave a negligible change in COD with a 2-d ASB retention time and a 15% decrease in COD with a 4-d retention time. Although it was not possible to do the experiment in the pilot plant, Figure 3 indicates increasing AD retention time to 15 to 20 d would significantly increase VS reduction to >75% with a concomitant increase in biogas. Figure 3 shows *C. bescii* pre-digested manure continued to produce biogas at a significant rate after 14 d in the bench-scale system.

A decrease in alkalinity in the ASB is a relative measure of the metabolic activity of *C. bescii* (see Eqs. 2-4). In the runs with *C. bescii* pre-digestion, alkalinity in the ASB averaged 20±5 mM, and alkalinity in the digesters averaged 40±5 mM. The alkalinity in the ASB is 20-30 mM lower than that in the digesters because *C. bescii* metabolism produced acetic and lactic acids that were neutralized to anions by bases in the feedstock. AD of acid anions produces an equivalent amount of bicarbonate, $HCO_3^-$ in the digesters, thus accounting for the increased alkalinity (Kateava et al., 2013).

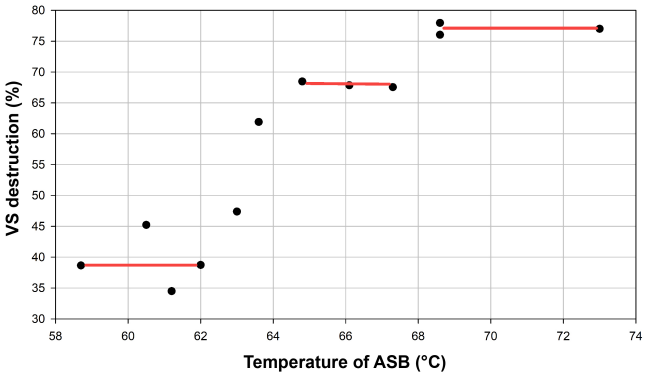


**Fig. 5.** The effect of decreasing ASB temperature on VS reduction. Temperature was started at 73°C and incrementally decreased to 59°C over 23 d. An Arrhenius plot of the data is shown in Figure S2.

Figure 5 provides the VS data from a run where the heaters on the ASB were turned down to separate the effects of enzyme-catalyzed and base-catalyzed hydrolysis and to determine the temperature dependence of the two processes. The initial temperature was 73°C, and over the next 23 d, the ASB cooled to 59°C. Cooling was primarily due to the injection of unheated feedstock, so the temperature decrease was approximately linear. Retention time in the ASB was 2 d and retention time in the digesters was 4 d. The ASB and digester effluents were sampled approximately daily. The initial VS reduction was near 75% when the ASB was operating at 73-69°C, just below the optimum temperature for *C. bescii* growth and enzyme

**Table 5.**
Average values of TS, VS, %VS reduction, % COD reduction, biogas production, and biogas composition in pilot plant runs with dairy manure with *C. bescii* pre-digestion and control runs without *C. bescii*.

| | Feedstock TS, g $L^{-1}$ | Feedstock VS, g $L^{-1}$ | %VS reduction | | %COD reduction | | Biogas, L $kg^{-1}$ VS[b] | Biogas, %$CH_4$ |
|---|---|---|---|---|---|---|---|---|
| | | | CSTR | IBR[a] | CSTR | IBR[a] | | |
| *C. bescii*[c] | 40.8 | 25.1 | 64±4 | 75±3 | 57±13 | 69±11 | 584 | 78 |
| Control[d] | 42.7 | 26.1 | 54±5 | 63±5 | 60±3 | 70±6 | 493 | 75 |
| *C. bescii*[e] | 33.8 | 20.7 | 54±5 | 69±5 | 58±4 | 66±2 | 493 | 76 |
| Control[f] | 35.8 | 21.5 | 31±5 | 51±5 | 35±5 | 52±5 | 283 | 74 |
| Rico et al. (2011)[g] | 57.6 | 45.0 | 36 | - | 38 | - | 289 | 69 |
| Rico et al. (2011)[h] | 57.6 | 45.0 | 43 | - | 46 | - | 304 | 69 |

[a] VS and COD reduction in the IBR are higher than in the CSTR because the IBR is not stirred and retains solids for a longer period of digestion.
[b] Because of incomplete measurements, total biogas was calculated from the reduction in VS in the CSTR with Equation 8. The VS was 48.9% carbon.
[c] Run 15 d with 2-d retention in the ASB and 4 d in AD.
[d] Run 23 d with 2-d retention at 75°C and 4 d in AD.
[e] Run 20 d with 4-d retention in the ASB and 8 d in AD.
[f] Run 14 d with 4-d retention at 75°C and 8 d in AD.
[g] Experiment done with no pretreatment and 10-d retention in a 1500-L digester very similar to the CSTR used in this study.
[h] Experiment done with no pretreatment and 20-d retention in a 1500-L digester very similar to the CSTR used in this study.

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activity. The approximate activation energies found from an Arrhenius plot (**Fig. S2** in **Supplementary Data**) of the data in **Figure 5**, assuming %VS destruction is proportional to the rate of hydrolysis reactions in th e ASB, are 24 kJ $mol^{-1}$ for the exozyme catalyzed reaction and 87 kJ $mol^{-1}$ for the base-catalyzed reaction.

### *3.4. Summary of results*

Pre-digestion with *C. bescii* increased biogas production from all the feedstocks tested. All of the feedstocks produced more biogas after *C. bescii* pre-digestion than the 75°C hydrolyzed controls, apparently because exozymes increased hydrolysis of polymeric materials to produce soluble, small molecules that were readily digested in AD. The data on dairy manure suggests an optimized commercial system using *C. bescii* pre-digestion would produce ≈600 L of biogas per kg VS with 75% methane and would destroy 75% of VS. As reference points, LTHP is estimated to increase VS reduction in dairy manure to 50-60% giving ≈450 L biogas per kg VS, and mesophilic AD of cow manure without pretreatment typically achieves 30-40% waste destruction and ≈300 L of biogas per kg VS with 60% methane (Rico et al., 2011; Nasir et al., 2012; Atelge et al., 2020). Mesophilic digestion of WAS without pretreatment achieves a similar level of waste destruction, biogas production, and biogas composition (Xu et al., 2020).

The increase in biogas methane content observed in nearly all experiments was most likely a consequence of the metabolism of some of the hydrolysis products to acetate and lactate by *C. bescii*. This increase in methane content can only be accounted for by digestion of acetate and/or lactate produced by *C. bescii* metabolism or by digestion of organic material with a low oxidation state, e.g., fats and oils. These results are in agreement with the results presented in Straub et al. (2019).

Pre-digestion of dairy manure with *C. bescii* resulted in increased VS destruction, increased biogas production, and a higher ratio of $CH_4/CO_2$ in the biogas. Optimizing the ASB retention time requires maintaining *C. bescii* in an actively growing state to maximize metabolism and enzyme activities (Lochner et al., 2011; Straub et al., 2019). But because the pilot plant was built with available parts and very little funding, equipment limitations prevented optimizing retention times in the ASB and AD for either the *C. bescii* or control runs. Also, ASB and AD retention times could not be changed independently. The results in **Table 5** show that decreasing retention time in the ASB from 4 d to 2 d did not significantly change VS reduction even though retention time in AD was decreased from 8 d to 4 d. Experiments in the batch process used in the bench-scale system showed 15-20 d AD retention time would be required to maximize biogas production. With four days in the ASB, *C. bescii* most likely reached a near stationary state that limits growth, metabolism, and exozyme production. With two days in the ASB, washout of *C. bescii* cells and exozymes is likely. Therefore, we conclude that the optimum time for pre-digestion of dairy manure is between 2 and 4 d.

### *3.5. Optimizing pre-digestion*

Our work highlights the need to optimize a *C. bescii* pre-digestion/AD system to achieve maximum benefits. Optimization requires quantifying the kinetics of the three processes that occur in the ASB tank as well as the rate of methanogenesis in the AD tank. Because an optimized continuous flow system should operate at a steady state near the inflection point in the growth curve, the growth rate of *C. bescii* can be described with a simple first-order equation (**Eq. 10**):

$$d[n]/dt = k_n[n] \text{ where } [n] \text{ is the concentration of } C.\ bescii \qquad \text{Eq.10}$$

Operating at this condition maximizes the rate of growth and exozyme production (Straub et al., 2019). The rate of enzyme-catalyzed hydrolysis of the substrate can be described with the Michaelis-Menten/Briggs-Haldane equation (**Eq. 11**):

$$d[\text{products}]/dt = k_{cat}[\text{exozymes}][\text{accessible sites}]/(K_M + [\text{accessible sites}]) \qquad \text{Eq. 11}$$

Base-catalyzed hydrolysis occurs both in the hydrolysis tank (**Fig. 1**) and in the pre-digestion tank in parallel with enzyme-catalyzed hydrolysis. The rate of base-catalyzed hydrolysis is described by a second-order rate law as shown in **Equation 12**:

$$d[\text{products}]/dt = k_h[\text{base}][\text{accessible sites}] \qquad \text{Eq. 12}$$

In **Equations 10**, **11**, and **12**, brackets indicate concentrations, and t is time. In **Equation 10**, [n] is the number concentration of *C. bescii*, and $k_n$ is the rate constant for growth. In **Equations 11** and **12**, [products] is the sum of the concentrations of hydrolysis products, $k_{cat}$ is the rate constant for enzyme-catalyzed hydrolysis, [exozymes] is the concentration of active hydrolases, [accessible sites] is the concentration of sites on the polymers that are accessible by the hydrolases, and $K_M$ is the Michaelis-Menten constant. The rate constant for base-catalyzed hydrolysis is $k_h$ and [base] is the concentration of bases in the solution. Accessible sites are the places on the polymeric materials where exozymes and bases catalyze the addition of water to split off small molecules. **Equation 11** indicates the rate of hydrolysis increases with the increasing concentration of exozymes, which is directly proportional to the growth rate of *C. bescii* (Lochner et al., 2011). Hydrolysis rates also increase with the accessibility of exozymes and bases to sites on the hydrolyzable material, i.e., the smaller the particles, the faster the reaction. The exozymes are only produced when *C. bescii* is actively growing, so to optimize the pre-digestion process, the culture must be continuously fed and diluted to prevent the culture from reaching a stationary state (Lochner et al., 2011; Straub et al., 2019). Metabolism of the products of hydrolysis to acetate and lactate also obviously depends on maintaining an actively growing *C. bescii* culture.

The exozyme-catalyzed hydrolysis of polymeric wastes creates small, soluble molecules and organic anions that are readily digested by methanogens in anaerobic digesters. Because AD of acetate and lactate from *C. bescii* metabolism produces nonvolatile bicarbonate as well as $CO_2$ and $CH_4$, digestion of pre-digested material produces biogas with reduced $CO_2$ content. Because the relative concentrations of sugar, lactate, acetate, and other digestible material in the effluent from pre-digestion depends on the relative rates of exozyme- and base-catalyzed hydrolysis and *C. bescii* metabolism, the methane content of biogas and the amount of biogas produced depend on the pre-digestion time and concentration of feedstock. **Figures 3b** and **c** indicate the higher rate of biogas production and methane content observed with pre-digested feedstock is likely because the small molecules and anions in pre-digested material favor free-living acetoclastic methanogens over biofilm-forming oxidative methanogens.

The 20 mM decrease in alkalinity and the difference in VS destruction between operating the ASB at 69-73°C and 67-65°C both suggest base-catalyzed hydrolysis at 75°C accounted for 80-90% of the hydrolysis products, and *C. bescii* metabolism accounted for 10-20% of the hydrolysis products being digested in the pilot plant with a 2-d ASB retention. **Table 6** summarizes the data on dairy manure from **Tables 2**, **3**, and **5**, comparing results from pre-digested and control experiments. The batch experiments done in the bench-scale system demonstrated a 37 and 82% increase in VS destruction in pre-digested manure compared with the LTHP control. Comparing the 4+8 d run with the 2+4 d run in the pilot plant shows *C. bescii* pre-digestion was more significant at a 4-d ASB retention than at a 2-d retention. However, there is very little difference in the VS destruction at the different ASB retention times even though the AD retention time was shorter. Therefore, VS destruction may be greater with the 2-d ASB retention. The COD ratios in **Table 6** show the same trends as the ratios of VS although the COD values are less accurate. The ratio of enzyme-catalyzed to base-catalyzed products being digested in the batch experiments is not subject to the washout expected at short AD retention times in the pilot plant. Washout of compounds that are more slowly digested by AD is likely the cause of the trend seen in **Table 6**. Unfortunately, this complication makes it impossible to identify an optimum ASB retention time, although it is clear that longer retention times in AD are necessary.

**Equations 1**, **2**, **10**, **11**, and **12** identify four variables that must be considered in designing the system in **Figure 1** for a particular feedstock; 1) concentration of volatile solids (VS) in the feedstock, 2) retention time in the hydrolysis tank, 3) retention time in the ASB, and 4) retention time in AD. Optimizing engineering design and operation for a given feedstock thus requires determination of the growth rate of *C. bescii*, exozyme activity, and the base-catalyzed hydrolysis rate as a function of feedstock

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**Table 6.**
Results of VS destruction in dairy manure expressed as the ratio of percent destruction in pre-digested to control.

| System | Ratio $\Delta VS_{CB}/\Delta VS_{cont}$ IBR | Ratio $\Delta VS_{CB}/\Delta VS_{cont}$ CSTR | Ratio $\Delta COD_{CB}/\Delta COD_{cont}$ IBR | Ratio $\Delta COD_{CB}/\Delta COD_{cont}$ CSTR |
|---|---|---|---|---|
| 30/60 L batch | 1.37 | NA[a] | NA[a] | NA[a] |
| 30/60 L batch | 44/24 = 1.83 | NA[a] | 52.2/44.7 = 1.17 | NA[a] |
| Pilot 4+8 d | 69/51 = 1.35 | 54/31 = 1.74 | 66/52 = 1.27 | 58/35 = 1.66 |
| Pilot 2+4 d | 75/63 = 1.19 | 64/54 = 1.19 | 69/70 = 0.99 | 57/60 = 0.95 |

[a] Not available

concentration. Since it provides the food for *C. bescii* growth, the rate of hydrolysis has to be faster than the growth rate, but hydrolysis may continue after *C. bescii* has reached a stationary state. **Equation 11** demonstrates that exozyme-catalyzed hydrolysis likely requires longer ASB retention times with increasing substrate concentration. However, the exozymes may become saturated, a condition set by the upper limit of the rate of exozyme production and VS concentration. For base-catalyzed hydrolysis, the rate depends on the concentrations and identity of the bases present in the system, e.g., $HCO_3^-$ and possibly $NH_3$, as well as the VS concentration. **Equation 12** indicates the rate of base-catalyzed hydrolysis increases linearly with increasing VS concentration.

To illustrate the effects of retention time and substrate concentration on *C. bescii* hydrolysis of the substrate, the following analysis uses data in *Figure 1* in Lochner et al. (2011) for *C. bescii* growing on Avicell, a crystalline cellulose, in standard media. Assuming the optimum conditions are near the inflection point in the growth curve where growth rate and enzyme production rate are maximal, growth rate, $dn/dt = 5x10^7$ cells $mL^{-1}$ $h^{-1}$ and the rate of exozyme production = d[exozymes]/dt = 6 μg $mL^{-1}$ $h^{-1}$. The optimum concentration of *C. bescii* is ≈$5x10^8$ cells $mL^{-1}$, and the optimum concentration of exozymes is ≈100 μg $mL^{-1}$. The retention time is given by **Equation 13**:

$$\text{Retention time} = \text{tank volume/influent rate} = V/(dV/dt) \quad \text{Eq.13}$$

The optimum retention time from the growth rate, $V/(dV/dt) = 5x10^8$ cells $mL^{-1}/5x10^7$ cells $mL^{-1}$ $h^{-1}$ = 10 h and from the exozyme production rate, V/(dV/dt) = 100 μg $mL^{-1}$/6 μg $mL^{-1}$ $h^{-1}$ = 17 h. So, the optimum retention time for growth and production of simple sugars from Avicell is between 10 and 17 h at the concentration of Avicell used in Lochner et al. (2011). Determining the dependence of optimum retention time on Avicell concentration would require data obtained at different Avicell concentrations.

The effects of substrate concentration on the rate of conversion of polymeric VS into small soluble molecules is described by the Michaelis-Menten/Briggs-Haldane equation (**Eq. 14**). At the optimum exozyme concentration from Lochner et al. (2011), and assuming the concentration of accessible sites is directly proportional to the VS concentration:

$$d[\text{products}]/dt = k_{cat}[100\ \mu g\ mL^{-1}][VS]/(K_M + [VS]) \quad \text{Eq. 14}$$

**Equation 14** shows the rate increases linearly with enzyme concentration, but because [VS] appears in both the numerator and denominator, the rate increases nonlinearly with increasing VS concentration. The rate reaches a maximum that depends on the value of $K_M$ relative to the VS concentration. The effect of increasing solids content of the feedstock was not evaluated in this study, but 8-10% solids will be required to take full economic advantage of *C. bescii* pre-digestion.

## 4. Conclusions and future directions

The focus of this study was to gain an understanding of the commercial potential of the *C. bescii* pre-digestion process. However, our study shows that taking advantage of thermophilic pre-digestion at a commercial scale requires further work to quantify the kinetics of the pre-digestion process. Characterizing the non-linear effects of feedstock concentration will be necessary to realize the full economic potential. The extent of base-catalyzed hydrolysis at the relatively low temperature used in this study was surprisingly high and needed further characterization. The products of *C. bescii* pre-digestion are very different than feedstocks typically used in AD, including those from other pretreatments, and as a consequence, further work is required to optimize the AD process.

Although more work is required to optimize engineering design and operation, this work demonstrates that pre-digestion with *C. bescii* has the potential to significantly increase biogas production from organic wastes at minimal additional operating cost. For example, an optimized system with *C. bescii* pre-digestion is expected to convert 75 to 85% of the VS in dairy manure and WAS into biogas with 75% methane. *C. bescii* pre-digestion thus has the potential to greatly increase the application of AD for the production of renewable energy.

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## Conflict of Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lee D. Hansen, Jaron C. Hansen, Zachary T. Aanderud, Conly L. Hansen, and L. Scott Rogers all have ownership in Verde, LLC that has licensed the pre-digestion technology described in this paper from BYU and is in the process of commercializing the Verde process. Jaron C. Hansen is the son of Conly L. Hansen, but Lee D. Hansen is not related.

## Author Contributions

LR and CB did data acquisition and curation and participated in developing methodology, software, and visualization. LDH, JCH, CLH, LSR, and ZTA all participated in conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing - original draft; and writing - review & editing. All authors read and approved the manuscript.

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