The effect of pH, temperature, and inoculum on the fermentation of pulp and paper biosludge: increasing the nutrient availability for rearing of black soldier fly larvae

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Abstract

Pulp and paper production is one of the largest global industries producing annually 400 million metric tons of pulp and paper products and 6 million tons of pulp and paper biosludge (PPBS). From a resource efficiency and sustainability perspective, there is a need for improving PPBS management. This study assessed fermentation of PPBS as pretreatment to improve PPBS feasibility as feed for black soldier fly larvae. The impact of temperature, pH, and inoculum on the concentration of soluble chemical oxygen demand (sCOD) and volatile fatty acids (VFA) was assessed. An initial pH of 10 and the addition of inoculum from an anaerobic digester substantially increased the concentration of sCOD. The obtained concentration of VFA was low compared to the VFA concentration needed to improve the growth of Black Soldier Fly Larvae (BSFL). The PPBS is recalcitrant to fermentation because of the high content of lignocellulose. Fermentation as done in this study does not convert PPBS to a feasible feed for black soldier fly larvae; thus, further research on improved fermentation is needed. However, fermentation at alkaline pH and addition of inoculum do increase the final pH of PPBS which improves its feasibility as feed for BSFL. Future studies should explore pH > 10 and temperatures > 55 °C to increase sCOD and improving generation of VFA by removal of inhibiting substances, testing other types of inoculum (rumen microorganisms) and co-fermentation.

Keywords Sludge · Recycling · Lignocellulose · Nutrient availability · Fermentation · Volatile fatty acids

1 Introduction

Pulp and paper production is one of the largest global industries producing 400 million metric tons of pulp and paper products annually [1] and 6 million tons of pulp and paper biosludge (PPBS) (calculated from [2]). Due to its low

biodegradability and low concentration of available nutrients or other valuable substances, PPBS is often incinerated or composted. From a resource efficiency and sustainability perspective, there is a need for other waste management options [3–5]. One option that has been suggested is using insects to convert this organic waste into protein and fat rich food and feed [6, 7]. However, PPBS contains high amounts of recalcitrant lignin (20–58%) and cellulose (2–28%) [2] and a previous study of PPBS as feed for Black soldier fly larvae (BSFL) identified the low availability of nutrients as an important obstacle to growth [3]. This article is one among several where methods to improve the nutrient availability of PPBS are explored.

Chemical and physical methods of improving nutrient availability as well as the technological methods to achieve it are summarized by Norgren et.al. [8]. Improving nutrient availability by anaerobic digestion is one method of pretreatment that has been discussed recently [8–16]. Disintegration, hydrolysis, acidogenesis (also called fermentation), acetogenesis, and methanogenesis are the steps in anaerobic digestion [17]: large organic compounds are first broken

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down into monomers, then further converted by microorganisms to volatile fatty acids (VFAs), alcohols, carbon dioxide, hydrogen, and methane [18]. Dissolved organic substances such as VFA are readily available for ingestion by insects such as BSFL in contrast to large pieces of insoluble lignocellulose [9]. Addition of VFAs as feed for BSFL improves growth of the larvae [19]; therefore, the focus of this study is on maximizing the yield of dissolved organic substances and VFAs. Examples of important factors affecting the anaerobic digestion process are as follows: type of inoculum, temperature, pH, carbon to nitrogen ratio, liquid to solid ratio, and duration [18, 20, 21].

The inoculum needs to contain the strains of microorganisms that can digest the recalcitrant substances in PPBS such as lignocellulose [22, 23]. The inoculum can be either a monoculture (containing one specific strain) or a mixed culture [22, 23]. However, anaerobic digestion by monoculture inoculate of specifically wastewater-derived sludge is problematic [23]. Monoculture inoculate results in low process stability and the added monoculture strain risk being out-competed by some of the many strains in PPBS if the PPBS is not hygienized. In addition, monocultures for anaerobic digestion of cellulose have low growth rates and low yield [22]. A mixed culture inoculum, on the other hand, provides diverse biochemical functions, improved process stability, is more robust, and therefore, allows the use of non-sterile conditions [23]. Digested sludge is one example of a mixed culture inoculum that contains a wide range of strains of anaerobic microorganisms [24, 25]. However, maintaining good conditions for the microorganisms, such as a suitable temperature and pH, is crucial for an efficient process.

Anaerobic digestion can be carried out at temperatures between 15 °C and 70 °C and pH higher than 4 [18, 25]. However, mesophilic (35–37 °C) or thermophilic (50–55 °C) temperatures, a pH of 5.2–7.0 for the acidogenic step, and a pH of 6.8–8.2 for the methanogenic step are common [18]. The temperature that is optimal for each of the strains varies and, depending on which temperature is used for the anaerobic digestion, some strains may thrive more than others thus changing the composition of the population [25] and affecting the rate of chemical reactions and the yield of, i.e., soluble COD (sCOD), VFA, and methane [25]. Wu et al. report how the population of microorganisms in digested sludge changes when the temperature increases [25]. The dominant phyla at 35 °C, *Proteobacteria*, is replaced as dominant phyla by *Firmicutes* when temperature increases to 55 °C. Furthermore, these changes in the microflora correlate with an increase in the hydrolysis rate and an decrease in the methanogenesis rate [25] thus showing the importance of temperature as a process control parameter. Control of pH is important for the growth of the microflora as well and effects the yield of for example methane. Methanogens are archaeal microorganisms that thrive at neutral to slightly alkaline pH

but are inhibited under extreme pH conditions, both acidic [26, 27] and alkaline [28].

The different steps of anaerobic digestion take up to several weeks; however, the VFA is produced in the acidogenic step (hereafter called fermentation) that takes place before the acetogenesis and methanogenesis. Reported process duration for maximal VFA concentration is 5–8 days [21, 29, 30]. The concentration of VFA increases rapidly during the first 4–8 days thereafter reaching a plateau then maintaining the concentration [31, 32]. Zhao et al. show that the production of short-chained fatty acids from waste activated sludge peaks at day 6–10 depending on pH [33]. The impact of varying duration of fermentation was not investigated in this study, and the fermentation was carried out for 10 days.

The disintegration and hydrolysis steps are considered to be rate limiting for fermentation because of the protection of extra cellular polymeric substances and cell envelope [17, 34] thus motivating the search for ways of improving the process. Previously published studies on anaerobic digestion of pulp and paper waste focused on the low biodegradability of lignocellulose, methods for accelerating disintegration and hydrolysis (mechanical, hydro-thermal, acid, alkali, enzymatic, advanced oxidation, ultrasonication) [2, 17, 35], and inhibiting substances created (phenolic compounds, furfural, hydroxymethylfurfural, organic acids) [2]. Furthermore, inhibition of methanogenesis is important for minimizing the methanogen consumption of fatty acid. This study focuses on alkaline methods (pH 8–10) for accelerating disintegration and hydrolysis and for inhibiting methanogenesis.

Alkaline methods cause the organic material to swell and become susceptible to enzymatic attack [35] thus facilitating formation of VFAs [34, 36–38]. Alkaline conditions can be achieved by the addition of NaOH, Ca(OH)₂, KOH, and NH₄OH [39]. Previously published studies on alkaline fermentation focused on production of fatty acids from municipal wastewater treatment, waste activated sludge [32, 40], and wetland plant litter [41]. The aspects that have been investigated are as follows: addition of sulfite [32]; characterization of dissolved organic matter [40]; the impact of ammonia and free ammonia [33]; addition of bio-surfactants [42]; and the contribution of biotic and abiotic factors at different pH [41]. However, to the best of our knowledge, there are no published studies on alkaline fermentation of PPBS for production of VFA.

The aim of the study was to assess fermentation of PPBS as pretreatment for improving PPBS feasibility as feed for BSFL. We used VFA as a measure of nutrient availability because it is a readily available feed for BSF; however, sCOD is faster to analyze than VFA; thus, we used the content of sCOD as precursor for VFA. The focus was on alkaline fermentation of PPBS at mesophilic and thermophilic temperatures, using a mixed culture as inoculum. The impact

of temperature, pH, and addition of inoculum on the concentration of sCOD and VFA were investigated.

2 Materials and methods

2.1 Materials

The material studied was pulp and paper biosludge (PPBS) from a chemical-thermomechanical pulp/ground wood pulp mill in Sweden. Forty litre of fresh PPBS were sampled and transported over night to our laboratory in Luleå, Sweden, and stored at 4 °C for 2 days until the start of the experiment. Quartering technique was used for sub-sampling. A liquid-to-solid ratio (L/S) of 10 was used to measure the electrical conductivity (EC) and pH of the PPBS. After adjusting L/S, the material was mixed for 1 h before measuring EC and pH. Representative samples of the material were sent to the accredited laboratory, Eurofins, for characterization.

In this study, the PPBS was not sterilized so contamination from the inoculum was not a concern; thus, a mixed culture inoculum was used to accelerate the fermentation process. The inoculum used was digested sludge collected from the mesophilic anaerobic digester at the municipal wastewater treatment plant in Luleå, Sweden. The total COD content of the inoculum used was 11.37 g L⁻¹. Total solids and volatile solids were measured at 105 °C for 24 h and 550 °C for 2 h, respectively, immediately after sludge sampling, and were 2.3% ± 0.0 and 1.4% ± 0.1, respectively, and pH was 7.4 ± 0.1. The inoculum was stored at 4 °C before starting the experiments.

2.2 Batch fermentation

Batch fermentation using 118-mL bottles sealed with air-tight rubber stoppers was used for the test. Each batch was filled with PPBS and inoculum or tap water. The triplicated control samples were prepared at each investigated temperature with PPBS and tap water without adjusting the initial pH. The triplicate batch samples were prepared in random order to minimize systematic errors. The PPBS-to-inoculum ratio applied was 1:2 g of VS to ensure that the amount of inoculum available was not a limiting factor. The pH was adjusted with 4 M NaOH solution. The batches were incubated in a heating cabinet for 10 days and continuously shaken. After fermentation, the gas production and final pH were measured. The volume of gas was quantified using a glass syringe. The liquid and solid phases were separated by centrifugation at 10,000 rpm for 10 min. The liquid was used for the sCOD measurement. The VFA was measured only in the experiments with the highest sCOD in order to access the extent of the acidogenesis.

2.3 Experimental design

The factors investigated were temperature, pH, and inoculum addition. A full factorial design of 23 with two central points was chosen for the **experimental design** [43] to identify which factors and their interactions increased the concentration of sCOD the most (Table 1). The experiment was fully randomized with replicates in a total of 48 runs. The main response was the concentration of sCOD in solution.

The samples are coded for clarity. The letters S and I identify the addition of PPBS and inoculum, respectively. The temperature is reported after the letters, and finally the initial pH. As an example, if the experiment was with PPBS and inoculum addition at 35 °C and initial pH 8, the sample is coded as SI35pH8. When the inoculum was not added, the letter I is not included in the sample name. As an example, the experiment with PPBS and without inoculum at 55 °C and initial pH 10 is named S55pH10.

2.4 Analysis

sCOD was measured in the liquid phase after centrifugation. In this paper, sCOD is defined as the COD in the liquid phase after centrifugation. The sCOD was measured using Merck vial test (range: 50–3000 and 500–10,000 mg COD L⁻¹). The samples were filtered through membrane filters with a 0.45-μm pore size before sending the samples for volatile fatty acid **analysis** [44] to the accredited laboratory, Eurofins (Sweden). To compare the results of VFAs production with those reported in the literature, the measured VFA concentrations were converted to g COD L⁻¹. The conversion factors used were 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric acid, and 2.04 for valeric acid. These factors were calculated based on the complete oxidation of each volatile fatty acid to carbon dioxide and water [45].

2.5 Statistical data evaluation

The results were statistically analyzed with one-factor ANOVA combined with the Tukey HSD/Kramer test using the Real Statistics Resource Pack software, Release 5.4 (Charles Zaiontz, www.real-statistics.com). The software

Table 1 Factors and their levels in the **experimental design**. For the inoculum factor, the central point is carried out at both high and low level

Level	Low (–)	High (+)	Central point (0)
Temperature	35 °C	55 °C	45 °C
pH	8	10	9
Inoculum	No	Yes	No/yes

MODDE (Umetrics) was used for response surface plotting and model validation.

2.6 Model validity and factor significance

Logarithmic transformation of the data was applied to improve the model because the data had positive skewness. After all non-significant factors and their interactions were removed, i.e., the interactions of temperature with and without inoculum, the model fits the measured responses well. The significance level of the model (R^2) was equal to 0.97, and the reproducibility was high ($Q^2 = 0.96$).

3 Results and discussion

The characteristics of the PPBS used are presented in Table 2. The contents of crude protein, crude fat, and volatile substances were within the typical ranges for PPBS [3, 46]; however, the content of dry matter was higher than the typical range (10–30%) reported by Norgren et al. [3].

3.1 Impact of pH, temperature, and inoculum on sCOD concentration

The concentration of sCOD obtained at the end of all the batch experiments are reported in Table 3. In the samples without inoculum addition, the final sCOD concentration

was not significantly impacted by the initial pH and did not exceed the 1.3 g COD L^{-1} under all the temperature investigated (Table 3). When inoculum was added, the effect of initial pH in the solubilization of the organic compounds became significant, resulting in a large increase in sCOD at alkaline conditions. The highest sCOD concentrations were achieved in the samples SI35pH10 and SI55pH10 with no statistically significant difference between the two temperatures (Table 3). The results show that the optimal conditions for generation of sCOD by fermentation of PPBS is pH 10 and addition of inoculum.

However, a major part of the sCOD might come from the inoculum. The sCOD produced from fermentation of inoculum only and PPBS only was measured at its initial pH (7.4 and 5.1, respectively). Table 3 shows the sCOD from the inoculum only that was 3.4–4.1 times higher than the sCOD from the PPBS only. A reasonable explanation for the low generation of sCOD from the PPBS compared to the inoculum is the PPBS content of lignocellulose being recalcitrant to digestion by the microbes available in the PPBS. Surface cellulose in the primary and secondary cell wall hydrolyzes but the gradual dissolution of these cell walls increases the fraction of lignin that is exposed. The lignin is recalcitrant to biodegradation; thus, the rate of the hydrolysis slows down and finally stops, and therefore, a major part of the lignocellulose remains undissolved [47]. These results show the low solubility of the PPBS compared to the inoculum at the initial pH; however, the sCOD from inoculum only and

Table 2 Summary of the main properties of PPBS ($n = 3$)

Parameter	Average	Parameter	Average
Total solids (%)	45.5 ± 1.0	Phosphorus (mg kg^{-1})	473 ± 12
Volatile solids (%TS)	84.2 ± 0.6	Calcium (mg kg^{-1})	413 ± 5
pH	5.1 ± 0.0	Potassium (mg kg^{-1})	61 ± 2
EC ($\mu\text{S s}^{-1}$)	624 ± 39	Copper (mg kg^{-1})	27 ± 0
Kjeldahl crude protein (g kg^{-1})	36.8 ± 0.0	Magnesium (mg kg^{-1})	65 ± 2
Crude fat (g kg^{-1})	31.0 ± 1.5	Manganese (mg kg^{-1})	55 ± 0
Crude fibre (g kg^{-1})	151.0 ± 2.9	Sodium (mg kg^{-1})	327 ± 196
Iron (mg kg^{-1})	727 ± 62	Zinc (mg kg^{-1})	184 ± 195
Nonfibrous carbohydrates (mg kg^{-1})	14.7 ± 0.4		

Table 3 Concentration of sCOD in the liquid phase after centrifugation of the sludge at the end of the experiments. sCOD values marked with the same lowercase letter do not differ significantly ($n = 3$)

Temperature ($^{\circ}\text{C}$)	pH	sCOD PPBS without inoculum (g sCOD L^{-1})	sCOD PPBS with inoculum (g sCOD L^{-1})	sCOD inoculum only (g sCOD L^{-1})
35	6.3 (control)	0.7 ± 0.0^a		2.4 ± 0.0^b
	8	0.6 ± 0.0^a	3.0 ± 0.6^b	
	10	1.3 ± 0.0^a	15.1 ± 0.9^c	
55	6.2 (control)	0.9 ± 0.0^a		3.7 ± 0.7^c
	8	1.0 ± 0.0^a	5.7 ± 0.1^d	
	10	1.1 ± 0.1^a	15.3 ± 0.4^c	

PPBS only were not measured at pH 8 or 10; thus, further research is needed to determine the contribution of sCOD from the PPBS and inoculum, respectively, at pH 8 and 10.

The concentration of sCOD obtained in SI55pH10 is consistent with earlier published studies ($6\text{--}14\text{ g L}^{-1}$) [40, 41, 48, 49]. However, even concentrations up to $23.5\text{ g sCOD L}^{-1}$ were obtained with a combined alkali and hydrothermal pre-processing [50]; thus, it is possible to further improve nutrient availability of PPBS. Reported values of optimal pH for maximizing sCOD are $10\text{--}12$ [40, 41]. However, to the best of our knowledge, the optimal temperature for maximizing sCOD by fermentation of PPBS has not been reported. The response surface plot for fermentation with inoculum (Fig. 1) shows a positive correlation between sCOD and pH. A reasonable explanation is that the alkaline conditions contributed to the bacterial cell disruption [51] and improve the digestibility of lignocellulose fraction by enzymes [52]. The positive correlation between sCOD and temperature at pH 8 becomes weaker at increasing pH. For improving fermentation the impact of $\text{pH} > 10$ and temperatures $> 55\text{ }^{\circ}\text{C}$ on sCOD concentration should be explored.

3.2 Acidification

Fermentation of organic material produces fatty acids that cause a decrease in pH. The initial pH and final pH were measured to assess the acidification (Table 4). The inoculum improved the alkalinity of the material, resulting in a 1.8 higher final pH in the experiments that had inoculum addition (Table 4). Moreover, the final pH at the initial pH of 10 was higher compared to the initial pH of 8 Table 4; thus, both the addition of inoculum and the adjustment of the initial pH from 8 to 10 increase the final pH. Furthermore, the initial pH of raw PPBS (5.1) (Table 2) is not optimal for BSFL. BSFL needs a neutral to alkaline pH for optimal growth [53]; thus, the increase of the initial pH and the

addition of inoculum improve the suitability of fermented PPBS as feed for BSFL.

3.3 Obtained concentration of volatile fatty acids

The samples with the two highest sCOD values, SI35pH10 and SI55pH10, were selected for VFA analysis (Fig. 2). The total VFA concentrations were $2.2 \pm 0.3\text{ g L}^{-1}$ and $2.7 \pm 0.0\text{ g L}^{-1}$ for fermentation at $35\text{ }^{\circ}\text{C}$ and $55\text{ }^{\circ}\text{C}$, respectively. Pang et al. [21] fed BSFL with a feed enriched with VFA solution at a concentration of 0, 15, 26, 37, and 48 g L^{-1} , respectively, to improve growth of the larvae. A VFA concentration of 26 g L^{-1} produced the heaviest larvae (17.8 mg) compared to 0 and 15 g L^{-1} (7.8 mg and 9.4 mg , respectively) [19]. The concentration of VFA obtained in this study is too low to obtain a substantial increase in the weight of larvae [8]; thus, further research on improved fermentation of PPBS is needed to increase the concentration of VFA. However, the acetic acid fraction of the total VFA obtained, i.e., 68% at $35\text{ }^{\circ}\text{C}$ and 63% at $55\text{ }^{\circ}\text{C}$ (Fig. 2), is close to the content of VFA of the solution used by Pang et al. (77%) [19] and in line with earlier published studies 42–86% [54, 55]. The optimal VFA speciation for BSFL has not yet been published; thus, further research is needed.

Figure 2 shows the total concentration of VFA obtained in this study, which is substantially lower than previously

Table 4 The impact of the addition of inoculum and adjustment of initial pH on the final pH after the fermentation. pH values marked with the same lowercase letter do not differ significantly ($n = 12$)

	Final pH
Without inoculum	5.9 ± 0.5^a
With inoculum	7.7 ± 1.0^b
Initial pH 8	6.3 ± 0.8^a
Initial pH 10	7.4 ± 1.3^b

Fig. 1 sCOD response surface plot for fermentation with inoculum (mg COD L^{-1}). pH and temperature ($^{\circ}\text{C}$) as the plot axes. Interpolation of the responses was performed using MODDE

Fig. 2 Speciation and corresponding concentration of different VFA produced during the fermentation in SI35pH10 and SI55pH10 ($n = 3$)

reported in the literature, i.e., 6–7 g L⁻¹ at 39–55 °C [48, 54]. A reasonable explanation is the generation of inhibiting substances during pretreatment. Pretreatment of lignocellulose at alkaline pH generates substances (such as, syringic acid, acetosyringone (phenolic compounds) [2], furfural, and benzoic acid [56]) that are inhibiting to microbial fermentation [2]. One example of microbes positively related to production of VFA is the genus *Clostridium* [57]; thus, a reasonable explanation for the low concentration of VFA is that inhibiting substances generated during the alkali pretreatment slow down the growth of VFA-producing microorganisms such as *Clostridium*.

For further comparison of the fermentation of PPBS with fermentation of other substrates, the VFA values have been converted to the corresponding g COD L⁻¹ values [45]. The obtained total VFA was 2.8 and 3.6 g COD L⁻¹ in SI35pH10 and SI55pH10, respectively, that is higher than for slaughterhouse wastewater (1.5 g COD L⁻¹), in the same range as paper mill wastewater (3.1 g COD L⁻¹), but lower than for winery wastewater, sewage sludge, crude glycerol, meat and bone meal, and the organic fraction of municipal solid waste (4–8.1 g COD L⁻¹) [58].

Biogas was measured to monitor inhibition of methanogenesis. At pH 8, the increase of temperature produced larger biogas volume, resulting in 17.8 ± 0.8 Nml gVS⁻¹ in SI35pH8 and 25.1 ± 5.2 Nml gVS⁻¹ in SI55pH8. However, at pH 10, the biogas production was inhibited; thus, loss of VFA by methanogenesis was constrained. In particular, the volume measured was 0.0 ± 0.0 Nml gVS⁻¹ in SI35pH10 and 0.1 ± 0.1 Nml gVS⁻¹ in SI55pH10.

Further research on improving nutrient availability of PPBS could follow two paths. The positive correlation of pH and sCOD motivate assessing sCOD production at pH > 10 and temperature > 55 °C, however, impairing the preconditions for generation of VFA. The tolerance of BSFL to substrate pH > 10 has not been assessed; however, the BSFL tolerate a wide range of pH. Published studies indicate that BSFL grow equal good on substrates with a pH around 6 as on substrates with a pH of 10 [53, 59]. This illustrates the possibility of fermenting PPBS at pH > 10. The second path focusing on improvement of nutrient availability by maximizing VFA would apply other measures than increase in pH such as co-fermentation and the effect of other types of inoculum. Lin et al. [48] and Li et al. [54] applied co-fermentation with food waste and use of rumen microorganisms as inoculate, respectively. Co-fermentation has been suggested as a method to improve pH and C/N-ratio of a fermentation substrate [48]. Co-fermentation of substrate with high pH or C/N-ratio with a substrate with a low pH or C/N-ratio could give a mixture with suitable pH and C/N-ratio. The C/N-ratio of PPBS was not analyzed in this study; however, reported C/N-ratios for PPBS in published studies is in the range of

8–50 [60–62]. A C/N-ratio of 20–30 is considered optimum for anaerobic digestion [63]. For instance, for co-fermentation of green waste and food waste and organic fraction of municipal solid waste the optimum C/N-ratio was 23 and 27, respectively [64, 65]. Lin et al. [48] found co-fermentation of paper sludge and food waste favorable for production of VFA compared to fermentation of paper sludge alone. In addition, rumen microorganisms digest lignocellulose well [66] and are therefore suggested as inoculum for fermentation of lignocellulosic wastes [54]. Furthermore, further research should focus on methods such as over-liming, two-phase separation, and advanced oxidation [2] for removal of inhibiting substances generated during pretreatment of PPBS.

4 Conclusions

This study assessed fermentation of PPBS as pretreatment to improve PPBS feasibility as feed for black soldier fly larvae by increasing content of sCOD and volatile fatty acids (VFA). The obtained concentration of VFA was too low compared to the VFA concentration needed to improve growth of BSFL according to earlier published rearing trials. An initial pH of 10 and inoculum addition substantially increased the concentration of sCOD. Fermentation as done in this study does not convert PPBS to a feasible feed for black soldier fly larvae; thus, further research on improved fermentation is needed. However, fermentation at alkaline pH and addition of inoculum increases the final pH of PPBS which improves its feasibility as feed for BSFL. Future studies should explore pH > 10 and temperatures > 55 °C to increase sCOD and improving generation of VFA by removal of inhibiting substances, testing other types of inoculum (rumen microorganisms), and co-fermentation.

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Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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