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MILLING STRATEGIES TO INCREASE MICROBIAL CARBOHYDRATE SOLUBILIZATION VIA COTREATMENT IN MULTIPLE SUBSTRATES

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ABSTRACT

The use of lignocellulosic biomass, such as sugarcane bagasse and switchgrass, for second-generation ethanol production often requires expensive pretreatments to overcome the recalcitrance of the cellulosic matrix to deconstruction and saccharification of fermentable carbohydrates. Consolidated bioprocessing with cotreatment is one approach for simplifying this conversion process by combining milling with direct microbial conversion (fermentation) of lignocellulose into ethanol in a single process step, thus eliminating thermochemical pretreatments and increasing ethanol productivity. The milling modality applied to this process will have a direct impact on both the effectiveness of increasing carbohydrate solubilization, but also the energetic and thus economic feasibility. This work evaluates the use of different ball-milling strategies in a cotreatment regime using thermophilic cellulolytic bacteria to improve biomass solubilization. The results show that milling between fermentations in a short milling time can increase solubilization in 20% for switchgrass and sugarcane bagasse.

Keywords: Cotreatment. Lignocellulosic biomass. Sugarcane bagasse. Switchgrass. Thermophilic bacteria.

1 INTRODUCTION

Agricultural byproducts, industrial wastes, non-edible plants, and any cellulose-containing biomass can be used as raw-material for second-generation ethanol (E2G) production. Lignocellulosic material presents the best potential due to its low-cost regarding transportation, industrial applicability, and environmental availability around the globe [1]. It is composed of cellulose, hemicellulose, and lignin, with the building blocks of these polysaccharides (hexoses and pentoses) being fermentable sugars for a variety of microorganisms [1–3]. However, it is necessary to process the biomass to facilitate the use of fermentable sugars in further processes. Lignocellulose conversion into ethanol involves four sequential major steps: (i) comminution; (ii) biomass pretreatment; (iii) enzymatic hydrolysis (saccharification) and (iv) fermentation [3,4]. Cotreatment and consolidated bioprocessing (C-CBP) is one approach to simplify this process by eliminating the pretreatment step and combine all the other unit operations into a single process step, with the inclusion of mechanical disruption and simultaneous microbial conversion [5–9]. This also eliminates the need for the addition of chemicals and is conducted at milder temperatures (55 °C) for peak microorganism performance [6]. Cotreatment processing platform must achieve three performance criteria: high carbohydrate solubilization, retained microbial activity, and low energy use (<10% the HHV of the biomass). It showed to be effective in the first two criteria using multiple feedstocks in a proof-of-concept bioreactor with *in-situ* continuous milling for ~160 h, however this setup was very energy intensive [5–7]. In this work we evaluated different ball milling conditions in an *ex*-situ cotreatment platform using one round of milling between fermentation of both sugarcane bagasse and switchgrass. We seek to determine the milling conditions which result in high carbohydrate solubilization in an *ex-situ* Ferment-Mill-Ferment configuration and finding ways to lower the overall value of energy to milling, which is crucial for a successful cotreatment regime.

2 MATERIAL & METHODS

Sugarcane bagasse harvested in Brazil during 2022 season at Alta Mogiana sugar/ethanol mill (São Joaquim da Barra, SP, Brazil) dried at room temperature was milled in a TREU hammer mill by ITAL (Campinas, SP, Brazil) until all material could pass a 2 mm sieve screen. Senescent lowland switchgrass from Ernst Seeds (Meadville, PA, USA) was planted in May 2016, harvested in December 2018, and dried at 150-200°C upon harvesting and milled to 0.6 cm for storage. Prior to fermentations, the switchgrass was milled in a Retsch ZM 200 centrifugal mill (Verder Scientific, Newton, PA, USA) to pass through a 2 mm sieve screen [10]. Both feedstocks were fermented by the thermophilic bacteria *Clostridium thermocellum* strain DSM1313 in a coculture with *Thermoanaerobacterium thermosaccharolyticum* strain HG-8 ATCC 31960 in an anaerobic environment, achieved by purging the reactor headspace with pure N2 gas overnight. To generate once-fermented material, large volume bioreactors were prepared at a 30g/L solid loading and fermentations were carried out at pH 6.5 to support *T. thermosaccharolyticum* growth. After fermentation, the bioreactor contents were harvested and centrifuged. A supernatant aliquot (50 mL) was collected directly from the reactor before residual solids recovery. Part of the wet solids were dried to constant mass and homogenized to undergo quantitative saccharification (QS) analysis as described in (Sluiter et al., 2008). The arabinose, glucose, and xylose contained in the solids (after QS) and supernatant (after mild acid hydrolysis), and fermentation products (acetate, ethanol, formate and lactate) were separated and quantified via HPLC. The data reported as fractional carbohydrate solubilization (FCS) was based on the carbohydrate loss from the solids according to equation 1. Moisture content of the once-fermented biomass was determined by gravimetry to ensure correct solid loading in the following step. The once-fermented material generated in the first fermentation was then used to test different milling conditions and generate enough material to be used in triplicated bottle fermentation at a 5 g/L glucan equivalent solid loading to verify total carbohydrate fractional solubilization (TCFS) after the second round of fermentation. A flow diagram for the experiments is explained in Figure 1. After 7 days of incubation, the bottles contents were

harvested and centrifuged to undergo quantitative analysis as above mentioned. The overall carbohydrate solubilization (TCFS) was calculated by the equation 2.

$$FCS = 1 - \frac{Final\ (m_{gluc} + m_{xyl} + m_{ara})}{Initial\ (m_{gluc} + m_{xyl} + m_{ara})} \quad (1)$$

$$TFCS = FCS\ 1\ ferm. + (1 - FCS\ 1\ ferm.) * FCS\ 2\ ferm. \quad (2)$$

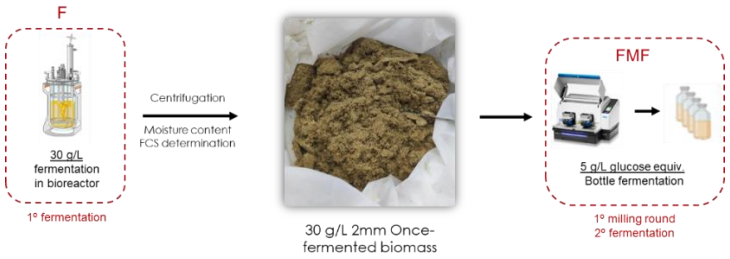

Figure 1: Schematic flow diagram of experiment.

The Retsch MM500 ball mill was used to test different milling conditions. The milling conditions that were considered effective enough for subsequent fermentation experiments were selected based on visual inspection of the milled material for evidence of particle size reduction. The different milling conditions were selected to include an exploration of multiple impact factors such as biomass moisture content during milling, milling time, frequency, chamber geometry, mass/size/material of milling media and milling mechanism (lateral shaking, oscillation).

Figure 2: Visual depiction of satisfactory and unsatisfactory milling conditions.

3 RESULTS & DISCUSSION

For switchgrass, the first round of fermentation (F) yielded 38% solubilization of carbohydrates, while for sugarcane bagasse it yielded 43% solubilization (Figure 1). For the second round of fermentation without milling (FF) these results increase to 57% for sugarcane bagasse and an 46% for switchgrass, which is considered a minimal increase. For milling before fermentation (MF), 57% solubilization was observed for sugarcane bagasse, being the highest FCS observed for one round of fermentation. When milling the biomass between fermentations (FMF), we can observe that at least 20% more carbohydrates were solubilized for both materials, observing 66% solubilization for sugarcane bagasse and 73% solubilization for switchgrass. The use of different milling media sizes and milling times also yielded different results for both sugarcane bagasse and switchgrass. For sugarcane bagasse, the best milling condition was milling the material for 10 minutes using 8 stainless steel beads of 20mm diameter. Milling the same material at the same solid loading in the chamber but for a longer time could have resulted in a higher solubilization, but this was not observed here. This could be due to the presence of metal particles in the biomass after milling that hinder fermentation or detain access for the microbes' enzymes that performs solubilization. It was also expected for the mix of beads to have provided a higher solubilization result than for just one bead size due to the mixed effects of friction and impact inside the milling chamber [11]. While this was not observed for the sizes of balls chosen, it is expected that the bead sizes/combinations could be further optimized. In the case of switchgrass, the use of milling increases solubilization under all conditions studied. As seen in Figure 3A, increasing the mass of the milling media (10mm steel balls) increases the solubilization, as does increasing milling time. Additionally, when comparing the MTI SFM-3 tabletop ball mill and the Retsch MM500 mill, the MTI outperforms when keeping milling time, the same, even with less overall mass of balls. An evaluation of the impact forces involved in milling of lignocellulosic biomass, such as shearing and friction forces, as well as an assessment of the resulting particle size reduction and degree of matrix defibrillation would provide an explanation for why the use of specific impact factors are useful for increasing carbohydrate solubilization. Previous data on continuous *in*-situ cotreatment shows that 160h of ball milling allowed 87% solubilization for switchgrass and 75% solubilization for sugarcane bagasse. With this FMF configuration we were able to provide noticeable

increase in solubilization for a short milling period, moving from 160h to 25 min of milling, thus providing a considerable reduction in energy demand for cotreatment.

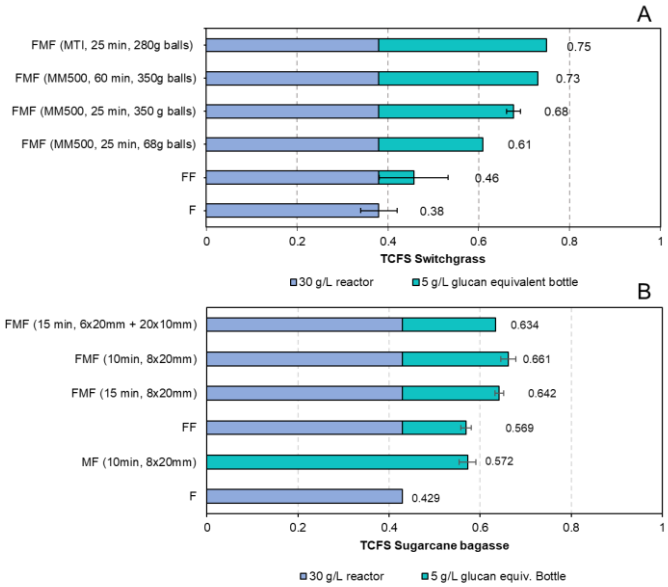

Figure 3: TFCS for both switchgrass (A) and sugarcane bagasse (B) fermentations.

4 CONCLUSION

In this work, we provide new insights into wet (moisture content >60%) biomass milling and ways to reduce milling time, and thus energy consumption, for biologically mediated carbohydrate solubilization. It is possible to obtain 70% solubilization of biomass with short (25 min) milling for switchgrass and 66% solubilization at 10 min for sugarcane bagasse. Future work will include an exploration of impact factors, such as optimizing the mix of milling media sizes in the chamber to improve carbohydrate solubilization at high moisture contents. Additionally, work is currently being done to characterize the physical changes that occur with milling fermented material.

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