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Liquefaction of sugarcane bagasse for enzyme production

F.M. Cunha [a,b,c], T. Kreke [a], A.C. Badino [b], C.S. Farinas [b,c], E. Ximenes [a], M.R. Ladisch [a,*]

[a] Laboratory of Renewable Resources Engineering and Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN 47907, USA
[b] Graduate Program of Chemical Engineering, Federal University of São Carlos, C.P. 676, 13565-905 São Carlos, SP, Brazil
[c] Embrapa Instrumentation, Rua XV de Novembro 1452, 13560-970 São Carlos, SP, Brazil

HIGHLIGHTS

- Submerged fermentation is made possible by liquefaction of biomass.
- Liquefaction gives a flowable slurry.
- Liquefied biomass is an effective fermentation media.
- Liquefied slurry gives 15× higher endoglucanase activity.

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ABSTRACT

The objective of this paper is to report liquefaction of pretreated and sterilized sugarcane bagasse for enhancing endoglucanase production through submerged fermentation by *Aspergillus niger*. After initial solid state fermentation of steam pretreated bagasse solids by *A. niger*, fed-batch addition of the substrate to cellulase in buffer over a 12 h period, followed by 36 h reaction, resulted in a liquid slurry with a viscosity of 0.30 ± 0.07 Pa s at 30% (w/v) solids. Addition of *A. niger* for submerged fermentation of sterile liquefied bagasse at 23% w/v solids resulted in an enzyme titer of 2.5 IU mL^{-1} or about 15× higher productivity than solid-state fermentation of non-liquefied bagasse (final activity of 0.17 IU mL^{-1}). Bagasse not treated by initial solid-state fermentation but liquefied with enzyme gave 2 IU mL^{-1}. These results show the utility of liquefied bagasse as a culture medium for enzyme production in submerged fermentations.

1. Introduction

The industrial competitiveness of the 2G (cellulose) ethanol depends on achieving efficient production and use of cellulase enzymes. Cellulase production by filamentous fungi may be achieved through either solid-state fermentation (SSF) or submerged fermentation (SmF). Despite many advantages of SSF over SmF, enzyme production in large-scale SSF bioreactors is hindered by low solids loadings, or if high solids are used, by solids handling and mass and heat transfer gradients during the cultivation process (Barrios-Gonzalez, 2012; Cunha et al., 2012; Esperança et al., 2014). Submerged cultivations with high solids loadings remain challenging since mass transfer and gas hold-up limitations are also compounded by viscosity increases that occur during the first hours of cultivation as a result of fungal growth.

Effects of the total solids loading on rheological behavior of cellulosic and lignocellulosic suspensions with the aim of improving the enzymatic hydrolysis of cellulose have been reported (Du et al., 2014; Jorgensen et al., 2007; Stickel et al., 2009). Rheological properties of sugarcane bagasse, specifically, were studied by Geddes et al. (2013) and Caldas Pereira et al. (2011), who demonstrated that a small amount of enzyme is able to decrease the viscosity of pretreated sugarcane bagasse slurries with improvements in hydrolysis coinciding with improved flow properties. Esperança et al. (2014) described hydrodynamic effects of carrying out microbial fermentation in a pneumatic bioreactor system at solids contents between 3% and 20% w/v. The current paper reports enzymatic and microbial liquefaction of steam exploded sugarcane bagasse in a fed-batch system followed by endoglucanase production at high solids loading by an *Aspergillus niger* wild type strain isolated from the Brazilian biome.

* Corresponding author. Tel.: +1 765 494 7022; fax: +1 765 494 7023.
E-mail address: ladisch@purdue.edu (M.R. Ladisch).

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2. Methods

2.1. Material

The inducer substrate for endoglucanase production was steam exploded sugarcane bagasse (particle size of 1 to 2 mm) kindly donated by the Sugarcane Research Center (CTC, Brazil). The steam explosion was conducted at 1667 kPa and 205 °C for 20 min. Composition of the pretreated bagasse, determined by standard NREL protocol (Sluiter et al., 2008) was 71% (w/w) glucan, 0.5% pentosan, 27.8% lignin and 3% ash. Sugarcane bagasse, before pretreatment, has a composition of 39% cellulose (glucan), 27.7 pentosans (including acetyl), 24.8% lignin, 3.9% ash, and 5.7% extractives (Ladisch et al., 2013).

2.2. Microorganism

A. niger wild type A12 strain, from Embrapa Food Technology collection (Rio de Janeiro, Brazil), isolated from black pepper (Couri and deFarias, 1995) and maintained at −18 °C in a 20% (w/w) glycerol/water solution, was activated in potato dextrose agar medium slants for 4 days at 32 °C. The spores were suspended by adding 0.3% Tween 80 (v/v) to the slants and their concentration was determined by counting in a Neubauer chamber.

2.3. Nutrient medium

Mandels nutrient medium was adapted from Mandels and Sternberg (1976) according to Cunha et al. (2012) and contained (w/v): 0.14% $(NH_4)_2SO_4$, 0.20% KH_2PO_4, 0.03% $CaCl_2$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.50% peptone, 0.20% yeast extract, 0.03% urea, 0.10% Tween 80 and 0.10% of salt solution (5 mg/L $FeSO_4 \cdot 7H_2O$, 1.6 mg/L, $MnSO_4 \cdot H_2O$, 1.4 mg/L $ZnSO_4 \cdot 7H_2O$, and 2.0 mg/L $CoCl_2$).

2.4. Solid state fermentation

The solid-state fermentation (SSF) cultivations were carried out for 72 h at static conditions and 32 °C in 250 mL Erlenmeyer flasks containing the steam exploded sugarcane bagasse to which 12 mL nutrient medium per 5 g bagasse (dry weight basis) was added. Enzymes were extracted at 35 °C by adding fermented solids at 1:10 (w/v) 50 mM sodium citrate buffer pH 4.8 and agitating at 120 rpm for 40 min. The extracts were vacuum filtered using Whatman glass microfiber filters and kept frozen at −20 °C until analysis. Runs were carried out in triplicate.

2.5. Liquefaction of sugarcane bagasse

Endoglucanase C, (Genencor Division of Danisco, Rochester, NY), 25 mL enzyme in 70 mL buffer was loaded into a 250 mL Erlenmeyer flask that was capped with a stopper. Pretreated bagasse was added in 4 g increments (dry weight basis) at 0, 1, 2, 3, 6, and 9 h with an additional 6 g at 12 h until the solids concentration was 30% w/v in 100 mL of 50 mM sodium citrate buffer, pH 4.8. Agitation was at 290 rpm in a bench top mixer (IKA, Wilmington, DE) for 24 or 48 h at either 32 °C or 50 °C. Bagasse treated for 12 h in a solid state fermentation was also liquefied using the same procedure.

2.6. Submerged fermentation with liquefied sugarcane bagasse

After the liquefaction, the slurry was sterilized at 121 °C for 30 min. *Modified* Mandels nutrient medium (Section 2.3) was added and the fungus *A. niger* A12 was inoculated at 10^7 spores per gram of dry biomass. Final solids concentration was 23% (w/v). The liquefied biomass was then fermented at 32 °C and 250 rpm for 72 h in an orbital shaker incubator (New Brunswick Innova 144). After fermentation, the remaining slurry was vacuum filtered using Whatman glass microfiber filters and kept frozen for analytical assays. Runs were carried out in triplicate.

2.7. Rheological measurements

Viscosity (μ_{app}) of the slurries was measured at 50 °C shear rates of 0.1 to 100 s^{-1} in a model AR-G2 rheometer (TA Instruments, USA) using a starch pasting impeller and cup geometry.

2.8. Analytical assay

Endoglucanase activity was measured with 1% (w/v) carboxymethyl cellulose in 50 mM sodium citrate buffer pH 4.8 (IUPAC, Ghose, 1987). One unit of endoglucanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min, using the DNS method.

3. Results and discussion

3.1. Liquefaction of sugarcane bagasse

The first liquefaction experiments used 30% solids, all added at $t = 0$. After 24 h of reaction, the stiffness of the sugarcane bagasse slurry was still so high that the measurement of the apparent viscosity was not possible. When the pretreated and fermented solids were added in a fed batch manner, the high initial enzyme to substrate ratio during the first hours of reaction allowed greater mixing and mass transfer. Bagasse in subsequent 4 g increments was added to a liquid slurry, thereby enabling mixing and mass transfer as the solids concentration increased and enzyme/solids ratio decreased over time. This approach was analogous to that of Rosgaard et al. (2007) for rice straw.

The resulting bagasse slurries showed non-Newtonian shear-thinning behavior. This is consistent with corn stover and 10% (w/v) sugarcane bagasse slurries (Du et al., 2014; Stickel et al., 2009; Caldas Pereira et al., 2011). The reduced viscosities are believed to reflect changes in structures of long-chain molecules and rearrangement of fibrous particles (Caldas Pereira et al., 2011; Du et al., 2014).

Liquefaction at 50 °C, which is optimal for enzyme activity, was compared to 32 °C which is suitable for *A. niger* A12 growth. The enzyme reaction results in a shear-thinning bagasse slurry (Fig. 1). *A. niger* growth caused additional production of enzyme due to the microorganism that was carried over from the solid state fermentation. The resulting slurry had an apparent viscosity of 0.87 Pa s at 100 s^{-1} shear rate. Slopes of the data sets were similar at 24 and 48 h for both enzyme and combined microbial and enzyme liquefaction, respectively. However, after 24 h. of reaction time, the viscosity was still too high for an efficient fermentation (upper curve in Fig. 1). A longer reaction time (48 h) decreased viscosity further (lower curve in Fig. 1).

The viscosity profiles are similar at both 32 and 50 °C, although enzyme stability is 4× higher at 32 °C than at 50 °C. Overall, the preferred conditions are a total liquefaction time of 48 h at 32 °C for bagasse that is treated through a combination of 12 h solid state fermentation and 36 h enzyme assisted liquefaction. The final apparent viscosity is 0.30 Pa s at 100 s^{-1} shear rate (Fig. 1). Liquefaction using bagasse treated with enzyme, only, corresponded to 0.48 Pa s at 100 s^{-1} shear rate.

3.2. Endoglucanase production using liquefied sugarcane bagasse

Endoglucanase production by submerged fermentation at 32 °C for 72 h using sugarcane bagasse, liquefied either in the absence or

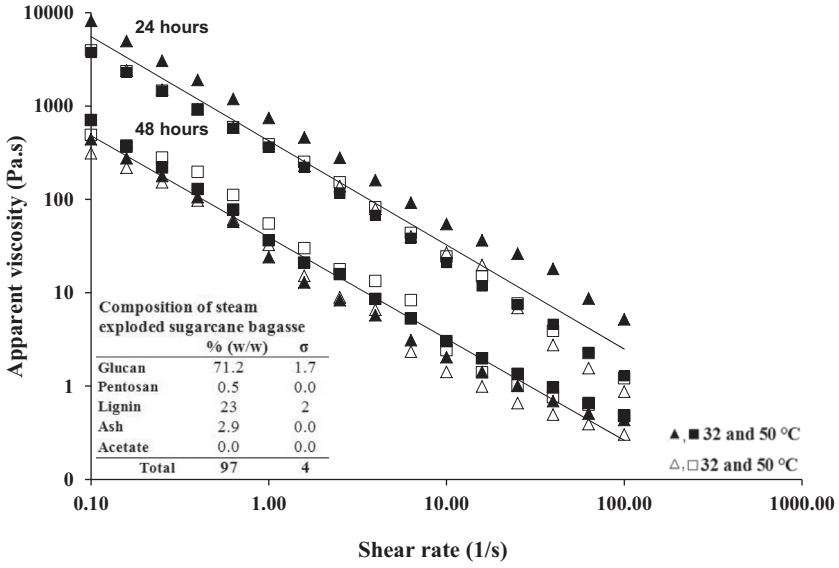

Fig. 1. Change in viscosity (η) as a function of shear rate (γ) after 24 and 48 h incubation with 301 IU endoglucanase per gram of dry bagasse. (▲, ■) Enzyme liquefaction at 32 and 50 °C or (Δ, □) enzyme combined with microbial liquefaction; -trend lines. Log $\eta = c + A \log \gamma$. $A_{24\,h} = -0.660$, $A_{48\,h} = -0.692$. Viscosity at $t = 0$ was not measureable due to solid characteristics of bagasse at 300 g/L. Initial composition of all samples as indicated in table (analysis by NREL standard procedure (Sluiter et al., 2008).

presence of *A. Niger* A12 corresponded to 2 to 2.5 IU mL^{-1}. Fermentation after liquefaction of this material resulted in endoglucanase activity of up to 15-fold higher than solid-state fermentation, with enzyme activities of 2.5 ± 0.3 IU mL^{-1} and 0.17 ± 0.3 IU mL^{-1} obtained in submerged and solid state cultivations, respectively.

The presence of *A. niger* A12 during liquefaction resulted in 22% higher endoglucanase titers compared to liquefaction in the absence of the fungus where endoglucanase titers were 2 IU mL^{-1}. In comparison, endoglucanase production on untreated bagasse obtained in this study was on the same order of that obtained by Delabona et al. (2012) after 96 h of solid-sate fermentation of untreated sugarcane bagasse by an *A. fumigatus* strain isolated from the Amazon forest. Delabona's organism gave 0.167 IU mL^{-1} endoglucanase production after 96 h of solid-state fermentation.

Liquid hot water pretreatment of sugarcane bagasse followed by additional substrate washing with distilled water gave an enzyme yield of 0.75 IU mL^{-1} which was higher than the 0.167 IU mL^{-1} for untreated substrate of Delabona et al. (2012) and Rodriguez-Zuniga et al. (2014). Liquid hot water pretreatment cooks the lignocellulose in hot, pressurized water causing release of inhibitors into the water (Kim et al., 2013a,b). In this case, removal of microbial and enzyme inhibitors (for instance phenolic compounds) by washing may help to achieve both higher enzyme activity and enzyme production (Ximenes et al., 2010, 2011).

This study has demonstrated, for the first time, the potential application of enzyme production after liquefaction of sugarcane bagasse in order to obtain high lignocellulose concentrations. High solids loading in large-scale cultivations combine the advantages of high enzyme productivity for solid-state fermentation and the scalability of submerged fermentation.

4. Conclusions

Enzyme catalyzed liquefaction of sugarcane bagasse enables submerged fermentation of *A. niger* and production of endoglucanase at a 12-fold higher yield than solid state fermentation. When a combined enzymatic and biological liquefaction promoted by *A. niger* A12 is used, the viscosity of 0.30 ± 0.07 Pa s is lower than bagasse liquefied using enzyme alone (0.48 ± 0.08 Pa s, at 100 s^{-1} shear rate). A 15-fold higher yield of endoglucanase is observed when using bagasse, processed through combined enzymatic and biological liquefaction, as culture medium in submerged fermentation.

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