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Second-generation ethanol production from elephant grass at high total solids

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HIGHLIGHTS

- Ethanol production was about double in the rotating hydrolysis reactor.
- Greatest glucose yield was obtained for 5% (w/v) untreated biomass.
- Highest glucose and ethanol concentrations were obtained using 20% (w/v) solids.

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ABSTRACT

The enzymatic hydrolysis of *Pennisetum purpureum* (elephant grass) was evaluated at high total solid levels (from 4% to 20% (w/v)) in a concomitant ball milling treatment in a rotating hydrolysis reactor (RHR). The greatest glucose yield was 20.17% when 4% (w/v) untreated biomass was employed. When sugars obtained from enzymatic hydrolysis were submitted to fermentation with *Saccharomyces cerevisiae*, the greatest ethanol yield was 22.61% when 4% (w/v) untreated biomass was employed; however, the highest glucose concentration (12.47 g/L) was obtaining using 20% (w/v) solids and highest ethanol concentration (6.1 g/L) was obtained using 16% (w/v) solids. When elephant grass was hydrolyzed in the rotating hydrolysis reactor, ethanol production was about double that was produced when the biomass was hydrolyzed in a static reactor (SR). These data indicate that it is possible to produce ethanol from elephant grass when milling treatment and enzymatic hydrolysis are performed at the same time.

1. Introduction

Energy consumption has increased, and petroleum has become the main resource to meet increasing energy demands. However, with the increased use of petroleum products such as gasoline, the amount of greenhouse gases released into the atmosphere has also increased, and it is known that fossil fuels cannot supply all of the world's energy requirements (Scholl et al., 2015). On the other hand, the use of alternative energy sources, such as ethanol, is increasing, and the use of renewable energy may lead to decreased greenhouse gas emissions (Bayrakci and Koçar, 2014).

In this sense, lignocellulosic biomass, mainly composed of cellulose, hemicellulose, lignin, extractives and ashes, is an abundant source of raw material that can be converted into useful materials and energy (Resch et al., 2013). The chemical properties of the components of lignocellulosics make them a substrate of biotechnological value. The biological conversion of these lignocellulosic materials into ethanol offers numerous benefits, but its development is hampered by economic and technical aspects (Sánchez and Cardona, 2008). Lignocellulosic biotransformation focuses on the enzymatic hydrolysis of cellulose to glucose, followed by fermentation to ethanol (Gan et al., 2002).

For ethanol production, an ideal lignocellulosic material should be employed. Elephant grass has high potential because of its productivity compared to other species, as it produces about 45 tons of dry matter per hectare per year. The production of sugarcane and corn is about 21 tons (sugar and bagasse) and 13 tons (grain and stover), respectively, on a dry matter basis (Menegol et al., 2014a). Additionally, untreated elephant grass is similar in cellulose composition to sugarcane bagasse, at around 36% cellulose (Menegol et al., 2014b), while sugarcane bagasse contains 34–45% cellulose (Szczerbowski et al., 2014). Additionally, the elephant grass contains approximately 22.5% of hemicellulose, 8% ash, 5.5% protein and 20% lignin. Due these characteristics, elephant grass is an interesting biomass to produce second generation ethanol.

The implementation of enzymatic hydrolysis using high solid concentrations is considered of fundamental importance for the reduction of costs for obtaining sugars from lignocellulosic

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biomass, especially regarding second generation ethanol production which involves fermentation and distillation (Lin and Tanaka, 2006). In conventional pretreatments, compounds are formed which negatively interfere in microbial metabolism, such as furfural, hydroxymethylfurfural and acetic acid. When high concentrations of raw material (substrate) are used, the sugar yield can usually be reduced, but the causes are not completely understood. It may be due to inefficient contact between the enzyme and the substrate, problems with homogenizing the lignocellulosic material and enzymes, as well as enzyme adsorption onto cellulosic or non-cellulosic substrate components such as lignin (Ramachandriya et al., 2013).

A range of microorganisms are able to produce an enzyme complex able to catalyze the enzymatic hydrolysis of different lignocellulosic materials. Mutant strains of *Penicillium echinulatum* are known to secrete high levels of enzymes, 35 FPU/g of substrate in solid-state culture and 8.3 FPU/mL in submerged culture (Camassola and Dillon, 2010; Dillon et al., 2011; dos Reis et al., 2013) and the potential of these strains for enzymatic hydrolysis has been previously investigated (Menegol et al., 2014a,b).

In addition to the cost of the raw material, the pretreatment and enzymatic hydrolysis steps are major contributors to the total production cost of ethanol from biomass. In this sense, the objective of this study was to evaluate concomitant ball milling treatment and enzymatic hydrolysis of elephant grass in order to obtain the highest release of glucose, using enzyme complexes of *P. echinulatum* produced in solid-state cultivation. These experiments were performed in a RHR (rotating hydrolysis reactor) in comparison to a SR (static reactor). Additionally, hexose fermentation by *Saccharomyces cerevisiae* was compared using the samples prepared in the RHR and SR.

2. Materials and methods

2.1. Microorganisms

The cellulolytic mutant *P. echinulatum* strain S1M29 was used in this study for enzyme production. This strain was obtained from strain 9A02S1 of *P. echinulatum*, with the use of hydrogen peroxide mutagenesis and the selection of mutants in 2-deoxyglucose medium. The strain was grown on C-agar slants for up to 7 days at 28 °C until conidia formed, and then stored at 4 °C until use (Dillon et al., 2011).

For fermentation, *S. cerevisiae* strain CAT-1 was used. This strain was kindly provided by Dr. Luiz Humberto Gomes of Escola Superior de Agricultura Luiz de Queiroz (ESALQ), University of São Paulo, Piracicaba, SP. The CAT-1 strain is a yeast that predominates during fermentation in ethanol plants in Brazil. The strain was grown on YPD (Yeast Extract Peptone Dextrose Agar) medium for up to 2 days at 28 °C, and then stored at 4 °C until use.

2.2. Substrate

The elephant grass samples were collected in the city of Nova Petrópolis, Rio Grande do Sul, Brazil. Plant harvesting was carried out 6 months after planting. This material was initially dried at 60 °C for 3 days and then triturated with a forage chopper (0.5–2 cm) and stored in polypropylene bags until used (Menegol et al., 2014a). The biomass composition was assessed as described by (Menegol et al., 2014b).

2.3. Scanning electron microscopy (SEM)

The changes in the physical biomass structure were observed by scanning electron microscopy performed according to the methodology used in the Materials Characterization Laboratory (LCMAT) of the University of Caxias do Sul. The methodology is based on a process called magnetron-sputtering plasma, known as physical vapor deposition (PVD), according to Schneider et al. (2014).

2.4. Enzyme production

Solid-state cultivations were conducted in 32 × 24 cm trays and were closed with a gauze-covered cotton wool plug containing 200 g of dry mass of the production media (50% (w/w) wheat bran, 25% (w/w) untreated elephant grass and 25% (w/w) untreated sugarcane bagasse) and 200 mL basal salt solution containing (in g/L) KH_2PO_4, 20; $(NH_4)_2SO_4$, 13; $CO(NH_2)_2$, 3; $MgSO_4{\cdot}7H_2O$, 3; $CaCl_2$, 3; $FeSO_4{\cdot}7H_2O$, 0.050; $MnSO_4{\cdot}H_2O$, 0.0156; $ZnSO_4{\cdot}7H_2O$, 0.014; and $CoCl_2$. 0.0020. The media were autoclaved at 121 °C for 1 h. Each medium was then inoculated with sufficient conidial suspension to give a final concentration of 1×10^6 conidia per gram of dry mass of production media. The moisture of the media was adjusted to 67% (w/w) by the addition of distilled water. The trays were incubated at 28 °C and 90% humidity for 4 days. To extract the enzymes after incubation, 600 mL of sodium citrate buffer (pH 4.8, 50 mmol/L) was added and incubated under agitation for 30 min at 4 °C, then filtered through polyester fabric.

2.5. Enzyme assay

The enzymatic activity of the enzyme broth was assessed to evaluate the stability of the enzymes during hydrolysis.

Enzymatic activity was analyzed on filter paper (filter paper activity – FPA) according to Ghose (1987). Endoglucanase activity was determined according to Ghose (1987), using 2% (w/v) carboxymethylcellulose solution in citrate buffer. Xylanase activity was determined in the same way as endoglucanase activity, but 1% (w/v) oat spelt xylan was used as the substrate in the place of carboxymethylcellulose. Reducing sugars were estimated as either xylose or glucose equivalents by the dinitrosalicylic acid (DNS) method (Bailey et al., 1992).

One international unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar from the appropriate substrate per minute under the assay conditions.

For β-glucosidase activity, ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) was used as the substrate. One unit of β-glucosidase activity (using the substrate ρNPG) was defined as the amount of enzyme required to hydrolyze 1 μmol of ρNPG per min (Daroit et al., 2008).

2.6. Determination of the adsorption of cellulases and xylanases by the substrate

In order to verify the adsorption of cellulases and xylanases in the lignocellulosic substrates, hydrolysis of different substrates was carried out in 50 mL Duran® flasks at 50 °C for 1 h and 6 h. After hydrolysis, these materials were filtered through polyester fabric and centrifuged for 20 min at 9800×*g*. Gels for total protein and zymograms for the detection of endoglucanase, exoglucanase, xylanase and β-glucosidase activities were prepared. First, 8% (w/v) substrate was tested using as the substrate elephant grass, sugarcane bagasse, Avicel®, while for 20% (w/v) substrate, only elephant grass was used. A control made from enzymatic extract and citrate buffer without addition of substrate (cellulose or elephant grass) was also used. It was used the same volume of sample containing enzymes for the hydrolysis carried out using 8% (w/v) and 20% (w/v) of substrate for endoglucanase, exoglucanase and xylanase zymograms, but for total proteins and β-glucosidase activities from hydrolysis using 20% (w/v) of substrate, it was used half the amount of sample than was used for 8% (w/v).