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Increased release of fermentable sugars from elephant grass by enzymatic hydrolysis in the presence of surfactants

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ABSTRACT

In the search for renewable energy sources, elephant grass is an alternative substrate for ethanol production, but this substrate must be hydrolyzed by cellulases and xylanases to liberate fermentable sugars. During enzymatic hydrolysis, cellulase activity is reduced by the irreversible adsorption of cellulase onto cellulose, decreasing the rate of hydrolysis. Adding surfactants during hydrolysis can improve the process. The effects of Tween® and Triton® surfactants on the enzymatic hydrolysis of elephant grass were evaluated in this context. The data indicate that pretreatment with sodium hydroxide, along with a smaller particle size (0.075–0.152 mm) and the use of Tween 80®, increased the efficiency of releasing reducing sugars from pretreated elephant grass biomass. Thus, it is possible to reduce grinding costs in second-generation ethanol production through the use of surfactants, as they allow efficient hydrolysis of larger biomass particles.

1. Introduction

The rising demand for liquid transportation fuels is placing increasing pressure on finite oil reserves, raising prices and driving the search for oil to increasingly remote locations, often in fragile ecosystems. Additionally, the planet's climate is affected by carbon dioxide emitted by the use of fossilized carbon as an energy source. The search for energy sources other than fossil fuels is ongoing. Within the alternative energy field, ethanol has become an attractive and promising energy source due to its production from renewable raw materials [1,2]. The tropical species *Pennisetum purpureum*, or elephant grass, is considered a new alternative energy crop expected to provide some countries with abundant and sustainable lignocellulosic biomass for biofuel production [3,4]. *P. purpureum* holds great potential for the efficient conversion of solar energy to biomass because, as a C4 plant, it possesses adaptations to suppress photorespiration [5]. It is also highly productive relative to other species, producing approximately 45 tonnes of dry matter per hectare per year. In comparison, the production of sugar cane and corn is approximately 21 tonnes (sugar and bagasse) and 13 tonnes (grain and stover), respectively [6]. Due to these characteristics, elephant grass has great potential for use in second-generation ethanol production.

Cost-effectively maximizing saccharification of cellulose and hemicellulose into fermentable sugars is crucial in bioconversion of lignocellulosic feedstocks to biofuels. One of the challenges facing the field is the impractically high cost of saccharification enzymes [7]. However, mutant strains of the microorganism *Penicillium echinulatum* [8] produce both cellulases and xylanases. Furthermore, their cellulolytic enzyme complex contains β-glucosidase in greater proportions than *Trichoderma reesei* [10] and is stable at 50 °C, making these mutants suitable for enzymatic hydrolysis [9].

Cellulase activity decreases over the course of enzymatic hydrolysis, partially due to the irreversible adsorption and inactivation of cellulases onto cellulose [11]. The addition of surfactants during enzymatic hydrolysis is a promising method to minimize this adsorption of cellulase onto cellulose. Surfactants are liquid soluble, surface-active agents that reduce the surface and interfacial tensions that cause adsorption at interfaces. Surfactants commonly used in enzymatic hydrolysis include polyoxyethylene sorbitan monolaurate (Tween 20®), polyoxyethylene sorbitan monooleate (Tween 80®) [12], polyoxyethylene glycol [13], polyoxyethylenesorbitan monooleate (Tween 81®), Emulgen 147® [14], sophorolipids, rhamnolipids and bacitracin [15]. Several authors have reported that the addition of surfactants to enzymatic hydrolysis mixtures increased cellulose hydrolysis [13,16].

Smaller sizes of the substrate solids increase the yield of hydrolysis, particularly at concentrations of less than 5% dry matter [17].

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However, obtaining reduced particle sizes requires higher energy expenses.

Thus, this study aimed to evaluate the effects of particle size and two different surfactants on the enzymatic hydrolysis of elephant grass biomasses. Enzyme complexes from *P. echinulatum* were produced in a solid-state cultivation medium, and elephant grass was used as both the inducer and carbon source of these cultures.

2. Experimental

2.1. Microorganisms

The cellulolytic mutant strain *P. echinulatum* 9A02S1 (DMS 18942) was used in this study. This strain was obtained by exposing wild type *P. echinulatum* strain 2HH to ultra-violet (UV) light and hydrogen peroxide (H_2O_2) [18]. These strains were obtained from the culture collection of the Enzymes and Biomass Laboratory of the Institute of Biotechnology at Caxias do Sul, Rio Grande do Sul, Brazil. The strain was grown on C-agar slants [19] for up to 7 days at 28 °C until conidia formed, then stored at 4 °C until use.

2.2. Substrates and pretreatments

Elephant grass samples were collected in the city of Nova Petrópolis, Rio Grande do Sul, Brazil. Plant cutting was carried out six months after planting. Grass was first dried at 60 °C for 3 days before trituration with a forage chopper. The samples were then separated into different particle sizes using pore sieves between 0.075 and 4.75 mm, corresponding to 4 and 200 mesh, respectively, and chemically pretreated. Pretreatment with sodium hydroxide was chosen for its effectiveness at removing lignin. A NaOH concentration of 2% was selected based on our previous testing and literature data [7].

Elephant grass samples were pretreated with 2% w/v NaOH solution at a ratio of 1:4, solid:liquid, then autoclaved at 121 °C for 15 min. The biomass was then washed until it reached a neutral pH.

After pretreatment, a small portion of substrate was dried to determine its moisture content, and the remainder reserved for enzymatic hydrolysis.

2.3. Enzyme production

Solid-state cultures of *P. echinulatum* were conducted in 32 × 24-cm trays. The trays were closed with gauze-covered cotton plugs and contained 200 g dry mass of the production media (50% wheat bran and 50% untreated elephant grass) and 200 mL of a basal salt solution (20 g/L KH_2PO_4, 13 g/L $(NH_4)_2SO_4$, 3 g/L $CO(NH_2)_2$, 3 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L $CaCl_2$, 0.050 g/L $FeSO_4{\cdot}7H_2O$, 0.0156 g/L $MnSO_4{\cdot}H_2O$, 0.014 g/L $ZnSO_4{\cdot}7H_2O$ and 0.0020 g/L $CoCl_2$). Untreated elephant grass was used, as our previous tests demonstrated its efficiency. The media was autoclaved at 121 °C for 20 min. Each medium was then inoculated with conidial suspension to a final concentration of 1×10^6 conidia per gram of dry production media. Media moisture was adjusted to 67% with distilled water. The trays were incubated at 28 °C and 90% humidity for 4 days. After incubation, enzymes were extracted by adding 600 mL of distilled water to the tray and mixing under agitation for 30 min at 4 °C before being filtered. The enzymatic activity was analyzed on filter paper (Filter Paper Activity – FPA), according to the method of Camassola and Dillon [20]. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per min under the assay conditions.

2.4. Enzymatic hydrolysis of elephant grass

Enzymatic hydrolysis was performed in vials; 50 mL of sodium citrate buffer (pH 4.8, 50 mM) was added to samples of elephant grass, either untreated or pretreated with 2% NaOH (w/v). Vials were incubated with enzyme broth in the presence of 0.01% (w/v) sodium azide at 50 °C and shaken at 150 rpm. Time points of 1 mL were collected periodically: a zero-hour time point, initially followed by one every four hours, and then at higher intervals (up to 72 h), to track biomass hydrolysis and the release of reducing sugars [17]. Hydrolysis time courses were performed in triplicate.

A surfactant concentration of 2.5 g/L (Tween 80® or Triton X-100®) and a substrate concentration of 2% were selected for hydrolysis. The enzyme extract from *P. echinulatum* had an activity of 2.3 FPU/mL, and a final enzyme concentration of 20 FPU per g of substrate was used for hydrolysis. The samples were centrifuged at 3000*g* for 15 min, and the reducing sugars present in the enzymatic hydrolysis solutions were measured by Miller's method [21]. Tween® surfactants were selected because they increase cellulase activity during enzymatic hydrolysis and do not inhibit cell growth during subsequent fermentation [15,17].

2.5. Determination of ash content in cellulosic materials

The ash content determinations were made according to the Standard Biomass Analytical Procedures, NREL/TP-510-42621 [22].

2.6. Analysis of reducing sugars

Reducing sugars present in enzymatic hydrolysis solutions were measured by Miller's method [21] and a glucose PAP Liquiform kit (Labtest®, Lagoa Santa, MG, Brazil), using 2 μL of sample and 200 μL of reagent. A standard curve was constructed with glucose solutions of 0, 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 mg/mL to quantify the released sugars.

2.7. Determination of extractable compounds

Determinations of extractable compounds were made according to the Standard Biomass Analytical Procedures, NREL/TP-510-42619 [23].

2.8. Chemical analysis of cellulosic substrates

The chemical compositions of the substrates were determined according to the Standard Biomass Analytical Procedures, NREL/TP-510-42618 [24].

2.9. Determination of total nitrogen and proteins

Kjeldahl analysis of total protein was performed by the Laboratory of Food Analysis and Research (LAPA) at the University of Caxias do Sul.

2.10. Statistical tests

Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's post-test for a $p < 0.05$ using the PrismGraphPad program.

3. Results and discussion

Converting cellulose to glucose is a persistent bottleneck in the production of biofuel. Raw material heterogeneity and the effects