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Effect of nutrient supplementation on ethanol production in different strategies of saccharification and fermentation from acid pretreated rice straw

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ABSTRACT

The effect of nutrient supplementation on ethanol production by recently selected thermotolerant yeast (*Kluyveromyces marxianus* NRRL Y-6860) was investigated in different strategies of saccharification and fermentation employing rice straw pretreated by dilute acid. Among the evaluated strategies, similar ethanol yields ($Y_{P/S}$ ~ 0.23 g g^{-1}) were obtained with or without nutrient addition. However, considering the whole process time, the strategy based on simultaneous saccharification and fermentation (SSF), without prehydrolysis, was assigned as the most suitable configuration due to the highest ethanol volumetric productivity (1.4 g L^{-1} h^{-1}), about 2-fold higher in relation to the others. The impact of enzymatic preparation employed in this study was also evaluated on glucose fermentation in semi-synthetic medium. The enzymatic preparation affected both glucose consumption and ethanol production by *K. marxianus* NRRL Y-6860, but just in the absence of nutrients. Therefore, the enzyme type and loading should be carefully defined, not only by the capital costs involved, but also by the possibility of increasing the fermentation inhibitors.

1. Introduction

Ethanol production from lignocellulosic biomass represents a potential alternative to petroleum based fuel due to its renewable nature and availability. Among these materials, agriculture residues with no food value (eg. sugar cane bagasse, rice straw, corn stover) are of major interest and are expected to be the main feedstocks in the near future [1,2]. Rice straw, the stalk of the plant that is left over on the field upon harvesting of the rice grain, is one of the main lignocellulosic residues worldwide, with estimated availability of 685 million tons per year [3]. As a lignocellulosic material, rice straw is mainly composed by cellulose (33%–43%), hemicellulose (23%–35%) and lignin (10%–22.3%), but differs from other lignocellulosics by its high content of ash (10%–12%) [1,3–5]. Since rice straw has a high content of sugary fractions (above 60%), constitutes a potential material for ethanol production by biotechnological route, collaborating with future energy needs.

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Pretreatment is required to decrease the crystallinity of cellulose and remove lignin and hemicellulose, which acts as a physical barrier to enzymes. As a result, biomass accessible surface area to enzymatic hydrolysis is increased, enhancing the conversion rate of carbohydrate polymers into monomeric sugars, which will be fermented into ethanol [6]. Dilute acid pretreatment removes mainly the hemicellulose, allowing high recovery of pentose sugars in the liquid fraction, which can also be converted into ethanol [7,8] and other valuables chemicals such as xylitol [9,10]. Thus, by using the hemicellulosic hydrolysate and the cellulosic fraction obtained in the dilute acid pretreatment, the carbohydrates from rice straw feedstock could be almost entirely used as a source of substrate for biotechnological products.

Taking into account the production of ethanol only from the cellulosic fraction, the glucose obtained by enzymatic conversion must be fermented by microorganisms. The simultaneous saccharification and fermentation (SSF) process, which combine in a single reactor the enzymatic hydrolysis of cellulose and the fermentation of the obtained sugars, has been indicated as a strategy to avoid inhibition of cellulolytic enzymes, since glucose is immediately consumed by the microorganism. In addition, the SSF also decreases process costs in comparison to the separate hydrolysis and fermentation [11,12]. Nevertheless, one of the greatest practical challenges of SSF is to match the temperature condition required for this simultaneous process, since there are significant differences between the optimum temperature of enzymatic hydrolysis (45 °C–50 °C) and fermentation (30 °C–37 °C), considering the most known ethanol producer *Saccharomyces cerevisiae* [13]. Therefore, the use of thermotolerant yeasts, such as some strains of *Kluyveromyces marxianus*, able to ferment glucose into ethanol at temperatures above 40 °C, has been recommended [12]. Recently, we have selected in our laboratories a thermotolerant *K. marxianus* strain (NRRL Y-6860) capable to ferment glucose at 45 °C with high yield and ethanol volumetric productivity [14].

Usually the media for ethanol production on bench scale are supplemented with peptone, yeast extract and salts to obtain rapid and efficient fermentation, but such addition is not feasible in industrial processes due to the high costs associated [15]. Thus, considering that the cost of culture medium is a decisive factor in determining the economic viability of bioconversion process, especially when lignocellulosic biomass is used as substrate, it is important to define the impact of nutrient requirements in fermentation, as well as investigate the yeast's behavior in absence of additional supplementation.

Another important factor to be considered in obtaining ethanol from cellulose is the enzyme solution. Commercial cellulase preparations employed in enzymatic hydrolysis of lignocellulosic biomass also contain a variety of noncellulase proteins as well as other metabolites and additives to increase the product's shelf-life [16], which can impact the fermentation medium since they remain in the reactor during the process. Cellulases are generally obtained by submerged cultivation of filamentous fungi belonging to the genera *Trichoderma* and *Aspergillus* in a complex medium based on the earlier studies published by Mandels and Reese [17], in which mineral and organic nutrients are used together with an inducer, which could be a lignocellulosic material or low molecular weight compounds, such as lactose [18]. However, the exact composition of fermentation media are not revealed by the commercial cellulases producers. According to Nieves et al. [19], bulking agents, such as low-cost, soluble carbohydrates and polyols, are ideal to stabilization and diminished microbial contamination of liquid enzyme preparations. Some additives were already reported in cellulase preparations as stabilizers, for example sorbitol, glycerol, arabinose and sucrose, but they could act as inhibitory substances for some microorganisms growth [16,19,20].

Within this context, the objective of the present work was to study the effect of nutrient supplementation on ethanol production by *K. marxianus* NRRL Y-6860 in different strategies of SSF and SHF and also to evaluate the impact of commercial enzyme preparation on yeast's fermentation parameters in semi-synthetic glucose medium.

2. Material and methods

2.1. Rice straw pretreatment

Rice straw (RS) was obtained from fields in Canas, São Paulo state, Brazil. The material was sun dried until 10% moisture content, milled in hammer mill (about 1 cm in length and 1 mm in thickness), weighed and bagged until further use. The acid pretreatment was performed in a 300 L stainless steel AISI 316 reactor, equipped with indirect heating by an electric resistance oil shirt. The material was submitted to a reaction with sulfuric acid (100 mg H_2SO_4 g^{-1} dry matter) in a 1:10 dry matter/acid solution ratio at 120 °C during 30 min, according to Roberto et al. [5]. The resulting solid material was washed and then dried by the sun until 10% moisture content, obtaining the rice straw cellulignin (RSC).

2.2. Enzymatic hydrolysis

For enzymatic hydrolysis, two commercial enzymes preparations from Novozymes (Denmark) were used, cellulase from *Trichoderma reesei* ATCC 26921 (Cellubrix) and β-glucosidase from *Aspergillus niger* (Novozyme 188). Total cellulase activity was determined according to National Renewable Energy Laboratory's (NREL) analytical procedures [21]. β-glucosidase activity was determined using *p*-nitrophenyl-*β*-D-glucosidase as substrate, according to Wood and Bhat [22]. The cellulolytic activity of Cellubrix was 40.2 filter paper units (FPU) per mL of solution. The β-glucosidase activities were 133 IU mL^{-1} for Cellubrix and 640 IU mL^{-1} for Novozyme 188.

The enzymatic hydrolysis assays were conducted in triplicate at 8% (w/v) RSC loading, at pH 4.8 (sodium citrate buffer 50 mM L^{-1}), final volume of 50 mL in 125 mL Erlenmeyer flasks at 100 rpm (orbital shaking) and 45 °C incubation temperature. In order to define the appropriate enzyme loading for further saccharification and fermentation experiments, were evaluated three loadings of cellulase (10, 25 and 40 FPU g^{-1} RSC), and the effect of supplementation with additional β-glucosidase (Novozyme 188) at 1:1 proportion (10:10, 25:25 and 40:40 FPU g^{-1} RSC: IU g^{-1} RSC for Cellubrix and Novozyme 188, respectively) on cellulose hydrolysis yield.