

ETHANOL PRODUCTION FROM ENZYMATICALLY PRETREATED WHEAT STRAW

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ABSTRACT

Lignocellulosic biomass can be utilized to produce ethanol, a promising alternative energy source for the limited crude oil. Wheat straw is an abundant agricultural residue which can be used as lignocellulosic raw material for bioconversion. There are mainly two processes involved in the bioconversion: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars, and fermentation of the sugars to ethanol. The current study involved the optimization of enzymatic hydrolysis of a wheat straw pretreated by acid hydrolysis, using a mixture of commercial cellulases: celluclast 1.5L + Novozym 188, with further fermentation of the hydrolysate' sugar content by three ethanologenic strains, namely two yeast of *Saccharomyces cerevisiae* (strains F and K) and a bacterial strain, *Zymomonas mobilis* (strain CP4). The fermentation assays, using undiluted hydrolysate with or without nutrient supplements, were monitored by the evaluation of glucose and ethanol yields. In the assays using no supplemented hydrolysate the results obtained for the two yeasts strains F and K, and *Zymomonas mobilis* were 74%, 79% and 58% of ethanol yield, respectively. However, when the hydrolysate was supplemented the fermentation results showed a better bioconversion process by the *Z. mobilis*, reaching 98% ethanol yield while the two strains of *S. cerevisiae* used maintained their behaviour. So, the fermentation results showed the necessity of the addition of nutrients for a good bioconversion process by the *Z. mobilis*, resulting in better ethanol yield than *S. cerevisiae* strains (F and K) from WSP hydrolysate.

Keywords: *Enzymatic hydrolysis; wheat straw pretreated; S. cerevisiae; Z. mobilis; bioethanol*

INTRODUCTION

Most of the fuel ethanol produced in the world is currently sourced from starchy biomass or sucrose (molasses or cane juice) but the technology for ethanol production from non-food plant sources is being developed rapidly so that large-scale production will be a reality in the coming years [1]. The increasing expansion of agro-industrial activity has led to the accumulation of a large quantity of lignocellulosic residues from wood, herbaceous, agricultural, forestry, solid wastes and various industrial wastes all over the world. The key obstacle for transitioning from starch-based to

lignocellulosic biofuels is the complicated structure of the cell wall, which is, by nature, resistant to breakdown.

Current bioconversion processes of these lignocellulosic residues to useful higher value products normally requires multi-steps processes: (i) pretreatment (mechanical, chemical or biological); (ii) hydrolysis of cellulose into fermentable reducing sugars; (iii) fermentation of sugars to ethanol and (iiii) ethanol recovery.

Pretreatment is an important tool for practical cellulose conversion processes and is required to alter the biomass size and structure as well as its microscopic chemical composition and structure to enhance digestibility and enzymatic hydrolysis of the fibers to produce fermentable sugars [2,3]. An effective pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzyme hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of by-products inhibitory to subsequent hydrolysis and fermentation processes; (4) be cost effective [4].

Cellulose can be hydrolytically broken down into glucose either enzymatically by cellulases or chemically by sulphuric or other acids. Utility cost of enzymatic hydrolysis is low compared to acid hydrolysis because is more efficient and proceeds under ambient conditions without generation of any toxic waste [1]. Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific and during its hydrolysis; cellulose is degraded to reducing sugars which can be fermented by yeasts or bacteria to ethanol. The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity and reaction conditions. Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of hydrolysis. However, high substrate concentration can cause substrate inhibition, which lowers the rate hydrolysis and the extent of substrate inhibition depends upon the ratio of total substrate to total enzyme. Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of hydrolysis, but would significantly increase the cost of the process. For these reasons and for process optimization it will be necessary to take into account the biomass feedstock, the pretreatment used, as well as the saccharification conditions (e.g. enzymatic cocktail, the ratio enzyme/substrate, pH, temperature).

Although the enzymatic hydrolysis rate of pretreated biomass and the composition of the resulting sugars in hydrolysates produced depend on the method of pretreatment (conditions employed) and the major constituents in enzymatic hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively [5].

The major sugar product from hydrolysis of most cellulosic biomass is D-glucose that is a six-carbon sugar readily fermentable by common industrial yeasts. Currently, the mainstream process of bioethanol production makes use of the basic yeast *Saccharomyces cerevisiae*. This organism is genetically tractable, has a proven trackrecord in industrial applications and fermentation, has superior conversion yields of ethanol from glucose, can tolerate ethanol and has been the organism of choice

for many years in fermentation processes. Bioprospecting to identify new attractive ethanologenic strains has focused on the isolation and characterization of several species, such as *Zymomonas mobilis*, *Pichia stipitis*, *Clostridium thermocellum*, etc, as well as their relative capacity for genetic manipulation [6]. Recently, research on ethanol fermentation technology has been developed using *Zymomonas mobilis*, a gram-negative bacterium, and several studies pointing out for the suitability of *Z. mobilis* as an alternative organism for large-scale fuel ethanol production over yeasts [7].

In this context, the present study evaluated the potential for ethanol production by fermenting wheat straw hydrolysate with two yeast strains of *S. cerevisiae* (strains F and K) and a bacterial strain of *Zymomonas mobilis* (strain CP4). Prior the fermentation assays, an optimization of wheat straw enzymatic hydrolysis using commercial cellulases was also carried out.

MATERIALS AND METHODS

Biomass feedstock and pretreatment

Wheat straw var Ardila was used as biomass feedstock. The straw was air dried, milled, and grinded to select the fraction of particles with a size lower than 0.5 mm and homogenized in a single lot. A sample of this feedstock was pretreated with diluted acid (Acid Hydrolysis: 130°C, 1.2% w/w H₂SO₄, RLS 7, pH 5, 150 min). The chemical characterization of the wheat straw pretreated (WSP) was: 67.55% of glucan, 4.85% of xylan and 29.17% of lignin. The WSP was either used immediately for enzymatic hydrolysis assays or stored in airtight containers at 4°C until used.

Commercial enzymes

The enzyme mixtures consisted of Celluclast 1.5 L derived from *T. reesei* supplemented with Novozym 188 derived from *A. niger* (Novozymes A/S, Denmark). The filter paper activity of the Celluclast was 14.7 U mL⁻¹ and 228.7 U mL⁻¹ of endo-β-1,4-xylanase activity. The activity of Novozym 188 was 0.6 U mL⁻¹ and 854.9 U mL⁻¹ of endo-β-1,4-xylanase activity.

Microorganisms and inoculum preparation

The *Saccharomyces cerevisiae* strains used were F and K, flocculent and no-flocculent strains, respectively. These yeast strains were isolated at our laboratory from corn fiber hydrolysate obtained in a Portuguese Distillery (DVT, Torres Novas). The *Z. mobilis* strain CP4 used was kindly provided by Prof. Lonnie Ingram from University of Florida (Florida, USA). Both yeasts were maintained on YEPD agar slants (10g/l yeast extract, 20 g/l peptone, and 50 g/l glucose, 20 g/l agar), and sub-cultured fortnightly. For preparation of yeast inocula the cultures were grown in YEPD broth for overnight at 30°C on a rotary shaker at 120 rpm. The bacterial culture, *Z. mobilis*, was maintained on agar plates containing (per liter): 20 g glucose, 10 g yeast extract, 2 g KH₂PO₄, 1 g MgSO₄·7H₂O and 1 g (NH₄)₂SO₄, at room temperature and sub-cultured fortnightly. For the bacterial inoculum preparation, the culture was grown in this medium but with glucose at 50g/l for 24 h at 30°C without agitation. The culture media were sterilized at 121°C for 15 min. These three precultures were used as inocula for bioethanol production from WSP hydrolysate

Enzymatic hydrolysis and ethanol fermentation

Enzymatic saccharification of wheat straw pretreated (WSP) biomass was performed by incubating about 10 g of WSP (~100 g/l polysaccharides) with different cellulose mixtures in a total volume of 50 ml made up with 50 mM acetate buffer (pH 4.8), in 250 ml flasks. The flasks were incubated in an orbital shaker at 55° at 130 rpm, for different combinations of enzyme loading (A to D): (A) Celluclast 10 FPU/g polys. + Novozym 0.1 mL/ g polys.; (B) Celluclast 10 FPU/g polys. + Novozym 0.2 mL/g polys.; (C) Celluclast 10 FPU/g polys. + Novozym 0.4 mL/g polys.; (D) Celluclast 20 FPU/g polys. + Novozym 0.2 mL/g polys. The best enzyme loading condition (B) was used to study the effects of temperature (50°, 55° and 60°C) and incubation time (24, 48 and 72 hours) on the hydrolysis process.

Ethanol production was studied using the enzymatic hydrolysate from WSP. The main fermentable sugar in this hydrolysate was glucose (~53g/l). Aliquots of WSP hydrolysate (50 ml) with different additional nutrients (peptone, yeast extract and mineral salts) were prepared and sterilized by filtration. These samples were inoculated using 10% v/v of each inocula (*S. cerevisiae* strains F and K; *Z. mobilis*) for the fermentations in batch. The incubation was carried out in stoppered flasks at 30°C, without and with agitation (130 rpm) for bacterium and yeasts, respectively. After 24h, samples of each experiment were filtered using 0.22 μ m filters and the ethanol and the remaining sugar content were analyzed by HPLC.

Table 1. Fermentation results from WSP hydrolysate, with or without nutrient supplement, by two *S. cerevisiae* strains, F and K; and the *Z. mobilis* strain CP4.

BIOETHANOL YIELD					
STRAINS	Culture Medium*	WSH	WSH+Min.Salts	WSH+YE	WSH + P
<i>S. cerevisiae</i> Strain F	73%	74%	---	75%	74%
	74%	79%		77%	78%
<i>Z. mobilis</i>	90%	58%	74.2%	98.2%	---

Bioethanol yield = ethanol produced/theoretic ethanol x 100

*Optimal culture medium for each strain containing 50 g/L glucose;

YE – Yeast Extract; P – peptone; Min. Salts – Mineral salts

Analytical methods

Sugars and ethanol were determined using Agilent 1100 HPLC system with a refractive index as a detector and a BIO-RAD Aminex HPX-87H column was used at 50°C. The eluent was 5mM H₂SO₄ at a flow rate of 0.6 mL/min and the injection volume was 5 μ L.

RESULTS AND DISCUSSION

Enzymatic hydrolysis optimization

The best results for the wheat straw hydrolysis were obtained for the enzymatic loading: celluclast 10 FPU/g polysaccharides + Novozym 188 0.2 mL/g polysaccharides. Using this mixture the effects of temperature and incubation time were studied appointing out for 55°C and 48 h as the optimal conditions for wheat straw pretreated saccharification, permitting a process yield of ~60% in hydrolysate sugar content.

WSP hydrolysate fermentation to bioethanol

The fermentation results from undiluted hydrolysate with or without nutrient supplements using two *S. cerevisiae* strains, F and K; and the *Z. mobilis* strain CP4, are presented in table 1. Fermentation results from direct WSP hydrolysates using two *S. cerevisiae* strains, F and K; and the *Z. mobilis* strain CP4, showed an ethanol yield of 74%, 79% and 58%, respectively.

In order to increase the ethanol yield several supplements were added to the hydrolysate. For the yeasts strains the supplements used (peptone and/or yeast extract) had no effect. However, the ethanol production by *Z. mobilis* was highly increased, presenting an increment of 29% when mineral nutrients were added and an increment of 70% when yeast extract was added to the hydrolysate, corresponding to an ethanol yield of 74.2% and 98.2%, respectively.

Control assays were carried out for yeasts and the bacterium using their optimal synthetic media with 50 g/l glucose, being the ethanol yields obtained in these culture media 73% and 74% for the yeasts strains F and K, and 90% for *Z. mobilis*. So, the fermentation results showed the necessity of the addition of nutrients for a good bioconversion process from WSP hydrolysate by the *Z. mobilis*, resulting in better ethanol yield than *S. cerevisiae* strains (F and K). Further studies for the fermentation of the WSP hydrolysate are being performed on MBR bioreactors using the different ethanologenic strains, as well as a sustainability assessment for the whole process.

ACKNOWLEDGEMENT

The authors acknowledge the financial support from FCT (BioRefino Project: PTDC/AGR-AAM/71533/2006).

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