

EFFECT OF TWEEN-80 ADDITION IN DILUTE ACID PRETREATMENT OF WASTE OFFICE PAPER ON ENZYMATIC HYDROLYSIS FOR BIOETHANOL PRODUCTION BY SHF AND SSF PROCESSES

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This paper describes the effect of the addition of Tween-80 in dilute sulfuric acid pretreatment on enzymatic hydrolysis of waste office paper. Lignocellulosic material was pretreated with dilute sulfuric acid (1% V/V) at 50 °C for 3 hours, with or without addition of Tween-80 (2.5 g/L). The pretreatment was able to remove hemicellulose and lignin from waste office paper, but the surfactant did not change the chemical composition of this material. In enzymatic hydrolysis (7 FPU/g substrate) with 5% of solids, the final glucose concentration was by about 50% higher (30 g/L for a duration of 72 hours) when the surfactant was added, irrespectively of whether the addition was done before or after the dilute sulfuric acid pretreatment. The SSF process was more efficient than the SHF process, since it required a shorter reaction time and similar ethanol concentration (about 12 g/L).

Keywords: hydrolysis, *Saccharomyces*, fermentation, non-ionic, ethanol

INTRODUCTION

Lignocellulosic material, such as waste paper, is particularly attractive as feedstock for bioethanol production because it is readily available.¹ Furthermore, a certain fraction of paper will always be sent to disposal,² since the maximum ratio of paper-to-paper recycling is estimated to be of 65%.³

A pretreatment before enzymatic hydrolysis is a necessary step to alter some structural characteristics of lignocellulose, increasing glucan and xylan accessibility to the enzymatic attack. Diluted acid pretreatment appears as a favorable method for industrial applications and has been studied for pretreating a wide range of lignocellulosic biomass.⁴

The conversion of lignocellulose can be improved by the addition of a surfactant by reducing the unproductive cellulases adsorption of the lignin part of the substrate.⁵ An investigation on the adsorption of cellulases during hydrolysis has shown that a Tween 20-assisted acid treated straw solution contained more free cellulases than individual acid treated straw solution.⁶ The use of

Tween 80 also increased the efficiency of hydrolysis of pretreated elephant grass biomass.⁷ Kaar and Holtzapfel⁸ suggested that Tween can protect enzymes from thermal denaturation in hydrolysis at higher temperatures, which was believed to be the dominating mechanism of the stimulatory effect of this surfactant. Polyethylene glycol (PEG 4000) had a positive effect on pure cellulose without lignin, as well as on lignocellulosic biomass. PEG 4000 contributed to preventing cellulose from deactivating on cellulosic substrates.⁹

Fermentations may occur separately from (SHF) or simultaneously (SSF) with the hydrolysis.¹⁰ The advantage of the SHF process is due to the possibility of optimizing the temperature both in the hydrolysis and in the fermentation.¹¹ However, this process can lead to feedback inhibition.¹² In order to avoid such inhibition, one can choose to perform the simultaneous hydrolysis and fermentation (SSF), as far as glucose is formed, it is consumed to produce ethanol.¹³ However, SSF conditions

require an intermediate temperature for the enzymes and yeast added, once the optimum temperature for the hydrolysis is 55 °C, and for the fermentation is 30 °C.¹³

The aim of the present study was to investigate the effect of addition of a non-ionic surfactant (Tween-80; 2.5 g/L) in dilute sulfuric acid (1% V/V) pretreatment on enzymatic hydrolysis of waste office paper for bioethanol production by *Saccharomyces cerevisiae* UFPEDA 1238 in separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

EXPERIMENTAL

Waste office paper and dilute acid pretreatment

Waste office paper collected at the Laboratory of Bioprocess and Bioproducts from the Federal University of Pernambuco, Brazil, was used. In acid pretreatment, 2.5 g of the paper was cut into pieces of 0.5 cm and mixed with 50 mL of sulfuric acid solution 1% V/V in 125-mL Erlenmeyer flasks. Acid pretreatment was carried out in a rotary incubator (TECNAL, TE-421) at 50 °C and 150 rpm, during 3 h. Tween 80 (3% V/V; Sigma–Aldrich, St. Luis, MO, USA) was also used in combination with sulfuric acid for the pretreatment of waste office paper. Pretreated waste office paper was recovered by filtration through qualitative filter paper. The solid fraction was used in the enzymatic hydrolysis. Cellulose, hemicellulose, total lignin, extractives and ash were quantified according to method developed by Gouveia *et al.*¹⁴

Enzymatic hydrolysis of waste office paper

A commercial enzymatic preparation of *Trichoderma reesei* (Celluclast 1.5 L: 35 FPU/mL, 0.27 U/mL cellobiase and 0.18 U/mL xylanase) from Novozymes A/S (Bagsværd, Denmark) was used. Batch enzymatic hydrolysis with addition of surfactant before or after the dilute acid pretreatment was carried out at 5% (w/V) consistency in a sodium citrate buffer (pH 4.8; 50 mM). The cellulase load was 7 FPU/g substrate in all the experiments at 50 °C and 150 rpm, and the hydrolysis was carried out for 72 h. In all enzymatic hydrolysis experiments, Erlenmeyer flasks of 125 mL, containing 50 mL buffer (in the SSF process: culture medium dissolved in sodium citrate buffer of pH 4.8) were used. Samples were withdrawn at regular intervals and filtered (0.45 µm) and the supernatant was analyzed for carbohydrate release. All the experiments were performed in duplicate.

Microorganism and inoculum

An industrial strain (UFPEDA 1238) of *Saccharomyces cerevisiae*, kindly provided by the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco, Brazil, was

used. The inoculum was prepared by transferring cells of *S. cerevisiae* UFPEDA 1238 into a 500 mL flask containing 100 mL of culture medium (20 g/L glucose, 3 g/L peptone, 4 g/L yeast extract; pH 7.0), and incubated at 30 °C for 12 h. Five milliliters were used to inoculate the fermentation medium. Cellulosic hydrolysate obtained from the hydrolysis was supplemented with 4 g/L yeast extract, 2 g/L (NH₄)₂SO₄, 2 g/L KH₂PO₄ and 0.75 g/L MgSO₄·7H₂O, and was used as fermentation medium. The pH was adjusted to 4.9.

Bioethanol production

In the SSF process, after a 12 h hydrolysis (50 °C and 150 rpm), the content of each Erlenmeyer flask was inoculated with yeast cells. All fermentations (SHF and SSF) were carried out at 37 °C, 80 rpm in 125 mL flasks with a working volume of 50 mL (SHF: nutrients dissolved in enzymatic hydrolyzed – liquid fraction; SSF: nutrients dissolved in sodium citrate buffer). Samples were withdrawn at 0, 12, 24, 36 and 48 h, filtered (0.45 µm filter), and the cell free supernatant was used to determine ethanol and glucose by high performance liquid chromatography. All experiments were carried out in duplicate.

Determination of substrates and products

Cellobiose, glucose, xylose, carboxylic acids, furfural, hydroxymethylfurfural and ethanol were quantified by HPLC on an Aminex HPX-87H+ (Bio-Rad, Hercules, CA, USA) column at 60 °C, using 5 mM H₂SO₄ at a flow rate of 0.6 mL/min as the mobile phase, and detected using an RI-detector (Shimadzu, RID 10A).

Fourier-transform infrared spectroscopy (FTIR)

FTIR was conducted using a Spectrum One FTIR system (Perkin Elemer) with a universal ATR (attenuated total reflection) accessory. 2.5 g waste office paper was incubated in 1% V/V sulfuric acid supplemented with Tween-80 (2.5 g/L) at 50 °C for 1 h, and filtered through qualitative filter. After the treatment, the sample was washed thoroughly with distilled water and dried at 45 °C in a vacuum oven. Pretreated waste office paper was pressed uniformly against the diamond surface using spring-loaded anvil. Sample spectra were obtained using an average of 32 scans over the range between 500 cm⁻¹ and 4000 cm⁻¹ with a spectral resolution of 4 cm⁻¹. Baseline and ATR corrections for penetration depth and frequency variations were applied using Spectrum One software supplied with the equipment. Control samples (without and with dilute acid pretreatment, but without surfactant) were also analyzed.

RESULTS AND DISCUSSION

The chemical composition of raw office paper was 63.11 ± 0.35% cellulose, 14.38 ± 0.38%

hemicellulose, $2.08 \pm 0.13\%$ total lignin, $2.20 \pm 0.01\%$ extractives, $9.96 \pm 0.07\%$ ash and 2.98 moisture (91.73% total content). Similar cellulose and hemicellulose contents were found in the literature for office paper.¹⁵

The effect of the addition of Tween-80 on the glucose concentration obtained in enzymatic hydrolysis of dilute acid pretreated waste office paper is summarized in Figure 1. Enhanced glucose concentrations were observed with the addition of the surfactant before or after dilute acid pretreatment. There was no significant difference in final glucose concentration when Tween-80 was added before or after the dilute acid pretreatment ($F = 0.13$; $p = 0.05$). Final glucose concentration was by about 50% higher when the surfactant was added. Carbohydrates were not detected in control experiments (without chemical pretreatment). A total of 30 g/L glucose was released in enzymatic hydrolysis with Tween-80 before or after the dilute acid pretreatment.

Glucose was the main product obtained, but cellobiose was also present in the hydrolyzate, as well as minor amounts of xylose (Fig. 2). Cellobiose concentration was below 3 g/L (Fig. 2), indicating that the amount of β -glucosidase (0.27 U/mL) in the Celluclast 1.5L was sufficient to hydrolyze cellobiose to glucose. Very low concentrations (<0.5 g/L) of xylose were detected in the hydrolyzates, due the absence of hemicellulose in the dilute acid pretreated waste office paper. This hypothesis was confirmed by the analysis of FTIR spectra.

The effect of Tween-80 on enzymatic hydrolysis of waste office paper was investigated at 50 °C. Tween-80 (2.5 g/L) was separately added into each hydrolysis mixture containing 2.5

g of cellulosic substrate. The results shown in Figure 3 demonstrate that this additive significantly enhanced the enzyme hydrolysis. With the addition of the surfactant, cellulose conversion was by 70% higher after 48 hours and by 45% after 72 hours compared to dilute acid pretreated waste office paper without any addition of surfactant, neither before nor after dilute acid pretreatment. Rocha *et al.*¹⁶ performed the enzymatic hydrolysis of discarded office paper, with acid pretreatment and without surfactant, with a biomass load equal to 4% m/V and obtained 20 g/L of glucose.

In this study, no inhibition of β -glucosidase occurred, since the yield of cellulose was of about 90% after 48 hours (Fig. 3), when the pretreatment with dilute acid and surfactant was used. Marques *et al.*¹⁷ also reported a cellobiose concentration of about 2 g/L in the solubilization of recycled paper sludge, resulting in yielded cellulose of 100%, after 72 hours. Chen *et al.*¹⁸ reported that the accumulation of cellobiose (about 12 g/L after 60 hours) caused severe feedback inhibition to the activities of β -glucosidase, resulting in low hydrolysis yield (about 70%). These authors only obtained an 85% higher hydrolysis yield when cellobiase from *Aspergillus niger* ZU-07 was added.

Tween-80 was confirmed to affect the interaction between cellulase and substrates, according to the above results, so that it enhanced the enzyme hydrolysis of waste office paper. To test if dilute acid and Tween-80 affect the structure of the cellulosic substrate or not, the waste office paper was treated with 1% V/V sulfuric acid and 2.5 g/L of Tween-80, and characterized by FTIR.

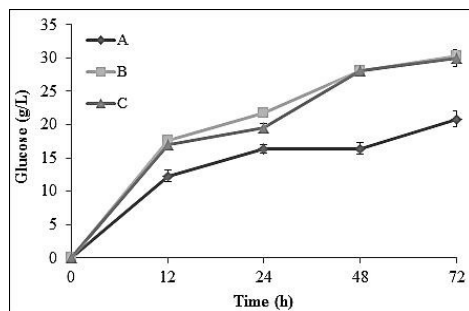


Figure 1: Evolution of glucose concentration during enzymatic hydrolysis of pretreated waste office paper; (A) sample pretreated with dilute acid; (B) sample pretreated with dilute acid with addition of Tween-80 (2.5 g/L) after pretreatment; (C) sample pretreated with dilute acid with addition of Tween-80 before pretreatment

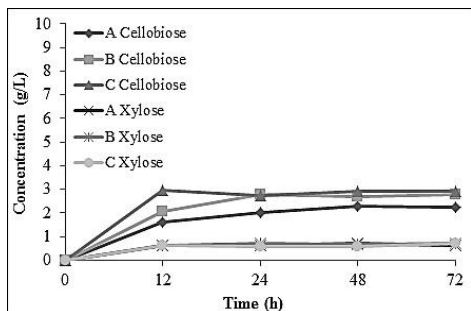


Figure 2: Evolution of xylose and cellobiose concentration during enzymatic hydrolysis of pretreated waste office paper; (A) sample pretreated with dilute acid; (B) sample pretreated with dilute acid with addition of Tween-80 (2.5 g/L) after pretreatment; (C) sample pretreated with dilute acid with addition of Tween-80 before pretreatment

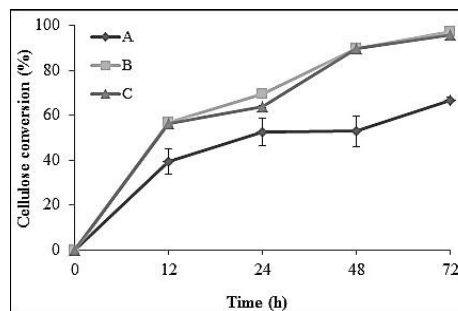


Figure 3: Cellulose conversion during enzymatic hydrolysis of pretreated waste office paper; (A) sample pretreated with dilute acid; (B) sample pretreated with dilute acid with addition of Tween-80 (2.5 g/L) after pretreatment; (C) sample pretreated with dilute acid with addition of Tween-80 before pretreatment

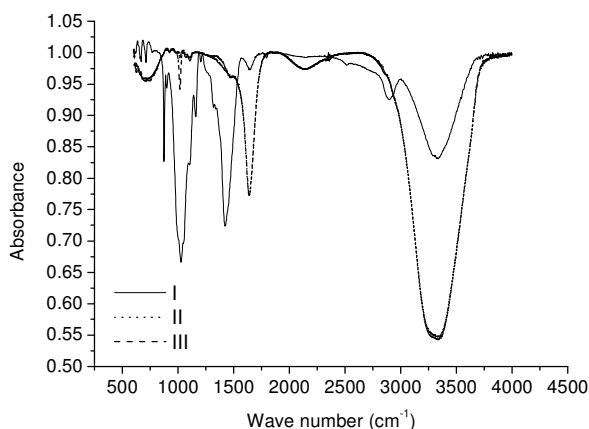


Figure 4: FTIR spectra of waste office paper; (I) untreated sample; (II) sample pretreated with dilute acid; (III) sample pretreated with dilute acid with addition of Tween-80 (2.5 g/L) before pretreatment

The spectra of waste office paper (I – untreated sample; II – sample pretreated with dilute acid; III – sample pretreated with dilute acid with addition of Tween-80 before the pretreatment) are shown in Figure 4. All samples exhibited a peak in the region of 3400 cm^{-1} . However, higher intensities were found in the sample pretreated with dilute acid, independently of the addition of Tween-80. This region refers to hydrogen bonded OH bond stretching vibration of α -cellulose.¹⁹ All treated samples exhibited peaks in the region of 1640 cm^{-1} , which represents the C=O stretching vibration in conjugated carbonyl of lignin.²⁰ The region near 1400 cm^{-1} indicates the presence of lignin.²¹ A peak in this region was observed only for the untreated sample. In the

region of 875 cm^{-1} , a peak was observed only for the untreated sample, indicating the presence of hemicellulose.²² Some regions (800 to 1500 cm^{-1}) present absorption bands associated with hemicelluloses due to the vast structural diversity.²³ The peaks in the spectra of the samples pretreated with dilute acid, with and without addition of surfactant, did not change significantly, which means the main structure of the cellulosic substrates was not altered by the treatment with Tween-80.

Figure 5 shows the variation of glucose and ethanol concentration over time during the SHF and SSF processes of pretreated waste office paper. Regarding longer periods (48 hours), no clear differences between the processes were

observed. However, starting from 24 h, the ethanol concentration was considerably higher in the SHF process than in SSF. For fermentation of

the enzymatic hydrolyzate of discarded office paper, Silva *et al.*²⁴ reported a fermentation efficiency of 40%, using the same yeast.

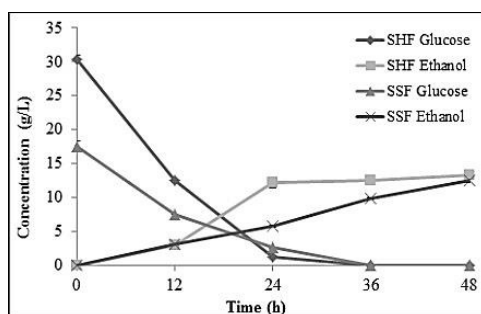


Figure 5: Evolution of glucose and ethanol concentration during SHF and SSF processes of pretreated waste office paper

The cellulose content (63%) was used to calculate the bioethanol yield, considering the loading raw waste office paper and the total time of process. Around 1000 kg of waste office paper will produce 337 L of ethanol using the SSF process (5.35 L/1000 Kg.h) developed, which reveals that waste office paper has a high potential for bioethanol production. Our results were consistent with those from Ouyang *et al.*²⁵ and Li *et al.*,⁹ when pure cellulose (Avicel) without lignin was used.

Surfactants, such as Tween 20, Tween 80, Triton X-100, polyethylene glycol and sodium dodecylsulphate, are not biodegradable and are chemically synthesized, which makes them more toxic to the environment. Biosurfactants, on the other hand, have attracted much attention due to their specificity, biodegradability and biocompatibility.²⁶ These studies should therefore be continued with a view to replacing surfactants by biosurfactants.

CONCLUSION

Dilute acid pretreatment has been shown to result in the degradation and dissolution of lignin and hemicelluloses. The addition of Tween-80 before or after pretreatment significantly improved the digestibility of enzymes on waste office paper. The presence of lignin was not essential to the positive effect of Tween-80 on enzyme hydrolysis, since the lignin was removed by the pretreatment. The SSF process can be preferred due to the minimum total time required (12 h for prehydrolysis and 48 h for fermentation) compared to the SHF process (72 h for hydrolysis and 24 h for fermentation). The processes

involving surfactants give higher glucose concentrations for the same enzymatic loading. Therefore, the addition of surfactants presents a promising approach to make large-scale saccharification economically feasible, since surfactants can be replaced by biosurfactants.

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