ALKALINE PRETREATMENTS EFFECTS IN ENZYMATIC HYDROLYSIS OF CORN COB AND CORN STOVER AND ETHANOL PRODUCTION

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ABSTRACT

This study investigated alkaline pretreatments (PT) with sodium hydroxide (NaOH), with and without anthraquinone (AQ), in the enzymatic hydrolysis of corn stover (CS) and cob (CC). Pretreated CS and CC were characterized and subjected to saccharification with Cellic CTEC 3 (enzyme loading of 12.5 FPU/g cellulose) in fed-batch mode to optimize the release of fermentable sugars. The NaOH pretreated CC showed the highest lignin removal (72.19%). Both methods effectively preserved cellulose in CC, but the use of AQ resulted in a higher content (57.61%), while in CS there was no significant variation between the pretreatments. The strategy of gradual substrate addition proved to be promising, with maximum release of total reducing sugars (TRS) in the hydrolysate of NaOH pretreated CC (67.91 g/L). High glucose concentrations were obtained in NaOH pretreated CC (47.05 g/L) and NaOH-AQ pretreated CS (52.66 g/L) hydrolysates. Low concentrations of inhibitors were observed in the pretreated hydrolysates, especially in NaOH pretreated samples, which also resulted in higher ethanol production compared to those pretreated with AQ, with maximum yields of 66.69% in NaOH pretreated CC and 77.27% in NaOH pretreated CS, indicating the superior effectiveness of pretreatment with only NaOH in the disruption and bioconversion of the biomasses.

Keywords: Alkaline Pretreatmentes. Lignocellulosic Biomass. Saccharification.

1 INTRODUCTION

Lignocellulosic wastes can be a valuable raw material in biorefineries (Cunha et al., 2021). Through enzymatic hydrolysis and microbial fermentation, these residues can be converted into second-generation biofuels, such as cellulosic bioethanol, which offers significant advantages in production efficiency and environmental impact reduction.[1] For an effective lignocellulose bioconversion, a step of pretreatment is indispensable for the enhancement of biomass digestibility by disrupting cellulose, hemicellulose, and lignin.[2] For that, alkaline pretreatment with sodium hydroxide (NaOH) has been employed.[1] Besides, the addition of anthraquinone (AQ) during this process can stabilize cellulose degradation.[3] In this regard, the present study aimed to investigate the effects of alkaline pretreatment with NaOH and AQ of two agricultural residues (corn stover and corn cob), followed by their respective chemical characterizations, hydrolysis with commercial enzyme extract (Cellic CTec3), and alcoholic fermentation.

2 MATERIAL & METHODS

Pretreatment and Biomass Characterization

Corn stover (CS) and corn cob (CC) were pretreated with a NaOH solution (1% w/v) and AQ (0.15% w/w). The reaction was carried out at 160°C for 1 hour, after 40 minutes of heating. Then, the samples were filtered, washed with deionized water, and stored. Pretreatments were performed separately for each biomass, with and without anthraquinone. Raw and pretreated samples were characterized to determine the contents of total Klason lignin (soluble and insoluble lignin in acid - TAPPI T 222 om-88 method), cellulose and hemicellulose (NREL LAP-002 method). The effectiveness of each pretreatment was evaluated by the degree of delignification of the pretreated samples.

Enzymatic Fed-batch Hydrolysis

The saccharifications of raw and pretreated CC and CS were conducted in 50 mL Erlenmeyer flasks with sodium citrate buffer (0.05 mol/L, pH 4.8) and a liquid-solid ratio of 10 mL/g, with additions of 5% of substrate every 12 hours until reaching 30% solids (at 12, 24, 36 and 48h). The assays were performed in triplicate with 12.5 FPU/g of cellulose of commercial enzyme solution (Cellic CTec3, Novozymes) at 150 rpm and 50°C for 72 hours. The resulting liquid fractions were filtered (0.20 μm) and centrifuged at 10,000 rpm for 10 minutes. The total reducing sugars (TRS) released in the hydrolysates were determined using the 3,5-dinitrosalicylic acid (DNS) method, as described by Rodrigues et al,[1]. The concentrations of glucose, organic acids (acetic and formic), furfural (FF), and 5-hydroxymethylfurfural (HMF) were determined by high-performance liquid chromatography (HPLC), according to Lamounier et al.,[4].

Alcoholic Fermentation

The commercial yeast *Saccharomyces cerevisiae* was aerobically cultured on a rotary shaker at 30°C, at 180 rpm for 24 hours in 5 mL of sterile YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and used as a pre-inoculum. Alcoholic fermentation were carried out in 50 mL Erlenmeyer flasks containing autoclaved hydrolysates and 5% (v/v) of the yeast pre-inoculum at 30°C without agitation for 48 hours, in triplicate.

Afterwards, the samples were centrifuged, filtered, and analyzed by high-performance liquid chromatography (HPLC) to determine the levels of glucose and ethanol.[1] The productivity and ethanol yield of the fermented hydrolysates were calculated using specific equations, considering the initial concentrations of glucose and ethanol produced.[2]

$$Q_P = \frac{Ethanol}{t} \qquad\qquad (1)$$

$$Y_{P/S} = \left[\frac{[Etha\quad]}{0,511 \; x \; [Glucose]} \right] x \; 100\% \qquad\qquad (2)$$

Where: QP represents ethanol productivity (g/L.h), Ethanol denotes the concentration of produced ethanol (g/L), t is the fermentation time (h), YP/S represents ethanol yield (%) and Glucose is the initial glucose concentration (g/L).

3 RESULTS & DISCUSSION

Total Klason lignin, cellulose and hemicellulose contents

For raw corn cob and corn stover, the lignin contents were 15.59 and 21.77%, the cellulose contents were 33.12% and 31.97%, and hemicellulose contents were 35.15% and 28.86%, respectively. After alkaline pretreatments, there was a significant reduction in lignin content in both samples, accompanied by a corresponding increase in the cellulose content.

Table 1 - Chemical composition of the biomasses and delignification rate.

Biomass	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Delignification (%)
Raw CC	15.59 ± 0.45	33.12 ± 0.56	35.15 ± 0.55	-
NaOH pretreated CC	9.72 ± 0.50	53.02 ± 0.99	43.08 ± 0.73	72.19 ± 0.65
NaOH-AQ pretreated CC	11.44 ± 0.76	57.61 ± 0.33	37.65 ± 0.31	66.39 ± 0.54
Raw CS	21.77 ± 0.37	31.97 ± 0.65	28.86 ± 0.37	-
NaOH pretreated CS	12.30 ± 0.51	64.99 ± 0.86	26.47 ± 0.65	67.01 ± 0.74
NaOH-AQ pretreated CS	16.30 ± 0.88	64.64 ± 0.98	28.75 ± 0.29	58.724 ± 0.45

The data showed that the exclusive use of NaOH resulted in higher delignification than the combination of NaOH and AQ, suggesting that anthraquinone did not have a significant impact on the lignin removal. Besides, the alkaline pretreatment significantly reduced the lignin content, making the biomasses more accessible for conversion while the structure of the cellulose fraction wwas preserved. On the other hand, the addition of AQ resulted in a higher cellulose content in NaOH-AQ pretreated CC. However, in CS, the presence of anthraquinone did not cause significant variations in cellulose content, possibly due to the higher lignin content in this biomass. These results highlight the complex interaction between anthraquinone, biomass composition, and alkaline methods, emphasizing the individual characteristics of each material in the optimization of bioconversions.

Enzymatic Batch-fed Hydrolysis

The results revealed that the gradual addition of substrate, until reaching 30% solids, promoted a significant increase in TRS levels, indicating an improvement of the efficiency of the enzymatic hydrolysis. Additionally, the presence of AQ in pretreatment did not have a substantial impact on TRS release compared to samples pretreated with only NaOH.

Table 2 - Total reducing sugars, glucose and inhibitors concentrations (g/L) after fed-batch enzymatic saccharifications. CC: corn cob, CS: corn stover. AQ: anthraquinone.

Biomass	TRS	Glucose	Formic Acid	Acetic Acid	HMF	FF
Raw CC	16.20 ± 2.69	11.14 ± 0.48	0.54 ± 0.33	1.19 ± 0.50	0.70 ± 0.01	0.40 ± 0.002
NaOH pretreated CC	67.91 ± 4.08	47.05 ± 0.71	0.27 ± 0.13	1.04 ± 0.37	0.71 ± 0.02	0.40 ± 0.005
NaOH-AQ pretreated CC	62.19 ± 3.20	39.60 ± 1.15	0.29 ± 0.02	0.84 ± 0.12	0.71 ± 0.01	0.39 ± 0.005
Raw CS	11.44 ± 0.71	4.82 ± 0.28	0.23 ± 0.11	2.64 ± 0.33	0.70 ± 0.01	ND
NaOH pretreated CS	61.58 ± 2.85	43.32 ± 0.95	0.14 ± 0.003	1.40 ± 0.10	0.79 ± 0.04	ND
NaOH-AQ pretreated CS	64.78 ± 1.66	52.66 ± 1.41	0.14 ± 0.002	1.50 ± 0.07	0.70 ± 0.01	ND

A considerable glucose release was observed after the hydrolyses performed with 30% solids of both pretreated biomasses (Table 2). In addition, degradation byproducts can be generated during pretreatments, which impair the subsequent enzymatic hydrolysis and fermentation. In this study, low concentrations of inhibitors were observed in the hydrolysates, except for CS, where FF was not detected (Table 2). No statistically significant differences were observed between raw and pretreated biomasses regarding the concentrations of these compounds, suggesting that the pretreatments did not affect their concentrations.The concentrations of organic acids decreased in the biomasses after pretreatments, with no significant differences between the methods (Table 2). The results highlight that the alkaline pretreatments employed in this study contributed to the reduction of inhibitors in the hydrolysates of CS and CC, compared to other pretreatment techniques.

Production of Ethanol

The CC and CS pretreated hydrolysates were used for alcoholic fermentation. The data revealed that the *S. cerevisiae* was able to consume all the glucose present in hydrolysates, indicating an effective fermentation regardless of the biomass source. Furthermore, the results demonstrated efficient ethanol production (Table 3), with the highest concentration, productivity, and ethanol yield in the biomasses pretreated with only NaOH, with no statistically significant differences between the biomasses. In despite of the absence of significant variation in inhibitor concentrations in the hydrolysates of CC and CS subjected to different pretreatments (Table 2), the lowest formic acid concentration in the NaOH-pretreated CS hydrolysate can explain the superior ethanol yield, as this acid has a stronger inhibitory effect than acetic acid. These data reinforce the superior efficiency of pretreatment with NaOH in preparing CC and CS biomasses for subsequent enzymatic saccharification and alcoholic fermentation. These consistent and favorable data support the viability of this method as an effective approach for the bioconversion of corn biomasses into ethanol.

Table 3 - Concentration, productivity, and ethanol yield resulting from fermentation.

Biomass	Consumption (%)	Ethanol (g/L)	Q_P (g/L/h)	$Y_{P/S}$ (%)
NaOH pretreated CC	100	16.11 ± 0.94	0.33 ± 0.02	66.69 ± 1.98
NaOH-AQ pretreated CC	100	10.18 ± 0.23	0.21 ± 0.005	50.37 ± 1.55
NaOH pretreated CS	100	16.47 ± 0.74	0.34 ± 0.02	77.27 ± 1.30
NaOH-AQ pretreated CS	100	15.35 ± 1.24	0.32 ± 0.03	56.15 ± 1.19

4 CONCLUSION

This study demonstrated the superior effectiveness of NaOH pretreatment in removing lignin, achieving maximum delignification rate (72.19%) in NaOH Pretreated CC. The fed-batch strategy, with gradual substrate addition up to 30% improved the hydrolytic process in pretreated biomasses, with maximum TRS release of around 67 g/L in NaOH-pretreated CC. High glucose concentrations (around 50 g/L in both biomasses) and low inhibitor formation were reached in the fed-batch hydrolysates, contributing to subsequent bioconversion into ethanol. Elevated ethanol yields were obtained in NaOH pretreated CC and CS (66.69% and 77.27%, respectively), indicating that the presence of anthraquinone (AQ) did not significantly influence the biomass digestibility. These findings emphasize the superiority of NaOH pretreatment, facilitating the production of fermentable sugars during enzymatic hydrolysis and contributing to the optimization of bioethanol production.

REFERENCES

[1] RODRIGUES, P. O., DA SILVA BARRETO, E., BRANDÃO, R. L., GURGEL, L. V. A., PASQUINI, D., BAFFI, M. A. 2022. *Waste and Biomass Valorization*, *13*(1), 95-106.
[2] RODRIGUES, P. O., MOREIRA, F. S., CARDOSO, V. L., SANTOS, L. D., GURGEL, L. V. A., PASQUINI, D., BAFFI, M. A. 2022. *Waste and Biomass Valorization*, *13*(6), 3085-3094.
[3] ANTUNES, F., MOTA, I. F., DA SILVA BURGAL, J., PINTADO, M., COSTA, P. S. 2022. *Biomass and Bioenergy*, *166*, 106603.
[4] LAMOUNIER, K. F. R., RODRIGUES, P. D. O., PASQUINI, D., DOS SANTOS, A. S., BAFFI, M. A. 2020. *Current microbiology*, *77*, 738-745.

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