Comparison of Dilute Organic Acid Pretreatment and a Comprehensive Exploration of Citric Acid Pretreatment on Corn Cob

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Abstract: As an important agricultural waste, corn cob is one of the most abundant lignocellulosic biomass. In this work, dilute acid pretreatment using seven different organic acids were compared for compositional analysis of corn cob. The first pKa of organic acids, influenced by the number and type of the side group and distance between two carboxylic groups, played a crucial role in the pretreatment effect. Citric acid was found to have better pretreatment performance than other tested dicarboxylic acids. Under the condition of 30 mM, 160°C and 60 min, the level of xylose reached 22.64 g/L in pretreatment liquid, and the pretreated corn cob demonstrated the high enhancement of enzymatic hydrolysis up to 93.4% and the ethanol yield was 73.6%.

Keywords: Corn cob; organic acid pretreatment; citric acid; enzymatic hydrolysis; bio-ethanol production

1 Introduction

As the most abundant biomass on the earth, lignocellulosic biomass is considered to be a highly sustainable feedstock for producing biofuels, platform chemicals, and biomaterial precursors [1,2]. As such, it has attracted worldwide attention. Corn cob is a kind of agricultural waste with high energy density [3]. It has advantages of uniform organization, suitable hardness, good toughness, strong water absorption and good wear resistance. Every year the output of corn cob is over 20 million tons in China [4] and most of them are discarded or burned, resulting in serious waste of resources. Therefore, the resource utilization of corn cob in China is very necessary.

During the bio-refinery process of corncob, pretreatment is an essential step to break down the biomass structure and allow bio-degradation of the polysaccharide in biomass [5,6]. Among all studied pretreatment methods, dilute acid pretreatment is probably the most commonly applied method [7-9]. Dilute sulfuric acid has been widely used in dilute acid pretreatment for its availability and high removal capacity for hemicellulose [10]. Hong et al. [11] and Zu et al. [12] pretreated corn stover with dilute sulfuric acid and hydrochloric acid respectively, and they both achieved good results. However, free sugars obtained by dilute inorganic acid pretreatment are more susceptible to degradation and become inhibitors, affecting subsequent fermentation [13,14]. Moreover, subsequent neutralization processes and removal to avoid negative effects on downstream processing will result in waste of resources and environmental pollution [15,16]. Thus, in order to overcome these disadvantages, people began to explore some alternative acid for pretreatment process. Because dicarboxylic organic acids have similar catalytic structure as enzymes, they were considered as alternative acid for sulfuric acid in pretreatment [10]. Over than that, organic acids have the advantage of environmentally friendly, and by-products treated with organic acids can be used as animal feed [17,18]. Because oxalic acid has been shown to offer much more

acidic than formic, acetic, and maleic acid, Qing et al. [19] used 0.5wt% oxalic acid to pretreat corn cob and obtained the highest xylose yield of 94.3% under the condition of 140 °C for 40 min at a solid loading of 7.5%. Subsequently, other dicarboxylic organic acids, such as maleic acid [20-22] and fumaric acid [23], have also been applied for biomass pretreatment due to their lower hazardous properties and lower inhibitory by-productions of fermentation. These studies showed that dicarboxylic organic acids can hydrolyze β-1,4 bonds more selectively than sulfuric acid [24]. Except to these dicarboxylic acids, citric acid was also used to pretreatment of oil palm trunk [25]. It also demonstrated a similar good performance of hemicellulose and lignin removal from oil palm trunk. Citric acid and its salt can be used as a pH buffer to be added in the enzymatic hydrolysis experiment without subsequent treatment during the whole enzymatic hydrolysis process [26]. Thus citric acid pretreatment offers good compatibility with downstream hydrolysis process.

Despite organic acid pretreatment has been proven as effective replacements of inorganic acid and produce less inhibitory by-products, a comparison of various organic acids in the pretreatment of biomass under the same condition has not previously been investigated. In the present study, seven organic acids (Oxalic acid, Malonic acid, Succinic acid, Maleic acid, Malic acid, Tartaric acid, Citric acid) were used and their pretreatment effects on corn cobs were compared. After that, citric acid was selected and the optimal pretreatment condition (concentration, time and temperature) was evaluated. Moreover, the pretreated solid residue after pretreatment was subjected to enzymatic hydrolysis and ethanol fermentation performance was evaluated by *Pichia stipitis*.

2 Experimental

2.1 Materials

Corn cob was harvested from Dongtai of Jiangsu province, China, in October 2017. After air-dried and crushed between 20 and 80 mesh. Cellulase (Cellic® CTec2) was purchased from Sigma-Aldrich (St. Louis, Mo). All organic acids were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd., China. The standard samples used in the analysis herein were purchased from Sigma-Aldrich (St. Louis, Mo).

2.2 Pretreatment

The laboratory scale pretreatment was carried out in an electrically heated oil bath. A total of 3 g of biomass sample and 24 mL organic acid was placed in a 30 mL sealed stainless steel tank at a solid/liquid ratio of 1:8 for pretreatment. After pretreatment, the reactor was cooled down by water immediately. The pretreatment liquid was collected by filtration and stored at -20°C for further analysis. The solid residue was washed with water to neutral and stored at 4°C. All experiments were carried out in two replicates.

2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in a closed 100 mL flask with a reaction volume of 20 mL, the substrate was 3% cellulose and cellulase dosage was 20 filter paper units (FPU) per gram glucan. 1 mol/L citric buffer was added in a ratio of 1:20 to maintain the solution pH around 4.8. The sealed flask was placed in a constant temperature shaker at 50°C and 150 rpm for 48 h. After enzymatic hydrolysis, the sample was withdrawn and centrifuged to remove the insoluble materials. All experiments were performed in replicates.

2.4 Fermentation

P. stipitis was used for enzymatic hydrolysate and pretreatment liquid fermentation. *P. stipitis* were grown in 250 mL conical flask containing 50 mL seed culture medium (3 g/L yeast extract, 5 g/L peptone, 30 g/L xylose) in a 30°C, 170 rpm shaking incubator for 24 h, then the yeast cells were harvested and transferred to a fresh culture medium (the same as seed culture medium) for incubation for another 24 h. The N source (5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$) was added to the enzymatic hydrolysate, and the pH was adjusted to 6.0.

2.5 Analysis Methods

The chemical compositions of the original and pretreated corn cob were determined according to the National Renewable Energy Laboratory (NREL) analytical methods for biomass [27]. The free fermentable sugars (glucose and xylose), several known inhibitors (acetic acid, furfural, 5-hydroxymethylfurfural (HMF)) and ethanol were directly quantified by HPLC (Aminex HPX-87H, Bio-Rad, USA). 5 mM H_2SO_4 as mobile phase was controlled at a flow rate of 0.6 mL/min at 55°C, analysis signal was detected by a refractive index detector.

2.6 Formula

The glucan recovery is:

Glucan recovery (%) = (G_p /G_u) × 100 (1)

where G_u represents the Glucan content in the untreated corn cob fraction and G_p represents the Glucan content in the pretreated corn cob fraction.

The xylan removal is:

Xylan removal (%) = ((H_u-H_p)/H_u) × 100 (2)

where H_u represents the xylan content in the untreated corn cob fraction and H_p represents the xylan content in the pretreated corn cob fraction.

The lignin removal is:

Lignin removal (%) = ((L_u - L_p)/L_u) × 100 (3)

where L_u represents the Lignin content in the untreated corn cob fraction and L_p represents the Lignin content in the pretreated corn cob fraction.

The enzymatic yield is:

Enzymatic yield (%) = ((C × 1.05 + G)/(Ccell × 1.1)) × 100 (4)

where *C* represents cellobiose concentration in the liquid after enzymatic hydrolysis, *1.05* represents conversion coefficient from cellobiose to glucose, *G* represents glucose concentration in the liquid after enzymatic hydrolysis, *Ccell* represents the cellulose concentration added in the enzymatic hydrolysis, *1.1* represents conversion coefficient from cellulose to glucose.

The ethanol yield is:

Ethanol yield (%) = (E/(G × 0.51 + X × 0.46)) × 100 (5)

where *E* represents ethanol concentration in the culture, *G* represents consumed glucose in the culture, *0.51* represents conversion coefficient from glucose to ethanol, *X* represents consumed xylose in the culture, *0.46* represents conversion coefficient from xylose to ethanol.

3 Results and Discussion

3.1 Physiochemical Properties of Different Organic Acids and Their Effect Evaluation for Pretreatment
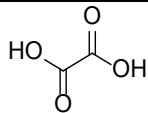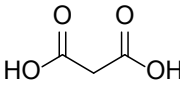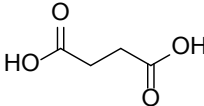
Recently, a few organic acids such as maleic, fumaric and oxalic acid were proven to be effective in biomass pretreatment [28]. Furthermore, dicarboxylic acid was found to have higher catalytic performance than monocarboxylic due to their difference in pKa [29]. Hence, we firstly investigated the pretreatment effect for corn cob using a divalent acid or trivalent acid without changing any other pretreatment conditions. The organic acids used for the pretreatment were listed in Tab. 1 with their pKa values. Six kinds of dicarboxylic acid contain different carbon chain lengths, structures and pKas. By comparison, oxalic acid (first pKa, 1.22, lower than a typical monocarboxylic acid) has the smallest molecular weight and is a stronger acid than other used acid. As chain length of acid increases from two carbons to four carbons, water solubility decreased and the first pKa of acid increased to 2.85(malonic acid) and 4.21(succinic acid). Among C4 organic acids, the value of the first pKa decreased along with hydroxyl number increasing, indicating that the hydroxyl numbers on side chain affect the acidic strength probably. In contrast, the existent of an unsaturated band brought more significant enhance for pKa
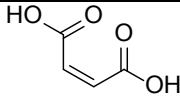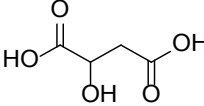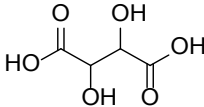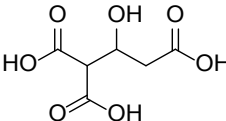
change and maleic acid demonstrated the second acidic strength, which was next to oxalic acid. As for citric acid, the first pKa is 3.13, which is lower than malic acid and higher than tartaric acid. The plausible reason attributed to the number and type of the side group and distance between two carboxylic groups.

Tab. 2 compared the effect of various organic acids used in the pretreatment of corn cob at 160°C and 1:8 solid/liquid ratio in 50 mM organic acid for 60 min. The initial glucan, xylan and lignin of raw material is 36.6%, 32.1% and 20.7% respectively. Firstly, oxalic, malonic, and succinic acid as dicarboxylic acids with different carbon chain lengths have been evaluated. The increasing in carbon chain length produced lower xylan and lignin removal performances. The largest xylan and lignin removal (95.3% and 23.4%) was achieved using oxalic acid due to its stronger acidity than malonic and succinic acid [30]. Maleic, malic and tartaric acid represent C4 organic acids with different spatial structure. Obviously, the effect of spatial structure on pretreatment reflected its enhancement for 1st pka value in C4 organic acids and the increase of 1st pKa value contribute to the pretreatment performance as expected. For maleic acid, an obvious enhance for pKa was found and hence the xylan and lignin removal after pretreatment could reach 93.4% and 25.2%, which was close to the effect of oxalic acid treatment [30] as reported. High xylose yield would be obtained from hemicellulose using the acid with pKa < 2.0 after pretreatment [31]. Moreover, it was found that pretreatment with malic acid and tartaric acid resulted in more significant pretreatment performance than malonic acid, although their 1st pKa value was numerically higher than that of malonic acid. It seemed reasonable that the existence of the hydroxyl group played an extra positive role in pretreatment. By comparison, although citric acid has a longer carbon chain length than other tested organic acids, there are three carboxyl groups and one hydroxyl group in its structural formula, which is a probable explanation for offsetting the disadvantage of its carbon chain length. Therefore, even at similar 1st pKa value (3.13) with malonic, tartaric and malic acid (2.85, 3.04 and 3.46), citric acid was found to have best pretreatment performance than other tested dicarboxylic acids. The xylan and lignin removal reach 90.2% and 26.5%. After pretreatment of citric acid, with the proportion of xylan in pretreated corn cob lower than 6.2%, the proportion of glucan and lignin reached 66.7% and 28.1% respectively.

Recently, organic acids were suggested as alternatives to inorganic ones in order to avoid the degradation of products to obtain more xylan sugars [32]. Compared with monocarboxylic acid, dicarboxylic acid was found to have better catalytic performance due to their enhancement in pKa [29]. In this study, the lower 1st pKa value of organic acid in similar structural dicarboxylic acids, relating to the stronger acidity, higher combined severity factor [29], and the higher xylan and lignin removal after pretreatment. This result was similar to previous studies [30]. In comparison with dicarboxylic acids, using citric acid as pretreatment agent led to a similar xylan removal and a higher lignin removal. Moreover, as metabolic intermediate in microbe cells, citric acid is often non-toxic for most microorganisms. Thus, it is further considered to be used for the pretreatment of corn cobs.

Table 1: Molecular weight, structural formula, chain length, number of carboxyl groups and pKa of seven organic acids

Name	Molecular weight	Structural formula	The number of carbon	The number of carboxyl	pKa	pH*
Oxalic acid	90.4		2	2	1.22 4.19	1.70
Malonic acid	104.06		3	2	2.85 5.66	2.26
Succinic acid	118.09		4	2	4.21 5.64	2.85

Maleic acid	116.07		4	2	1.92 6.23	2.60
Malic acid	134.09		4	2	3.46 5.13	1.90
Tartaric acid	150.09		4	2	3.04 4.37	2.27
Citric acid	192.14		6	3	3.13 4.76 6.4	2.30

*: pH of the solution at the concentration of 50 mM

Table 2: Compositional analysis of pretreated corn cob with different organic acids (oxalic acid, malonic acid, succinic acid, maleic acid, malic acid, tartaric acid and citric acid) under the condition of 50 mM, 160°C, 60 min and 1:8

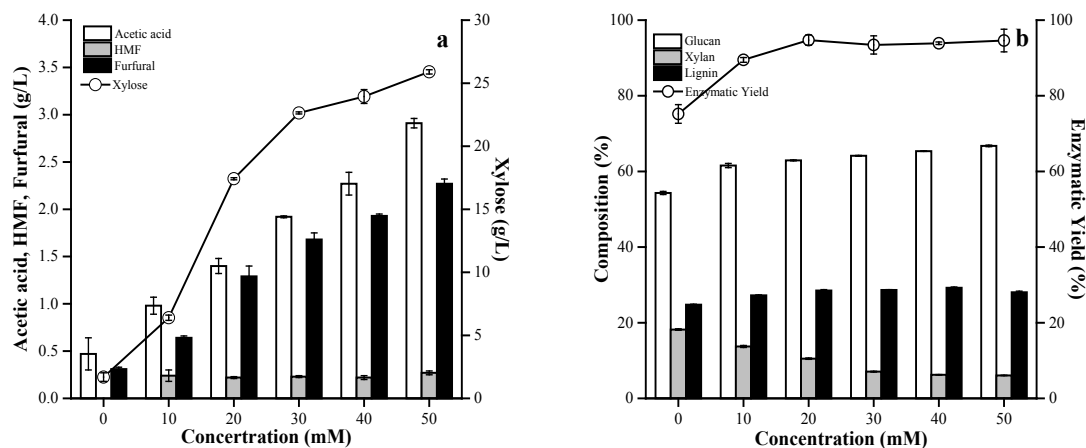| Organic Acid | Compositional analysis | | | | | |
	Glucan(%)	Xylan(%)	Lignin(%)	Glucan recovery(%)	Xylan removal(%)	Lignin removal(%)
Oxalic acid	69.8	3.2	30.4	95.3	95.3	23.4
Malonic acid	60.6	16.1	28.0	97.3	72.8	16.1
Succinic acid	61.3	13.8	28.9	97.4	76.7	14.4
Maleic acid	68.2	4.6	30.2	91.4	93.4	25.2
Malic acid	64.6	10.3	29.7	96.3	83.2	16.1
Tartaric acid	66.0	6.3	29.5	94.5	90.0	24.7
Citric acid	66.8	6.2	28.1	94.4	90.2	26.5

3.2 Pretreatment of Corn Cob Biomass Using Citric Acid

The citric acid concentration, pretreatment temperature and time played important roles in pretreatment efficiency of corncob. Therefore, their effects were evaluated to for optimizing the pretreatment conditions.

3.2.1 Citric Acid Concentration

The effect of citric acid concentration on corn cob under the 160°C, 1:8, 60 min condition was evaluated from 0-50 mM. Fig. 1(a) showed the contents of xylose and inhibitor (furfural, acetic acid and HMF) in the pretreatment liquid after pretreatment. Fig. 1(b) showed the compositions of glucan, xylan and lignin in the pretreated solid residue after pretreatment and their enzymatic hydrolysis efficiencies. As blank control, despite the least xylose and inhibitor content was found in the hydrolysate after hot water pretreatment, the proportion of xylan in the pretreated corncob decreased from 32.1% to 18.2% and the proportion of glucan significantly increased from 36.6% to 54.3%. This result suggested that part of xylan was still removed from the solid and it might be converted into soluble xylooligosaccharides due to lack of strong acid environment [32]. Using citric acid pretreatment, the xylose concentration linearly

increased up to 30 mM citric acid, beyond which it only slightly increased. Additionally, the level of contents of acetic acid and furfural in the hydrolysate linearly increased until 50 mM citric acid. Meanwhile, the HMF content almost had no changes with the increase of citric acid concentration and remained below 0.3 g/L. These results confirmed that citric acid could selectively hydrolyze xylan into xylose and harsh condition would lead to degradation of xylose into furfural during the pretreatment [33]. The highest xylose level was 25.91 g/L which was achieved at 50 mM citric acid. Meanwhile, the maximum content of acetic acid and furfural reached 2.91 g/L and 2.27 g/L respectively. After pretreatment, the pretreated corncob residue was analyzed and used for subsequent enzymatic hydrolysis. Citric acid concentration demonstrated the important effect on the xylan content and sequent hydrolysis performance. At 20 mM, the hydrolysis yield reached a maximum of 94.7%, while the proportion of xylan decreased to 10.5%. After that, raising the acid loading continually did not increase hydrolysis yield, although the proportion of glucan still gradually decreased. Among tested pretreatment conditions, no notably influence on the removal of lignin was found, indicating that the lignin has a small negative impact on the enzymatic hydrolysis of the material when the removal rate of lignin could reach about 20.0% [34]. In this work, taking into account acid cost, xylose and enzymatic hydrolysis, further experiments were performed under 30 mM citric acid concentration.
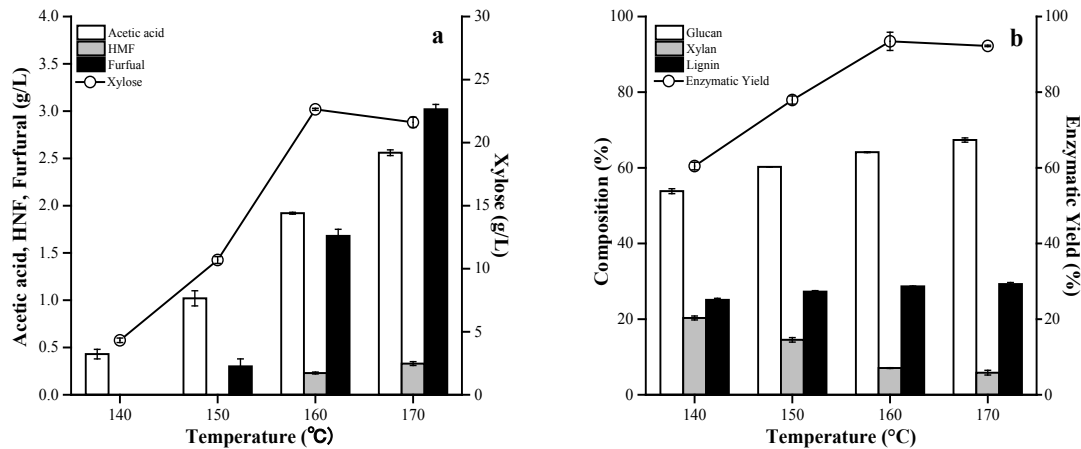

Figure 1: Effect of citric acid concentration on pretreatment under the condition of 160°C, 1:8 for 60 min. (a) The concentration of inhibitor (acetic acid, HMF, furfural) and xylose in the pretreatment liquid after pretreatment; (b) The composition and enzymatic yield of pretreated corn cob
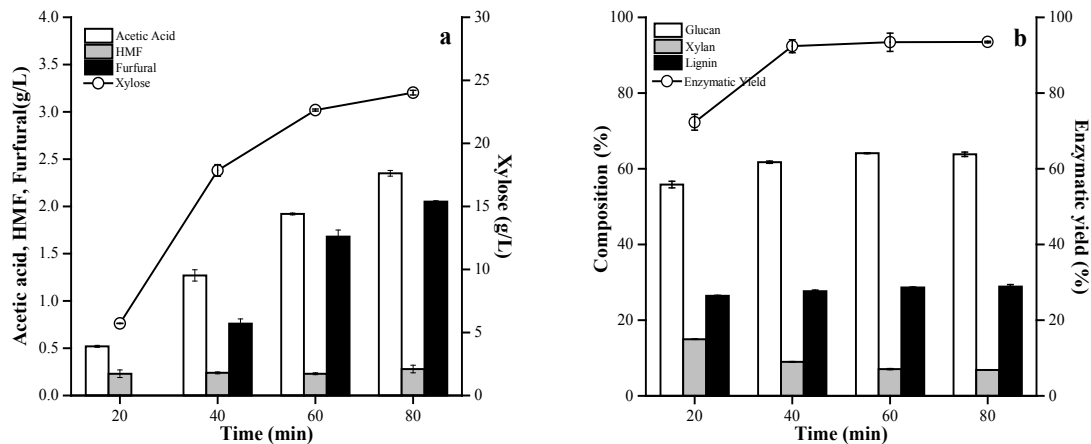
3.2.2 Pretreatment Temperature and Time

To investigate the effect of pretreatment temperature on citric acid pretreatment, various temperatures within the range of 140-170°C was tested under the conditions of 30 mM citric acid concentration, 60 min reaction time and solid-liquid ratio 1:8. In general, increasing the temperature was known to increase the pretreatment severity [34]. As observed from Fig. 2(a), only a relatively small part of acetic acid and xylose were detected in the pretreatment liquid at 140°C and no HMF and furfural were found. With an increase of temperature, a sharp increase in xylose yield occurred from 140°C to 160°C, indicating that higher temperature contributes to xylan hydrolysis. A little of furfural was simultaneously produced at 160°C and increasing temperature to 170°C resulted in twice the amount of furfural produced (3.28 g/L). This phenomenon has been studied by several studies [23]. The highest xylose concentration (22.64 g/L) was obtained under 160°C. When the temperature increased to 170°C, xylose concentration decreased to 21.61 g/L. A probable explanation is due to the degradation of xylose monosaccharide at higher temperature. Fig. 2(b) showed the effect of pretreatment temperature on the changes of chemical composition in pretreated residues and the post-pretreatment enzymatic hydrolysis. With the increase of pretreatment temperature, the proportion of glucan and lignin in residues increased slowly, while the

proportion of xylan decreased from 20.3% at 140°C to 5.8% at 170°C. The pretreatment temperature has a visibly influence on the enzymatic hydrolysis of pretreated materials. Similar to the situation of xylose in Fig. 2(a), with the increased reaction temperature, the enzymatic hydrolysis yield of pretreated solid increased up to 160°C, beyond which it only slightly decreased. The maximum enzymatic hydrolysis yield, obtained at 160°C, was 93.5%. In view of the higher xylose and enzymatic hydrolysis yields, 160°C was set as suitable pretreatment temperature in the next study.

Figure 2: Effect of temperature on pretreatment under the condition of 30 mM, 1:8 for 60 min. (a) The concentration of inhibitor (acetic acid, HMF, furfural) and xylose in the pretreatment liquid after pretreatment; (b) The composition and enzymatic yield of pretreated corn cob

Under the condition of 30 mM citric acid, 1:8 and 160°C, corncob samples were pretreated under in the time range of 20-80 min. The influences of retention time on pretreatment efficiency were shown in Fig. 3(a) and Fig. 3(b). In the Fig. 3(a), with an increase of time, acetic acid and furfural production sharply increased up to 2.35 and 2.05 g/L (80 min), due to removal of acetyl groups from hemicellulose and lignin and degradation of xylose into furfural [31]. As for xylose accumulation, there was only 5.72 g/L xylose determined after 20 min pretreatment. A sharp rise occurred on xylose from 20 min to 60 min, indicating that longer pretreatment time was conducted to the xylooligosaccharide degradation [32]. The highest xylose production was 24.02 g/L at 80 min. The analysis of the pretreated residues showed that part of xylan was already broken into the hydrolysate and hence the xylan content reduced to 15.0% after 20 min pretreatment. When the time is prolonged to 40 min, the proportion of xylan decreased to less than 10.0%, while the proportion of glucan increased to more than 60.0%, and the proportion of lignin also increased slightly. But after that, there was no significant change in compositions of pretreated residues. Meanwhile, trend of the enzymatic hydrolysis yield was accordance with that of composition change. A higher enzymatic hydrolysis yield was obtained at 40 min and prolonging pretreatment time did not bring an evident increase in the enzymatic hydrolysis yield. These results suggested that 40 min of pretreatment time was adequate for acquiring an easily digestible substrate, but prolonging the pretreatment time to 60 min was in favor of xylose formation efficient. Under the premise of cost, the optimal condition of 30 mM, 160°C, 1:8 and 60 min could be obtained from the two aspects of xylose concentration and enzymatic hydrolysis rate. We could get 22.64 g/L xylose and the enzymatic yield is 93.4% on this condition.
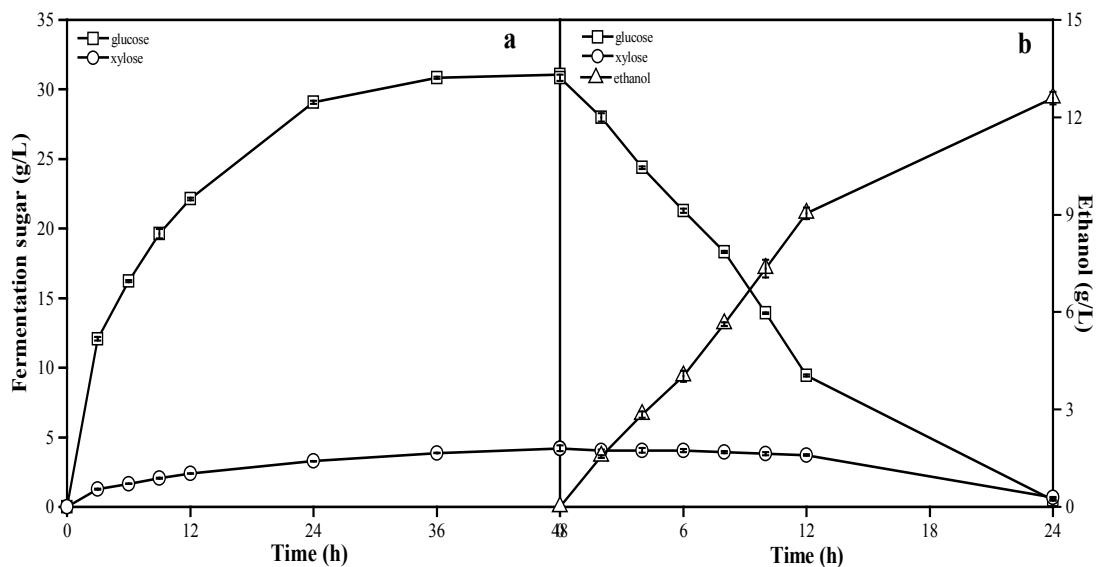
Figure 3: Effect of reaction time on pretreatment under the condition of 30 mM, 1:8 and 160°C. (a) The concentration of inhibitor (acetic acid, HMF, furfural) and xylose in the pretreatment liquid after pretreatment; (b) The composition and enzymatic yield of pretreated corn cob

3.3 Enzymatic Hydrolysis and Ethanol Production

Citric acid is a food industrial grade acid, and its salts can be used as a pH buffer in enzymatic hydrolysis [26]. To demonstrate the influence of citric acid pretreatment on hydrolysis and fermentation, the pretreated corn cobs after pretreatment were enzymatically hydrolyzed and sequent fermented as substrate for ethanol fermentation by *P. stipites* (Fig. 4). Fig. 4(a) exhibited fermentable sugars (glucose and xylose) amount obtained by enzymatic hydrolysis of corn cobs. The enzymatic hydrolysis yield of pretreated corn cob reached 88.2% at 24 h and the enzymatic hydrolysis rate reached 0.97 g/g (glucose/cellulose), Which is faster than that pretreated with oxalic acid (89.2% at 96 h) under the optimized conditions [19], During 48 hours, 31.05 g/L glucose and 4.21 g/L xylose could be obtained through the enzymatic hydrolysis. The ethanol production and consumption of fermentation sugars of *P. stipites* using the above enzymatic hydrolysates within 24 h were shown in Fig. 4(b). After 24 hours of fermentation, glucose and xylose could be almost completely metabolized and the ethanol concentration was 12.59 g/L, corresponding to an ethanol yield of 73.6%. It has been reported that oil palm trunk treated with different organic acid were enzymatically hydrolyzed and used as substrate for ethanol fermentation, citric acid was the most efficient organic acid for pretreatment to enhance ethanol production compared to acetic acid and oxalic acid [25].

Figure 4: Fermentable sugar content in enzymatic hydrolysate and ethanol fermentation by *P. stipitis*. (a) Glucose and xylose obtained by enzymatic hydrolysis; (b) Consumption of sugars and ethanol production during fermentation of enzymatic hydrolysate

4 Conclusions

In this work, the pretreatment effects of seven organic acids on corn cobs were studied. It was found that the first pKa of organic acids was influenced by the number and type of the side group and distance between two carboxylic groups, which played a crucial role in the pretreatment effect. In comparison with dicarboxylic acids, using citric acid as pretreatment agent led to a similar xylan removal and a higher lignin removal than other tested dicarboxylic acids. After corncob was pretreated at 30 mM citric acid, 1:8, 160°C, and 60 min, 22.64 g/L xylose was produced in the pretreatment liquid and the enzymatic yield was 93.4%. In the subsequent enzymatic hydrolysate fermentation experiment, 12.59 g/L of ethanol was obtained, with the ethanol yield of 73.6%. These results suggested that citric acid pretreatment is a potential method for bioethanol production from corncob.

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