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Reinforced acid-pretreatment of *Triarrhena lutarioriparia* to accelerate its enzymatic hydrolysis

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ABSTRACT

In this study, physical method reinforced chemical pretreatments of *Triarrhena lutarioriparia* (TL) were compared to explore the higher reducing sugar yield in subsequent enzymatic hydrolysis. Four different pretreatments, namely hydrothermal pretreatment (HTP), dilute acid pretreatment (HCl-P), ultrasound assisted acid pretreatment (HCl + U) and microwave assisted acid pretreatment (HCl + M) were conducted on TL. The structural features of TL after different pretreatments were investigated by Scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and Fourier transformed infrared (FT-IR) techniques. The fractal-like theory was introduced to study the enzymatic hydrolysis kinetics of TL. Results showed that the reducing sugar yield of HCl-P reached 100.14 mg/g, showing 1.34 times higher than that of the raw. Due to the hemicellulose and lignin removal and lignocellulosic structure destruction, ultrasound and microwave irradiation strengthened the HCl-P, leading to a great enhancement of enzymatic hydrolysis of TL, especially the microwave irradiation. Moreover, the fractal-like theory was confirmed to be satisfactory for studying the enzymatic hydrolysis kinetics of lignocellulosic biomass.

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Introduction

Over the past few decades, an increase in excessive and unsustainable fossil fuel use has led to serious environmental problems, such as large amounts of carbon dioxide emissions and global warming [1]. In order to counteract this serious situation, production of environmental-friendly energy including renewable lignocellulosic biomass has a high priority to ensure global energy security and environmental protection. Fortunately, in more recent years, more attention has been focused on biofuel production from lignocellulosic

biomass [2–4]. *T. lutarioriparia* (TL) is a species of perennial herb with a well-developed rhizome, adapts well to warm and humid environments, and does not compete for land with food with a high biomass productivity more than 22.5 t/(a*ha) [5,6]. This plant has become a notable source of energy plants, and has a great potential for biofuel production.

The conversion of lignocellulosic biomass to biofuel is currently a topic of great interest around the world. However, this technology is limited by the poor deconstruction and enzymatic hydrolysis. This resistance is mainly caused by the stable chemical structure and components of raw biomass,

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which build a dimensional and irregular layer as a protective network [7]. Inexpensive and efficient pretreatments for biomass conversion are required to break the recalcitrance of lignocellulosic biomass for improving the amount of biomass accessible surface to enzyme, and decreasing the loss of reducing sugar during the pretreatment process [8].

In previous studies, numerous promising pretreatment approaches including chemical, physical, biological, and combined pretreatments have been applied to decrease the recalcitrance and enhance the enzymatic saccharification of lignocellulosic biomass, such as hydrothermal, microwave irradiation, high-pressure homogenization, acid, alkaline and white-rot fungi pretreatment [9–13]. Among these pretreatments, dilute acid pretreatment has received a lot of attention and has a wide range of applications [14,15]. Main objective of dilute acid pretreatment is to solubilize the hemicellulosic fraction from biomass, thus making the biomass more susceptible to enzymatic hydrolysis [16,17]. Some studies reported that more than 80% hemicellulose could be solubilized from the lignocellulosic feedstock by dilute acid pretreatment, which facilitated the subsequent enzymatic hydrolysis of biomass [18].

Microwave heating has been proved an effective and promising method to pretreat the lignocellulosic biomass, increasing reaction temperature and disrupting biomass structure for biofuel production [19]. Ultrasound pretreatment is used to improve chemical reactions and industrial processes by shearing action, cavitation effect and reactive radical oxidation [20,21]. Various pretreatments have different effects on lignocellulosic biomass. Furthermore, combined pretreatments (such as microwave + alkali and microwave + acid) have an obvious effect on the enzymatic hydrolysis of lignocellulosic biomass [22,23]. Singh et al. reported that a maximum reducing sugar reached 1334.79 $\mu\text{g/ml}$ after microwave assisted NaOH pretreatment [24]. However, the effect of assisted physical methods on reinforcing dilute acid pretreatment of TL for enzymatic hydrolysis is rarely reported.

Therefore, the objective of this study was to compare the effectiveness of reinforcing dilute acid pretreatment by various physical approaches for enzymatic hydrolysis of TL, and analyze the composition and structure change of TL after pretreatments by scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and Fourier transformed infrared (FT-IR) techniques. Meanwhile, the fractal-like theory was introduced to estimate the enzymatic hydrolysis kinetics of TL after different pretreatments.

Materials and methods

Materials

TL was collected from Beijing, China. The air-dried sample was milled to pass 20 mesh size by a laboratory mill (DF-25S, Dade, China), then stored in plastic bags at room temperature. All chemicals were of analytical grade and purchased from Beijing Chemical Industry Group Co., China. The commercial enzyme was purchased from Hunan province, China, which was generated by *Trichoderma reesei*.

Methods

Hydrothermal and acid pretreatments

For hydrothermal pretreatment (HTP) and dilute hydrochloric acid pretreatment (HCl-P), 10 g dried TL was immersed in 100 ml of distilled water and HCl solution of 10% (w/v) at 120 °C for 1 h with agitation in a round-bottom flask, respectively. After the pretreatment, the solid fraction was separated by filtering, and then washed with tap water until a neutral pH. The obtained solid fraction was dried at 65 °C and then used as substrate for the subsequent enzymatic hydrolysis.

Microwave assisted acid pretreatment (HCl + M)

Microwave irradiation was carried out in a domestic microwave oven (Samsung, S7A73). After HCl pretreatment, the flask containing substrate mixture was placed into the microwave reactor. The microwave treatment time lasted 6 min with a power of 400 W. After that, the flask was taken out from the microwave oven, and the solid fraction was separated and dried according to Section “Hydrothermal and acid pretreatments”.

Ultrasound assisted acid pretreatment (HCl + U)

Ultrasound was generated by a HD2070 Sonopuls Bandelin Ultrasonic Homogenizer device, equipped with MS 73 titanium microtip probe, working with an operating frequency of 20 kHz. Ultrasonication was performed with an output power of 200 W and an exposure time of 30 min. After ultrasonication, the solid fraction was also separated and dried according to Section “Hydrothermal and acid pretreatments”.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out with a solid loading of 2.5% (w/v) at pH 4.8, a temperature of 50 °C and a rotation speed of 150 r/min for 72 h in 250 ml flasks. The enzyme-loading was 30 FPU/g biomass. The reaction mixture was filtered and the liquid was used to analyze the reducing sugar production.

Fractal-like kinetics model

The fractal-like kinetics model can describe the reactions of materials that are prepared as fractals. This model supposes that the reducing sugar yield consists of several components to form a single combined effect on enzymatic hydrolysis. The reactions fit the fractal-like kinetics model and the reducing sugar yield can be deduced as Eq. (1):

$$P = \frac{S_0}{0.9} \left[1 - \exp\left(-\frac{0.9k}{1-h} t^{1-h}\right) \right] \quad (1)$$

where P is the concentration of reducing sugar, S_0 is the initial concentration of reducing sugar, h is the fractal dimension describing the substrate fractal, k is the rate constant presenting the reaction rate between substrates and enzyme, and t is the enzymatic hydrolysis time. The fractal-like kinetics model presents some advantages, such as, effective analytical solution, a good convergence for product formation and a simple equation [25].

Analysis methods

The chemical components of raw and pretreated TL were analyzed by a fiber analyzer (A200i, Ankom, USA). The reducing sugar concentration was measured using the 3, 5-dinitrosalicylic acid method.

FT-IR spectra was recorded in the range from 4000 to 400 cm^{-1} on a Perkin Elmer Spectrophotometer Spectrum Two (USA) fitted with ATR device. The surface morphology and characteristics of the different residual solids were observed by SEM (S-3400N II, Hitachi, Japan) and performed at a beam accelerating voltage of 10 kV. All samples were sputter-coated with gold before scanning. XRD of samples was analyzed using an XRD instrument (D8 Advance, Bruker, Germany). The determination was performed with Ni-filtered Cu K α radiation ($\lambda = 1.54 \text{ \AA}$) at 40 kV and 40 mA. Specimens were scanned at 6 s/step from $2\theta = 5^\circ$ – 35° with a step of 0.2° . The sample crystallinity was quantified from XRD data using a crystallinity index by Eq. (2):

$$\text{CrI} = \frac{I_{002} - I_{\text{amorphous}}}{I_{002}} \times 100\% \quad (2)$$

where I_{002} is the diffraction intensity of crystalline structure ($2\theta = 22.6^\circ$), and $I_{\text{amorphous}}$ is the diffraction intensity of amorphous fraction ($2\theta = 18.0^\circ$) [26].

Results and discussion

Effect of pretreatment on surface structure of TL

SEM reveals the superficial physical change of biomass [27]. The structure change after different pretreatment is shown in Fig. 1. The raw TL showed a smooth and compact surface morphology without any traces of erosion, suggesting that the TL was tightly and orderly integrated together. There was no obvious difference compared with the surface of control and that after HTP (Fig. 1B). However, after HCl-P the tight structure of TL disappeared and some small fragments were

produced. When microwave or ultrasound was applied the dilute acid pretreatment, the surface structure was further eroded (Fig. 1C and D). Microwave irradiation significantly stripped the outer layer of biomass and altered the organized polymer structure with more porous grooves, and the sample was disrupted more severely than that with other pretreatments. As a result, more holes, cracks and erosion troughs in biomass surface might increase the accessible surface area of cellulose, further improving the subsequent enzymatic hydrolysis. Ultrasound could disrupt the bond between the cellulose and hemicellulose by heating and cavitation effects [20,28]. The similar effects were also observed in other publications [29,30].

Effect of pretreatment on chemical composition of TL

The raw TL consisted of 44.48% cellulose, 28.38% hemicellulose and 17.60% lignin (as shown in Table 1). Compared with the control, no significant change in hemicellulose and lignin content was observed when the TL was pretreated with hydrothermal process. The addition of HCl had a positive influence on hemicellulose removal. After HCl-P, HCl + U and HCl + M, the hemicellulose fraction dramatically decreased to 18.14%, 16.74% and 16.53%, respectively. The combined pretreatments could more effectively solubilize the hemicellulose and lignin than the single HCl-P, keeping the majority of cellulose in the solid residues. As a result, the cellulose could be largely exposed to enzyme and the subsequent enzymatic hydrolysis could be improved. The solid yield after pretreatments is shown in Table 1. After different pretreatments, the solid yield decreased by 65.55%–83.50%, which was mainly attributed to the degradation and solubilization of lignin and hemicellulose. Only 65.72% solid residues were obtained after HCl + M, which was just because more hemicellulose was dissolved.

Moreover, the chemical and physical pretreatments can change the crystalline region or amorphous region of cellulose. To gain insight into the possible structural features affecting the cellulose hydrolysis, the untreated and

Fig. 1 – SEM images of different samples (A) raw, (B) after HTP, (C) after HCl-P, (D) after HCl + U and (E) after HCl + M.

Table 1 – Chemical composition and CrI value of before and after pretreatments.

Pretreatment	Composition (%)				CrI (%)
	Solid yield	Hemicellulose	Cellulose	Lignin	
Raw	100	28.38	44.48	17.60	37.50
HTP	83.50	28.34	42.23	18.42	36.60
HCl-P	69.55	18.14	45.57	16.54	39.79
HCl + U	67.88	16.74	46.13	15.56	41.09
HCl + M	65.72	16.53	46.95	15.09	41.50

pretreated TL were conducted by X-ray diffraction (XRD) analysis to illustrate the crystal structural change, and the CrI of samples is listed in Table 1. The CrI of control and after HTP showed no difference, because the HTP was mild, showing insignificant effect, which was similar to that reported by Sun et al. [31]. The CrI after HCl-P was higher than that of the raw, which might be attributed to the main HCl attack to the amorphous region of cellulose [32,33]. Microwave and ultrasound reinforced the HCl-P, resulting in an increase of CrI. The obvious increase in crystallinity indicated that dilute acid pretreatment might mainly disrupt the amorphous zone of cellulose, inducing the peak at $2\theta = 22.6^\circ$ higher than the control. The CrI increased by 10.67% as a result of HCl + M, making the pretreated TL more accessible to enzyme. This finding agreed with the report from Romaní et al. [34].

The main characteristics of FT-IR spectra are assigned to observe functional groups presented in cellulose, hemicellulose, and lignin [35,36]. As shown in Fig. 2, when the sample was pretreated, some obvious differences could be observed as expected. Fig. 2 shows several intense bands for hemicellulose or lignin at 1725, 1600, 1505 and 1374 cm^{-1} , and the broad peak at 3200–3500 cm^{-1} was associated with O–H stretching vibration, which was attributed to carbohydrate and aromatic ring. The FT-IR spectrum of control and sample after HTP was similar, which indicated that the mild pretreatment insignificantly changed the functional groups of TL [37]. It could be observed that the intensity of peak at

Fig. 2 – FT-IR spectra of samples before and after pretreatments.

3200–3500 cm^{-1} reduced, and this region became wide after HCl-P, which suggested that a certain number of hydroxyl bonds were broken and the structure of cellulose became much looser. It was also found that the characteristic peak at 1725 cm^{-1} nearly disappeared. This peak was associated with C=O (carbonyl) and C–O (carbohydrate) stretching vibration. The C=O and C–O groups were from aromatic skeletal of hemicellulose or lignin, and their peak disappearance indicated the HCl-P removed the hemicellulose and lignin,

Fig. 3 – Reducing sugar production of TL before and after pretreatments. **Fig. 4 – Fitted reducing sugar concentration by fractal-like kinetics model.**

Table 2 – Parametric values fitted by fractal-like kinetics model.

Pretreatment	Enzymatic hydrolysis		
	k	h	R ²
Raw	0.005790	0.471463	0.9761
HTP	0.006144	0.559825	0.9879
HCl-P	0.012532	0.655716	0.9693
HCl + U	0.014905	0.710745	0.9793
HCl + M	0.018370	0.655527	0.9780

Table 3 – Correlation of component and enzymatic hydrolysis of TL.

Component	Enzymatic hydrolysis	
	Equation	R ²
Cellulose	$Y_1 = 15.33X_1 - 592.81$	0.8557
Hemicellulose	$Y_2 = -4.39X_2 + 193.30$	0.8054
Lignin	$Y_3 = -21.20X_3 + 451.04$	0.9415
CrI	$Y_4 = 0.07X_4 - 32.56$	0.9135

corresponding to the results in Table 1. For the HCl + U and HCl + M, there was no significant change compared to the spectrum of sample after HCl-P, which indicated that ultrasound and microwave irradiation did not significantly change the chemical structure of TL pretreated by dilute acid. The hemicellulose removal led to a great improvement of the interaction between enzyme and TL. The similar results have been obtained for pretreated poplar and corn stover [38].

Effect of pretreatment on enzymatic hydrolysis and reducing sugar production of TL

The enzymatic hydrolysis of raw and pretreated TL is comparatively shown in Fig. 3. After enzymatic hydrolysis for 72 h, the reducing sugar production only reached 74.90 and 64.44 mg/g for the raw TL and TL after HTP, respectively. The lower reducing sugar yield after HTP might be caused by the insufficient cellulose dissolution in a relatively low temperature and the integrated lignocellulosic structure [37]. What is more, the lignin might absorb a part of enzyme, which was inconducive to the subsequent enzymatic hydrolysis [39]. After HCl-P, the reducing sugar yield reached 100.14 mg/g, which could be mainly attributed to the reduction of

hemicellulose and lignin corresponding to an increase of cellulose exposure and the increase of sample surface area. When Microwave or ultrasound was used to assist the HCl-P, the hemicellulose and lignin content further decreased, especially showing a lowest hemicellulose and lignin content for the HCl + M. The reducing sugar yield of HCl + M was the highest with 140.67 mg/g, which was 1.9 times than that of the raw TL.

To further clarify the kinetics of enzymatic hydrolysis, the reducing sugar production was fitted into fractal-like kinetics models according to Eq. (1) with nonlinear regression (Fig. 4). The values of k , h and correlation coefficients (R^2) are shown in Table 2. The experimental data was very close to the modeled curves, and the R^2 3 were all above 0.96, which indicated that the fractal-like theory was suitable for studying the cellulose enzymatic hydrolysis kinetics [25]. The larger the rate constant, the greater the binding capacity between substrates and enzymes [40]. After HCl-P, the rate constant k was obviously higher than that of the control. The rate constant k of HCl + U and HCl + M was higher than that of HCl-P. The probable reason might be that the HCl + M was able to greatly remove hemicellulose and efficiently improve the access of enzyme, comparing to other pretreatments.

Fig. 5 – Relationship between component and enzymatic hydrolysis of TL.

Fig. 6 – Schematic illustration and mass balance for pretreatment and enzymatic hydrolysis based on 100 g TL.

To deeply understand the effect of chemical component change of TL on the enzymatic hydrolysis, the relationship between the enzymatic digestibility and component change was studied. After pretreatments, the enzymatic hydrolysis was mostly influenced by the content of cellulose and lignin as shown in Fig. 5 and Table 3. The cellulose content showed a positive correlation with the enzymatic hydrolysis, while the hemicellulose and lignin had a negative correlation with the enzymatic hydrolysis. The increase of hemicellulose and lignin removal helped accelerate enzymatic hydrolysis of cellulose and produce more reducing sugar. This is similar to the finding in previous study [8].

The relationship between the CrI and enzymatic hydrolysis has been extensively investigated [41]. The CrI was positively related to enzymatic hydrolysis and the R^2 reached 0.9135, which might resulted from the disruption of amorphous cellulose after pretreatments (Fig. 5 and Table 3).

Schematic illustration and mass balance

Fig. 6 presents a clear schematic illustration and an overall mass balance based on 100 g raw TL and provides an overview of complete process of pretreatment and enzymatic hydrolysis. The raw TL consisted of 44.80 g cellulose, 28.38 g hemicellulose and 17.60 g lignin, respectively. After different pretreatments the composition of cellulose, hemicellulose and lignin ranged 30.86–35.25 g, 10.86–23.66 g and 9.92–15.38 g, respectively.

After pretreatments, the TL was subsequently enzymatically hydrolyzed at 50 °C for 72 h with a cellulase loading of 30 FPU/g. The direct enzymatic hydrolysis of raw TL generated 7.49 g reducing sugar, and After HTP, HCl-P, HCl+U and HCl+M the reducing sugar was 6.44, 10.01, 11.12 and 14.07 g, respectively. The maximum reducing sugar yield of 14.07 g/100 g raw TL was achieved after HCl+M, which indicated that the biorefinery process based on HCl+M for TL utilization is promising in the future.

Conclusions

This research compared four pretreatments, HTP, HCl-P, HCl+U and HCl+M for enhancing the enzymatic hydrolysis of TL. HCl-P was very effective in solubilizing the hemicellulose and lignin, changing the cellulose structure and further improving enzymatic hydrolysis. After 72 h enzymatic hydrolysis, the reducing sugar yield reached 100.14 mg/g, increasing by 34% compared with that of the control. Microwave and ultrasound assistance further improved the enzymatic hydrolysis efficiency of TL, especially the HCl+M. The results of fractal-like kinetics model showed that HCl+M pretreatment is more advantageous for acceleration and improvement of enzymatic hydrolysis of TL. Above all, microwave assisted HCl pretreatment is the most suitable for improving the bioconversion of TL.

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