Recycling of Nitric Acid Solution in Chemical Pretreatment of Oat Hulls for Biorefining

Ekaterina I. Kashcheyeva,* Ekaterina A. Skiba, Vladimir N. Zolotukhin, and
Vera V. Budaeva

The pretreatment of lignocellulosic bioresources is a key step in obtaining fermentable sugars. The present study explored the recycling of an HNO_3 cooking solution during one-step chemical pretreatment of oat hulls and examined the enzymatic hydrolysis performance of the resultant substrates. Two series of experiments on solution recycling were performed: stabilization and experimental elevation of HNO_3 concentration by adding 70 wt% HNO_3. When the HNO_3 concentration was stabilized at 4.4 wt%, the solution could be used five times. Meanwhile, when the HNO_3 concentration was experimentally elevated from 4.4 wt% to 5.6 wt% (at the tenth pretreatment) by adding 70 wt% HNO_3, the cooking solution could be used ten times. The yield of reducing sugars on a hydrolyzables content basis was 95% to 99% for ten substrates. In this case, the lignin contents of the substrates did not increase and had no adverse effect on the enzymatic hydrolysis performance.

Keywords: Bioresources; Biomass; Oat hulls; Pretreatment; Nitric acid; Recycling; Enzymatic hydrolysis; Biorefining

*Contact information: Bioconversion Laboratory, Institute for Problems of Chemical and Energetic Technologies, Siberian Branch of the Russian Academy of Sciences (IPCET SB RAS), Biysk 659322, Altai Krai, Russia; *Corresponding author: makarova@ipcet.ru*

INTRODUCTION

Biorefining (comprehensive conversion) of cellulosic biomass (Hillson *et al*. 2019; Sunwoo *et al*. 2019) is a rapidly advancing trend at the interface of chemistry and industrial biotechnology. It is applied to the annual renewability of cellulosic feedstocks on an industrial scale and, hence, to the sustainability of this resource (Wan *et al*. 2016). Approximately 1,000 tons of cellulose are synthesized per year *via* the photosynthetic pathway (Goodger 1975); therefore, the sustainable conversion of this resource into high-value-added products will become increasingly attractive economically. As glucose is an elementary unit of cellulose, biotechnological approaches for conversion have the greatest growth potential.

Hydrolysis is a universal technological operation and is the basis of any biotechnological productions. Enzymatic hydrolysis is preferred, as it proceeds under mild conditions (unlike chemical hydrolysis) and does not result in byproducts (such as furfural, hydroxymethylfurfural, acids, and phenols); this is an essential trait for further use of the resultant hydrolyzate in microbiological processes (Jönsson and Martín 2016). However, no type of lignocellulosic biomass (neither wood nor grass) can directly be subjected to enzymatic hydrolysis, as the polymers composing lignocellulosic biomass (including cellulose, hemicelluloses, and lignin) are bound to one another in a recalcitrant composite matrix, which should be broken down to access the individual polymers.

Thus, pretreatment of lignocellulosic biomass is a key step in obtaining fermentable sugars and is applied to enhance the yield of sugars from cellulose and hemicelluloses in the subsequent enzymatic hydrolysis of the resultant substrates (Asgher *et al*. 2014; You *et al*. 2016; Chen *et al*. 2017; Farías-Sánchez *et al*. 2017; Ullah *et al*. 2018; Vergara *et al*. 2019). Chemical pretreatment methods (including acid, alkali, and ozone) are the most commonly used techniques (Feng *et al*. 2014; Rezania *et al*. 2017). Still, other well-known methods include physical treatments (including comminution and thermal hydrolysis) (Sindhu *et al*. 2018; Lü *et al*. 2019), physicochemical treatments (including steam explosion and ammonia fiber explosion (AFEX)), mechanochemical treatments (Bychkov *et al*. 2019), and biological treatments (De Bhowmick *et al*. 2018). Among the chemical techniques, acid pretreatment of biomass is widely used and comprises treatment with dilute acid solution or strong acid (Feng *et al*. 2014; Rabemanolontsoa and Saka 2016; Farías-Sánchez *et al*. 2017; Germec *et al*. 2017; Sunwoo *et al*. 2019).

Pretreatment efficiency is characterized by several criteria. These include the destruction of the barrier to enzymatic hydrolysis of celluloses and hemicelluloses, the absence of byproducts that inhibit enzymes and microorganisms used in microbiological synthesis of target products, environmental friendliness, reduced power and reagent consumption, and low cost (Vergara *et al*. 2019). In light of this, few pretreatment methods of lignocellulosic biomass are considered attractive for industrial application.

In this study, oat hulls (*Avena sativa*), an annual raw material, were used as lignocellulosic biomass. Because oat hulls are a byproduct of the food industry, they can be viewed as a zero-cost feedstock. Oat hulls are widely available in agricultural regions worldwide. World production of oats accounts to approximately 11 million metric tons annually (FAOSTAT 2017). Thus, 2.75 million tons to 3.3 million tons of oat hulls are generated as grain-processing waste (Chaud *et al*. 2012). The proportion of hulls accounts for 25% to 30% of the dry grain weight (Chaud *et al*. 2012; Demirel *et al*. 2018). The high sugar content of hulls (as great as 70%) determines their potential use in biotechnological processes for the manufacture of high-value-added products such as ethanol (Germec *et al*. 2017; Demirel *et al*. 2018) and bacterial cellulose (Sakovich *et al*. 2017).

Treatment of oat hulls with dilute HNO_3 under atmospheric pressure has been shown to afford substrates with high reactivity to enzymatic hydrolysis, despite the presence of water-insoluble and acid-insoluble lignin at 12.5% ± 0.1% (Skiba *et al*. 2017). The unique fact that the reactivity to enzymatic hydrolysis is independent of the presence of quite high lignin concentrations is explained by a conceptual distinction of HNO_3 action from all other acids, because the HNO_3 chemical treatment combines three functions: hydrolysis, nitration, and oxidation. Nitric acid not only hydrolyzes polysaccharides but also nitrates and oxidizes lignin. However, it is important that the residual cooking solution is washed from the substrate because lignin nitration products that are present as water-soluble fractions inhibit enzymatic hydrolysis even when the residual concentration of the inhibitor contained in the substrate is as low as 0.019 wt% ± 0.001 wt% (in this case, the yield of sugars declines by a factor of 1.4). Adherence to thorough washing conditions, to remove the residual cooking solution from the substrate, prevents inhibitory effects during both enzymatic hydrolysis and alcohol fermentation. Ethanol is not the only high-value-added product that can be produced from an oat-hull enzymatic hydrolyzate. These products could include many compounds of industrial biotechnology (alcohols, amino acids, organic acids, *etc*.), as the glucose medium is versatile in microbiological synthesis. Thus, the HNO_3 pretreatment method suggested herein for preparation of a substrate for enzymatic hydrolysis may become an efficient technological solution to manufacturing needs.

Despite the use of such a corrosive reagent as HNO_3, this chemical pretreatment method for lignocellulosic biomass is eco-friendly and quite productive, as the process is performed under atmospheric pressure using low-concentration acid (4 wt%) and necessarily employs hermetically sealed equipment to entrap nitrogen oxides, which return to the cooking solution and play an important part in chemical processes. Furthermore, the spent cooking solution is neutralized by ammonia to furnish a complex of lignin-humic fertilizers (Skiba *et al.* 2017).

The most important aspect of pretreatment is its cost, associated with expenses for power and reagents. These expenses are important factors in the development of a cost-effective method. One proposed method to reduce the chemical pretreatment cost is recycling, that is, the repeated use of a spent cooking solution for subsequent treatment cycles (Han *et al*. 2014; Gao *et al*. 2019; Vergara *et al*. 2019). Recycling solutions resulting from pretreatment of lignocellulosic biomass reduces water usage, wastewater, and costs for power and reagents, thereby enhancing the environmental sustainability of the process. However, toxic degradation products (hydroxymethylfurfural, 2-furaldehyde, acetic acid, levulinic acid, formic acid, *etc*.) may build up in the cooking solution and adversely affect subsequent treatment cycles using the same solution (Diaz *et al*. 2018). In this case, a successful chemical solution does not guarantee the success of the biotechnological process in biorefining.

The present study explored the recycling of an HNO_3 cooking solution with addition of 70 wt% HNO_3 during chemical pretreatment of oat hulls and examined the enzymatic hydrolysis performance of the resultant substrates.

EXPERIMENTAL

Feedstock and Pretreatment

Oat hulls of the 2018 harvest were acquired from the Biysk Elevator Company (Biysk, Russia) and used as a feedstock in this study. For pretreatment, 200 g of oat hulls were loaded into a 4-L round-bottom flask containing a 4.4% HNO_3 solution and fitted with a reflux condenser (with a solid-to-liquid mass ratio of 1:15). The flask contents were heated with an LTHS 4000 heating mantle (Brněnská Drutěva, Brno, Czech Republic) to 90 °C to 95 °C and held for 8 h. Then, the reaction mixture was filtered using a Laboport N811 vacuum pump (KNF, Freiburg, Germany). The resultant nitric acid treatment product (NATP) of the oat hulls was washed on a filter until wash waters were colorless.

The total titratable acidity (mass fraction of chiefly nitric acid in solution) of the filtered cooking solution was measured by potentiometric titration. On the basis of the data obtained, the spent solution was then strengthened with a 70% HNO_3 (70 wt% HNO_3) solution to start a new treatment cycle of a fresh oat hull batch. The amount of required 70 wt% HNO_3 was calculated by Eq. 1. The cooking solution was reused ten times in total (treatment cycles I to X). The experiments were performed in two series: In the first experimental series, the HNO_3 concentration was stabilized at 4.4 wt% prior to cooking for each fresh batch of oat hulls; in the second experimental series, the HNO_3 concentration was empirically elevated for each fresh batch of oat hulls.

$$m = (V(C_{cs} - C_{ss}))/0.7 \quad (1)$$

In Eq. 1, *m* is the amount of 70 wt% HNO_3 required to concentrate the spent solution (g); *V* is the cooking solution volume (L); C_{cs} is the cooking solution concentration (g/L); C_{ss} is the spent solution concentration (g/L); and 0.7 is the conversion factor for 70 wt% HNO_3.

The experiments of the first and second series were performed in duplicate and expressed as statistically average data.

Chemical Composition of NATPs

Some NATP samples were air-dried for chemical analysis, and the remainder were subjected to enzymatic hydrolysis in the wet state. The moisture contents of the oat-hull NATP samples were measured using an Ohaus MB23 moisture analyzer (Parsippany, NJ, USA). Oat hulls and their pretreatment products were analyzed by standard procedures, as described below.

Kürschner cellulose was measured by extraction with mixed alcohol and nitric acid in a ratio of 4:1 for 4 h (Kurschner and Hoffer 1993). Klason (acid-insoluble) lignin and acid-soluble lignin were quantified pursuant to TAPPI T222 om-02 (2002). Pentosans were transformed in boiling 13% HCl solution to furfural, which was collected in the distillate and determined using a xylose-calibrated UNICO UV-2804 spectrophotometer (Dayton, NJ, USA) (at a wavelength of 630 nm) with the orcinol / ferric chloride reagent (Obolenskaya *et al*. 1991). The ash content was quantified by incinerating the substrates at 600 °C for 3 h according to TAPPI T211 om-85 (1985).

Enzymatic Hydrolysis

For enzymatic hydrolysis of untreated oat hulls and ten wet NATP samples, a multi-enzyme cocktail was used. It was composed of CelloLux-A (Sibbiopharm, Berdsk, Russia) and BrewZyme BGX (Polfa Tarchomin S.A., Warsaw, Poland) and standardized against cellulase, xylanase, and β-glucanase activities in compliance with the certificates of analysis. The multi-enzyme cocktail was injected as follows: CelloLux-A 40 FPU/g solid and BrewZyme BGX 15 FPU/g solid.

For enzymatic hydrolysis, the substrate (weighing 4.5 g on an oven-dry basis) was put into an Erlenmeyer flask and poured over with acetate buffer (0.1 M, pH = 4.7) containing the dissolved enzymes. The required volume of acetate buffer was calculated with allowance for the initial substrate concentration (30 g/L) and water present in the wet substrate. The flask containing the suspension was placed onto a PE 6410M shaking platform (Ecros, St. Petersburg, Russia) operating at 150 rpm. The hydrolysis was performed under operating parameters that provide the maximum yield of reducing sugars (RS): a temperature of 45 ± 2 °C and a pH value of 4.6 ± 0.3. Sampling was performed every 8 h from 0.005-L hydrolyzates to measure the RS concentrations using the spectrophotometer. The RS concentration on a glucose-content basis was determined using the 3,5-dinitrosalicylic acid reagent (Miller 1959). The suspension was filtered *in vacuo* after 72 h of hydrolysis, and the RS concentration in the filtrate was analyzed. The analysis results were employed to estimate the final yields of RS on a substrate-weight basis and on a hydrolyzables-content basis in the substrate (Kashcheyeva *et al*. 2019). The experiment was performed in duplicate for each substrate.

The work was conducted on equipment of the Biysk Regional Center for Shared Use of Scientific Equipment (IPCET SB RAS, Biysk, Russia).

RESULTS AND DISCUSSION

The results for the chemical composition measurements of the oat hulls revealed a cellulose content of 39.1%. The oat hulls had a high ash content (5.1%) and a high pentosan content (33.1%), with the lignin content being 18.3%.

Table 1 shows the changes in the total titratable acidity of the HNO_3 cooking solution when reused ten times in the first experimental series. During the HNO_3 treatment, partial cellulose oxidation, acid hydrolysis, and partial lignin destruction and nitration occur (Zhang *et al*. 2019). Organic acids, chiefly formic and acetic acids, accumulate in the spent solution as a result of oxidative processes (Skiba *et al*. 2017). The total titratable acidity of the cooking solution after treatment decreased to 2.4% to 2.5%. Therefore, in the first experimental series, the cooking solution after each treatment was increased to an initial HNO_3 concentration of 4.4% and used for the next cycle.

Table 1. Experimental Series I: Change in Total Titratable Acidity during Ten Cycles

Treatment Cycle	Total Titratable Acidity (%)	
	Before Treatment	After Treatment
I	4.40 ± 0.2	2.51 ± 0.1
II	4.39 ± 0.2	2.47 ± 0.1
III	4.41 ± 0.2	2.38 ± 0.1
IV	4.40 ± 0.2	2.42 ± 0.1
V	4.39 ± 0.2	2.51 ± 0.1
VI	4.41 ± 0.2	2.54 ± 0.1
VII	4.42 ± 0.2	2.49 ± 0.1
VIII	4.40 ± 0.2	2.44 ± 0.1
IX	4.40 ± 0.2	2.50 ± 0.1
X	4.41 ± 0.2	2.47 ± 0.1

Table 2 summarizes the measurement results for solids yields and chemical compositions of the NATPs of the oat hulls obtained after ten treatment cycles with the same cooking solution. The NATP yield increased by 15.3% from treatment cycle I to X, and the greatest increase was from treatment VI to X (by 11.6%). This result indicates NATP quality impairment from treatment VI to X. Treatments I to V afforded NATPs similar in chemical composition, in which cases the cellulose contents of NATPs I to V increased by 25.9% to 27.0% compared with the untreated feedstock. Meanwhile, the lignin and pentosan contents decreased by 3.9% to 4.2% and 22.1% to 23.0%, respectively. During treatments VI to X, the quality of the resultant NATPs worsened, as compared to NATPs I to V. For the NATP X sample, the cellulose content decreased by 8.6%, and the lignin and pentosan contents increased by 4.6% and 3.1%, respectively, as compared to NATP V. The ash contents of the NATP samples after all ten treatment cycles were similar (9.8% to 10.5%).

It was thus established that the 4.4% HNO_3 solution could not be reused more than 5 times, and a decrease in cellulose content and an increase in lignin and pentosan contents were observed for the NATP samples during the subsequent treatment runs.

Table 2. Experimental Series I: Solids Yields and Chemical Compositions of NATPs of Oat Hulls

NATPs	Solids Yield (%)	Content (%)			
		Kürschner Cellulose	Lignin	Ash	Pentosans
I	41.3 ± 0.2	66.1 ± 0.4	14.1 ± 0.1	9.9 ± 0.001	10.1 ± 0.1
II	41.9 ± 0.2	66.4 ± 0.4	14.2 ± 0.1	9.8 ± 0.001	10.3 ± 0.1
III	42.3 ± 0.2	66.2 ± 0.4	14.3 ± 0.1	9.9 ± 0.001	10.3 ± 0.1
IV	43.0 ± 0.2	65.4 ± 0.4	14.3 ± 0.1	10.3 ± 0.001	10.7 ± 0.1
V	43.9 ± 0.2	65.0 ± 0.4	14.4 ± 0.1	10.0 ± 0.001	11.0 ± 0.1
VI	45.0 ± 0.2	62.8 ± 0.4	15.1 ± 0.1	10.3 ± 0.001	12.1 ± 0.1
VII	47.1 ± 0.2	60.9 ± 0.4	16.2 ± 0.1	10.0 ± 0.001	12.9 ± 0.1
VIII	49.9 ± 0.2	58.8 ± 0.4	17.5 ± 0.1	10.2 ± 0.001	13.5 ± 0.1
IX	53.5 ± 0.2	57.1 ± 0.4	18.7 ± 0.1	10.4 ± 0.001	13.8 ± 0.1
X	56.6 ± 0.2	56.4 ± 0.4	19.0 ± 0.1	10.5 ± 0.001	14.1 ± 0.1

The oat hull NATP samples appeared to be a loose mass. With each successive cooking run, the hue became deeper, and the color turned from orange-yellow to brown. The colors of the NATP samples correlated to their lignin contents (Fig. 1).

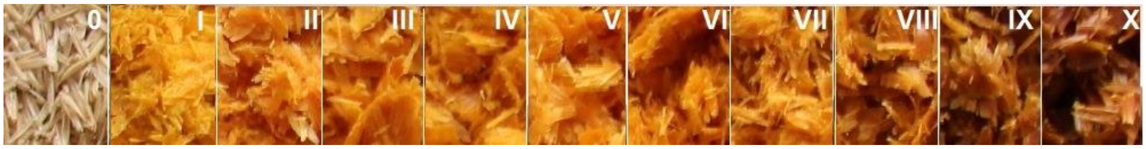

Fig. 1. The colors of the NATP samples depending on HNO_3 solution recycles: (0) oat hulls and (I to X) NATPs of oat hulls

The enzymatic hydrolysis efficiency results for the NATP samples obtained in the first experimental series are given in Table 3. Nitric acid treatment products I to V exhibited the greatest reactivities to enzymatic hydrolysis: The RS yields were 95% to 99% on a hydrolyzables content basis in the substrate.

Table 3. Experimental Series I: Enzymatic Hydrolysis Results for NATPs of Oat Hulls

Substrate	RS Concentration (g/L)	RS Yield on a Substrate Weight Basis (%)	RS Yield on a Hydrolyzables Content Basis (%)
Oat hulls	4.4 ± 0.1	11.9 ± 1.0	15.4 ± 1.1
NATP I	25.0 ± 0.5	75.1 ± 1.6	98.9 ± 1.8
NATP II	25.1 ± 0.5	75.2 ± 1.6	98.8 ± 1.8
NATP III	25.0 ± 0.5	75.0 ± 1.6	98.9 ± 1.8
NATP IV	24.9 ± 0.5	74.8 ± 1.6	99.2 ± 1.8
NATP V	24.0 ± 0.5	72.0 ± 1.5	95.2 ± 1.6
NATP VI	22.0 ± 0.5	66.0 ± 1.5	88.5 ± 1.6
NATP VII	21.5 ± 0.5	64.5 ± 1.5	87.4 ± 1.6
NATP VIII	20.6 ± 0.5	61.8 ± 1.5	85.5 ± 1.6
NATP IX	18.7 ± 0.2	56.1 ± 0.6	79.1 ± 0.7
NATP X	16.5 ± 0.2	49.5 ± 0.6	70.0 ± 0.7

The pretreatment of the oat hulls (I to V) with dilute HNO_3 increased the RS yield by a factor of 5.5 to 5.7 compared with the untreated feedstock, indicating the treatment's efficiency. However, for NATPs VI to X, the RS yield decreased to 70.0% to 88.5% during the enzymatic hydrolysis, suggesting inefficient treatment of oat hulls with the same cooking solution containing 4.4% HNO_3 when used more than five times.

Nitrogen oxides released during the nitric acid pretreatment are known to catalyze the oxidation of lignin and hemicelluloses, leading to removal of non-cellulosics. The presence of nitrogen oxides was evaluated visually from the formation of yellow vapors above the surface of the reaction mass (red smoke). With each subsequent treatment, the release of nitrogen oxides occurred later and was less vigorous, thereby slowing the treatment overall. For instance, in treatment cycle I, nitrogen oxides appeared upon heating beyond 80 °C (1 h after the heating onset), while in treatment run V, they appeared upon heating to 90 °C to 95 °C (4 h to 5 h after the heating onset). This result was likely due to acetic and formic acids absorbing nitrogen oxides and retarding the oxidative process. In run VI, nitrogen oxides did not emerge after 5 h of treatment. Therefore, in the second experimental series, the HNO_3 concentration was increased to 4.9% during treatment run VI, which immediately resulted in nitrogen oxides in 1 h. Because the concentration of organic acids in the spent solution increased after each treatment cycle, the HNO_3 concentration was elevated experimentally, too, in the second experimental series: It was increased to 5.4% in treatment cycles VII to IX and to 5.6% in treatment cycle X. The changes in total titratable acidity of the HNO_3 solution during ten cycles are shown in Table 4.

Table 4. Experimental Series II: Change in Total Titratable Acidity during Ten Cycles

Treatment Cycle	Total Titratable Acidity (%)	
	Before Treatment	After Treatment
I	4.40 ± 0.2	2.52 ± 0.1
II	4.40 ± 0.2	2.48 ± 0.1
III	4.42 ± 0.2	2.40 ± 0.1
IV	4.39 ± 0.2	2.41 ± 0.1
V	4.42 ± 0.2	2.49 ± 0.1
VI	4.91 ± 0.2	2.84 ± 0.1
VII	5.42 ± 0.3	3.20 ± 0.2
VIII	5.43 ± 0.3	3.31 ± 0.2
IX	5.43 ± 0.3	3.50 ± 0.2
X	5.61 ± 0.3	3.58 ± 0.2

Table 5 summarizes the solids yields and chemical compositions of the NATP samples obtained in the second experimental series. The elevated HNO_3 concentrations of the spent cooking solution (starting from sample VI) were able to improve quality attributes of the NATP samples. For instance, the chemical compositions of samples I to X were very similar. Therefore, the multiple reuse of the HNO_3 solution by elevating the acid concentration seems to be appropriate for application. It follows from Table 5 that ten recycles of the HNO_3 cooking solution do not constitute a limit. However, the real limit to the number of recycles of the spent solution can be determined only under production conditions, as bottle-necks of this process can be revealed particularly during scale-up.

Table 5. Experimental Series II: Solids Yields and Chemical Compositions of NATPs of Oat Hulls

NATP	Solids Yield (%)	Content (%)			
		Kürschner Cellulose	Lignin	Ash	Pentosans
I	41.0 ± 0.2	66.9 ± 0.4	13.9 ± 0.1	9.8 ± 0.001	9.4 ± 0.1
II	42.2 ± 0.2	66.2 ± 0.4	14.1 ± 0.1	9.8 ± 0.001	9.9 ± 0.1
III	42.9 ± 0.2	65.7 ± 0.4	14.3 ± 0.1	9.8 ± 0.001	10.2 ± 0.1
IV	43.8 ± 0.2	64.6 ± 0.4	14.4 ± 0.1	10.2 ± 0.001	10.8 ± 0.1
V	44.2 ± 0.2	63.9 ± 0.4	14.6 ± 0.1	10.3 ± 0.001	11.2 ± 0.1
VI	43.9 ± 0.2	64.0 ± 0.4	14.6 ± 0.1	10.2 ± 0.001	11.2 ± 0.1
VII	43.1 ± 0.2	64.9 ± 0.4	14.4 ± 0.1	10.2 ± 0.001	10.5 ± 0.1
VIII	42.6 ± 0.2	65.7 ± 0.4	14.2 ± 0.1	10.0 ± 0.001	10.1 ± 0.1
IX	41.8 ± 0.2	65.9 ± 0.4	14.0 ± 0.1	10.3 ± 0.001	9.8 ± 0.1
X	41.5 ± 0.2	66.3 ± 0.4	13.9 ± 0.1	10.3 ± 0.001	9.5 ± 0.1

The assessment of the enzymatic hydrolysis performances of the oat hull NATPs in 72 h (Table 6) showed that NATP samples I to X had similar reactivities, that is, the RS yields were 73% to 76% on a substrate weight basis.

Table 6. Experimental Series II: Enzymatic Hydrolysis Results for NATPs of Oat Hulls

Substrate	RS Concentration (g/L)	RS Yield on a Substrate Weight Basis (%)	RS Yield on a Hydrolyzables Content Basis (%)
NATP I	25.2 ± 0.5	75.6 ± 1.6	99.1 ± 1.8
NATP II	25.0 ± 0.5	75.0 ± 1.6	98.6 ± 1.8
NATP III	24.8 ± 0.5	74.4 ± 1.6	98.0 ± 1.8
NATP IV	24.9 ± 0.5	74.7 ± 1.6	99.1 ± 1.8
NATP V	24.3 ± 0.5	72.9 ± 1.6	97.1 ± 1.8
NATP VI	24.8 ± 0.5	74.4 ± 1.6	98.9 ± 1.8
NATP VII	24.5 ± 0.5	73.5 ± 1.6	97.5 ± 1.8
NATP VIII	24.1 ± 0.5	72.2 ± 1.6	95.2 ± 1.8
NATP IX	24.7 ± 0.5	74.1 ± 1.6	97.9 ± 1.8
NATP X	24.8 ± 0.5	74.3 ± 1.6	98.0 ± 1.8

Fig. 2. Illustration of the conversion of oat hulls into reducing sugars through HNO_3 recycling

The RS yields of 95% to 99% on a hydrolyzables content basis suggested complete hydrolysis of the accessible parts of the substrates into sugars, in which case the lignin contents (Table 5) of NATPs I to X were also similar, and the oxidized nitrated lignin did not negatively affect the enzymatic hydrolysis performance.

The overall results are illustrated in Fig. 2. The data indicated that the HNO_3 cooking solution could efficiently be recycled ten times for successful conversion of oat hulls into a substrate for enzymatic hydrolysis.

CONCLUSIONS

1. When the cooking solution was controlled to maintain the HNO_3 concentration (4.4%), at the start of each cycle it was possible to perform five cycles. The color of the substrate (NATP) served as the visual control, *i.e.*, the color must have been gold-orange, and turning brown was not acceptable. Meanwhile, the lignin content increase of the substrate served as the instrumental control (greater than 14.5% was not admissible). The reactivity to enzymatic hydrolysis declined from 98.9% when the cooking solution was used once to 70.0% when the cooking solution was used ten times.
2. When the HNO_3 cooking solution was controlled against the nitrogen oxide concentration, it was possible to perform ten cycles, in which case the HNO_3 concentration in the cooking solution was elevated to 5.6% in the tenth cycle. The reactivity to enzymatic hydrolysis was stable after the cooking solution was used ten times. The RS yield (95% to 99%) on a hydrolyzables content basis suggested complete hydrolysis of the accessible parts of the substrates into sugars. In this case, the lignin contents of the substrates did not increase and had no negative impact on the enzymatic hydrolysis efficiency.
3. The HNO_3 cooking solution could effectively be used ten times under lab-scale conditions for the chemical pretreatment of the oat hulls to produce substrates for enzymatic hydrolysis. Under pilot production conditions, the number of recycles could be even greater, with the use of hermetically sealed equipment and supply lines.

ACKNOWLEDGMENTS

This study was conducted under the State Assignment Program with Theme Registration No. AAAA-A17-117011910006-5.

REFERENCES CITED

Asgher, M., Bashir, F., and Iqbal, H. M. N. (2014). "A comprehensive ligninolytic pre-treatment approach from lignocellulose green biotechnology to produce bio-ethanol," *Chemical Engineering Research and Design* 92(8), 1571-1578. DOI: 10.1016/j.cherd.2013.09.003

Bychkov, A., Podgorbunskikh, E., Bychkova, E., and Lomovsky, O. (2019). "Current achievements in the mechanically pretreated conversion of plant biomass," *Biotechnology and Bioengineering* 116(5), 1231-1244. DOI: 10.1002/bit.26925

Chaud, L. C. S., da Silva, D. D. V., de Mattos, R. T., and Felipe, M. d. G. d. A. (2012). "Evaluation of oat hull hemicellulosic hydrolysate fermentability employing *Pichia stipites*," *Brazilian Archives of Biology and Technology* 55, 771-777. DOI: 10.1590/S1516-89132012000500017

Chen, H., Liu, J., Chang, X., Chen, D., Xue, Y., Liu, P., Lin, H., and Han, S. (2017). "A review on the pretreatment of lignocellulose for high-value chemicals," *Fuel Processing Technology* 160, 196-206. DOI: 10.1016/j.fuproc.2016.12.007

De Bhowmick, G., Sarmah, A. K., and Sen, R. (2018). "Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products," *Bioresource Technology* 247, 1144-1154. DOI: 10.1016/j.biortech.2017.09.163

Demirel, F., Germec, M., Coban, H. B., and Turhan, I. (2018). "Optimization of dilute acid pretreatment of barley husk and oat husk and determination of their chemical composition," *Cellulose* 25(11), 6377-6393. DOI: 10.1007/s10570-018-2022-x

Diaz, A. B., Blandino, A., and Caro, I. (2018). "Value added products from fermentation of sugars derived from agro-food residues," *Trends in Food Science & Technology* 71, 52-64. DOI: 10.1016/j.tifs.2017.10.016

FAOSTAT (2017). "Crops," (http://faostat3.fao.org/download/Q/QC/E), accessed 20 June 2019.

Farías-Sánchez, J. C., Velázquez-Valadez, U., Pineda-Pimentel, M. G., López-Miranda, J., Castro-Montoya, A. J., Carrillo-Parra, A., Vargas-Santillán, A., and Rutiaga-Quiñones, J. G. (2017). "Simultaneous saccharification and fermentation of pine sawdust (*Pinus pseudostrobus* L.) pretreated with nitric acid and sodium hydroxide for bioethanol production," *BioResources* 12(1), 1052-1063. DOI: 10.15376/biores.12.1.1052-1063

Feng, L., Qin, L., Liu, Z.-H., Dong, C.-Y., Li, B.-Z., and Yuan, Y.-J. (2014). "Combined severity during pretreatment chemical and temperature on the saccharification of wheat straw using acids and alkalis of differing strength," *BioResources* 9(1), 24-38. DOI: 10.15376/biores.9.1.24-38

Gao, J., Chen, C., Wang, L., Lei, Y., Ji, H., and Liu, S. (2019). "Utilization of inorganic salts as adjuvants for ionic liquid–water pretreatment of lignocellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle," *3 Biotech* 9, 264. DOI: 10.1007/s13205-019-1788-3

Germec, M., Demirel, F., Tas, N., Ozcan, A., Yilmazer, C., Onuk, Z., and Turhan, I. (2017). "Microwave-assisted dilute acid pretreatment of different agricultural bioresources for fermentable sugar production," *Cellulose* 24(10), 4337-4353. DOI: 10.1007/s10570-017-1408-5

Goodger, E. M. (1975). *Hydrocarbon Fuels: Production, Properties and Performance of Liquids and Gases*, Wiley, New York, NY, USA.

Han, M., Moon, S.-K., and Choi, G.-W. (2014). "Pretreatment solution recycling and high-concentration output for economical production of bioethanol," *Bioprocess and Biosystems Engineering* 37(11), 2205-2213. DOI: 10.1007/s00449-014-1198-1

Hillson, N., Caddick, M., Cai, Y., Carrasco, J. A., Chang, M. W., Curach, N. C., Bell, D. J., Le Feuvre, R., Friedman, D. C., Fu, X., *et al*. (2019). "Building a global alliance of biofoundries," *Nature Communications* 10. DOI: 10.1038/s41467-019-10079-2

Jönsson, L. J., and Martín, C. (2016). "Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects," *Bioresource Technology* 199, 103-112. DOI: 10.1016/j.biortech.2015.10.009

Kashcheyeva, E. I., Gladysheva, E. K., Skiba, E. A., and Budaeva, V. V. (2019). "A study of properties and enzymatic hydrolysis of bacterial cellulose," *Cellulose* 26(4), 2255-2265. DOI: 10.1007/s10570-018-02242-7

Kurschner, K., and Hoffer, A. (1993). "Cellulose and cellulose derivative," *Fresenius Journal of Analytical Chemistry* 92(3), 145-154.

Lü, H., Zhou, J., Liu, J., Lü, C., Lian, F., and Li, Y. (2019). "Optimization of hydrothermal pretreatment for co-utilization of xylose and glucose of cassava anaerobic residue for producing ethanol," *Chinese Journal of Chemical Engineering* 27(4), 920-927. DOI: 10.1016/j.cjche.2018.08.025

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry* 31(3), 426-428. DOI: 10.1021/ac60147a030

Obolenskaya, A. V., Yelnitskaya, Z. P., and Leonovich, A. A. (1991). *Laboratornye Raboty po Khimii Drevesiny i Tsellyulozy* [*Laboratory Works on Wood and Cellulose Chemistry: Textbook for Higher Educational Institutions*], Ekologiya, Moscow, Russia.

Rabemanolontsoa, H., and Saka, S. (2016). "Various pretreatments of lignocellulosics," *Bioresource Technology* 199, 83-91. DOI: 10.1016/j.biortech.2015.08.029

Rezania, S., Md Din, M. F., Eva Mohamad, S., Sohaili, J., Mat Taib, S., Mohd Yusof, M. B., Kamyab, H., Darajeh, N., and Ahsan, A. (2017). "Review on pretreatment methods and ethanol production from cellulosic water hyacinth," *BioResources* 12(1), 2108-2124. DOI: 10.15376/biores.12.1.Rezania

Sakovich, G. V., Skiba, E. A., Budaeva, V. V., Gladysheva, E. K., and Aleshina, L. A. (2017). "Technological fundamentals of bacterial nanocellulose production from zero prime-cost feedstock," *Doklady Biochemistry and Biophysics* 477(1), 357-359. DOI: 10.1134/S1607672917060047

Sindhu, R., Binod, P., Mathew, A. K., Abraham, A., Pandey, A., Gnansounou, E., and Castro, G. E. (2018). "An effective surfactant-assisted hydrothermal pretreatment strategy for bioethanol production from chili post-harvest residue by separate hydrolysis and fermentation," *Bioprocess and Biosystems Engineering* 41(4), 565-571. DOI: 10.1007/s00449-018-1891-6

Skiba, E. A., Budaeva, V. V., Baibakova, O. V., Zolotukhin, V. N., and Sakovich, G. V. (2017). "Dilute nitric-acid pretreatment of oat hulls for ethanol production," *Biochemical Engineering Journal* 126, 118-125. DOI: 10.1016/j.bej.2016.09.003

Sunwoo, I., Kwon, J. E., Nguyen, T. H., Jeong, G.-T., and Kim, S.-K. (2019). "Ethanol production from water hyacinth (*Eichhornia crassipes*) hydrolysate by hyper-thermal acid hydrolysis, enzymatic saccharification and yeasts adapted to high concentration of xylose," *Bioprocess and Biosystems Engineering* 42(8), 1367-1374. DOI: 10.1007/s00449-019-02136-3

TAPPI T211 om-85 (1985). "Ash in wood, pulp, paper and paperboard: Combustion at 525 °C," TAPPI Press, Atlanta, GA, USA.

TAPPI T222 om-02 (2002). "Acid-insoluble lignin in wood and pulp," TAPPI Press, Atlanta, GA, USA.

Ullah, K., Sharma, V. K., Ahmad, M., Lv, P., Krahl, J., Wang, Z., and Sofia. (2018). "The insight views of advanced technologies and its application in bio-origin fuel synthesis from lignocellulose biomasses waste, a review," *Renewable and Sustainable Energy Reviews* 82, 3992-4008. DOI: 10.1016/j.rser.2017.10.074

Vergara, P., García-Ochoa, F., Ladero, M., Gutiérrez, S., and Villar, J. C. (2019). "Liquor re-use strategy in lignocellulosic biomass fractionation with ethanol-water mixtures," *Bioresource Technology* 280, 396-403. DOI: 10.1016/j.biortech.2019.02.057

Wan, Y. K., Sadhukhan, J., and Ng, D. K. S. (2016). "Techno-economic evaluations for feasibility of sago-based biorefinery, part 2: Integrated bioethanol production and energy systems," *Chemical Engineering Research and Design* 107, 102-116. DOI: 10.1016/j.cherd.2015.09.017

You, Y., Zhou, Z., Zhao, P., Bu, L., Jiang, J., and Zhang, W. (2016). "Comparison of pretreatment methods for production of ethanol from sugarcane bagasse," *BioResources* 11(1), 2297-2307. DOI: 10.15376/biores.11.1.2297-2307

Zhang, W., Fei, B., Polle, A., Dejuan Euring, D., Tian, G., Yue, X., Chang, Y., Jiang, Z., and Hu, T. (2019). "Crystal and thermal response of cellulose isolation from bamboo by two different chemical treatments," *BioResources* 14(2), 3471-3480. DOI: 10.15376/biores.14.2.3471-3480

Article submitted: September 17, 2019; Peer review completed: January 10, 2020; Revised version received: January 14, 2020; Accepted: January 15, 2020; Published: January 16, 2020.
DOI: 10.15376/biores.15.1.1575-1586