Waste and Biomass Valorization (2020) 11:121–131
https://doi.org/10.1007/s12649-018-0400-0

ORIGINAL PAPER

Enhanced Bioethanol Production from Waste Paper Through Separate Hydrolysis and Fermentation

Neelamegam Annamalai[1,2] · Huda Al Battashi[1] · S. Nair Anu[1] · Ahlam Al Azkawi[1] · Saif Al Bahry[1] · Nallusamy Sivakumar[1]

Received: 13 December 2017 / Accepted: 14 July 2018 / Published online: 21 July 2018

Abstract
The effect of various pretreatments for efficient hydrolysis of waste office paper and newspaper into fermentable sugars and subsequent production of bioethanol through fermentation was investigated. Pretreatment with H_2O_2 (0.5% v/v) at 121 °C for 30 min was considered as the most effective method for this kind of soft biomass like waste paper due to the considerable increase in available cellulose and sugar yield in addition to efficient delignification. Under optimized conditions, enzymatic hydrolysis of pretreated office paper and newspaper resulted the sugar yield of 24.5 and 13.26 g/L with hydrolysis efficiency of 91.8 and 79.6%, respectively. Further, ethanol production using the hydrolysate by *Saccharomyces cerevisiae* was about 11.15 and 6.65 g/L with the productivity of 0.32 and 0.28 (g ethanol/L/h), respectively. The improved yields achieved through the pretreatment and subsequent ethanol production suggested that the waste paper could be a potential feedstock for the production of bioethanol.

Keywords Waste paper · Pretreatments · Enzymatic hydrolysis · Sugars · Ethanol · Fermentation

Statement of Novelty

Development of feasible bioprocess for the improved production of bioethanol from waste paper which would enhance the utilization of waste paper biomass and also reduces the cost of production for bioethanol.

Introduction

There is an increased production of biomass—derived biofuel which can overcome fossil fuel depletion and other related environmental issues in the past decades [1, 2]. Bioethanol production has increased rapidly as many countries targeted towards reducing oil imports, boosting rural economies along with improving the quality of air [3]. Until now, most of bioethanol is still produced from food-based crops like sugarcane and maize, raising the debates concerning competition with food supply and arable lands [4, 5]. Therefore, the development of second generation bioethanol from lignocellulosic biomass serves many advantages from both economic and environmental point of views [6]. Bioethanol derived from lignocellulosic biomass is a renewable energy source that is being rapidly developed and commercialized as a substitute for fossil fuels in many countries [7, 8]. Lignocellulosic biomasses are mainly composed of cellulose (40–60%), hemicellulose (20–30%), and lignin (15–30%) which is considered as a promising alternative source as it is inexpensive, renewable and most abundant raw materials [9–12].

Waste paper is considered as one of the major components of municipal and industrial wastes [13]. Despite the awareness of recycling, waste papers are still available as a major municipal waste due to the constraints in recycling of paper fibers which turned into low quality paper products [14] and also the process is more difficult when the papers mixed with other wastes [9, 15]. However, waste paper has the potential to be used as an excellent alternative feedstock for ethanol production due to its relative abundance and low cost

✉ Neelamegam Annamalai
annabact@gmail.com

✉ Nallusamy Sivakumar
apnsiva@squ.edu.om

[1] Department of Biology, College of Science, Sultan Qabbos University, PO Box 36, Musact 123, Oman

[2] Hawaii Natural Energy Institute, University of Hawaii at Manoa, 1680, East-West Road, Honolulu, HI 96822, USA

(average $55/ton). Utilization of waste paper for the production of biofuel is a much valuable alternative route for waste management [16]. The efficient conversion of waste paper is still remains as a challenge due to its recalcitrant structure as the cellulose chains interact with hemicellulose and lignin to form a lignin–carbohydrate complex, making it difficult to depolymerize into fermentable sugars [10]. Leu and Zhu [17] suggested that the factors like (1) substrate accessibility to cellulose—the roles of component removal and size reduction by pretreatments, (2) substrate and cellulase reactivity limited by component inhibition, and (3) reaction conditions-substrate-specific optimization which affects bioconversion of waste paper to sugar production. Furthermore, there are two fundamental issues unique to bioconversion of waste paper to ethanol; (1) the composition of waste paper and (2) the effect of fiber hornification caused by drying in the paper production process on enzymatic hydrolysis of waste paper cellulose [18]. Drying induced fiber hornification of waste paper cause changes in fiber structure leads to low enzymatic saccharification efficiency, which reduced the enzyme accessibility to cellulose and substrate enzyme digestibility (SED) [18, 19].

Several pretreatment methods were used, including steam explosion, acid, alkali, organic solvents, alkaline hydrogen peroxide, ammonia and hot water treatments [20–22]. However, choice of pretreatment process is the most important factor in ethanol production process because it influences waste treatment, cellulose conversion rates, performance of hydrolytic enzymes and ethanol fermentation [23]. Thus, an appropriate pretreatment method is essentially required for the efficient conversion of waste paper into ethanol in a short time with a high yield. The present study was aimed to investigate the effect of various pretreatments on waste office paper (OP) and newspaper (NP) and subsequent production of bioethanol by *Saccharomyces cerevisiae* through separate hydrolysis and fermentation (SHF).

Materials and Methods

Materials and Microorganism

All the chemicals and materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated. Cellulase from *Trichoderma reesei* ATCC 26921 (C2730) and β-glucosidase from *Aspergillus niger* (49291) were purchased from Sigma-Aldrich (MO, USA). The activity of cellulase and β-glucosidase were estimated as 185 FPU/mL and 500 CBU/mL respectively. The cellulase activity was determined by standard filter paper assay [24]. One unit of enzyme activity (FPU) is defined as the amount of enzyme required to liberate 1 µmol of glucose from filter paper in 60 min at 50 °C and pH 4.8. The β-glucosidase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) [25]. One unit of CBU activity is defined as the amount of enzyme required to produce 1 µmol of *p*-nitrophenol from *p*NPG per minute at 50 °C and pH 5.0.

The yeast, *S. cerevisiae* DSM 70449 was purchased from DMSZ *(Leibniz Institute DSMZ*: German collection of microorganisms and cell cultures), stored at 4 °C and propagated every 2 weeks on yeast malt peptone (YMP) agar slants (yeast extract 3; malt extract 3; peptone 3; glucose 10; agar 15 g/L, pH 6.0).

Preparation of Waste Paper

Waste OP and NP collected from the local market were shredded in to small pieces (2×6 mm) using a mechanical shredder (Atlas, China). The shredded papers were soaked in deionized water (5% w/v), milled and dried at 60 °C for 24 h. Then, the dry matter was milled again to remove most physical barriers of cellulose structure and used for further analysis.

Pretreatment of Waste Paper

The waste OP and NP prepared as described earlier were subjected to pretreatment with sulphuric acid, sodium hydroxide, phosphoric acid and hydrogen peroxide at a concentration of 0.1, 0.5 and 1% (v/v). Briefly, the milled OP and NP was added in a 500 mL screw cap bottle with a solid: liquid ratio of 1: 20 (5%, w/v) with H_2SO_4, NaOH, H_3PO_4 and H_2O_2 and then autoclaved at 121 °C for 30 min. After cooling, the solid residue was collected by filtration through muslin cloth, washed several times with deionized water till obtain neutral pH and dried at 50 °C for 24 h. The liquid fraction was collected and stored at 4 °C for further analysis. The dried materials were milled and used as a substrate for further enzymatic hydrolysis studies.

Crystallinity Analysis of Waste Paper by XRD

The crystallinity of the cellulose in untreated and pretreated OP and NP (0.1% H_2SO_4, 0.1% NaOH, 0.1% H_3PO_4 and 0.5% H_2O_2) was evaluated through X-Ray Diffraction (Panalytical, X' Pert PRO X-Ray Diffraction, The Netherlands). Copper Kα radiation (1.54 Å), 40 kV of voltage and 40 mA of electric current, and a rate of 2.0° per minute for a 2θ continuous scan from 10 to 70° were applied. This analysis allowed the detection of amorphous and modification of the crystalline structure of the cellulose in the waste paper. The crystallinity index (*CrI*) was obtained from the ratio of the maximum peak intensity 002 (I_{002}, 2θ = 22) and minimal depression (I_{am} 2θ = 18.5) between peaks 001 and 002 [26] according to the following equation.

$$CrI(\%) = \left[\left(I_{002} - I_{am}\right) / I_{002}\right] \times 100$$

where *CrI* is the crystallinity index, I_{002} is the maximum intensity at 2θ − 22°, and I_{am} is the minimum intensity corresponding to the amorphous content at 2θ − 18.5°.

Enzymatic Digestibility of Untreated and Pretreated Waste Paper

The enzymatic digestibility (ED) study was carried out in 500 mL hydrolysis flasks containing 100 mL of 50 mM citrate buffer (pH 4.8) with pretreated OP and NP (2%, w/v) and 0.02% (w/v) sodium azide. The enzyme loadings used were cellulase (37 FPU/g solids) + β-glucosidase (25 CBU/g solids). After solid and enzyme loading, flasks were incubated at 50 °C for 120 h at 160 rpm and the aliquots withdrawn at regular intervals (24 h) were immediately heated to 100 °C for 3 min to prevent further hydrolysis, centrifuged at 5000×*g* for 10 min and the supernatant was subjected to sugar analysis. The ED is defined as the percentage of substrate glucan enzymatically hydrolyzed to glucose.

Effect of Solid Loadings

Effect of solid loadings on sugar yield and hydrolysis was carried out with 1–4% (w/v) of pretreated OP and NP with the enzyme loading of cellulase (37 FPU/g solid) + β-glucosidase (25 CBU/g solid). The flasks were incubated at 50 °C for 120 h at 160 rpm and the aliquots withdrawn at regular intervals (24 h) were subjected to sugar analysis as mentioned above. The percentage of hydrolysis was calculated as follows:

$$\text{Hydrolysis (\%)} = \text{Glucose (g)} \times 0.9 \times 100/\text{Cellulose in the initial substrate}$$

Enzymatic Hydrolysis of Pretreated Waste Paper Under Optimum Conditions

Enzymatic hydrolysis of 0.5% H_2O_2 pretreated OP and NP under optimized conditions was carried out in 500 mL stoppered conical flasks containing 100 mL of 50 mM citrate buffer (pH 4.8) with 3% (w/v) solids and 37 FPU/g + 25 CBU/g solids of enzyme loadings. The sugar yield and percentage hydrolysis were estimated as mentioned above.

Ethanol Fermentation Using Waste Paper Hydrolysate

The yeast, *S. cerevisiae* DSM 70449 was grown in YMP (yeast extract 3; malt extract 3; peptone 3; glucose 10 g/L) medium for 12 h at 30 °C with 160 rpm and used as inoculum. Ethanol production experiments were performed in 500 mL Erlenmeyer flasks containing 100 mL of filter sterilized fermentation medium [yeast extract 5; $(NH_4)_2SO_4$ 1; KH_2PO_4 2; $MgSO_4 \cdot 7H_2O$ 1 g/L] prepared using waste OP and NP hydrolysate (pH 5.0). The flasks were seeded with 5% (v/v) inoculum and incubated in a shaker incubator with 160 rpm at 30 °C for 48 h. Cell growth was monitored directly by measuring the optical density at 600 nm. The samples collected at regular intervals (12 h) were centrifuged at 10,000×*g* for 10 min and the cell-free supernatants were used to determine the ethanol and residual sugar concentration.

Analytical Methods

The moisture and ash contents were determined using NREL/TP-510-42621 [27] and NREL/TP-510-42622 [28] methods, respectively. The structural carbohydrates (cellulose and hemicellulose) and acid soluble and insoluble lignin of untreated and pretreated solids and liquids were determined by NREL/TP-510-42618 method [29]. The cellulose (as glucose), hemicellulose (as xylose), hydroxymethylfurfural (HMF), furfural and ethanol were analyzed by HPLC (Shimadzu; LC10AD) equipped with Aminex HPX-87H (Bio-Rad) column at 65 °C using 5 mM sulfuric acid as mobile phase (0.6 mL/min) with refractive index detector (Shimadzu; RID10A) and the sugars (glucose and xylose) were quantified by external calibration with standards. The total phenolic content was determined by the Folin–Ciocalteu method [30] with gallic acid as standard. All the experiments were performed in triplicate and the results are presented as mean ± standard deviation.

Statistical Analysis

The mean values and standard deviations were calculated from the data obtained from three independent experiments. Analysis of variance was performed by one way ANOVA followed by Tukey's HSD Post Hoc multiple comparison analysis using IBM SPSS statistics package, version 21. Statistical differences at $p < 0.05$ were considered as significant.

Results and Discussion

Compositional Analysis of Untreated and Pretreated Waste Papers

In Table 1, composition of various pretreated solids and sugars released in pretreatment liquid were presented and the results suggested that there was significant variations

Table 1 Components in solid and liquid fractions of untreated and various pretreated OP and NP

Pretreatment	%	Components of the solid residue (%)						Sugars in pretreatment liquid (%)			
		Cellulose (as glucose)		Hemicellulose (as xylose)		Lignin		Cellulose (as glucose)		Hemicellulose (as xylose)	
		OP	NP	OP	NP	OP	NP	OP	NP	OP	NP
Untreated	–	52.42 ± 1.24^{e}	$34.97 \pm 1.48^{e, f}$	9.48 ± 0.86^{a}	9.55 ± 0.63^{a}	15.08 ± 1.32^{a}	21.72 ± 1.26^{a}	–	–	–	–
H_2SO_4	0.1	66.99 ± 1.08^{c}	$38.28 \pm 0.78^{c, d}$	6.77 ± 0.28^{b}	$6.35 \pm 0.14^{e, f}$	9.68 ± 0.25^{c}	$20.73 \pm 0.62^{a, b}$	$0.56 \pm 0.08^{f, g}$	$0.50 \pm 0.12^{e, f}$	$1.12 \pm 0.13^{e, f}$	$1.22 \pm 0.12^{f, g}$
	0.5	60.77 ± 0.81^{d}	$32.83 \pm 0.62^{f, h}$	4.62 ± 0.34^{c}	5.05 ± 0.23^{h}	8.04 ± 0.58^{d}	$17.58 \pm 0.48^{d, e}$	2.08 ± 0.23^{c}	$1.21 \pm 0.26^{c, d}$	$1.46 \pm 0.20^{c, d}$	$1.43 \pm 0.08^{e, f}$
	1	60.13 ± 1.22^{d}	$30.89 \pm 0.54^{i, j}$	2.64 ± 0.23^{e}	4.14 ± 0.32^{i}	$6.99 \pm 0.42^{d, e}$	$16.44 \pm 0.52^{e, f}$	$2.41 \pm 0.16^{b, c}$	2.92 ± 0.32^{b}	2.92 ± 0.32^{a}	2.50 ± 0.26^{c}
NaOH	0.1	46.74 ± 0.78^{f}	$32.27 \pm 1.02^{h, i}$	$4.16 \pm 0.33^{c, d}$	$4.53 \pm 0.23^{h, i}$	5.33 ± 0.28^{f}	$18.25 \pm 0.63^{c, d}$	$1.21 \pm 0.05^{d, e}$	1.74 ± 0.08^{c}	$1.69 \pm 0.16^{b, c}$	$2.10 \pm 0.17^{c, d}$
	0.5	46.56 ± 0.52^{f}	$32.23 \pm 0.83^{h, i}$	$3.47 \pm 0.26^{d, e}$	2.45 ± 0.15^{j}	2.74 ± 0.24^{g}	14.66 ± 0.58^{f}	2.64 ± 0.10^{b}	2.48 ± 0.34^{b}	2.89 ± 0.12^{a}	3.24 ± 0.23^{b}
	1	42.34 ± 0.72^{g}	30.08 ± 0.67^{i}	2.69 ± 0.19^{e}	2.33 ± 0.34^{j}	2.38 ± 0.31^{g}	10.54 ± 0.72^{h}	3.77 ± 0.15^{a}	3.62 ± 0.15^{a}	3.00 ± 0.06^{a}	4.07 ± 0.22^{a}
H_3PO_4	0.1	58.56 ± 1.32^{d}	$40.59 \pm 0.78^{b, c}$	6.83 ± 0.32^{b}	8.39 ± 0.16^{b}	12.68 ± 0.28^{b}	$20.54 \pm 0.33^{a, b}$	$0.89 \pm 0.09^{e, f}$	$0.41 \pm 0.06^{e, f}$	$1.31 \pm 0.10^{d, e}$	0.75 ± 0.06^{h}
	0.5	52.49 ± 1.14^{e}	$36.64 \pm 0.56^{e, f}$	6.53 ± 0.23^{b}	$7.99 \pm 0.21^{b, c}$	12.31 ± 0.31^{b}	$19.90 \pm 0.26^{b, c}$	1.34 ± 0.12^{d}	$0.92 \pm 0.12^{d, e}$	$1.81 \pm 0.18^{b, c}$	$1.21 \pm 0.12^{f, g}$
	1	48.73 ± 1.22^{f}	$33.35 \pm 0.42^{i, j}$	6.10 ± 0.15^{b}	$7.59 \pm 0.13^{c, d}$	10.31 ± 0.22^{d}	$19.78 \pm 0.42^{b, c}$	2.15 ± 0.21^{c}	1.62 ± 0.08^{c}	2.15 ± 0.23^{b}	$1.54 \pm 0.12^{e, f}$
H_2O_2	0.1	71.01 ± 1.32^{b}	$42.24 \pm 0.70^{a, b}$	6.74 ± 0.30^{b}	$7.10 \pm 0.13^{e, f}$	$6.24 \pm 0.26^{e, f}$	$18.94 \pm 0.62^{b, c}$	$0.36 \pm 0.02^{g, h}$	$0.30 \pm 0.06^{f, g}$	0.80 ± 0.05^{f}	$0.84 \pm 0.02^{g, h}$
	0.5	$73.35 \pm 1.08^{a, b}$	$42.85 \pm 1.02^{a, b}$	5.88 ± 0.34^{b}	$6.01 \pm 0.21^{f, g}$	2.52 ± 0.30^{g}	15.62 ± 0.42^{f}	0.93 ± 0.08^{e}	$0.78 \pm 0.22^{d, e}$	$1.06 \pm 0.08^{e, f}$	$1.20 \pm 0.04^{f, g}$
	1	74.25 ± 0.87^{a}	43.27 ± 0.56^{a}	$4.26 \pm 0.22^{c, d}$	$5.25 \pm 0.18^{g, h}$	2.14 ± 0.21^{g}	12.52 ± 0.35^{g}	1.53 ± 0.06^{d}	$1.30 \pm 0.31^{c, d}$	$1.78 \pm 0.16^{b, c}$	$1.75 \pm 0.02^{d, e}$

The results were presented as mean ± SD, n = 3

Values with different alphabets in the same column are significantly different ($p < 0.05$)

observed between untreated and pretreated substrates ($p < 0.05$). The cellulose, hemicellulose and lignin content of the untreated OP was 52.42 ± 1.24, 9.48 ± 0.86 and 15.08 ± 1.32%, whereas it was 34.97 ± 1.48%, 9.55 ± 0.63 and 21.72 ± 1.26% with untreated NP, respectively. The results suggested that the NP contains significantly higher lignin and hemicellulose, substantially low cellulose than OP and this structural feature may limits the extent of cellulose hydrolysis of NP. The results obtained from this study on components of OP and NP were quite comparable with the earlier reports [15, 31].

The amount of cellulose, hemicellulose and lignin in dilute H_2SO_4 pretreated OP varied between 60.13–66.99, 2.64–6.77 and 6.99–9.68%, whereas it was about 30.89–38.28, 4.14–6.35 and 16.44–20.73% with NP, respectively (Table 1). The results suggested that the amount of cellulose, hemicellulose and lignin in the solid residue was significantly decreased with increasing the acid concentrations between 0.1 and 1% ($p < 0.05$). Further, there was a considerable amount of glucose (OP 0.56–2.41%, NP 0.5–2.92%), and xylose (OP 1.12–2.92%, NP 1.22–2.50%) were detected in the pretreatment liquids. It is suggested that the sugars in the hydrolysis liquid was significantly increased with increasing concentration of acid used for pretreatment which caused more sugar loss due to solubilization ($p < 0.05$). Likewise, Byadgi and Kalburgi [32] recovered the maximum cellulose (55%) with 1.5% dilute sulphuric acid with a heating period of 45 min at 121 °C. Several reports suggested that the dilute sulphuric acid (< 4%) was considered as an effective and inexpensive pretreatment process; however, the loss of sugar caused by solubilization was higher than that of other pretreatments [13, 33].

In case of NaOH pretreatment, amount of cellulose, hemicellulose and lignin in pretreated OP was about 42.34–46.74, 2.69–4.16 and 2.38–5.33%, whereas it was about 30.08–32.27, 2.33–4.53 and 10.54–18.25% in NP, respectively. Similar to acid pretreatment, increase in concentration of NaOH used for pretreatment significantly decreased the cellulose, hemicellulose and lignin content of both OP and NP ($p < 0.05$). Further, the glucose and xylose in the pretreatment liquid were about 1.21–3.77 and 1.69–3.00% in OP, and 1.74–3.62 and 2.10–4.07% in NP, respectively. It is suggested that the sugars released during pretreatment was increased significantly with increasing concentration of NaOH between 0.1 and 1% ($p < 0.05$). The amount of hemicellulose and lignin removed from the OP and NP was significantly higher with NaOH pretreatment than other pretreatments; however, sugars released in pretreatment liquid was significantly high compared with other pretreatments ($p < 0.05$). Several other studies also suggested that the pretreatment using dilute NaOH caused significant loss of cellulose and hemicellulose in addition to the higher removal of lignin [9, 34, 35].

The cellulose, hemicellulose and lignin of dilute H_3PO_4 pretreated OP was varied between 48.73–58.56, 6.10–6.83 and 10.31–12.68%, whereas it was about 33.35–40.59, 7.59–8.39 and 19.78–20.54% in NP, respectively. Similar to other pretreatment, increase in concentration of acid significantly affect the composition of the OP and NP ($p < 0.05$). Furthermore, glucose and xylose in the liquid fraction was about 0.89–2.15 and 1.31–2.15%, and 0.41–1.62 and 0.75–1.5% in OP and NP, respectively. It is clearly evidenced that sugars released in the pretreatment liquid was comparatively lower than the other pretreatments ($p < 0.05$). However, there was no significant difference was noted between the components of untreated and pretreated OP and NP ($p > 0.05$). Similarly, Brummer et al. [21] also reported that no significant increase in cellulose in waste paper after pretreatment with 0.25% H_3PO_4; however, considerable increase was noticed after the subsequent addition of 2% HNO_3.

The results of dilute hydrogen peroxide pretreatment revealed that the cellulose, hemicellulose and lignin content were ranged between 71.10–74.25, 4.26–6.74, and 2.14–6.24%, whereas it was about 42.24–43.27, 5.25–7.10 and 12.52–18.94% in OP and NP, respectively. The amount of cellulose was increased with increasing the concentrations of H_2O_2 used (0.1–1%), whereas the lignin content was decreased. In addition, the hemicellulose content in pretreated OP and NP was significantly lower than the other pretreated substrates ($p < 0.05$). In pretreatment liquid, the amount of glucose and xylose released were about 0.36–1.53 and 0.80–1.78, 0.30–1.30 and 0.84–1.75% in OP and NP, respectively. The results revealed that the sugars released in pretreatment liquid was increased with increasing the concentrations (0.1–1%); however, it was significantly lower than the sugar loss from other pretreatments ($p < 0.05$). Among the various pretreatment, 0.5% hydrogen peroxide was significantly increased the cellulose and removed the lignin in addition less sugar loss in pretreatment liquid from both OP and NP ($p < 0.05$). Likewise, Gellerstedt and Perersson [36] suggested that the hydrogen peroxide promotes rapid oxidative depolymerization of the lignin in lignocellulosic materials which would be useful in pretreatment of biomass. Kim et al. [20] reported that there was a significant increase in cellulose and 40–60% removal of lignin in NP pretreated with 5% H_2O_2.

Crystallinity Analysis of Pretreated Waste Paper by XRD

The XRD analysis suggested that there was a significant difference was observed in the crystallinity of untreated and pretreated waste paper (Fig. 1). The *CrI* of untreated, H_2SO_4, H_3PO_4, NaOH and H_2O_2 pretreated OP and NP was 77.93 and 66.15, 66.05 and 60.84, 58.39 and 58.13, 61.90

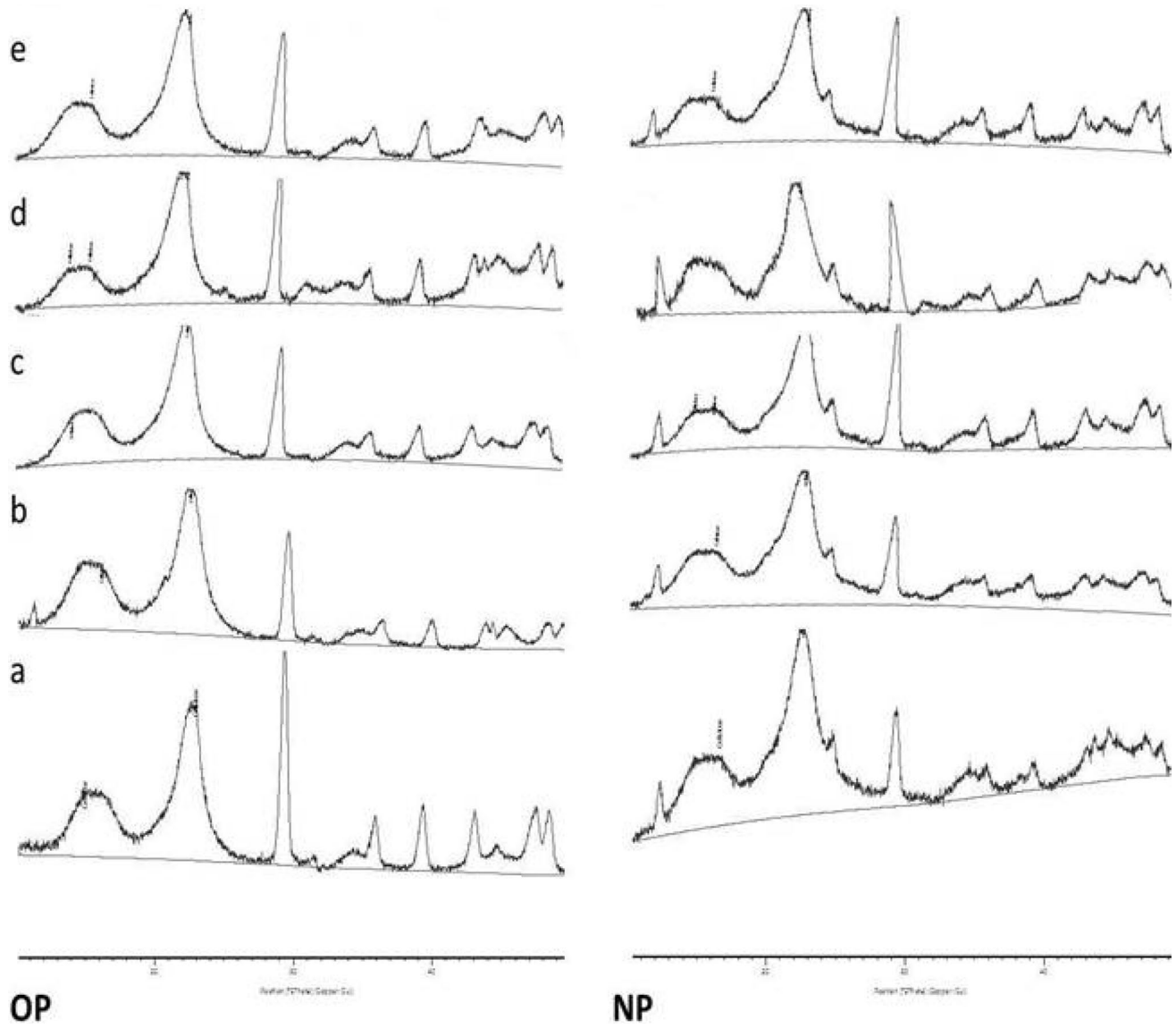

Fig. 1 X-ray diffractograms of pretreated OP and NP *a* untreated, *b* 0.1% H_2SO_4 pretreated, *c* 0.1% NaOH pretreated, *d* 0.1% H_3PO_4 pretreated and *e* 0.5% H_2O_2

and 65.10, and 55.32 and 51.79%, respectively (Table 2). The results suggested that the *CrI* of the untreated waste OP and NP was significantly higher than the other pretreated waste OP and NP ($p < 0.05$) and it is confirmed that the pretreatments significantly affected the crystalline region of cellulose than the amorphous structure. Likewise, several other studies also suggested that the pretreatment with acid, alkaline and other chemicals significantly decreased the crystallinity index of the cellulosic biomass [37, 38]. Contrarily, Dubey et al. [13] reported that the crystallinity of the waste paper treated with 0.5N H_2SO_4 (81.63%) was considerably increased than untreated paper (76.32%), due to the breakdown of amorphous cellulose under acidic condition.

Table 2 Crystallinity index (*CrI*) and enzymatic digetability (ED) of untreated and pretreated OP and NP

Pretreatment	*CrI* (%)		ED (%)	
	OP	NP	OP	NP
Untreated	77.93^{a}	66.15^{a}	57.57 ± 0.85^{d}	52.90 ± 0.82^{d}
0.1% H_2SO_4	66.05^{b}	60.84^{b}	82.27 ± 0.72^{b}	70.26 ± 0.65^{c}
0.1% NaOH	58.39^{d}	58.13^{c}	81.36 ± 1.32^{b}	71.49 ± 1.12^{c}
0.1% H_3PO_4	61.90^{c}	65.10^{a}	75.38 ± 0.63^{c}	74.70 ± 0.85^{b}
0.5% H_2O_2	55.32^{d}	51.79^{d}	91.29 ± 0.64^{a}	78.75 ± 0.81^{a}

Values with different alphabets in the same column are significantly different ($p < 0.05$)

The ED was 57.5 and 52.9, 82.2 and 70.2, 81.3 and 7.4, 75.3 and 74.7, and 91.2 and 78.75% with untreated, 0.1% H_2SO_4, 0.1% NaOH, 0.1% H_3PO_4 and 0.5% H_2O_2 pretreated OP and NP, respectively (Table 2). The ED of 0.5% H_2O_2 pretreated office and NP (91.2 and 78.7%) was significantly higher than that of untreated and other pretreatments ($p < 0.05$). The results clearly evidenced that the ED was significantly increased due the high cellulose accessibility caused by the significant removal of lignin from pretreated OP and NP. Leu and Zhu [17] reported that the pretreatment removes cell wall components which result in a substrate with a relatively open and porous structure which increases the cellulose accessibility (CAC) for effective lignocellulose saccharification. Luo and Zhu [18] suggested that the pretreatment significantly affects the fiber hornification which increased the enzyme accessibility to cellulose and ED. Several studies suggested that the increase in ED of the pretreated substrate was due to significant removal hemicellulose improved fiber porosity [39] and enzyme access to the cellulose component [40].

Sugar Yield and Inhibitors in Untreated and Pretreated Waste Paper Hydrolysate

The enzymatic saccharification of untreated and pretreated OP and NP were presented in Table 3. The sugar yield obtained from this study was about 8.06 and 5.29, 12.98 and 6.87, 9.04 and 5.56, 10.05 and 7.47, and 16.23 and 8.75 with untreated, dilute H_2SO_4, NaOH, H_3PO_4 and H_2O_2 pretreated OP and NP, respectively. The results revealed that the pretreatment significantly increased the sugar yield and the hydrolysates were mainly consists of glucose and xylose; however, sugar yield was significantly higher with 0.5% H_2O_2 pretreated OP and NP than other pretreated substrates ($p < 0.05$) and also the sugar yield achieved from this study was comparatively higher than the previous reports [31, 33]. Rocha et al. [22] achieved about 15 g/L of glucose and 2.5 g/L of xylose from 2% untreated paper biomass even with high enzyme dosage (71 FPU/g of cellulose and 35 CBU/g) and the yield was reduced after pretreatment with 1% H_2SO_4.

In general, the inhibitors such as furfural and 5-hydroxymethylfurfural (5-HMF) produced from the decomposition of sugar during pretreatment of biomass limits efficient utilization of the hydrolysate to produce products through fermentation [41, 42]. However, amounts of inhibitors produced are mostly depends on reaction conditions such as temperature, concentration of acid/alkali and hydrolysis time [43]. The results suggested that there was no furfural and HMF was detected in hydrolysate except dilute H_2SO_4; however, phenolics were present in almost all the hydrolysate due to the lignin degradation (Table 3). The amount of furfural and HMF in dilute H_2SO_4 pretreated OP and NP hydrolysate

Table 3 Sugar yield and inhibitors in the hydrolysates of untreated and pretreated OP and NP

Pretreatment	Sugar yield (g/L)						Inhibitors (g/L)					
	Glucose		Xylose		Total Sugars		HMF		Furfural		Total phenolics	
	OP	NP	OP	NP	OP	NP	OP	NP	OP	NP	OP	NP
Untreated	6.59 ± 0.24^{d}	3.91 ± 0.16^{d}	1.44 ± 0.15^{a}	1.35 ± 0.08^{b}	8.06 ± 0.32^{d}	5.29 ± 0.42^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.29 ± 0.03^{c}	0.54 ± 0.03^{c}
0.1% H_2SO_4	12.06 ± 0.28^{b}	6.12 ± 0.24^{b}	0.92 ± 0.13^{b}	0.75 ± 0.14^{c}	12.98 ± 0.28^{b}	6.87 ± 0.18^{b}	0.08 ± 0.03^{a}	0.18 ± 0.02^{a}	0.12 ± 0.03^{a}	0.23 ± 0.02^{a}	0.82 ± 0.02^{b}	1.32 ± 0.02^{b}
0.1% NaOH	8.52 ± 0.16^{c}	5.01 ± 0.15^{c}	0.52 ± 0.14^{c}	0.53 ± 0.13^{d}	$9.04 \pm 0.62^{c, d}$	5.56 ± 0.23^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.95 ± 0.03^{a}	1.56 ± 0.03^{a}
0.1% H_3PO_4	8.71 ± 0.24^{c}	5.97 ± 0.17^{b}	1.32 ± 0.13^{a}	1.50 ± 0.08^{a}	10.05 ± 0.42^{c}	7.47 ± 0.31^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.32 ± 0.02^{c}	0.44 ± 0.04^{d}
0.5% H_2O_2	15.05 ± 0.36^{a}	7.52 ± 0.10^{a}	$1.18 \pm 0.15^{a, b}$	1.20 ± 0.08^{b}	16.23 ± 0.28^{a}	8.75 ± 0.24^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.14 ± 0.04^{d}	0.21 ± 0.03^{e}

The results were presented as mean ± SD, n = 3

Values with different alphabets in the same column are significantly different ($p < 0.05$)

was about 0.08 and 0.18, and 0.12 and 0.23 g/L, respectively. However, the amount of phenolics in the hydrolysates were 0.29 and 0.54, 0.82 and 1.32, 0.95 and 1.56, 0.32 and 0.44, and 0.14 and 0.21 g/L with untreated, dilute H_2SO_4, NaOH, H_3PO_4 and H_2O_2 pretreated OP and NP, respectively. The amount of phenolics was significantly higher in the hydrolysate of NaOH pretreated OP and NP mainly due to more removal of lignin from the biomass. Similarly, Rocha et al. [22] reported that the furfural and 5-HMF were not detected in 1% H_2SO_4 pretreated waste OP. Based on results, pretreatment of waste OP and NP using 0.5% H_2O_2 was considered as an effective method for the enhanced utilization of waste paper for production of bioethanol and biofuels.

Effect of Solid Loadings

Several reports suggested that the high solid loadings significantly decreases the sugar yield as the viscosity of the biomass system increases abruptly at increased loadings, which affects the uniform mixing and mass transfer of the enzymes and also results in feedback inhibiton by increased concentration of sugars [44, 45]. The effects of solid loadings on enzymatic hydrolysis of waste OP and NP were investigated and presented in Fig. 2. The results suggested that the sugar yield was significantly increased while increasing the solid loadings from 1 to 3% and decreased further with 4% ($p < 0.05$). The sugar yield obtained from the OP and NP was maximum with 3% (23.48 and 13.12 g/L) and minimum with 1% solid loadings (8.82 and 4.3 g/L). It is suggested that the decreased sugar yield with 4% solid loadings due to hard mixing and enzyme dosage makes the solids remain intact during hydrolysis. Rocha et al. [22] obtained about 42 and 8 g/L of glucose and xylose respectively with 8% solid loading of acid pretreated waste OP and also Wu et al. [33] achieved 12 g/L sugar yield from 2% solid loading of pretreated waste NP.

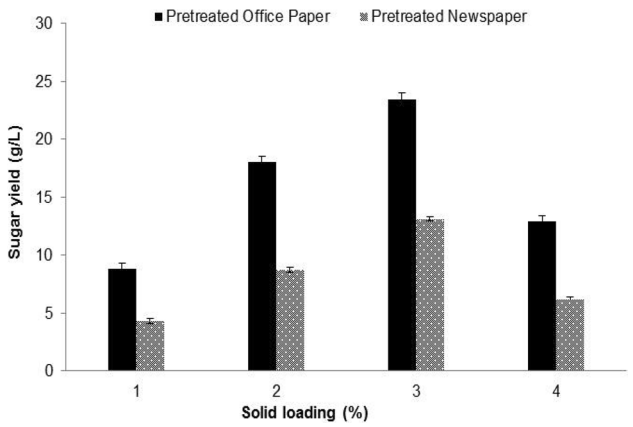

Fig. 2 Effect of solid loadings on sugar yield of pretreated **a** OP, **b** NP. The results were presented as mean ± SD, n = 3

Enzymatic Saccharification Under Optimum Conditions

Enzymatic hydrolysis of pretreated waste OP and NP was carried out under optimum condition [3% solids and enzyme loading (37 FPU/g + 25 CBU/g of solids)] and the results were presented in Fig. 3. The sugar yield and percentage hydrolysis was increased significantly upto 72 h and no further significant increase afterwards ($p < 0.05$). Under optimized conditions, the sugar yield and hydrolysis achieved from this study was about 24.5 and 13.26 g/L, and 91.8 and 79.6% with pretreated OP and NP, respectively. The yield achieved from the present study was comparatively higher than the previous studies [31, 33]. However, the sugar yield of pretreated OP was considerably higher than that of NP since it contains relatively more cellulose with less amount of lignin. Likewise, several other studies also suggested that the OP has more potential than NP due to the high sugar yield and conversion rate [31, 46].

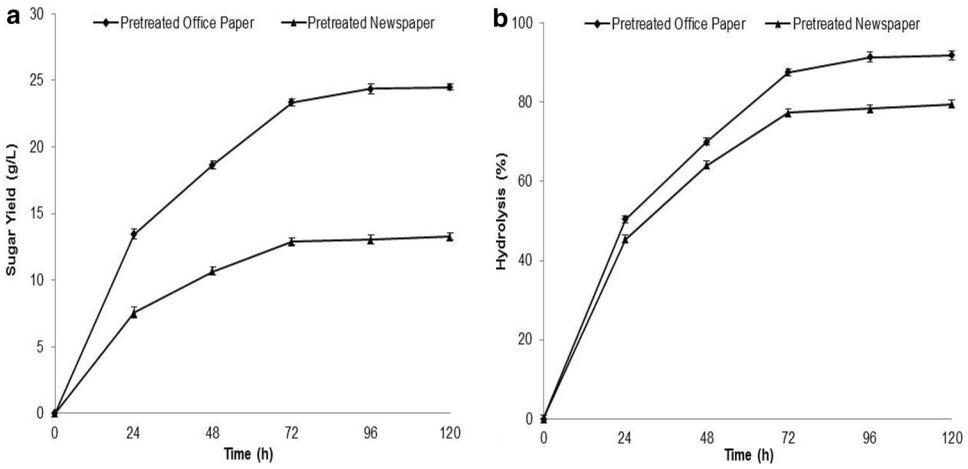

Fig. 3 The sugar yield (**a**) and hydrolysis (**b**) obtained from pretreated waste office and NP. The results were presented as mean ± SD, n = 3

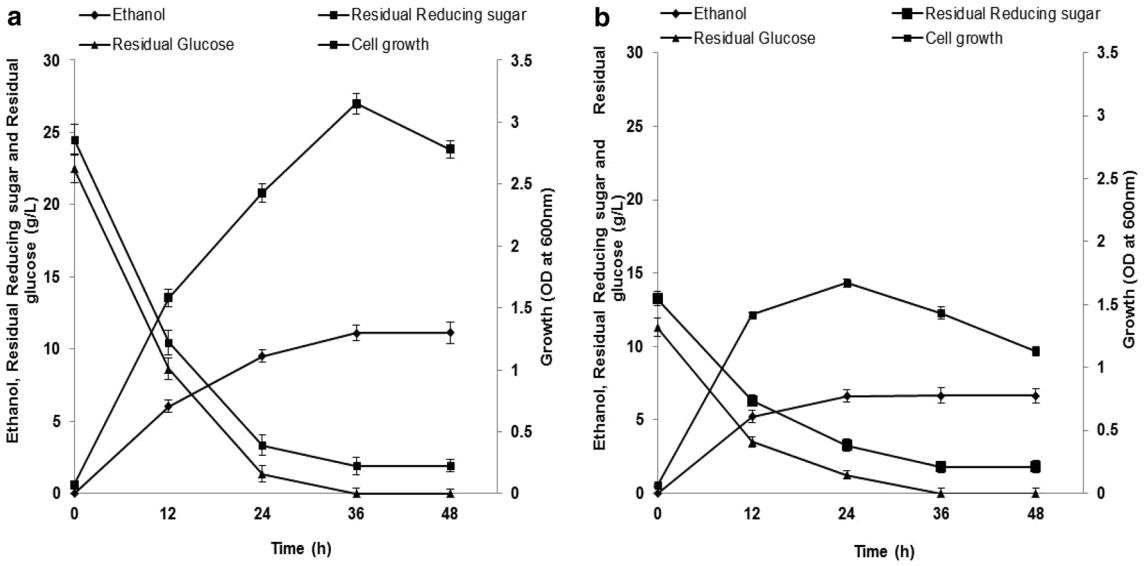

Fig. 4 Ethanol fermentation by the yeast, *S. cerevisiae* DSM 70449 using waste **a** OP and **b** NP hydrolysate

Ethanol Fermentation Using Waste Paper Hydrolysate

The ethanol fermentation was carried out using the hydrolysate of waste OP (24.5 g/L sugar with 22.5 g/L glucose) and NP (13.2 g/L sugar with 11.4 g/L glucose) and the results were presented in Fig. 4. The cell growth and ethanol production in OP hydrolysate was significantly increased with fermentation time, reached maximum at 36 h and no further significant increase afterwards ($p < 0.05$); whereas it reached maximum at 24 h and no significant increase afterwards with NP hydrolysate. The ethanol production and yield was about 11.15 and 6.65 g/L, and 0.51 and 0.58 (g ethanol/g sugar) with the corresponding productivity of 0.32 and 0.28 (g ethanol/L/h) from OP and NP, hydrolysate. The results suggested that 0.479 and 0.277 g of ethanol could be produced from 1 g of waste OP and NP, respectively. The residual reducing sugar and residual glucose analysis revealed that that there was a significant decrease in sugar due to the high degree of conversion of sugar into ethanol. The results suggested that the glucose from OP and NP hydrolysates were completely utilized and xylose remained unutilized since the yeast, *S. cerevisiae* does not utilize pentose sugars. Guerfali et al. [46] obtained about 8.8 g/L ethanol in 36 h with a yield of 0.38 g ethanol/g sugar from the hydrolysates of dilute phosphoric acid pretreated OP using *S. cerevisiae* CTM-30101. Thus, the results confirmed that the ethanol yield achieved from OP and NP hydrolysate in this study was comparatively higher than the previous studies [9, 13, 33]. Further, the ethanol yield could be increased further with some genetically modified strains which utilize both hexose and pentose sugar is in progress.

Conclusion

Pretreatment with hydrogen peroxide (0.5% v/v) was considered as an effective method as it considerably increased the cellulose in addition to the significant removal of lignin from waste OP and NP. The waste OP and NP hydrolysates obtained from this study resulted in high sugar yield with less inhibitors and the sugars were subsequently bioconverted into ethanol using *S. cerevisiae* suggested that the waste paper could be a potential alternative feedstock to develop a feasible and economical process for the future scale-up production of bioethanol.

Acknowledgements The authors are greatly thankful to The Research Council (TRC), Oman for financial support (ORG/EBR/14/003) and also thankful to Central Analytical and Applied Research Unit (CAARU) for XRD and GC analysis.

References

1. Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G.: Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches. Renew. Sust. Energy Rev. **66**, 751–774 (2016)
2. Lee, W.H., Jin, Y.S.: Improved ethanol production by engineered *Saccharomyces cerevisiae* expressing a mutated cellobiose transporter during simultaneous saccharification and fermentation. J. Biotechnol. **245**, 1–8 (2017)
3. Asgher, M., Bashir, F., Iqbal, H.M.N.: A comprehensive ligninolytic pretreatment approach from lignocellulose green biotechnology to produce bioethanol. Chem. Eng. Res. Des. **92**, 1571–1578 (2013)

4. Li, S.Z., Chan-Halbrendt, C.: Ethanol production in (the) People's Republic of China: potentials and technologies. Appl. Energy **86**, 162–169 (2009)
5. Behera, S., Kar, S., Mohanty, R.C., Ray, R.C.: Comparative study of bioethanol production from mahula (*Madhuca latifolia*L.) flowers by *Saccharomyces cerevisiae* cells immobilized in agar and Ca-alginate matrices. Appl. Energy **87**, 96–100 (2010)
6. Zhang, Y.H.P., Berson, E., Sarkanen, S., Dale, B.E.: Sessions 3 and 8: pretreatment and biomass recalcitrance: fundamentals and progress. Appl. Biochem. Biotechnol. **153**, 80–83 (2009)
7. Goldemberg, J.: Ethanol for a sustainable energy future. Science **315**, 808–810 (2007)
8. Service, R.F.: Biofuel researchers prepare to reap a new harvest. Science **315**, 1488–1491 (2007)
9. Bilal, M., Asgher, M., Iqbal, H.M.N., Ramzan, M.: Enhanced bio-ethanol production from old newspapers waste through alkali and enzymatic delignification. Waste Biomass Valor. **8**, 2271–2281 (2017)
10. Karim, M.N., Ryu, S.: A whole cell biocatalyst for cellulosic ethanol production from dilute acid pretreated corn stover hydrolyzates. Appl. Microbiol. Biotechnol. **91**, 529–542 (2011)
11. Yucel, Y., Goycıncık, S.: Optimization and modelling of process conditions using response surface methodology (RSM) for enzymatic saccharification of spent tea waste (STW). Waste Biomass Valoriz. **6**, 1077–1084 (2015)
12. Isikgor, F.H., Becer, C.R.: Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym. Chem. **6**(25), 4497–4559 (2015)
13. Dubey, A.K., Gupta, P.K., Garg, N., Naithani, S.: Bioethanol production from waste paper acid pretreated hydrolyzate with xylose fermenting *Pichia stipites*. Carbohydr. Polym. **88**, 825–829 (2012)
14. Jung, J.Y., Choi, M.S., Yang, J.K.: Optimization of concentrated acid hydrolysis of waste paper using response surface methodology. J. Korean Wood Sci. Technol. **41**, 87–99 (2013)
15. Wang, L., Templer, R., Murphy, R.J.: High-solids loading enzymatic hydrolysis of waste papers for biofuel production. Appl. Energy **99**, 23–31 (2012)
16. Wang, L., Sharifzadeh, M., Templer, R., Murphy, R.J.: Bioethanol production from various waste papers: economic feasibility and sensitivity analysis. Appl. Energy **111**, 1172–1182 (2013)
17. Leu, S.Y., Zhu, J.Y.: Substrate related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding. Bioenerg. Res. **6**, 405–415 (2013)
18. Luo, X., Zhu, J.Y.: Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. Enzyme Microb. Technol. **48**, 92–99 (2011)
19. Chandra, R., Ewanick, S., Hsieh, C., Saddler, J.N.: The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis. Part 1: a modified Simons' staining technique. Biotechnol. Prog. **24**, 1178–1185 (2008)
20. Kim, J.S., Lee, Y.Y., Park, S.C.: Pretreatment of wastepaper and pulp mill sludge by aqueous ammonia and hydrogen peroxide. Appl. Biochem. Biotechnol. **84–86**, 129–139 (2000)
21. Brummer, V., Jurena, T., Hlavacek, V., Omelkova, J., Bebar, L., Gabriel, P., Stehlik, P.: Enzymatic hydrolysis of pretreated waste paper: source of raw material or production of liquid biofuels. Bioresour. Technol. **152**, 543–547 (2014)
22. Rocha, J.M.T.S., Alencar, B.R.A., Mota, H.G., Gouveia, E.R.: Enzymatic hydrolysis of waste office paper for ethanol production by *Spathaspora passalidarum*. Cellulose Chem. Technol. **50**, 243–246 (2016)
23. Ranjithkumar, M., Ravikumar, R., Sankar, M.K., Kumar, M.N., Thanabal, V.: An effective conversion of cotton waste biomass to ethanol: a critical review on pretreatment processes. Waste Biomass Valoriz. **8**, 57–68 (2017)
24. Ghose, T.K.: Measurement of cellulase activities. Pure Appl. Chem. **59**, 257–268 (1987)
25. Parry, N.J., Beever, D.E., Owen, E., Vandenberghe, I., Van Beeumen, J., Bhat, M.K.: Biochemical characterization and mechanism of action of a thermostable β-glucosidase purified from *Thermoascus aurantiacus*. Biochem. J. **353**, 117–127 (2001)
26. Segal, L., Creely, J.J., Martin, A.E., Conrad, C.M.: An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Textile Res. J. **29**, 764–786 (1959)
27. Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J.: Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples. Technical Report-NREL/TP-510-42621, National Renewable Energy Laboratory, Golden (2008)
28. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeto, D.: Determination of Ash in Biomass. Technical Report-NREL/TP-510-42622, National Renewable Energy Laboratory, Golden (2008)
29. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D.: Determination of Structural Carbohydrates and Lignin in Biomass. Technical Report -NREL/TP-510-42618, National Renewable Energy Laboratory, Golden (2012)
30. Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M.: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods Enzymol. **299**, 152–178 (1999)
31. Chu, K.H., Feng, X.: Enzymatic conversion of newspaper and office paper to fermentable sugars. Process Safety Environ. Prot. **91**, 123–130 (2013)
32. Byadgi, S.A., Kalburgi, P.B.: Production of bioethanol from waste newspaper. Procedia Environ. Sci. **35**, 555–562 (2016)
33. Wu, F.C., Huang, S.S., Shih, I.L.: Sequential hydrolysis of waste newspaper and bioethanol production from the hydrolysate. Bioresour. Technol. **167**, 159–168 (2014)
34. Toquero, C., Bolado, S.: Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. **157**, 68–76 (2014)
35. Annamalai, N., Sivakumar, N.: Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. J. Biotechnol. **237**, 13–17 (2016)
36. Gellerstedt, G., Perrersson, I.: Chemical aspects of hydrogen peroxide bleaching. Part II. The bleaching of Kraft pulps. J. Wood Chem. Technol. **2**(3), 231–250 (1982)
37. Zhang, J., Wang, Y., Zhang, L., Zhang, R., Liu, G., Cheng, G.: Understanding changes in cellulose crystalline structure of lignocellulosic biomass during ionic liquid pretreatment by XRD. Bioresour. Technol. **151**, 402–405 (2014)
38. Singh, S., Cheng, G., Sathitsuksanoh, N., Wu, D., Varanasi, P., George, A., Balan, V., Gao, X., Kumar, R., Dale, B.E., Wyman, C.E., Simmons, B.A.: Comparison of different biomass pretreatment techniques and their impact on chemistry and structure. Front. Energy Res. **62**, 1–12 (2015)
39. Christov, L., Biely, P., Kalogeris, E., Christakopoulos, P., Prior, B.A., Bhat, M.K.: Effects of purified endo-b-1, 4-xylanases of family 10 and 11 and acetyl xylan esterases on eucalypt sulfite dissolving pulp. J. Biotechnol. **83**, 23–244 (2000)
40. Jeoh, T., Ishizawa, C., Davis, M.F., Himmel, M.E., Adney, W.S., Johnson, D.K.: Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnol. Bioeng. **98**, 112–122 (2007)
41. Taherzadeh, M., Karimi, K.: Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. J. Biosci. Bioeng. **87**, 169–178 (1999)
42. Franden, M.A., Pienkos, P.T., Zhang, M.: Development of a high-throughput method to evaluate the impact of inhibitory

compounds from lignocellulosic hydrolysates on the growth of *Zymomonas mobilis*. J. Biotechnol. **144**, 259–267 (2009)
43. Lenihan, P., Orozco, A., Onill, E., Ahmad, M.N.M., Rooney, D.W., Walker, G.M.: Dilute acid hydrolysis of lignocellulosic biomass. Chem. Eng. J. **56**, 395–410 (2010)
44. Kristensen, J.B., Felby, C., Jorgensen, H.: Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol. Biofuel **2**, 1–11 (2009)
45. Modenbach, A.A., Nokes, S.E.: The use of high-solids loadings in biomass pretreatment-a review. Biotechnol. Bioeng. **109**, 1–13 (2012)
46. Guerfali, M., Saidi, A., Gargouri, A., Belghith, H.: Enhanced enzymatic hydrolysis of waste paper for ethanol production using separate saccharification and fermentation. Appl. Biochem. Biotechnol. **175**, 25–42 (2015)