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Bioethanol production from waste paper acid pretreated hydrolyzate with xylose fermenting *Pichia stipitis*

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ABSTRACT

This study evaluates acid pretreated waste paper hydrolyzate as feedstock for bioethanol production. The presence of 70.12 ± 4.88% carbohydrates (holocellulose) makes waste paper a prospective and renewable biomass for bioethanol production. The waste paper was found to contain α-cellulose (61.5 ± 3.49%), pentosans (7.42 ± 0.36%), lignin (16.33 ± 0.96%), ash (12.50 ± 0.33%) and moisture (8.28 ± 0.63%). Conditions for the dilute acid pretreatment of waste paper were optimized by varying solid/liquid ratio 1:8–1:14 (w/v), reaction time 01–06 h, and sulfuric acid concentration 0.005–1.00 N at 120 °C in an autoclave. The conditions optimized for acid hydrolysis of waste paper were 0.50 N H_2SO_4 at 120 °C for 02 h reaction time keeping biomass:acid ratio 1:10 (w/v) for recovery of reducing sugars from waste paper hydrolyzate. Fermentation of acid hydrolyzate of waste paper with *Pichia stipitis* under optimum condition resulted in ethanol production 3.73 ± 0.16 g/l with 77.54 ± 4.47% of fermentation efficiency.

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1. Introduction

The conversion of lignocellulosic biomass to liquid fuel, i.e. bioethanol has long been pursued for its potential to provide an alternative renewable energy source that substitute the fossil fuels. Conventionally, bioethanol is produced from the fermentation of starchy materials or sucrose-containing feedstocks, such as corn, sugar cane, and honey. The yield of bioethanol from corn and sugar are high and the techniques are mature, however, it increases the risk of causing global food shortage (Chen, Tu, & Sheen, 2009). In this context, lignocellulosic biomass such as weed, grasses, saw dust, municipal solid waste, waste paper or pulp and paper mill wastes acquires significant importance as an alternative to the conventional raw materials for ethanol production.

The current study is based on ethanol production from waste paper because waste paper constitutes a considerable share of municipal and industrial waste even thought recycling efforts have been strengthened in recent years by legal provisions like the Packaging Directive (99×10^6 tons of paper were produced in 2008 in the Confederation of European Paper Industries, with a recycling rate of 66%, CEPI, 2008). When paper materials are recycled, they usually turn into lower grade paper products. With further recycling of paper, fiber length in the paper becomes shorter. Since the shortening of paper fibers decreases the quality of paper, the maximum ratio of paper-to-paper recycling is said to be 65% (Ikeda, Park, & Naoyuki, 2006). This means that a certain fraction of paper would always be sent to disposal. This fraction contains a significant and underutilized source of sugars/cellulose and could be converted to ethanol and used for energetic proposal achieving both environmental and energy benefits. Therefore, waste paper could be used as an excellent source of lignocellulosic biomass for sugars and ethanol production.

Currently, the technology for lignocellulosic ethanol production relies mainly on pre-treatment, chemical or enzymatic hydrolysis, fermentation and product separation or distillation. An appropriate pretreatment strategy is essential for the efficient enzyme hydrolysis of lignocellulosic biomass as lignin hinders the saccharification process. Various pre-treatment approaches have been exploited in the past such as acid or alkali pretreatment, hydrogen peroxide pretreatment, steam explosion, liquid hot water, ammonia fiber expansion pretreatment (Teymouri, Laureano-Perez, Alizadeh, & Dale, 2005), sodium chlorite pretreatment (Gupta, Sharma, & Kuhad, 2009) and biological pretreatment. The purpose of dilute acid pretreatment is the removal of hemicelluloses and the recovery of the component sugars. Among all pretreatment methods, the acid pretreatment methods of biomass with dilute sulfuric acid has long been recognized as a critical step for removing the hemicellulosic fraction from the lignocellulosic substrate to economize the biological conversion of cellulosic biomass to ethanol (Kuhad, Gupta, Khasa, & Singh, 2010).

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However, pre-treatment with dilute acid is a fast and rapid method to release hemicellulosic sugars but it leads to the accumulation of toxic byproducts such as furfural and phenolics. These toxics products are known to affect yeast cell metabolism during fermentation (Chandel, Kapoor, Singh, & Kuhad, 2007; Palmqvist & Hahn-Hagerdal, 2000). Although various detoxification methods have been investigated for the removal of fermentation inhibitory compounds, but overliming and activated charcoal adsorption methods are widely recommended (Gupta et al., 2009; Miyafuji et al., 2003). The detoxified hemicellulosic sugars obtained through acid hydrolysis can be used efficiently for the production of ethanol by suitable fermentative micro-organism.

The prime interest of this investigation is to optimize the conditions for acid treatment of waste paper and production of waste paper hydrolyzate containing mono-sugars such as glucose and xylose and its fermentation using the yeast species *Pichia stipitis* for ethanol production.

2. Materials and methods

2.1. Raw material

Waste paper was collected from the paper plant of Cellulose and Paper Division, Forest Research Institute, Dehra Dun (India). The waste paper was processed using valley beater (Vally Iron Work Co., Appleton, WI, USA) at room temperature for defiberization and production of pulp. Pulp was air dried and powdered in a Willey mill (A. Gallenkamp Co., Ltd., London) to 60 mesh size. The powdered material was used for the study.

2.2. Biomass composition analysis

The composition of waste paper was analyzed for holocellulose, lignin, pentosans, ash and moisture content following TAPPI protocols.

2.3. Dilute acid pretreatment

Conditions for the dilute acid pretreatment of waste paper were optimized by varying solid/liquid ratio 1:8–1:14, treatment time 01–06 h, and sulfuric acid concentration 0.005–1.00 N in a 1.0 l reaction vessel at 120 °C in an autoclave. The acid hydrolyzates after treatment were recovered by filtering through double-layered muslin cloth. The liquid hydrolyzate filtrate was analyzed for total reducing sugars, xylose and phenolics released.

2.4. Detoxification of acid hydrolyzate

The acid hydrolyzate of waste paper was detoxified at room temperature by mixing dried calcium oxide (CaO) till the pH raised to 7.0, proceeded with continuous stirring for 60 min and then centrifuged (15,000 × *g*, 15 min) to remove the precipitate. The CaO treated hydrolyzate was further detoxified by adding 2% (w/v) activated charcoal with continuous stirring at room temperature for 30 min and the detoxified hydrolyzate was recovered using vacuum filtration.

2.5. Fermentation of detoxified hydrolyzate

2.5.1. Microorganism

P. stipitis NCIM-3499 was procured from National Chemical Laboratory (NCL), Pune (India) for the current study. It was cultivated on agar medium containing (g/l); xylose 20, yeast extract 3.0, malt extract 3.0, peptone 5.0, agar 20 at pH 5.0 and temperature 30 °C. Maintained the culture on agar slants and preserved at 4 °C.

Table 1
Dilute acid pretreatment of waste paper with different solid:liquid ratios.

Bath ratio	TRS (g/l)	Xylose (g/l)	Phenolics (%)
1:8	2.88 ± 0.133	0.422 ± 0.021	0.016 ± 0.001
1:10	**3.27 ± 0.162**	**0.759 ± 0.039**	**0.022 ± 0.001**
1:12	2.74 ± 0.164	0.432 ± 0.032	0.017 ± 0.002
1:14	2.34 ± 0.117	0.347 ± 0.021	0.014 ± 0.001

Table 2
Dilute acid pretreatment of waste paper with different reaction time.

Time (h)	TRS (g/l)	Xylose (g/l)	Phenolics (%)
1 h	1.022 ± 0.042	0.514 ± 0.022	0.012 ± 0.001
2 h	**2.542 ± 0.097**	**1.213 ± 0.032**	**0.017 ± 0.001**
3 h	2.615 ± 0.096	1.223 ± 0.030	0.019 ± 0.001
4 h	2.855 ± 0.138	1.350 ± 0.029	0.036 ± 0.002
5 h	3.187 ± 0.183	1.931 ± 0.062	0.042 ± 0.001
6 h	3.601 ± 0.196	2.111 ± 0.054	0.059 ± 0.003

2.5.2. Fermentation

The fermentation of the detoxified acid hydrolyzate was carried out using bench top fermentor (5 l, Sartorius, Germany). The acid hydrolyzate (10.79 g/l sugars) supplemented with (g/l) NH_4Cl 0.5, KH_2PO_4 2.0, $MgSO_4{\cdot}7H_2O$ 0.5, yeast extract 1.5, $CaCl_2{\cdot}2H_2O$ 0.1, $FeCl_3{\cdot}2H_2O$ 0.1, $ZnSO_4{\cdot}7H_2O$ 0.001 was inoculated with 10% (v/v) inoculum of *P. stipitis* at pH 5.0 and incubated at 30 °C with 150 rpm of agitation. The pH of the medium was adjusted with 2 N HCl and 2 N NaOH. The dissolved oxygen concentration was monitored continuously throughout the process using dissolved oxygen probe and air flow of 0.4 l/min was maintained throughout the study. Samples were withdrawn at regular intervals of 4 h and centrifuged for 15 min. The supernatant was used to determine the ethanol and residual sugar concentration.

2.6. Analytical methods

The concentration of total reducing sugars (TRS), phenolic content and ethanol was determined using dinitrosalicylic acid (DNS) method (Miller, 1959), Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) and chromic acid method (Caputi, Ueda, & Brown, 1968) respectively. The xylose concentration was estimated by p-bromoaniline method (Bala et al., 2004).

2.7. Wide angle X-ray diffractions (WAXDs)

WAXDs of solid samples were recorded using a Bruker AXS D8 Advance X-ray powder diffractometer, Wisconsin, USA, with a Cu Kα target.

2.8. Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean ± standard deviation.

Table 3
Dilute acid pretreatment of waste paper with different acid concentrations.

Acid concentration (N)	TRS (g/l)	Xylose (g/l)	Phenolics (%)
0.005	0.088 ± 0.004	0.048 ± 0.002	0.011 ± 0.001
0.01	0.104 ± 0.007	0.062 ± 0.002	0.012 ± 0.001
0.05	0.789 ± 0.042	0.400 ± 0.012	0.016 ± 0.001
0.10	2.542 ± 0.118	1.213 ± 0.034	0.022 ± 0.001
0.50	**12.422 ± 0.792**	**8.680 ± 0.223**	**0.120 ± 0.004**
1.00	14.456 ± 0.859	8.809 ± 0.267	0.129 ± 0.004

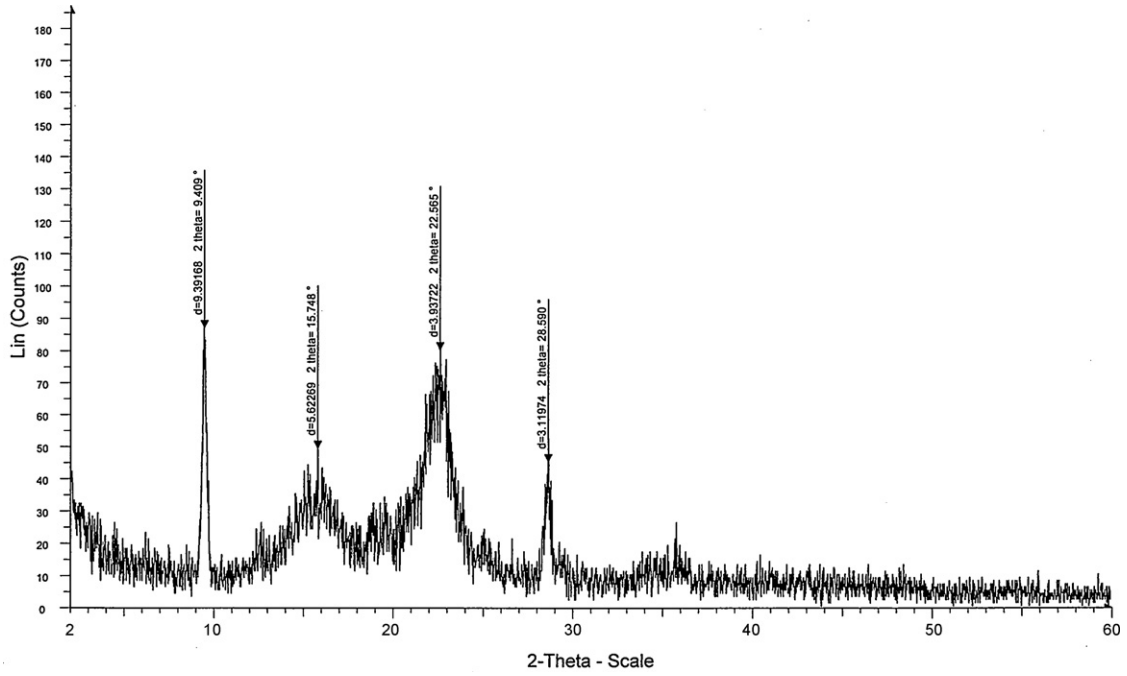

Fig. 1. WAXD of untreated waste paper.

3. Results and discussion

3.1. Waste paper compositional analysis

The oven dried waste paper dust (60 mesh size) was found to contain holocellulose (70.12 ± 4.88%) comprising α-cellulose (61.5 ± 3.49%), pentosans (7.42 ± 0.36%), lignin (16.33 ± 0.96%), ash (12.50 ± 0.33%) and moisture (8.28 ± 0.63%). The presence of 70.12 ± 4.88% of total carbohydrates (holocellulose) makes waste paper a prospective and renewable biomass for bioethanol production.

3.2. Dilute acid pretreatment

The dilute acid pretreatment of waste paper was carried out with different solid/liquid ratios (1:8–1:14), different reaction time (01–06 h) and different sulfuric acid concentrations (0.005–1.00 N) at 120 °C temperature in an autoclave.

3.2.1. Effect of different bath ratio

Pretreatment with different bath ratios (1:8–1:14) was carried out with sulfuric acid concentration 0.10 N for 02 h at 120 °C temperature. The maximum total reducing sugars yield obtained was

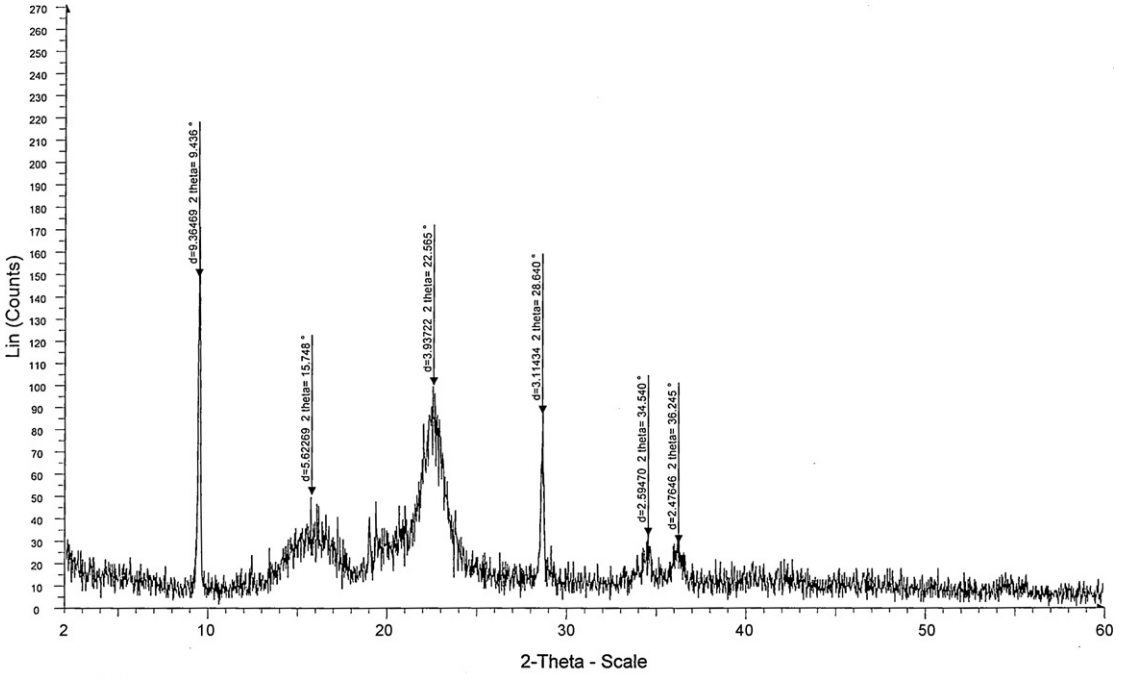

Fig. 2. WAXD of acid pretreated waste paper.

03.27 ± 0.162 g/l at 1:10 bath ratio (Table 1). The biomass when treated with 1:12 and 1:14 there was slightly decrease in concentration of total reducing sugars, xylose and phenolics.

3.2.2. Effect of different reaction time

The treatment of waste paper was carried out with different time periods (01–06 h) under optimized bath ratio (1:10), with sulfuric acid concentration 0.10 N. The maximum total reducing sugars yield was 3.601 ± 0.196 g/l and xylose was 2.111 ± 0.054 g/l when biomass was treated with 06 h (Table 2). As evident from the results, there was sharp increase in the total reducing sugars and xylose content up to 02 h and thereafter there was not much increase, also the phenolics were low up to 02 h and thereafter their content was increased. As the desirable combination would be high total reducing sugars and xylose with the low phenolics for better fermentation efficiency, therefore time taken is 02 h for further study.

3.2.3. Effect of different acid concentrations

Among the different acid concentrations (0.005–1.00 N), the maximum sugar yield 14.456 ± 0.859 g/l and xylose 8.809 ± 0.267 g/l was obtained when waste paper was hydrolyzed with 1.0 N H_2SO_4 at 120 °C for 02 h with 1:10 bath ratio (Table 3). The data revel that there was substantial increase up to 0.50 N in total reducing sugars (12.422 ± 0.792 g/l) and xylose (8.680 ± 0.223 g/l) content, but thereafter there was not much difference from 0.50 N to 1.00 N acid hydrolysis, therefore, 0.50 N was taken as optimized condition for further reaction.

3.3. Effect of detoxification of acid hydrolyzate

The phenolics are lignin degradation byproducts are known to decrease fermentability by reducing the activities of yeasts fermentation efficiency. Among different detoxification treatments, overliming (Chandel et al., 2007) and activated charcoal (Miyafuji et al., 2003) are commonly used. However, no single method is efficient at removing all the inhibitors present in acid hydrolyzates. Therefore, we attempted a sequential treatment of overliming with CaO and activated charcoal adsorption for the detoxification of acid hydrolyzate. Overliming with CaO followed by activated charcoal reduced phenolics by 82.11 ± 5.31%. Our findings are in agreement with earlier work reported on the detoxification of acid hydrolyzates by overliming followed by activated charcoal adsorption (Mussatto, Santos, & Roberto, 2004).

3.4. Wide angle X-ray diffraction (WAXD) of untreated and acid pretreated waste paper

The X-ray diffractogram of the untreated waste paper and acid pretreated waste paper are sketched in Figs. 1 and 2. In the study of Mulinari et al. (2009), it was described that a major diffraction peak for 2Φ ranging between 22° and 23° corresponds to the cellulose crystallographic planes. From the distributions shown in Figs. 1 and 2 the crystal structures of cellulose in the untreated waste paper and pretreated materials are clearly obtained. Therefore, the peak movements show that there is increase in the crystallinity in acid (0.50 N H_2SO_4) pretreated waste paper. X-ray diffractogram of untreated and acid pretreated waste paper indicate that crystallinity index of untreated waste paper is 76.32% and that acid pretreated waste paper under optimum condition (0.50 N H_2SO_4) is 81.63%. Converse, Matsuno, Tanaka, and Taniguchi (1988) observed an increases in crystallinity index of pretreated samples after dilute acid pretreatment, may be because more amorphous cellulose breaks down under acid condition (Kumar, Singh, & Ghose, 2009). Our results on crystallinity index were in agreement with previous reports on crystallinity index of acid pretreated biomass (Satyanagalakshmi et al., 2011).

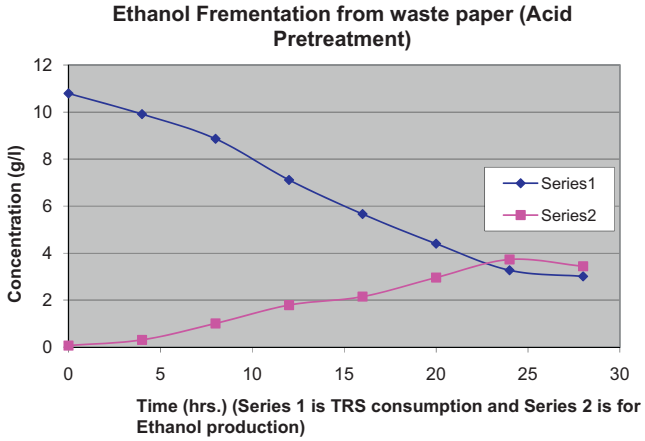

Fig. 3. Ethanol fermentation and consumption of sugars by *Pichia stipitis*.

3.5. Fermentation of detoxified acid hydrolyzate

The optimized acid hydrolyzate was fermented with *P. stipitis*. The detoxified xylose rich hydrolyzate obtained after dilute acid hydrolysis (10.79 ± 0.73 g/l sugars), when fermented with *P. stipitis*, resulted in ethanol production of 3.73 ± 0.16 g/l with fermentation efficiency 77.54 ± 4.47% after 24 h. The fermented broth was distilled to obtain ethanol. The consumption of sugars and production of ethanol during fermentation at different time intervals varied from 0 h to 28 h. The rate of fermentation of alcohol was dependent upon the period of fermentation. The concentration of ethanol from dilute acid hydrolyzate increased from 0.07 ± 0.01 g/l to 3.73 ± 0.16 g/l when the fermentation was carried out up to 24 h and thereafter decreased. Our results were in agreement with previous reports on fermentation with *P. stipitis* (Kapoor, Nair, & Kuhad, 2008) (Fig. 3).

4. Conclusion

The presence of 70.12 ± 4.88% of carbohydrates (holocellulose) makes waste paper a prospective and renewable biomass for bioethanol production. The waste paper was found to contain α-cellulose (61.5 ± 3.49%), pentosans (7.42 ± 0.36%), lignin (16.33 ± 0.96%), ash (12.50 ± 0.33%) and moisture (8.28 ± 0.63%). Conditions for the dilute acid pretreatment of waste paper were optimized by varying solid/liquid ratio 1:8–1:14 (w/v), reaction time 01–06 h, and sulfuric acid concentration 0.005–1.00 N in a 1.0 l reaction vessel at 120 °C in an autoclave. The conditions optimized for acid hydrolysis of waste paper were 0.50 N H_2SO_4 at 120 °C for 02 h reaction time keeping biomass:acid ratio 1:10 (w/v) for recovery of reducing sugars from waste paper hydrolyzate. The X-ray diffractogram show an increase in the crystallinity in acid pretreated waste paper. The acid hydrolyzate of waste paper was detoxified with dried calcium oxide (CaO) and further by adding 2% (w/v) activated charcoal at room temperature for 30 min. Fermentation of acid hydrolyzate of waste paper with *P. stipitis* under optimum condition resulted in ethanol production 3.73 ± 0.16 g/l with 77.54 ± 4.47% of fermentation efficiency.

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