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EFFECT OF ACID HYDROLYSIS AND FUNGAL BIOTREATMENT ON AGRO-INDUSTRIAL WASTES FOR OBTAINMENT OF FREE SUGARS FOR BIOETHANOL PRODUCTION

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ABSTRACT

This study was designed to evaluate selected chemical and microbiological treatments for the conversion of certain local agro-industrial wastes (rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse) to ethanol. The chemical composition of these feedstocks was determined. Conversion of wastes to free sugars by acid hydrolysis varied from one treatment to another. In single-stage dilute acid hydrolysis, increasing acid concentration from 1 % (v/v) to 5 % (v/v) decreased the conversion percentage of almost all treated agro-industrial wastes. Lower conversion percentages for some treatments were obtained when increasing the residence time from 90 to 120 min. The two-stage dilute acid hydrolysis by phosphoric acid (1.0 % v/v) followed by sulphuric acid (1.0 % v/v) resulted in the highest conversion percentage (41.3 % w/w) on treated sugar beet waste. This treatment when neutralized, amended with some nutrients and inoculated with baker's yeast, achieved the highest ethanol concentration (1.0 % v/v). Formation of furfural and hydroxymethylfurfural (HMF) were functions of type of acid hydrolysis, acid concentration, residence time and feedstock type. The highest bioconversion of 5 % wastes (37.8 % w/w) was recorded on sugar beet waste by *Trichoderma viride* EMCC 107. This treatment when followed by baker's yeast fermentation, 0.41 % (v/v) ethanol and 8.2 % (v/w) conversion coefficient were obtained.

Key words: Bioethanol, agro-industrial wastes, acid hydrolysis, biotreatment.

INTRODUCTION

In view of continuously rising petroleum costs and dependence upon fossil fuel resources, considerable attention has been focused on alternative energy resources. The reliance on bioethanol as an alternative energy resource is one way to reduce both the consumption of crude oil and environmental pollution (27). In general, the potential global demand for biofuel is disproportion with the current world production (51). About 60 % of the world's bioethanol is produced from sugarcane and 40 % from other crops (15). However, the use of sugar or starch as raw materials for fuel production competes with their use as food (35). The less expensive lignocellulosic biomass is an attractive alternative material for bioethanol

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production that would minimize the potential conflict between land use for food (and feed) production and energy feedstock production. Lignocellulose is the most abundant renewable resource on earth. For example, high yielding maize and rice can produce field residues as much as 11 t/ha and 25 t/ha annually in most developing countries (17). Lignocellulosic biomass could produce up to 442 billion liters per year of bioethanol (7, 24). Many lignocellulosic materials have been tested for bioethanol production (41), but the large-scale commercial production has not been yet implemented due to the high cost of production based on current technologies. This is because the rate and yield of lignocellulose conversion to fermentable sugars is low, due to the resistant crystalline structure of cellulose and the physical barrier formed by the lignin surrounding the cellulose (14). Dilute acid hydrolysis has been successfully developed for pretreatment of different lignocellulosic materials. Dilute sulphuric acid has been the most commonly used so far for converting hemicellulose into fermentable sugars with high reaction rates, especially as it is a low cost technology (21). At moderate temperature**,** direct saccharification suffers from low yields because of sugar decomposition. Developed dilute acid hydrolysis processes using less severe conditions and achieving higher sugar conversion yields are needed. Roberto et al. (37) studied the effects of H_2SO_4 concentration and residence time on the production of sugars from rice straw at 121°C and found 1.0 % acid for 27 min attained the highest yield of xylose (77 %). Cara et al. (8) analyzed the production of fermentable sugars from olive tree biomass by dilute acid pretreatment under different pretreatment conditions (0.2 %, 0.6 %, 1.0 % and 1.4 % w/w sulphuric acid concentrations with temperatures in the range of 170 – 210°C). The best result of sugars recovered in the hydrolysate was obtained at 170°C, 1.0 % sulphuric acid. Diaz et al. (13) showed that *Pichia stipitis* can be used for the effective fermentation of sugars contained in the hydrolysates from dilute (1.0 % w/w) sulphuric acid pretreatment at 190°C for 10 min of olive tree biomass.

Alternatively, biological pretreatment processes of agro-industrial wastes have additional promising results, which are performed by using microorganisms such as brown, white and soft-rot fungi to degrade lignocellulosic materials in either submerged or solid-state cultures. Although biological pretreatment process requires low energy and less environmental conditions, the pretreatment process is time consuming (44). Itoh et al. (20) combined pretreatment of wood chips by *Ceriporiopsis subvermispora* with ethanolysis**,** achieving an overall ethanol yield of 62 % (v/w). In another study, 63 % (v/w) ethanol yield was obtained when rice straw was pretreated by *Phanerochaete chrysosporium* under submerged cultivation (4). Patel et al. (34) examined five different fungi viz. *Aspergillus niger*, *A. awamori*, *Trichoderma reesei*, *Phenerochaete chrysosporium* and *Pleurotus sajor-caju*, for pretreatment of wheat straw and rice straw. Pretreatment with *A. niger* and *A. awamori*, followed by fermentation using *Saccharomyces cerevisiae* (NCIM 3095), yielded 2.5 gl^{-1} and 2.2 gl^{-1} ethanol from wheat straw and rice straw, respectively.

A great number of microorganisms are capable of bioethanol formation among which *Sacch. cerevisiae* (baker's yeast) is the most frequently and traditionally used organism (5). Manjunath and Geeta (30) tested *Zymomonas mobilis*, *Sacch. cerevisiae* (CFTRI 101), *Candida shehatae* (NCIM 3500), *Pachysolen tannophilus* (NCIM 3445) and *P. stipitis* for ethanol production on biotreated sugarcane bagasse, paddy straw and wheat straw and found *Z. mobilis* to give the highest ethanol concentration (0.08 % w/w) from all the substrates.

The work reported here is on the investigation of either acid hydrolysis or fungal biotreatment of some agro-industrial wastes related to ethanol fermentation with different microorganisms.

MATERIALS AND METHODS

Agro-industrial wastes

Rice straw and corn stalks were collected from farms of rice and corn in El-Beheira governorate (located in Lower

Egypt in the Delta of the Nile north of Cairo, lat. 30.921076375384878, long. 30.2728271484375). Sugar beet waste and sugarcane bagasse were obtained from the Sugar Refinery Factory at El-Beheira governorate and sugarcane fresh syrup shops in Cairo, respectively. Sawdust was gathered from the local joinery atelier at Heliopolis University, Cairo, Egypt. Corn stalks were coarsely crushed using a laboratory hammer mill (Retsch GmbH & Co. KG, Germany). The other agro-industrial wastes were chopped into small pieces using a shredder and then ground to pass through 1.5 mm screens. All samples were homogenized and oven-dried at 45°C prior to characterization and pretreatment assays. The dried materials were stored in air tight containers at room temperature before use.

Microorganisms

Cellulose degrading fungi: *Aspergillus niger* EMCC 72 and *Trichoderma viride* EMCC 107 were obtained from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Ethanol producing organisms: *Saccharomyces cerevisiae* ATCC 9763, *Sacch. cerevisiae* ATCC 7754 and *Sacch. cerevisiae* DSM 70487 were obtained from The Department of Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, while *Candida utilis* EMCC 41, *Kluyveromyces marxianus* EMCC 11, *K. marxianus* EMCC 12 and *Zymomonas mobilis* EMCC 1543 were obtained from Cairo MIRCEN. Fresh commercial baker's yeast was purchased from a local bread bakery in Cairo. Active dry yeast was kindly provided from the Egyptian-Belgian Yeast Company (EgyBel) at El-Noubarya Industrial City, El-Beheira governorate, Egypt.

Analytical methods

Cellulose and hemicelluloses content of agro-industrial wastes was determined using the standard laboratory analytical procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL, USA) and methods developed by the Association of Official Analytical Chemistry (AOAC) for biomass analysis. Determination of total solids in samples was carried out according to the laboratory analytical procedure for determination of total solids in biomass (42). Triplicate samples were dried at 105°C to constant weight to determine the biomass moisture content (18). The ash determination was done by burning of triplicate samples at 550°C in a muffle furnace (Blue M Electric Company, Blue Island, USA) for 3 h, according to the laboratory analytical procedure for determination of ash in biomass (43). Lignin content in samples was determined by performing a two-step acid hydrolysis (47). The total nitrogen content of wastes was determined by using the Kjeldahl method (2). Total sugars in biomass and total free sugars in hydrolysates filtrates were determined according to AOAC (3). For the determination of furfural and hydroxymethylfurfural (HMF) in the acid hydrolyzates filtrates, samples were analyzed by HPLC equipped with UV/VIS and RI detectors (Jasco International Co., Tokyo, Japan) using an Aminex HPX-87H column (Bio-Rad) at 60°C with 0.6 ml/min eluent of 0.005M sulfuric acid. Furfural and HMF were quantified on UV chromatograms at 210 nm (23). Analytical grade furfural and HMF (Sigma Chemical Co. Ltd., USA) were used.

Hydrolysis of agro-industrial wastes

Two treatments were conducted for converting agro-industrial wastes to fermentable sugars using dilute acid hydrolysis or fungal biotreatment and their potential efficacy were compared.

Acid hydrolysis of agro-industrial wastes

Single-stage acid hydrolysis assays were performed in an autoclave at 120°C in 250 mL flasks with H_3PO_4, HCl or H_2SO_4 at the concentrations of 1.0 % and 5.0 % (v/v) for residence time ranged from 15 to 120 min. Solid : liquid ratio was adjusted to 5 % (w/v). The best conditions obtained from single-stage hydrolysis were applied to the two-stage hydrolysis. For operating the two-stage acids hydrolysis (one different acid for each stage), the first-stage liquor was drained

and the residual materials were then hydrolyzed by the second acid. After hydrolysis, solid materials were removed from flasks by filtration and the supernatant was neutralized with NaOH adjusting the pH between 5.0 and 5.5. Hydrolysates filtrates were then kept refrigerated at 5 – 7°C until used for ethanol production.

Biotreatment of agro-industrial wastes

Cellulose degrading fungi were grown and maintained on potato dextrose agar (PDA) slants. Fungal cultures were inoculated onto PDA medium in Petri plates. After 4 – 5 days of incubation at 28°C, cultures were used for inoculation. Five discs (5 mm diam.) of the plate cultures were inoculated into 50 mL of 2.0 % (w/v) malt extract medium in 250 mL cotton-plugged Erlenmeyer flasks and incubated in shaker incubator at 28°C and 150 rpm for 7 d. The liquid cultures were used for inoculation of solid-state cultivation.

The biotreatment of agro-industrial wastes prior to ethanol production was conducted either in submerged or solid-state cultures by growing *A. niger* EMCC 72 and *T. viride* EMCC 107 either individually or in mixed culture using the medium reported by Juhász et al. (22) with the following composition (g/L): urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4, 2.0, $CaCl_2$ 0.3, $MgSO_4$ 0.3, yeast extract 0.25, peptone 0.75, pH=7.0. The medium was also supplemented with the following trace elements (mg/L): $FeSO_4.7H_2O$ 5, $CoCl_2$ 20, $MnSO_4$ 1.6, $ZnSO_4$ 1.4. In submerged cultivation, 5 g of each residue was placed in 250 mL conical flasks containing 100 mL of the above medium. The flasks were plugged with cotton and sterilized at 121°C for 20 min. In single culture treatments, each flask was inoculated with 4 discs of each fungus, while in the mixed culture treatment, each flask was inoculated with 2 discs of each fungus. All flasks were incubated at 28°C in an orbital shaking incubator at 150 rpm. After seven days of incubation, the residual materials were separated by filtration through filter cloth then through Whatman filter paper No.1. The filtrates were used for total free sugars determination and ethanol production. In solid-state cultivation, 5 g of each residue were placed in 250 mL Erlenmeyer flasks and then conditioned with DI water to obtain moisture content of 60 % (wet basis). Flasks containing wet materials were autoclaved (121°C, 20 min) and cooled prior to inoculation. A homogeneous liquid culture (2 mL) of each fungus was inoculated on the top of the substrate in each flask. Pretreatment was carried out in an incubator at 28°C in static conditions for 25 d. Flasks without fungal inoculation were used as controls. All tests were performed in triplicates.

Ethanol production

Ethanol production from acid hydrolyzed agro-industrial wastes: The optimal acid hydrolysis condition, defined as yields of liberated total free sugars, was used for ethanol production by the ethanol producing organisms mentioned above. All microorganisms were maintained on MGYP agar slants containing (g/L): malt extract 3.0, glucose 10.0, yeast extract 3.0, peptone 5.0, agar 20, and pH 6.4. Stock cultures were stored at 5°C till used. The inocula for ethanol production of these microorganisms (except active dry yeast and baker's yeast, were inoculated by 2.0 % w/v) were prepared by harvesting (13000 *x g* for 5 min) the cells grown on MGYP broth for 48 h at 30°C. The cells were washed with DI water twice by centrifugation (6600 *x g* for 5 min) and used for inoculating (2 % v/v) the neutralized filtrates taken from the acid hydrolysis (as described previously). Prior to inoculation, the neutralized filtrates (100 mL in 250 mL conical flasks) were amended with the following components (g/100 mL): yeast extract 1, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.1, $MgCl_2$ 0.1, and pH was adjusted to 5.0. All flasks were incubated in an anaerobic incubator (Hirayama Manufacturing Corp., Tokyo, Japan) at 30°C for 4 d. The overall ethanol concentration was determined colormetrically according to the method of Lau and Luk (28).

Ethanol production from biotreated agro-industrial wastes: Ethanol production on different biotreated agro-industrial wastes was conducted in 250 mL Erlenmeyer flasks

containing 100 mL of filtrates taken from fungal biotreatments in submerged cultures. In solid-state cultures obtained from biotreated agro-industrial wastes, no filtrates could be recovered, therefore DI was added to flasks to build up a 100 mL volume. All flasks were amended with the following components (g/100 mL): yeast extract 1, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.1, $MgCl_2$ 0.1, and pH was adjusted to 5.0. Baker's yeast was inoculated at 2 % w/v. Ethanol was determined in flasks after 4 d of anaerobic incubation at 30°C.

Statistical analysis

Duncan`s Multiple Range Test was used to test significance of means according to IBM® SPSS® statistics software (19).

RESULTS AND DISCUSSION

Chemical composition of agro-industrial wastes

Agro-industrial wastes have to be comminuted by a combination of chipping, grinding and milling to reduce their size and cellulose crystallinity prior to any processing. The size of the materials is usually 10 – 30 mm after chipping and 0.2 – 2 mm after milling or grinding. The chemical composition of the five different agro-industrial residues investigated in this work is shown in Table 1. The highest fraction of cellulose (61.2 % w/w), hemicellulose (56.7 % w/w) and lignin (36.1 % w/w) (based on dry weight) was recorded in corn stalks, sugarcane bagasse and rice straw, respectively. The lowest content of lignin was recorded in sugar beet waste (2.5 % w/w). These results were in accordance with previous reports on analyses of different lignocellulosic materials (6, 11, 12, 36). The highest moisture content was recorded in sugar beet waste (12.4 % w/w), followed by sugarcane bagasse (8.34 % w/w), while the lowest was recorded in sawdust (1.12 % w/w). The highest percentage of total solids was recorded in rice straw and sawdust (98.62 % and 98.54 % (w/w), respectively), while, sugar beet waste recorded the lowest content (87.5 % w/w). Likewise, Patel et al. (33) found rice straw to contain 98.62 % (w/w) total solids. Furthermore, comparative information on chemical composition of different biomass materials is often based on reports from different laboratories. As can be seen (Table 1), rice straw contained the highest ash content (12.4 % w/w). Ash per se is a drawback for ethanol production, when mineral components have neutralizing ability, which could increase the pH during acid hydrolysis, and higher temperatures or longer time would be required to achieve the desired hydrolyzing effect (32). Sugar beet waste recorded the highest total sugars and nitrogen contents (0.83 % and 1.84 % (w/w), respectively). Preliminary, these results suggests that sugar beet waste will be the most suitable for ethanol production due to its high total sugars and nitrogen contents**.**

Table 1. Chemical composition of agro-industrial wastes.

Agro-industrial wastes	Chemical composition (% w/w)*								
	Moisture	Total solids	Ash	Cellulose	Hemicellulose	Lignin	Total sugars	Total carbon	Total nitrogen
Rice straw	1.83	98.62	12.4	39.2	23.5	36.1	0.071	41.8	0.457
Corn stalks	1.92	97.78	10.8	61.2	19.3	6.9	0.22	50.3	1.05
Sawdust	1.12	98.54	1.2	45.1	28.1	24.2	0.025	37.8	0.24
Sugar beet waste	12.4	87.5	4.8	26.3	18.5	2.5	0.83	44.5	1.84
Sugarcane bagasse	8.34	91.66	1.9	30.2	56.7	13.4	0.55	36.45	0.448

*(% w/w) = Percentage based on dry weight.
The values are mean of three replicates. Standard deviation was within 10 %.

Acid hydrolysis of agro-industrial wastes

The aim of these experiments was to optimize the acid hydrolysis conditions of agro-industrial wastes to obtain high

sugar yields. Among hydrolyzing acids, sulphuric acid, phosphoric acid and hydrochloric acid are the most favored for cellulose hydrolysis. Therefore, these acids at different concentrations and variable residence times were investigated. After each feedstock hydrolysis treatment, the liquid fraction was analyzed for total free sugars content. In general, sulphuric acid hydrolysis treatment was more effective than other acids hydrolysis treatments. A proportional relationship between the amount of sugars recovered after acid treatment and residence time was observed in sawdust and with less extent in rice straw (Table 2). Thus, the longer the treatment is, the more sugars are released. However, 90 min of residence time was efficient to obtain the maximum conversion percentage from the other cellulosic residues. For the hydrolysis of rice straw, sulphuric acid (1 % v/v) for 120 min achieved the maximum conversion percentage (18.2 % w/w) to total free sugars. Regardless the type of acid, corn stalks hydrolysis achieved higher levels of releasing sugars comparing with rice straw. This might be attributed to their higher levels of initial total sugars, cellulose content and lower lignin content comparing with rice straw (Table 1). Sulphuric acid (1 % v/v) achieved the highest conversion percentage (27 % w/w) from corn stalks for 90 min. Sugar beet waste hydrolysis using sulphuric acid (1 % v/v) for 90 min achieved the highest level of conversion (34.6 % w/w) among the other agro-industrial wastes used, followed by sugar beet waste (33.1 % w/w) hydrolyzed with hydrochloric acid (1 % v/v). Sugarcane bagasse hydrolysis with sulphuric acid (1 % v/v) for 90 min achieved the highest conversion percentage (30.2 % w/w). Dilute acid hydrolysis of different cellulosic materials was frequently used by many authors; Chamy et al. (9) found the best conditions for sugar beet pulp hydrolysis to be 1.1 g H_2SO_4/g sugar beet pulp, 90 min at 80°C. Under such conditions, 86.3 % and 7.8 % of cellulose and hemicellulose hydrolysis, respectively, were obtained. Yáñez et al. (49) reported a dilute acid pretreatment (180 min with 3 % w/w of H_2SO_4) of residual corrugated cardboard, rendering the pretreated waste susceptible to enzymatic hydrolysis. Mtui and Nakamura (31) applied a pre-hydrolysis stage (carried out with dilute strong acid followed by steam treatment at 120°C for 15 min) before the enzymatic hydrolysis achieved a glucose concentration of 0.13 and 0.05 g/L (corresponding to 1 g of pretreated lignocellulosic material). Li et al. (29) examined a pre-hydrolysis treatment consisted of dilute acid (H_2SO_4, HNO_3 or HCl, 1 and 4 %, 180 min, 60°C) before the enzymatic hydrolysis with cellulases from *T. reesei* and *T. viride*. The highest glucose yield (73 %) was obtained with 1 % H_2SO_4 followed by steam treatment at 121°C, and enzymatic hydrolysis with *T. viride*.

Table 2. Effect of using different acids (sulphuric, phosphoric and hydrochloric at the concentration of 1 % v/v) and variable residence times (15, 30, 60, 90, and 120 min) at 120°C on the conversion percentage (% w/w)* of agro-industrial wastes to total sugars.

Agro-industrial wastes	Hydrolysis treatments					
	Acids used for hydrolysis (1 % v/v)	Residence time (min)				
		15	30	60	90	120
Rice straw	H_3PO_4	3.6^{F}	4^{DEF}	5.2^{BCD}	6.3^{AB}	9.2^{xy}
	HCl	9^{xy}	10.4^{w}	11^{vw}	9.1^{xy}	12^{uv}
	H_2SO_4	15^{qr}	14.2^{rs}	17^{nop}	17.3^{mno}	18.2^{klmn}
Corn stalks	H_3PO_4	3^{F}	9^{xy}	15^{qr}	18^{lmn}	15.1^{qr}
	HCl	12^{uv}	15.2^{qr}	22^{j}	24^{i}	21^{j}
	H_2SO_4	14^{rs}	18^{lmn}	25.1^{h}	27^{g}	18.3^{klm}
Sawdust	H_3PO_4	3.1^{F}	3^{F}	4^{DEF}	4.8^{CDE}	5.4^{ABC}
	HCl	5.1^{CD}	5.4^{ABC}	6.5^{A}	7.6^{z}	7.7^{z}
	H_2SO_4	8.3^{yz}	10.5^{w}	13.4^{st}	15.1^{qr}	16^{pq}
Sugar beet waste	H_3PO_4	5.6^{ABC}	12.6^{tu}	18^{lmn}	21.3^{j}	18.7^{kl}
	HCl	15.2^{qr}	19.3^{k}	30.3^{d}	33.1^{b}	31.5^{c}
	H_2SO_4	18.5^{klm}	24.6^{hi}	31.5^{c}	34.6^{a}	32.1^{bc}
Sugarcane baggase	H_3PO_4	4.9^{CDE}	10.1^{wx}	14.3^{rs}	18.3^{klm}	17.5^{lmn}
	HCl	12.5^{tu}	16.2^{opq}	24.6^{hi}	28.6^{e}	26.5^{g}

	H_2SO_4	17^{nop}	21.3^{j}	27.5^{fg}	30.2^{d}	28.5^{ef}

(% w/w)* = Percentage based on dry weight.
The values are mean of three replicates. Standard deviation was within 10 %.
Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5 % level.

Increasing hydrolyzing acids concentration up to 5 % v/v (Fig. 1) for treated rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, did not enhance the sugars availability in liquid fraction comparing with the results obtained by the dilute acids hydrolysis of these feedstocks (1 % v/v, Table 2). On the contrary, increasing phosphoric acid concentration to 5 % v/v increased the conversion percentage of sawdust to total sugars (24 % w/w) by 50 % as compared with that obtained using 1 % v/v sulphuric acid (16 % w/w, Table 2). The highest conversion percentage was obtained from sugar beet waste (27 % w/w) hydrolyzed with sulphuric acid followed by sawdust (24 % w/w), sugar beet waste (24.2 % w/w) and sugarcane bagasse (24 % w/w) hydrolyzed with phosphoric acid, hydrochloric acid and phosphoric acid, respectively. Based on the observed data, hydrolysis with 1 % acid was more efficient than hydrolysis with 5 % acid to obtain high conversion percentages. In a comparable study, Yu and Zhang (50) hydrolyzed a cellulosic cotton waste with 0.2 mol/l H_2SO_4 at 121°C for 20 min and a maximum glucose yield of 17.35 % was obtained. Romero et al. (38) reported on the fermentation of hydrolysates obtained from olive tree pruning residues pretreated with different sulphuric acid concentrations (0.5 – 4 N) at 90°C for 240 min. The maximum ethanol yield (0.38 g/g) was reached with the hydrolyzate obtained with 0.75 N sulphuric acid. Higher acid concentrations allowed total hydrolysis of the hemicellulose, but the ethanol yields resulting from the fermentation were lower. Furfural and hydroxymethylfurfural (HMF) are the most important inhibitors during fermentation of dilute-acid hydrolyzates, therefore lower amounts of these substances are desirable in the hydrolyzates. As observed from our present study, the negative effect of either longer residence time of single-stage dilute acids treatment (Table 2) or increasing acids concentrations (Fig. 1), to some agro-industrial wastes on total free sugars availability, may be attributed to the degradation of the released sugars and the increased concentrations of furfural and HMF in the hydrolyzates that might occur. Such an observation was evidenced by the HPLC analysis of feedstocks hydrolyzates. A gradual increase was observed in furfural and HMF concentrations by increasing the residence time of single-stage dilute acids hydrolysis of different feedstocks. After 120 min of residence time of sulfuric acid hydrolysis, the highest concentrations of furfural were obtained (0.04 %, 0.052 %, 0.065 %, 0.081 % and 0.07 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively), while HMF concentrations were 0.01 %, 0.021 %, 0.041 %, 0.022 % and 0.03 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively. Increasing the hydrolyzing acids concentration up to 5 % v/v, exhibited a proportional increase in furfural and HMF concentrations in hydrolyzates of different feedstocks. In this treatment, furfural concentrations were 0.064 %, 0.072 %, 0.09 %, 0.12 % and 0.1 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively, while HMF concentrations were 0.022 %, 0.033 %, 0.055 %, 0.03 % and 0.042 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively. The observation of increased concentrations of furfural and HMF in the hydrolyzates due to elevated hydrolyzing acids concentrations and increased residence time was evidenced in previous related work (10, 16).

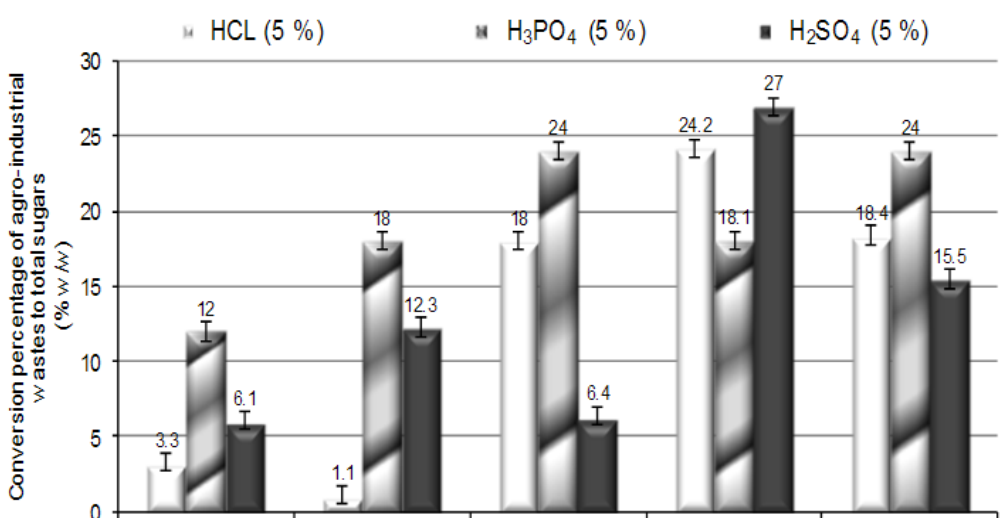

Figure 1. Hydrolysis effect of different acids (hydrochloric, phosphoric and sulphuric at the concentration of 5 % v/v) and 90 min of residence time on the conversion percentage of various agro-industrial wastes to total sugars.

Two-stage dilute acids hydrolysis of agro-industrial wastes were tested using phosphoric acid (1 % v/v), hydrochloric acid (1 % v/v) and sulphuric acid (1 % v/v) in different combinations (Fig. 2). This treatment was thought to improve the yield of total sugars and to reduce the formation of furfural and HMF. The two-stage hydrolysis of sugar beet waste by phosphoric acid followed by sulphuric acid, resulted in the maximum conversion percentage 41.3 % (w/w), which represents 1.19 folds of the maximum conversion percentage obtained from the single-stage dilute acid hydrolysis of sugar beet waste using sulphuric acid (34.6 % w/w, Table 2). Similarly, the two-stage hydrolysis of corn stalks by phosphoric acid followed by hydrochloric acid, resulted in a conversion percentage 34.4 % (w/w), which represents 1.27 folds of the maximum conversion percentage obtained from the single-stage acid hydrolysis of corn stalks using sulphuric acid (27 % w/w, Table 2). Likewise, the two-stage hydrolysis of rice straw and sugarcane bagasse using either of phosphoric acid followed by hydrochloric acid or phosphoric acid followed by sulphuric acid achieved higher conversion percentages (30 %, 37.7 % w/w, respectively). These figures represent 1.65, 1.25 folds of the maximum conversion percentage obtained by the single-stage acid hydrolysis of rice straw or sugarcane bagasse, respectively, using sulphuric acid. The two-stage dilute acid hydrolysis of all agro-industrial wastes increased the conversion percentage of these materials to total sugars as compared with single-stage hydrolysis using dilute acids. However, this increase was varied greatly among the agro-industrial wastes used, which may be attributed to the diversity in the chemical properties of each lignocellulosic material. Estimations of furfural in the most efficient treatment of two-stage dilute acids hydrolysis mentioned above were 0.051 %, 0.058 %, 0.072 %, 0.091 % and 0.083 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively, while HMF concentrations in the same treatments were 0.016 %, 0.028 %, 0.05 %, 0.03 % and 0.038 % w/v for rice straw, corn stalks, sawdust, sugar beet waste and sugarcane bagasse, respectively. Moreover, the two-stage dilute acid hydrolysis exhibited lower furfural and HMF concentrations comparing with those obtained in single-stage elevated acids hydrolysis (5 % v/v) and slightly higher concentrations than those obtained in single-stage dilute acids hydrolysis (1 % v/v). The two-stage dilute acid hydrolysis not only attained moderate furfural and HMF concentrations, but also helped the bioethanol producing organism to tolerate these inhibitors in ethanol production stage. Karimi et al. (23) used high pressure two-stage dilute acid hydrolysis (1.0 % H_2SO_4 in the first stage and 0.5 % in the second) to obtain high conversion of 189 g xylose and 29 g glucose per kg and considerable amounts of furfural and HMF of the rice straw used. Kumar et al. (26) hydrolyzed sugarcane bagasse by sulphuric acid treatment in two-stages. In the first stage, the sugarcane bagasse was soaked into 2 – 10 % (w/w) sulphuric acid with a solid-to-liquid ratio of 1:10 at 100°C for 1 h. In the second stage, 18 – 65 % (w/w) sulphuric acid was added to residual bagasse taken from first stage hydrolysis. The overall ethanol yield on the basis of total fermentable sugars present in hydrolyzate was 35 % with a negligible furfural concentration. Saha et al. (39, 40) accomplished a two-stage dilute acid pretreatment to rice hulls and wheat straw. A first stage was carried out at 140°C during 15 min, followed by a second stage at 190°C for 10 min. These authors pointed out that dilute-acid pretreatment at low temperatures (121°C) helped avoiding the degradation of sugars to furfural and HMF.

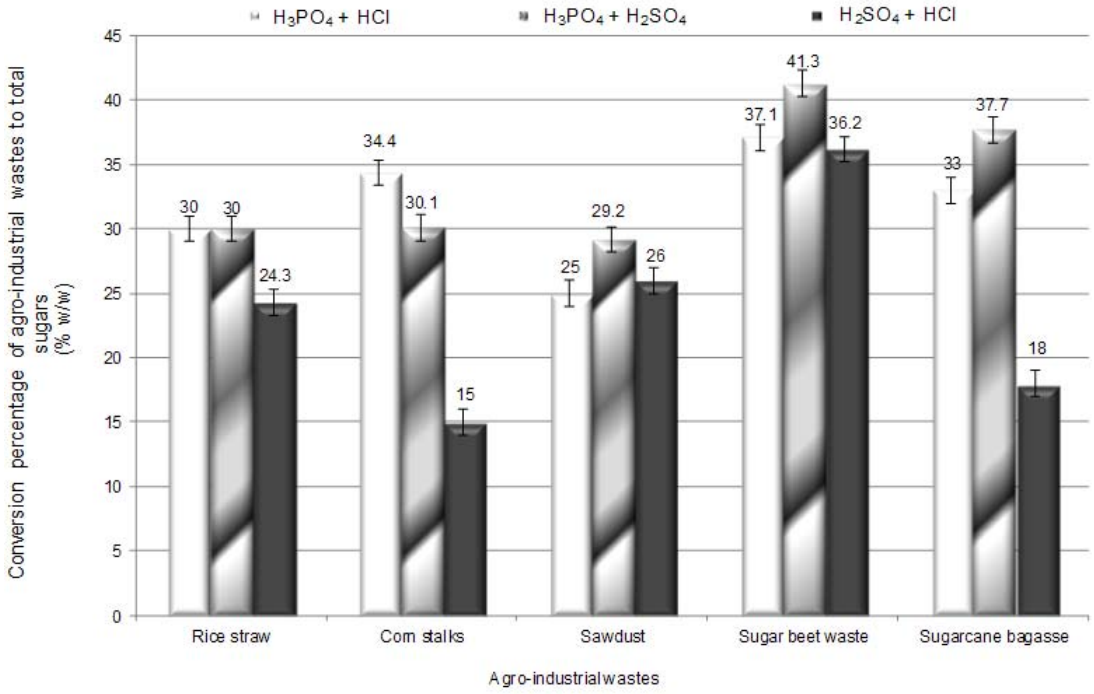

Figure 2. Effect of two-stage dilute acid hydrolysis (1 % v/v) by sulphuric acid, phosphoric acid and hydrochloric acid in different combinations and 90 min residence time on the conversion percentage of agro-industrial wastes to total sugars.

Ethanol production on acid hydrolyzed agro-industrial wastes

Different ethanol producing organisms were tested for ethanol production on 5 % w/v of each agro-industrial waste hydrolyzed with the most efficient treatment (two-stage dilute acids hydrolysis with 1 % v/v phosphoric acid followed by 1 % v/v sulphuric acid for 90 min at 120°C) (Table 3). Active dry yeast and baker's yeast achieved the highest concentrations of ethanol on all hydrolyzed agro-industrial wastes. The highest record was achieved on sugar beet waste (1.0 % v/v), followed by *Z. mobilis* EMCC 1543 and *Sacch. cerevisiae* ATCC 9763 on sugar beet waste (0.8 % v/v). In case of rice straw, active dry yeast, baker's yeast and *Z. mobilis* EMCC 1543 recorded the highest ethanol concentration (0.5 % v/v). The lowest concentrations of ethanol were obtained using *K. marxianus* EMCC 11 and *K. marxianus* EMCC 12 on all tested residues.

Table 3. Ethanol production (% v/v) on two-stage* acid hydrolyzed agro-industrial wastes using different organisms.

Agro-industrial wastes (5 % w/v)	Ethanol producing organisms								
	***K. marxianus* EMCC 11**	***K. marxianus* EMCC 12**	***C. utilis* EMCC 41**	***Sacch. cerevisiae* ATCC 9763**	***Sacch. cerevisiae* ATCC 7754**	***Sacch. cerevisiae* DSM 70487**	**Active dry yeast**	**Baker's yeast**	***Z. mobilis* EMCC 1543**
Rice straw	0.01^{l}	0.02^{kl}	0.08^{jkl}	0.09^{jkl}	0.06^{jkl}	0.2^{i}	0.5^{e}	0.5^{e}	0.5^{e}
Corn stalks	0.01^{l}	0.01^{l}	0.13^{ij}	0.12^{ij}	0.11^{ijk}	0.12^{ij}	0.61^{c}	0.6^{cd}	0.52^{de}
Sawdust	0.01^{l}	0.01^{l}	0.02^{k}l	0.02^{kl}	0.04^{jkl}	0.04^{jkl}	0.12^{ij}	0.11^{ijk}	0.05^{jkl}
Sugar beet waste	0.3^{h}	0.2^{i}	0.5^{e}	0.8^{b}	0.41^{fg}	0.6^{cd}	1.0^{a}	1.0^{a}	0.8^{b}

Sugarcane baggase	0.11^{ij}	0.12^{ij}	0.3^{h}	0.66^{c}	0.34^{gh}	0.45^{ef}	0.82^{b}	0.8^{b}	0.67^{c}

* Hydrolyzed in the first-stage by phosphoric acid 1 % v/v and in the second-stage by sulphuric acid 1 % v/v for 90 min at 120°C.
The values are mean of three replicates. Standard deviation was within 10 %.
Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5 % level.

Abbi *et al*. (1) studied the fermentation of rice straw hydrolyzate to ethanol by *Candida shehatae*. They reported ethanol yields of 0.37 – 0.50 g/g substrate utilized by free and immobilized *C. shehatae*, while different types of acid-hydrolysis of the straw were used. In another attempt, Krishnan et al. (25) tried to ferment rice straw hydrolyzates with a recombinant strain of *Z. mobilis*, which resulted in 0.46 g/g of ethanol per sugars utilized. On a chemically hydrolyzed cellulosic cotton waste, Yu and Zhang (50) produced ethanol by *Sacch. cerevisiae*, *Pichia* sp. YZ–1 and *Z. mobilis* and obtained a maximal ethanol yield of 0.45 g/g glucose by *Sacch. cerevisiae*. Tang et al. (45) used acid hydrolyzate of wood biomass for ethanol production in a continuous process using *Sacch. cerevisiae* strain KF-7 and observed a high ethanol productivity of over 20 g/L/h. Karimi et al. (23) obtained a better performance in ethanol production (using *P. stipitis* than *Mucar indicus*) at identical conditions with ethanol yield 0.38 g/g of the sugars within a dilute acid hydrolyzed rice straw**.**

Ethanol production on biotreated agro-industrial wastes

The degradation of agro-industrial wastes using different fungi was examined as a cheap and useful tool for converting cellulosic materials to free sugars for ethanol production. *Aspergillus niger* EMCC 72 and *Trichoderma viride* EMCC 107 were used individually or in mixed culture for treating the feedstocks in either submerged or solid-state cultures. Ethanol production using baker's yeast was conducted after the biotreatment is completed. In general, higher conversion percentages to total sugars were obtained from fungal growth on different agro-industrial wastes in submerged cultures than in solid-state cultures (Table 4). The propagation of *T. viride* EMCC 107 on rice straw in submerged culture or on corn stalks in solid-state culture recorded the highest conversion percentage (25.2 % w/w). Neither the single culture treatment using *A. niger* EMCC 72 nor the mixed culture treatment enhanced the sugars release comparing with *T. viride* EMCC 107 treatment. The highest conversion percentage was obtained by biotreatment of sugar beet waste with *T. viride* EMCC 107 in submerged culture (37.8 % w/w) followed by the mixed culture treatment of sugar beet waste in submerged culture (35.2 % w/w). The highest ethanol concentration was obtained on either sugar beet waste or sugarcane bagasse (0.41 % and 0.4 % v/v, respectively). Similar work was done by many authors; Zayed and Meyer (52) obtained an ethanol yield of 1.18 % by using *T. viride* and the yeast *Pachysolen tannophilus* for the biotreatment of wheat straw and ethanol production, respectively. Taniguchi et al. (46) found that 33 % of cellulose in wheat straw was converted to glucose after a 60-d pretreatment with *P. ostreatus*. Zhang et al. (53) observed a maximum saccharification yield of 37 % (w/w) from *Coriolus versicolor* pretreated bamboo residues. Manjunath and Geeta (30) pretreated sugarcane bagasse, paddy straw and wheat straw with the fungi *Phanerochaete chrysosporium* and *Pleurotus* spp. The filtrate fermentation of these materials showed maximum production of ethanol (0.08 % w/w) and *Z. mobilis* was efficient (comparing with *Sacch. cerevisiae* (CFTRI 101), *Candida shehatae* (NCIM 3500), *P. tannophilus* (NCIM 3445) and *P. stipitis*) in conversion to ethanol from all the substrates. Among the substrates, bagasse recorded maximum release of ethanol compared to paddy straw and wheat straw. Wan and Li (48) pretreated corn stover with the fungus *Ceriporiopsis subvermispora* for 18 d at 28°C with 75 % moisture content. The overall glucose yield of 57.67 % was obtained and the highest overall ethanol yield of 57.80 % was obtained with 35 d pretreated corn stover**.**

By studying the best results of either acids hydrolysis treatments or biotreatment of different agro-industrial wastes conducted through this investigation it could be concluded that

on all agro-industrial wastes, the two-stage dilute acid hydrolysis achieved higher conversion percentages to total sugars and subsequently higher ethanol concentrations as compared with the biotreatment using *T. viride* EMCC 107. The two-stage hydrolysis of sugar beet waste by phosphoric acid followed by hydrochloric acid or phosphoric acid followed by sulphuric acid resulted in the highest conversion percentage, ethanol concentration and conversion coefficient (41.3 % w/w, 1.0 % v/v and 20 % v/w, respectively). In spite of the reduction in conversion percentages of agro-industrial wastes to total sugar by biotreatment, comparing with acid hydrolysis treatment, which led to a decrease in ethanol concentration, this may be a useful tool for lowering the production costs of ethanol from lignocellulosic feedstocks.

Table 4. Effect of biotreatment of agro-industrial wastes using single or mixed fungi on the conversion percentage to total sugars and the equivalent ethanol production by baker's yeast.

Fungi used for biotreatment	Agro-industrial wastes (5 % w/v)	Conversion percentage to total sugars (% w/w)		Ethanol concentration (% v/v)	
		Submerged culture	Solid-state culture	Submerged culture	Solid-state culture
Aspergillus niger EMCC 72	Rice straw	12^{jk}	15^{i}	0.1^{e}	0.1^{e}
	Corn stalks	15.1^{i}	12.5^{ij}	0.1^{e}	0.11^{e}
	Sawdust	8.1^{lm}	7.6^{lm}	0.02^{f}	0.01^{f}
	Sugar beet waste	30.3^{c}	22.2^{efg}	0.4^{a}	0.22^{cd}
	Sugarcane bagasse	28^{c}	24^{def}	0.31^{b}	0.2^{d}
Trichoderma viride EMCC 107	Rice straw	25.2^{d}	15.4^{i}	0.2^{d}	0.1^{e}
	Corn stalks	15^{i}	25.2^{d}	0.1^{e}	0.21^{cd}
	Sawdust	9.2^{kl}	8.6^{lm}	0.05^{f}	0.03^{f}
	Sugar beet waste	37.8^{a}	25^{de}	0.41^{a}	0.22^{cd}
	Sugarcane bagasse	34.4^{b}	22^{fg}	0.4^{a}	0.2^{d}
Aspergillus niger EMCC 72 + *Trichoderma viride* EMCC 107	Rice straw	15^{i}	6^{m}	0.12^{e}	0.11^{e}
	Corn stalks	18^{h}	15^{i}	0.21^{cd}	0.12^{e}
	Sawdust	8.3^{lm}	6.7^{lm}	0.03^{f}	0.01^{f}
	Sugar beet waste	35.2^{b}	22.6^{defg}	0.4^{a}	0.24^{cd}
	Sugarcane bagasse	30.2^{c}	20.4^{gh}	0.32^{b}	0.25^{c}

(% w/w) = Percentage based on dry weight.
Zero ethanol was detected in control flasks without fungal inoculation.
The values are mean of three replicates. Standard deviation was within 10 %.
Values in the same column followed by the same letter(s) do not significantly differ from each other according to Duncan's at 5 % level.

CONCLUSION

This work investigated the production of ethanol on either acid hydrolyzed or biotreated agro-industrial wastes. The five kinds of agro-industrial wastes analyzed in this paper correspond to resources that are present in almost all countries, relatively inexpensive and are expected to lower the cost of producing ethanol as well as providing stability to supply. Techno-economic studies are required to determine the feasibility of utilizing these agro-industrial wastes for ethanol production. In general, sulphuric acid hydrolysis was more effective than other acid hydrolysis treatments. The formation of furfural and HMF gradually increases when the acids concentration or hydrolysis residence time are increased, regardless of the type of feedstock. The two-stage dilute acid hydrolysis by phosphoric acid followed by sulphuric acid increased the conversion percentage of all agro-industrial wastes to total sugars and reduced the formation of furfural and HMF comparing with single-stage acid hydrolysis. The fungal biotreatment of agro-industrial wastes as an alternative to the acid hydrolysis attained less released sugars; nevertheless it could be a promising choice on the economic basis.

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