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Evaluation of hardboard manufacturing process wastewater as a feedstream for ethanol production

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Abstract Waste streams from the wood processing industry can serve as feedstream for ethanol production from biomass residues. Hardboard manufacturing process wastewater (HPW) was evaluated on the basis of monomeric sugar recovery and fermentability as a novel feedstream for ethanol production. Dilute acid hydrolysis, coupled with concentration of the wastewater resulted in a hydrolysate with 66 g/l total fermentable sugars. As xylose accounted for 53 % of the total sugars, native xylose-fermenting yeasts were evaluated for their ability to produce ethanol from the hydrolysate. The strains selected were, in decreasing order by ethanol yields from xylose ($Y_{p/s}$, based on consumed sugars), *Scheffersomyces stipitis* ATCC 58785 (CBS 6054), *Pachysolen tannophilus* ATCC 60393, and *Kluyveromyces marxianus* ATCC 46537. The yeasts were compared on the basis of substrate utilization and ethanol yield during fermentations of the hydrolysate, measured using an HPLC. *S. stipitis*, *P. tannophilus*, and *K. marxianus* produced 0.34, 0.31, and 0.36 g/g, respectively. The yeasts were able to utilize between 58 and 75 % of the available substrate. *S. stipitis* outperformed the other yeast during the fermentation of the hydrolysate; consuming the highest concentration of available substrate and producing the highest ethanol concentration in 72 h. Due to its high sugar content and low inhibitor levels after hydrolysis, it was concluded that HPW is a suitable feedstream for ethanol production by *S. stipitis*.

Keywords Hemicellulose · Hardboard process water · Xylose · Ethanol · Dilute acid hydrolysis

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Introduction

Hardboard is a lumber product made through the deconstruction of wood and rearrangement of the resulting fibers into panels [15]. The process is achieved by steaming and then grinding woodchips into softened fibers, and then using high pressure (several 1,000 tons/panel) and temperatures (>175 °C), the fibers are pressed into board [15]. Removal of water from the wood itself and that used in processing is essential to the panel-making process [15]. Wastewater produced as a result of hardboard manufacturing must undergo traditional wastewater treatment prior to being released into the environment. However, waste streams of this type from the forest product industry can be rich in woody biomass and other organic materials [2, 40]. As a result, a potentially attractive alternative to wastewater treatment is to integrate these wastes into feedstreams for liquid biofuels production [43]. In addition to avoiding costly wastewater treatment, this process fills a secondary need of identifying alternative sources for biomass feedstreams. However, varying compositions and physio-chemical properties from one another and from traditional sources of biomass pose a challenge [40]. Just as the composition and characteristics of the hardboard can vary based on the type of manufacturing process used, starting material, and additives, so can the wastewater [3].

The process water produced as a result of hardboard manufacture contains dissolved hemicelluloses that can be hydrolyzed into 5-carbon (xylose and arabinose) and 6-carbon (glucose, galactose, and mannose) [33]. Hemicellulose may be converted directly to monomeric sugars through dilute acid hydrolysis. This method employs the use of high temperatures (up to 200 °C) and low concentrations of acid ($\sim$1 % wt.) to solubilize the hemicellulose sugars [43]. Xylose, the most abundant of the sugars in hardwood hemicellulose, can be converted to ethanol by wild-type *Scheffersomyces stipitis*, *Pachysolen tannophilus*, and *Kluyveromyces marxianus* [13, 19, 28]. Dilute acid hydrolysis of hemicellulose also results in the production of potentially inhibitory compounds for yeast [2]. The presence and concentration of inhibitory compounds affect the fermentability of hydrolysates [40].

Evaluating hardboard manufacturing process wastewater (HPW) as a feedstream for ethanol production is the first step in determining the feasibility of integrating this material into a feedstream for commercial ethanol production and/or value-added products [5]. The purpose of this research is to determine the composition and fermentability of a dilute acid pretreated hydrolysate from HPW. A comparison of xylose-fermenting yeast provides insight into potential products yields and toxicity of using this material.

Methods and materials

Cultures

Scheffersomyces (*Pichia*) *sti*pitis ATCC 58785 (CBS 6054); *P. tannophilus* ATCC 60393; and *K. marxianus* ATCC 46537 were used in this study. All but *S. stipitis* were obtained from the American type culture collection (ATCC); *S. stipitis* CBS 6054 was obtained from Dr. Thomas Jeffries, USDA Forest Products Laboratory Madison, WI.

Media and fermentation conditions

All yeast cultures were maintained on yeast extract, peptone, and xylose (YPX) agar plates (yeast extract 10 g/l, peptone 20 g/l, and xylose 20 g/l) [16]. All inocula were prepared in 125-ml Erlenmeyer flasks with 25 ml of YPX broth (yeast extract 10 g/l, peptone 20 g/l, and xylose 50 g/l). All fermentations were carried out in 125-ml Erlenmeyer flasks with a 25-ml working volume at 30 °C on a gyratory shaker at 160 rpm unless otherwise specified. The cultures were incubated for 72 h prior to all fermentations in order to avoid diauxic lag [39]. The media used during the fermentation were YPX broth, hydrolysate, and a synthetic hydrolysate. The synthetic hydrolysate (SH) contained the five sugars present in the hydrolysate at their respective concentrations and supplemented with YP (yeast extract 10 g/l and peptone 20 g/l). A 10 % (v/v) inoculum was used and fermentations were carried out over a 72-h time period. The flasks were sealed using aluminum foil and parafilm. Samples were taken at 24-h intervals for sugar and product analysis.

Feedstream and dilute-acid hydrolysate

The starting feedstream, process wastewater from a hardboard manufacturing plant, was received after dilute sulfuric acid hydrolysis (1 % w/v) and concentration by a third party. Information on the exact hydrolysis and concentration methods is limited to acid concentration (1 % w/v), hydrolysis time (60 min), and concentration technique (evaporation). The composition of the hydrolysate (HPW) was determined by HPLC (see below) prior to fermentation (Table 1).The hydrolysates were neutralized to a pH of 5.5 using calcium oxide (CaO). The media were pre-filtered using a Whatman 1 filter and a 0.5-µm glass fiber filter. The hydrolysate was supplemented with YP (peptone, 20 g/l; and yeast extract, 10 g/l). It was then filter-sterilized using a 0.2-µm nylon filter prior to the fermentation.

Analysis of fermentation

High-performance liquid chromatography (HPLC) was used to determine ethanol, carbohydrate, and organic acid levels in all samples following the methods described by Cho et al. [9]. Agilent (Santa Clara, CA, USA) HPLC (1100) with a Bio-Rad (Hercules, CA, USA) Aminex HPX-87H column and a Bio-Rad cation H+ guard column was

Table 1 Composition of the hardboard manufacturing process waste water (HPW) effluent and dilute-acid hydrolysate as determined by HPLC analysis

Compound	Effluent[a] (g/l)	Hydrolysate[a] (g/l)	Fermentation media[a,b] (g/l)
Glucose	0.22 ± 0.00	15.06 ± 0.06	13.70 ± 0.05
XGM[c]	0.29 ± 0.02	54.94 ± 0.08	51.55 ± 0.10
Arabinose	0.23 ± 0.01	8.16 ± 0.67	7.64 ± 0.03
Acetic acid	0.57 ± 0.42	4.75 ± 0.01	4.48 ± 0.02
5-HMF	0.03 ± 0.03	0.50 ± 0.08	0.47 ± 0.15
Furfural	<MDL[d]	0.46 ± 0.11	0.68 ± 0.06
Total sugars	0.74 ± 0.21	78.15 ± 0.60	72.88 ± 0.17

[a] Mean ($n = 3$) ±2 SD

[b] Hydrolysate supplemented with yeast extract and peptone

[c] XGM—Xylose (76.8 %), galactose (13.7 %), and mannose (6.8 %)

[d] Below minimal detectable limits

used for the analysis of sugars (xylose, glucose, galactose, mannose, and arabinose) as well as ethanol. Xylose, galactose, and mannose co-elute on the column and are referred to as XGM. HPW hydrolysate degradation products, Acetic acid, furfural, and 5-hydroxymethylfurfural (5-HMF), were also measured using this method. Individual sugar concentrations for the effluent and the hydrolysate were measured using Bio-Rad Aminex HPX-87P column using the methods described by Jensen et al. [20]. Ethanol yields were calculated on the basis of gram of ethanol produced per gram of substrate consumed (Y g_p/g_s) [46]. All percent theoretical yields were calculated from ethanol yields based on consumed sugars [37].

Results and discussion

Effluent and hydrolysate composition

Wastewater from the forest products industry has the potential to be a valuable feedstream for commercial ethanol production. In this study, process wastewater from hardboard manufacturing was evaluated as a novel feedstream for ethanol production. The effluent used in this study was produced as a result of the manufacture of hardboard made from 100 % hardwood. Cellulose and hemicellulose account for 58–89 % (w/w) of hardwoods' overall composition [17]. Hemicellulose represents a 33–40 % of the available fermentable sugars. Based on the general hardboard manufacturing process, it was assumed that all of the material converted to fermentable sugars after dilute acid hydrolysis of the HPW was from hemicellulose [43]. The effluent used in this investigation contained a total of 5.0 g/l soluble monosaccharide sugars prior to pretreatment. Dilute sulfuric acid pretreatment (1 % w/v) resulted in the production of a hydrolysate rich in fermentable sugars (Table 1). Xylose made up 53 % of the total sugars followed by glucose, at 20 %. As expected, sugar loss was observed during neutralization with CaO but was not considered significant (Table 1) [38]. The sugar yield results were similar to other reported hemicellulose hydrolysates from hardwoods and hardwood extracts [19, 31].

In addition to the sugars, several organic acids and other organic compounds were detected in the HPW and the hydrolysate (Table 1). These compounds can potentially negatively affect growth, substrate utilization, and ethanol production [4, 12, 27, 32]. In this study, acetic acid, furfural, and 5-HMF where quantified due to their status as common by-products of dilute acid hydrolysis [21, 38]. All three compounds are known to inhibit both biomass and ethanol production [6, 38]. Acetic acid is released from the acetyl groups present on the hemicellulose, and furfural as well as 5-HMF is produced as a result of xylose and glucose degradation, respectively [2]. Although dilute acid hydrolysis of the HPW resulted in an increased amount of inhibitors (acetic acid, 4.5 g/l; furfural, 0.7 g/l; and 5-HMF, 0.5), their concentrations in the hydrolysate were low compared to previously reported levels in hardwood hydrolysates [23, 26, 42]. The effect of these compounds is dependent on many factors, ranging from their concentration to the fermentation conditions and type of organism [6, 22]. During the course of this study, furfural and 5-HMF were completely absent from the media after the first 24 h and acetic acid concentrations did not change throughout the fermentation (data not shown). The disappearance of furfural and 5-HMF in the fermentation media may be a result of the yeasts metabolism [26, 27]. As a result, it was assumed that based on the initial concentrations of acetic acid, furfural, and 5-HMF in the hydrolysate that they would impose little to no inhibitory effect on the yeast [2, 12, 29, 34, 38]. The presence of additional inhibitors produced as a result of dilute acid hydrolysis (other organic acids, aldehydes, and phenolic compounds) was not quantified during this initial study. Based on the HPLC analysis of sugars and main inhibitors, dilute sulfuric acid hydrolysis (1 % w/v) of the effluent resulted in a high total sugar yield and low inhibitor levels, allowing HPW hydrolysate to meet the standards for a promising hemicellulose feedstream [14].

Baseline fermentation of YPX and SH

The ability of *S. stipitis*, *P. tannophilus*, and *K. marxianus* to ferment xylose (YPX) and the mixed sugars present in the synthetic hydrolysate (SH) (which contained only the five sugars present in the hydrolysate at their respective concentrations) was evaluated in liquid batch fermentations. YPX and SH were used to establish baseline performance of each strain. As suggested by Nigam [31], substrate utilization, ethanol concentration, and ethanol yield were used as the fermentation parameters to compare the yeast (Table 2). All inocula were grown on xylose prior to fermentation. This method has been shown to induce the activity of xylose reductase (XR) and xylitol dehydrogenase (XDH) in *S. stipitis* and *P. tannophilus*, thus decreasing fermentation times, substrate inhibition, and diauxic lag [39].

With xylose as the sole carbon source, all the yeasts were able to produce ethanol under microaerophilic batch fermentation conditions. *S. stipitis* and *P. tannophilus* were able to utilize all of the detectable xylose within 72 h. *S. stipitis* produced the most ethanol from xylose. The results of this study are well within the reported ranges for each of these yeasts. When compared to the other yeast, *K. marxianus* will not perform as well during the fermentation

Table 2 Fermentation results for three xylose-fermenting yeasts on xylose (YPX), synthetic hydrolysate (SH), and HPW hydrolysate

Yeast strain	Substrate utilization (%)			Max. ethanol conc. (g/l)			Ethanol yield (g/g)		
	YPX	SH	HPW	YPX	SH	HPW	YPX	SH	HPW
S. stipitis	100.0 ± 0.00	85.0 ± 0.00	75.0 ± 0.01	15.2 ± 0.34	20.4 ± 0.62	18.8 ± 0.04	0.42 ± 0.03	0.37 ± 0.01	0.34 ± 0.01
P. tannophilus	100.0 ± 0.00	69.8 ± 0.03	68.7 ± 0.00	12.4 ± 2.69	14.9 ± 0.45	15.9 ± 0.82	0.27 ± 0.06	0.33 ± 0.02	0.31 ± 0.02
K. marxianus	60.9 ± 0.01	52.1 ± 0.03	58.4 ± 0.00	2.4 ± 0.12	12.1 ± 1.4	10.0 ± 0.17	0.11 ± 0.01	0.24 ± 0.01	0.36 ± 0.01

Fermentations were carried out over 72 h (30 °C, pH 5.5). Mean ($n = 3$) ±2 SD

of xylose [11]. *K. marxianus* utilized only 61 % of the available substrate. The rate of xylose consumption was slower compared to the other yeasts [11, 36]. The incomplete substrate utilization observed in *K. marxianus* may be a result of the microaerophilic conditions used in this study. *K. marxianus* has a greater affinity for xylose under aerobic conditions compared to microaerophilic; this however, does not result in an increase in ethanol production [41].

During the fermentation of SH all the yeasts consumed the sugars sequentially. Glucose was completely consumed within the first 24 h. The other sugars were consumed after glucose at decreased rates, with the exception of arabinose for *S. stipitis* and *P. tannophilus*. Arabinose can be utilized by *S. stipitis* and *P. tannophilus* for biomass production but is not converted to ethanol [28, 39]. During the 72-h fermentation, arabinose was not utilized nor transported by *S. stipitis* or *P. tannophilus*. *K. marxianus* utilized some of the available arabinose. *K. marxianus* has a high affinity transport system for arabinose and is able to use the sugar simultaneously with xylose [24]. Glucose and mannose will be used simultaneously followed by galactose utilization in *K. marxianus* [36]. Xylose utilization is not observed until the available hexose sugars have been exhausted [36]. The presence of hexose sugars also had implications for the other yeasts as well. Maximum ethanol concentrations for *S. stipitis* and *P. tannophilus* were observed at 72 h (Fig. 1). *K. marxianus* reached maximum ethanol yields within the first 24 h and quickly diminished after the disappearance of glucose (Fig. 1) [35]. Fermentation of the SH resulted in higher total ethanol concentrations for all of the yeast compared to xylose fermentations.

The fermentation profiles for these yeasts for single substrate and mixed substrate fermentations were in agreement with previous studies of these organisms under these conditions. Based on that fact, the YPX and SH fermentation results provided a baseline for each of the yeasts to be evaluated against during fermentation of the hydrolysate.This information also establishes the potential ethanol yield for each of the yeasts in the absence of any known or unknown inhibitors present in the HPW hydrolysate.

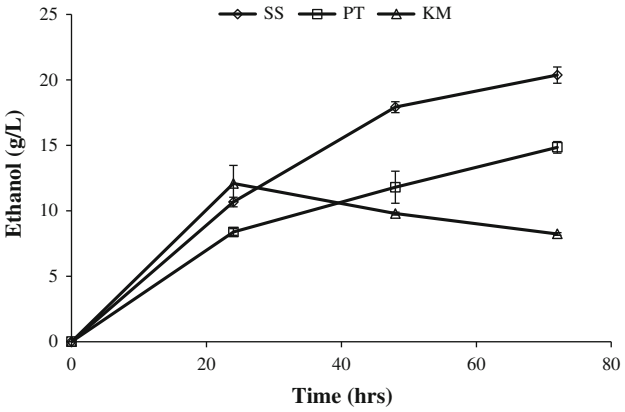

Fig. 1 Ethanol production by *S. stipitis* CBS 6054 (*SS*), P. *tannophilus* ATCC 60393 (*PT*), and *K. marxianus* ATCC 46537 (*KM*) during the fermentation of synthetic HPW hydrolysate. Fermentations were carried out over 72 h (30 °C, pH 5.5). Mean ($n = 3$) ±2 SD

Fermentation of HPW hydrolysate

Results for the fermentation of the hydrolysate by the native xylose-fermenting yeasts have a profile similar to that observed with the synthetic hydrolysate (Fig. 2a–c). *S. stipitis*, *P. tannophilus*, and *K. marxianus* all utilized a percentage of the available sugars and produced ethanol. Similar results have been observed in previous studies for these organisms on various hemicellulose hydrolysates, ranging from hardwoods, softwoods, and herbaceous feedstocks/feedstream [1, 8, 10, 25, 28, 47]. With the degradation products below inhibitory levels and the high total sugar concentration, these results were not unexpected. During the fermentation of the hydrolysate, *S. stipitis* consumed the majority (75 %) of the available substrate within 72 h. *S. stipitis* was able to produce a maximum ethanol concentration that represented 67 % of theoretical yield from 100 % HPW hydrolysate (Fig. 2). *P. tannophilus* was able to utilize 69 % of the available substrate and produce 61 % theoretical yield (Fig. 2). *K. marxianus* utilized 53.0 % of the available substrates and produced a maximum ethanol concentration within 24 h; the ethanol concentration decreased in all subsequent samples (Fig. 2c). The low substrate utilization and ethanol production observed for *K. marxianus* has been reported elsewhere [47].

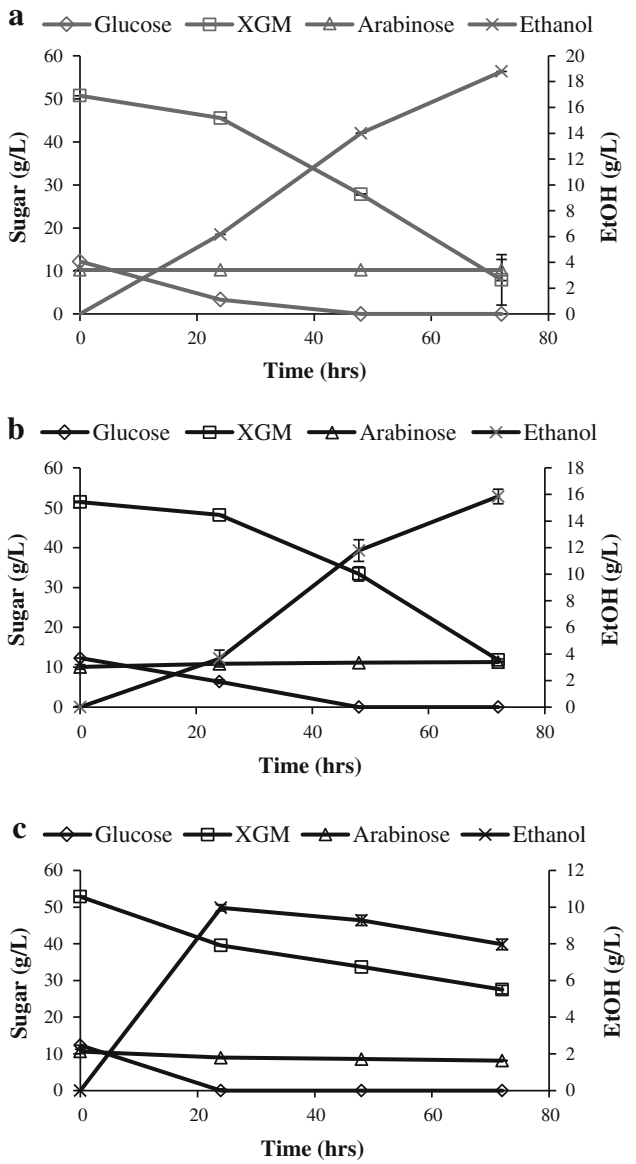

Fig. 2 Fermentation of hydrolysate by *S. stipitis* CBS 6054 (**a**), *P. tannophilus* ATCC 60393 (**b**), and *K. marxianus* ATCC 46537 (**c**). Fermentations were carried out over 72 h (30 °C, pH 5.5). Mean ($n = 3$) ±2 SD. *XGM* xylose, galactose, and mannose

One important difference observed during the fermentation of the HPW and the SH is that all the yeast had a decreased rate of substrate utilization. This may be due to the presence of the degradation products or other unknown compounds present in the HPW that could be potential inhibitors. Even though acetic acid, furfural, and 5-HMF were present below known inhibitory concentrations, there is evidence that inhibitors that result from dilute acid hydrolysis can have a synergistic effect [18, 23, 30].

S. stipitis and *P. tannophilus* exhibited a high conversion efficiency of HPW hydrolysate to ethanol. Based on the literature and the result of this study, *K. marxianus* is not a suitable organism for this application. *S. stipitis* was able to produce the highest ethanol concentration during the fermentation of the dilute acid pretreated hydrolysate (Fig. 3). The yield results achieved during the fermentation of the hydrolysate were comparable to those reported in the literature for hydrolysates that had been detoxified or with low inhibitor concentrations after dilute acid hydrolysis [7, 38].

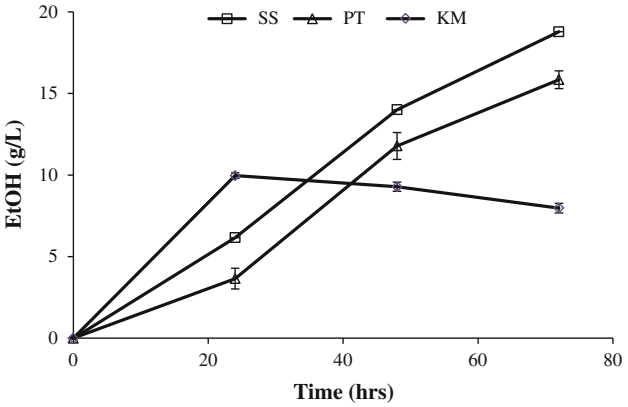

Fig. 3 Ethanol production by *S. stipitis* CBS 6054 (*SS*), *P. tannophilus* ATCC 60393 (*PT*), and *K. marxianus* ATCC 46537 (*KM*) during the fermentation of HPW hydrolysate. Fermentations were carried out over 72 h (30 °C, pH 5.5). Mean ($n = 3$) ±2 SD

Conclusions

Dilute acid hydrolysis of HPW produced a hydrolysate high in total sugars and low in inhibitors, making it a good feedstream for lignocellulosic ethanol production. The hydrolysate consisted of primarily a mixture of hemicellulosic sugars and was readily fermentable. Due to the high xylose content of the HPW hydrolysate, native xylose-fermenting or genetically modified organisms are essential for efficient conversion of the hydrolysate to ethanol. Of the yeasts evaluated, all were able to convert the hydrolysate to ethanol with yields ranging from 52.9–77.1 % of theoretical. The most complete substrate utilization and highest ethanol concentration was obtained by *S. stipitis* CBS 6054. This organism appears to be the most suitable yeast for conversion of HPW hydrolysate to ethanol. However, its performance in the synthetic hydrolysate was slightly better than the actual hydrolysate. The process may be improved through decreased fermentation times and increased yields. Adaptation of the organisms to the conditions present in the hydrolysate is a possible option for achieving such improvement.

Hardboard manufacturing process wastewater meets the characteristics of a promising feedstream for commercial ethanol production. Ethanol production per hardboard

manufacturing plant is estimated at between 2.3 and 3.4 million l/year, based on wastewater data from the US EPA and the most recent hardboard market data from the United Nations [44, 45]. As an industry in the U.S., hardboard manufacturing has the potential to contribute 32-42-million l of ethanol annually from their wastewater streams. Doing this will reduce costs for wastewater treatment and aid in achieving the goals set by the US Renewable Fuels Standards program.

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