# Pretreatment and Fractionation of Wheat Straw for Production of Fuel Ethanol and Value-added Co-products in a Biorefinery

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## ABSTRACT

An integrated process has been developed for a wheat straw biorefinery. In this process, wheat straw was pretreated by soaking in aqueous ammonia (SAA), which removed lignin but preserved most of the carbohydrate fractions. The pretreated material was hydrolyzed with a commercial hemicellulase to provide a solution rich in xylose and low in glucose. This solution then was used for production of value-added products. In this investigation xylitol and astaxanthin were used to demonstrate the fermentability of the xylose-rich hydrolysate. The cellulose-enriched residue obtained after the enzymatic hydrolysis of the pretreated straw was used for ethanol production in a fed-batch SSF process. In this process, a commercial cellulase was used for hydrolysis of the glucan in the residue and the yeast *S. cerevisiae*, which is the most efficient commercially suitable ethanol-producing organism, was used for ethanol production. Final ethanol concentration above 50 g/l was obtained.

***Keywords***: wheat straw, fuel ethanol, biomass bioconversion, biorefinery, value-added products

## 1 INTRODUCTION

Ethanol has attracted considerable attention worldwide as a clean and renewable liquid fuel. The two main feedstocks currently used successfully for commercial ethanol production are corn and sugarcane. However, to meet the projected demand for ethanol other sources of fermentable sugars are needed. In attempts to reach this objective significant efforts have been made to develop technologies for production of ethanol from lignocellulosic feedstocks (LCF), which are available in very large quantities. Whereas in the U.S. corn stover is considered the most important LCF wheat straw is its counterpart in other regions such as Europe and Asia. China is the largest wheat producer in the world. In 2011 China produced 117 million metric tons (MT) of wheat, which accounted for 17 % of the total world production [1]. Using a ratio of 0.623 kg straw per kg grains [2] the quantity of wheat straw generated in China in that year was estimated as 72.9 million MT. Wheat straw typically contains 39.6 wt% cellulose and 26.4 wt% hemicellulose on a dry basis [3]. Thus, about two-third of the total mass of wheat straw can be converted biochemically to useful products. Because of its potential as a very important LCF many studies have been performed on bioconversion of wheat straw to ethanol [4-9]. Conversion of a LCF to ethanol as the only major product is a very expensive process, which requires large capital investments. In addition, the microorganisms that have been developed for fermentation of both C5 and C6 sugars derived from a LCF to ethanol have many disadvantages such as low ethanol tolerance and yields. Thus, the concept of a LCF biorefinery has recently been proposed. The products of a LCF biorefinery include ethanol and value-added products. In the LCF biorefinery instead of fermenting both C5 and C6 sugars to ethanol only glucose is used for ethanol production whereas the C5 sugars are used for production of other products. Aside from the potential economic benefit of generating a suite of products with higher profit margins than ethanol, a major technical advantage is, with glucose as the substrate, the yeast *Saccharomyces cerevisiae*, which is the most efficient commercial ethanol-producing organism, can be used to carry out the fermentation. The biorefinery process recently developed at the ERRC using wheat straw as feedstock is described in this report.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Pioneer 34R65 wheat straw was harvested at Hougar Farms, Coatesville, Pennsylvania in 2012 and kindly donated to the Sustainable Biofuels and Co-Products Research Unit at the ERRC for research purposes. *Phaffia rhodozyma* ATCC 74219 (astaxanthin producer) and *Candida mogii* NRRL Y-17032 (xylitol producer) were stored in a glycerol medium (25 % v/v glycerol) at -70$^{o}$C. Active Dry Ethanol Red yeast culture (*Saccharomyces cerevisiae*) was provided by Lesaffre Yeast Corporation. The biomass enzyme products ACCELLERASE® 1500 and MULTIFECT® Xylanase were kindly provided by DuPont Industrial Biosciences. All chemicals were of reagent grades and purchased from various suppliers.

### 2.2 Methods

*Wheat straw pretreatment and hydrolysis*

 CTSI-Cleantech 2013, www.ct-si.org, ISBN 978-1-4822-0594-7

Ground wheat straw having particle size of <1 mm (70 g, dry basis) was pretreated in a closed container using 700 ml of 15 wt% $NH_4OH$ (i.e. a solid:liquid ratio of 1:10) at 65°C for 15 h. The pretreated solids were recovered by vacuum filtration and washed with deionized (DI) water until the pH of the wet cake was about 7. Several batches of wheat straw were pretreated as described. To prepare the xylose-rich hydrolysate pretreated wheat straw was hydrolyzed with MULTIFECT® Xylanase at 0.2 ml/g dry biomass in 50 mM citrate buffer at pH 4.8. The solid loading was 10 wt % (dry basis). The hydrolysis was performed in a 2.5-liter New Brunswick Scientific BIOFLO® 410 fermentor maintained at 55°C and agitated at 250 rpm for 72 h. The residual solids were recovered by vacuum filtration, dried in a 55°C oven, and ground with a small coffee grinder (Krups, model F 203) for further processing. The hydrolysate was stored frozen until use.

*Astaxanthin fermentation*

Astaxanthin production using the wheat straw hydrolysate was performed in 250-ml flasks. Amberex 695 AG yeast extract was added to the hydrolysate at 5 g/l to provide the required nutrients. The nutrient-enriched medium then was adjusted to pH 5 with 2 M $H_2SO_4$ and transferred to the flasks at 25 ml/flask. The inoculum was prepared in YM medium (21 g/l). Each 250-ml flask containing 25 ml YMB medium was inoculated with 0.5 ml thawed glycerol stock culture. The inoculum medium was sterilized by autoclaving at 121°C for 20 minutes whereas the fermentation medium was filter-sterilized. The inoculum flask was incubated at 22°C and 250 rpm for 3 days before 1 ml culture was used to inoculate the production flasks. Following inoculation the production flasks also were incubated at 22°C and 250 rpm. Samples were taken at intervals for astaxanthin and sugar analysis. The astaxanthin production experiment was performed in triplicates and the average results are reported.

*Xylitol fermentation*

Xylitol production experiments were performed in a similar manner using the same nutrient-enriched hydrolysate as described for astaxanthin fermentation. The only differences were: 1. The pH of the medium was adjusted to 6.5; 2. The inoculum flask was inoculated with 0.2 ml thawed glycerol stock culture and the culture was grown for 24 hours prior to inoculation of the production flasks; and 3. The inoculum and production flasks were incubated at 30°C. The xylitol production experiment was performed in duplicates and the average results are reported.

*Ethanol fermentation*

Fermentation of the cellulose-enriched residue was performed using a fed-batch SSF protocol. The dried residue was ground in a small coffee grinder for two minutes prior to use in the fermentation experiment. The first day of the experiment was dedicated solely to enzyme hydrolysis. Thus, 2.00 g cellulose-enriched residue was added to a 250-ml flask containing 18.5 ml 50 mM citrate buffer at pH 4.8, which was previously sterilized by autoclaving at 121°C for 20 minutes. Next, 500 µl ACCELLERASE® 1500 was added. The flask was securely capped with a rubber stopper and incubated at 50°C and 250 rpm. After 24 hours a 0.5 ml sample was removed from the flask. The sample was centrifuged on a microcentrifuge (Eppendorf, model 5415D) for 3 minutes. The supernatant was filtered using a 0.2-micron syringe filter and saved for sugar analysis. The solid pellet was recovered from the microcentrifuge tube with a small spatula and added back to the flask. A volume of citrate buffer equal to the volume of the removed supernatant was also added to the flask. After the sampling was completed, the following were added to the flask: 1.00 g cellulose-enriched residue, 375 µl ACCELLERASE® 1500, and 1.0 ml stock solution of Amberex 695 AG yeast extract containing 191.5 g/l, which was previously sterilized as described for the citrate buffer. The inoculum was prepared by suspension of 0.25 g Dry Ethanol Red yeast powder in 5 ml buffer and stirring for 30 minutes. The SSF was initiated by addition of 1.0 ml yeast culture to the flask. The flask was capped with a rubber stopper which had an 18-gauge hypodermic needle punctured through to provide pressure relief for the $CO_2$ produced. The flask finally was incubated in a shaker maintained at 32°C and 175 rpm. Progress of ethanol fermentation was followed by weight loss due to production of $CO_2$. Every day 1.00 g cellulose-rich residue and 375 µl ACCELLERASE® 1500 were added to the flask. An Excel spreadsheet was set up to calculate ethanol concentrations using the weight loss data. Details of the calculation were published previously [10]. At the end of the experiment a final sample was removed from the flask and processed as described previously for analysis of residual sugars, ethanol, and other metabolites. The SSF experiment was performed in duplicates and the average results are reported.

*Analytical methods*

Astaxanthin was determined as total carotenoid with pure astaxanthin used as standard as described previously [11]. Samples taken for analysis of other metabolites were centrifuged on a microcentrifuge and the supernatants were filtered through a 0.2 µm filter. The metabolites were analyzed by HPLC as described previously [11]. The carbohydrate contents of the wheat straw were determined by the NREL method [12].

# 3 RESULTS AND DISCUSSION

## 3.1 Wheat straw pretreatment/hydrolysis

The untreated wheat straw contained 34.5 wt % glucan and 19.3 wt % xylan. After pretreatment and xylanase hydrolysis the glucan content of the residual solid was enriched to 46.3 wt % whereas the xylan content decreased to 14.6 wt %. The high glucan level in the solid made it a suitable feedstock for ethanol production by *S. cereviaise*. The hydrolysate obtained by xylanase hydrolysis contained 12.5 g/l xylose and 1.5 g/l glucose. Low glucose levels made it a suitable fermentation medium for xylose-metabolizing microorganisms.

## 3.2 Production of value-added products

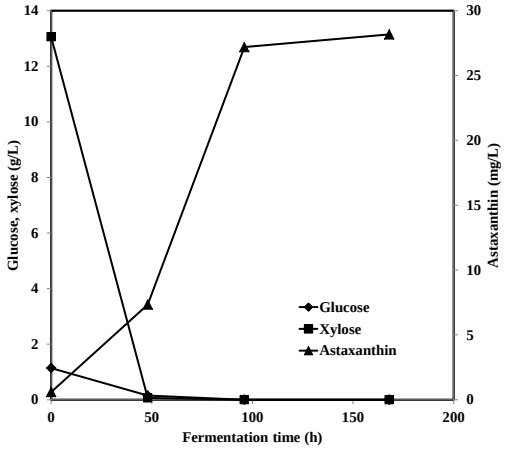


Figure 1. Production of astaxanthin from xylose-rich hydrolysate obtained from SAA-treated wheat straw.

The results of astaxanthin fermentation are shown in Figure 1. The results clearly demonstrated that the wheat straw hydrolysate was a good fermentation medium for astaxanthin production. It was observed in previous investigation that high glucose concentrations suppressed utilization of other sugars by *P. rhodozyma* [11]. The hydrolysate obtained by hydrolysis of the SAA-treated wheat straw contained very low glucose concentrations, thus, qualified itself for a suitable fermentation medium for conversion of xylose to astaxanthin. The final yield was 1.98 mg astaxanthin/g total sugars consumed, which was similar to the yield observed previously [11]. The results of xylitol fermentation are shown in Figure 2. The results again demonstrated the suitability of the hydrolysate obtained from the SAA-treated wheat straw for production of xylitol, similar to the case of astaxanthin. Xylitol was produced at relatively high rates and reached 5.8 g/l after the first day. However, when both substrates (glucose and xylose) were depleted some of the xylitol produced was consumed probably for cell growth and maintenance via the pentose-phosphate pathway [13]. Xylitol consumption by *C. mogii* upon substrate depletion is an important factor that should not be overlooked in development of fermentation processes for its production. The maximum xylitol yield was 0.51 g/g xylose consumed, which is similar to the value observed by others [13].

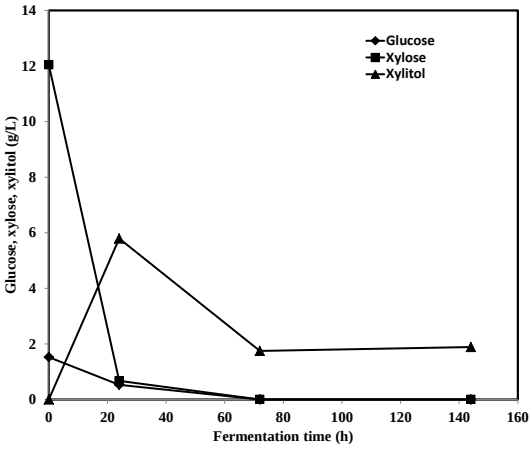


Figure 2. Production of xylitol from xylose-rich hydrolysate obtained from SAA-treated wheat straw.

## 3.3 Production of ethanol

The results of the fed-batch SSF of the cellulose-rich solid residue are shown in Figure 3. As discussed previously, these results were calculated using the actual data on weight loss due to $CO_2$ production. At the end of the first 24-hour period during which the hydrolysis was performed at the optimum temperature of the enzyme the concentration of glucose in the liquid was 23.4 g/l, which was equivalent to 51 % of the theoretical yield. As expected, the availability of readily fermentable glucose allowed ethanol production to proceed at high rates during the first day after the SSF was initiated. The ethanol production rate then dropped by about 40% on the next day, presumably due to near exhaustion of the initial glucose. The rate then continued to decrease, but only gradually. The final solid loading was 27.2 wt. % and the final ethanol concentration calculated from the weight loss data was 56 g/l. The final ethanol yield was equivalent to 63 % of the theoretical value, based on the glucan content of the cellulose-enriched solid residue. Recovery of ethanol by distillation is a very energy-intensive process, thus a major item of the overall operating costs, especially for dilute ethanol solutions. Normally the target for cellulosic ethanol fermentation is 50 g/l since at this concentration the benefit of distillation energy reduction with respect to increase in ethanol concentration starts to become less significant [14]. The final ethanol concentration achieved in the fed-batch SSF of the cellulose-enriched solids was above that target. The average concentrations of glucose and xylose in the final samples were 2.0 g/l and 17.9 g/l, respectively. Low glucose concentrations indicated a highly efficient SSF process. The yeast strain used in this experiment was capable of metabolizing only glucose, thus resulting in accumulation of xylose. Even without xylose conversion, to the best of our knowledge, the final ethanol concentration obtained in this work was still among the highest values

reported for biochemical conversion of lignocellulosic biomass to ethanol.

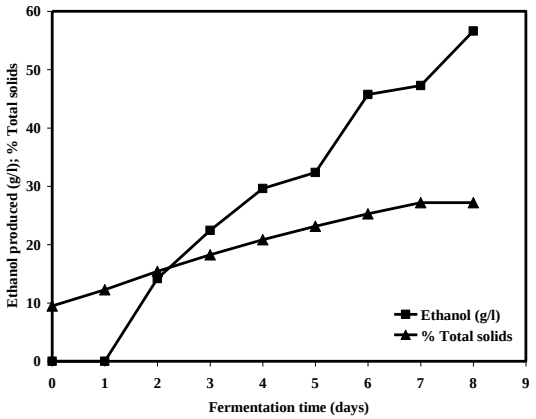


Figure 3. Ethanol production in the fed-batch SSF of the cellulose-enriched solids.

## 3.4 The wheat straw biorefinery

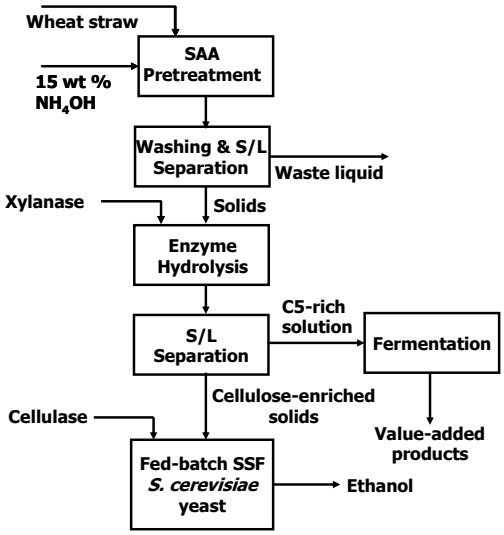


Figure 4. The integrated process for production of ethanol and value-added products in a wheat straw biorefinery.

The simplified process flow diagram (PFD) of the biorefinery is shown in Figure 4. In this process, wheat straw first is pretreated by the SAA or other ammonia-based process that remove lignin but preserve most of the carbohydrate fractions. The pretreated material is hydrolyzed with a commercial hemicellulase product to provide a solution rich in xylose and low in glucose. This solution then is used for production of value-added products in fermentation processes. In this investigation we demonstrated the production of two such products, i.e. xylitol and astaxanthin. However, other industrially chemicals can also be produced using the xylose-rich solution and suitable microorganisms. The residue obtained after the enzymatic hydrolysis of the pretreated straw, in which the glucan contents have been concentrated by extraction of lignin in the pretreatment step and conversion of xylan to xylose in the hydrolysis step, is used for ethanol production in a fed-batch SSF process. In this process, a commercial cellulase product is used for hydrolysis of the glucan in the residue and the yeast *S. cerevisiae*, which is the most efficient commercially suitable ethanol-producing organism, is used for ethanol production.

# 4 CONCLUSIONS

An integrated process for production of fuels and chemicals in a wheat straw biorefinery has been demonstrated. The results shown here are only the proof of concept for such process. Further process development and optimization followed by process validation in pilot plants are needed before the developed process can be implemented in commercial practice.

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