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Dilute phosphoric acid pretreatment of wheat bran for enzymatic hydrolysis and subsequent ethanol production by edible fungi *Neurospora intermedia*

Ramkumar B. Nair*, Magnus Lundin, Tomas Brandberg, Patrik R. Lennartsson, Mohammad J. Taherzadeh

Swedish Centre for Resource Recovery, University of Borås, SE-50190 Borås, Sweden

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ABSTRACT

The use of an underutilized and abundant lignocellulosic feedstock residue, wheat bran, was studied for ethanol production using dilute phosphoric acid pretreatment followed by fermentation using edible fungi *Neurospora intermedia*. Wheat bran was subjected to dilute acid pretreatment at varying acid concentrations (0.5–3.0% w/v), temperature (150–210 °C), and reaction time (5–20 min). The interaction of multiple factors showed the optimum pretreatment conditions at acid concentration of 1.75% (w/v), at 190 °C for 10 min. The maximum total polysaccharide yield of 0.27 ± 0.01 g/g dry biomass loading, corresponding to 66% of the theoretical maximum was observed. Subsequent fermentation with *N. intermedia* showed 85% of the theoretical maximum ethanol yield from the untreated bran glucose. The effect of the dilute acid pretreatment on the functional groups of the wheat bran cellulose was determined with 78% reduction in the cellulose crystallinity index. The validation of the dilute phosphoric acid pretreatment in a demo plant is also reported for the first time. Enzymatic hydrolysis of pretreated slurry from the demo plant showed 85% total theoretical yield of polysaccharides. Compared to the untreated bran biomass, an increase of 51% was observed in the ethanol yield following pretreatment, with a total ethanol yield of 95% theoretical maximum. Higher yield of ethanol is also attributed to the xylose fermenting capability of the fungi.

1. Introduction

Bioethanol is the dominating biofuel for transportation, with an annual world production increasing from 28.5 billion liters in 2004 to 87.2 billion liters in 2013 (REN21, 2014). The overall economic status of the current starch-based bioethanol production (first generation) is greatly dependent on the by-products sold as animal feed, usually referred to as DDGS (dried distillers grains with solubles), together with the mainstream ethanol (Zarrinbakhsh et al., 2013). Recently, Lennartsson et al. (2014) proposed a new process integration model where the first and second generation ethanol processes could be integrated to improve the quality of the DDGS using edible filamentous fungi. This integration is based on the introduction of the pretreated lignocelluloses to the thin stillage (residuals after the fermentation and distillation of ethanol in the first generation ethanol plant), and fermentation together with the edible fungi. Being a rich source of protein, edible fungi has the potential to be sold separately as animal feed (Ferreira et al., 2014) or as dietary supplements (Bellou et al., 2012). In this aspect, they possess several advantages over conventional industrial ethanol producing microorganisms.

Currently, in most of the first generation ethanol plants, grains such as corn or wheat are converted into ethanol, while fibers or bran remain unutilized (Palmarola-Adrados et al., 2005). It is estimated that 150 million tons of wheat bran are produced per year worldwide (Prückler et al., 2014). Industrial wheat bran usually accounts for about 25% of the grain (das Neves et al., 2006) and consists mainly of starch, significant amount of sugars such as hemicellulose, residual cellulose, protein, and lignin and has the potential to serve as a low-cost feedstock for fuel ethanol production (Favaro et al., 2013). Considering these facts, a promising approach to introduce the integration process in existing wheat-to-ethanol plants is to use wheat bran as a substrate for ethanol and fungal biomass production, improving the overall economic status of the first generation ethanol process plants.

* Corresponding author. Tel.: +46 33 4355903; fax: +46 33 4354003.
E-mail address: ramkumar.nair@hb.se (R.B. Nair).

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One of the major challenges, however, is to obtain sufficiently high sugar concentrations from wheat bran for efficient growth of fungal biomass. Enzymatic hydrolysis of bran is not enough to degrade the hemicellulose and cellulose into simple sugars; therefore, chemical or physical pretreatment methods are required (Favaro et al., 2013). Development of effective pretreatment procedures is thus crucial for utilizing wheat bran to produce ethanol. However, the pretreatment method should be specifically chosen so that it does not negatively influence the quality of the animal feed product (DDGS) or produce large amounts of inhibitors. The use of dilute acids such as sulfuric acid results in problems associated with sulfur contamination of the animal feed products. An alternative strategy leads to the possibility of using weaker acids such as dilute phosphoric acid (Castro et al., 2014). In spite of its higher cost, phosphoric acid has several advantages such as being less corrosive and less toxic, representing a reduction in the cost of the plant construction (López-Linares et al., 2013). Using phosphoric acid also means a lower environmental impact; furthermore, it has the advantage of being a source of phosphorous, a nutrient for microorganisms (de Vasconcelos et al., 2013). The presence of phosphorus in the DDGS serves as a source of nutrients, thus, improving its quality. There are studies where dilute phosphoric acid for pretreatment such as e.g., corn stover (Avci et al., 2013), sugarcane bagasse (Nieves et al., 2011), and soft wood (Castro et al., 2014) were reported.

The present study, to the best of our knowledge, is the first time that phosphoric acid is being used for the pretreatment of wheat bran biomass and for ethanol production using edible fungi, *Neurospora intermedia.* The conditions were optimized for efficient pretreatment to produce the maximum sugar and the minimum inhibitor concentrations. The influence of §various factors on the pretreatment reactions were determined experimentally on a lab-scale and validated in a demo plant. The structural and physiological changes in the bran biomass during the pretreatment process were also studied. Enzymatic hydrolysis and a subsequent fermentation using *N. intermedia* for the bioethanol production were carried out.

2. Materials and methods

2.1. Substrates and enzymes

Commercial wheat bran (88.6 ± 0.1% dry matter) was supplied by Lantmännen Agroetanol (Norrköping, Sweden) for both laboratory and demonstration experiments. Cellulase enzyme Cellic Ctec2 (Novozymes, Denmark) with 94 FPU mL^{-1} activity (Adney and Baker, 2008) was used for the hydrolysis. Amyloglucosidase from *Aspergillus niger* (300 U mL^{-1} activity) and α-amylase from Aspergillus oryzae (100 U/mg activity) were supplied by Sigma–Aldrich Co. (Germany).

2.2. Microorganisms

An edible Ascomycetes fungus, *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, Netherlands) was maintained on potato dextrose agar (PDA) slants. Spore solution was prepared from PDA plates after 48 h incubation at 35 °C. The yeast strain, *Saccharomyces cerevisiae* CBS 8066, was maintained on the YPD agar plate and stored at 4 °C. Cells were grown in 100 mL YPD broth media, and cell biomass was harvested after 48 h incubation at 35 °C.

2.3. Experimental design and statistical analysis

The full-factorial experimental design was developed using MINITAB® 17 (Minitab Inc., State College, PA, U.S.A.). Factors examined were phosphoric acid concentration (0.5%, 1.75% and 3% w/v), reaction temperature (150, 170, 190, and 210 °C), and pretreatment duration (5, 10, 15, and 20 min). Solid loading was kept constant at 15%. The selection of factors and the range of variables were based on the preliminary experiments conducted (data not shown). A split-plot interaction model was developed from a full factorial model, with each group having an identical temperature and time but with varying acid concentrations. The results were analyzed with ANOVA (analysis of variance) – the general linear model and the optimization of the pretreatment, hydrolysis, and fermentation conditions were carried out using ANOVA-response optimizer. The response variables included, yield (g/g dry biomass) of ethanol, individual polysaccharides- xylan, arabinan, glucan, and inhibitors- furfural, hydroxymethyl furfural (HMF), and acetic acid.

To evaluate the role of each individual response variable in determining the best pretreatment conditions a composite desirability (D) value was calculated. Composite desirability (D) evaluates how the settings optimize a set of responses overall by determining a combined effect of individual response variables. For each individual response a desirability value was specified by defining an upper and lower limit value to calculate the individual desirability function (d). Based on the range of results obtained in the present study, upper limit for desirability of the individual total hydrolyzed polysaccharides was set to their maximum produced level (g/g dry biomass loading of glucan 0.13, xylan 0.09, and arabinan 0.06). A weightage point of 5 (that defines the significance of a response) in a scale of 1–5, was assigned to each response. For the inhibitors, the individual desirability decreases with the increase in value of the response, a value above the upper limit is outside the acceptable limit, thus having desirability of 0. Hence the upper limit of desirability value of the individual inhibitors was set to their maximum produced level (g/g dry biomass loading of furfural 0.19, HMF 0.13 and acetic acid 0.01) with a weightage point of 5 (in a scale of 1–5). Minitab's response optimizer calculates composite desirability using a composite desirability function (also called utility transfer function)

$$D = (d_1 \times d_2 \times d_3 \times \ldots \times d_m)^{1/m}$$

where *d* is the individual desirability value that evaluates how the settings optimize a single response. Composite desirability value has a range of zero to one were one represents the ideal case; zero indicates that one or more responses are outside their acceptable limits. A composite desirability value fairly close to 1 indicates the settings appear to achieve favorable results as specified in the individual desirability function.

2.4. Dilute acid pretreatment of wheat bran

Dilute phosphoric acid (H_3PO_4) pretreatment of wheat bran biomass was carried out in 150 mL tubular stainless steel reactors (Swagelok, U.S.A.) with a working volume of 100 mL. Reactors were heated with circulating oil maintained at a set temperature and quenched in an ice bath to terminate the pretreatment reactions. The pretreated biomass was then used for the subsequent enzymatic hydrolysis and fermentation experiments.

2.5. Enzymatic hydrolysis and fermentation of pretreated wheat bran

The enzymatic hydrolysis with subsequent fermentation of the selected pretreated bran biomass was carried out with solid loading of 7.5% (working volume 100 mL) at pH 5.5 ± 0.1. Hydrolysis with enzyme loading 10 FPU/g dry matter was carried out at 35 °C, 125 rpm, for 48 h, after which the fermentation was initiated by adding fungal spores (2.5×10^5 spores mL^{-1}). The untreated wheat