Optimizing dilute phosphoric acid pretreatment of wheat straw in the laboratory and in a demonstration plant for ethanol and edible fungal biomass production using *Neurospora intermedia*

Short title: Ethanol and edible filamentous fungal biomass from pretreated wheat straw

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Abstract

BACKGROUND: We report a method that uses dilute phosphoric acid for wheat straw pretreatment and subsequent ethanol and fungal biomass production with the edible fungus *Neurospora intermedia*. Dilute phosphoric acid pretreatment of wheat straw was optimized at a laboratory scale, and the results were validated in a biorefinery demonstration plant for the first time. The various conditions for the dilute acid pretreatment include such factors as phosphoric acid concentrations (0.5–3.0% w/v), temperature (150–210°C), and reaction time (5–20 min).

RESULTS: The optimal pretreatment conditions were determined as an acid concentration of 1.75% (w/v) at a temperature of 190°C for 15 min, based on the maximum enzymatic digestibility with the minimum inhibitor release. The efficiency of enzymatic polysaccharide hydrolysis was 36% for untreated straw and 86% for straw pretreated with dilute phosphoric acid. Scale up of the pretreatment at a biorefinery demonstration plant improved the process, with the subsequent efficiency of polysaccharide hydrolysis being 95% of the theoretical maximum. Ethanol fermentation of enzymatically hydrolyzed wheat straw using *N. intermedia* showed an improvement in the ethanol yield from 29% (with untreated straw) to 94% (with dilute phosphoric acid pretreated straw) of the theoretical maximum.

CONCLUSION: This study opens up an alternative strategy for the efficient use of wheat straw for the production of ethanol and edible fungal biomass in existing wheat-to-ethanol plants.

Keywords: Dilute phosphoric acid; Pretreatment; Optimization; Ethanol; Filamentous fungi; Fermentation

1. INTRODUCTION

The use of lignocellulosic waste materials as an ethanol feedstock has been considered for several decades. Integrated use of wheat straw at existing first generation wheat-to-ethanol facilities can be a promising approach for improving the overall ethanol production capacity of the plant. The International Grain Council reported that the annual world wheat production was approximately 720 million tons in the year 2014/15 (Dec/Nov)¹, with a straw to grain ratio of 1.3 (for most wheat varieties), thus highlighting the availability of straw as an abundant substrate for various applications. The overall economic status of current wheat-based bioethanol production (first generation) depends greatly on DDGS (distillers dried grains with solubles), a byproduct that is sold separately as animal feed². Hence, any approach to introducing ethanol production processes based on wheat straw at existing wheat-to-ethanol facilities should also maintain the current DDGS quality. Wheat straw contains approximately 35%–45% cellulose, 20%–30% hemicellulose and 8%–15% lignin and its recalcitrant nature underscores the need for a pretreatment process prior to its sugar hydrolysis³. In this regard, acid treatment is the most widely used pretreatment method. However, the use of concentrated acids, such as hydrochloric acid or sulfuric acid, poses several disadvantages, such as corrosion of the reactor⁴ or high sulfur contamination of feed residue, thus affecting the quality of the DDGS⁵. The use of other acids, such as phosphoric acid⁶, is a viable option for overcoming the problem of feed contamination. Moreover, the residual phosphate in the residue serves as a nutrient source and improves the quality of the DDGS⁷. However, the use of concentrated phosphoric acid (83– 85.9%) necessitates an additional phosphorus recovery step, which uses organic solvents such as acetone⁸ and could potentially results in phosphorus driven eutrophication of terrestrial, fresh- water and marine ecosystems. Hence, the use of a dilute form of phosphoric acid would be a viable option for overcoming these problems. There are only a few studies that have used dilute phosphoric acid for pretreatment, for substrates such as corn stover⁹, sugarcane bagasse¹⁰, soft wood⁶ and wheat bran¹¹.

The commercialization of lignocellulosic ethanol production is also influenced by various processes other than the pretreatment, for example the fermentation. The microorganisms that are conventionally used for ethanol fermentation, such as *Saccharomyces cerevisiae*, are often unable to utilize the hemicellulose sugars that are liberated from lignocellulosic substrates. The use of genetically modified strains for this process often leads to problems associated with DDGS feed quality. Therefore, the use of naturally existing microorganisms for lignocellulose fermentation is critical. Several edible filamentous fungi have recently gained attention for their use in ethanol and fungal biomass production as they are capable of utilizing hemicellulosic sugars ^{12, 13}. The presence of fungal biomass in the feed residue from the ethanol plants enriches the protein content in the DDGS ¹³, thus improving its nutritional quality. Edible fungi, such as *Neurospora intermedia*, have historically been used for oncom (peanut press cake) fermentation in Indonesia and were recently studied for their contribution to ethanol and fungal biomass production using thin stillage ¹⁴. However, there are limited studies on the use of *N. intermedia* for ethanol and fungal biomass production from lignocellulosic substrates.

Hence, the primary objective of the present study was to develop a process involving the pretreatment of wheat straw with dilute phosphoric acid, with a subsequent novel fermentation process using the edible fungus, *N. intermedia*. Various process parameters that affect the pretreatment and hydrolysis were optimized at laboratory scale, and the optimized conditions were validated in a biorefinery demonstration plant. This study widens the possibilities for the use of phosphate-rich, acid-pretreated straw as a feedstock for the production of ethanol and edible fungal biomass at existing wheat-to-ethanol plants.

2. MATERIALS AND METHODS

2.1. Materials

Commercial wheat straw (92.4% dry content) for both laboratory and demonstration experiments was supplied by Lantmännen Agroetanol (Norrköping, Sweden). Prior to its use, straw, with the composition (g/g, dry basis with SE of mean) of arabinan 0.048 ± 0.013 , galactan 0.0053 ± 0.0015 , glucan 0.315 ± 0.061 , mannan 0.0047 ± 0.0011 , and xylan 0.24 ± 0.08 , was milled (0.2 to 0.25 mm size) using a laboratory rotor beater mill (SM 100, Retsch Technology GmbH, Germany). Cellulase Cellic CTec2 (Novozymes, Denmark) with 94 FPU/mL activity¹⁵ was used for the hydrolysis.

2.2. Fungal strain

An edible ascomycete, *Neurospora intermedia* CBS 131.92 (*Centraalbureau voor Schimmelcultures*, Netherlands), was maintained on potato dextrose agar (PDA) plates containing (in g/L) potato extract 4; dextrose 20; agar 15. For preparing spore suspension, fungal plates were flooded with 20 mL sterile distilled water and the spores were released by gently agitating the mycelium with a disposable cell spreader. An inoculum of 50 mL spore suspension (with a spore concentration of 3.1×10^5 spores/mL) per liter of medium was used for the cultivations. For preparing fungal biomass inoculum, the spores were inoculated into 100 mL YPD broth containing (in g/L) dextrose 20; peptone 20; yeast extract 10. The culture was incubated for 48 h at 35°C and 125 rpm. The fungal biomass was harvested at the end of the cultivation and used as the inoculum, with the dry weight calculated by drying the biomass at 105°C overnight.

2.3. Experimental design for dilute acid pretreatment

The statistically developed experimental design was used for the pretreatment-conditions optimization. A full-factorial experimental model was designed using MINITAB® 17 (Minitab Inc., State College, PA, USA) software. The phosphoric acid concentration (0.5%,

1.75% and 3% w/v), the reaction temperature (150, 170, 190, and 210°C), and the duration of the pretreatment process (5, 10, 15, and 20 minutes) were the factors examined. Initial solid loading was 15% w/v (based on wheat straw dry weight content). The full-factorial design developed 96 individual test runs. A split plot interaction design was later generated from the full-factorial model making 16 groups. Each of the group had constant temperature and time parameters, although acid concentration varied from one test run to another. The results were analyzed with an ANOVA (analysis of variance) general linear model. The relationship between various factors and the responses were modeled, generating the main effects and the interaction plots. The main effect examines how the different levels of the factors affect the response (such as example sugar or inhibitor yield) differently. The interaction plot examines whether the data means for one factor (such as example, time or temperature or acid conc) at each level interact with another factor. The process optimization (for pretreatment, hydrolysis, and fermentation) was carried out using an ANOVA response optimizer. The Minitab response optimizer tool shows how different experimental settings affect the predicted responses for factorial, response surface, and mixture designs. The optimization plot shows the effect of each factor on the responses. The yields (g/g substrate) of ethanol, released polysaccharides (xylan, arabinan, glucan), inhibitors (furfural, hydroxymethyl furfural (HMF)) and acetic acid were selected as the response variables. For individual response, a desirability value was specified by defining an upper and lower limit value to calculate the individual desirability function. The upper limit for the desirability of the individual total hydrolyzed polysaccharides was set to their maximum produced level and the individual inhibitors were set to their minimum produced level. A weightage point of 5 (which defines the significance of a response) in a scale of 1 to 5 was assigned to each response. A composite desirability (D) value was calculated. Composite desirability (D) evaluates how the settings optimize a set of overall responses by determining the combined effect of individual response

variables. Minitab's response optimizer calculates composite desirability using a composite desirability function (also called utility transfer function). $D = (d_1 \times d_2 \times d_3 \times \dots \times d_m)^{1/m}$, where d is the individual desirability value that evaluates how the settings optimize a single response. A composite desirability value with a range of zero to one represents the ideal case; zero indicates that one or more responses are outside their acceptable limits. A composite desirability value that is fairly close to 1 indicates that the settings appear to achieve favorable results as specified in the individual desirability functions.

2.4. Experimental setup for bench scale dilute acid pretreatment and enzymatic hydrolysis

Tubular (150 mL) stainless steel reactors (Swagelok, U.S.A.), with a working volume of 100 mL, were used for dilute phosphoric acid pretreatment at bench scale with 15% solid loading. Pretreatment was carried out at various combinations of conditions of acid concentration (0.5, 1.75 and 3.0%), temperature (150, 170, 190 and 210°C) and holding time (5, 10, 15 and 20 min). A circulating oil bath provided temperature control for the reactors and pretreatment reactions were quenched by immersing the reactor in an ice bath. Enzymatic hydrolysis was carried out for all the pretreated samples from the 96 individual test runs. Enzymatic hydrolysis of the pretreated slurry (with the solid and liquid unseparated) was carried out with a solid loading of 7.5% (working volume 100 mL) at pH 5.5±0.1. Enzyme loading of 10 FPU/g dry substrate initiated the hydrolysis under conditions of 35°C, 125 rpm for 48 h. Enzymatic hydrolysis of the wheat straw that was pretreated in the demonstration plant was carried out under identical conditions. Untreated straw was used as a control.

2.5. Dilute acid pretreatment in the biorefinery demonstration plant

Dilute acid pretreatment of straw was scaled up using the previously optimized conditions at the Biorefinery Demo Plant (BDP) operated by SP (Technical Research Institute of Sweden) in Örnköldsvik, Sweden. Based on the laboratory results, the optimum pretreatment conditions (acid concentration of 1.75%, residence time of 10 min and temperature of $190\pm 2^{\circ}\text{C}$) were maintained for the test runs. The reactions were performed in a vertical plug-flow continuous reactor (30 L) with the acid continuously added in the feed screw prior to reaching the reactor. Two test runs of the pretreatment were performed with 1,000 L of pretreated material being produced for each run. Enzymatic hydrolysis and subsequent fermentation of the pretreated slurry were conducted at laboratory scale under controlled conditions.

2.6. Fermentation experiments

Separate hydrolysis and fermentation (SHF) experiments were performed using acid-pretreated and enzymatically hydrolyzed (48 h) straw from the demonstration plant (without subjecting it to any pre-washing). The pH of the slurry was adjusted to 5.5 using 1 M NaOH. Addition of *N. intermedia* spores (3.1×10^5 spores/mL) initiated the fermentation at varying solid (substrate) loading concentrations (2.5, 5, 7.5%). Untreated wheat straw was used as a control. The straw hydrolysate was supplemented with salts (in g/L) $(\text{NH}_4)_2\text{SO}_4$ 7.5, KH_2PO_4 3.5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.75; trace metals (10 mL/L); and vitamin (1 mL/L), as modified from Sues et al.¹⁶. Various sets of minimal media were formulated to determine the effect of medium components on fungal growth, with one individual component being excluded at a time. Non-sterilized and sterilized processes were also compared.

2.7. Analytical methods

Initial spore concentration was measured using a Bürker counting chamber (with a depth of 0.1 mm) under a light microscope (Carl Zeiss AxioStar plus, Germany). The spore solution was diluted ten-fold before the measurement, and the spores were counted in a volume of 1/250 μL each. The total solid, suspended solid and sugar contents in the untreated and phosphoric acid pretreated straw were measured according to the National Renewable Energy Laboratory (NREL) methods ^{17, 18}. HPLC (Waters 2695, Waters Corporation, U.S.A.) was used to analyze all liquid fractions. A hydrogen-based ion-exchange column (Aminex HPX-87H, Bio-Rad Hercules, CA, U.S.A.) at 60°C with a Micro-Guard cation-H guard column (Bio-Rad) and 0.6 mL/min 5 mM H_2SO_4 , used as eluent, was used for the analyses of glucose, ethanol, acetic acid, furfural, and 5-hydroxymethyl-furfural. For the separation of glucose, mannose, galactose, cellobiose, xylose, and arabinose, a lead (II) based column (Aminex HPX-87P, Bio-Rad) was used. A set of two Micro-Guard De-ashing column, followed by a Micro-Guard Carbo-P guard column (Bio-Rad) was coupled with the lead (II) based column. Structural changes in the pretreated straw were investigated using a Fourier Transform Infrared (FTIR) spectrometer (Impact 410 iS10, Nicolet Instrument Corp., Madison, WI, U.S.A.). The spectral data were generated by Nicolet OMNIC 4.1 software (Nicolet Instrument Corp.) and analyzed by eFTIR (Essential FTIR, U.S.A.). The crystallinity index (CI) was calculated as the ratio of the absorbances at wavenumbers 1429 cm^{-1} and 893 cm^{-1} ¹⁹. The viscosity of the pretreated slurry was determined using a Brookfield digital viscometer-model DV-E (Chemical Instruments AB, Sweden). All experiments and analyses were carried out in duplicate and results are reported with error bars and intervals representing two standard deviations.

3. RESULTS

3.1. Optimization of the conditions - bench scale dilute acid pretreatment

Dilute phosphoric acid pretreatment of wheat straw at bench scale yields 0.441 ± 0.003 g polysaccharide hydrolyzed per g dry straw substrate. The yield of polysaccharides that are hydrolyzed during the pretreatment process was determined by measuring the individual mono-sugar released, followed by calculating the respective polysaccharide content (conversion factor, 0.9 and 0.88). The maximal observed polysaccharide hydrolysis yield, in the present study (theoretical maxima) were 76% for xylan, 68% for arabinan, and 41% for glucan. The increasing trend for glucan yield was observed as a function of the factorial interaction between pretreatment time and temperature (Fig. 1(a)). However, for xylan (Fig. 1(b)) and arabinan (Fig. 1(c)), there was a decrease in yield at higher pretreatment time (15, 20 min) (p -value range: 0.00 -0.016) and temperatures (190, 210°C) (p -value: 0.01 -0.023). Efforts were made to determine the pretreatment conditions that would generate minimum inhibitors. Furfural yield showed an initial increase (Fig. 2(b)) when the temperature rose from 150 to 170°C, however, a considerable decrease in its yield was observed while increasing the temperature range from 190 to 210°C. Unlike furfural, the HMF yield remains low (Fig. 2(a)) except for the highest pretreatment conditions (3% acid, 210 °C, 20 min). Although the acetic acid yield increased steadily with an increase in the severity of the pretreatment conditions, a change in acid concentration from 1.75 to 3% resulted in only a slight increase in its yield (Fig. 2(c)). The various pretreatment conditions resulted in a wide range of polysaccharides degradation and inhibitors release. For all treatment combinations, the highest values of polysaccharide degradation were found for the time range of 10 to 20 min (p -value: 0.00- 0.032) and temperatures 170 to 210°C (p -value: 0.001 – 0.043) (Fig 1). In addition, there was a considerable increase in inhibitor yield (p -value: 0.016 – 0.033) at a high acid concentration (3%) (Fig. 2). Hence, for the statistical optimization of the pretreatment conditions, a model was developed with the range of the factorial set limited to 0.5 to 1.75%

acid, temperatures of 170 to 210°C, and a time frame of 10 to 20 min. The response-optimizer prediction model considers all the possible factorial combinations (pretreatment conditions). Based on the results obtained in the present study, the response optimization was carried out with the yield of the polysaccharides hydrolyzed set to the maximum value, g/g dry biomass loading of glucan 0.08, xylan 0.16, and arabinan 0.032 and the inhibitors released being set to the minimum value, g/g dry biomass loading of furfural 0.004, HMF 0.002 and acetic acid 0.014. The composite desirability value (D) that defines the extent of optimum fit for the combinational effect of the pretreatment conditions was calculated using the preset response set. The response optimizer model predicted three combinations as the best pretreatment conditions: a) 0.5% acid, 190°C, 15 min; b) 0.5% acid, 190°C, 20 min; and c) 1.75% acid, 190 °C, 15 min (Table 1), with composite desirability (D) factors of 0.73, 0.77, and 0.84, respectively. A higher composite desirability value represents the optimum condition based on the desired responses set ¹¹.

3.2. Effect of dilute acid pretreatment on functional groups and viscosity

The effects of pretreatment reactions on the structural changes in straw were determined by measuring the crystallinity index (CI) value (ratio of absorbances at wave numbers 1429 cm⁻¹ and 893 cm⁻¹). The CI value of untreated straw was 0.79±0.25. Overall, the dilute acid pretreatment resulted in a reduction in the CI value by 76 percentage points, with the lowest value (0.19±0.01) measured for treatment conditions 190°C, 5 min, and 3% acid concentration. However, at a lower acid concentration (0.5%) the CI value remained constant, irrespective of the pretreatment time (Fig. 3(a)). At a higher acid concentration (3%), there was a decrease in the CI value at the increased temperature (210°C). In the present study, the viscosity of pretreated slurry decreased when each of the parameters of the pretreatment conditions was increased. When the acid concentration was increased from 0.5 to 1.75%, the

viscosity decreased by 52%. The function of the factorial interaction between acid concentration and time (Fig. 3(b)) showed that there was a considerable decrease in viscosity (by approximately 60%) when the time was increased from 15 to 20 min (at different acid and temperature combinations). The low viscosity of the pretreated slurry aids mixing (mass and heat transfer) during the subsequent enzymatic hydrolysis processes. Similar observations were also made regarding the interaction of temperature with acid concentration and time (Fig. 3(b)), where a decrease in viscosity was again observed.

3.3. Enzymatic hydrolysis of laboratory scale pretreated wheat straw

Enzymatic hydrolysis (following acid pretreatment) of straw pretreated under a range of conditions at laboratory scale yielded hydrolyzed polysaccharides at (g/g dry substrate loading) 0.31 ± 0.02 glucan, 0.18 ± 0.01 xylan, and 0.023 ± 0.003 arabinan. The maximum glucan hydrolysis after enzymatic hydrolysis occurred for wheat straw pretreated with 0.5% acid, 15 min, and 190°C. On the other hand, hydrolysis of xylan and arabinan was maximal for wheat straw pretreated with 1.75% acid, 15 min and 190°C (Fig. 4). A maximum total polysaccharide hydrolysis of 0.52 ± 0.03 g/g dry substrate loading, which corresponded to 86% of the theoretical maximum, was observed after hydrolysis following acid pretreatment. In terms of overall efficiency of polysaccharide hydrolysis, enzymatic hydrolysis of the acid pretreated wheat straw gave 86% of the theoretical maximum while enzymatic hydrolysis of untreated wheat straw gave only 36% (19% of the glucan, 12% of the xylan and 5% of the arabinan) of the theoretical maximum. The maximum release of free sugars from polysaccharides after enzymatic hydrolysis was obtained under the pretreatment conditions a) 1.75% acid, 15 min, and 190°C; b) 0.5% acid, 15 min and 190°C and c) 0.5% acid, 20 min and 190°C (Table 2).

3.4. Validation of dilute phosphoric acid pretreatment of wheat straw at the biorefinery demonstration plant

The validation of the optimum conditions for dilute phosphoric acid pretreatment of wheat straw at the biorefinery demonstration plant is reported here for the first time. The optimum pretreatment conditions (an acid concentration of 1.75%, a residence time of 10 min and a temperature of $190\pm 2^{\circ}\text{C}$), obtained from the laboratory results, was used for the test runs in the biorefinery plant. The optimum acid concentration of 1.75% was chosen since the hydrolysis of xylan and arabinan was maximal for the wheat straw pretreated with 1.75% acid, 15 min and 190°C (Fig. 4) at the laboratory scale. A reduced residence time (10 min, compared to the optimum of 15 min identified at laboratory scale in Section 3.4) was chosen at the demonstration scale as the longer heating and cooling periods of the reactor could also contribute to the hydrolysis process. The straw slurry was added to the reactor continuously at a flow rate between 0.7 and 1.7 L/h, resulting in a pretreated slurry with a pH of 2.25 ± 0.05 , a total solids content of 24% and a suspended solids content of 16%. The pretreated wheat straw slurry had a viscosity of 82.3 ± 0.6 cP. This is lower than that of the slurry produced in the laboratory-scale experiments (108.5 ± 0.4 cP), which might be due to the improved efficiency of heat transfer and mixing at the larger scale compared to the non-mixed conditions at the laboratory scale. The pretreated solid fraction had a CI value of 0.58 ± 0.01 , which is in accordance with the results obtained from the laboratory-scale experiments (0.63 ± 0.03). Based on composition analysis of the pretreated sample (Table 3), the total hydrolysis yield was calculated to be 0.310 ± 0.003 g polysaccharides per g dry straw substrate. Similar conditions (1.75% acid, 15 min and 190°C) in the laboratory-scale experiments showed a considerably lower yield of 0.192 ± 0.008 g polysaccharide hydrolyzed per g of dry straw substrate. Hence, the demonstration-scale pretreatment improved the polysaccharide yield by 38% compared to the laboratory-scale process. In addition, the pretreatment process

at the demonstration plant resulted in approximately 4% reduction in the inhibitor yield compared to the laboratory process (with a p -value in the range 0.00–0.031, proving it to be significant). The liquid prehydrolysate from the laboratory pretreatment process performed under similar conditions (1.75% acid, 15 min and 190°C) contained (g/L): furfural 4.1 ± 0.1 ; HMF 0.16 ± 0.02 ; and acetic acid 1.32 ± 0.01 . The enzymatic hydrolysis (for 48 h) of the straw pretreated in the demonstration plant resulted in hydrolysis of 0.578 ± 0.039 g total polysaccharide per g dry substrate straw, which was 95% of the theoretical maximum (based on the composition of untreated wheat straw).

3.5. Fermentation using *N. intermedia*

Fermentation of the enzymatically hydrolyzed pretreated straw from the demonstration plant showed a maximum ethanol yield of 0.072 ± 0.004 g/g dry substrate loading, after processing at laboratory scale. Fermentation with *N. intermedia* mycelial biomass (with an initial concentration of 8.1 ± 0.3 mg dry weight per L) resulted in an increased amount of ethanol, 0.127 ± 0.002 g/g dry substrate loading, corresponding to 71% of the theoretical maximum based on the glucan content of the substrate wheat straw (Fig. 5(a)). In contrast, fermentation after the enzymatic hydrolysis of untreated straw (control) gave 0.052 ± 0.001 g ethanol/g dry substrate loading (29% of theoretical maximum). At solids loadings of 5% and 2.5% of pretreated wheat straw, ethanol yields of 0.102 ± 0.002 and 0.082 ± 0.005 g/g dry substrate, respectively, were obtained (Fig. 5(a)).

Fermentation was also done with pretreated substrate supplemented with specific (minimal) media components to determine whether leaving out individual components of the medium would have detrimental effects on fungal growth. With the complete minimal media, the maximum ethanol production was 0.086 ± 0.002 g/g dry substrate loading. However, there

was no significant fungal growth in samples that lacked any one of the medium components, except in the case of vitamins (Fig. 5(b)). An ethanol concentration of 0.056 ± 0.0024 g/g dry substrate loading was obtained without vitamins. The ethanol yield increased to 0.171 ± 0.01 g/g dry substrate loading (94% of the theoretical maximum) with the addition of nutrients to the non-sterilized pretreated straw substrate (containing initial polysaccharides and an inhibitor composition similar to the sterilized substrate) and with the fermentation being initiated by the inoculation of the mycelium of *N. intermedia*. A similar experiment was performed, except that the fermentation was carried out with the inoculation of *N. intermedia* spores; this gave a lower ethanol concentration of 0.083 ± 0.004 g/g dry substrate (Fig. 5(b)).

4. DISCUSSION

In this work, the optimum conditions for dilute phosphoric acid pretreatment of wheat straw was determined in the laboratory scale and subsequently validated in the demonstration scale for the first time. The work also describes a novel fermentation process using the edible filamentous fungus *N. intermedia*. Dilute acid pretreatment has become a state of the art technology for pretreating lignocellulosic biomass and it has the advantage of not only solubilizing hemicellulose but also converting solubilized hemicellulose to fermentable sugars²¹. The dilute acid pretreatment, thus, eliminates or reduces the need for use of hemicellulase mixtures. In the present study, we used dilute phosphoric acid, avoiding the use of the more popular sulfuric acid. However, the use of phosphoric acid has some limitations as the cost of industrial grade acid (approx. U.S. \$600–750/metric ton for 85% H_3PO_4) is higher than that of other conventionally used acids, such as industrial grade sulfuric acid (approx. U.S. \$200–400/metric ton for 92.5% H_2SO_4)¹¹. Nevertheless, the higher cost of phosphoric acid is offset by its advantages in biomass pretreatment (described in section 1).

In this study, an improved polysaccharide hydrolysis yield was obtained after dilute acid pretreatment followed by enzymatic hydrolysis, as compared to the previous studies on wheat straw pretreatment with other dilute acids. For example, Saha et al.²¹ reported that the maximum yield of sugars was 76% after enzymatic saccharification of 0.5% H₂SO₄ (v/v) pretreated wheat straw for 15 min at 180 °C. This is lower than that reported in the present study, where a polysaccharide hydrolysis of 95% of the theoretical maximum was observed. However, there is a critical problem for the fermentation of dilute acid hydrolysates, namely the inability of the fermentative microorganism to withstand inhibitory compounds produced during pretreatment. This usually means that a detoxification step is necessary to improve the fermentability²⁴. In this study, no additional detoxification step (except for pH adjustment) for neutralizing the effect of acid or inhibitors was necessary since the filamentous fungus used, *N. intermedia*, tolerated the inhibitors present in the hydrolysate slurry. However, at higher pretreatment temperatures used in the present study, particularly at 190°C, inhibitors such as furfural reached its maximum level. High furfural levels were also observed for other lignocellulosic substrates pretreated with dilute phosphoric acid. For example, a similar change in the levels of furfural and acetic acid at a high temperature and acid concentration has been reported previously for wheat straw pretreatment using 0.5% H₂SO₄ (v/v) for 15 min at 180°C²¹, namely 0.032 g furfural and 0.027 g acetic acid per gram of dry wheat straw substrate.

Using *N. intermedia*, in the present study, a higher ethanol yield was obtained compared to the previous studies that used genetically-modified ethanol-fermenting microorganisms. Recently, while studying the pilot-scale conversion of wheat straw to ethanol, and fermentation using recombinant *Escherichia coli* FBR5, Saha et al.³ reported a maximum ethanol yield of 0.29 g/g of wheat straw (86% of the theoretical ethanol yield based on

substrate wheat straw), which was comparatively lower than the values reported in the present study (94% of the theoretical maximum ethanol yield). Their study also used a detoxification step (overliming with calcium hydroxide) prior to fermentation. Similarly, Zhu et al.²⁵ used 1% sulfuric acid to pretreat wheat straw at 100°C and 2 h and obtained an ethanol yield of 88% of the theoretical maximum (based on substrate wheat straw), using *Saccharomyces cerevisiae*. However, ethanol yields that are higher than those obtained in the present study have been previously reported for other lignocellulosic substrates pretreated with dilute H₃PO₄. For example, in a recent study, Avci et al.²³ reported an ethanol yield of 96% of the theoretical maximum, from dilute-H₃PO₄-pretreated corn stover using the recombinant strain *E. coli* FBR5. The optimum pretreatment conditions reported in their study were 161°C, 0.78% acid, and 9.7 min. Although these ethanol yields are higher than that obtained in the current work, the present study uses an edible ascomycete fungus, enriching the nutritional value of the solid residue after ethanol fermentation, increasing its suitability for use as animal feed.

5. CONCLUSIONS

To the best of our knowledge, this report describes the first use of dilute phosphoric acid for the pretreatment of wheat straw. The acid pretreatment process improved the overall enzymatic hydrolysis efficiency by increasing the polysaccharide hydrolysis from 36% to 86% of the theoretical maximum based on the initial straw substrate loading. Wheat straw pretreated at the optimum conditions in a biorefinery demonstration plant showed a total polysaccharide hydrolysis of approximately 95% of the theoretical maximum after enzymatic hydrolysis (based on the composition of substrate straw). An ethanol yield of 94% of the theoretical maximum (based on glucan content of straw) was achieved in a non-sterile fermentation experiment with the addition of mycelial biomass of *N. intermedia*, and the utilization of nutrient supplements together with straw hydrolysate. The present work hence

outlines the potential of using phosphoric acid as an efficient pretreatment agent, thereby eliminating problems associated with sulfur contamination of feed stock when sulfuric acid is used. With the use of an edible ascomycete, the study also produced nutritionally enriched straw fermentation residue, which could be sold separately as an animal feed.

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TABLES

Table 1. Polysaccharide hydrolysis and inhibitor yield at the optimum conditions (0.5% acid, 190 °C, and 20 min) of wheat straw pretreatment.

Response	Optimization	Yield (g/g dry substrate loading)	95% limits	Confidence	95% limits	Prediction
			Lower	Upper	Lower	Upper
Xylan	Maximum	0.162	0.140	0.184	0.125	0.200
Glucan	Maximum	0.08	0.009	0.026	0.003	0.032
Arabinan	Maximum	0.032	0.026	0.039	0.021	0.043
Furfural	Minimum	0.004	0.003	0.005	0.001	0.024
HMF	Minimum	0.002	0.001	0.002	0.0004	0.008
Acetic acid	Minimum	0.014	0.011	0.016	0.009	0.018

Table 2. Optimum conditions for the maximum polysaccharide hydrolysis yield after dilute acid pretreatment and enzymatic hydrolysis of wheat straw.

Pretreatment conditions			Glucan		Xylan		Arabinan				
Acid load (% w/v)	Temp (°C)	Time (min)	Yield (g/g) initial dry substrate loading	95% Confidence limits		Yield (g/g dry substrate loading)	95% Confidence limits		Yield (g/g) initial dry substrate loading	95% Confidence limits	
				Lower	Upper		Lower	Upper		Lower	Upper
0.5	190	15	0.305	0.281	0.329	0.135	0.102	0.168	0.015	0.005	0.041
0.5	190	20	0.280	0.256	0.303	0.151	0.118	0.184	0.013	0.003	0.023
1.75	190	15	0.240	0.216	0.264	0.189	0.156	0.222	0.023	0.012	0.035

Table 3. Composition of the solid and liquid fractions of the pretreated wheat straw substrate from the demonstration plant

Component	Composition of liquid fraction (g /L)	Composition of solid fraction (g/g dry matter substrate)
Arabinan	15.02± 0.14	0.022± 0.002
Glucan	11.53± 0.06	0.235 ± 0.01
Xylan	16.1± 0.1	0.037± 0.006
Acetic acid	1.82± 0.03	
Furfural	2.71± 0.12	
HMF	0.89± 0.03	

Table 4. Studies on the acid pretreatment of wheat straw and use of phosphoric acid for pretreatment of lignocellulosic substrates

Substrate	Acid	Pretreatment conditions (temp; time)	Polysaccharide hydrolysis yield	Microorganism – Ethanol fermentation	Ethanol yield	Reference
Wheat straw	Sulfuric acid 0.75% (v/v)	121°C; 1h	74% total polysaccharide hydrolysis yield	Recombinant <i>Escherichia coli</i> FBR5	0.24 g ethanol/g dry substrate	Saha et al. ²¹
Wheat straw	Sulfuric acid 1 % (w/w)	100 °C; 2 h	86.4% xylose recovery yield	<i>Saccharomyces cerevisiae</i> YC-097	0.334 g ethanol /g wheat straw	Zhu et al. ²⁵
Corn stover	Phosphoric acid 0.5% (v/v)	180°C; 15 min	85% glucose yield	Recombinant <i>Escherichia coli</i> FBR5	0.49 ethanol/g sugar	Avci et al. ²³
Corn stover	Phosphoric acid 1% (v/v)	160 °C; 10 min	91.4% xylose recovery yield	Recombinant <i>Escherichia coli</i> FBR5	0.49 ethanol/g sugar	Avci et al. ²³
Sugarcane bagasse	Phosphoric acid 1% (w/w)	160°C; 10 min	76% xylan hydrolysis yield	<i>Escherichia coli</i> LY160	16.4 g /L ethanol (89% theoretical)	Geddes et al. ²²
Sugarcane bagasse	Phosphoric acid 0.20% (w/v)	186°C; 8 min and 186°C; 24 min	96% and 98% total hemicellulose hydrolysis yeild	-	-	de Vasconcelos et al. ⁷
Corn stover	Phosphoric acid 0.75% (v/v)	180 °C; 10 min	70.3% total sugar yield	<i>S. cerevisiae</i> NRRL Y-2034	0.13 g ethanol/g corn stover	Avci et al. ⁹
Wheat straw	Phosphoric acid 1.75% (v/v)	190 °C; 15 min	95% of theoretical maximum	<i>Neurospora intermedia</i>	0.17 g ethanol/g dry substrate (94% theoretical)	Present study

FIGURES

Figure 1. Impact of various pretreatment conditions on the polysaccharide hydrolysis yield (g/g dry substrate loading) of a) glucan, b) xylan, and c) arabinan.

Figure 2. Impact of various pretreatment conditions on the yield (g/g dry substrate loading) of a) HMF, b) furfural and c) acetic acid

Figure 3. Impact of pretreatment conditions on the a) change in the crystallinity index value of wheat straw cellulose and b) viscosity value of the prehydrolyzate

Figure 4. Polysaccharides hydrolysis yield (g/g dry straw substrate) of arabinan (■), glucan (■), and xylan (■) after dilute phosphoric acid pretreatment and subsequent enzymatic hydrolysis (48 h)

Figure 5. Fermentation profile of *N. intermedia* in dilute acid pretreated wheat straw substrate with a) addition of yeast extract with fungal biomass (●) and spores (○); and control fermentation using acid untreated but enzymatically hydrolyzed straw (◆) b) Complete minimal media with addition of fungal spores - sterilized (Δ) and non-sterilized (▲) pretreated slurry; fungal biomass to non-sterilized (■) pretreated slurry; Minimal media with the absence of vitamins (■)