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# Optimization of pH as a Strategy to Improve Enzymatic Saccharification of Wheat Straw for Enhancing Bioethanol Production

--Manuscript Draft--

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| **Manuscript Number:** | JANS-D-19-00282R1 |
| **Full Title:** | Optimization of pH as a Strategy to Improve Enzymatic Saccharification of Wheat Straw for Enhancing Bioethanol Production |
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| **Author Comments:** | This paper is a part of The first author (Mohammed O. Abdulsattar) thesis held and accepted in Hull University 2019. Supervised by the last author Dr. Sherif H. Zein. E-mail: S.H.Zein@hull.ac.uk. |
| **Response to Reviewers:** | Dear Prof. Cheong<br><br>Thank you very much for sending the reviewers comments on our manuscript. We also would like to thank the reviewers' precious time to review our manuscript. We have prepared a new version of this manuscript, which has been substantially modified addressing all points raised by the reviewers. We feel that the current version has been markedly improved and hope that it would be now acceptable for publication. We |

provide below a detailed explanation on how we have addressed the points raised by the reviewers.

Thank you very much.

Yours Sincerely,

Corresponding Author

Reviewer #1: There is no comment on this paper.
This paper is that describes how to increase the productivity of bioethanol and is thought to be helpful to researchers who study Wheat Straw as a biomass.

Authors respond: The authors would express their gratitude to the reviewer for his/her time taken to review the paper.

Reviewer #2: The authors described how they found the new optimized conditions of pH range for highest yield of total reduced sugar from enzymatic saccharification of WS. Based on this optimization, they observed higher optimized pH range than the widely reported values. The results seem to be of considerable interests for the related field and the manuscript is relatively well written.

However, I believe that the manuscript should be clear for the following questions and comments prior re-review. The authors can just reply to the comments if they think no revision of manuscript is required.

1. pH's of the suspensions (buffer solutions and WS) after adding enzyme were adjusted to the original pH values (3.00, 3.50, 4.00, and so on). This made the suspension hold substantial amount of buffer capacity (in other words, comming back to the buffer solution with enough buffer capacity), which means that real pH changes (after time went on) were screened by the buffer solutions. Do the author ensure that the pH changes after 72 hrs reflected the real change in pH ?

Author respond: The authors would express their gratitude to the reviewer for his/her time taken in improving the paper. The authors have checked during the research work the pH changes after 72 hours and it reflect the real change in pH.

2. The authors did not describe why the optimized pH ranges for WS are shifted for higher values than those for pure cellulosic substrates.

Authors respond: The authors included the reason in the conclusion (Paragraph 3):

(Reducing the acidity in lignocellulosic substrates enzymatic hydrolysis might have an effect on reducing lignin inhibition of the activity of the enzyme, by reducing the lignin absorption of enzymes or affecting the lignin-cellulose binding and interaction by affecting the electrostatic charge of the lignocellulose, changing the pH could also have an effect on the lignin-derived phenols).

Minor corrections:

1. The font size of sub-titles can make the reader confused. For example, the font (or its size) of "Raw wheat straw composition using HPLC" and "Sugar analysis" should be differ from "Analytical methods".

Authors respond: The font of titles and subtitles were changed and underline was added to the Analytical method and the sugar analysis was removed.

2. Please double check if any period mark or comma should be added in line 32 in

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| | page 3 (in Materials and Methods section).<br><br>Authors respond: Thanks. The commas are inserted. |
| **Additional Information:** | |
| **Question** | **Response** |

# Optimization of pH as a Strategy to Improve Enzymatic Saccharification of Wheat Straw for Enhancing Bioethanol Production

## Abstract

In this work, wheat straw (WS) was used as a lignocellulosic substrate to investigate the influence of pH on enzymatic saccharification. The optimum enzymatic hydrolysis occurred at pH range 5.8 – 6.0, instead of 4.8 - 5.0 as has been widely reported in research. Two enzymes cocktails, Celluclast® 1.5L with Novozymes 188, Cellic® CTec2 and endo-1, 4-β-Xylanase, were used for the pH investigation over a pH range of 3.0 – 7.0. The highest concentration of total reduced sugar was found at pH 6.0 for all the different enzymes used in this study. The total reduced sugar produced from the enzymatic saccharification at pH 6.0 was found to be 7.0, 7.4 and 10.8 (g $L^{-1}$) for Celluclast® 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic® CTec2, respectively. By increasing the pH from 4.8 to 6.0, the total reduced sugar yield increased by 25% for Celluclast® 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase and 21% for Cellic® CTec2. The results from this study indicate that WS hydrolysis can be improved significantly by elevating the pH at which the reaction occurs to the range of 5.8 to 6.0.

**Keywords:** Wheat straw, Hydrolysis, pH effect, Sugar yield.

## Introduction

Environmental degradation and the universal need for energy has raised the demand for clean, easily available and renewable energy sources to replace fossil fuel. The use of conventional fossil fuels as a major energy source has increased greenhouse gas emissions leading to global warming (Talebnia et al., 2010, Yang et al., 2013). Among the renewable energy sources, bioethanol has been of great interest in recent decades. There are many raw materials which can be used as resources for bio-ethanol production such as; molasses, corn and sugarcane. With the rising debate of food versus fuel, lignocellulosic waste present a very good raw material for bioethanol production (Govumoni et al., 2013, Sarkar et al., 2012). Bioethanol fuel production from lignocellulosic waste obtained from crops, wood and agricultural residues represent a promising resource for a sustainable bioethanol fuel production due to the low cost and large quantity available worldwide (Avci et al., 2013, Talebnia et al., 2010). Among the variety of lignocellulosic materials, agricultural residues such as wheat straw (WS) stands as an important candidate for large scale bioethanol production. This can be attributed to its sustainability, abundance and the large content of cellulose contrasted with a low lignin content (Qiu et al., 2017). According to statistics, WS which is a by-product from wheat production is one of the largest biomass feedstock in the world with a total production of approximately 690 kilotons in 2009, reaching 730 million tons in 2014 (Zheng et al., 2018). As a result, WS serves as a main appropriate lignocellulosic feedstock for bioenergy in the 21$^{st}$ century.

WS cells mainly consist of three different polymers namely cellulose, hemicellulose and lignin (de Assis Castro et al., 2017). The bioconversion of lignocellulosic to cellulosic biofuel *via* a reduced sugar (fermentable sugars) platform involves three key steps. The first step is pre-treatment, followed by enzymatic saccharification or catalytic conversion of reduced sugar and finally fermentation, the last step in the ethanol production line (Lan et al., 2013).

Enzymatic saccharification has been considered as a fundamental and the highest cost step in bioconversion of lignocelluloses. Few studies have been carried out using lignocellulosic substrates (instead of standard cellulose substrates) to find the optimum pH value for enzymatic hydrolysis (Lan et al., 2013). The majority of studies conducted on the enzymatic hydrolysis of lignocelluloses using *Trichoderma reesei* (i.e Celluclast® 1.5L) were performed at pH 4.8 and at a temperatures around 50 °C. These conditions were considered as the optimum condition for hydrolysis based on laboratory enzyme activities using model substrates, i.e., pure cellulose (Lan et al., 2013). The condition used for lignocelluloses enzymatic hydrolysis with endo-1, 4-β-Xylanase are quite similar to those commonly reported for Celluclast® 1.5L with Novozymes 188, which include a temperature of 50 °C and pH 4.8-5.0 (Yang et al., 2015, Maitan-Alfenas et al., 2015).

Similarly, although the recommended pH range for Cellic Cellic® CTec2 by Sigma Aldrich (Novozymes) is 5.0 – 5.5, pH 4.8 or 5.0 is the most commonly reported in the literature (Procentese et al., 2017, Sun et al., 2018). Celluclast® 1.5L with Novozymes 188 and Cellic Cellic® CTec2 are among the most used enzymes for cellulose hydrolysis, whiles endo-1, 4-β-Xylanase is for hemicellulose hydrolysis (Avci et al., 2013, Oladi and Aita, 2018, Jørgensen et al., 2007, Kumar et al., 2008).

Lignocellulosic substrates differ from pure cellulosic substrates in terms of physical and chemical compositions and structures. The presence of the hydrophobic lignin is considered a vital factor which inhibits the enzymatic hydrolysis of cellulose (Rajput and Visvanathan, 2018). The mechanism by which lignin alters the hydrolysis process depends on the adsorption of cellulase on to lignin rather than cellulose via ionic bond interactions, hydrogen bond interactions and hydrophobic interactions (Nakagame et al., 2011). To solve this problem some researchers have modified the lignin surface using acid groups such as carboxylic and sulfonic to increase the hydrophilicity of the lignin (Nakagame et al., 2011). This reduces the non-productive (non-specific) binding to cellulase which limits the yield of cellulose hydrolysis during the biochemical reaction of the lignocellulosic biomass (Mansfield et al., 1999).

Lignin is considered as a phenolic polymer with three main hydroxycinnamoyl alcohols: sinapyl, coniferyl and p-coumaryl alcohols. During the pre-treatment, these alcohols might be polymerized to guaiacyl, syringyl and p-hydroxyphenyl moieties (Bonawitz and Chapple, 2010). Both cellulases and hemicellulases are affected by lignin-derived phenols during enzymatic hydrolysis (dos Santos et al., 2018). Moreover, the exposed lignin present in the lignocellulosic biomass after pre-treatment affects the enzymes by absorbing them (Selig et al., 2007). Many binding mechanisms between enzymes and lignin have been suggested related to hydrophobic, electrostatic and carbohydrate interactions (Sammond et

al., 2014). pH is an important factor as it alters the surface hydrophobicity by inducing a surface charge, this can also affect electrostatic interaction between lignin and cellulose. (Lan et al., 2013).

The aim of the research in this paper was to investigate the optimal pH range for different commercial enzyme cocktails that gives maximal lignocellulosic saccharfication during the enzymatic hydrolysis for the WS as a lignocelluloses substrates instead of the pure cellulosic substrate. The optimum pH for pure cellulosic substrate is established to be 4.8 which is also widely used as the optimum pH for lignocellulosic substrates during enzymatic hydrolysis. This study highlights that the optimum pH for pure cellulosic substrate (i.e whatman filter paper) is not necessarily the optimum pH for lignocellulosic materials during enzymatic hydrolysis.

# Experimental

## Materials and Methods

Celluclast® ® 1.5 L, Novozyme 188 (β-glucosidase) and endo-1, 4-β-Xylanase were purchased from Sigma-Aldrich Co., UK, while Cellic® CTec2 enzyme was garrulously provided by Novozymes Biotechnology Co., Ltd (Tianjin, China). The enzymes activities were measured according to the standard procedure (Ghose, 1987). The Celluclast® ® 1.5 L and Cellic® CTec2 cellulase activities were and found to be 74 filter paper unit (FPU) $mL^{-1}$ and 140 (FPU) $mL^{-1}$ respectively, the Novozyme activity was 760 cellobiase unit (CBU) $mL^{-1}$ and the endo-1, 4-β-Xylanas activity is 7700 $Ug^{-1}$ Sodium citrate buffer, sugar standards (glucose, xylose, galactose, mannose, arabinose, cellobiose), hydrochloric acid, sodium hydroxide, sodium azide, Whatman no. 1 filter paper strip, 3, 5-dinitrosalicylic (DNS) acid and Rochelle salt (sodium potassium tartrate tetrahydrate) were purchased from Fisher Scientific, UK. All experiments in this study were conducted using WS generously supplied from a local farm in Driffield, East Riding of Yorkshire, UK (Harvest Summer 2017).

## Raw material preparation

To remove the surface dirt the WS was washed with distilled water several times until the residue colour become white. The washed WS was then dissected into smaller parts using a knife blender (Luvele Power-Plus Blender | 2200w, UK) and milled using a laboratory ceramic desk grinder (Waldner, Biotech GMBH). The milled straw was then sieved (AS-200 control, Retsch GmbH) to get uniform particle sizes within a range more than 2000 to less than 250 µm and dried at 35º C ± (2 °C) in a drying cabinet for 24 hrs. The moisture content was determined according to NREL protocol and found to be in the range of 8-10 % (Sluiter et al., 2008).

## Enzymatic hydrolysis assay

The dried WS biomass was enzymatically hydrolysed to release monomeric sugars from cellulosic materials. This was achieved using 1g of dried WS in 50 mL of buffer solution (sodium citrate 0.05 M) allowing a total working volume of 50 mL. Prior to hydrolysis, 0.02% w/w sodium azide was added to the samples, before addition of the enzyme, to inhibit the microbial growth as this may consume the monomeric sugar produced and

inhibit the enzyme's activity (da Costa Lopes et al., 2013, Qi et al., 2009). The samples were incubated for 60 minutes at (50 ºC, 200 rpm) in a laboratory shaker/incubator (Orbital incubator SI 500, Stuart, UK). The hydrolysis proceeded under mild conditions (50 ºC, 200 rpm) in the shaker/incubator for 94 hrs. The pH of the buffer solution was adjusted between 3.0 – 7.0 using 1M sodium hydroxide and 1M hydrochloric acid.

To investigate the pH effect on different enzymes, a cocktail of Celluclast® 1.5 L with an activity loading of 15 FPU $g^{-1}$ DM and Novozyme 188 with an activity loading of 30 CBU $g^{-1}$ DM was used. Additionally, xylanase enzyme with an activity loading of 1540 U $g^{-1}$ DM was used for the pH investigation. The commercial cellulose enzyme cocktail Cellic® CTec2 with an activity loading of 15 FPU $g^{-1}$ DM was also selected for these experiments. The enzymatic hydrolysis was carried for 94 hrs, but it was found that after 72 hrs the total reduced sugar yield did not change, therefore 72 hrs was used as the end of the hydrolysis instead of 94 hrs. Aliquots of the hydrolysate were withdrawn every 24 hrs from the hydrolysis to check the total reduced sugar residue. These aliquots were boiled for 5 minutes to stop the enzymes activity and were then centrifuged (centrifuge 5702, Eppendorf, UK) at 4500 rpm for 5 minutes. The supernatants were sampled for total reduced sugar analysis using 3,5-dinitrosalicylic acid (DNS) reagent as described below (Miller, 1959). All the hydrolysis experiments were carried out in triplicate to ensure reproducibility.

## Analytical methods

The raw WS carbohydrate composition, reduced sugar yield and carbohydrate composition in different WS samples were determined with the help of standard laboratory analytical procedure. The details of the analysis are as follows:

### Raw wheat straw composition using HPLC

The carbohydrate composition of raw WS was determined by the NREL standard protocol (Sluiter et al., 2010). Oven-dried WS (0.3 g) was hydrolyzed with 3 mL of 72 % sulfuric acid for 60 minutes at 30 ºC in a water bath. The samples were then diluted with 84 mL of deionized water to an acid concentration of 4 % and autoclaved for another 60 minutes at 121 ºC. The hydrolysis liquor was neutralized using solid calcium carbonate to pH (5.0 – 6.0) and centrifuged for 10 minutes at 4400 rpm. The supernatant was filtered by passing through a 2 µm filter paper and collected for the determination of the carbohydrates and lignin composition. High performance liquid chromatography (HPLC, Nexera-1, Shimadzu) with a UV detection at 280 nm was used to determine the carbohydrates composition. The instrument was equipped with a Shodex sugar SP0810 column, the separation was carried out at 80º C. Deionized water was used as an eluent in a flow rate of 0.6 mL $minute^{-1}$ with 20 µm injected sample volume. The WS composition was 41 % cellulose, 33 % hemicellulose, 18 % lignin and 8% others.

~~Sugar analysis~~

### Total reduced sugar yield using UV/Visible

The DNS method was used to measure the reduced sugar yield, by mixing 3 mL of DNS reagent and 1 mL of sodium citrate buffer (0.05M) with 0.5 mL of hydrolysate

supernatants. This mixture was submerged into a boiling water bath for 5 minutes then cooled to room temperature in a water-ice bath. 1.5 mL of sodium citrate buffer (0.05 M) and 3 mL of DNS reagent was used as a blank. All analyses were carried out in triplicate using Bibby Scientific™ 7305 Model UV/Visible Spectrophotometer at 540 nm wavelength. A calibration curve was obtained for glucose as it is the major product from WS. The calibration curve equation is $Y = 0.3098 X + 0.0618$ with $R^2 = 0.9957$, where Y represents absorbance and X represents the total reduced sugar concentration (1mg/0.5mL).

**Composition analysis using GC-MS**

The sugar extracted at the end of hydrolysis was centrifuged at 4400 rpm for 5 minutes and filtered through 0.2 µm filter paper. The samples were then evaporated to dryness, treated with 300 µL of methoxyamine hydrochloride solution in pyridine at a concentration of 20 mg $mL^{-1}$ and incubated at 37º C for 90 minutes. Aliquots equal to 300µL of n-Methyl-n-(trimethylsilyl) trifluoroacetamide (MSTFA) were added and incubated for another 60 minutes at the same temperature. The reduced sugar was then analyzed by gas chromatography–mass spectrometry (GC-MS) using an Agilent 6890 plus GC with a 5973N MS, (Agilent Technologies, Palo Alto, CA, USA) equipped with a Restek column (30 m × 0.25 mm × 0.25 µm, RxI-5MS, Bellafonte, PA, USA). The GC oven temperature was kept constant for 1 minute at 70 °C and gradually increased at a fixed rate of 5 °C $minute^{-1}$ until 320 °C. The injection port and transfer line temperatures were 260 °C and 280 °C, respectively. The carrier gas (helium) flow rate was 1 mL $minute^{-1}$. The injection volume was 1.0 µL with a split injection ratio of 50:1. The data were recorded in the mass range of 50 – 500 m/z and the results were specified by comparison (cross match) with standards sugars (Yang et al., 2013). The average results of duplicate runs were reported.

## Results and Discussions

**pH evaluation before and after enzyme addition**

The main objective of this study was to investigate the effect of changing the pH during the enzyme hydrolysis, therefore pH values were measured before and after addition of the enzymes. The measured pH values are reported in Table 1 for both before and after addition of the enzymes to the suspension (buffer solution and WS). Since the pH value increased as a result of adding the enzymes, the pH of the solutions were adjusted back to the original pH values, this is reported as pH-adjusted in Table 1. The results reported in Table 1 are the average of three replicates for each enzymes and pH- value.

It was found that at low pH values, the change was higher after adding the enzymes than at high pH values due to the low acidity of the enzymes (pH 6.0-6.5). The highest increase in the pH value was noticed after adding the Ctec 2 to the pH 3 solution, with the pH value increasing from 3.0 to 3.61. Whiles the lowest change occurs after adding the Cellic® CTec2 to the solution with pH 7.0, the increase was very low and was neglected.

**Table 1:** Measured pH values before and after addition of the enzymes and adjusted

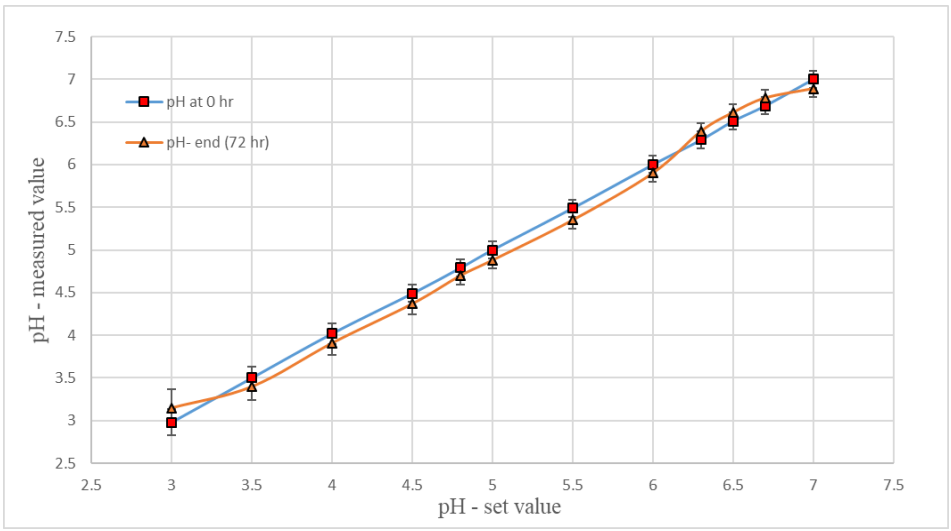
| | pH- After adding the enzymes | | | pH- Adjusted | | |
|---|---|---|---|---|---|---|
| pH before adding the enzymes | Celluclast® 1.5L + Novozymes 188 | endo-1, 4-β-Xylanase | Cellic® CTec2 | Celluclast® 1.5L + Novozymes 188 | endo-1, 4-β-Xylanase | Cellic® CTec2 |
| 3.00 | 3.55 ± 0.1 | 3.41 ± 0.07 | 3.61 ± 0.08 | 3.00 ± 0.01 | 2.99 ± 0.01 | 3.00 ± 0.01 |
| 3.50 | 3.96 ± 0.07 | 3.85 ± 0.05 | 4.0 ± 0.1 | 3.49 ± 0.01 | 3.5 ± 0.01 | 3.50 ± 0.01 |
| 4.00 | 4.40 ± 0.08 | 4.32 ± 0.08 | 4.42 ± 0.07 | 4.05 ± 0.01 | 4.02 ± 0.01 | 4.00 ± 0.01 |
| 4.50 | 4.79 ± 0.05 | 4.79 ± 0.06 | 4.70 ± 0.09 | 4.52 ± 0.01 | 4.49 ± 0.01 | 4.50 ± 0.01 |
| 4.80 | 5.10 ± 0.07 | 5.10 ± 0.06 | 5.20 ± 0.05 | 4.8 ± 0.01 | 4.79 ± 0.01 | 4.80 ± 0.01 |
| 5.00 | 5.24 ± 0.07 | 5.20 ± 0.06 | 5.18 ± 0.05 | 5.00 ± 0.01 | 5.00 ± 0.01 | 5.03 ± 0.01 |
| 5.50 | 5.70 ± 0.05 | 5.68 ± 0.04 | 5.63 ± 0.03 | 5.50 ± 0.01 | 5.49 ± 0.01 | 5.48 ± 0.01 |
| 5.70 | 5.88 ± 0.05 | 5.81 ± 0.02 | 5.80 ± 0.03 | 5.69 ± 0.01 | 5.70 ± 0.01 | 5.70 ± 0.01 |
| 6.00 | 6.15 ± 0.02 | 6.15 ± 0.02 | 6.13 ± 0.03 | 6.01 ± 0.01 | 6.00 ± 0.01 | 5.99 ± 0.01 |
| 6.30 | 6.43 ± 0.04 | 6.40 ± 0.02 | 6.39 ± 0.02 | 6.30 ± 0.01 | 6.29 ± 0.01 | 6.29 ± 0.01 |
| 6.50 | 6.60 ± 0.02 | 6.59 ± 0.01 | 6.55 ± 0.02 | 6.49 ± 0.01 | 6.51 ± 0.01 | 6.49 ± 0.01 |
| 6.70 | 7.10 ± 0.02 | 6.77 ± 0.01 | 6.75 ± 0.03 | 7.00 ± 0.01 | 6.69 ± 0.01 | 7.00 ± 0.01 |
| 7.00 | 7.20 ± 0.03 | 7.05 ± 0.01 | 7.03 ± 0.01 | 6.99 ± 0.01 | 7.00 ± 0.01 | 7.03 ± 0.01 |

**pH value at the end of enzymatic hydrolysis**

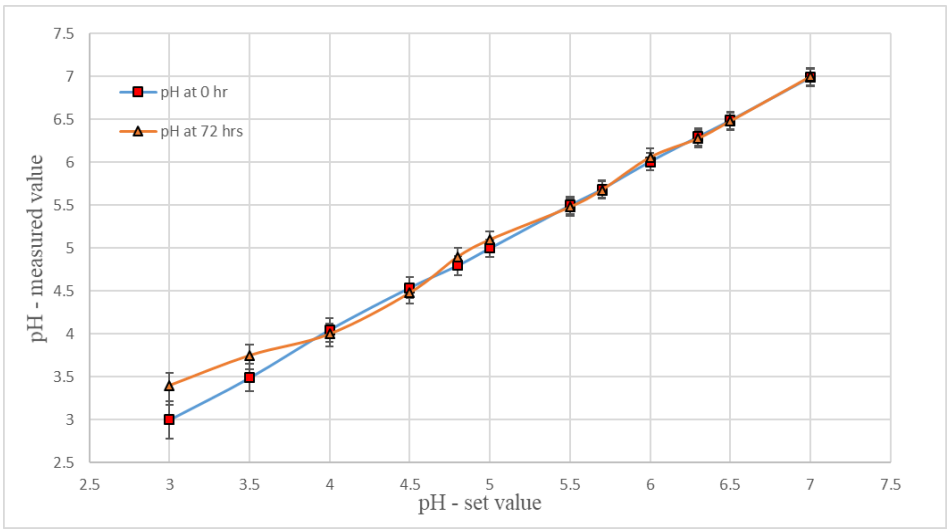
Due to the importance of the pH value, the pH at the start (0 hr) and at the end (72 hrs) of the enzymatic hydrolysis are shown in Figure 1 (a-c), for Celluclast® 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic® CTec2 respectively. The experiments were repeated three times with the average results presented graphically in Figure 1.

Figure 1(a) shows that for Celluclast® 1.5 l with Novozymes 188 there was a minor increase in pH at the end of the enzymatic hydrolysis. The difference in pH values were less than 0.17% at the maximum difference. On the other hand with endo-1, 4-β-Xylanase (Figure 1 (b)) there was an increase of 0.26% at the end of the enzymatic hydrolysis. A negligible change was observed after pH 4.0, and for pH 5.0 - 7.0, the pH value remained the same. The greatest change was seen for Cellic® CTec2 as shown in Figure 1 (c). Between pH 3.0 – 4.8, there was an increase in pH at the end of the enzymatic hydrolysis. The highest increase was found at pH 3.0 where the pH increased from 3.0 at (0 hr) to 3.41 at (72 hrs). This means that there was approximately 13% increase in the pH value at the end of hydrolysis.

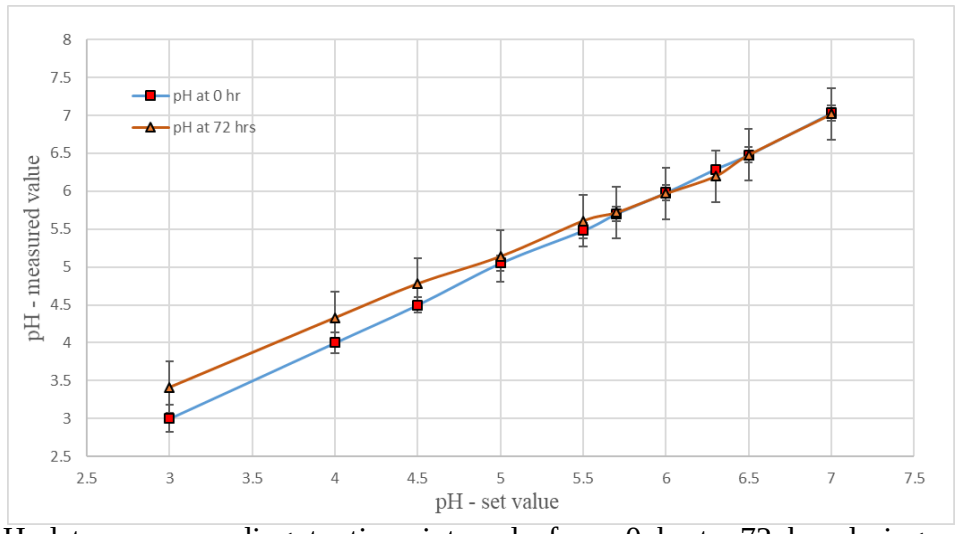
Although both endo-1, 4-β-Xylanase and Ctec 2 show the highest difference in pH value at pH 3.0, 3.5 and pH 3.0, 3.5, 4.0, 4.5 respectively, the difference is not very high and is within the error bar. Therefore, the adjusted pH value at (0 hr) and the final pH measured at each different pH point studied show no significant difference and can be assumed to be the same.




(a)




(b)




(c)

**Fig. 1.** pH data corresponding to time intervals from 0 hr to 72 hrs during enzymatic hydrolysis using three different enzymes ((a) Celluclast® 1.5L with Novozymes 188, (b) endo-1, 4-β-Xylanase and (c) Cellic® CTec2).

**Influence of WS particle size on reduced sugar yield**

WS particle size is a fundamental parameter that effects biomass digestion efficiency. It has been suggested that grinding the WS to a fine size, breaks down the lignin as well as increasing the surface area of the sample which gives the enzymes more accessibility and facilitates the biomass digestion (Hu et al., 2017).

The WS was ground using a ceramic disk and sieved to get different particle sizes ranging from less than 250 to more than 2000 µm. Then the range of samples with different particle size was subjected to enzymatic hydrolysis at pH 4.8 using (Celluclast® 1.5L + Novozymes 188, endo-1, 4-β-Xylanase and Cellic® CTec2) for 94 hrs respectively. The hydrolysis was carried out for 24 hrs longer than the normal 72 hrs to ensure reaction completion.

The reduced sugar yield was found to increase with smaller particle size as shown in Table 2. Grinding the WS to reduce the particle size increased the surface area and reduced the degree of crystallinity which gives more accessibility for enzymes and therefore increases the total reduced sugar yield (Silva et al., 2012). The total reduced sugar yield increased rapidly with time up to about 50 hrs then it begins to level out. After 72 hrs, there was no significant increase in the total reduced sugar yield. It can clearly be seen that higher reduced sugar yield was obtained from the finest particle size for all the enzymes. Therefore the sample which gave the highest reduced sugar yield (less than 250 µm) at pH 4.8 was chosen to study the pH effect on total reduced sugar yield during enzymatic hydrolysis.

**Table 2:** Influence of different particle size on total reduced sugar yield (g $L^{-1}$) at pH 4.8.

| | | Total reduced sugar produced (g $L^{-1}$) | | | |
|---|---|---|---|---|---|
| | | **24 hrs** | **48 hrs** | **72 hrs** | **94 hrs** |
| Celluclast® 1.5L + Novozymes 188 | >2000 µm | 1.6 ± 0.28 | 3.2 ± 0.27 | 3.7 ± 0.28 | 3.7 ± 0.28 |
| | 200-1000 µm | 1.8 ± 0.27 | 3.3 ± 0.29 | 3.9 ± 0.33 | 3.9 ± 0.33 |
| | 1000-710 µm | 2.5 ± 0.29 | 3.6 ± 0.29 | 4.1 ± 0.32 | 4.1 ± 0.32 |
| | 710-500 µm | 2.7 ± 0.31 | 3.8 ± 0.28 | 4.2 ± 0.3 | 4.2 ± 0.3 |
| | 500-250 µm | 2.9 ± 0.25 | 4.1 ± 0.3 | 4.8 ± 0.34 | 4.8 ± 0.34 |
| | <250 µm | 3.2 ± 0.3 | 4.8 ± 0.38 | 5.2 ± 0.4 | 5.2 ± 0.4 |
| endo-1, 4-β- Xylanase | >2000 µm | 1.8 ± 0.26 | 3.2 ± 0.31 | 3.8 ± 0.25 | 3.8 ± 0.25 |
| | 200-1000 µm | 1.9 ± 0.26 | 3.3 ± 0.3 | 3.9 ± 0.3 | 3.9 ± 0.3 |
| | 1000-710 µm | 2.6 ± 0.28 | 3.7 ± 0.25 | 4.3 ± 0.35 | 4.3 ± 0.35 |
| | 710-500 µm | 2.7 ± 0.33 | 3.9 ± 0.28 | 4.4 ± 0.22 | 4.4 ± 0.22 |
| | 500-250 µm | 3.1 ± 0.32 | 4.2 ± 0.3 | 4.9 ± 0.29 | 4.9 ± 0.29 |
| | <250 µm | 3.5 ± 0.3 | 4.9 ± 0.27 | 5.5 ± 0.26 | 5.5 ± 0.26 |
| Cellic® CTec2 | >2000 µm | 4.1 ± 0.51 | 5.2 ± 0.6 | 5.8 ± 0.54 | 5.8 ± 0.54 |
| | 200-1000 µm | 4.3 ± 0.58 | 5.4 ± 0.59 | 6.0 ± 0.62 | 6.0 ± 0.62 |
| | 1000-710 µm | 4.4 ± 0.62 | 5.9 ± 0.65 | 6.5 ± 0.6 | 6.5 ± 0.6 |
| | 710-500 µm | 5.2 ± 0.62 | 6.7 ± 0.45 | 7.2 ± 0.52 | 7.2 ± 0.52 |
| | 500-250 µm | 6.1 ± 0.56 | 7.5 ± 0.4 | 8.0 ± 0.51 | 8.0 ± 0.51 |
| | <250 µm | 6.5 ± 0.64 | 8.5 ± 0.6 | 8.8 ± 0.58 | 8.8 ± 0.58 |

**Influence of pH on WS enzymatic hydrolysis using Celluclast® 1.5L with Novozymes 188**

Using the smallest particle size (less than 250 µm) 1 g of WS was enzymatically hydrolyzed using 50 mL of various pH (3.0 – 7.0) solutions at 50 ºC and 200 rpm. Celluclast® 1.5L with Novozymes 188 was subjected to pH study since they are widely used for lignocellulosic enzymatic hydrolysis (Hu et al., 2015, Lan et al., 2013). The total reduced sugar yield was obtained using the DNS method and plotted against the pH at the end of hydrolysis (72 hrs) as shown in Figure 2.

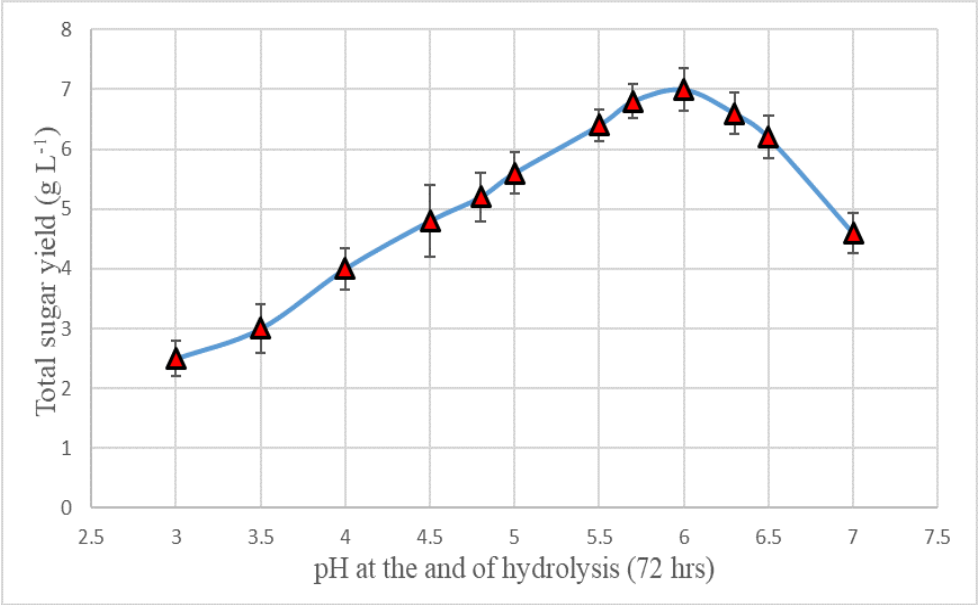



**Fig. 2.** Total reduced sugars concentration for different pH solution at the end of the hydrolysis (72 hrs).

As can be seen from Figure 2, the highest reduced sugar yield was achieved between pH 5.7 – 6.3 rather than at 4.8 as cited by most researchers (Lan et al., 2013). The total reduced sugar yield increased from 5.2 (g $L^{-1}$) to 7.0 (g $L^{-1}$) by changing the pH value from 4.8 to 6.0 respectively.

To give further confirmation, the total reduced sugar yield was observed between 0 – 72 hrs at pH 4.8 and 6.0 and shown in Figure 3, which clearly indicates that the total reduced sugar yield for the WS substrate increased from 5.1 (g $L^{-1}$) to 7.1 (g $L^{-1}$) (approximately 28 %).

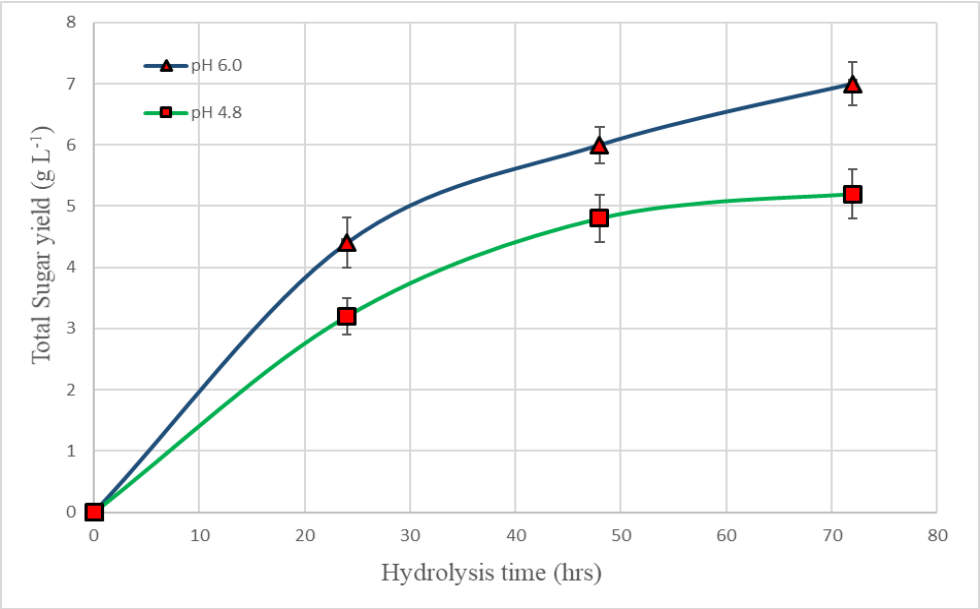



**Fig. 3.** Total reduced sugars yield at the end of Hydrolysis (72 hrs) for pH 4.8 and 6.0.

**Influence of pH on WS enzymatic hydrolysis using endo-1, 4-β-Xylanase**

The experiment was repeated using the same conditions for the endo-1, 4-β-Xylanase enzyme as shown in Figure 4. Similarly to the previous enzyme cocktail, pH 4.8 – 5.0 is currently the preferred value for enzymatic hydrolysis (Avci et al., 2013). It can be seen that there was a detectable increase in total reduced sugar yield efficiency from 3.1 – 7.4 (g $L^{-1}$) in the pH range of 3.0 – 6.0 with the optimum range being pH 5.7 – 6.0 instead of 4.8 as widely used by researchers.

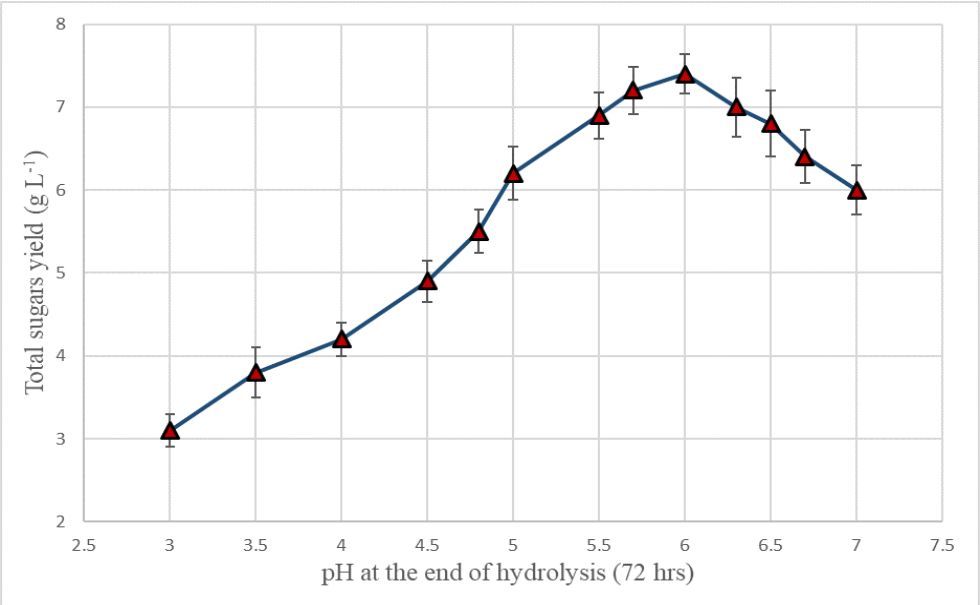



**Fig. 4.** Total reduced sugars concentration for different pH solution at the end of the hydrolysis (72 hrs).

Figure 5 shows a similar trend in the change of total reduced sugar yield with time by using endo-1, 4-β- Xylanase. The total reduced sugar yield increased from 5.5 (g $L^{-1}$) to 7.4 (g $L^{-1}$) at pH 4.8 and 6.0, respectively.

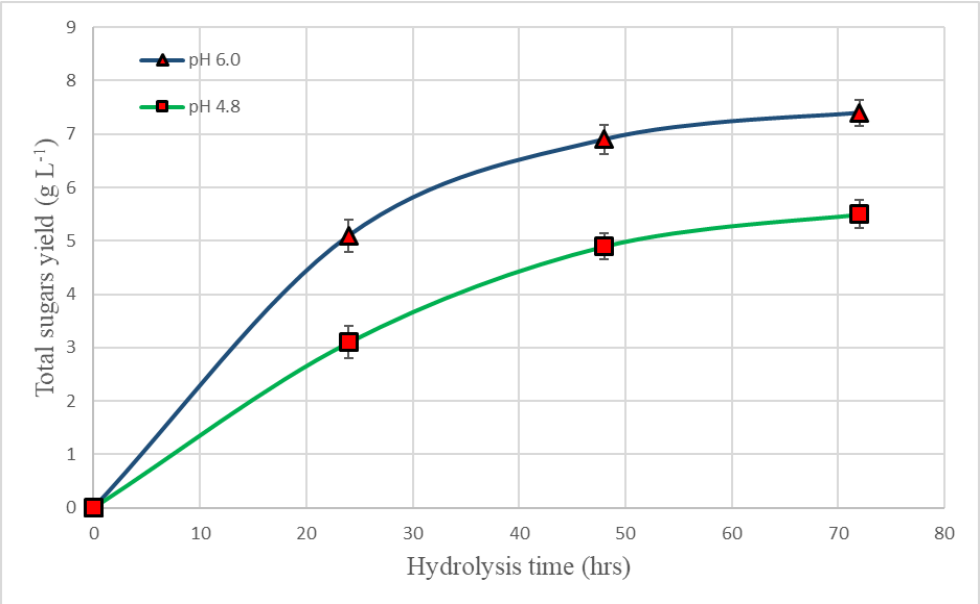



**Fig. 5.** Total reduced sugars yield at the end of hydrolysis (72 hrs) for pH 4.8 and 6.0

**Influence of pH on WS enzymatic hydrolysis using Cellic Cellic® CTec2**

Cellulase Cellic® CTec2 is a commercial enzyme cocktail which was also subjected to the optimum pH investigation. The WS was enzymatically hydrolyzed under the same experimental conditions as for the previous enzymes (Celluclast® 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase). Figure (6) shows the total reduced sugar yield plotted against the pH value at the end of enzymatic hydrolysis (72 hrs).

By increasing the pH from 4.8 to 6.0, the total reduced sugar yield increased from 8.5 (g $L^{-1}$) to 10.8 (g $L^{-1}$). The total reduced sugar yield for both pH 4.8 and 6.0 was also monitored with time during the hydrolysis, and the results are shown in Figure 7. The total reduced sugars yield from pH 4.8 and 6.0 behave similarly with time. The gap between the reduced sugar concentration was almost constant at 2.2 (g $L^{-1}$) during the hydrolysis. Therefore, it is recommended to use pH 6.0 to achieve high reduced sugar yield from WS.

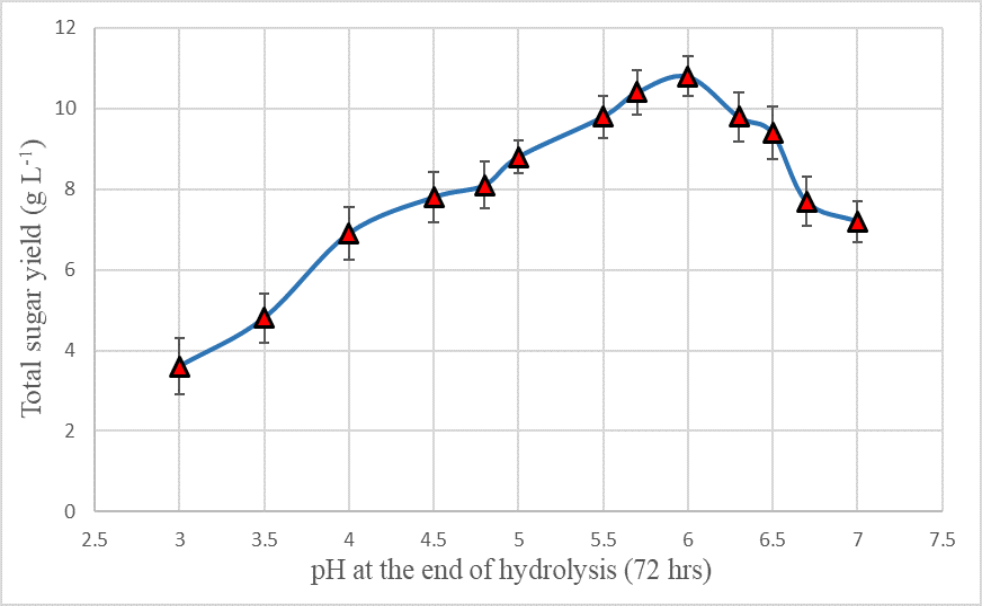



**Fig. 6.** Total reduced sugars concentration for different pH solution at the end of the hydrolysis (72 hrs).

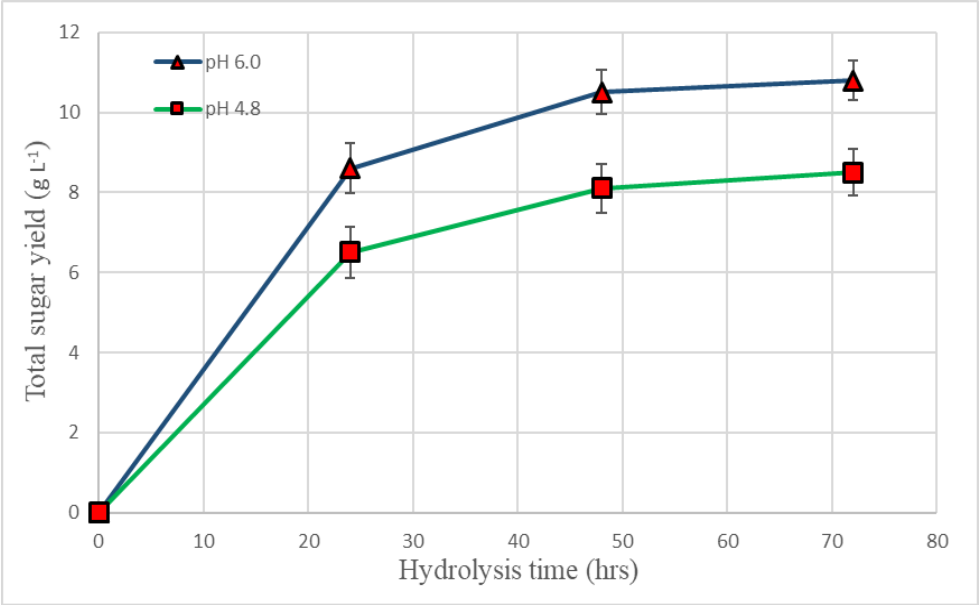



**Fig. 7.** Total reduced sugars yield at the end of Hydrolysis (72 hrs) for pH 4.8 and 6.0

In summary, all the enzymes used in this study show an improvement after changing the pH. Figure 8 illustrates the total reduced sugar yield after enzymatic hydrolysis for the enzymes at pH 4.8 and 6.0. By changing the pH of the solution from 4.8 to 6.0, Celluclast® 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase show an increase in the total reduced sugar yield from 5.2 (g $L^{-1}$) to 7.0 (g $L^{-1}$) and 5.5 (g $L^{-1}$) to 7.4 (g $L^{-1}$) respectively. In the case of Cellic Ctec 2, the total reduced sugar increased from 8.5 (g $L^{-1}$) to 10.8 (g $L^{-1}$).

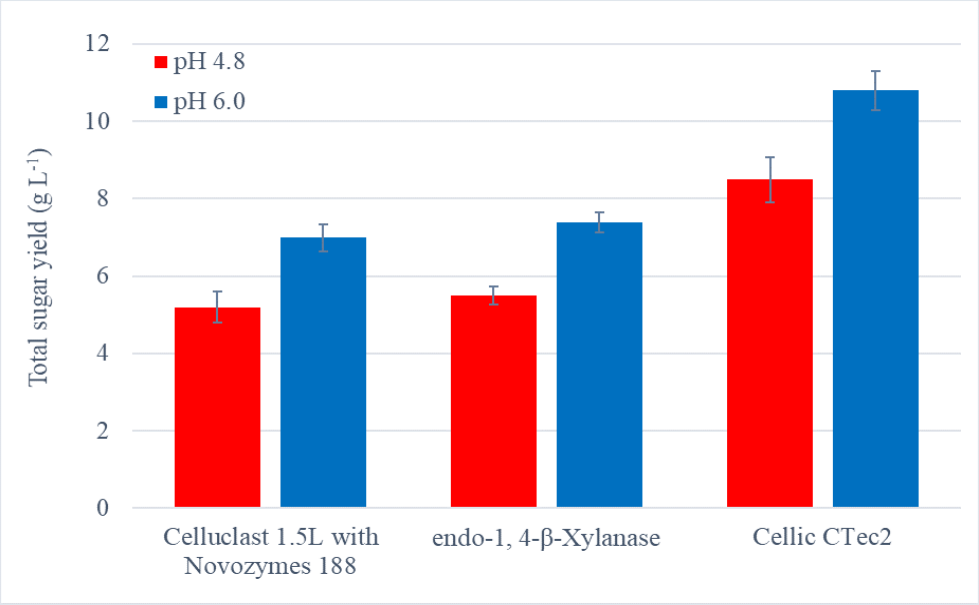



**Fig. 8.** Total reduced sugar yield for the enzymes at pH 4.8 and 6.0

## Conclusion

The results of the present study indicate the optimum pH for enzymatic hydrolysis using different enzymes (Celluclast® 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic® CTec2) is different from the range pH 4.8 – 5.0 used in most studies. The enzymatic hydrolysis was carried out for 94 hrs in the beginning, however, since there was no change in the reduced sugar yield after 72 hrs, here was no need to continue with enzymatic hydrolysis and the enzymatic hydrolysis was stopped after 72 hrs.

The results obtained from this study indicate that the optimum pH for WS as a lignocellulosic substrate is higher than pH 4.8 which is exclusively used by almost all the existing literature. The enzymes activity test based on using pure cellulose substrate (Whatman paper) at pH 4.8 as an optimum pH suggested by cellulase manufacturers is not necessarily the same optimum value for lignocellulosic (i.e. WS) substrate.

Reducing the acidity in lignocellulosic substrates enzymatic hydrolysis might have an effect on reducing lignin inhibition of the activity of the enzyme, by reducing the lignin absorption of enzymes or affecting the lignin-cellulose binding and interaction by affecting the electrostatic charge of the lignocellulose, changing the pH could also have an effect on the lignin-derived phenols.

All the enzymes which were used in this study show a significant improvement in total reduced sugar yield after changing the pH from 4.8 to 6.0, both Celluclast® 1.5L with Novozymes 188, endo-1, 4-β-Xylanase shows an increase of (25%) while Ctec 2 shows an increase of (21%).

Based on the results presented in this study, it is recommended that future work on enzymatic hydrolysis of WS as a lignocellulose substrate be conducted at a pH range of 5.8 – 6.0.

**List of abbreviations**

**WS** Wheat Straw

**FPU** Filter Paper Unit

**DM** Dry Matter

**CBU** Cellobiase Unit

**DNS** 3, 5-Dinitrosalicylic acid

**HPLC** High Performance Liquid Chromatography

**GC-MS** Gas chromatography–mass spectrometry

**UV** Ultraviolet

**MSTFA** n-Methyl-n-(trimethylsilyl) trifluoroacetamide

**List of nomenclature**

**Y** Absorbance

**X** Concentration **(**mg $0.5mL^{-1}$)

## Declarations

## Availability of data and material

All raw data used in this manuscript are available and could be supplied upon request

## Competing interests

There are no conflicts to declare.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## Authors' contributions

All authors approved the final version of the manuscript for publication


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**References**


AVCI, A., SAHA, B. C., KENNEDY, G. J. & COTTA, M. A. 2013. Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant Escherichia coli FBR5 without detoxification. *Bioresource technology,* 142**,** 312-319.

BONAWITZ, N. D. & CHAPPLE, C. 2010. The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annual review of genetics,* 44**,** 337-363.

DA COSTA LOPES, A. M., JOÃO, K. G., RUBIK, D. F., BOGEL-ŁUKASIK, E., DUARTE, L. C., ANDREAUS, J. & BOGEL-ŁUKASIK, R. 2013. Pre-treatment of lignocellulosic biomass using ionic liquids: wheat straw fractionation. *Bioresource technology,* 142**,** 198-208.

DE ASSIS CASTRO, R. C., FONSECA, B. G., DOS SANTOS, H. T. L., FERREIRA, I. S., MUSSATTO, S. I. & ROBERTO, I. C. 2017. Alkaline deacetylation as a strategy to improve sugars recovery and ethanol production from rice straw hemicellulose and cellulose. *Industrial Crops and Products,* 106**,** 65-73.

DOS SANTOS, A. C., XIMENES, E., KIM, Y. & LADISCH, M. R. 2018. Lignin–Enzyme Interactions in the Hydrolysis of Lignocellulosic Biomass. *Trends in Biotechnology*.

GHOSE, T. 1987. Measurement of cellulase activities. *Pure and applied Chemistry,* 59**,** 257-268.

GOVUMONI, S. P., KOTI, S., KOTHAGOUNI, S. Y., VENKATESHWAR, S. & LINGA, V. R. 2013. Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production. *Carbohydrate polymers,* 91**,** 646-650.

HU, J., CHANDRA, R., ARANTES, V., GOURLAY, K., VAN DYK, J. S. & SADDLER, J. N. 2015. The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. *Bioresource technology,* 186**,** 149-153.

HU, J., JING, Y., ZHANG, Q., GUO, J. & LEE, D.-J. 2017. Enzyme hydrolysis kinetics of micro-grinded maize straws. *Bioresource technology,* 240**,** 177-180.

JØRGENSEN, H., KRISTENSEN, J. B. & FELBY, C. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining,* 1**,** 119-134.

KUMAR, R., SINGH, S. & SINGH, O. V. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of industrial microbiology & biotechnology,* 35**,** 377-391.

LAN, T., LOU, H. & ZHU, J. 2013. Enzymatic saccharification of lignocelluloses should be conducted at elevated pH 5.2–6.2. *BioEnergy Research,* 6**,** 476-485.

MAITAN-ALFENAS, G. P., VISSER, E. M. & GUIMARÃES, V. M. 2015. Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products. *Current Opinion in Food Science,* 1**,** 44-49.

MANSFIELD, S. D., MOONEY, C. & SADDLER, J. N. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnology progress,* 15**,** 804-816.

MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry,* 31**,** 426-428.

NAKAGAME, S., CHANDRA, R. P., KADLA, J. F. & SADDLER, J. N. 2011. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnology and bioengineering,* 108**,** 538-548.

OLADI, S. & AITA, G. M. 2018. Interactive effect of enzymes and surfactant on the cellulose digestibility of un-washed and washed dilute ammonia pretreated energy cane bagasse. *Biomass and Bioenergy,* 109**,** 221-230.

PROCENTESE, A., RAGANATI, F., OLIVIERI, G., RUSSO, M. E. & MARZOCCHELLA, A. 2017. Pre-treatment and enzymatic hydrolysis of lettuce residues as feedstock for bio-butanol production. *Biomass and bioenergy,* 96**,** 172-179.

QI, B., CHEN, X., SHEN, F., SU, Y. & WAN, Y. 2009. Optimization of enzymatic hydrolysis of wheat straw pretreated by alkaline peroxide using response surface methodology. *Industrial & Engineering Chemistry Research,* 48**,** 7346-7353.

QIU, J., MA, L., SHEN, F., YANG, G., ZHANG, Y., DENG, S., ZHANG, J., ZENG, Y. & HU, Y. 2017. Pretreating wheat straw by phosphoric acid plus hydrogen peroxide for enzymatic saccharification and ethanol production at high solid loading. *Bioresource Technology,* 238**,** 174-181.

RAJPUT, A. A. & VISVANATHAN, C. 2018. Effect of thermal pretreatment on chemical composition, physical structure and biogas production kinetics of wheat straw. *Journal of environmental management,* 221**,** 45-52.

SAMMOND, D. W., YARBROUGH, J. M., MANSFIELD, E., BOMBLE, Y. J., HOBDEY, S. E., DECKER, S. R., TAYLOR, L. E., RESCH, M. G., BOZELL, J. J. & HIMMEL, M. E. 2014. Predicting enzyme adsorption

to lignin films by calculating enzyme surface hydrophobicity. *Journal of Biological Chemistry*, jbc. M114. 573642.

SARKAR, N., GHOSH, S. K., BANNERJEE, S. & AIKAT, K. 2012. Bioethanol production from agricultural wastes: an overview. *Renewable energy,* 37**,** 19-27.

SELIG, M. J., VIAMAJALA, S., DECKER, S. R., TUCKER, M. P., HIMMEL, M. E. & VINZANT, T. B. 2007. Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. *Biotechnology progress,* 23**,** 1333-1339.

SILVA, G. G. D., COUTURIER, M., BERRIN, J.-G., BULÉON, A. & ROUAU, X. 2012. Effects of grinding processes on enzymatic degradation of wheat straw. *Bioresource technology,* 103**,** 192-200.

SLUITER, A., HAMES, B., HYMAN, D., PAYNE, C., RUIZ, R., SCARLATA, C., SLUITER, J., TEMPLETON, D. & WOLFE, J. 2008. Determination of total solids in biomass and total dissolved solids in liquid process samples. *National Renewable Energy Laboratory, Golden, CO, NREL Technical Report No. NREL/TP-510-42621*, 1-6.

SLUITER, A., HAMES, B., RUIZ, R., SCARLATA, C., SLUITER, J., TEMPLETON, D. & CROCKER, D. 2010. Determination of structural carbohydrates and lignin in biomass. *Laboratory analytical procedure*.

SUN, F., MUKASEKURU, M. R., TAN, L., REN, J., HUANG, Z., REN, H. & ZHANG, Z. 2018. Optimization of on-site cellulase preparation for efficient hydrolysis of atmospheric glycerol organosolv pretreated wheat straw. *Journal of Chemical Technology & Biotechnology*.

TALEBNIA, F., KARAKASHEV, D. & ANGELIDAKI, I. 2010. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology,* 101**,** 4744-4753.

YANG, M., KUITTINEN, S., ZHANG, J., KEINÄNEN, M. & PAPPINEN, A. 2013. Effect of dilute acid pretreatment on the conversion of barley straw with grains to fermentable sugars. *Bioresource Technology,* 146**,** 444-450.

YANG, M., ZHANG, J., KUITTINEN, S., VEPSÄLÄINEN, J., SOININEN, P., KEINÄNEN, M. & PAPPINEN, A. 2015. Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone–butanol–ethanol fermentation. *Bioresource technology,* 189**,** 131-137.

ZHENG, Q., ZHOU, T., WANG, Y., CAO, X., WU, S., ZHAO, M., WANG, H., XU, M., ZHENG, B., ZHENG, J. & GUAN, X. 2018. Pretreatment of wheat straw leads to structural changes and improved enzymatic hydrolysis. *Scientific Reports,* 8**,** 1321.

# Optimization of pH as a Strategy to Improve Enzymatic Saccharification of Wheat Straw for Enhancing Bioethanol Production

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