Expression of Serpins by *Clostridium thermocellum* and Simultaneous Saccharification of Lignocellulosic Biomass to Enhance Ethanol Production

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Utilization of biomass for production of second generation bioethanol was considered as a way to reduce burdens of fossil fuel in Pakistan. The materials wheat straw, rice straw, cotton stalk, corn stover, and peel wastes were used in this experiment. Various parameters, such as acidic and alkali pretreatment, enzymatic hydrolysis by cellulases, and effect of proteases inhibitors on ethanol production, were examined. Fermentation was completed by the yeasts *Saccharomyces cerevisiae* and *Clostridium thermocellum* separately, and their ethanol production were compared and maximum ethanol yield was obtained with wheat straw *i.e*.,11.3 g/L by *S. cerevisiae* and 8.5 g/L by *C. thermocellum*. Results indicated that a higher quantity of sugar was obtained from wheat straw (19.6 ± 1.6 g/L) followed by rice straw (17.6 ± 0.6 g/L) and corn stover (16.1 ± 0.9 g/L) compared to the other evaluated biomass samples. A higher yield of ethanol (11.3 g/L) was observed when a glucose concentration of 21.7 g/L was used, for which yeast fermentation efficiency was 92%. Results also revealed the increased in ethanol production (93%) by using celluases in combination with recombinant Serine protease inhibitors from *C. thermocellum*. It is expected that the use of recombinant serpins with cellulases will play a major role in the biofuel production by using agricultural biomass. This will also help in the economics of the biofuel.

Keywords: Biomass; Cellulose; Fermentation; Alcoholic fuels; Proteases inhibitors

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INTRODUCTION

Non-food biomass, one of the most abundant classes of organic substances on Earth, is mainly composed of lignocellulosic material and consists of cellulose (35 to 50%), hemicellulose (20 to 35%), and lignin (5 to 30%) (Huber *et al.* 2006). Different agricultural substances such as green leaves, fruit shells, various straws, nutshells, fruit seeds (Demirbas 2001) and feedstocks; wheat straw, wheat bran, corn stover, corn steep liquor, and apple pomace (Kim and Dale 2004; Ejezi *et al*. 2007) have been used as a renewable energy resource. Today, agricultural as well as municipal wastes are being used to produce biofuels (biodiesel, bioethanol, biohydrogen, and biomethane) from agricultural residues. A large amount lignocellulosic biomass is available that can be used to obtain bio-based products including bioethanols, *etc.* (Mahro and Timm 2007; Gomez *et al.* 2008).

Exploration of various sources for alternate energy has increased because of increasing concerns about energy security and climate change (Singhvi and Gokhale 2019). The transportation sector plays an important role in the emission of greenhouse gases due to the use of fossil fuels. However, replacement of oil-derived fuels with ethanol could reduce greenhouse gases and be helpful to improve the current situation of the environment as well as give advantages on social and economic levels (Humbird *et al.* 2011). Various alternatives to generate sustainable biofuels from biomass have been investigated for the biological energy resources including bioelectricity, biogases, biodiesel, and bioalcohols. Among these sources, bioethanol shows a great potential to reduce the emission of greenhouse gases, decrease the dependence on fossil fuel, and act as potential fuel for the transport sector (Dhamole *et al.* 2015). The production of bioethanol has been greatly improved because many countries are trying to reduce the import of oil, improve the quality of air, and grow rural economics. The global ethanol production is 29,000 million liters (National Renewable Fuels Association 2019). Ethyl alcohol has some advantages as a fuel, such as it has a higher oxygen content. The high oxygen level permits the improved oxidation of hydrocarbons with successive reduction in emissions of aromatic compounds and carbon monoxide, while ethanol has greater octane rating properties (Thomas and Kwong 2001).

Simultaneous saccharification and fermentation is a one-step process that includes polysaccharide hydrolysis and fermentation by exogenously produced enzymes. This process is more appealing because of low concentration of monomeric sugar, which is needed for the enzyme activity and ultimately it reduces cost by decreasing the amount of enzyme needed for the process (Lin and Lee 2011; Mohapatra *et al.* 2019).

Ethanol production from lignocellulosic biomass involves four main steps: 1. Pretreatment, 2. Hydrolysis, 3. Fermentation, and 4. Ethanol recovery by distillation. Various pretreatment methods have been suggested, depending upon the removal of hemicellulose or lignin from the biomass (Carvalho *et al.* 2017; Nargotra *et al.* 2018). Dilute acid pretreatment is the more promising pretreatment method as it increases the solubilization and digestibility of hemicellulose by breaking hydrogen bonds, as well as the partial degradation of cellulose and lignin (Mikulski and Kłosowski 2018). In addition, acid pretreatment makes the cellulose more accessible to enzymatic hydrolysis to convert fractions into glucose (Rezania *et al.* 2017). The choice of pretreatment technology for a particular raw material depends on several factors, some of them are directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate in addition to the pretreatment conditions has a great influence on biomass digestibility (Du *et al.* 2016; Zhu *et al.* 2018; Mikulski and Kłosowski 2020).

Saccharomyces cerevisiae (commonly known as baker's yeast) is a single-celled eukaryote that is frequently used in scientific research. *S. cerevisiae* is an attractive model organism because i) of its complete sequenced genome, ii) its genetics can be easily manipulated, and iii) it is easy to maintain in the lab. Most yeasts require oxygen for their growth; therefore continuous oxygen supply must be regularly checked. Some yeasts can ferment sugars to alcohol and carbon dioxide in the absence of air; therefore they need sugar as a substrate in addition to oxygen (Woo *et al.* 2014). They produce ethyl alcohol and carbon dioxide from simple sugars such as glucose and fructose. Yeasts are active in a broad temperature range from 0 to 50 °C, with an optimum temperature range of 20 to 30 °C. The optimum pH for most micro-organisms that are usually acid tolerant is near the neutral point (pH 7.0). Yeasts can grow in a pH range of 4 to 4.5 (Mountney and Gould 1988).

C. thermocellum is the most investigated and efficient biomass degrader. It is an anaerobic bacterium that can convert cellulose into ethanol directly. It can grow on different substrates besides cellulose (*e.g.*, cellobiose, xylose, and hemicellulose). Its growth temperature range is 50 to 68 °C, which is suitable for industrial processes (McBee 1954). Despite features that make *C. thermocellum* an ideal candidate for fermentation, its most important characteristic is the presence of cellulosome, a discrete enzyme unit and a multienzyme machinery comprising 20 different enzymes that aid in cellulose degradation (Artzi *et al.* 2017; Sanrattana *et al.* 2019).

Although *C. thermocellum* is a proven industrial ethanol producer in traditional starch-based processes, it will be no easy task to provide this microorganism with the ability to convert lignocellulosic biomass to ethanol. The carbohydrate components of lignocellulose (cellulose and hemicellulose) are tightly bound to lignin, making the sugars largely inaccessible to enzymes (Artzi *et al.* 2017). In addition, cellulosome contains serpins (serine protease inhibitors). The primary function of serpins is to neutralize the effect of serine proteases. The authors investigated the genome of *C. thermocellum* and found three serpin genes, two of them were present inside the cellulosome. The structure and function of serpins were poorly characterized. The presence of two of serpin genes can indicate their importance in cellulose degradation that can be helpful in the protection of cellulases against proteases attack. Therefore, in the current study, the amount of cellulase was supplemented with recombinant serpins to increase ethanol yield (Johnston and McAloon 2014).

Keeping in view the importance of bioethanol as a fuel, this study was conducted with the following objectives: (i) to treat lignocellulosic biomass with acid/alkali to obtain maximum amount of sugars; (ii) to optimize various conditions for yeast and bacterial fermentation process; and (iii) to study the effect recombinant protease inhibitor from *C. thermocellum* on ethanol production

EXPERIMENTAL

Collection of Agricultural Substrates

Various biomass samples (wheat and rice straws, cotton stalks, corn stover, and peel wastes) were collected from different areas of Punjab province (Pakistan). The samples were dried, ground to powder form, passed through a 40-mesh sieve and stored in fine plastic bags at lower temperature until use.

Analysis of Biomass Samples

Proximate analysis of all biomass samples was performed following methods reported in Association of Official Analytical Chemists (AOAC 1990). Percentages of moisture, ash, dry matter, crude protein, crude fiber, and crude fat were determined (Sluiter *et al.* 2008b). Cellulose, hemicellulose, and lignin contents were quantified using the standard method described by Su *et al.* (2015).

Acidic and Alkaline Pretreatment of Biomass Samples

Pretreatment process was performed using H_2SO_4 and NaOH (1.0, 1.5, and 2%) at diverse temperatures of 100 °C, 110 °C, and 120 °C for different durations (10, 20, and 40 min). Solid sample (10%) (w/v) in reagent bottle was utilized during the experiment. After

pretreatment, the vacuum filtration assembly was used for filtration of samples in each bottle and the contents were emptied onto filter paper. After filtration, the solid was washed away with 300 mL distilled water to neutralize the pH and the filter paper was then dried at 105 ℃ and weighed.

Enzymatic Hydrolysis

The biomass samples after pretreatment was hydrolyzed with cellulase and acid protease (Novozyme A/S (Bagsvaerd, Denmark) at 50 ℃ and 160 rpm for 72 h in a water bath shaker with 0.05 M buffer (sodium citrate) (Precision SWB 27; Pittsburgh, PA, USA) at 4.8 pH (Kim and Holtzapple 2005; Sun and Cheng 2005). Chloromphenicol (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and ampicillin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were also added during reaction to inhibit microbial growth. Cellulases from *Trichoderma reesei*, cellobiase from *Aspergillus niger*, and Novozyme 188 were delivered by Novozyme A/S (Bagsvaerd, Denmark) with an activity of (30 FPU g^{-1}). Serpin 191 and 1270 (Chemical Engineering Lab, University of Rochester, NY, USA) from *C. thermocellum* genome were cloned, purified, and characterized in the lab. The samples were withdrawn from reagent bottle every 12 h to determine the concentration of sugar.

Saccharification

The agro and municipal waste samples (wheat, cotton, rice straws, corn stover, and peel wastes) were taken as a solid loading of 5% (w/v) and then autoclaved. The enzymes were added to substrate with the ratio of substrate to enzyme 1:1 and placed for 72 h at 50 ℃. Both of the enzymes were added in a separate reaction mixture to check the individual enzymatic activity. After saccharification, the sugar contents were determined (Moretti and Thorson 2008). In a separate flask, purified recombinant serpin 191 and 1270 (0.75 mL) were added in addition to the enzyme mixture to study its effect on ethanol production.

Culture Conditions for Growth

Saccharomyces cerevisiae strain was maintained on YPD; Yeast Extract Peptone Dextrose (yeast extract 1% (w/v), peptone 2% (w/v), and glucose 2% (w/v)) agar medium at 4 ℃. Culturing of yeast cells was performed in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30 ℃ for 16 h on a rotary shaker (100 rpm) according to Alfenore *et al.* (2002). Cultural conditions for *C. thermocellum* were maintained according to method described by McBee (1954).

Separate Hydrolysis, Fermentation, and Recombinant Serpin (191 and 1270)

Each fermentation experiment was completed using *Saccharomyces cerevisiae* and *C. thermocellum* grown in broth medium for 48 h, and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature (Jiang *et al.* 2015). Exactly 0.75 mL of Serpin 191 and 1270 solution were added in a separate flask with *C. thermocellum* to study its effect on ethanol fermentation. Fermentation experiment was performed at 50 ℃ and 120 rpm for 72 h under anaerobic conditions. After completion of fermentation reaction, the obtained mixture containing methanol, butanol, ethanol, and acetone was removed by fractional distillation process in a fractional distillation apparatus (Quickfit SH4/33; SciLabware Stoke-on-Trent, Midlands, UK), on the basis of boiling point. Because butanol has a higher boiling

point (118 °C) than water (100 °C), butanol can be condensed and then separated. The boiling point of ethanol is lower (78.3 °C) in comparison with water, which is why it can be condensed earlier than water (Amiri and Karimi 2018).

High-performance Liquid Chromatography (HPLC) Analysis of Enzymatic Hydrolysate

The fermentation products, such as monomer sugars (hexoses and pentoses), acetone- butanol, and ethanol as well as other byproducts, were determined using the method reported by Haifeng *et al.* (2015). The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stover were further analyzed by HPLC. For this purpose, the samples that showed a higher amount of glucose at optimized conditions were used for analysis. The samples that were withdrawn at different times during enzymatic hydrolysis were centrifuged at 14,000 rpm, at 4 °C for 15 min. Supernatant was separated and then filtered using a 0.22-μm syringe filter. An aliquot of the sample (500 μL) was diluted with 1 mL methanol to bring the concentrations of the samples within the range of calibration curve. Methanol was used due to the solubility of the sugars. The samples and standard solution of glucose were passed through the 0.22-μm filter prior to analysis. A total of 20 μL of sample was injected through an injection loop into the HPLC system with acetonitrile:water (80:20) as mobile phase. The HPLC was performed on SHIMAZDU LC-20AT model having C-18 column of 25 mm length with internal diameter of 4.6mm. The analysis was performed in isocratic and reverse phase mode at 1 mL/min flow rate for 10 min. (Shields and Cathcart 2010).

Statistical Analysis

Data generated through various analyses were statistically analyzed for mean and standard deviation using an analysis of variance (ANOVA 1) and Graph Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used in this experiment.

RESULTS

Results regarding the physical and chemical analysis of biomass samples, pretreatments, enzymatic analysis, yeast, and bacterial fermentation as well as quantification of end products using HPLC are mentioned in following sections.

Proximate Analysis of Various Biomass Samples

Various samples of biomass were analyzed for the quantification of dry matter, moisture, crude protein, lipid ash, and fiber contents (Table 1). Analysis of these parameters (moisture, crude protein, lipid, ash, and fiber contents) will help in designing the better pretreatments processes with following phases *e.g.*, enzymatic hydrolysis for ethanol production

Pretreatment of Agricultural Substrates

Samples of wheat, cotton, rice (straws), and corn stover as well as peel wastes were used for the pretreatment process. A maximum amount of sugar (19.6 ± 1.6 g/L) was found in wheat straw when the sample was treated with 2% H_2SO_4 at 120 °C compared to rice straw (17.6 ± 0.6 g/L), corn stover (16 ± 0.9 g/L), and compared to other samples analyzed

(Table 2). During alkali pretreatment, a higher amount of sugar (18.5 ± 1.2 g/L) was released from wheat straw followed by rice straw (16.4 ± 1.5 g/L) and corn stover (15.3 ± 1.7 g/L) when these samples were treated with 2% NaOH at 120 ℃ for 20 min (Table 3).

Comparative Study of Treatments Using Various Substrates

It was observed that the amount of sugar released in both chemical treatments depended on the nature of substrates used for analysis. Both the agricultural waste (Wheat straw, Cotton stalk, Corn Stover, Rice straw and peel wastes) and municipal waste (peel waste) used in present study contains a good amount of sugar (Tables 2 and 3) and can be used for ethanol production on commercial scale. Similar results were also reported by Zhao *et al.* (2012).

It was observed that a significant amount of sugar was obtained when biomass samples were treated with dilute acid concentration (2%) for 20 min at 120 ℃ (Table 2) compared to when similar samples were treated with equal quantity of alkali. It has been shown that moderate temperature along with acidic pretreatment play a key role in increasing the production of the glucose; this may be due to increasing the surface area of substrate and availability of substrate to fermentative organisms. Depending upon the biomass composition most of the time pre-treatment methods are used in combinations. In the present study the variables of temperature and acidic pretreatments were combined so that cellulose would be easily available for the enzymatic hydrolysis that would ultimately results into higher glucose production. A similar finding on acid hydrolysis of various biomass samples has been reported by Tao *et al.* (2014).

Table 1. Proximate Analysis (%) of Biomass Samples

Parameters	Cotton Stalks	Corn Stover	Wheat Straw	Rice Straw	Peel Wastes
Dry Matter	93.5 ± 2.6	89.8 ± 3.1	92.4 ± 1. 5	91.6 ± 0.5	94.6 ± 3.5
Moisture Contents	6.5 ± 0.8	7.3 ± 0.9	7.8 ± 0.6	5.7 ± 0.6	6.9 ± 0.7
Volatile Matter	77.6 ± 1.2	75.8 ± 2.6	89.3 ± 2.5	90.6 ± 1.5	91.5 ± 2.6
Fixed Carbon Content	17.5 ±1.2	19.5 ± 2.3	18.7 ± 1.3	17.5 ± 1.6	16.8 ± 1.5
Ash Contents	8.7 ± 0.5	6.2 ± 0.4	5.1 ± 0.7	3.4 ± 0.6	4.6 ± 0.8
Crude Fat Content	3.6 ± 0.2	3.8 ± 0.8	3.7 ± 0.3	2.8 ± 0.4	1.9 ± 0.7
Crude Protein Content	4.2 ± 0.6	6.8 ± 0.9	9.6 ± 0.4	4.7 ± 0.5	2.8 ± 0.3
Cellulose Content	37.5 ± 1.5	33.6 ± 2.1	38.5 ± 2.7	34.8 ± 1.7	29.6 ± 1.6
Hemicellulose Content	29.5 ± 2.5	25.5 ± 2.8	27.8 ± 3.1	26.7 ± 2.8	24.3 ± 1.4
Lignin Content	14.8 ± 2.6	19.5 ± 1.5	13.7 ± 2.4	15.8 ± 1.9	17.5 ± 1.4

Mean ± SD (standard deviation, n = 3) and on the basis of dry weight (%)

Saccharification of Biomass Samples with Enzymes and Proteases

The saccharification process of various biomass samples was completed after acid/ alkali treatment with cellulase and acid proteases. It was observed that a higher amount of sugar was produced when wheat straw was treated with the enzymes cellulase and recombinant serpins. Results indicated that wheat straw released a higher quantity of glucose followed by rice straw and corn stover compared to other samples assessed (Table 2 and 3) at variable experimental conditions. In another study when all the fermentation conditions (time period, pH, temperature, substrate concentration, and inoculum size) were optimized by using central composite design (CCD), higher yields of both sugars and ethanol were obtained.

Table 2. Chemical Pretreatment of Biomass Samples with Different Concentrations (%) of H_2SO_4 for Sugars Release (%) From Different Resources after 72 h Duration

H_2SO_4 (%)	Temp (℃)	Time (min)	Glucose Concentration (g/L)				
			Wheat Straw	Rice Straw	Corn Stover	Cotton Stalk	Peel Wastes
1	100	10	11.9 ± 0.3	7.8 ± 0.5	14.9 ± 0.4	6.7 ± 0.8	8.6 ± 0.6
		15	11.8 ± 0.4	9.8 ± 0.4	15.2 ± 0.7	9.2 ± 0.5	9..1 ± 0.2
		20	11.5 ± 0.8	8.5 ± 1.5	15.1 ± 0.7	9.8 ± 0.6	8.6 ± 0.5
	110	10	8.6 ± 0.5	11.5 ± 0.4	13.8 ± 0.5	10.3 ± 0.5	7.8 ± 0.9
		15	7.9 ± 0.2	12.8 ± 0.3	13.9 ± 0.4	11.7 ± 0.3	9.2 ± 0.6
		20	8.1 ± 0,7	9.5 ± 0.1	10.8 ± 0.7	9.8 ± 0.1	8.5 ± 0.7
	120	10	12.1 ± 0.7	13.8 ± 0.2	14.9 ± 0.1	10.8 ± 0.2	7.4 ± 0.3
		15	12.7 ± 0.8	14.2 ± 0.4	14.8 ± 0.1	12.90 ± 0.1	8.5 ± 0.6
		20	15.6 ± 0.7	9.1 ± 0.7	15.1 ± 0.3	15.01 ± 0.5	9.1 ± 0.2
1.5	100	10	11.2 ± 0.7	15.3 ± 0.6	14.9 ± 0.1	12.6 ± 0.1	8.1 ± 0.6
		15	11.9 ± 0.5	13.5 ± 0.6	14.2 ± 0.4	13.1 ± 0.4	8.7 ± 0.5
		20	9.5 ± 0.6	12.0 ± 0.8	15.1 ± 0.3	13.5 ± 0.7	7.1 ± 0.9
	110	10	11.6 ± 0.8	12.8 ± 0.6	13.2 ± 0.0	13.0 ± 0.2	6.9 ± 0.8
		15	9.5 ± 0.3	16.2 ± 0.5	9.2 ± 0.1	9.1 ± 0.5	8.6 ± 0.5
		20	12.5 ± 0.7	14.6 ± 0.1	16.6 ± 0.7	10.6 ± 0.2	9.8 ± 0.2
	120	10	14.6 ± 0.8	11.2 ± 0.6	14.8 ± 0.5	14.1 ± 0.6	9.6 ± 0.5
		15	16.5 ± 0.9	12.8 ± 0.7	15.9 ± 0.2	9.1 ± 0.2	10.7 ± 0.8
		20	15.6 ± 0.6	11.6 ± 0.2	16.1 ± 0.9	10.5 ± 1.0	11.8 ± 0.5
2.0	100	10	13.2 ± 0.5	13.2 ± 0.6	15.1 ± 0.6	14.2 ± 0.4	10.3 ± 0.4
		15	14.3 ± 0.7	11.9 ± 0.5	14.0 ± 0.7	12.2 ± 0.6	9.5 ± 0.8
		20	12.6 ± 0.9	11.3 ± 0.6	14.5 ± 0.9	**16.4 ± 1.0**	**11.9 ± 0.5**
	110	10	12.3 ± 0.8	16.1 ± 0.2	15.1 ± 0.1	12.2 ± 0.4	8.3 ± 0.3
		15	13.7 ± 0.5	12.2 ± 0.7	15.2 ± 0.6	12.6 ± 0.5	8.5 ± 0.6
		20	14.5 ± 0.6	16.4 ± 1.0	14.9 ± 0.5	13.1 ± 0.8	9.8 ± 0.4
	120	10	14.6 ± 0.3	14.0 ± 0.3	15.1 ± 0.2	12.8 ± 0.7	8.9 ± 0.3
		15	16.7 ± 0.8	15.1 ± 0.1	14.8 ± 0.1	14.2 ± 0.5	10.6 ± 0.3
		20	**19.6 ± 1.6**	**17.6 ± 0.1**	**16.1 ± 0.9**	14.3±0.3	11.3 ± 0.7

Chemical treatment of biomass samples for sugar, Mean ± SD (Standard Deviation, n=3)

The fermentation process was completed by C. *thermocellum* using wheat straw sample with addition of the recombinant serpins solution (0.75 mL). Results were compared for both samples with and without serpins and it was concluded that ethanol production increased *via* the addition of serpins compared to the reaction that was carried out without serpins (Fig. 1). The reason behind higher achieved saccharification was because there was no accumulation of sugar, like cellobiose, beside the availability of cellobiose in the reaction mixture. Furthermore, Xue *et al.* (2012) has also pointed out that the performance of cellulase was actually enhanced (due to the absence of cellobioses), and the results in higher sugar recovery after enzymatic hydrolysis are possible.

Table 3. Chemical Pretreatment of Biomass Samples with Different Concentrations (%) of NaOH to Release of Sugars after 72 h Duration

NaOH (%)	Temp. (℃)	Time (min)	Glucose Concentration (g/L)				
			Wheat Straw	Rice Straw	Cotton Stalk	Corn Stover	Peel Wastes
1	100	10	12.5 ± 0.4	5.2 ± 0.7	3.7 ± 0.6	4.9 ± 0.4	11.6 ± 0.3
		15	12.8 ± 0.3	9.01 ± 0.1	5.2 ± 0.5	5.6 ± 0.4	11..8 ± 0.2
		20	13.6 ± 0.8	11.5 ± 1.3	10.5 ± 0.2	11.1 ± 0.1	11.3 ± 0.5
	110	10	8.7 ± 0.4	4.5 ± 0.9	13.1 ± 0.1	12.1 ± 0.3	11.8 ± 0.6
		15	7.9 ± 0.2	5.8 ± 0.3	13.7 ± 0.3	12.9 ± 0.4	12.2 ± 0.6
		20	8.1 ± 0,6	9.5 ± 0.1	9.8 ± 0.1	9.8 ± 0.7	12.5 ± 0.7
	120	10	10.6 ± 0.7	13.2 ± 0.2	12.8 ± 0.2	13.1 ± 0.1	12.8 ± 0.3
		15	12.6 ± 0.8	14.2 ± 0.4	13.90 ± 0.1	14.8 ± 0.1	13.5 ± 0.6
		20	16.3 ± 0.5	9.1 ± 0.7	15.01 ± 0.5	12.1 ± 0.3	13.8 ± 0.2
1.5	100	10	13.6 ± 0.7	12.8 ± 0.1	13.6 ± 0.1	12.9 ± 0.1	12.1 ± 0.6
		15	15.5 ± 0.5	14.5 ± 0.1	14.01 ± 0.4	13.2 ± 0.4	13.7 ± 0.5
		20	16.8 ± 0.9	15.0 ± 0.3	15.5 ± 0.4	15.1 ± 0.3	13.1 ± 0.9
	110	10	11.6 ± 0.8	12.5 ± 0.1	13.0 ± 0.2	13.2 ± 0.0	13.9 ± 0.8
		15	15.5 ± 0.3	13.2 ± 0.5	9.1 ± 0.5	9.2 ± 0.1	13.6 ± 0.7
		20	17.5 ± 0.7	14.6 ± 0.1	10.6 ± 0.2	10.6 ± 0.2	13.8 ± 0.2
	120	10	13.6 ± 0.8	11.2 ± 0.0	14.1 ± 0.6	13.8 ± 0.5	13.6 ± 0.5
		15	14.5 ± 0.9	9.8 ± 0.1	9.1 ± 0.2	8.9 ± 0.2	13.7 ± 0.8
		20	17.6 ± 0.6	11.6 ± 0.2	10.5 ± 1.0	10.1 ± 0.9	13.8 ± 0.3
2.0	100	10	13.5 ± 0.8	14.2 ± 0.5	14.2 ± 0.4	13.9 ± 0.5	14.3 ± 0.4
		15	14.6 ± 0.4	8.9 ± 0.1	12.2 ± 0.6	11.0 ± 0.8	14.5 ± 0.7
		20	15.3 ± 0.5	10.3 ± 1.0	**16.4 ± 1.0**	**15.1 ± 0.9**	14.9 ± 0.5
	110	10	14.2 ± 0.6	14.1 ± 0.2	13.2 ± 0.3	12.1 ± 0.1	14.3 ± 0.6
		15	15.8 ± 0.9	12.2 ± 0.7	13.6 ± 0.1	12.2 ± 0.1	14.5 ± 0.8
		20	16.2 ± 0.7	15.4 ± 1.0	12.1 ± 0.2	11.9 ± 0.1	14.8 ± 0.7
	120	10	16.6 ± 0.5	13.0 ± 0.3	10.8 ± 0.3	10.1 ± 0.2	13.9 ± 0.3
		15	18.7 ± 0.9	13.1 ± 0.1	13.2 ± 0.2	12.8 ± 0.1	13.6 ± 0.7
		20	**18.5 ± 1.2**	**16.4 ± 1.5**	12.3 ± 0.3	**15.3 ± 1.7**	13.3 ± 0.8

Chemical treatment of biomass samples for sugar, Mean ± ST

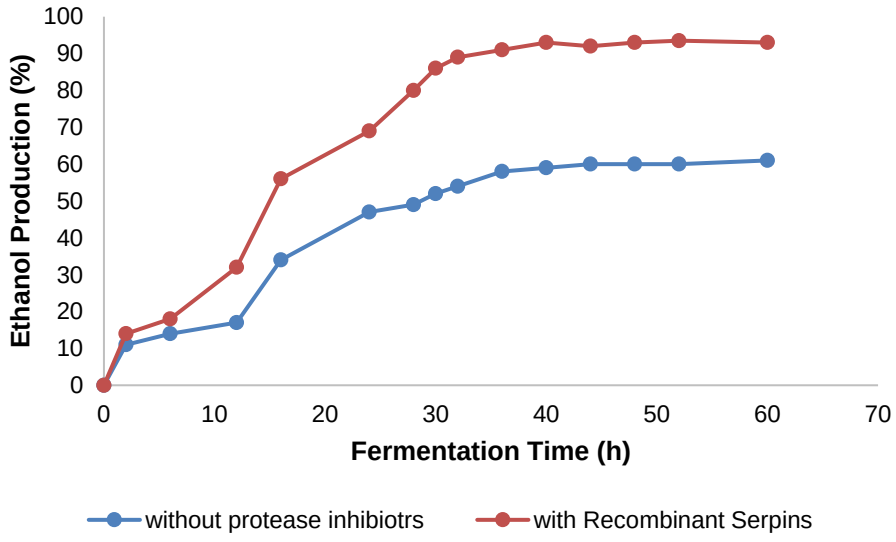

Fig. 1. Effect of recombinant serpins on glucose production

Comparison of Yeast and Bacterial Fermentation

After acid/alkali pretreatment and enzymatic hydrolysis, both yeast (*S. cerevisiae*) and bacterial (*C. thermocellum*) fermentation processes were completed separately to efficiently compare both organisms (Tables 4 and 5). It was observed that *S. cerevisiae* had higher yields of ethanol with wheat straw as substrate followed by rice straw and other substrates (Table 4). Meanwhile, sugars from similar substrates when fermented with *C. thermocellum* provided lower quantity of glucose and ethanol compared to yeast fermentation (Table 5).

Table 4. Ethanol Yield by *Saccharomyces cerevisiae* Fermentation

Substrate	Total Concentration (g/L)	Total Theoretical Yield of Ethanol (g/L)	Actual Yield of Ethanol (g/L)	Fermentation Efficiency (%)
Wheat Straw	21.7	12.7	11.3	92.3
Rice Straw	17.8	9.6	10.6	82.8
Corn Stover	16.5	7.8	9.5	91.5
Cotton Stalk	18.6	10.8	9.7	90.5
Peel Wastes	14.5	6.5	7.8	89.3

Mean values of yeast fermentation products

Table 5. Ethanol Yield by *C. thermocellum* Fermentation

Substrate	Total Concentration (g/L)	Total Theoretical Yield of Ethanol (g/L)	Actual Yield of Ethanol (g/L)	Fermentation Efficiency (%)
Wheat Straw	19.3	9.3	8.5	88.5
Rice Straw	16.4	8.5	7.8	87.9
Corn Stover	14.3	7.9	6.8	87.3
Cotton Stalk	13.2	7.6	6.3	86.6
Peel Wastes	12.6	6.3	5.9	86.4

Mean values of bacterial fermentation products

Analysis of Sugars with HPLC

The HPLC analysis of the various sugar content is presented in Table 6, which indicates that a higher amount of glucose was present in wheat straw (28.3 g/L) compared to rice straw (22.5 g/L), corn stover (17.7 g/L), cotton stalk (16.5 g/L), and peel wastes (12.4 g/L), when these samples were treated with acid compared to those treated with alkali (Table 6).

Wheat straw provided a higher yield of glucose and ethanol after enzymatic hydrolysis was conducted for 72 h. Amount of the sugar released was effected by increasing in the concentration of H_2SO_4 from 1 to 2%. In all the experiments, the yield of sugars was checked for 50 min. For acidic pretreatment conditions of wheat straw, the conditions were optimized at 120 °C with 2% sulphuric acid. At this level, glucose concentration was maximum. During alkali pretreatment conditions, the glucose yield was increased by increasing the temperature, and a higher yield was recorded at 120 °C. Through increasing the time of enzymatic hydrolysis from 0 to 48 h, the sugar yield increased but further increasing time up to 72 h reduced the sugar concentration. The decrease in glucose concentration was probably due to the production of inhibitors under

higher acid concentrations. Rice straw has shown a higher glucose yield in acidic pretreatment conditions at 110 ℃, with an acid concentration of 1.5%. The optimum conditions used for rice straw analysis for alkaline pretreatment were 100 ℃ and a sodium hydroxide concentration of 0.5%. A higher yield was obtained after 72 h of enzymatic hydrolysis. During acidic pretreatment, a high yield of glucose was obtained at a temperature of 120 ℃ and H_2SO_4 concentration of 2.0%.

The identification of glucose concertation in five different samples (wheat straw, rice straw corn stover, cotton stalk, and peel wastes) was performed based on its retention time t_R and compared with standard. There was only one prominent peak observed for glucose having retention time 8.6 min.

Table 6. Analysis of Different Sugars in Sample with HPLC

		Sugars Concentration (g/L)				
Components	Retention Time (Mins.)	Rice Straw	Wheat Straw	Cotton Stalk	Corn Stover	Peel Wastes
Glucose	8.6	22.52	28.3	16.5	17.7	12.4
Cellobiose	7.1	1.02	1.05	1.3	1.4	1.5
Xylose	11.6	4.3	5.6	4.7	4.5	3.8
Arabinose	12.0	1.4	1.8	1.4	1.6	1.2
Mannose	13.2	1.5	2.8	2.1	2,5	1.8
Galactose	15.5	1.2	1.5	1.3	1.4	1.1
Furfural	42.5	1.4	2.65	1.3	1.5	1.2
HMF	28	1.2	2.84	1.6	1.7	1.4

Analysis of sugar with HPLC

DISCUSSION

The production of alcoholic fuels from lignocellulosic feedstock requires various steps such as acid/alkali pretreatment, enzymatic saccharification, and fermentation. In the past, many countries have considerably improved their techniques for the production alcoholic fuels by refining these processes for a higher level of ethanol recovery (Zhao *et al.* 2012). The popular cases of biomass-based fuels production in developed countries may be good references for the developing countries. In addition, many novel ideas, such as biorefinery and the concept of oriented conversion of classified composition have been investigated for ethanol production (Gregg and Saddler 1996; Demirbas 2009; García *et al.* 2011). The cost of fuels may further decrease when it is produced on an industrial scale and efficient combination of these processes will result in competitive biofuel production from plant biomass, which is currently not being utilized effectively (Tao *et al*. 2014).

Fermentation of available sugars in cellulosic biomass has potential to provide important products such as acetone, butanol, ethanol, and other similar alcohols that could be used as liquid fuels. The most available source of biomass containing carbohydrates are wood wastes, agriculture crops, like wheat, rice, and cotton straws, corn covers, sorghum straws, fruit and vegetable wastes, and other similar substrates. Cellulose is considered as a major sugar for alcohol (fuel) production and cellulose is a complex sugar present in plant materials. This complex cellulosic material is broken down into smaller units with the help of acid treatment and enzymatic hydrolysis as well as bacterial/fungal fermentation

(Nargotra *et al.* 2018). These forms of alcohol are important because that may be used as fuels. Therefore, biofuels may provide the solutions of: (1) Combating climate change, as it helps to reduce the level of carbon emission; (2) Biofuel is able to respond to the growing demand of fossil fuel and energy; (3) Biofuels secure energy supply as it provides security to challenges rising from fuels globally; (4) Reducing the amount of waste and utilizing natural resources. Therefore, biofuels are an excellent example to provide an answer for the circular economy.

In the current study, various cellulosic materials were used to produce bioethanol. Among all substrates of biomass, wheat straw provided better ethanol yield compared to the other materials used. The use of certain proteases in ethanol fermentation has been demonstrated to improve fermentation by digesting the proteins into peptides and make the cellulose accessible to cellulases activity, which can provide the nutrient source for yeast and bacterial growth and reduce sugars in thin stillage. Yeast requires certain nutrients to grow and maintain their population to convert glucose into ethanol. If yeast nutrition is not maintained, then the fermentation will suffer and result in lower rates and yield of ethanol formation. Nitrogen sources, such as urea, ammonia, *etc.*, can be added. In the conventional process of producing ethanol biofuel from various biomass samples, the recovery of ethanol from the fermentation broth is accomplished using different parameters for maximum recovery of ethanol. These types of study are useful for the conversion of organic waste materials into industrial important products for economic development of developing countries, like Pakistan (Johnston and McAloon 2014).

CONCLUSIONS

1. Acid and basic pretreatments condition were optimized for the hydrolysis of lignocellulosic biomass including rice straw, wheat straw, corn stalk, corn stover and peel wastes. Enzymatic hydrolysis was carried out after the pretreatment. Under optimized condition, higher amount of glucose was obtained from wheat straw (28.3 g/L).
2. As compared to *Clostridium thermocellum*, *Saccharomyces cerevisiae* released a high concentration of ethanol 21.7g/L by wheat straw.
3. Two serpin genes 191 and 1270 were identified in the *C. thermocellum* genome. Among these two serpins, Serpin 191 is present inside the cellulosmal complex. These two genes were cloned, purified, and characterized in the lab.
4. The effect of the purified recombinant Serpins 191 and 1270 has been evaluated for the degradation of cellulose in combination with cellulases.
5. The highest percentage of ethanol was obtained (93%) in the presence of recombinant Serpin 191, which can indicate the importance of serpins for the activity of cellulases.
6. Use of pretreatment method, followed by enzymatic hydrolysis, yeast and bacterial fermentation and the presence of serine protease inhibitors can provide an optimized and an ideal environment for higher production of ethanol.

ACKNOWLEDGMENTS

The authors are thankful to the Higher Education Commission of Pakistan (HEC) for financial support. Contribution of all co-works are highly appreciated.

REFERENCES CITED

Alfenore, S., Molina-Jouve, C., Guillouet, S. E., Uribelarrea, J. L., Goma, G., and Benbadis, L. (2002). "Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process," *Applied Microbiology and Biotechnology* 60, 67-72. DOI: 10.1007/s00253-002-1092-7

Amiri, H., and Karimi, K. (2018). "Pretreatment and hydrolysis of lignocellulosic wastes for butanol production: Challenges and perspectives," *Bioresource Technology* 270, 702-721. DOI: 10.1016/j.biortech.2018.08.117

Artzi, L., Bayer, E. A., and Morais, S. (2017) "Cellulosomes: Bacterial nanomachines for dismantling plant polysaccharides," *Nature Reviews Microbiology* 15, 83-95. DOI: 10.1038/nrmicro.2016.164

Association of Official Analytical Chemists (AOAC) (1990). *Official Methods of Analysis of the AOAC*, 15th Ed., Methods 920.85, Association of Official Analytical Chemists, Arlington, VA, USA.

Carvalho, D. M., Queiroz, J. H., and Colodette, J. L. (2017). "Hydrothermal and acid pretreatments improve ethanol production from lignocellulosic biomasses," *BioResources* 12(2), 3088-3107. DOI: 10.15376/biores.12.2.3088-3107

Demirbas, A. (2001). "Biomass resource facilities and biomass conversion processing for fuels and chemicals," *Energy Conservation and Management* 42(11), 1357-1378. DOI: 10.1016/S0196-8904(00)00137-0

Demirbas, A. (2009). "Biorefineries: Current activities and future developments," *Energy Conversion and Management* 50(11), 2782-2801. DOI: 10.1016/j.enconman.2009.06.035

Dhamole, P. B., Mane, G. R., and Feng, H. (2015). "Screening of non-ionic surfactant for enhancing biobutanol production," *Applied Biochemistry and Biotechnology* 177, 1272-1281. DOI: 10.1007/s12010-015-1812-y

Du, S. K., Su, X., Yang, W., Wang, Y., Kuang, M., Ma, L., Fang, D., and Zhou, D. (2016). "Enzymatic saccharification of high pressure assist-alkali pretreated cotton stalk and structural characterization," *Carbohydrate Polymers* 20(140), 279-286. DOI: 10.1016/j.carbpol.2015.12.056

Ejezi, T. C., Qureshi, N., and Blaschek, H. P. (2007). "Bioproduction of butanol from biomass: From genes to bioreactor," *Current Opinion in Biotechnology* 18(3), 220-227. DOI: 10.1016/j.copbio.2007.04.002

García, V., Päkkilä, J., Ojamo, H., Muurinen, E., and Keiski, R. L. (2011). "Challenges in biobutanol production: How to improve the efficiency?," *Renewable and Sustainable Energy Reviews* 15(2), 964-980. DOI: 10.1016/j.rser.2010.11.008

Gomez, L. D., Steele-King, C. G., and McQueen-Mason, S. J. (2008). "Sustainable liquid biofuels from biomass: The writing's on the walls," *New Phytologist Trust* 178(3), 473-485. DOI: 10.1111/j.1469-8137.2008.02422.x

Gregg, D., and Saddler, J. N. (1996). "A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process," *Applied Biochemistry and Biotechnology* 57-58(1), 711-727. DOI: 10.1007/BF02941753

Haifeng, S., Gang, H., Mingxiong, and Furong, T. (2015). "A biorefining process: Sequential, combinational lignocellulose pretreatment procedure for improving biobutanol production from sugarcane bagasse," *Bioresource Technology* 187, 149-160. DOI: 10.1016/j.biortech.2015.03.107

Huber, G., Iborra, W. S., and Corma, A. (2006). "Synthesis of transportation fuels from biomass: Chemistry, catalysts and engineering," *Chemical Reviews* 106(9), 4044-4098. DOI: 10.1021/cr068360d

Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A., Schoen, P., Lukas, J., Olthof, B., and Worley, M., *et al.* (2011). *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol* (NREL/TP-5100-47764), National Renewable Energy Laboratory, Golden, CO, USA.

Jiang, Y., Liu, J., Jiang, W., Yang, Y., and Yang, S. (2015). "Current status and prospects of industrial bio production of n-butanol in China," *Biotechnology Advances* 33(7), 1493-1501. DOI: 10.1016/j.biotechadv.2014.10.007

Johnston, B. D., and McAloon, A. J. (2014) "Protease increases fermentation rate and ethanol yield in dry-grind ethanol production," *Bioresource Technology* 154, 18-25. DOI: 10.1016/j.biortech.2013.11.043

Kim, S., and Dale, B. E. (2004). "Global potential of bioethanol production from wasted crops and crop residues," *Biomass and Bioenergy* 26(4), 361-375. DOI: 10.1016/j.biombioe.2003.08.002

Kim, S., and Holtzapple, M. T. (2005). "Lime pretreatment and enzymatic hydrolysis of corn stover," *Bioresource Technology* 96(18), 1994-2006. DOI: 10.1016/j.biortech.2005.01.014

Lin, Y. S., and Lee, W. C. (2011). "Simultaneous saccharification and fermentation of alkali-pretreated Cogongrass for bioethanol production," *BioResources* 6(3), 2744-2756.

Mahro, B., and Timm, M. (2007). "Potential of biowaste from the food industry as a biomass resource," *Engineering in Life Sciences* 7(5), 457-468. DOI: 10.1002/elsc.200620206

McBee, R. H. (1954). "The characteristics of *Clostridium thermocellum*," *Journal of Bacteriology* 67(4), 505-506.

Mikulski, D., and Kłosowski, G. (2018). "Efficiency of dilute sulfuric acid pretreatment of distillery stillage in the production of cellulosic ethanol," *Bioresource Technology* 268, 424-433. DOI: 10.1016/j.biortech.2018.08.005

Mikulski, D., and Kłosowski, G. (2020). "Hydrotropic pretreatment on distillery stillage for efficient cellulosic ethanol production," *Bioresource Technology* 300, Article ID 122661. DOI: 10.1016/j.biortech.2019.122661

Mohapatra, S., Mishra, S. S., Bhalla, P., and Thato, H. (2019). "Engineering grass biomass for sustainable and enhanced bioethanol production," *Planta* 250, 395-412. DOI: 10.1007/s00425-019-03218-y

Moretti, R., and Thorson, J. S. (2008). "A comparison of sugar indication enables a universal high throughout sugar-1-phosphate nuclotidyltransferase assay," *Analytical Biochemistry* 377(2), 251-258. DOI: 10.1016/j.ab.2008.03.018

Mountney, G. J., and Gould, W. A. (1988). "Low-temperature food preservation," in: *Practical Food Microbiology and Technology*, 3rd Edition, Van Nostrand Reinhold Company, New York, NY, USA, pp. 112-115

Nargotra, P., Sharma, V., Gupta, M., Kour, S., and Bajaj, B. K. (2018). "Application of ionic liquid and alkali pretreatment for enhancing saccharification of sunflower stalk biomass for potential biofuel-ethanol production," *Bioresource Technology* 267, 560-568. DOI: 10.1016/j.biortech.2018.07.070

National Renewable Fuels Association (2019). "Industry statistics," Renewable fuel association, Annual Fuel Ethanol Production (https://ethanolrfa.org/statistics/annual-ethanol-production/), Accessed 13-04-2020.

Rezania, S., Md Din, M. F., Eva Mohamad, S., Sohaili, J., Mat Taib, S., Mohd Yusof, M. B., Kamyab, H., Darajeh, N., and Ahsan, A. (2017). "Review on pretreatment methods and ethanol production from cellulosic water hyacinth," *BioResources* 12(1), 2018-2024. DOI: 10.15376/biores.12.1.Rezania

Shields, P., and Cathcart, L. (2010). "Oxidase test protocol," *American Society for Microbiology*, (https://asm.org/getattachment/00ce8639-8e76-4acb-8591-0f7b22a347c6/oxidase-test-protocol-3229.pdf), Accessed 11 Nov 2010.

Singhvi, S. M., and Gokhale, V. D. (2019). "Lignocellulosic biomass: Hurdles and challenges in its valorization," *Applied Microbiology and Biotechnology* 103(23-24), 9305-9320. DOI: 10.1007/s00253-019-10212-7

Sluiter, A., Hames, B. R., Ruiz Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008b). *Determination of Structure Carbohydrates and Lignin in Biomass* (NREL/TP-51 0-42618), National Renewable Energy Laboratory, Golden, CO, USA.

Su, H. F., Liu, S., He, M. X., and Tan, F. R. (2015). "A biorefining process: Sequential, combinational lignocellulose pretreatment procedure for improving biobutanol production from sugarcane bagasse," *Biores*ource *Technology* 187, 149-160. DOI: 10.1016/j.biortech.2015.03.107

Sun, Y., and Cheng, J. J. (2005). "Dilute acid pretreatment of rye straw and Bermuda grass for ethanol production," *Bioresource Technology* 96(14), 1599-1606. DOI: 10.1016/j.biortech.2004.12.022

Tao, L., Tan, E. C. D., McCormick, R., Zhang, M., Aden, A., He, X., and Zigler, B. T. (2014). "Techno economic analysis and life-cycle assessment of cellulosic isobutanol and comparison with cellulosic ethanol and n-butanol," *Biofuels Bioproducts & Biorefining* 8(1), 30-48. DOI: 10.1002/bbb.1431

Thomas, V., and Kwong, A. (2001). "Ethanol as a lead replacement: Phasing out leaded gasoline in Africa," *Energy Policy* 29(13), 1133-1143. DOI: 10.1016/S0301-4215(01)00041-6

Woo, J. M., Yang, K. M., Kim, S. U., Blank, M. L., and Park, J. B. (2014). "High temperature stimulates acetic acid accumulation and enhances the growth inhibition and ethanol production by *Saccharomyces cerevisiae* under fermenting conditions," *Applied Microbiology and Biotechnology* 98(13), 6085-6094. DOI: 10.1007/s00253-014-5691-x

Xue, C., Zhao, J. B., Lu, C. C., Yang, S. T., and Bai, F. W. (2012). "High-titer n-butanol production by *Clostridium acetobutylicum* JB200 in fed-batch fermentation with intermittent gas stripping," *Biotechnology and Bioenergy* 109(11), 2746-2756. DOI: 10.1002/bit.24563

Zhao, X. Q., Zi, L. H., Bai, F. W., Lin, H. L., Hao, X. M., Yue, G. J., and Ho, N. W. Y. (2012). "Bioethanol from lignocellulosic biomass," in: *Biotechnology in China III: Biofuels and Bioenergy*, Springer, Berlin, Heidelberg, Germany, pp. 25-51. DOI: 10.1007/10_2011_129

Zhu, S., Yang, M., Luo, F., Yang, X., and Xue, Y. (2018). "Engineering cell wall-degrading enzymes into growing plants to improve lignocellulosic ethanol production," *BioResources* 13(1), 3-5. DOI: 10.15376/biores.13.1.3-5

Article submitted: July 16, 2020; Peer review completed: September 5, 2020; Revised version received and accepted: September 22, 2020; Published: September 25, 2020.
DOI: 10.15376/biores.15.4.8662-8676