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ENZYMATIC DEPOLYMERISATION OF WHEAT STRAW INTO FERMENTABLE SACCHARIDES BY THERMOPHILIC CELLULASES

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Abstract

Bioethanol is in limelight these days being safe and renewable. Its production from plant feed stock is getting considerable importance as it does not pose food competition. Therefore, current study was focused on the enzymatic conversion of wheat straw in to fermentable saccharides which can further be used for bioethanol formation. Thermophilic cellulases i.e. Endo-1,4-β-glucanase, Exo-1,4-β-glucanase and β-1,4-glucosidase were used for this purpose. Differently pretreated i.e. acid and alkali wheat straw samples were analysed for their effective enzymatic conversion to less complex sugars. Two different strategies (Simultaneous and Sequential) for cellulase action on substrate were assessed. Furthermore, effect of different reaction conditions such as buffer, pH, temperature and substrate concentration was also evaluated. Maximum saccharification i.e. 43.78% (p<0.05) have been recorded using alkali treated wheat straw substrate in a concentration of 1.5% (w/v) by sequential addition of cellulases in a citrate phosphate buffer of pH 7 at an incubation temperature of 55°C.The microscopic examination of saccharified biomass showed clear disruption in the cellulose fibres. Moreover, thin layer chromatography of saccharified slurry showed the presence of variable fermentable sachharides.

Key words: Cellulase, Biomass, Renewable energy, Reducing sugars.

Introduction

Over the decades, the harmful environmental effects, rising prices and inevitable depletion of fossil fuels have led scientists to search for alternative energy sources. Bioethanol is amongst such renewable fuels and has been proved environmental friendly (Limayem &Ricke, 2012). It can be produced from plant material that contains high amount of cellulose or starch but the use of such material may lead to a problem of food security, so agricultural waste that is composed of lignocellulose can be used (Saini *et al.*, 2015). The ease of availability of this material and low cost makes it an ideal substrate for bioethanol production (Kazi *et al*., 2010). Additional advantage includes the use of locally available resources without the requirement of a huge capital investment. Considering Pakistan's agro-based economy, the utilization of this waste which is otherwise hard to dispose of, adds extra benefit (Goldemberg, 2007).

Amongst the various lignocellulosic substrates that can be used for bioethanol production wheat straw consists of a cellulosic content ranging between 30 to 50% with low lignin amount making it superior to others (Passoth *et al.*, 2013). The structural and morphological analysis of wheat straw shows that the cellulose present has the same characteristic arrangement throughout and possesses low crystallinity making it feasible for reproducible bio-processing. This quality, along with its high cellulosic content and relatively abundant supply in Pakistan, makes wheat straw an ideal substrate for the production of bioethanol (Sana *et al*., 2017).

The prime step for the production of ethanol from lignocellulosic biomass is saccharification which may be carried out chemically, thermally or enzymatically (Heng *et al.*, 2017). Enzymes offer several advantages over other processes, such as high efficiency, mild working conditions, low process energy requirements and easy recovery of products (Pancha *et al.*, 2016). It is the synergistic action of cellulases on the lignocellulosic material that defines the effectiveness of saccharification which in turn determines the yield of ethanol (Van Dyk & Pletschke, 2012). Therefore, it is essential to understand the role of each enzyme in the process and devise a solution to overcome the loss of effectiveness of the enzyme with time. The sequential addition of each enzyme is one way to increase the efficiency of the process (Dutta *et al*., 2014).

In the view of challenges encountered during bioethanol production, the effects of different components of the cellulase complex on the saccharification of wheat straw were analysed. This included saccharification of alkali treated wheat straw by the use of thermostable cellulases, produced by genetically modified strains of *E. coli*. Different factors such as substrate concentration, temperature, pH, enzyme activity and time have an impact on the bioconversion process and their optimization makes the process economically practicable (Dhabhai *et al*., 2012).

Material and Methods

Thermophilic cellulases: Thermophilic cellulases i.e. Endo-1, 4-β-glucanase (EC 3.2.1.4) from *Clostridium thermocellum,* Exo-1, 4-β-glucanase (EC 3.2.1.91) and β-1,4-Glucosidase (EC 3.2.1.21) from *Anaerocellum thermophilum* cloned in BL 21 *E. coli* strain were obtained from the principal investigator of the project entitled 'Production of bioenergy from plant biomass' taken place at Institute of Industrial Biotechnology, GC University Lahore, Pakistan.

Substrate: Wheat straw untreated and pretreated samples were delivered by Pakistan council of scientific and industrial research (PCSIR) Lahore Pakistan. The lignocellulosic composition of the samples was provided as shown in Table 1.

Table 1. Composition of variably pretreated wheat straw.

Biomass	Cellulose (%)	Lignin (%)	Delignification (%)	Pretreatment conditions
Wheat Straw (Control)	54.0	19.0	-	
Wheat Straw Sample I (Pretreated)	56.0	3.0	84.0	2% HCl, 30minutes, Steaming temperature 200 ± 2℃, Mesh size 2mm
Wheat Straw Sample II (Pretreated)	60.0	2.0	84.2	2.5% NaOH, 10minutes, Steaming temperature 200 ± 2℃, Mesh size 2mm

Saccharification of wheat straw: Wheat straw was saccharified in air-tight culture bottles (25 ml) using both control and pretreated samples. 1g of each sample and 20 ml of buffer having pH 6 was taken to which cellulases (50U/mg each) were added in a sequential as well as a simultaneous manner to analyse the effect of both strategies. Saccharification was carried out at 45℃ and 100rpm in a water-bath shaker. 1mL sample was withdrawn after every hour and the reducing sugar was estimated applying DNS method devised by Miller (Miller, 1959).

Optimization of factors affecting saccharification: Screening and selection of best pre-treated wheat straw sample was performed using constant enzyme units and physical parameters. Different factors affecting the bioconversion process were optimized including, mode of cellulose addition, saccharification buffer and its pH(sodium acetate, sodium phosphate, potassium phosphate, sodium citrate and citrate phosphate); (5.0–8.0), temperature (40-60℃) and amount of substrate (0.5–2%).

Calculation of percentage saccharification: The percentage saccharification was determined using method **of** Ahmed *et al.***,** (2012).

Thin layer chromatography: Thin layer chromatography of the saccharification mixture was performed for the qualitative estimation of the fermentable sugars following the procedure developed by Farag (1979).

Statistical analysis: An SPSS version 16.00 was used to statistically analyze the data obtained. Significant probability value (P) was calculated by subjecting replicates to one way ANOVA. Error bars in figures of result section represents the standard deviation (±SD) among replicas, which differ significantly at $p<0.05$.

Results and Discussion

Screening of wheat straw samples: Three wheat straw samples were screened; best results were obtained from wheat straw sample II with bioconversion of 17.26% ($p<0.05$) after 12 hours as shown in Fig. 1. It is widely understood that the pretreatment process can disrupt the covalent linkages between lignin, hemicellulose and cellulose. The removal of both lignin and hemicellulose increases the availability of cellulose and reduces its recalcitrance to water, thereby assisting its enzymatic hydrolysis (Saini *et al*., 2016). Acid pretreatment only results in the partial removal of lignin and hemicelluloses. On the other hand alkali pretreatment leads to the disruption of cell wall and not only solubilizes lignin and hemicellulose, but also silica and uronic acid esters (Rishi *et al*., 2009).

Comparison of sequential and simultaneous addition of cellulases: Wheat straw sample II was saccharified by both sequential and simultaneous addition of enzymes. Figure 2 shows that saccharification of 12.52% ($p<0.05$) was obtained after 5 hours when all the enzymes were added at the same time. Sequential addition of enzymes led to a better saccharification i.e.17.26% ($p<0.05$) after 12 hours. During saccharification, the phenomenon of feed-back inhibition operates for cellulases whereby glucose can inhibit beta-glucosidase which consequently increases the concentration of cellobiose in the saccharification mix. This in turn inhibits the activity of exoglucanase (Binod *et al*., 2011). In sequential addition of enzymes, each enzyme is allowed to act maximally before the addition of next enzyme. Therefore, the problem of feed-back inhibition can be avoided (Pihlajaniemi *et al*., 2014).

Effect of buffer and pH: Citrate phosphate buffer having pH 7.0 had the most positive effect on bioconversion rate (22.3 %; $p<0.05$) that is clearly evident from Figures 3 and 4. These cellulases worked best at neutral pH and gave a higher saccharification yield compared to other acidophilic and thermophilic cellulases (Oraby *et al*., 2007). Buffers contain salts consisting of ions that may interact with the enzyme by altering their conformational stability. Alterations in the conformational stability of the enzyme have an inherent effect on its activity, so, the buffer containing ions that interact minimally with the enzymes is best for saccharification (Zheng *et al*., 2013). The higher the pH, the more negatively charged lignin becomes. Consequently, the ability of lignin to bind non-specifically to cellulases is diminished leading to an increase in the efficiency of the saccharification process (Lou *et al*., 2013).

Role of incubation temperature: The temperature of the saccharification mix was varied from 40ºC to 60ºC (Fig. 5). The best results amongst the five temperatures was obtained at 55ºC with a hydrolysis percentage of 29.43% ($p<0.05$). Thermophilic cellulases carried out saccharification much more efficiently at higher temperatures compared to mesophilic enzymes that barely gave a saccharification of 20% (Baig *et al*., 2004). At temperatures above optimum value, the enzyme activity tends to decrease due to alteration in protein structure (Sharma & Horn, 2016). Moreover, lower temperatures favoured the adsorption of cellulases on lignin residues that remained after pretreatment in wheat straw which led to a reduction in the amount of enzyme available for hydrolysis (Kumar *et al*., 2008).

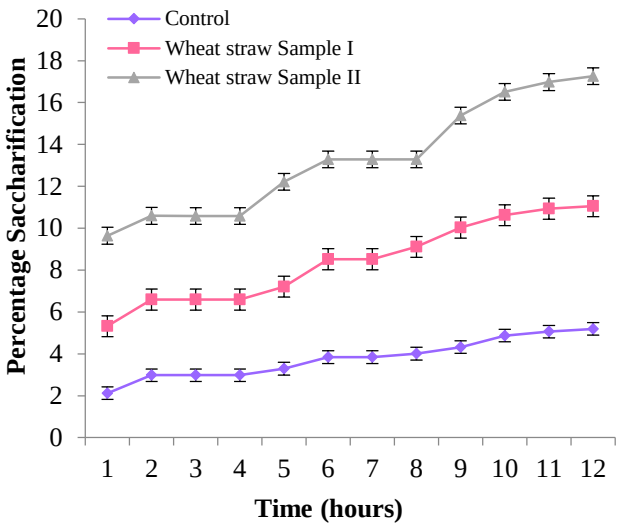

Fig. 1. Comparison of wheat straw samples for best bioconversion ability.

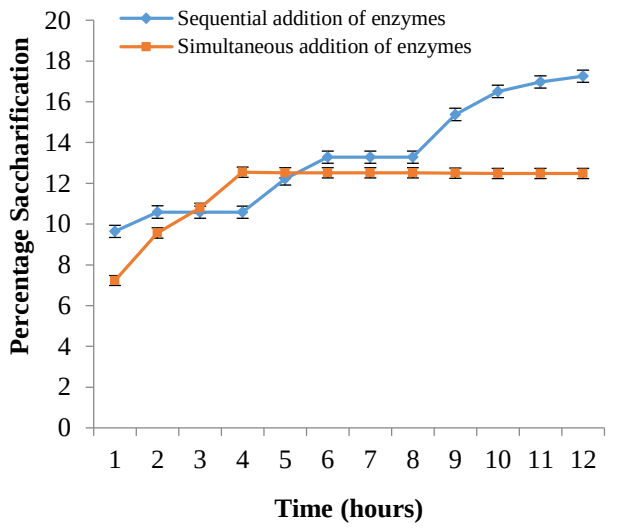

Fig. 2. Comparison of saccharification obtained by sequential and simultaneous addition of cellulases.

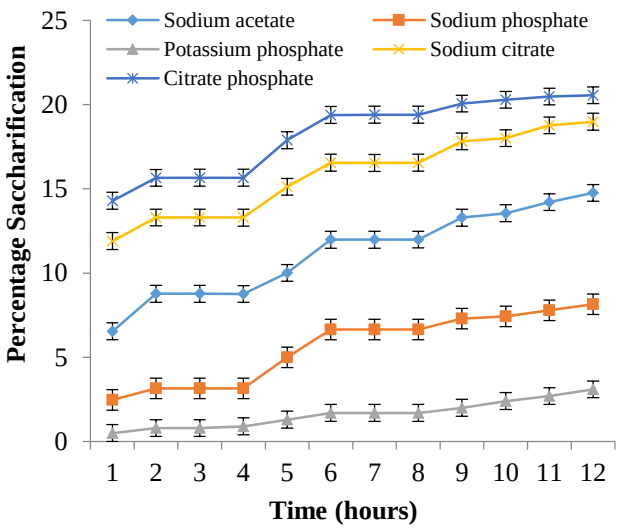

Fig. 3. Effect of various buffers on the saccharification of wheat straw.

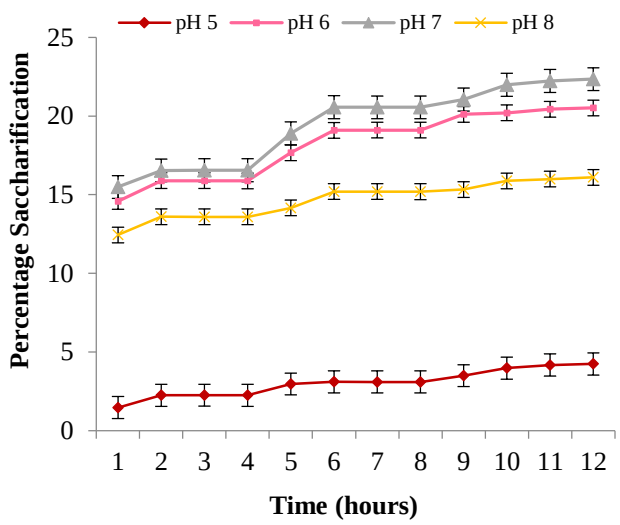

Fig. 4. Impact of Citrate phosphate buffers pH on hydrolysis of wheat straw

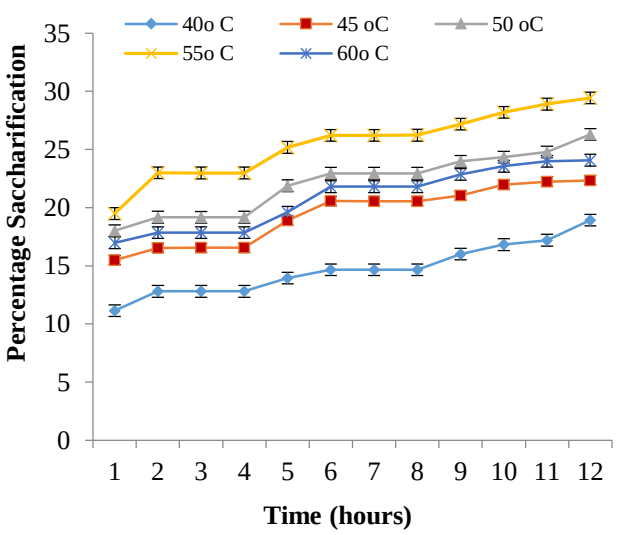

Fig. 5. Effect of incubation temperature in effective bioconversion of wheat straw.

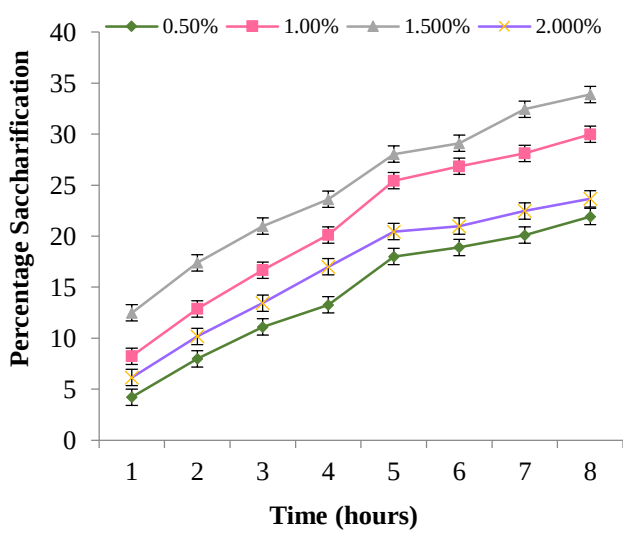

Fig. 6. Sacchrification of wheat straw in different biomass to reaction mixture concentrations.

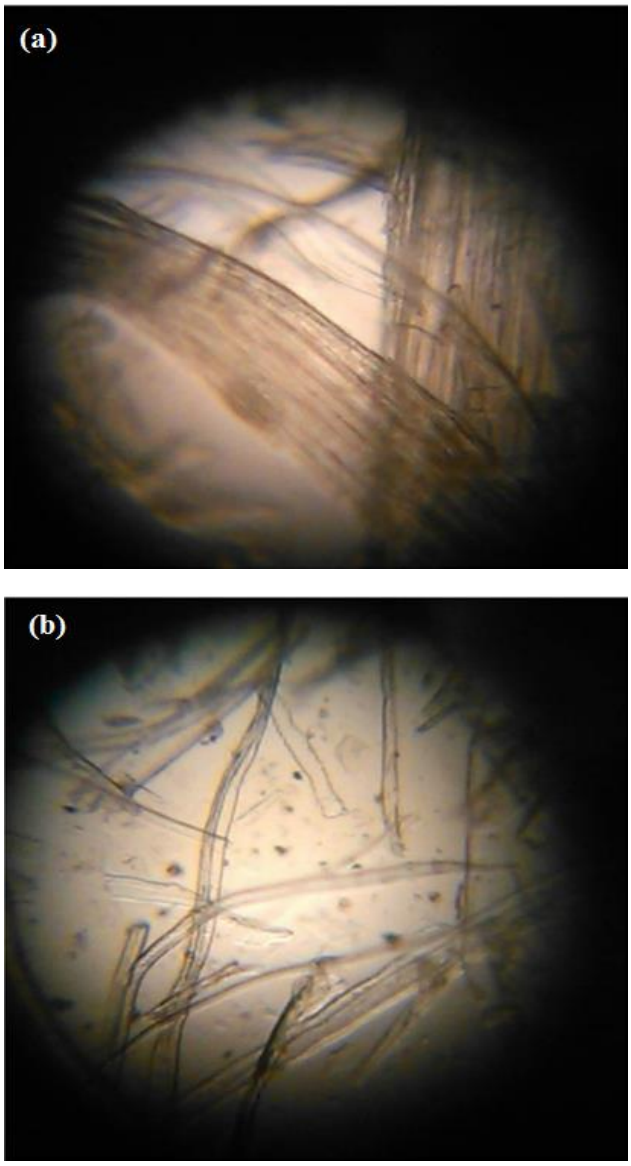

Fig. 7. Microscopic structure of wheat straw (a) before and (b) after enzymatic hydrolysis (40X Magnification of Light Microscope).

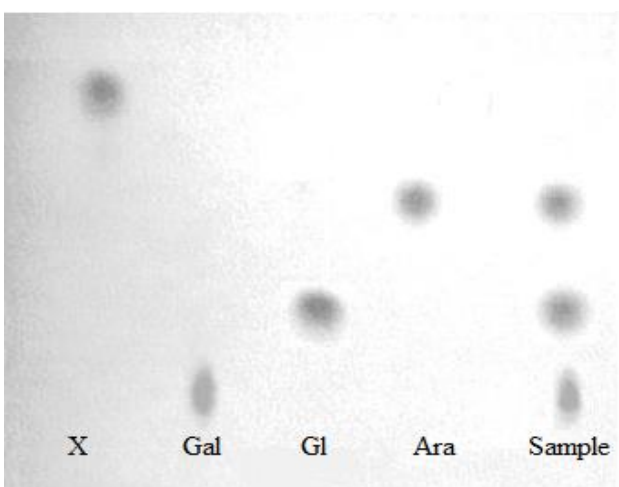

Fig. 8. Thin layer chromatography for the qualitative estimation of sugars produced after the bioconversion of wheat straw. The sugars i.e. X: Xylose, Gal: Galactose, Gl: Glucose, Ara: Arabinose were used as standards.

Substrate to reaction mixture ratio: Wheat straw was hydrolysed in variable quantities i.e. 0.5, 1, 1.5 and 2.0% to investigate the suitable substrate concentration for achieving higher bioconversion rate keeping the stable concentration of cellulases. The results are shown in Figure 6 whereby, substrate concentration of 1.5 % with respect to the reaction mixture produced highest yield 43.5% (p<0.05) from wheat straw hydrolysis. This yield is comparable to that of the widely used commercial fungal cellulases, whereby the use of 3% substrate results in 43.78% hydrolysis. (Zhang *et al*., 2012). Therefore it can be concluded that these thermophilic cellulases are equally efficient. This finding might owe to the fact that at higher concentration of biomass, the amount of substrate may become a limiting factor and may not lead to any increase in the yield or rate since all the active sites of the enzyme may get occupied. This inhibition depends upon the ratio of the total substrate to total enzyme volume (Lai & Idris, 2016).

Microscopy of wheat straw before and after enzymatic hydrolysis: The microscopy of wheat straw sample II was done before and after it was subjected to saccharification and it was found that changes in the structure occurred during the enzymatic breakdown of cellulose (Fig. 7). Pretreated wheat straw depicted a smooth structure consisting of unidirectional fibres. The fibres appeared to be intact mainly owing to the elongated layers of parenchymal cells exposed as a result of the partial removal of the cell wall during the pretreatment process (Hansen *et al*., 2011). The microscopy of wheat straw after enzymatic hydrolysis showed that whilst the initial compact structure had broken down, remnants of the elongated fibres were still present. These were probably the non-hydrolysed portions of xylem and phloem vessels. The results suggested that a major portion of the wheat straw had been broken down into its constituent monosaccharide units by the synergistic action of cellulases (Kristensen *et al*., 2008).

Thin layer chromatography: The results obtained after thin layer chromatography showed that the saccharified mix contained three fermentable sugars namely glucose, galactose and arabinose (Fig. 8). Xylose was not produced during the bioconversion of wheat straw by cellulolytic enzymes. The enzymatic saccharification of wheat straw often yields glucose, galactose and arabinose (Adiguzel &Tuncer, 2015).

Conclusion

The efficient management of cellulases was found to have a significant impact on bioconversion process of wheat straw. Optimization of different parameters such as buffer, pH, mode of cellulose addition, substrate pretreatment type and concentration resulted in 2 times increase in saccharification yield. The results of this study can be employed for development of economical saccharification process using wheat straw.

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