***Pak. J. Bot.*, 49(6): 2491-2494, 2017.**

REMOVAL OF PHENOLIC INHIBITORS FROM PRETREATED SUGARCANE BAGASSE FOR ENHANCED ENZYMATIC RECOVERY OF FERMENTABLE SUGARS

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Abstract

Phenolic inhibitory compounds are produced during an essential pre-treatment process carried out for removal of lignin from biomass for making the cellulose and hemicelluloses accessible to cellulases. Phenolic compounds produced, acts as cellulase inhibitors, make the process of biomass conversion in to biofuels less efficient and economical. Keeping in mind this challenge, a strategy was designed to remove the phenolic compounds from pre-treated sugarcane bagasse to evaluate its effect on saccharification rate. The pretreated sugarcane bagasse sample having phenolic compounds, considered as control, showed 19±0.04% of saccharification and Total Phenolic Content (TPC) as 254.71±0.10 mg Gallic Acid Equivalent /gram of Dry Weight of biomass. Removal of phenolic compounds was carried out by treating with organic solvents (Isopropanaol, acetone and methanol) and Alkalis i.e. Sodium hydroxide and calcium hydroxide. It was observed that overliming resulted in the better removal of phenolic compounds (Residual TPC = 154.06±0.02 mg GAE/g DW) as compared to Na(OH) and organic solvents employing 30 min incubation time at 60°C. Overliming using $Ca(OH)_2$ solution of pH 12, for 120mins at 75°C showed significant removal of phenolic compounds (119.43±0.09 mg GAE/g DW) and enhanced saccharification by almost 2.21 folds (42.06±0.07%). This removal of toxic phenolic componds will enhance the saccharification yield and substrate efficient recovery of fermentable saccahrides for economical and feasible biofuel production.

Key words: Agricultural waste; Biomass; Renewable energy; Detxoification; Green biotechnology.

Introduction

Bio-renewable energy source such as bioethanol is considered as a reliable alternative of fossil fuels (Gurram *et al*., 2011). Scientists all over the world are now looking to produce biofuels from agricultural waste products instead of competing with food crops. These agricultural waste products can be converted into fermentable saccharides enzymatically after making the cellulose part of biomass accessible to cellulases by applying a suitable pretreatment strategy (Berlin *et al*., 2006). Pretreatment process mainly involves the degradation of lignin portion which leads to the release of phenolic compounds which can act as enzyme inhibitors (Harmsen *et al*., 2012).

Phenolic components are renowned for their adverse effect on cellulase activity even at low concentrations. Multiple phenolic compounds once get attached to proteins results in their denaturation. They deactivate the enzyme by getting proteins adsorb on their hydroxyl group (Ximenes *et al*., 2011). These compounds specifically hinder the catalytic action of cellulases and cause the overall reduction in the reaction rate with low product yield (Sineiro *et al*., 1997).

Physiochemical and biological strategies can be used for elimination of phenolic compounds that can reduce the enzyme loading required for saccharification process (Sindhu *et al*., 2011). Among the different methods i.e. adsorption, solvent extraction etc. use of alkali (Calcium hydroxide) is reported as most appropriate. Calcium hydroxide reacts with the different phenolic and converts them into such forms that cannot bind or react with the cellulases resulting in their enhanced catalytic action on lingo-cellulosic substrates (Asgher *et al*., 2013).

Keeping in mind the above mentioned challenges, production of phenolic compounds during pretreatment of lingo-cellulosic biomass was estimated. Effect of different strategies on removal of phenolic inhibitory compounds was analyzed. Optimization of different physiochemical properties was carried out to enhance removal rate of poly-phenols. Effect of removal of inhibitory compounds on the saccharification was carried out to access the improvement in the product yield.

Material and Methods

Ligno-cellulosic biomass: Sugarcane bagasse sample was collected from agricultural field in the vicinity of Lahore, Punjab, Pakistan. The biomass was subjected to pretreatment with 3% of NaOH for 60 minutes under steaming temperature of 120^{o}C. The mesh size of 2 mm was applied for crushing the pretreated substrate (Huang *et al*., 2008). Cellulosic content was estimated applying the method used by Gopal & Ranjhan (1980).

Removal of phenolic compounds

Treatments: Sugarcane bagasse sample was incubated with organic solvents i.e., Isopropanaol, acetone, methanol and alkalis i.e., $Ca(OH)_2$, and Na(OH) at 60^{o}C for an incubation period of half an hour. After treatments, samples were centrifuged, separated and washed twice with distilled water.

Effect of physical parameters on Overliming: Over liming of the substrate was further investigated. Different physical parameters such as pH of alkali solution (8 to 12), reaction incubation temperature (60^{o}C to 80^{o}C) and incubation period (30-120 min) were optimized.

Estimation of phenolic compounds in biomass: Total phenolic contents estimation was carried out using the Folin–Ciocalteu (Folin & Ciocalteu, 1927) assay. After the assay, solid biomass was removed by centrifugation at 6000 rpm for 10 min and absorbance values of supernatant were recorded at 765nm. Total phenolic Contents (TPC) expressed in Gallic acid equivalent (GAE)/ gram dry weight (DW) of biomass.

Enzymatic depolymerization comparison of substrates: Treated and untreated biomass samples for removal of phenolic compounds were saccharified by using thermophilic cellulases taken from the project "Production of Bioenergy from Plant Biomass" at Institute of Industrial Biotechnology, GC University Lahore. Three air tight culture bottles were used; Two were labeled as experimental and the one as control. All the three enzymes i.e. Endo-1,4-β-glucanase (EC 3.2.1.4), Exo-1,4-β-glucanase (EC 3.2.1.91) and β-1,4- Glucosidase (EC 3.2.1.21) were added in concentration of 50 U each in all three bottles. Untreated substrate for phenolic compounds removal (sugarcane bagasse) was loaded in experimental bottles 1 and substrate (sugarcane bagasse) treated for removal of phenolic compounds was added to experimental bottle 2. In control bottle, no substrate was added. Saccharification was carried out at 80°C for three hours with agitation speed of 50 rpm. Total sugar was estimated by DNS method (Miller, 1959). Saccharification percentage was determined by using the following equation proposed by Vallander & Eriksson (1985).

$$\% \text{ Saccharification} = \frac{\text{R.S x V x F1}}{\text{M} \times \text{F2}} \times 100$$

R.S. = Sugar concentration in hydrolysis estimated as total reducing sugar (mg/ml)
V = Total volume of the reaction mixture (ml)
F1= Factor used for the conversion of monosaccharide to polysaccharide due to water uptake during hydrolysis (0.9 for hexoses)
M= Amount of substrate (mg) dry weight
F2= Factor for carbohydrate content of substrate (total carbohydrate, mg/ total substrate, mg)

Statistical analysis: Statistical analysis was carried out by using SPSS version 16.00 (2007). One-way ANOVA was applied on replicates to observe the significant difference with the probability (P).

Results and Discussion

Pretreatment and cellulosic content: Sodium hydroxide in combination with steam was applied for pretreatment of sugarcane bagasse. Cellulosic content availability was visibly increased from 48.73% to 56.23% with a visible decrease in lignin content from 23.56% to 6.15% (Table 1). This reduction in lignin content results in the formation of a phenolic compounds which stays with the biomass and interfere with the activity of cellulases (Dent, 2013).

TPC reduction strategy: Phenolic compounds removal ability of organic solvents i.e., Isopropanaol, acetone, methanol and alkalis i.e., $Ca(OH)_2$, and Na(OH) from sugarcane baggse was analyzed by estimating TPC and effect on saccharification yield. Reduction in TPC was observed in all samples treated for removal of polyphenols. However, $Ca(OH)_2$treated substrate showed maximum reduced in TPC (154.06±0.02 mg GAE/g DW) as compared to untreated sample (254.71±0.10 mg GAE/g DW). Saccharification yield of all the substrates, which showed reduction in TPC, increased with maximum yield (35.13±0.12%) recorded in the case of $Ca(OH)_2$ treated substrate as shown in Fig. 1. This might be due to the divalent nature of calcium ions (Ca^{2+}) that they convert phenolic inhibitors more efficiently to compounds that have less ability of hindering enzyme activity. Due to the ionization of their hydroxyl groups (Martin *et al*., 2007). Less saccharification instead of decrease in TPC applying organic solvents might be due to the reason that biomass become more compact than before treatment and thus more resistant to the enzymatic hydrolysis (Cantarella *et al*., 2004). Findings of this study are in accordance with results of Dent *et al*., 2013 who found that organic solvents showed little decrease in phenolic content.

Effect of pH on over-liming: Over-liming of sugarcane bagasse was carried out at different pH (8-12) to understand the effect on TPC reduction and saccharification. Increase in pH of lime solution used for polyphenols removal resulted in gradual decrease in TPC and reached to maximum at pH 12 (134.08±0.02 mg GAE/g DW) with increased saccharification yield (37.88±0.12%) shown in Fig. 2. This might be due to the reason that pH directly effects the reactivity of the phenolic compounds by bringing some conformational changes in their structures. These changes might lead to the conversion of these phenolics in to such components which could not affect the activity of cellulases. Findings of the current study are contrary to the results of Guo *et al*., 2013 who recorded pH 10 of overliming as best for TPC reduction in spruce hydrolysate.

Overliming in relation to temperature: Over-liming of substrate was conducted at five different temperatures i.e., 65, 70, 75, 80 and 85°C and maximum saccharification (39.13±0.06%) was estimated in a sample over-limed at 75°C with maximum decrease in TPC i.e., 124.37±0.01 mg GAE/g DW. High temperature along with higher pH might help converting polyphenols vigorously into their respective phenolate ions. Phenolate ions are less reactive towards the cellulases as compared to the original phenolic contents (Martin, 2007). Cantarella *et al*. (2004) also showed increase in saccharification i.e. 31 % after treating their biomass by over-liming at 75°C (Fig. 3).

Incubation period for over-liming: Treated and untreated sample for phenolic compounds elimination were incubated with $Ca(OH)_2$ for different intervals of time i.e., 30, 60, 90 and 120 minutes. Substrate over-limed for incubation period of 90 mins at pH 12 and 75°C resulted in maximum conversion into fermentable saccharides (42.06±0.07%) due to higher removal of phenolic compound 119.43±0.09 mg GAE/g DW. However, further increase in incubation time of over-liming yielded little more amount of TPC might be due to formation of more complex compounds which can hinder with the action of cellulases. On the contrary, Dent *et al*. (2013) suggested that treatment of biomass for a lesser time period was not enough to remove the sufficient number of phenolic compounds which in result could increase the saccharification rate (Fig. 4).

Table 1. Estimation of cellulose and lignin content in sugarcane bagasse before and after pretreatment.

Serial No.	Pretreatment	Cellulosic content (%)	Hemicellulose (%)	Lignin content (%)
1.	Untreated	48.73	26.72	23.59
2.	Alkali + Steaming temperature	56.23	23.21	6.15

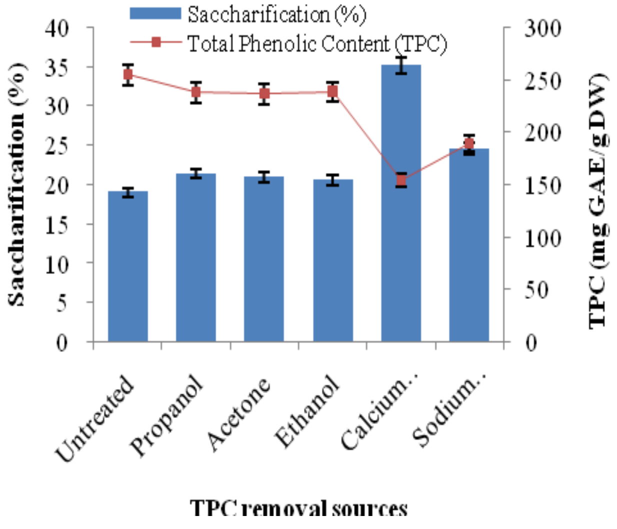

Fig. 1. TPC reduction assessment applying different organic solvents and alkalis along with its effect on saccharification yield.
Y error bars indicates the standard deviation (±SD) among the three replicates, which differs significantly at p≤0.05.

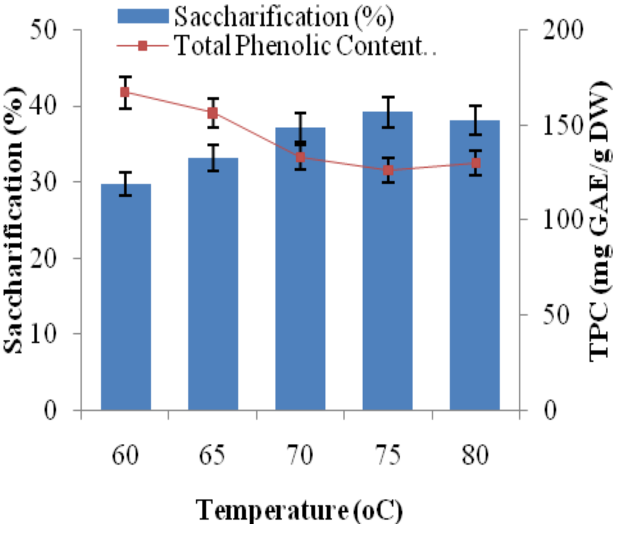

Fig. 3. Over-liming effect in relation to temperature on polyphenols reduction and saccharification yield.
Y error bars indicates the standard deviation (±SD) among the three replicates, which differs significantly at p≤0.05.

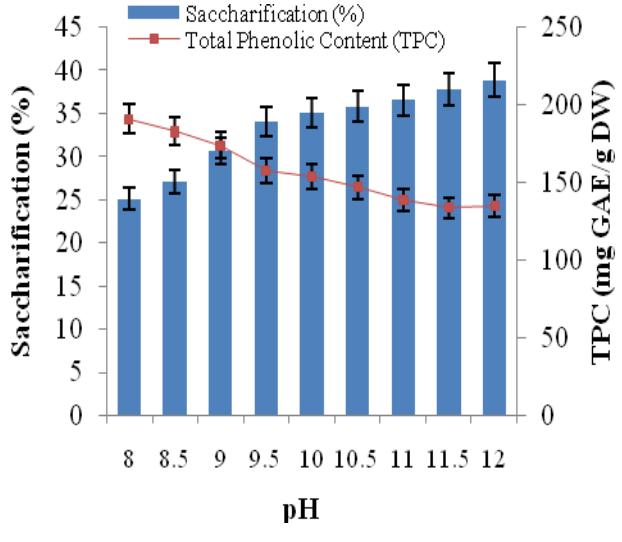

Fig. 2. Effect of overliming pH on TPC reduction and saccharification yield.
Y error bars indicates the standard deviation (±SD) among the three replicates, which differs significantly at p≤0.05.

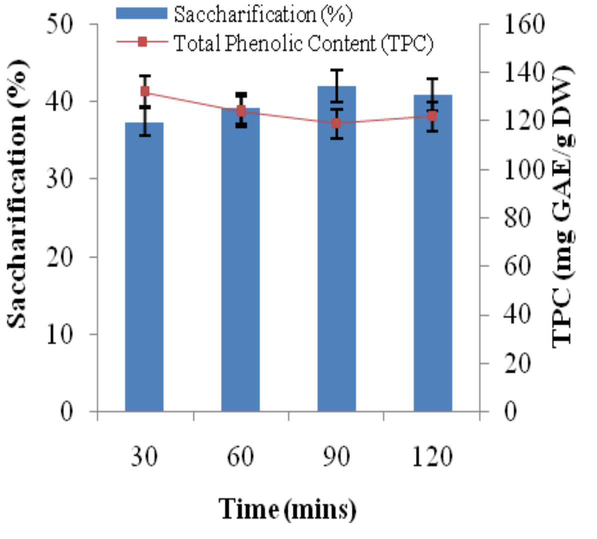

Fig. 4. Incubation time analysis of over-liming for polyphenols removal and its effect on saccharification yield.
Y error bars indicates the standard deviation (±SD) among the three replicates, which differs significantly at p≤0.05.

Conclusion

It was concluded that removal of phenolic compounds from sugarcane bagasse produced during pre-treatment significantly affect the saccharification yield. Maximum removal of these compounds through optimization of different parameters is significant for efficient recovery of saccharified sugars.

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(Received for publication 14 December 2016)