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COMPARATIVE EVALUATION OF DETOXIFICATION STRATEGIES FOR SUGARCANE BAGASSE HYDROLYSATE

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ABSTRACT

Lignocellulosic agricultural biomass generated by agriculture sector remains underutilized which can be a key source for bio-development. Biomass from dilute acid hydrolysate comprise a considerable quantity of fermentable sugars while carbonyl degradation of compounds (Furans, acetic acid, and phenolic compounds) exhibit severe toxicity to fermentation microbes. The study was carried out to hydrolyse the sugarcane bagasse and to optimize different detoxification strategies. Sugarcane bagasse was hydrolysed with acid at 121°C for 20, 40 and 60 minutes. Afterwards analysis of detoxification methodologies like over-liming (pH 6; pH 9.0), vacuum evaporation (3-fold; 5-fold) and charcoal treatment (1%, 30 min; 5%, 60 minutes) were carried out. The results indicate that concentrations of sugars and degradation products was observed to enhance with an increase of hydrolysis time. Overliming utilizing calcium hydroxide resulted in 50% decline of furfurals while substantial decrease of phenolic compounds was observed. Five-fold vacuum evaporated concentration of hydrolysate exhibit considerable decrease in the concentration of acetic acid and furans. Furfural and hydroxymethyle furfural were removed completely removal with 5% treatment of charcoal at 60°C while acetic acid and phenolics also reduced. The detoxified hydrolysate can be applied in industry for production of several important products.

Keywords: Agricultural biomass, Comparative evaluation, Detoxification, Fermentation inhibitors, Sugarcane bagasse.

INTRODUCTION

Agricultural lignocellulosic waste materials, agro-industrial wastes and forestry residues are renewable and abundant sources of polysaccharides (Asada *et al.,* 2015). Fractionation by hydrolysis of lignocellulose waste materials is core of "biomass refinery" theory, which can isolate cellulose, hemicelluloses and lignin components i.e 34-50%, 19-34% and 11-30% respectively, transforms into commercial products like xylitol (Bajpai, 2016). Hemicellulose generates different sugars while cellulose polymer of glucose only. Xylans including galactans, arabinogalactans, mannans, glucomannans are the present in hemicelluloses (He *et al.,* 1993).

Agricultural biomass has been utilized for bio-development as it is a great potential substrate bio-development (Lynd, 2017). Dilute acid pretreatment is conducted to break down the lignocellulosic material recalcitrance and improve the accessibility of cellulose for enzymatic hydrolysis (Yang and Wyman, 2008;). After dilute acid pretreatment hemicellulose and cellulose can be hydrolyzed to corresponding monomers in the hydrolysates and is accessible for further bioproduction (Martinez *et al.,* 2007). Though, significant amount of inhibitors have been produced in the hydrolysate, which impede fermentation microbes (Jonsson *et al.,* 2013). Furfural, hydroxymethylfurfural (HMF), phenolics and aliphatic acids are the lignocellulosic-derived fermentation inhibitors (Sharma *et al.,* 2009). Research have been conducted on the quantification of fermentation inhibitors (Sharma *et al.,* 2009; Klinke *et al.*, 2002). In a corn stover hydrolysate 32 phenolic compounds, aliphatic acids, aldehydes and aromatic acids were identified by Chen *et al.* (2006). Similarly, Sharma *et al.* (2009) characterized 40 potential fermentation inhibitors in lignocellulosic hydrolysate. However, with alkali treatment several degradation products are produced from wheat straw including furans, acids, and phenols (Klinke *et al.,* 2002). Although much study have been carried out to find the most potential inhibitors on fermentation, yet not a single compound is considered a dominant inhibition compound (Xie *et al.,* 2015). Furfural and hydroxymethyle furfural have been indicated as the important compound for hydrolysate toxicity but both are not the only main inhibitors (Wang *et al.,* 2016). Aromatic aldehydes (syringaldehyde and 4-hydroxybenzaldehyde) and aromatic alcohols (coniferyl alcohol and catechol) also exhibit to impede fermentation rate of *Escherichia coli* LY01 (Zaldivar *et al.,* 2000; Zaldivar *et al.,* 1999) and this inhibition was related to the hydrophobicity (Wang *et al.,* 2018). Indeed, ketones and aldehydes are usually considered as main toxic compounds to the fermenting microorganisms. Ando *et al.* (1986) in the poplar hydrolysates identified 12 aromatic degradation compounds and explored the impact on yeast fermentation which conclude that ketones and aldehydes were more inhibitory compounds than alcohols and acids. Therefore, the quantification and detoxification

of fermentation inhibitor in the hydrolysates are required for bio-development.

Sugarcane is cultivated in colossal quantities in countries like China, Brazil, Pakistan, Mexico, India, Colombia, Thailand, Philippines, Indonesia, and the United States. It is estimated that 280 Kilogram of bagasse can be obtained from 1 ton of sugarcane. Sugarcane bagasse is a considered as a lignocellulosic waste that is rich in polysaccharides, which makes it a promising raw material for attaining products under biorefinery concept (Serna *et al.,* 2018). It is produced in huge quantity in Pakistan, according to Pakistan Bureau of statistics Pakistan produced 81.102 million tons during 2017-18. Most of sugarcane bagasse produced as byproduct can be utilized in value addition like single cell protein, organic acids, amino acid, enzymes, ethanol or xylitol. The purpose of the study was to hydrolyse the sugarcane bagasse and to carry out the comparative analysis of different available detoxification methods to improve the ferment ability of hydrolysate.

MATERIALS AND METHODS

Sugarcane (*Saccharum officinarum*) bagasse was purchased from market, converted into powder by 1mm screens and stored in air tight bags. Cellulose, lignin and xylan content of sugarcane bagasse was determined by the Van Soest method (1987). Moisture was determined by method No. 44-15A AACC (2000). Ash contents were determined by AACC (2000) method No.08-01.

Substrate's Pre-treatments: The grinded sample was added in a dilute H_2SO_4 solution (98%) to make concentration of 2mL of acid per100g of biomass with ratio of 10:1(liquid to solid) (Sreenivas *et al.,* 2006).

Autoclaving: Autoclave was utilized for hydrolysis. The process was carried out for 20, 40 or 60 min at 121°C. Electricity was turned off when the desired processing time reached. Samples were collected from autoclave after cooling (Martin *et al.,* 2007).

Filtration: The hydrolyzed samples were filtered by Whattman filter paper.

Detoxification Methodologies

Over-liming: pH of filtered hydrolysates was adjusted to pH 9.0 and pH 6 at 6°C by adding Ca $(OH)_2$. Afterwards filtration and centrifugation of hydrolysate was carried out at 2000 rpm to remove of precipitates of calcium hydroxide (Martinez *et al.,* 2001).

Vacuum Evaporation: To increase sugar contents and reduction of volatile inhibitors hydrolysate under vacuum at 40°C was concentrated three-fold and five-fold (Larsson *et al.,* 1999).

Charcoal Treatment: Powdered charcoal was added to hydrolysate i.e. 1% and 5% for 30 and 60 minutes respectively and stirring was carried out at 30°C. Filtration was carried out to obtain hydrolysate and analysed chemically (Villarreal *et al.,* 2006).

Analysis of Sugars: Derivatization of glucose, xylose, galactose and arabinose were carried out by gas chromatograph identification. Oximation-tri-methyl silyl process was used for quantification (Li, 1996). Sugars were estimated by Gas Chromatography (GC-17A Shimadzu, Japan) by FID detector. Temperature of detector was 300°C and injector temperature was set at 280°C.Carrier gas (Helium) was utilized at rate of 30 mL min^{-1} and pressure set at 150 psi. Internal standard used was Phenyl β-D-glucopyranoside. Data assimilated and examined with the HP-Chemstation software (Winterova *et al.,* 2008).

Determination of Furfural, HMF, Acetic acid and Phenolic Components: HPLC (10A) is used for the estimation of furfural and hydroxymethyl furfural (HMF). C_{18} column was used for the purpose in which UV-detector with 276nm. Acetonitrile-water was utilized as eluent and rate of flow was 1mL/min. C_{18} column was used for acetic acid estimation with having wavelength of 210nm. Mobile phase used was 0.045N H_2SO_4 having flow of 1mL/min (Carvalho *et al.,* 2006). Total phenolic contents were determined by using UV Spectrophotometer (Model U2020) at 280nm (Rocha, 2000).

Statistical Analysis: The significance level was determined by Analysis of Variance Technique (Completely Randomized Design) and used to estimate the significance level (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

Characterization of Substrate: Cellulose lignin and xylan of sugarcane bagasse were estimated on a dry weight basis. Cellulose is found to be abundantly present (42.8±3.26%) in comparison to other compounds. Lignin and xylan components were 22.1±2.03% and 20.8±1.42%, respectively. Unlike cellulose, hemicelluloses have various pentosans and hexoses sugars and different sugar acids. However, xylan is considered to be widely distributed polysaccharide in lignocellulosic substrates (Saha, 2003). The bagasse contained moisture and ash contents (6.88±0.28%) (1.40±0.13%) respectively.

Chemical Analysis of Substrate after Acid Hydrolysis (20, 40 and 60 minutes): Various hydrolysis time show pronounced effect on production of sugars (Fig. 1A) and byproducts from sugarcane bagasse. Rate and yield of hydrolysis were noticed to enhance with an increase of

temperature and hydrolysis time. Hydrolysis for 60 minutes considerably improved sugar contents and by-products in substrate when compared to 40 and 20 minutes (Table 1). Xylose content raised to 19.1g/L at 60 minutes as compared to hydrolysis conducted for 40 minutes i.e. 18.8g/L. Similar trend of sugar generation can be observed for the other sugars in Figure 1. Similarly, generation of carbonyle degradation compounds such as phenolic and acetic acid compounds were highest 0.28g/Land 2.75g/L , respectively when hydrolysis conducted for 60 minutes while 0.24g/Land 2.65g/L, respectively when hydrolysed for 40 minutes (Table 1).Fractionation of lignocellulosic material depend on many factors like temperature, acidity, reaction time and sugar contents in bagasse (Taherzadeh and Karimi, 2007).The sugar contents in hydrolysate increased with hydrolysis time (Martin *et al.,* 2007). However, prolonged hydrolysis reaction times i.e., more than 60 minutes, sugar contents may reduce because of generation of further degraded products (Cruz *et al.,* 2000). Moreover, during hydrolysis high pressure and temperature application during degrades xylose to furfural and glucose to hydroxymethyl furfural, respectively. However, incomplete lignin breakdown produces phenolic compounds (Mussatto and Robert, 2003).

Substrate properties including neutralizing capacity, degree of polymerization of cellulose chain, proportion of easily hydrolysable hemicellulose and cellulose, hydrolysis condition may effect generation of sugars. However, presence of cellulose with other polymer such as proteins, mineral elements and lignin within plant cell wall may also influence the hydrolysis and generation of different sugars. Particle size is also one of the effective factors (Taherzadeh *et al.,* 1997).

The hydrolysis process may influence from the diffusion of acid into the biomass and its dispersion in the reactor. Diffusion rate of acid catalyst depends on the nature of biomass that would be pointedly more in agricultural biomass than in hardwood (Kim and Lee, 2002).On the other hand, arabinose hydrolysis is easy because of its position in the arabinoxylan structure as the glycosidic bonding (Sjostrom, 1993).These degradation products which are toxic for fermentation microbes generated at maximum hydrolysis time may be decreased by adopting various detoxification strategies (over-liming, vacuum evaporation, charcoal treatment). The main purpose was the reduction of degraded products to such a level that they may not inhibit the growth of fermentation microorganism.

Effect of Over-liming on the Sugars and Degradation Products: After acid hydrolysis the hydrolysate was treated with calcium hydroxide for over-liming to detoxify fermentation inhibitors. Over-liming at pH 9 causes 50% decline of furfurals (Table 1). Similarly, there was a substantial decline of sugars (Fig. 1B) and phenolic components in detoxified hydrolysate than un-detoxified hydrolysate (Table 1). Over-liming in combination with high pH and temperature is considered as a favorable detoxification procedure for dilute sulfuric acid-treated hydrolysate of lignocellulosic material [Martinez *et al.,* 2001]. Over liming remove volatile inhibitory compounds such as furfural and (HMF) from the hydrolysate additionally causing a sugar loss (~10%) by adsorption. In a study carried out by Zhang *et al.,* (2018), detoxification carried out by over liming reduced 75.6% of furfural and derivatives while 68.1% of aromatic monomers decreased. The study concludes that neutralization with calcium hydroxide under certain pH could be due to formation of precipitates of toxic fermentation inhibitor and ionization of some these inhibitors reduced toxicity (Misra *et al.,* 2013). It is observed that some inhibitors are not stable at a certain pH, pH modification with calcium hydro-oxide (lime) is a common detoxification method for a various of hydrolysates. In general, lime $Ca(OH)_2$ is mixed in hydrolysate to modify the pH to 9-10, and then acid mosty H_2SO_4 or HCl is mixed to readjust pH to 5.5-6.5. It was noted that detoxification with over liming decreased furans (51%), phenolic compounds (41%) and sugars (8.7%). Chemical modifications of furfural and hydroxymethyle furfural by $Ca(OH)_2$ proved to lower toxicity and enhance the fermentation ability of hydrolysates (Palmqvist *et al.,* 1999).

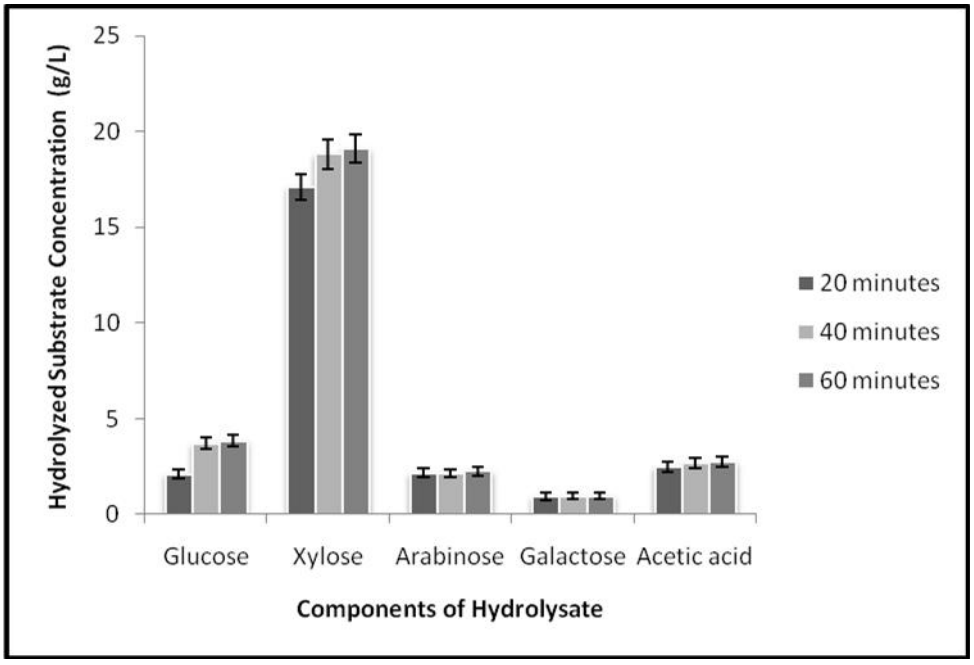
Effect of Vacuum Evaporation on the Detoxification of Inhibitors: Vacuum evaporation for hydrolysate detoxification is a process to decrease the volatile detoxification inhibitors contents. There was substantial decrease in furfural, hydroxymethyle furfural and acetic acid concentration of hydrolysate. The hydrolysates concentrated after vacuum evaporation did not exhibit more concentration of acetic acid. The contents raised 2.72g/L in hydrolysate to 5.89g/L in 5-foldhydrolysate. Furfural is found to be among volatile components. In 5-fold vacuum evaporated acid hydrolysate furfural concentration increased0.083 g/L to 0.12 g/L. 5-fold hydrolysate concentration effect lower rise of HMF from 0.03 to 0.13g/L (Table 1). The major disadvantage related to detoxification process with vacuum evaporation was considerable rise in consents of phenolic contents. Phenolic concentration raised 0.19 to 69.1g/L in acid treated hydrolysate. The comparative high yield of glucose, xylose, galactose, arabinose was observed in five-folds concentrated hydrolysate (Fig. 1C).

The study recommends the hydrolysate detoxification by vacuum concentration favours biotransformation which increases sugars content (Felipe, 2004). Furthermore, vacuum evaporated concentration can effective procedure for exclusion of various volatile components. Though, this method enhances non-volatile

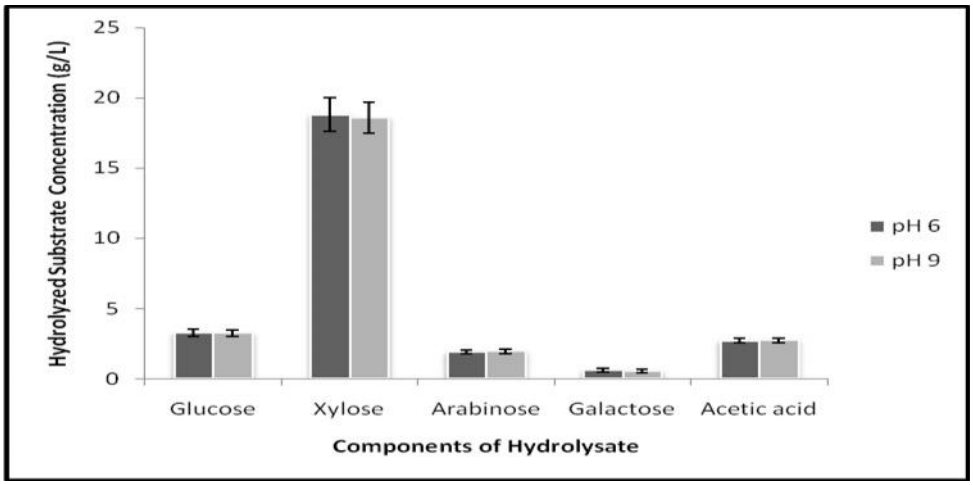
components concentration that raised substantially (Palmqvistand and Hahn-Hagerdal, 2000; Rodrigues, 2001). Vacuum concentration removed acetic acid and furfural derivatives and in the hydrolysates detoxification. It is considered more beneficial for removal of furfural as it removes about 90% of furfural. Furfural is found to be a most volatile component which at high temperature could polymerized (Lee *et al.,* 1999). Furfurals completely eliminated due to vacuum concentration from a hydrolysate (Rodrigues, 2001). HMF and acetic acid are relatively lower volatile as compared to furfural, although, decline to a lower degree. However, on the hand non-volatile compounds such as phenolic contents and sugars raised consistently by increasing the concentration of hydrolysate (Girion *et al.,* 1996).

Effect of Charcoal Treatment on Hydrolysate: Two different detoxification process were carried out with charcoal. First process was conducted with 1% and 5% charcoal at 30°C and at 60°C respectively. Both treatments substantially effected the elimination of fermentation inhibitors and sugars (Table 1). The reduction of sugar contents, phenolic compounds removal and acetic acid removal was determined increased with 5%, 1%charcoal at 60°Cand 30°C respectively. However, furfural and hydroxymethyle furfural were exhausted with hydrolysate detoxified at 60°C by 5% charcoal. The study reveals that 50% reduction of phenolic components was observed that by applying1% charcoal at 30°C. However, at 60°C with 5% charcoal eliminated 90% of phenolic components. The study concludes that activated charcoal at 60°C with 5% concentration is a more suitable step for detoxification of hydrolysate as this process increase exclusion of biotransformation inhibitors. The reason could be faster adsorption of compounds at elevated temperature which increase diffusion rate of adsorbant molecule (Bernardin, 1985). The temperature also had considerable impact on the absorption of color components on charcoal. These particles are bigger in comparison and their direction on charcoal surface may be longitudinal under definite temperatures and transversal in other condition, the adsorption capacity of activated charcoal might enhance or decline (Moreira *et al.,* 2000). Study suggest higher charcoal concentration of15 g/L is most suitable value considered for clarification of fermented broth (Misra *et al.,* 2011). It shows true that with increased concentrations of charcoal, the effect of contact time reduces. However, with an elevated temperature, rates reduction of these components raised 6-folds because of comparatively higher appropriate packaging of the components in pores of charcoal pores at elevated temperatures (Santana *et al.,* 2018). Hydrolysate treated with activated charcoal with 1% was found suitable to exclude 94% of phenolic components and causing lower sugar losses (Silva *et al.,*1998). It was observed from the study that charcoal treatment could reduce remarkably more phenolic compounds than over liming. It was concluded from the study that effect of over liming, vacuum evaporation and charcoal treatment on inhibitors removal was related to their chemical structures.

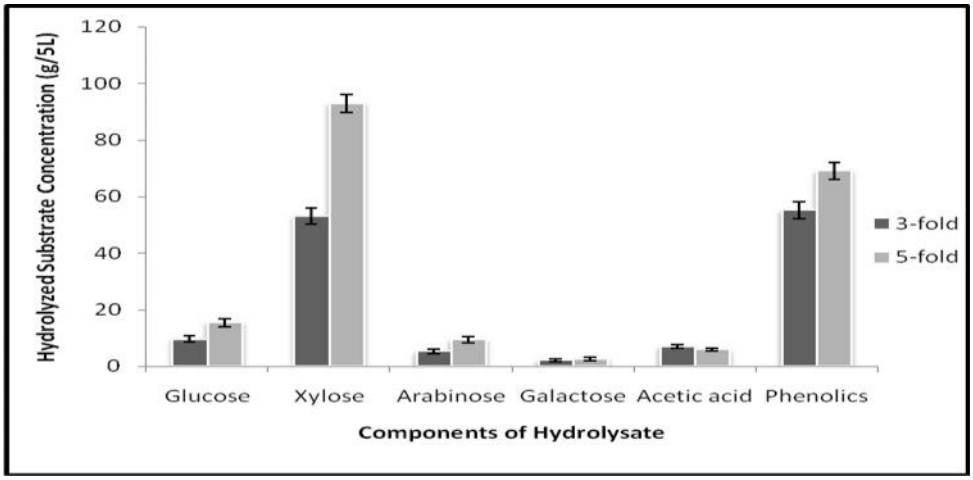
(A)



(B)



(C)



(D)

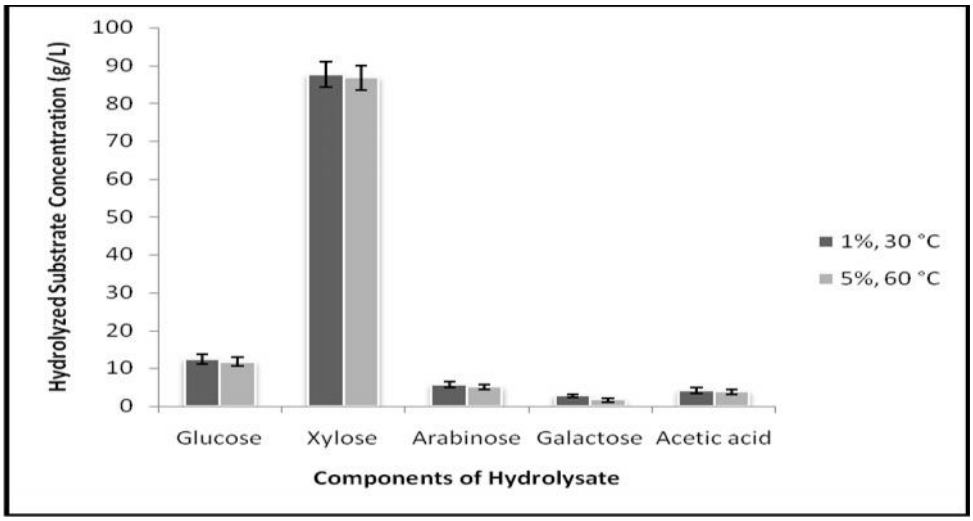


Figure 1. Chemical analysis of sugarcane bagasse substrate after (A) acid hydrolysis (20, 40 and 60 minutes) (B) over-liming (pH 6; pH 9) (C) vacuum evaporation (3-fold; 5-fold) (D) charcoal treatment (1%, 30 °C; 5%, 60 °C).

Table 1. Effect of hydrolysis and detoxification techniques on hydrolysed substrate concentration.

	Hydrolysis (g/L)			Over Liming (g/L)		Vacuum Evaporation (g/5L)		Charcoal (g/L)	
	20 minutes	**40 minutes**	**60 minutes**	**6 pH**	**9 pH**	**3-fold**	**5-fold**	**1%, 30 ˚C**	**5%, 60 ˚C**
Glucose	2.1 ±0.23	3.7 ±0.06	3.83±0.09	3.28±0.04	3.25 ±0.03	9.65±0.01	15.5 ±0.01	12.3±0.26	11.7±0.15
Xylose	17.1 ±0.20	18.8 ± 0.06	19.1±0.06	18.8± 0.12	18.6 ± 0.11	53.2± 0.18	93.0± 0.23	87.6± 1.67	86.8±0.874
Arabinose	2.15± 0.24	2.13± 0.09	2.25±0.02	1.90± 0.026	1.95± 0.028	5.24±0.011	9.40±0.014	5.60±0.017	5.03± 0.036
Galactose	0.71± 0.04	0.75± 0.02	0.76±0.02	0.60± 0.03	0.56± 0.02	1.60± 0.04	2.58± 0.06	1.72± 0.017	0.66±0.012
Acetic acid	2.46±.088	2.65 ± 0.03	2.75 ±.03	2.70± 0.07	2.72 ± 0.08	7.01± 0.05	5.89± 0.03	4.1±0.017	3.74±0.023
Furfural	0.09 ±0.01	0.11 ± .003	0.13±0.002	0.1± 0.003	0.08 ± 0.001	0.20±0.003	0.12±0.002	0.018±0.0008	ND
HMF	0.03 ±0.01	0.04 ±0.001	0.05±.001	0.04± .001	0.03 ± .001	0.18±0.004	0.13±0.002	0.014±0.002	ND
Ph. C	0.22±0.01	0.24± 0.009	0.28±0.003	0.25± 0.003	0.19± 0.002	55.2±0.31	69.1±0.38	0.22± 0.017	0.061±.001

Conclusions: Presence of inhibitors in lignocellulosic hydrolysate is an industrial malaise. The present study concludes that hydrolysis conducted for 60 minutes found to be more significant in definition of generating xylose and fermentable sugars. Over liming, vacuum evaporation and charcoal treatment for hydrolysate detoxification decreased the concentration of undesirable reaction products. Considering these preliminary assays, other new effective methodologies should be investigated to release fermentable sugars from hemicellulosic hydrolysate for their subsequent bio-based conversion into products of commercial significance.

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