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ENZYMATIC HYDROLYSIS OF *SACCHARUM OFFICINARUM* LIGNOCELLULOSIC BIOMASS BY GENETICALLY MODIFIED HYPERTHERMOPHILIC CELLULASES

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Abstract

The rapid exhaustion of fossil fuels and increasing environmental pollution due to burning of traditional fuels urge the scientists to look for alternative renewable energy resources. One of the important renewable energy sources is bioethanol. Bioethanol production from lignocellulosic substrate comprises of different steps. One of the important part of these steps is saccharification which yield fermentable sugars that can later be converted into ethanol by fermentation. In the present study, hyperthermophilic cellulolytic enzymes (Endoglucanase, Exoglucanase and β-Glucosidase) produced in genetically modified mesophilic host *Escherichia coli* BL-21 strain were used for hydrolysis of untreated and pretreated sugarcane bagasse samples. Alkaline peroxide treated bagasse was screened among differently pretreated sugarcane bagasse samples and saccharification yield of 26.64% was achieved by simultaneously adding 150 Units of Endo-1,4-β-glucanase, 300 Units of Exo-1,4-β-glucanase and 600 Units of β-1,4- Glucosidase for 3h at 80°C. Moreover, substrate concentration of 0.75% (w/v) yielded best hydrolysis rate of 35.19%. This optimization study resulted in 8.05 folds increase in the saccahrification yield which is an important step forward towards cheap production of fermentable saccharides.

Key words: Agricultural residue, Saccharification, Bioenergy, Catalysis, Thermophiles.

Introduction

Increased prices, understanding of environmental hazards and depletion of fossil fuels has led to a better apprehension of energy crisis and importance to look for renewable alternatives energy sources known as biofuels. Bioethanol is one such type of biofuel which is extensively used as an alternative having advantages such as its renewable, eco-friendly and sustainable nature; use as a gasoline enhancer, having high oxygen content and lesser requirement of additives (Balat *et al.,* 2008; Carodona & Sanchez, 2008). The production of bioethanol is a multi-step process, out of these hydrolysis or saccharification of cellulose into monomeric sugars is the most important one. In this process, β-1,4 glycosidic linkage in cellulose is broken down and several different sugars are produced (Liu *et al.,* 2018). Different types of substrates can be used for the production of bioethanol but lignocellulosic biomass is a preferred source because of low cost, easy availability and non-competitive nature with food sources, (Kumar *et al.,* 2009; Zhu & Pan, 2010). Sugarcane bagasse is one such substrate which is used in the present study because of high regeneration capacity, cheap availability and low ash content.

There are different ways of which hydrolysis can be achieved such as chemical, thermal or enzymatic. Enzymatic saccharification is preferred as it takes place under milder conditions. It is also non-toxic, gives high product yield and produces lesser inhibitors. It is carried out by a group of enzymes known as cellulases. They catalyze conversion of cellulose with the help of water thus releasing shorter oligomeric chains and ultimately soluble, fermentable sugars (Alvira *et al.,* 2010). Cellulases are substrate specific and mainly three different types of enzymes includes; endoglucanase, exoglucanase and β-glucosidase, each has its particular function. Along with these enzymes other accessory enzymes can be used in enzymatic saccharification for complete conversion of substrate. All these enzymes work in a synergistic way to break down the complex cellulose molecule (Verardi *et al.,* 2012).

Hyper thermophilic bacterial cellulases (capable of growing at temperature > 60°C) were used in this study. Despite the evident benefits of thermostable enzymes there are some problems associated with their production such as requirement of complex cultivation media for growth of thermophiles and hyperthermophiles. This problem has been overcome by cloning the desired gene into a suitable host especially mesophilic bacterial system which has high expression and growth (Goyal *et al.,* 2001). In this study genetically modified strain of *Escherichia coli* is used for this purpose.

Enzymatic hydrolysis is a complex process and there are some factors which should be considered while designing the process of enzymatic hydrolysis. Some factors inhibit while others enhance the rate of hydrolysis (Farinas *et al.,* 2018; Verardi *et al.,* 2012). The present study focuses on such factors like concentration of substrate and enzyme, pH, temperature, reaction and reaction time to achieve maximum hydrolysis of sugarcane bagasse.

Materials and Method

Chemicals: All the chemicals used in current study were of analytical grade and obtained from authentic suppliers of Merck and Sigma Ltd**.**

Hyperthermophilic Cellulases: Genetically modified strains of *E. coli* BL 21 containing genes of *Thermotoga petrophila* were used to produce Endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). The mesophilic host *Escherichia coli* BL21 containing gene of *Caldicellulosiruptor* spp. was used for Exoglucanase (EC

3.2.1.91) production. These thermophilic enzymes were obtained from a project "Production of bioenergy from plant biomass" carried out at Institute of Industrial biotechnology, GC University Lahore, Pakistan with the permission of principle investigator.

Substrate: Both control (untreated) and pretreated samples of sugarcane bagasse (*Saccharum officinarum*) with lignocellulosic composition were taken from Pakistan Council of Scientific and Industrial Research, Lahore (Table 1).

Enzymatic depolymerization of sugarcane bagasse: Pre-weighed quantity (0.5% w/v) of sugarcane bagasse was added in air-tight culture bottles. Specific units (50 U/mg) of each enzyme i.e. Endoglucanase, Exoglucanase and β-Glucosidase were added along with 25 mL of citrate phosphate buffer having pH 6. Enzymatic depolymerization was performed at 70˚C and 100 rpm in water bath shaker (Model: WSB-30, Wisd, WiseBath, Germany). Sample (1ml) on regular intervals (30 min) was withdrawn and DNS method was used for calculation of reducing sugars (per ml) and percentage saccharification. Control was also run along with the test sample. Method devised by Vallander and Eriksson, (1989) was used for percentage saccharification calculation.

Optimization of factors affecting enzymatic hydrolysis of bagasse: After screening and selection of pretreated sugarcane bagasse conditions of hydrolysis were examined to maximize the conversion of substrate into reducing sugar. The parameters which were assessed include: incubation time (0.5-5hrs), temperature (60°C-90°C), enzyme concentration, and substrate concentration (0.25-1%) (Saratale *et al*., 2018)

Scanning electron microscopy: Microscopic comparison of the samples before and after saccharification was conducted using Scanning Electron Microscope (JSM-6480, Tokyo, Japan) at Center of Advance Studies in Physics (CASP) GC University Lahore, Pakistan.

Statistical analysis: 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.

Results and Discussion

Selection of substrate: Control and pre-treated samples of sugarcane bagasse were assessed for enzymatic hydrolysis. All the substrate samples gave maximum hydrolysis after an incubation period of 3 h. However, among the samples, Bagasse A pretreated with 5% H_2O_2 and 2.5% KOH showed maximum saccharification of 13.21±0.04%, which was followed by Bagasse B and control sample with saccharification of 11.02±0.07% and 4.37±0.01%, respectively, as shown in figure 1. Therefore, Bagasse A was selected for further experiments. Alkaline hydrogen peroxide pretreatment may have resulted in decreasing the crystallinity of cellulose by selectively removing lignin. The recalcitrance of bagasse was also reduced, increasing cellulose accessibility for enzyme action. Alkali also tends to produce a more open cellulosic structure by penetrating in the inner layers via removing hemicelluloses and hydrolyzing lignin (Iroba *et al.*, 2013). Matins *et al*., (2015) and Supranto *et al*., (2014) reported used of 7% H_2O_2 in combination with KOH and 30% H_2O_2 for the pretreatment of sugarcane bagasse, respectively. These pretreated substrates also showed higher conversion rate in response to enzymatic hydrolysis compared to substrates pretreated with other strategies which shows the efficiency of H_2O_2+KOH pretreatment to achieve better conversion of cellulose to fermentable saccharides.

Effect of incubation time: Effect of incubation time on depolymerization of Bagasse A was monitored every 30 min for a period of 5 h. Initially with increase in time, gradual rise in saccharification i.e. 5.87±0.12%, 7.72±0.07%, 8.96±0.01%, 11.89±0.01%, 12.51±0.07% after 0.5, 1, 1.5, 2 and 2.5 h, respectively, was observed which reached to maximum saccharification of 13.24±0.05% after 3 h of incubation. However, further increase in incubation time resulted in linear decrease in saccharification yield (Fig. 2). This was might be due to the fact that rate of enzymatic hydrolysis is rapid during the primary hydrolysis phase as the substrate is readily available for enzyme action. While, it slows down in the secondary hydrolysis phase because of significant product inhibition and enzyme inactivation with the passage of time. With the passage of time pH changes are also likely to be produced because of the formation of phenolic compounds, resulting in their inactivation of cellulases (Verardi *et al.*, 2012). Sukumaran *et al*., (2009) and Sindhu *et al*., (2011) in comparison to our findings obtained maximum sachharification of 17.23% and 1.23% respectively using commercial cellulases. This difference in findings can be attributed to the use of thermophilic cellulases in current study which might have better catalytic activity but shorter stability period compared to commercial cellulases.

Table 1. Lignocellulosic composition of untreated and pretreated sugarcane bagasse.

Biomass	Cellulose (%)	Lignin (%)	Delignification (%)	Pretreatment conditions
Bagasse (control)	5%	16%	-	
Bagasse A (pretreated)	60%	3%	80%	5% H_2O_2 + 2.5% KOH, 120°C, 30 minutes
Bagasse B (pretreated)	53 %	6%	75%	2% HCl 120°C, 30 minutes

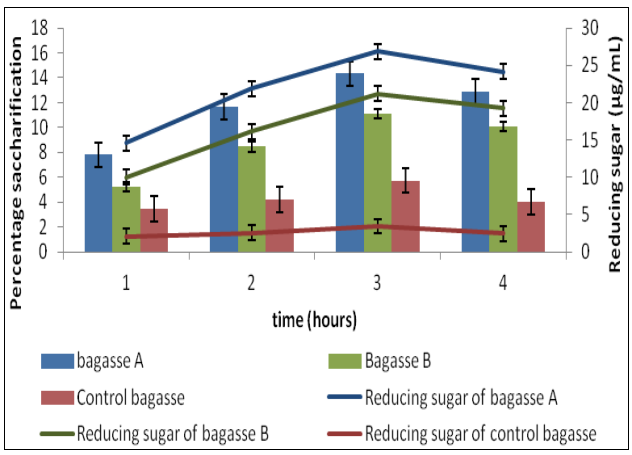

Fig. 1. Screening of pretreated sugarcane bagasse samples by enzymatic hydrolysis. Error bars indicate standard deviation (±SD) among three replicates.

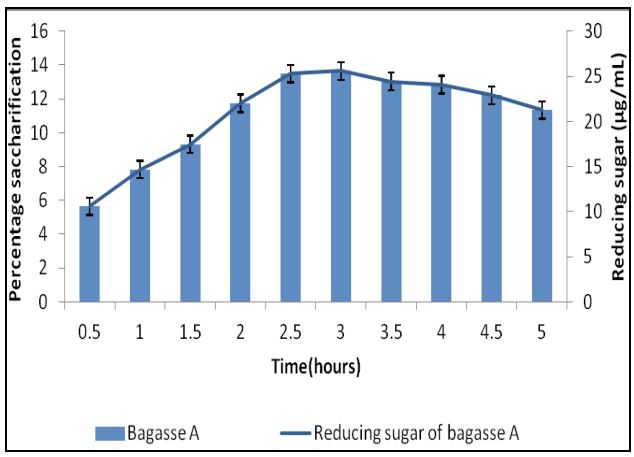

Fig. 2. Effect of incubation time on saccharification of Sugarcane Bagasse A. Error bars indicate standard deviation (±SD) among three replicates.

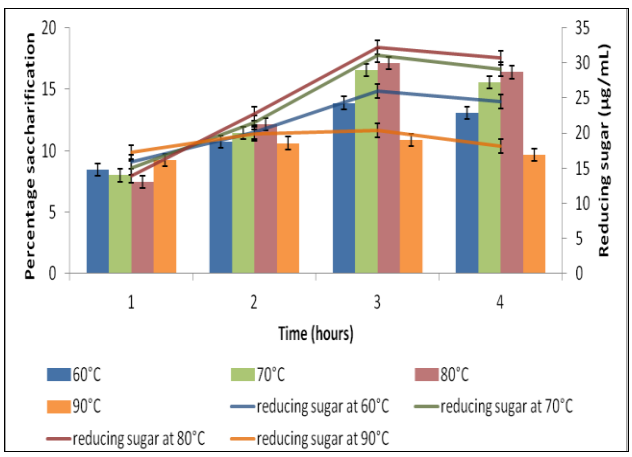

Fig. 3. Effect of different temperatures on the enzymatic saccharification of sugarcane bagasse A. Error bars indicate standard deviation (±SD) among three replicates.

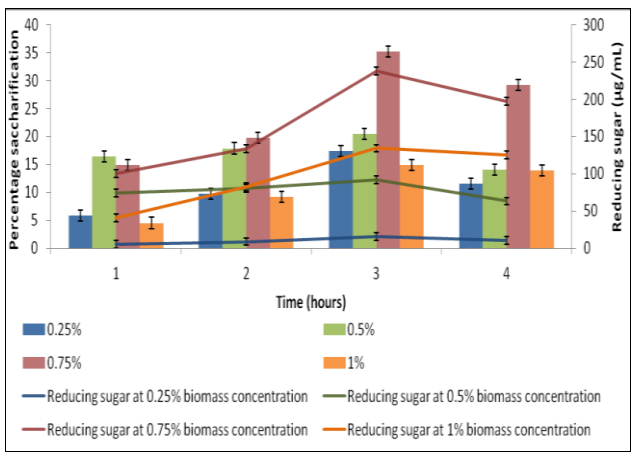

Fig. 4. Impact of various biomass concentrations on enzymatic hydrolysis of sugarcane bagasse. Error bars represent the significance of three determinants with a probability of ≤ 0.05.

Role of temperature in the enzymatic hydrolysis: Variable temperatures such as 60°C, 70°C, 80°C and 90°C were tested to maximize the hydrolysis of Bagasse A into fermentable saccharides. After 1 h of incubation maximum saccharification i.e., 8.58±0.05% was noted at 90°C. However, increase in incubation time resulted in increased saccharification at all tested temperature up to 3 h but hydrolysis at 90°C was remain constant after 2 and 3 h. Maximum saccharification of 17.68±0.03% was observed at 80°C (Fig. 3). This is might be due to increase in cellulose adsorption with increasing temperature (Solomon *et al.*, 2010). High temperature affect the viscosity of the reaction mixture and decrease viscosity increase cellulose solubilization, thereby increasing the chances of bioconversion. High temperature also decreases the risk of contamination (Bergquist *et al*., 2004). However, stability of the thermophilic cellulases may decrease with increase in temperature for a longer period of time. Pereira *et al*., (2015) and Kazeem *et al*., (2016) put forward 15.6% and 1.92 % saccharification of sugarcane bagasse using fungal and bacterial cellulases at 70 and 80°C, respectively.

Optimization of enzyme units: In order to enhance the hydrolysis yield of sugarcane bagasse A, endoglucanase, exoglucanase and β-Glucosidase enzyme was applied. Concentration of Endo β-1,4- glucanase was varied in the range of 50-200 Units. While, for Exo β-1,4-glucanase and β-1,4- Glucosidase units were varied in a range of 100-400 and 200-800 Units, respectively. Maximum percentage saccharification was obtained using synergy containing 150 Units of endoglucanase (22.45%), 300 Units of exoglucanase (25.71%) and 600Units of β-glucosidase (29.64%) as indicated in Table 2. At constant substrate concentration hydrolysis rate is directly proportional to enzyme concentration i.e. greater the enzyme concentration greater will be the hydrolysis of bagasse however this trend is followed up to a certain value, after that increasing enzyme concentration do not enhance the product yield as all of the enzyme's active sites get saturated with substrate molecules. As the time passes substrate will get converted into product making enzyme's active site available. Substrate concentration now acts as a rate limiting factor and further increase in enzyme concentration will no longer increase the saccharification yield (Yang *et al*., 2006). Hence, an optimum amount of each of the three enzymes is needed to elevate the percentage saccharification, avoiding the phenomenon of feedback inhibition.

Table 2. Optimization of cellulase units for improved saccharification of sugarcane bagasse.

Enzyme optimized	Endo β-1,4-glucanase (U)	Exo β-1,4-glucanase (U)	β-1,4-Glucosidase (U)	Saccharification (%)			
				1 h	2 h	3 h	4 h
Endo β-1,4- glucanase	50	100	200	4.36	9.84	17.97	16.34
	100	100	200	6.12	12.40	20.02	19.31
	150	100	200	8.39	14.89	22.45	21.83
	200	100	200	7.34	13.02	20.76	19.46
Exo β-1,4- glucanase	150	100	200	8.41	14.67	22.46	21.66
	150	200	200	9.67	15.28	23.98	22.10
	150	300	200	11.28	17.89	25.71	24.39
	150	400	200	10.01	16.20	24.09	22.78
β-1,4- Glucosidase	150	300	200	11.20	17.21	25.72	24.38
	150	300	400	13.20	20.29	27.37	26.40
	150	300	600	15.39	24.39	29.64	28.89
	150	300	800	13.29	23.19	29.01	28.01

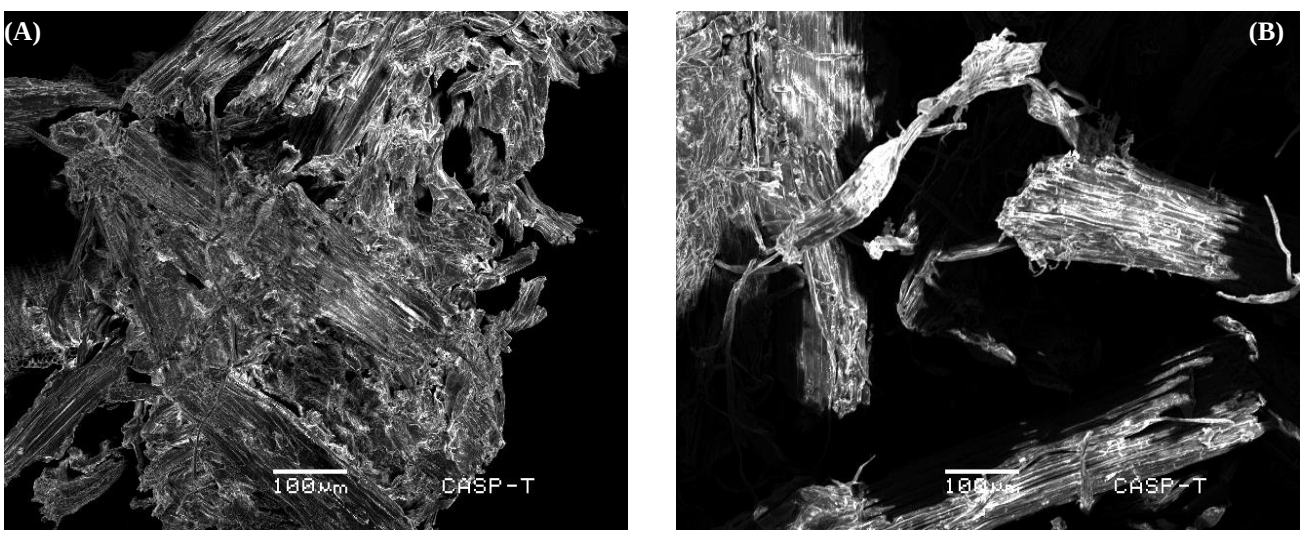

Fig. 5. Scanning electron micrograph of Sugarcane bagasse (A) before hydrolysis (b): after hydrolysis.

Effect of biomass concentration: Different biomass concentrations including 0.25%, 0.5%, 0.75% and 1% (w/v) of sugarcane bagasse A were evaluated for maximum saccharification yield. Among these, maximum percentage saccharification i.e., 35.19±0.12% was achieved using 0.75% (w/v) sugarcane bagasse A (Fig. 4). Nature and concentration of substrate can both have an important role in bioconversion process (Yang *et al*., 2006). At constant enzyme concentration, rate of hydrolysis increases by increasing the substrate concentration because all of the enzyme's active sites are accessible for catalysis but this only occurs to a certain limit i.e. until saturation level is reached, after that increasing the substrate concentration will not enhance the process of enzymatic hydrolysis. More than one substrate molecule may bind to the active site of enzyme at higconcentration of substrate resulting in active site blockage (Kristensen *et al*., 2009). High substrate concentration may also become problematic as it hinders in proper mixing and mass transfer resulting in reduced hydrolytic yield. Huang *et al*., (2015) and Rodrigues *et al*., (2017) employed 6% and 2.2% concentration of sugarcane pulp and sugarcane bagasse, respectively to obtain the maximum saccharification.

Scanning electron microscopy: Microscopic analysis of Sugarcane bagasse A was performed before and after the process of hydrolysis. Differences in structure and morphology of bagasse were observed under scanning electron microscope. Before hydrolysis densely packed fibers were, they depicted rigid and compact morphology but after depolymerization, the fiber bundles were present in a disintegrated and fractured form. As depicted in figure 5(a), the structure of the cell wall is conserved; cellulose microfibrils being arranged in elongated layers, parallel to one another. After being enzymatically hydrolyzed the initial compact structure of bagasse was dismantled in the form of detached fibers as evident from figure 5(b). In accordance with the current study Rodrigues *et al.,* (2017) also used scanning electron microscopy to analyze the structural variance in sugarcane bagasse.

Conclusion

It became evident from current study that pretreatment strategy affects the enzymatic hydrolysis of sugarcane bagasse directly. Moreover, it was also concluded that different reaction parameters such as incubation time, temperature, enzymes and biomass concentration have a significant effect on the depolymerization of sugarcane bagasse by cellulases. Structural variance in biomass before and after enzymatic hydrolysis can be observed significantly using scanning electron micrography.

These findings can be a step forwards towards a process development for the production of fermentable saccharides to produce bioethanol at larger scale.

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References

Alvira, P., E.T. Pejo, M. Ballesteros and M.J. Negro. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Biores. Technol.*, 101: 4851-4861.

Balat, M., H. Balat and C. Oz. 2008. Progress in bioethanol processing. *Prog. Energ. Combust. Sci.*, 34: 551-573.

Bergquist, P.L., V.S.J. Teo, M.D. Gibbs, N.C. Curah and K.M.H. Nevalainen. 2004. Recombinant enzymes from thermophilic micro-organisms expressed in fungal hosts. *Biochem. Soc. Trans.*, 32(2): 293-297.

Cardona, C.A. and O.J. Sanchez. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Biores. Technol*., 99(1): 5270-5295.

Farinas, C. S., J.M. Marconcini and L.H.C. Mattoso. 2018. Enzymatic conversion of sugarcane lignocellulosic biomass as a platform for the production of ethanol, enzymes and nanocellulose. *J. Renew. Mater.*, 6(2): 203-216.

Goyal, K., P. Selvakumar and K. Hayashi. 2001. Characterization of a thermostable β-glucosidase (BglB) from *Thermotoga maritime* showing transglycosylation activity. *J. Mol. Catal. B: Enzymatic.*, 15(3): 45-53.

Huang, Yeping, X. Qin, X. M. Luo, Q. Nong, Q. Yang, Z. Zhang and Y. Gao. 2015. Efficient enzymatic hydrolysis and simultaneous saccharification and fermentation of sugarcane bagasse pulp for ethanol production by cellulase from *Penicillium oxalicum* EU2106 and thermotolerant *Saccharomyces cerevisiae* ZM1-5. *Biomass Bioen.*., 77: 53-63.

Iroba, K.L., L.G. Tabil, T. Dumonceaux and O. Baik. 2013. Effect of alkaline pretreatment on chemical composition of lignocellulosic biomass using radio frequency heating. *Biosys. Eng.*, 116(4): 385-398.

Kazeem, M.O., U.K.M. Shah and A.S. Baharuddin. 2016. Enhanced cellulase production by a novel thermophilic *Bacillus licheniformis* 2D55: characterization and application in lignocellulosic saccharification. *Bio. Resour.*, 11(2): 5404-5423.

Kristensen**,** J.B., C. Felby and H.J. Orgensen. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocelluloses. *Biotechnol. Biofuels*, 2(11): 122-128.

Kumar, S., S.P. Singh, I.M. Mishra and D.K. Adhikari. 2009. Recent advances in production of bioethanol from lignocellulosic biomass. *Chem. Eng. Technol.*, 32(4): 517-526.

Liu, G., Q. Zhang, H. Li, A.S. Qureshi, J. Zhang, X. Bao and J. Bao. 2018. Dry biorefining maximizes the potentials of simultaneous saccharification and co-fermentation for cellulosic ethanol production. *Biotechnol. Bioeng.*, 115(1): 60-69.

Martins, L.H.D.S., S.C. Rabelo and A.C.D. Costa. 2015. Effects of the pretreatment method on high solids enzymatic hydrolysis and ethanol fermentation of the cellulosic fraction of sugarcane bagasse. *Biores. Technol.*, 191: 312-321.

Pereira, J.D.S., N.P. Marques, A. Rodrigues, T.B.D. Oliveira, M. Boscolo, R.D. Silva, E. Gomes and D.A.B. Martins. 2015. Thermophilic fungi as new sources for production of cellulases and xylanases with potential use in sugarcane bagasse saccharification. *J. Appl. Microbiol.*, 118(4): 928-939.

Rodrigues, P.D.O, J.D.S. Pereira, D.Q.D. Santos, L.V.A. Gurgel, D. Pasquini and M.A. Baffi. 2017. Synergistic action of an *Aspergillus* (hemi-) cellulolytic consortium on sugarcane bagasse saccharification. *Ind. Crops Prod.*, 109: 173-181.

Saratale, G.D., R.G. Saratale, S.H. Kim and G. Kumar. 2018. Screening and optimization of pretreatments in the preparation of sugarcane bagasse feedstock for biohydrogen production and process optimization. *Int. J. Hydrog. Energy.*, 43(25): 11470-11483.

Sindhu, R., M. Kuttiraja, P. Binod, K.U. Janu, R.K. Sukumaran and A. Pandey. 2011. Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. *Biores. Technol.*, 102 (23): 10915-10921.

Solomon, M.M., S.A. Umoren, I.I. Udosoro and A.P. Udoh. 2010. Inhibitive and adsorption behaviour of carboxymethyl cellulose on mild steel corrosion in sulphuric acid solution. *Corros. Sci.*, 52(4): 1317-1325.

Sukumaran, R.K., R.R. Singhania, G.M. Mathew and A. Pandey. 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renew. Energy.*, 34(2): 421-424.

Supranto, T. and D.E. Yunanto. 2014. Sugarcane bagasse conversion to high refined cellulose using nitric acid, sodium hydroxide and hydrogen peroxide as the delignificating agents. In: *Proceeding of the 27th Regional Symposium on Chemical Engineering*, 29-30.

Vallander, L. and K.E. Eriksson. 1985. Enzymatic saccharification of pretreated wheat straw. *Biotechnol. Bioengg.*, 27(5): 650-659.

Verardi, A., I.D. Bari, E. Ricca and V. Calabro. 2012. Hydrolysis of lignocellulosic biomass: current status of processes and technologies and future perspectives, Bioethanol. Prof. Marco Aurelio Pinheiro Lima (Ed.), ISBN: 978-953-51-0008-9.

Yang, S., W.Y. Ding and H. Z. Chen. 2006. Enzymatic hydrolysis of rice straw in a tubular reactor coupled with UF membrane. *Proc. Biochem.*, 41(3): 721-725.

Zhu, J.Y. and X.J. Pan. 2010. Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. *Biores. Technol.*, 101(13): 4992-5002.

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