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ARTICLE

Valorization of wheat bran for cost-effective production of cellulolytic enzymes by *Aspergillus fumigatus* SKH2 and utilization of the enzyme cocktail for saccharification of lignocellulosic biomass

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ABSTRACT Production of cellulolytic enzymes like CMCase (endoglucanase), FPase, and xylanase by *Aspergillus fumigatus* SKH2 under solid state fermentation was carried out employing wheat bran as low cost substrate. Fermentation time, medium pH and incubation temperature were optimized at 48 h, pH 5.0 and 35 °C, respectively. At optimized state, CMCase (endoglucanase), FPase and xylanase of 826, 102 and 1130 U/gds yield was noticed, respectively. Crude enzyme cocktail was assayed at varied pH and temperature, and pH 5.0 and 35 °C were proved to be optimal for the studied enzyme activities. Fourier transform infrared spectroscopic FTIR analysis attested that NaOH was a good delignifying agent for sugarcane bagasse and grass *Aristida* sp., which enhanced subsequent saccharification efficiency of cellulolytic enzyme cocktail. By correlating FTIR analysis with saccharification profile it was found that highest saccharification was achieved after 16 h and 48 h after treating with 1M and 3M NaOH for sugarcane bagasse and *Aristida* sp., respectively. The present investigation validates eco-friendly and cost effective production of *A. fumigatus* SKH2 cellulolytic enzyme cocktail using agricultural waste materials, and subsequent application of this cellulase mixture for saccharification of lignocellulosic biomass, which collectively endorse the employment of the bioprocess to produce biofuel in future.

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cellulolytic enzyme
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Introduction

From the last few years, there has been an increasing research importance in the worth of lignocellulosic biomass. These are the maximum promising feedstock as natural and renewable reserve. Among many of the developing countries, it's a routine practice that such agricultural wastes are not been fully castoff and then have turn out to be a major cause of environmental pollution (Soudham 2015). Naturally, hemicellulose, cellulose and lignin are the major constituents of plant cell walls and among all of them, cellulose is the most conjoint and profuse component of all plant matter.

Since the last decade, demand for some industrially important enzymes increases and among them cellulases are pioneer. Cellulase is being employed in many industrial applications mainly but not limited in the field of cotton processing, paper salvaging, agronomy and in the field of research and development (Dong et al. 2015). Besides, the production of fuel ethanol from lignocellulosic biomass through cellulase hydrolysis is a most promising tool of the modern world. The most efficient technology for the conversion of the lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulolytic enzymes (Das et al. 2013).

India is an agricultural land that produced a large magnitude of lignocellulosic wastes which can be utilized for production of useful industrial enzymes or enzyme-based products. Enzymatic hydrolysis of such agricultural wastes provides an ecofriendly means of depolymerizing cellulose and other carbohydrates at high yields (Das et al. 2013). But the most important barrier to produce low cost fermentable sugar from biomass is that the cellulose component of lignocellulosic materials is tightly bound to hemicelluloses and lignin. Lignin protects from degradation and confers hy-

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drolytic stability and structural robustness to the cell walls of the plants. To stunned lignocellulose resistance, pretreatment is required to alter the structure as well as its submicroscopic chemical composition, so that enzymatic hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more quickly with greater yields. Till date, the most widely familiar pretreatment techniques include liquid hot water, steam explosion, treatment with alkali, dilute acid, sulphur dioxide, hydrogen peroxide, organosolve, ammonia fiber explosion (AFEX), CO_2 explosion, ionic liquids etc. (Jena et al. 2016). Considering these, alkali, acid and peroxide treatments are widely applied in industries, but the suitability and the concentration of the chemicals depend on the type of substrate utilized (Chiaramonti et al. 2011).

Literature review attested that cellulolytic filamentous fungi belonging to the genus *Trichoderma* have widely been considered for production of cellulolytic enzymes and also preferred by many companies worldwide (Kovács et al. 2009; Sørensen et al. 2013; Rani et al. 2014). However, wild types *T. reesei* is generally characterized as high endoglucanase and cellobiohydrolase producer, but it secretes the β-glucosidase in minimum quantity and thus, additional supplementation of β-glucosidase is need to carry out saccharification of cellulosic substrates efficiently (Lynd et al. 2002; Rani et al. 2014). Generally, the production of cellulases accounts for about 40% of total cost in bioethanol production (Gray et al. 2006). To minimize the production cost, attempt should be taken for utilization of zero-valued/low cost easily available lignocellulosic materials for the production of cellulolytic enzymes. Moreover, the microorganisms capable to utilize the complex lignocellulosic materials also liberates other hydrolyzing enzymes like xylanase, ligninase etc. along with cellulolytic enzymes. Presence of these accessory enzymes enhances the biodegradation of the complex substrates. So, the approach for production of crude enzyme cocktail from low cost substrates and its subsequent application in saccharification is more viable than commercially available cellulase due to their reasonable cost and presence of other accessory enzymes in the crude preparation which enhances the saccharification efficiency.

The solid-state fermentation (SSF) can be of special interest in bioprocesses where crude fermented product could be used directly as enzyme source. This technology is most efficient, because this processes requires lower energy, high product concentration, lower input of infrastructure and skill, produce less wastewater and are ecofriendly as they resolve the problem of solid waste disposal (Halder et al. 2016). Currently, industrial demand for cellulases is being met by production methods using submerged fermentation (SmF) processes. The cost of production through SmF systems is, however, very high and uneconomical. Therefore, it is necessary to reduce the production cost by deploying alternative production methods such as SSF. In the arena of cellulase production and saccharification for cost-effective bioethanol production, lingo-cellulosic biomass may contributes significantly as substrate which replenished constantly in nature, available as wastes in the form of pre- and post-harvest agricultural losses as well as wastes of food processing industries and therefore considered as world's largest reusable reservoir of potentially fermentable carbohydrates (Begum and Alimon 2011; Pandey 2015).

Among the various types of lingo-cellulosic biomass, wheat bran was employed as substrate for production of cellulolytic enzymes, whereas sugarcane bagasse and grass *Aristida* sp. were employed for generation of fermentable reducing sugar through saccharification. Sugarcane bagasse is the residual biomass (fiber) waste left after juice extraction while *Aristida* is abundant in the local area and remain unutilized. It also has nearly cosmopolitan distribution and rarely reported to be used as substrate for saccharification but have immense potential to be used for the same.

Thus, the aims of the current study were to optimize the production of low cost cellulolytic enzymes through solid state fermentation by fungal isolate *Aspergillus fumigatus* SKH2 and optimization of production of fermentable reducing sugar by enzymatic saccharification of the pretreated sugarcane bagasse and *Aristida* grass for better yield.

Materials and Methods

Microorganism cultivation and inoculum preparation

Aspergillus fumigatus SKH2 was obtained from the culture collection of Department of Microbiology, Vidyasagar University (West Bengal, India). The strain was grown on potato dextrose agar (PDA) slants at 30 °C for 5 days until good sporulation occurred and stored at 4 °C until use. Inoculum was prepared by adding 5 ml of sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture slants. The spores were dislodged by gentle pipetting under strict aseptic conditions and the number of spores in the suspension was determined using a Neubauer chamber. The concentration of the spore suspension was adjusted ~5 $\times 10^9$ spores/ml. Then the spore solutions were collected in a sterilized bottle and stored in refrigerator for future use. For detection of cellulolytic activity in agar plate, the spore suspension was plated on Mandels's agar media [having the composition of KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4 \cdot 7\ H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.4; $FeSO_4 \cdot 7\ H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.0016; $ZnSO_4 \cdot 7\ H_2O$, 0.0014; $CoCl_2$, 0.0020; carboxymethyl cellulose (CMC), 10; and agar 17.5 g/l; pH 5.0]. After 3 days, the plates were flooded with Congo red solution (1%), after 5 min it was discarded, and the plates were washed with

1 M NaCl solution allowed to stand for 15 min. The clear zone around the colony indicates the cellulolysis. Cellulase activity on CMC agar was recorded as the Index of Relative Enzyme Activity (I_{CMC}) which is a ratio of clear zone diameter / colony diameter.

Optimization of cellulolytic enzymes production

Wheat bran (WB) was procured from local market in Midnapore (West Bengal, India). Cellulolytic crude enzyme cocktail was produced by using WB as substrate through solid state fermentation (SSF). SSF was carried out in 250-ml Erlenmeyer flask containing 5 g of WB moistened with modified Mandels's salt medium (Mandels et al. 1976) composed of $(NH_4)_2SO_4$ 1.4%, KH_2PO_4 2.0%, $CaCl_2$ $2H_2O$ 0.4%, $MgSO_4$ 0.3%, peptone 1.0%, NaCl 0.3%, $FeSO_4$ 0.005%, $MnSO_4$ 0.0016%, $ZnSO_4$ $7H_2O$ 0.0014%, and $CoCl_2$ 0.002%. The resultant mixture had an initial moisture content of 50%. Sterilized medium in the flask was homogeneously mixed with 0.5 ml spore suspension, and then it was subjected for fermentation. Effects of pH, temperature and fermentation time were optimized by one variable at a time (OVAT) approach.

Extraction and condensation of cellulolytic enzymes

After fermentation, enzymes were extracted with sterile distilled water (1:10 substrate to volume ratio) by shaking for 1 h at 120 rpm, followed by centrifugation at 5000 g. The supernatant was used as crude enzyme source. The crude enzyme solution was condensed by rotary evaporator (Eyela, Japan).

Optimization of pH and temperature of crude enzyme activity

To determine the optimum cellulolytic condition, activity of the different cellulolytic enzymes was checked at varied pH and temperature. To determine the pH optimum of the cellulolytic activity, citrate buffer (pH 3, 5, and 6), phosphate buffer (pH 7) and TRIS buffer (pH 8, 9) in 0.05 M concentration were used. Optimum temperature for the activity was determined by incubating the crude enzyme mixture at varied temperature conditions (20, 30, 40, 50, and 60 °C).

Pretreatment of lignocellulosic biomass

Grass *Aristida* sp. and sugarcane bagasse were collected from the adjoining area and market of Midnapore town (West Bengal). The residues are washed, dried and cut into small pieces (~5 mm in diameter). Alkaline (NaOH), acidic (H_2SO_4), oxidizing (H_2O_2) agent mediated pretreatments were carried out to make the substrate more accessible to enzymatic saccharification. Briefly, grass samples and sugarcane bagasse (10%, w/v) were boiled with sulphuric acid (1-5 M), sodium hydroxide (1-5 M) or hydrogen peroxide (1-5%, w/v) for 30 min. After cooling, the samples were washed with tap water and air dried.

Enzymatic saccharification

Untreated (native) samples, and the water-insoluble residue of pretreated grasses and sugar cane bagasse were subjected to enzymatic hydrolysis (substrate:enzyme:buffer = 1:2:3). Enzymatic saccharification was performed in a rotary shaker at 100 rpm up to 48 h. Samples were withdrawn periodically and the amount of reducing sugar released was estimated colorimetrically by dinitrosalicylic acid method (Miller 1959).

Enzyme activity assays

Endoglucanase or CMCase (E.C. 3.2.1.4) activity

Endoglucanase or CMCase (E.C. 3.2.1.4) activity was determined by incubating 0.5 ml of enzyme solution with 0.5 ml of 1.0% (w/v) CMC (carboxymethyl cellulose) (Sigma, St. Louis, MO, USA) under buffered conditions (Das et al. 2013). After incubation at optimum temperature for 30 min, the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid reagent. Liberated reducing sugars were estimated colorimetrically according to the method of Miller (1959) using glucose as standard.

Filter paper degrading activity (FPase)

Filter paper degrading activity (FPase) was determined using processed Whatman No 1 filter paper as substrate (50 mg/ml) according to the method of Wood and Bhat (1988). After incubation at 50 °C for 30 min, the reactions were stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS) reagent. Liberated reducing sugar was estimated colorimetrically according to the method of Miller (1959) using glucose as standard.

Xylanase (E.C. 3.2.1.8) activity

Xylanase (E.C. 3.2.1.8) activity was determined by measuring the release of reducing sugars from 1% (w/v) birch wood xylan under optimum assay condition (Bailey et al. 1992). Free sugar released was analyzed by the DNS method. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars (measured as monosaccharide content) by hydrolyzing respective raw substrate per minute under specified assay conditions.

Fourier Transform Infrared Spectroscopic of native and pretreated biomass

The infrared spectral analysis of the untreated and treated grasses and sugar cane bagasse were studied using Fourier Transform Infrared Spectroscopy (Schimadzu, Japan), and the Essential FTIR (Operant LLC) software was used to record and analyze the FTIR data. Biomass of 3 mg was dispersed in 300 mg of spectroscopic grade potassium bromide (KBr) and subsequently pressed into disks at 10 MPa for 3 min (Binod et al. 2012). The spectra were obtained in transmission mode with an average of 30 scans and a resolution of 4 cm^{-1} in the range of 4000-400 cm^{-1}.

Results and Discussion

Currently, the researchers are paying more interest to the utilization of plant-derived waste residues. In this study, we have chosen sugarcane bagasse and grass *Aristida* sp. for this purpose because they are arisen as plant-waste annually in high amount. These wastes consist of lignin and complex polysaccharides (*e.g.*, cellulose, hemicellulose), which can be converted into fermentable sugars by pretreatment and saccharification processes. For cost effective production of fungal cellulolytic enzymes, wheat bran can be considered as good supporting substrate probably due to the presence of various available nutrients, good porosity, suitable particle size and consistency required for fungal anchorage and enzyme excretion (Sun et al. 2008; Kar et al. 2013). Its texture remains loose even in moist condition, thereby provides a large surface area and increased water holding capacity (Das et al. 2013). Considering these properties, wheat bran was applied as substrate in this study to produce cellulolytic enzymes in high yield using the cellulolytic fungal isolate *Aspergillus fumigatus* SKH2. The fungal strain was previously isolated from paper mill effluent and maintained in slant culture. During the evaluation of cellulolytic activity in CMC containing agar plate, I_{CMC} value (clear zone diameter / colony diameter) of 1.69 was estimated after 3 days of incubation. The result revealed that the fungal strain has the potentiality to produce cellulolytic enzymes which degrade the CMC around the colony.

Production of crude A. fumigatus SKH2 cellulases and xylanases

Optimization of fermentation conditions

For enhancement of productivity of any microbial metabolite in laboratory, the levels of the physico-chemical factors influencing the bioprocess should be optimized. The optimization and designing of fermentation conditions is an important footstep to develop economically feasible bioprocesses. In this context, solid state fermentation (SSF) has been considered as the cheapest and environment-friendly approach. In the present study, wheat bran substrate moistened with Mandel's mineral salt solution (Mandels et al. 1976) was used as fermentation medium, and the physical conditions like fermentation time, medium pH and incubation temperature were optimized sequentially following one variable at a time approach (OVAT) to enhance the production yield of *A. fumigatus* SKH2 cellulolytic and xylanolytic enzymes (CMCase, FPase and Xylanase).

Production of cellulolytic enzymes increased during the first phase of SSF, and the highest yield was obtained at the 48^{th} h of fermentation (Fig. 1a). Enzyme yield was decreased after 48 h which may be due to nutrient depletion, toxic end products accumulation, change in medium pH or loss of moisture in the medium (Hosamani and Kaliwal 2011).

Growth, development and metabolic activities of an organism are significantly influenced by the temperature used for incubation. Hence, it is essential to optimize this parameter for maximal cellulolytic production. As can be seen on Figure 1b, the production of *A. fumigatus* SKH2 CMCase, FPase and xylanase under SSF condition was highest at 35 °C. The low enzyme yield below this temperature is possibly due to alteration of membrane permeability which restricts flow of nutrients inside the cells (Iftikhar et al. 2008; Halder et al. 2014). On the contrary, at higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of the enzymes of the metabolic pathway, resulting in lower production of the metabolites (Das et al. 2013; Dutta and Kumar 2014). At optimized state, 826, 102 and 1130 U/gds maximal yields were noticed for the CMCase (endoglucanase), FPase and xylanase enzymes, respectively.

The production yield of the studied cellulolytic enzymes was higher at slightly acidic pH; the best yield could be achieved at pH 5 (Fig. 1c). Enzyme production decreased at alkaline pH which probably due to the proteolytic inactivation of the cellulose (Dutta and Kumar 2014). Our results showed that slightly acidic conditions supported the cellulase and xylanase production, while at higher pH, the activities decreased gradually. It is supposed that the H^+ concentration in the fermentation medium had a significant effect on the enzyme production. The optimal pH for cellulase production from *Aspergillus niger* and *Aspergillus phoenicis* were reported between 6.0-7.0 and 4.5-4.8, respectively (Akiba et al. 1995; Yeoh et al. 1986).

Temperature and pH optimum of the crude enzyme activities

Temperature and pH are key factors that affect the catalytic

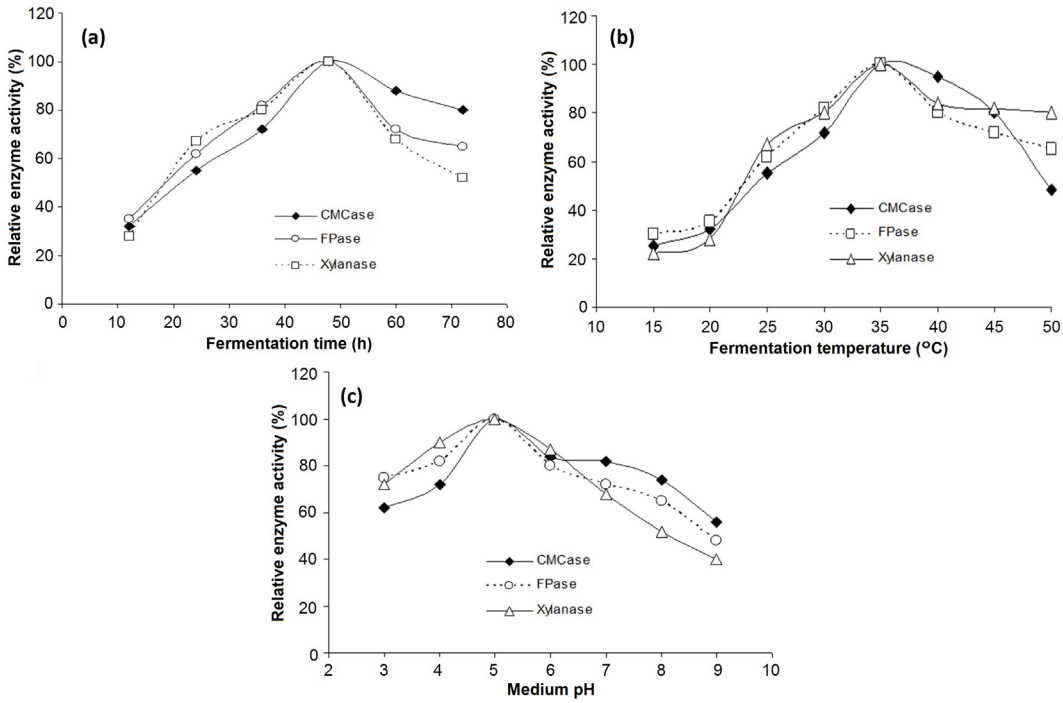

Figure 1. Effect of fermentation time (a), temperature (b) and medium pH (c) on production of cellulolytic enzymes by *Aspergillus fumigatus* SKH2 during SSF.

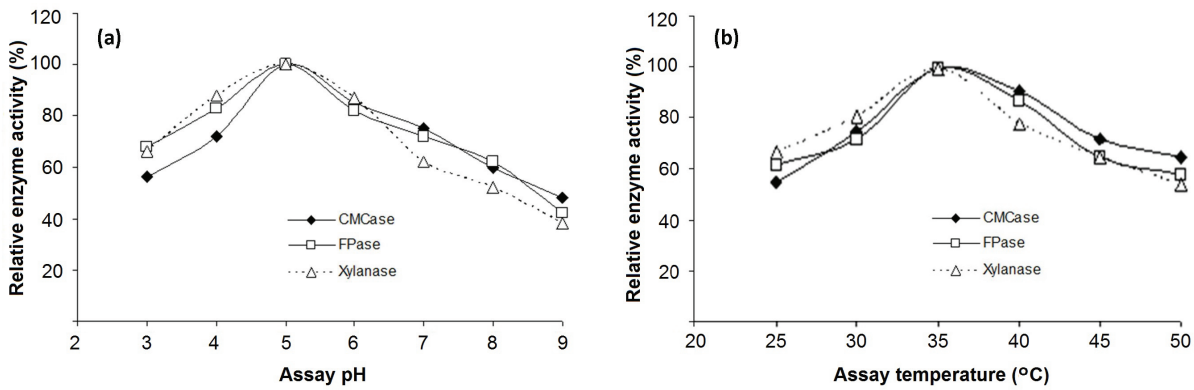

Figure 2. Effect of pH (a) and temperature (b) on activity of cellulolytic enzymes of *Aspergillus fumigatus* SKH2.

efficacy and stability of an enzyme, hence, for practical applications, determination of the optimal pH and temperature conditions for the activity is obligatory. Here we assayed the cellulolytic and xylanolytic activities in the *A. fumigatus* SKH2 crude enzyme preparation in different pH and temperature conditions. It was found that all three studied enzymes (CMCase, FPase and xylanase) were stable in the pH range of 4-6. The optimal pH condition for their activity was around pH 5 (Fig. 2a). It may be due to the acidic nature of the enzymes and abundance of acidic amino acid at the active site, which undergoes protonation and deprotonation by the influence of environmental pH and in turn regulate the enzyme activity (Jana et al. 2013).

Stability of cellulases and xylanases in a temperature range is one of the important parameters which determine their application in various industrial processes. In the present study, all three investigated crude enzymes retained more than 70% of their original activity between 30-45 °C presenting maximal activity values at 35 °C (Fig. 2b).

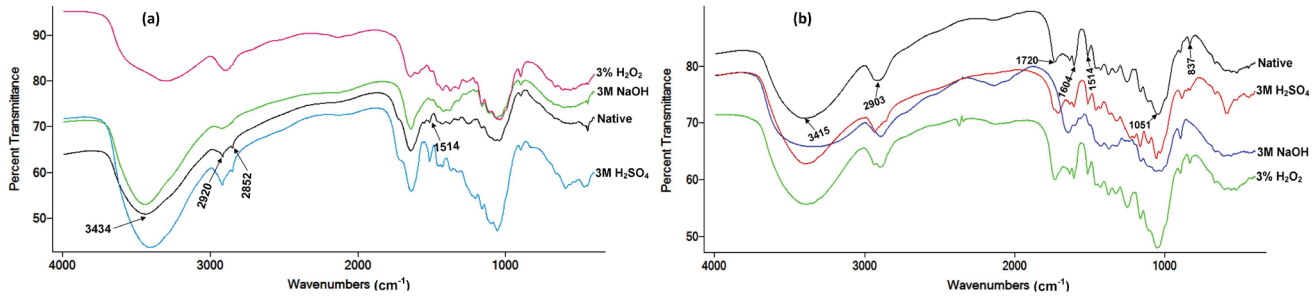

Figure 3. Comparisons of FTIR spectra of pretreated grass *Aristida* sp. (a) and sugarcane bagasse (b) with their native (untreated) form.

Saccharification of sugarcane bagasse and grass samples

Pretreatment and delignification

It is known that the lignin content of a plant-derived residue may affect the action of cellulolytic and xylanolytic enzymes which is required to achieve higher fermentable sugar yields from the substrate. Therefore, we tested oxidizing (H_2O_2), acidic (H_2SO_4) or alkaline (NaOH) agents in varied concentrations to delignify the substrates. After treatment, FTIR analysis was performed to find out the best agents for delignification. Figure 3 shows the comparison of FTIR spectra of native (untreated) and treated (in 3 M or 3% concentrations) grass (Fig. 3a) and sugarcane bagasse (Fig. 3b) samples.

The absorption band of 3434 cm^{-1} assigned for -OH stretching indicates the alcoholic and phenolic hydroxyl groups in the lignin polymer (Fig. 3a). The absorption was reduced after H_2O_2 and NaOH treatment in contrast with untreated grass (Fig. 3a). This is may be due to de-lignification which subsequently increased accessibility of cellulose by the enzymes. The C-H stretching vibrations of methoxyl group of lignin were assigned to 2920 and 2852 cm^{-1} (Hergert 1960); these distinct peaks were disappeared after NaOH and H_2O_2 treatment. The semi-circle stretch of para-substitute benzene rings was assigned to 1514 cm^{-1} (Adapa et al. 2011). This peak was disappeared after NaOH treatment indicating the removal of aromatic benzene rings from lignin. Moreover, the intensity of this band decreased after H_2SO_4 and H_2O_2 treatment which also indicates benzene ring rupture.

In the case of sugarcane bagasse, the intensity of absorption band at 3415 cm^{-1} represents the stretching of –OH groups. The absorption at 3415 cm^{-1} was increased after H_2O_2 treatment which indicated that partial hydrogen bonds within and between cellulose fibers were destroyed. On the contrary, opposite result was found after NaOH treatment which is in accordance with the results of grass.

Carbonyls as important functional groups mainly occur in the side chains of lignin structural units. These aldehyde and keto groups are lying in C-γ or C-β position, respectively. The disappearance of spectral band at 1720 cm^{-1} assigned to the carbonyl (C=O) stretching of unconjugated ketones indicated that the side chain of lignin was also broken down during NaOH and H_2SO_4 treatment and removal of lignin (Bykov 2008; Das et al. 2013). The small absorption bands of native form at 1604, 1514, 1051 and 837 cm^{-1} represents typical lignin structures (Bykov 2008; Adapa et al. 2011). The absorption band at 1514 cm^{-1} assigned to semi-circle stretch of para-substitute benzene rings disappeared after NaOH treatment, which indicates the removal of aromatic benzene rings from lignin polymer. After H_2SO_4 and H_2O_2 treatment, the intensity of this band decreased which suggests the rupture of the benzene rings (Adapa et al. 2011). The bands at 1604 and 1051 cm^{-1} are attributed to C-Ph and O-H stretching of primary alcohol vibration, respectively. These bands are generally found in the lignin aromatic structure. After pretreatment with NaOH and H_2SO_4, these bands are changed or disappeared.

Anyway, our results indicate that NaOH is better delignifying agent than the other compounds tested when grass

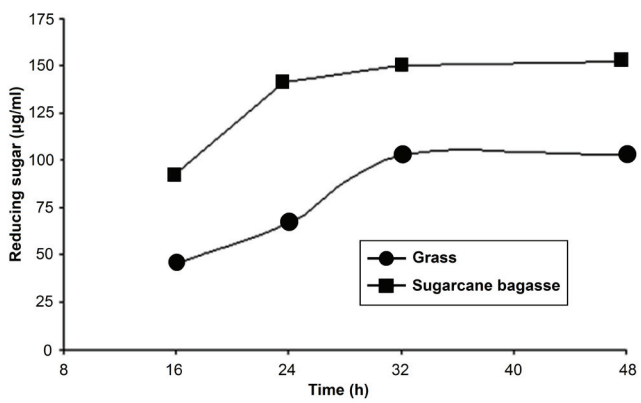

Figure 4. Time dependent saccharification of native grass and sugarcane bagasse by crude cellulolytic enzyme.

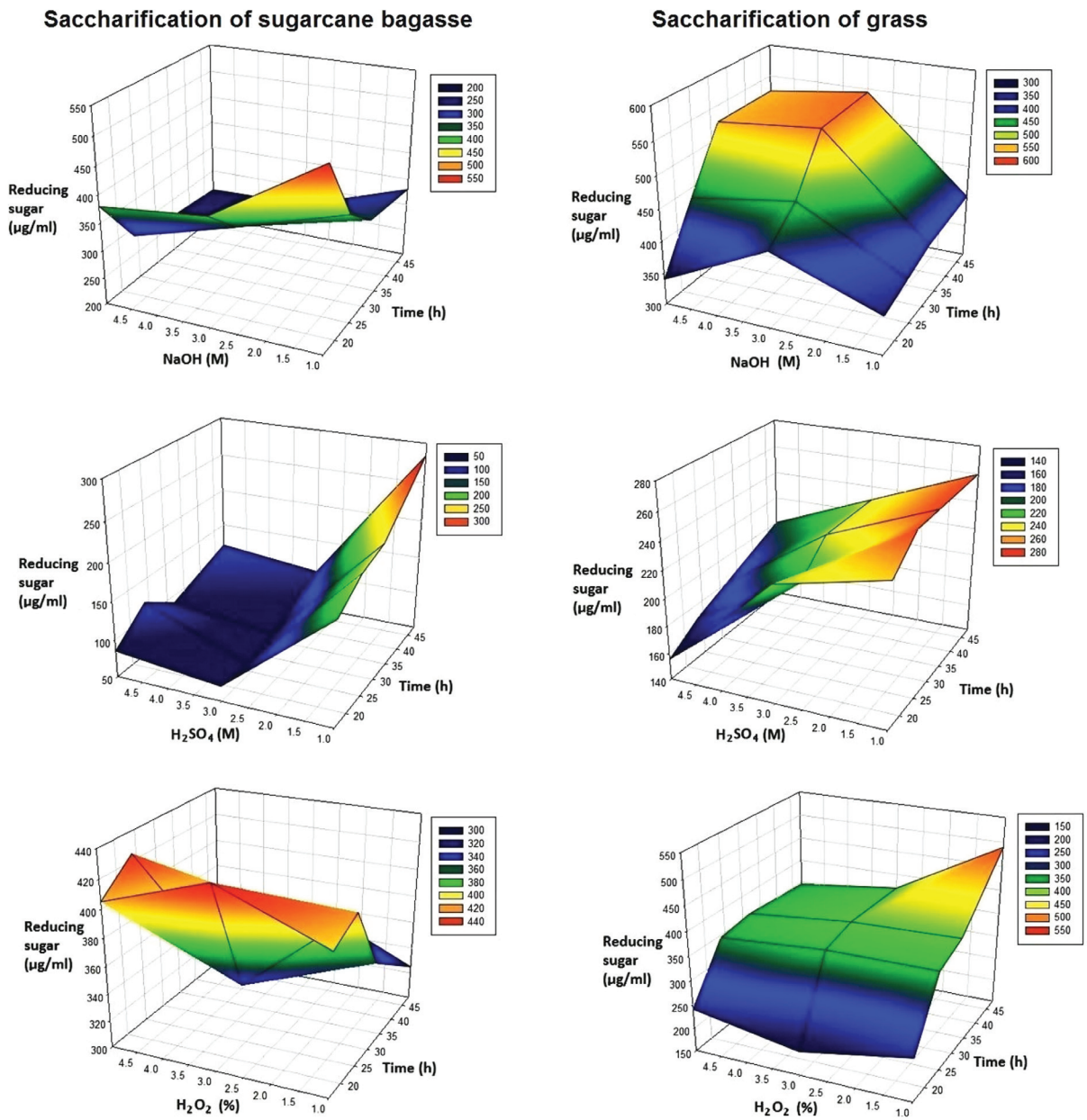

Figure 5. Dose and time dependent saccharification (reducing sugar yield) profile of lignocellulosic substrates treated with various agents under investigation.

(Aristida sp.) and sugarcane bagasse samples were used as substrate.

Optimization of pretreatment agent dose and treatment time on saccharification

Enzymatic hydrolysis of sugarcane bagasse and grass substrates in native and after pretreated form by crude enzyme cocktail (containing CMCase endoglucanase, FPase, and xylanase in 7.69:1:11.69 ratio) was carried out at 48 h. It was found that the sugar yield from both pretreated residues was varied in dose and time dependent manner. Untreated (native) samples from both residues were also subjected for saccharification, in which moderate increase in the reducing sugar content was detected (Fig. 4). It indicates that the crude enzyme preparation may contain some ligninolytic enzymes like lignin peroxidase, manganese peroxidase, laccase etc., which facilitate the action of cellulolytic enzymes.

In case of sugarcane bagasse, highest reducing sugar yield was achieved after 16 h incubation with the samples treated with 1 M NaOH. In grass samples, it was maximal with 3 M NaOH treated residues after 48 h incubation (Fig. 5). Based on these results, it can be concluded that NaOH is a useful agent to remove most part of the lignin polymer, thus, the subsequent saccharification process has been enhanced.

Conclusion

A reduction of the cost of fermentable reducing sugar production can be achieved by reducing the cost of raw materials. Using zero valued lignocellulosic materials such as agricultural residues, grasses, forestry wastes, sugarcane bagasse and other low-cost biomass can significantly reduce the cost of raw materials. Apart from that, reduction of the cost of cellulolytic enzyme production is also a key issue in the enzymatic hydrolysis of lignocellulosic materials. The above study theorized that *Aspergillus fumigatus* SKH2 is a potent cellulolytic fungal isolate which utilized wheat bran as robust anchorage cum substrate for production of cellulolytic enzymes like CMCase (endoglucanase), FPase, and xylanase and hence make the bioprocess proficient. Moreover, NaOH is found as good delignifying agent for sugarcane bagasse and grass *Aristida* sp., which enhances the saccharification efficiency of cellulolytic enzyme cocktail by many folds. As a whole, the present investigation validates eco-friendly and cost effective production of cellulolytic enzyme cocktail by *Aspergillus fumigatus* SKH2 from agricultural waste and subsequent application of the same for saccharification of lignocellulosic biomass. Considering the simplicity and proficiency, production of bioethanol from the generated reducing sugars by the action of yeast or bacteria in single or in combination will explore a new horizon in upcoming eons as an alternative sustainable approach to replenish the ever increasing demand of biofuels.

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