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Development of Thermophilic Fungal Consortium for Saccharification of Rice Straw and Its Utilization for Bioethanol Production

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ABSTRACT

The rice straw is considered to be a promising agricultural byproduct for bioethanol production. The saccaharification of cellulose to fermentable sugars using commercial enzymes is a major deterrent to the economic viability of bioethanol production process. Therefore, use of indigenous microbial enzymes has been suggested. A thermophilic consortium was developed by co-culturing two Aspergillus sp. for the saccharification of rice straw. The fungal consortium exhibited 29.0 U/gdry substrate (gds) filter paper, 90.0 U/gds carboxymethyl cellulase and 22.3 U/gds cellobiase activity significantly higher than the individual Aspergillus sp. The saccharification of two step chemical pretreated rice straw (dilute sulphuric acid followed by sodium hydroxide) with fungal consortium cellulase resulted in higher reducing sugars (24.9 g/100gds) as compared to individual Aspergillus sp. The fermentation of saccharified and pretreated hydrolysate resulted in a combined ethanol content of 13.2 g100/g.

Key words: *Rice straw, Aspergillus sp., Thermophilic, Co-culturing, Ethanol*

INTRODUCTION

Depleting fossil fuel reserves, dwindling fuel prices, increased concerns over climate change, and most importantly the search for energy security have led the global research community to look for renewable, environment friendly and sustainable energy sources. Lignocellulosic biomass from crops, whose main components are polysaccharides polymers as cellulose and hemicellulose (75-80%) is an important renewable energy source. Cellulose, a structural component of lignocellulosic biomass, is the most abundant feedstock used for the production of alternative liquid fuels, mainly bioethanol. However, cellulose and hemicelluloses is intertwined with lignin, which require an additional input as pretreatment for its removal[1].The main goal of pretreatment is to break lignin seal, disrupt crystalline structure of cellulose, and increase biomass porosity[2].

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Many physical, chemical and biological pretreatment methods have been studied in the past, but each of these treatments has limitations in technical and economical aspect. A two step pretreatment using mild acid followed by alkali can lead to overall increased pretreatment efficiency as well as saves on chemical input and energy. Dilute acid pretreatment effectively hydrolyzes the hemicellulose component of the lignocellulosic biomass[3] while alkali like sodium hydroxide leads to delignification thus improving the biomass digestibility[4].

Degradation of cellulose to its constituent monosaccharides has attracted considerable attention for the production of biofuels. The degradation of cellulose to glucose is achieved by the cooperative action of endoglucanases (EC 3.1.1.4), exoglucanases (cellobiohydrolases, CBH, 3.2.1.91; glucanohydrolases, 3.2.1.74) and beta-glucosidases[5]. The cellulase can be produced via biological route by means of bacterial or fungal fermentation. Although many cellulolytic bacteria produce cellulases with high specific activity, they do not produce high enzyme titres. Therefore most research for commercial cellulase production has been focused on fungi[6]. Thermophilic fungi which grow at a maximum temperature of 50°C or above have received significant attention in recent years as a source of new thermostable enzymes for hydrolysis of lignocellulosic biomass[7]. The advantages of conducting hydrolysis at elevated temperatures is swelling of cellulose at higher temperatures, thereby becoming easier to break down, reducing the risk of contamination by mesophilic microorganisms, and decrease in viscosity and higher mass-transfer rates leading to better substrate solubility[8,9]. A number of thermophilic fungi have been isolated in recent years and the cellulases produced by them have been purified and characterized at both structural and functional level.

In lieu of expensive and energy intensive commercial enzyme preparations, there has been a need to have a simplified and inexpensive method of cellulase production. Cellulase is produced by growing cellulolytic fungi either in submerged liquid fermentation or in solid-state fermentation. The submerged liquid fermentation usually involves mixing, forced aeration, control and monitoring of temperature, pH, dissolved oxygen and gas flow rates while solid-state fermentation (SSF) has low risk of contamination and is less energy intensive as low grade lignocellulosic substrates can be used[10]. Co-culture is beneficial in cellulase production via SSF as the fungi are normally co-existed symbiotically on solid substrates in nature[11]. Besides that, co-culture also offers advantages such as higher productivity, adaptability and substrate utilization compared to pure and monoculture[12]. Moreover, enzymes system produced by co-culturing different fungi could complement each other and form a complete cellulase system that is favorable for lignocellulosic substrate hydrolysis. Keeping in mind the aforementioned facts present study was performed to develop cellulase producing thermophilic fungal consortium from strains isolated from diverse natural sources under solid state fermentation conditions. The cellulolytic enzyme extracted from consortium was concentrated and characterized according to various parameters, evaluated for saccharification of two-step pretreated (acid followed by alkali) rice straw and finally utilized for bioethanol production.

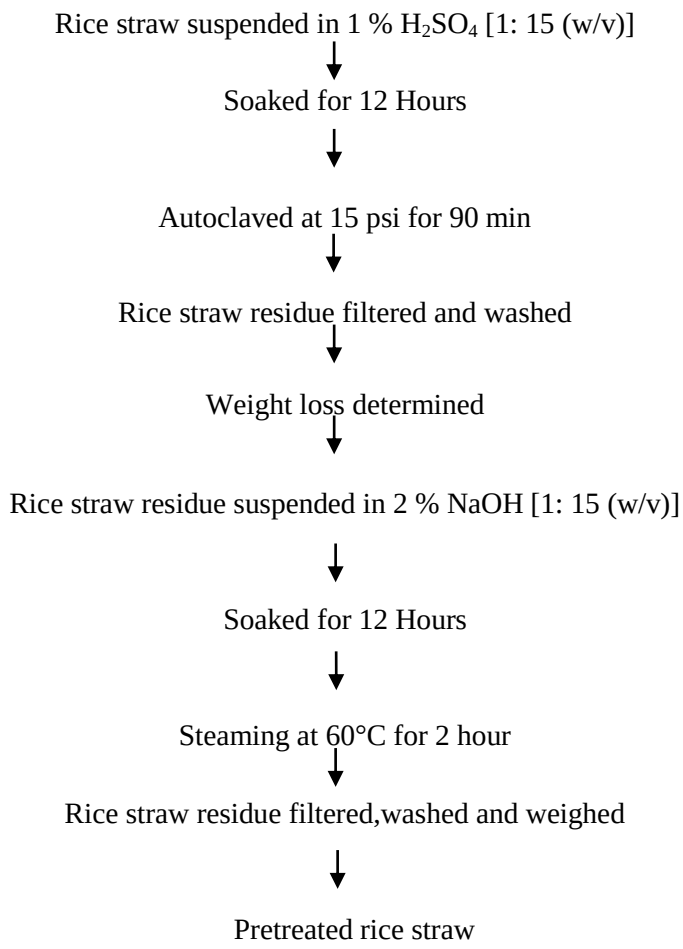
MATERIAL AND METHODS

Collection of sample

The rice straw was collected from School of Renewable Energy Engineering, PAU Ludhiana. The raw material was washed thoroughly with tap water until clean and colourless and then dried in air, ground and sieved to 30 mesh size for further treatment.

Pretreatment of rice straw Two-step pretreatment

The process flow diagram of rice straw pretreatment is shown as follows:



Isolation of cellulase producing thermophilic fungal strains

The samples were collected from diverse sources such as spent mushroom compost, ruminant cud, and rice cultivated soil. Serially diluted samples (10^{-3} and 10^{-6}) were spread on Potato dextrose agar (PDA) medium supplemented with chloramphenicol (20 mg/L). The plates were incubated in a BOD incubator at 45°C for 4-6 days and observed daily for the growth of fungal colonies.

Qualitative screening of thermophilic fungal strains for cellulose degradation

Isolated fungal strains were tested for cellulose degradation by culturing on carboxymethyl cellulose (CMC) agar medium with the following composition (g/L): Sodium nitrate: 2.0; Monopotassium phosphate: 1.0; Magnesium sulphate heptahydrate: 0.5; Potassium chloride: 0.5; Carboxymethyl cellulose sodium salt: 10.0; Peptone: 0.2; and Agar: 20.0. The plates were incubated at 45°C for 3-4 days. After incubation, the plates were stained with 1.0 % Congo red solution followed by destaining with 1.0 M NaCl solution. The halo formation around the fungal colony, regarded as a positive test for cellulase production was measured. The cellulolytic index (CI) was expressed as the ratio between the diameter of halo and diameter of the colony[13]. The screened fungal strains were identified by observing their macroscopic (mycelium colour, spore colour, margins and colony elevation) and microscopic (microstructures) characteristics according to Gilman[14].

Quantitative cellulase production

Inoculum preparation

The mycelia of screened fungi were grown on PDA medium. The spores were obtained from

one week old Petri plates by rinsing the plates with sterile distilled water and collecting the spores in sterile vials. The fungal spore suspension of $1x10^8$ spores/ml was used for inoculation.

Enzyme production

The cellulase enzyme was produced under two sets of conditions, with untreated rice straw and pretreated rice straw as substrate. The experimental flask (250ml) containing rice straw and wheat bran in the ratio of 4:1 (w/w) was moistened with Mandels and Weber (MW) Medium to achieve a moisture content of 80%[15,16]. The composition (g/L) of MW medium as follows: Monopotassium phosphate: 2.0; Magnesium sulphate heptahydrate: 0.3; Calcium chloride dehydrate: 0.3; Ammonium sulphate: 1.4; Ferrous sulphate heptahydrate: 0.005; Manganese sulphate: 0.016; Zinc sulphate heptahydrate: 0.014; Cobalt chloride hexahydrate: 0.002; Peptone: 1.0; Tween 80: 1.0; pH: 5.0. The experimental flasks were autoclaved at 121°C for 15 min, inoculated with 10% (v/v) fungal spore suspension and incubated at 45°C.

Enzyme extraction

To extract the enzyme, the contents of the flask were mixed properly with 0.1 M sodium citrate buffer (pH 5) to achieve a solid /liquid ratio of 1:10 (w/v). The suspension was stirred in an orbital shaking incubator at 120 rpm for 30 min. The extract was filtered through double layered muslin cloth and centrifuged at 6,000 rpm for 10 min. The supernatants obtained were stored in sterilized glass bottles at 4° C and used as crude enzyme extracts for assaying the cellulase enzyme activity.

Formulation of cellulase consortium

The cellulase consortium was formulated using the potential isolates screened on the basis of qualitative and quantitative screening.

Inoculum preparation

The mycelia of fungal combinations selected for consortium development were grown on PDA medium. The spores were obtained from one week old Petri plates by rinsing the plates with sterile distilled water and collecting the spores in sterile vials. For inoculum preparation, half the number of spores from each strain was mixed to maintain a total spore count of 1×10^8 spores/ml.

Enzyme production

The enzyme production was carried out in 250 ml Erlenmeyer flask containing 5 g of pretreated rice straw and moistened with MW medium to achieve moisture content of 80%. The experimental flasks were autoclaved at 121°C for 15 min, inoculated with 10% (v/v) fungal spore suspension and incubated at 45°C.

Enzyme extraction

To identify the optimum production of enzymes, the cellulase enzyme assay was carried out every 24 h. For the assay, the contents of the experimental flasks were harvested by 0.1 M sodium citrate buffer (pH 5), added to achieve a solid/ liquid ratio of 1: 10 (w/v). The suspension was stirred in an orbital shaking incubator at 120 rpm for 30 min. The biomass was filtered using double layered muslin cloth and the filtrates were centrifuged at 6,000 rpm for 10 min. The supernatants obtained were stored in sterilized glass bottles at 4° C and used as crude enzyme extracts for assaying the cellulase enzyme activity.

Enzyme assay

The crude enzyme extract was assayed for Filter paper activity by the method of Mandels *et al.*[17]using Whatman No.1 filter paper strip (1×6 cm) dipped in 0.9 ml of sodium citrate buffer (pH 5.0) and 0.1 ml of enzyme extract. The tubes were vortexed to coil filter paper at the bottom of the tube and incubated in a water bath incubator at 50°C for 1 h. The quantification of reducing sugars was carried out by the DNS method[18]. Carboxymethyl was assayed by the method of Mandels *et al.*[17] in a reaction mixture containing 0.9 ml of 1.0% carboxymethyl cellulose solution and 0.1 ml of enzyme extract. The reaction mixture was incubated at 50°C for 30 min in a water bath incubator and reducing sugars estimated. Cellobiase activity was assayed in a reaction mixture contained 0.9 ml of 0.05 % cellobiose solution and 0.1 ml of enzyme extract[19]. The reaction mixture was incubated in a water bath incubator at 50°C for 2 h and reducing sugars

estimated. One unit (U) of filter paper, carboxymethyl cellulase and cellobiase activity was defined as amount of an enzyme needed to liberate 1 µmol of reducing sugar equivalents in one minute. Protein concentration was determined by method described by Lowry[20] using bovine serum albumin (BSA) as a standard for the calibration curve.

Membrane concentration of enzyme

The membrane concentration of the crude cellulase enzyme was performed using 10 kDa Polyethersulphone (PES) membrane with molecular weight cuttoff of 10 kDa procured from CleaNsep Pvt. Ltd., Mumbai. The enzyme was concentrated 10 folds and assayed for cellulase enzyme activity and total proteins.

Polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 12.5% resolving and 4% stacking gels according to the method of Laemmli[21]. After electrophoresis, the gel was stained with Coomassie Brilliant blue.

Scanning electron microscope analysis

Scanning electron microscope (SEM) images of untreated and pretreated rice straw samples were obtained to analyze morphological effect of the pretreatment step. The samples were mounted with double coated carbon conductive tape on pin-mount specimen holder made of gold and palladium and analyzed by SEM (Hitachi S-3400 N).

Biochemical characterization of fungal consortium cellulase

Determination of optimum pH

To 0.9 ml of 1.0 % carboxymethyl cellulose in various pH (pH 3.0-7.0, 0.1 M sodium citrate buffer; pH 6.0-9.0, 0.1 M potassium phosphate buffer; pH 8-11, 0.1 M glycine-NaOH buffer), 0.1 ml of concentrated cellulase was added. The reaction mixture was incubated at 50°C for 30 min in a water bath incubator and reducing sugars estimated.

Determination of optimum temperature

To 0.9 ml of 1 % carboxymethyl cellulose solution in 0.1 M sodium citrate buffer (optimum pH), 0.1 ml of concentrated cellulase was added and incubated in a water bath incubator at various temperatures (20-90°C) for 30 min and reducing sugars estimated.

Determination of temperature stability

Thermal stability of carboxymethyl cellulase was determined by incubating the reaction mixture (0.9 ml of 1 % carboxymethyl cellulose solution in 0.1 M sodium citrate buffer and 0.1 ml of concentrated cellulose) in a water bath incubator at its optimum temperature for various time durations (30-180 min) and reducing sugars estimated.

Effect of activators/inhibitors

One percent carboxymethyl cellulose solution in 0.1 M sodium citrate buffer and concentrated cellulase was incubated with 1 mM (final concentration) of $CaCl_2$, $CoCl_2$ $CuSO_4$, $MgCl_2$, $MnCl_2$, $FeCl_2$, SDS and EDTA in a water bath incubator under optimized conditions for 30 min and reducing sugars estimated.

Determination of kinetic parameters

The kinetic parameters (K_m and V_{max}) of carboxymethyl cellulase were determined by varying the concentration of carboxymethyl cellulose from 2 to 20 mg/ml in 0.1 M sodium citrate buffer. The reaction was carried out in a water bath incubator under optimized conditions and reducing sugars estimated. The kinetic parameters were determined from double reciprocal plot[22].

Saccharification of pretreated rice straw

The saccharification of pretreated rice straw was performed by 30 filter paper unit per gram of dry substrate (FPUg/ds) of concentrated cellulase from thermophilic fungal consortium. The pretreated straw was suspended in 0.1 M sodium citrate buffer to obtain a solid/liquid ratio of 1: 20 (w/v) after the enzyme addition[23].To the reaction mixture, 0.3% sodium azide and 0.2% Tween 20 were added and saccharification performed in a water bath incubator at 50°C, with continuous shaking at 120 rpm for 72 h. The aliquots were withdrawn after every 24 h, centrifuged at 6,000 rpm for 10 min and analyzed for reducing sugars.

Fermentation studies

Fermentation of saccharified rice straw hydrolysate

A 10% (v/v) suspension of *Saccharomyces cerevisiae* (MTCC 11815) and *Pachysolen tannophilus* (NCIM 3502) inoculated into saccharified hydrolyaste supplemented with 0.2% diammonium hydrogen phosphate was incubated at 30±2°C for glucose and xylose fermentation, respectively[24].The aliquots were periodically collected after at an interval of 24 h, centrifuged at 6,000 rpm for 10 min and analysed for reducing sugars and ethanol[25].

Fermentation of pretreated rice straw hydrolysate

The fermentation of pretreated rice straw hydrolysate was carried out with *S. cerevisiae* and *P. tannophilus* under the conditions as described above. Sequential fermentation was carried out by the method of Kocher and Uppal[26]. Firstly, the fermentation medium was inoculated with *S.cerevisiae* at 10 % (v/v) and incubated in a BOD incubator at 30±2°C till complete sugar utilization. The *S.cerevisae* cells were inactivated by keeping the flasks at 50°C for 6 h. Subsequently, the fermentation medium was inoculated with 10% (v/v) *P.tannophilus* and incubated in a BOD incubator at 30±2°C till complete xylose utilization. The aliquots periodically collected after at an interval of 24 h were analysed for reducing sugars and ethanol.

RESULTS AND DISCUSSION

Effect of pretreatment of rice straw on its weight loss and chemical constituents

The weight loss and chemical composition change of rice straw are important indices for the effectiveness of its pretreatment. The two step pretreated rice straw revealed a 60% weight loss which signifies loss of integrity of rice straw due to solubilization of hemicellulose sugars and loss of lignin and other inorganic components. Moreover, rice straw after pretreatment turned a little lighter in colour and texture changed to crumpled and fragile from a rigid structure. As seen in Figure 1, pretreated rice straw contains 62.3% cellulose, 9.5% hemicellulose, 2.0% lignin. Compared with the chemical components in the raw/untreated straw, it was clear that two-step pretreatment relatively increased cellulose by 54.83%, and decreased hemicellulose and lignin by 36.6 % and by 82.6%, respectively. It can be inferred from the results that step1 (acid pretreatment) successfully removed most of the hemicellulose and step 2 (alkali pretreatment) mainly removed lignin, besides cellulose enrichment in the whole pretreatment process. The relative increase in cellulose and decrease in hemicellulose and lignin has been reported in previous studies also. Kim *et al.*[27]reported an increase in cellulose to 52.5 g/100g from 35.9 g/100g dry weight and decrease in hemicellulose to 1.3 g/100g from 24.4 g/100g and lignin to 5.3 g/100g from 15.9 g/100g dry weight from two-stage pretreatment using aqueous ammonia and dilute acid.

Isolation and screening of cellulase producing thermophilic fungal strains

The fungal strains were isolated from diverse habitats and their cellulolytic potential was determined by Congo red plate assay. Among the forty seven fungal strains isolated, two strains viz. CTS1 and CTS2 were found to be cellulolytic as well as thermophilic in nature. The results of macroscopic and microscopic characteristics reveal that fungal strain CTS 1 and CTS 2 belong to *Aspergillus* sp.

Development of thermophilic fungal consortium

The thermophilic fungal consortium for cellulase production was developed using *Aspergillus* sp. CTS 1 and *Aspergillus* sp. CTS 2 on pretreated rice straw under solid state fermentation conditions. The filter paper activity, carboxymethyl cellulase and cellobiase activities of the consortium were observed to be 10.2, 30.0 and 7.9 U/gds, respectively at 72 h of incubation which were significantly higher than the enzymatic activities of *Aspergillus* sp. CTS 1 and *Aspergillus* sp. CTS 2 (Table 1). The phenomena of higher yield in co-cultures can be attributed to production of all the cellulase components in significant amounts as well as their synergistic functioning in the form of a

base complex. Vyas and Vyas[28] evaluated cellulase production from a co-culture of *Aspergillus terreus* and *Trichoderma viride* on alkali treated ground nut shells under solid state fermentation conditions. The co-culture was reported to produce higher endoglucanase (4.56 U/ml) and exoglucanse (0.45 FPU/ml) as compared to the monocultures. A co-culture of *T. reesei* and *Aspergillus oryzae* for the solid-state fermentation of soybean hulls supplemented with wheat bran was investigated by Brijwani *et al.*[15]. A significant increase in enzyme activities and volumetric productivities were observed in mixed-culture fermentation as compared to mono-culture, due to augmentation of lower beta-glucosidase activity in *T. reesei* cultures by co-cultured *A. oryzae.*

Membrane concentration of fungal consortium cellulase

The Table 2 presents the comparison of enzyme activities of crude and 10 folds concentrated enzyme extracts. The concentration resulted in enhancement of filter paper activity to 2.9 U/ml from 1.0 U/ml, carboxymethyl cellulase activity to 9.0 U/ ml from 3.0 U/ml and cellobiase activity to 2.23 U/ml from 0.79 U/ml. A decline in specific activity of the concentrated enzymes suggests that purification of the enzyme could not be achieved with the membrane concentration method as it excluded only low molecular weight compounds and salts. In line with the present study, crude celluase filtration through membrane with molecular weight cutoff of 10 kDa has been reported in various studies[29,30,31].

Biochemical characterization of cellulase enzyme

The concentrated cellulase of fungal consortium was biochemically characterized with respect to carboxymethyl cellulase activity using carboxymethyl cellulose as substrate. The carboxymethyl cellulase specifically measures the endo-1,4-β glucanase activity therefore it has widely been used for the measurement of cellulase activity using carboxymethyl cellulose, which is a soluble and highly amorphous derivative of cellulose[32].

Effect of pH

The pH of the concentrated carboxymethyl cellulase was determined by measuring the enzyme activity at varying pH values ranging from 3–10 using different buffers (Figure 2). The results indicate that carboxymethyl cellulase has an acidic range of pH optimum and the enzyme was most active at pH 5. Nascimento *et al.*[33]have reported an acidic pH range of 2.0-5.0 with maximum activity at pH 4.0 for cellulase produced by *Streptomyces malaysiensis* under submerged fermentation. The maximum carboxymethyl cellulase activity at pH 2.0 was reported for *Aspergillus fumigatus*[34].

Effect of temperature

Effect of temperature on concentrated carboxymethyl cellulase was recorded over a broad range of temperatures (30-90°C). The carboxymethyl cellulase activity increased with increase in temperature up to 60°C and there was a drop afterwards (Figure 3). The result was similar as the other *Aspergillus* sp. including *Aspergillus terreus*[35], *A. fumigatus*[36], *A. niger*[37] with optimum temperature range reported between 50 to 70°C.

Thermostability of carboxymethyl cellulase

For the saccharification of lignocellulosic biomass which is carried out at 50-55° C, the thermosatbility of cellulase enzyme at elevated temperature plays a pivotal role, since at high temperature a rapid reaction rate can be maintained and contamination can be prevented[38]. The thermostability of carboxymethyl cellulase was determined at 60°C for 180 min. The enzyme was completely stable up to 120 min and about half of the activity was present up to 150 min (Figure 4). Crude enzyme from *Trichoderma* sp. strain IS-05 was able to retain 59.6% residual activity at 60°C for 4 h; with a half-life of 5 h at 60°C[39].

Effect of activators/inhibitors

The carboxymethyl cellulase was induced by 1.0 mM Co^{2+}, Ca^{2+}, Mg^{2+} with maximum enhancement of 20% in the presence Ca^{2+} and inhibited by EDTA, SDS, Mn^{2+}, Fe^{2+} and Cu^{2+} to a variable extent (Figure 5). The most cited inhibitors of cellulases are Cu^{2+} and Mn^{2+}[40,41]. A remarkable loss in the activity of β-

glucosidase (retained activity 1.61%) from *Aspergillus niger* was reported by Ahmed *et al.*[42].

Effect of substrate concentration

Determination of K_m and V_{max}

The kinetic parameters K_m and V_{max} of carboxymethyl cellulase was determined at 60°C and pH 5.0, using various concentrations of carboxymethyl cellulose as substrate (2.0 - 10.0 mg/ml). The carboxymethyl cellulase showed Michaelis–Menten constant, K_m of 10 mg/ml and maximum velocity, V_{max} of 9.9 U/mg protein (Figure 6). Pham et al.[43] demonstrated K_m value of 4.08 mg/ml for endoglucanse of *Aspergillus niger* VTCC-F021. Bagewadi and Ninnekar[44] reported high affinity of endoglucanase towards sodium carboxyl methyl cellulose (CMC) with K_m of 1.11 mg/ml and V_{max} of 50 U/ml.

Molecular weight determination

The cellulase enzyme extracts were concentrated and dialysed before molecular weight determination. The SDS- PAGE revealed eight bands each in the range of 16-124 kDa and 10-124 kDa for *Aspergillus* sp. CTS 1 and *Aspergillus* sp. CTS 2 respectively. For the consortium, protein profile different from individual strains was obtained, with ten bands in the range of 10-250 kDa (Figure 7). Similarily, Hu *et al.*[45] reported a combined protein profile of *A. niger* and *A. oryzae* co-cultivation as compared to individual cultures.

Scanning electron microscope (SEM) analysis

The SEM images of untreated rice straw showed smooth and rigid surface with highly ordered structures such as large fibrils, lumps, papillae and silica layer. The pretreatment resulted in extensive damage of the microfibrils and cell structures deformation thus rendering an increased surface area and greater accessibility to the cellulase enzymes for high saccharification performance (Figure 8).

Saccharification of pretreated rice straw

The concentrated cellulase from thermophilic fungal consortium was used for saccharification of pretreated rice straw. The Table 3 presents the saccharification of pretreated rice straw using concentrated cellulase obtained from different sources viz. *Aspergillus* sp. CTS1, *Aspergillus* sp. CTS 2 and cellulase from *Aspergillus* sp. CTS1, *Aspergillus* sp. CTS 2 consortium. The sacccharification using consortium cellulase resulted in a maximum of 24.9 g/100gds reducing sugars at 48 h of incubation after which a decline in reducing sugar content was observed which may be due to product inhibition by glucose. In the previous studies, the consortium of *Pholiota adiposa* and *Armillaria gemina* lignocellulases gave a maximum 84.3 % saccharification yield from NaOH pretreated sunflower stalk biomass using 10 FPU/g enzyme with 5.5 % substrate concentration, equivalent to that obtained using a mixture of commercial enzymes Celluclast and Novozyme β-glucosidase[46]. Similarily, Dhiman *et al.*[47]reported that hydrolysis of laccase pretreated rice straw and willow using a fungal consortium of *A. gemina and P. adiposa* resulted in saccharification yield of up to 74.2% and 63.6%, respectively.

Fermentation of pretreated rice straw hydrolysate

The time course of sugar utilization and ethanol production from saccharified rice straw hydrolysate is presented in Table 4. The fermentation of hydrolysate resulted in 8.4 g/100g ethanol at 120 h of incubation. The pretreated rice straw hydrolysate contains both hexoses and xyloses, therefore it was fermented sequentially by *S.cerevisiae* and *P.tannophilus*, besides separate fermentation with *S.cerevisiae* and *Pachysolen tannophilus* (Table 5). The fermentation by *S.cerevisiae* produced 4.1 g/100g ethanol at 72 h of incubation. The fermentation by *P.tannophilus*, produced 3.3g/100g at 72 h. The sequential fermentation resulted in significantly higher production of ethanol i.e. 4.7g/100g at 144 h of incubation. The Table 6 presents the combined bioethanol production from pretreated and saccharified hydrolysates. The combined bioethanol production from two step pretreated rice straw saccharified by indigenous cellulase was observed to be 13.2 g/100g ethanol with a fermentation efficiency

of 70.9%. Abedinfar *et al.*[48] have investigated the fermentation of rice straw (pretreated with diluted acid and subsequent enzyme treatment) rice straw using *Mucor indicus* and *Rhizopus oryzae*. An ethanol yield of 0.36-0.43 g/g using *Mucor indicus* was obtained which was comparable with the corresponding yield by *S. cerevisae* (0.37-0.45 g/g). The combined method of acid pretreatment with ultrasound and subsequent enzyme treatment of rice straw resulted in ethanol yield of 10 and 11 g/L after 7 days fermentation with *S. cerevisiae*[49].

Table 1: Cellulolytic activities of fungal consortium and individual fungi

Fungal consortium and individual fungi	Enzyme activity (U/gds)		
	Filter paper	Carboxymethyl cellulase	Cellobiase
Fungal consortium	10.20±0.56	30.00±1.65	7.90±0.43
Aspergillus sp. CTS1	4.10±0.23	5.82±0.32	2.70±0.15
Aspergillus sp. CTS 2	4.44±0.24	9.19±0.5	3.79±0.21
$CD_{5\%}$	2.07	0.690	0.483

Values are mean± standard deviation; CD5%: Critical difference at 5% level

Table 2: Effect of membrane concentration on cellulase enzyme activities of fungal consortium

Enzyme type	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Filter paper			Carboxymethyl cellulase			Cellobiase		
				Enzyme activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Enzyme activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Enzyme activity (U/ml)	Total activity (U)	Specific activity (U/mg)
Crude	1000	0.95±0.05	950	1.00±0.06 (10.0)	1000	1.10	3.00±0.17 (30.0)	3000	3.15	0.79±0.04 (7.9)	790	0.83
Concentrated	100	3.0±0.16	300	2.90±0.16 (29.0)	290	0.97	9.00±0.5 (90.0)	900	3.0	2.23±0.12 (22.3)	223	0.74
$CD_{5\%}$	NA	0.28	89.3	0.27	91.8	0.13	0.84	1950.0	3.08	0.21	506.5	0.79

Enyme activities in paranthesis are expressed as U/gds

Table 3: Saccharification of pretreated rice straw

Incubation period (h)	Reducing sugars (g/100gds)		
	***Aspergillus* sp. CTS1 cellulase**	***Aspergillus* sp. CTS 2 cellulase**	**Consortium cellulase**
24	10.5±0.58	11.4±0.63	15.8±0.86
48	15.4±0.85	16.3±0.90	24.9±1.39
72	12.5±0.69	13.0±0.72	17.6±0.96
$CD_{5\%}$	13.9	15.9	21.4

Total reaction volume: 20 ml; Enzyme loading: 30 FPU/gds; temperature: 55°C;
Values are mean± standard deviation ; CD5% Critical difference at 5% level

Table 4: Fermentation of saccharified rice straw hydrolysate

Parameter	Incubation period (h)				$CD_{5\%}$
	48	72	96	120	
Initial sugars (g/100g)	24.9 ±1.36				
Residual reducing sugars (g/g)	11.5±0.63	6.2±0.34	4.1±0.26	3.9±0.21	0.07
Reducing sugars utilized (g/g)	13.4±0.74	18.7±1.02	20.8±1.14	21.0±1.16	0.19
Ethanol (g/g)	3.6±0.19	6.2±0.34	8.1±0.46	8.5±0.47	0.07

Incubation temperature: 30° C; pH 5**;** Values are mean± standard deviation

Table 5: Fermentation of pretreated rice straw hydrolysate

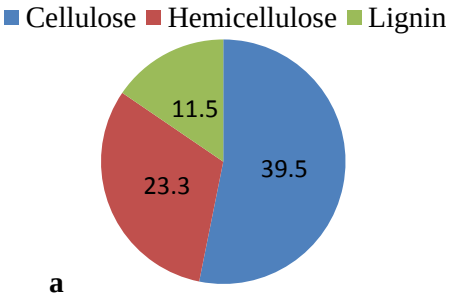
Fermentative culture	Initial reducing sugars (g/100g)	Residual reducing sugars (g/100g)	Reducing sugars utilized (g/100g)	Ethanol yield (g/100g)
[1]*S.cerevisiae*	18±0.01	2.9±0.16	15.1±0.83	4.1±0.23
[2]*P. tannophilus*		3.0±0.17	15.0±0.82	3.3±0.18
[3]*S.cerevisiae* followed by *P.tannophilus*		2.6±0.14	15.4±0.85	4.7±0.26
CD $_{5\%}$		0.27	1.37	0.36

Fermentation at 1, 2 and 3 at 72 h and 4 at144 h of incubation; Incubation temperature: 30° C; pH: 5 (*S.cerevisiae*) and pH:7 (*Z.mobilis*)**;**Values are mean± standard deviation; CD5%: Critical difference at 5% level

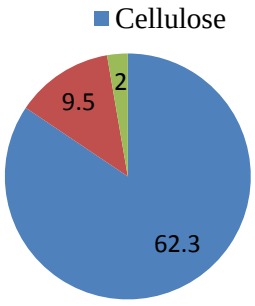
Table 6: Combine ethanol production from pretreated rice straw

Parameter	Saccharified hydrolysate (I)	Pretreated hydrolysate (II)	Combined ethanol production (Hydrolysate I and II)
Initial reducing sugars (g/100g)	24.9 ±1.36	18.0±0.99	42.9±2.35
Residual reducing sugars (g/100g)	3.8±0.21	2.6±0.14	6.4±0.35
Reducing sugars utilized (g/100g)	21.1±1.16	15.4±0.85	36.5±2.00
Ethanol produced (g/100g)	8.5±0.47	4.7±0.26	13.2±0.73

Values are mean± standard deviation



a



b

Figure 1: Chemical composition of a) untreated rice straw b) two-step pretreated rice straw

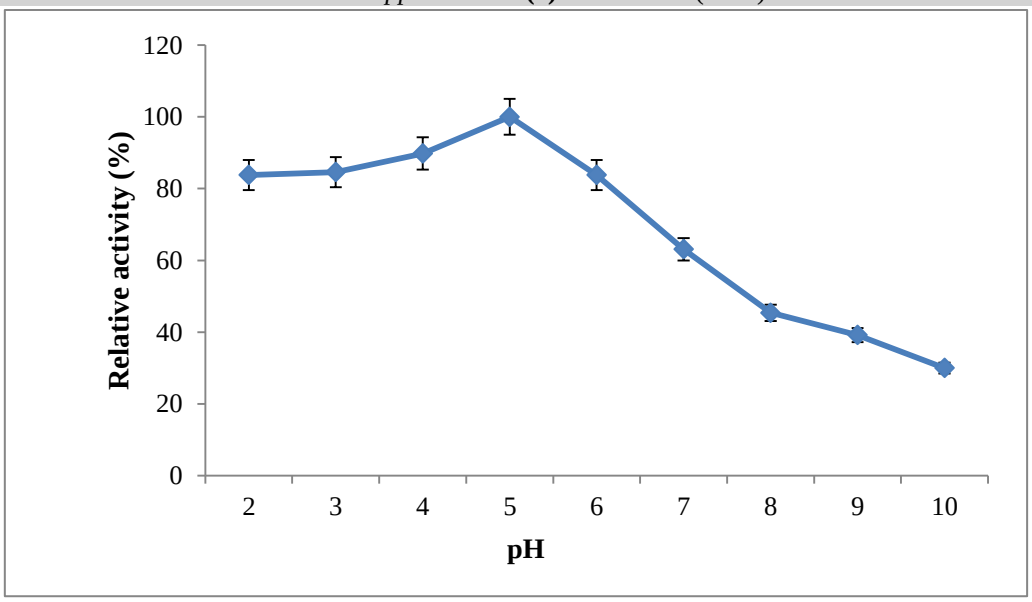


Figure 2: Effect of pH on carboxymethyl cellulase activity

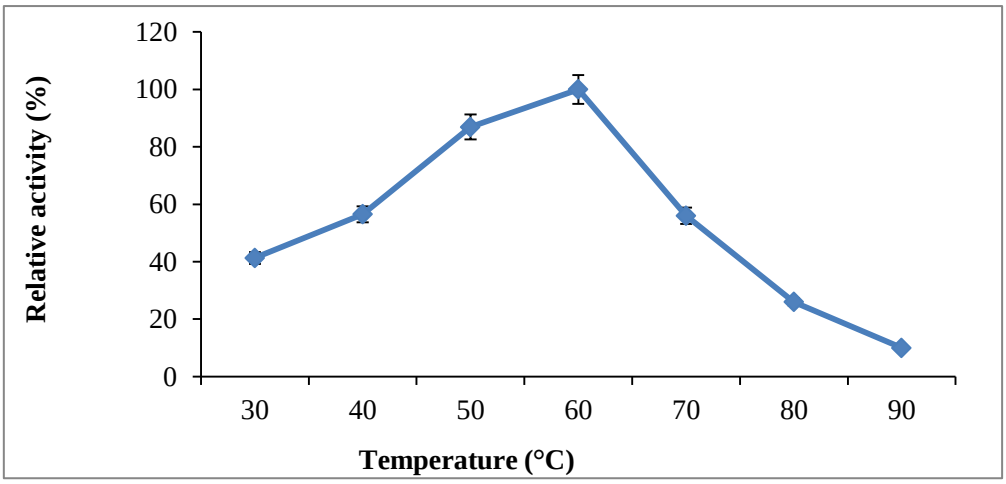


Figure 3: Effect of temperature on carboxymethyl cellulase activity

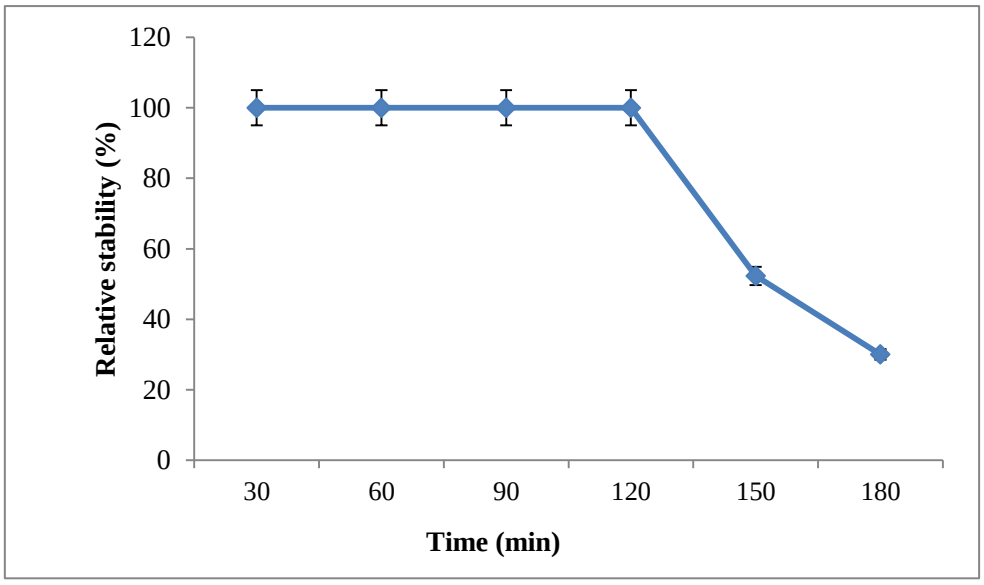


Figure 4: Thermal stability of carboxymethyl cellulase

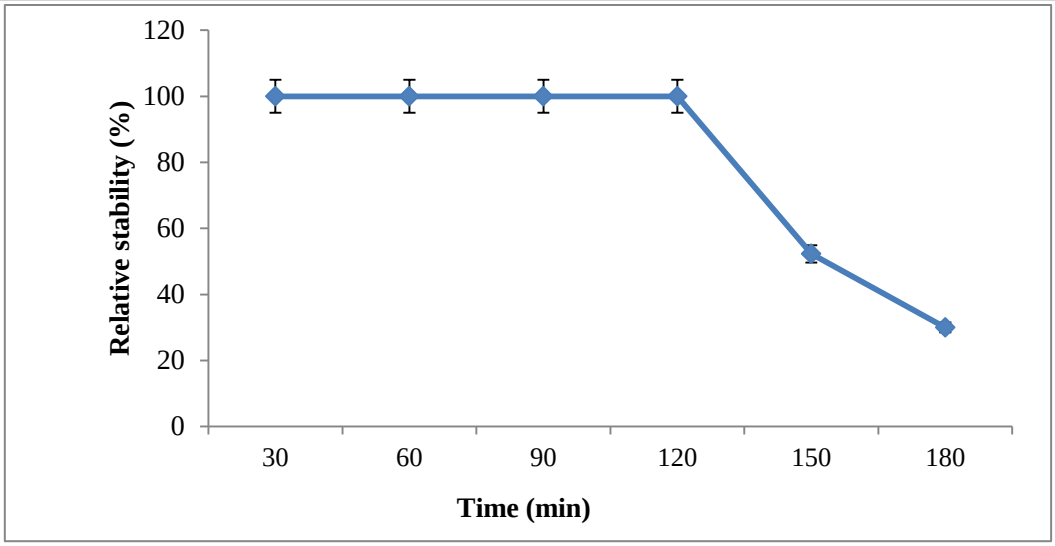


Figure 5: Effect of activators/inhibitors on carboxymethyl cellulase activity

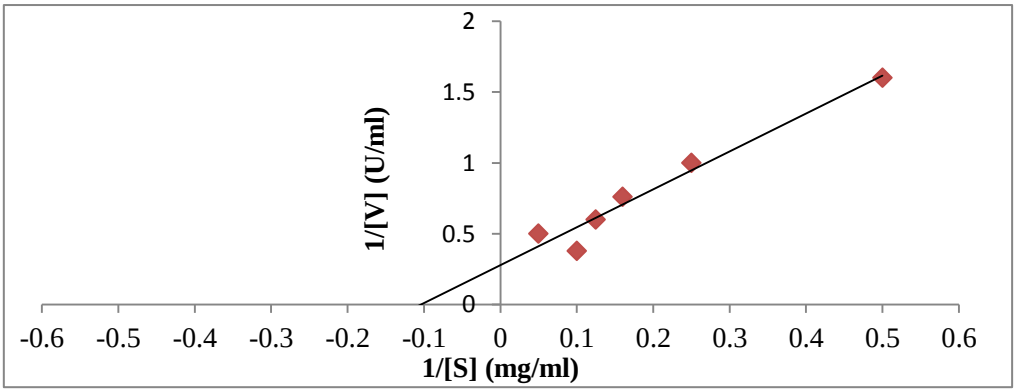


Figure 6: Lineweaver-Burk double reciprocal plot for the determination of K_m and V_{max} values of carboxymethyl cellulase

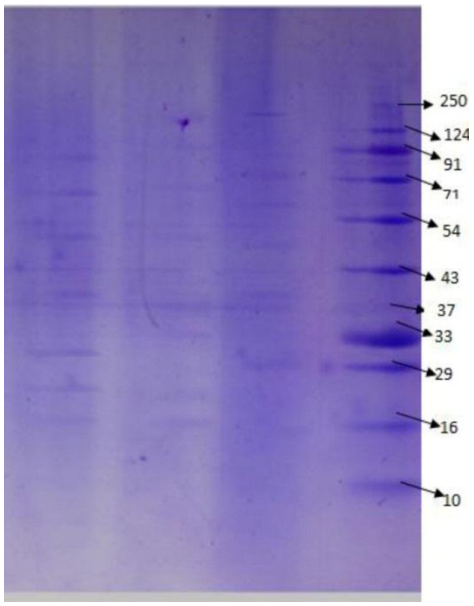
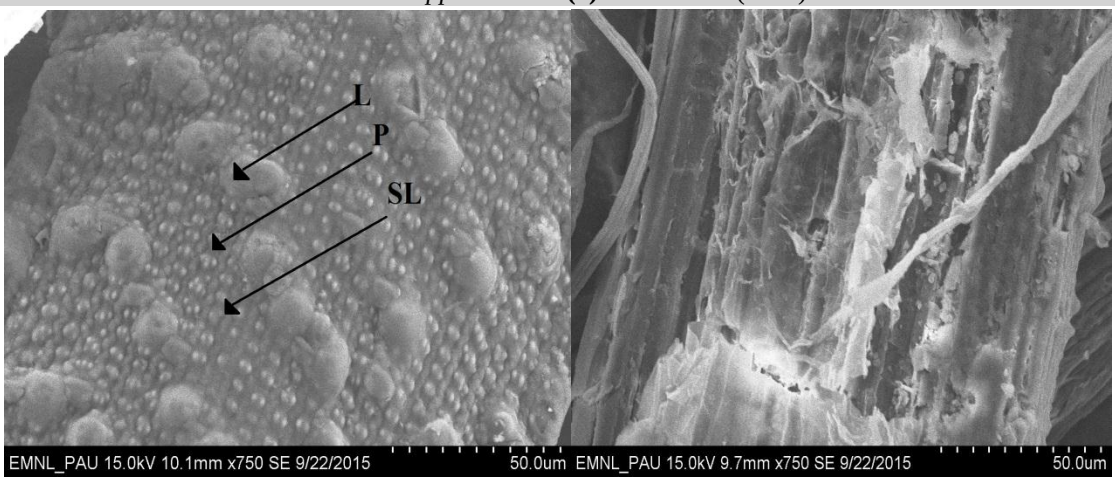


Figure 7: SDS-PAGE profile of concentrated and dialyzed cellulase enzyme: (L-R) Lane 1: *Aspergillus* sp. CTS1; Lane 2: *Aspergillus* sp. CTS 2; Lane 3: Consortium of *Aspergillus* sp. CTS1 and *Aspergillus* sp. CTS 2; Lane 4: Reference molecular weight marker



(a) **(b)**

Figure 8: Scanning electron microscope (SEM) images of untreated rice straw (a) and pretreated rice straw (b): (a) Prominence of Lumps (L), Papillae (P) and Silica layer (SL) on the surface (10.1mm x 750); (b) Extensive damage of the fibrils and cell structures deformation (9.7mm X 750)

CONCLUSION

The two-step i.e. dilute acid followed by alkali pretreatment for delignification of rice straw and suitability of co-culture of thermophilic fungi for the production of an efficient cellulase enzymes complex on pretreated rice straw is demonstrated in the present study. The enhancement in enzyme activity by the consortium- over the mono-culture could chiefly be attributed to the synergistic action of different types of cellulolytic enzymes. The subsequent hydrolysis of pretreated rice straw and use of rice straw hydrolysate for bioethanol production was shown to be successful. The results proved that indigenously developed concentrated cellulase from *Aspergillus* sp. could be a potent candidate for the enzyme cocktail preparation for biomass hydrolysis in lignocellulosic bioethanol programme.

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REFERENCES

1. Wyman, C. E. and Yang, B., Cellulosic biomass could meet California's transportation fuel needs, *California Agri.*, **63:** 185–190 (2009).
2. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. and Ladisch, M., Features of promising technologies, *Bioresour. Technol.*, **96(6):** 673-686 (2005).
3. Hsu, T. C., Guo, G. L., Chen, W. H. and Hwang, W. S., Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis, *Bioresour. Technol.*,**101(13):** 4907-4913 (2010).
4. Zhu, J., Wan, C. and Li, Y., Enhanced solid state anaerobic digestion of corn stover by alkaline pretreatment, *Bioresour. Technol.*, **101(19):** 7523-7528 (2010).
5. Vlasenko, E., Schulein, M., Cherry, J. and Xu, F., Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases, *Bioresour. Technol.*, **101(7):** 2405–2411 (2010).
6. Duff, S. J. B. and Murray, W. D., Bioconversion of forest product industry waste cellulosics to fuel ethanol: A review, *Bioresour. Technol.*,**55:** 1-33 (1996).
7. Maheshwari, R., Bharadwaj, G. and Bhat, M. K., Thermophilic fungi: their physiology and enzymes, *Microbiol. Mol. Biol. Rev.*, **64(3):** 461–488 (2000).
8. Li, D. C., Li, A. N. and Papageorgiou, A., Cellulases fromthermophilic fungi: recent insights and biotechnological potential, *Enzyme Res.*, **2011:** 1-9 (2011).

9. Martins, E. S., Silva, D., Leite, R. S. R., Da Silva, R. and Gomes, E., Purification and characterization of polygalacturonase produced by thermophilic *Thermoascus aurantiacus* CBMAI-756 in submerged fermentation, *Antonie Van Leeuwenhoek*, **91:** 291-299 (2007).
10. Lever, M., Goen, Ho. and Cord-Ruwisch, R., Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation, *Bioresour. Technol.*, **101:** 7083–7087 (2010).
11. Holker, U., Hofer, M. and Lenz, J. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, *Appl. Microbiol. Biotechnol.*, **64(2):** 175-186 (2004).
12. Dashtban, M., Schraft, H. and Qin, W., Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *Int. J. Biol. Sci.*, **5(6):** 578-595 (2009).
13. Febiyanto, A., Rusmana, I. and Raffiudin, R., Characterization and identification of cellulolytic bacteria from gut of worker *Macrotermus givus*, *Hayati J. Biosci.,* **22:** 197-200 (2015).
14. Gilman, J. C., *A Manual of Soil Fungi*. 2nd Ed. Iowa State College Press, Ames, Iowa (1971).
15. Brijwani, K., Oberoi, H. S. and Vadlani, P. V., Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran, *Process Biochem.*, **45:** 120-128 (2010).
16. Mandels, M. and Weber, J. The production of cellulases, *Adv. Chem.*, **95:** 395–414 (1969).
17. Mandels, M., Andreotti, R. and Roche, C., Measurement of saccharifying cellulase, *Biotechnol. Bioeng. Symposium*,**6:** 21-23 (1976).
18. Miller, G. L., Use of dinitrosalicyclic acid reagent for determination of reducing sugar, *Anal. Chem***., 31:** 426-428 (1959).
19. Srivastava, S. K., Gopalakrishnana, S. K. and Ramachandran, K. B., The production of β-glucosidase in shake flask by *Aspergillus wentii*, *J. Ferment. Technol.*, **65:** 95-99 (1987).
20. Lowry, O. H., Rosenbrough, N. J., Farr, A. and Randall, R. J. Protein measurement with the Folin phenol reagent, *J. Biol. Chem.,* **193:** 265-275 (1951).
21. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature,* **227:** 680-685 (1970).
22. Lineweaver, H. and Burk, D., The determination of enzyme dissociation constant, *J. Am. Chem. Soc*. **56:** 658-666 (1934).
23. Hideno, A., Inoue, H., Yanagida, T., Tsukahara, K., Endo, T. and Sawayama, S., Combination of hot compressed water treatment and wet disk milling for high sugar recovery yield in enzymatic hydrolysis of rice straw, *Bioresour. Technol.*, **104:** 743–748 (2012).
24. Vaithanomsat, P., Chuichulcherm, S. and Apiwatanapiwat, W., Bioethanol production from enzymatically saccharified sunflower stalks using steam explosion as pretreatment, *Int. J. Biol. Biomol., Agric. Food Biotechnol. Eng.,***3:** 88-91 (2009).
25. Caputi, A. Jr . and Wright, D., Collaboration study of the determination of ethanol in wine by chemical oxidation. *J. Assoc. Off. Anal. Chemists*, **52:** 85-88 (1969).
26. Kocher, G. S. and Pooja, Status of wine production from guava (*Psidium guajava* L.): A traditional fruit of India, *African J Food Sci*, **5:** 851-860 (2011).
27. Kim, J., Kim, K. S., Lee, J., Park, S. M., Cho, H., Park, J.C. and Kim, J. S., Two-stage pretreatment of rice straw using aqueous ammonia and dilute acid, *Bioresour.Technol.*,**102:** 8992–8999 (2011).
28. Vyas, A. and Vyas, D., Production of fungal cellulases by solid state bioprocessing of groundnut shell wastes, *J. Sci. Indus. Res.*, **64:** 767-770 (2005).
29. Llewellyn, D. A., Marston, T. T., Teutemacher, K. L., Higgins, J. J. and Melgarejo, T., Evaluation of low molecular weight fractions and crude

enzyme preparation from a *Trichoderma* cellulase complex as a treatment for fibrous feeds, *Animal Feed Sci. Technol.,* **160:** 39-48 (2010).

30. Inoue, H., Decker, S. R., Taylor, L. E., Yano, S. and Sawayama, S., Identification and characterization of core cellulolytic enzymes from *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) critical for hydrolysis of lignocellulosic biomass, *Biotechnol. Biofuels*, **7:** 151-164 (2014).
31. Adiguzel, A. O. and Tuncer, M., Production and characterization of partially purified thermostable endoxylanase and endoglucanase from novel *Actinomadura geliboluensis* and the biotechnological applications in the saccharification of lignocellulosic biomass, *Bioresources,* **12:** 528-547 (2017).
32. Wood, T. M. and Bhat, K. M., Methods of measuring cellulase activities, *Methods Enzymol.,***160:** 87-117 (1988).
33. Nascimento, R. P., Junior, N. A., Pereira, Jr. N., Bon, E. P. S. and Coelho, R. R. R., Brewer's spent grain and corn steep liquor as substrates for cellulolytic enzymes production by *Streptomyces malaysiensis*, *Lett. Appl. Microbiol.,* **48:** 529-535 (2009).
34. Grigorevski-Lima, L., Da Vinha, F. N. M., Souza, D. T., Bispo, A. S. R., Bon, E. P. S., Coelho, R. R. R. and Nascimento, R. P., *Aspergillus fumigatus* thermophilic and acidophilic endoglucanases, *Appl. Biochem. Biotechnol.,* **155:** 321-329 (2009).
35. Sohail, M., Ahmad, A. and Khan, S. A., Production of cellulase from *Aspergillus terreus* MS105 on crude and commercially purified substrates, *3 Biotech,* 6: 103 (2016).
36. Das, A., Paul, T., Halder, S. K., Jana, A., Maity, C., Das Mohapatra, P. K., Pati, B. R. and Mondal, K. C., Production of cellulolytic enzymes from *Aspergillus fumigatus* AB K9 in wheat bran- rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp, *Bioresour. Technol.,* 128: 290-296 (2013).
37. Sohail, M., Ahmad, A. and Khan, S. A., Comparative studies on production of cellulases from three strains of *Aspergillus niger*, *Pakistan J. Botany,* 46: 1911-1914 (2014).
38. Rivera, E. C., Rabelo, S. C., Dos Reis Garcia, D., Maciel Filho, R. and Da Costa, A. C., Enzymatic hydrolysis of sugarcane bagasse for bioethanol production: determining optimal enzyme loading using neural networks, *J. Chem. Technol. Biotechnol.,* 85: 983-992 (2010).
39. Andrade, J. P., Bispo, A.S.R., Marbach, P.A.S. and Nascimento, R. P., Production and partial characterization of cellulases from *Trichoderma* sp. IS-05 isolated from sandy coastal plains of northeast Brazil, *Enzyme Res.,* 2011: 1-7 (2011).
40. Shanmughapriya, S., Kiran, G., Selvin, J., Thomas, T. and Rani, C., Optimization, purification, and characterization of extracellular mesophilic alkaline cellulase from sponge-associated *Marinobacter* sp. MSI032, *Appl. Biochem. Biotechnol.,* **16:** 625-640 (2010).
41. Tao, Y. M., Zhu, X. Z., Huang, J. Z., Ma, S. J., Wu, X. B., Long, M. N. and Chen, Q. X., Purification and properties of endoglucanase from a sugarcane bagasse hydrolyzing strain, *Aspergillus glaucus* XC9. *J. Agric. Food Chem*., **58:** 6126-6130 (2010).
42. Ahmed, S. A, El-Shayeb, N. M. A., Hashem, A. G. M., Saleh, S. A. A. and Abdel-Fattah, A. F., Chemical modification of *Aspergillus niger* β-glucosidase and its catalytic properties, *Brazilian J. Microbiol*., **46:** 23-28 (2015).
43. Pham, T. H., Quyen, D. T. and Nghiem, N. M. Purification and properties of an endoglucanase from *Aspergillus niger* VTCC F021, *Turkish J Biol.,* **36:** 694-701 (2012).
44. Bagewadi, Z. K. and Ninnekar, H. Z., Production, purification and characterization of endoglucanase from

Aspergillus fumigatus and enzymatic hydrolysis of lignocellulosic waste, *Int. J. Biotechnol. Biomed. Sci.*, **1:** 25-32 (2015).

45. Hu, H. L, van den Brink, J., Gruben, B. S., Wösten, H. A. B., Gu, J. D. and de Vries, R. P., Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi, *Int. Biodeterior. Biodegrad.*,**65:** 248–252 (2011).
46. Ramachandran, P., Kim, T. S., Dhiman, S. S., Li, J., Park, J. H., Choi, J. H., Kim, J. Y., Kim, D. and Lee, J. K, Saccharification of sunflower stalks using lignocellulases from a fungal consortium comprising *Pholiota adipose* and *Armillaria gemina, Bioprocess Biosys. Eng.*, **38(9):** 1645-1653 (2015).
47. Dhiman, S. S., Haw, J. R., Kalyani, D., Kalyani, V. C., Kang, V.C. and Lee, J. K., Simultaneous pretreatment and saccharification: green technology for enhanced sugar yields from biomass using a fungal consortium. *Bioresour, Technol.*,**179:** 50-57 (2015).
48. Abedinfar, S., Karimi K., Khananhmadi, M. and Taherzadeh, M., Ethanol production by *Mucor indicus* and *Rhizopus oryzae* from rice straw by separate hydrolysis and fermentation, *Biomass Bioenerg.*, **33:** 828-833 (2009).
49. Belal, E. B., Bioethanol production from rice straw, *Brazilian J. Microbiol.*,**44:** 225-234 (2013).