BIOCONVERSION OF SUGARCANE BAGASSE INTO SECOND GENERATION BIOETHANOL AFTER ENZYMATIC HYDROLYSIS WITH IN-HOUSE PRODUCED CELLULASES FROM *Aspergillus* sp. S_4B_2F

Sanjeev Kumar Soni,[a]* Navdeep Batra,[a] Namita Bansal,[a] and Raman Soni [b]

An integrated approach was studied for in-house cellulase production, pretreatment, and enzymatic conversion of sugarcane bagasse into glucose followed by the production of second generation bioethanol. Solid state cultures of *Aspergillus* sp. S_4B_2F produced significant levels of cellulase complex on wheat bran, supplemented with 1% (w/w) soyabean meal, moistened with 1.5 parts of distilled water after 96 h of incubation at 30°C. The highest productivities of endoglucanase, exoglucanase, and β-glucosidase were 66, 60, and 26 IU/g of fermented dry bran, respectively. The enzyme components had a temperature and pH optima at 50°C and 4.0, respectively and revealed high thermostability at 50°C, retaining 66, 54, and 84% residual activities after 72 h. Pretreatment with 2% alkali in combination with steam was the most efficient pre-hydrolysis method for enzymatic bioconversion and fermentation of cellulosic residue of sugarcane bagasse, which produced the highest cellulose conversion (67%), with glucose and alcohol yields of 323 mg and 175 μl respectively per dry gram of bagasse.

Keywords: Cellulases; Aspergillus; sugarcane bagasse; enzymatic hydrolysis; second generation ethanol

*Contact information: a: Department of Microbiology, Panjab University, Chandigarh 160014 INDIA; b: Department of Biotechnology, D.A.V. College, Sector 10, Chandigarh 160011 INDIA; *Corresponding author: sonisk@pu.ac.in*

INTRODUCTION

Starchy grains, tubers and sugarcane juice are currently being exploited worldwide for the production of ethanol. As these starchy products compete with human food, there is a growing interest to replace them with lignocellulosic biomass, which represents the most abundant renewable resource and does not compete with human food. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about 200×10^9 tons/year, where about $8–20\times10^9$ tons of the primary biomass remains potentially accessible. Lignocellulose is a much more complex substrate than starch and is composed of a mixture of polysaccharides (cellulose and hemicellulose) and lignin. A great deal of research has been focused on the enzymatic bioconversion of lignocellulosic polysaccharides into monomer sugars that can then be converted into second generation ethanol (Sanchez 2009; Kovacs et al. 2009). Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds, forming the dimer cellobiose. A major portion of cellulose exists as long chains linked together by hydrogen and covalent bonds which give them a crystalline form, while a small amount of

unorganized cellulose chains forms amorphous cellulose (Laureano-Perez et al. 2005). Cellulose appears in nature associated with other complex plant compounds such as hemicellulose and lignin, and this association affects its biodegradation. Hemicellulose, a polysaccharide with a lower molecular weight than cellulose, is composed of various components including xylan, glucuronoxylan, arabinoxylan, mannan, glucomannan, and xyloglucan formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic, and D-glucuronic acids linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds (Sanchez 2009).

The complete hydrolysis of cellulose and hemi-cellulose requires a well designed cocktail of enzymes consisting of endoglucanases, cellobiohydrolases, β-glucosidases, xylanases, mannanases, and various enzymes acting on side chains of xylans and mannans. Due to the recalcitrant structure of lignocelluloses, a pretreatment step is essential to disrupt lignin prior to enzymatic hydrolysis in order to disorganize the crystalline structure for releasing the polymer chains of cellulose, so that it becomes more accessible to the enzymes (Galbe and Zacchi 2002). As the end product of cellulose hydrolysis is easily fermentable sugar in the form of glucose, while the hemi-cellulose yields a mixture of pentoses including xylose, arabinose and mannose, which are difficult to ferment, the major area of interest in the first stage of global research is the bioconversion of cellulose into glucose for the production of second generation biofuels in the form of alcohol.

For the commercial scale, conversion of cellulosic biomass requires the use of cellulases including endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.91), and β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21), which act synergistically for complete depolymerization into glucose. The commercial cellulases are relatively costly; hence, cellulase production from a wide range of microorganisms has been studied extensively to make the enzyme production process feasible (Lynd et al. 2005). Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity. Fungal cellulases have been the major subjects of investigation over the years, as these catalyze the decay of lignocellulosic material in our ecosystem and some fungi produce extracellular cellulases in significant amounts. Commercial cellulase preparations from *Trichoderma reesei* are popular, as they contain high activities of both exo-glucanase and endo-glucanase, but have low levels of β-glucosidases (Rosgaard et al. 2006). Attention has recently been diverted to other microorganisms, including the members of genus *Aspergillus*, which possess all the three essential components of cellulase system (Sohail et al. 2009) including endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-1,4-glucosidase. Therefore, to obtain maximum yields of cellulase enzymes using abundantly available and the cost effective lignocellulosic substrates, fermentation studies are necessary and are demanding attention worldwide (Mekala et al. 2008).

In the present study an attempt has been made for the optimization of solid state fermentation process for the production of complete cellulase complex having endo-β-1,4-glucanase, exo-β,1-4-glucanases and β-glucosidase from a fungal isolate and its application in the bioconversion of sugarcane bagasse into glucose for the production of second generation bioethanol.

EXPERIMENTAL

Microorganism and Inoculum

A new strain of *Aspergillus* sp. S_4B_2F, used in the present study, was isolated from local soil after an extensive screening of various natural isolates of fungal strains on yeast extract-malt extract (YM) agar plates containing carboxymethyl cellulose (CMC) as the carbon source and identified on the basis of macroscopic and microscopic observations. The strain was capable of producing appreciable levels of endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase activities and was maintained on PDA slants and stored at 4°C after overlaying with sterile mineral oil.

Cellulase Production by Solid State cultures of *Aspergillus* sp. S_4B_2F

Five grams of wheat bran, moistened with 7.5 ml of distilled water, was used as the basal medium for SSF. It was taken in different sets of 250 ml Erlenmeyer flasks, autoclaved, cooled, and then inoculated with five discs (7mm diameter) cut from the periphery of 72 h-old culture of *Aspergillus* sp. S_4B_2F actively growing on PDA plates. The flasks were then incubated at 30°C in stationary state.

Cellulase Extraction

The cellulase enzymes were extracted by adding 100 ml of distilled water to the flask of solid state culture after mixing on a rotary shaker (150 rpm) for 30 min at room temperature. The clear supernatant obtained after centrifugation of contents (10,000 rpm; 4°C; 20 min) was used as a source of extracellular cellulases.

Enzyme Assays

All the three components of cellulase complex were assayed as reported earlier (Soni et al. 1999). Endo-β-1, 4-glucanase (EC 3.2.1.4, Carboxy- methyl- cellulase, CMCase activity), Exo-β-1,4-glucanase (EC 3.2.1.91; filter paper; FPase activity), and β-1,4-glucosidase (EC 3.2.1.21) activities were determined at 50°C using 0.5% CMC, Whatman filter paper no. 1 (1×6 cm strip), and 0.5% salicin in 0.1M acetate buffer, pH 5.0 as the respective substrates. The reducing sugars liberated in the enzyme reactions were determined by the dinitrosalicylic acid reagent method (Miller 1959), and the enzyme activities were expressed in terms of International Units (IU) equivalent to the μmoles of glucose liberated in 1 min. The enzyme yields were expressed as IU/g of fermented dry matter.

Pattern of Cellulase Production

Different sets of 250 ml Erlenmeyer flasks containing basal media were incubated at 30°C under stationary conditions for 144 h. The flasks, in duplicate, were withdrawn at regular intervals of 24 h to study the production profiles of all the three cellulase components.

Optimization of Cellulase production

This was studied by separately supplementing various additional nutrients as carbon and nitrogen sources (Fig. 2) as well as by varying the moisture content in the

wheat bran based basal media. The cellulase productivities were estimated after 96 h of incubation at 30°C.

Scale-up of Cellulase Production

The scale-up of enzyme production was carried out in different sets of specially designed wooden trays (30cm×45cm×5cm), having sides made up of 1cm thick wooden frame and base fitted with the wire mesh. The different batches of the trays containing 200, 500, and 750 g of wheat bran moistened with an equal amount of tap water and covered with aluminium foil, were autoclaved and inoculated with 100, 200, and 300 ml of spore suspensions (spore count of 1.50×10^5/ml) made from 1, 2, and 3 Erlenmeyer flasks (250 ml) containing the 4-day-old sporulated solid state culture of *Aspergillus* sp. S_4B_2F grown on 5 g of wheat bran based basal solid media. The trays were then incubated at 30°C as static cultures after covering with aluminium foil for 5 days, and the enzymes were extracted in water equal to 10 times of the wheat bran used, after properly mixing the contents in a blender.

Characterization of the Cellulase Components

The crude cellulase preparation from solid state cultures, having endoglucanase, exoglucanase, and β-glucosidase activities, were characterized by studying the effect of temperature & pH versus activities and stabilities in addition to the effect of various metal ions on enzyme activities. Temperatures that were optimal for various enzyme activities were determined by assaying at different temperatures (30, 40, 50, 60, 70, and 80°C), keeping the pH at 5.0, while the pH optima were determined by assaying at different pH (3.0-9.0), while keeping the temperature at 50°C.

The temperature stability profiles of the cellulase components were studied by incubating different sets of enzyme preparation over a temperature range of 50-70°C, in the presence of 3% finely ground sugar cane bagasse. The residual activities were determined by the standard assays under normal conditions (50°C; pH 5.0) at regular intervals of 24 h till 72 h of incubation.

The pH stability profiles were studied by incubating the enzyme preparations, separately in buffers ranging from pH 4.0 to pH 9.0 at room temperature and determining the residual activities by the standard assay under normal conditions at regular intervals of time till 24 h of incubation.

The requirement of metal ions for all the three cellulolytic components, if any, was studied by supplementing various metal salts, individually, in the reaction mixtures and determining the relative activities under normal assay conditions.

Bioconversion of Sugar Cane Bagasse into Bioethanol Using the Cellulase Preparation Produced In-house

As the sugarcane bagasse residues are very complex and are difficult to hydrolyze, probably because of the integration of lignin, attempts were made to make cellulose in this residue accessible to enzymatic hydrolysis after physico-chemical pretreatments.

Physical Pretreatment

Sugarcane bagasse was soaked in water for 3-4 h, washed under running water, and then dried in hot air oven at 70°C. This was then ground, screened (16 mesh), and stored in a dark and dry environment at room temperature before the treatment.

Pre-hydrolysis Treatment

Various pre-hydrolysis treatments with dilute H_2SO_4 or dilute NaOH and their combinations with steam treatment were applied consecutively. Biomass and acid/ alkali (1-2%) were mixed, separately at a solid**:**liquid ratio of 1.0 g in 50 ml during 180 min at 90°C. Additionally acid and alkali pre-treatment methods were combined with steam treatment by treating the biomass, separately with 1 and 2% H_2SO_4 and NaOH for 180 min at 90°C first, followed by steam treatment, undertaken in an autoclave, at a constant temperature (121°C) for 15 min. After the respective treatments, samples were drained, and then washed with deionised water until the pH of the filtrate remained constant at an approximate value of 5.0. The pre-treated samples were drained, and air-dried overnight to a final moisture content of 45-50%.

Enzymatic Hydrolysis

Enzymatic hydrolysis of 1.0 g of pre-hydrolysed sugarcane bagasse was carried out at 50°C in 100 ml Erlenmeyer flasks placed in an orbital agitator at 150 rpm for 120 h using the crude cellulase preparation from the laboratory culture of *Aspergillus* sp. S_4B_2F having all the three components, viz. endoglucanase, exoglucanase, and β-glucosidase. All the experiments were performed at enzyme to substrate ratios of 40 IU each of endoglucanase and exoglucanase and 20 IU β-glucosidase/g substrate, and 0.3 mg/ml chloramphenicol for 96 h. The total volume of the reaction mixture was set at 25 ml and the pH kept at 4.0 using 0.1 M acetate buffer. Samples were withdrawn from the reaction mixture after every 3, 6, 24, 46, 72 and 96 h for kinetic studies.

Fermentation Studies

The enzymatically hydrolyzed reaction mixtures were autoclaved at 121°C for 15 min so as to inactivate the residual enzymes and the contents of each set were fermented with distillers's yeast strain of *Saccharomyces cerevisiae* MTCC 786, procured from Microbial Type Culture Collection and Gene Bank at Institute of Microbial Technology, Chandigarh, India. To 15 ml filtrate, taken in 50 ml Erlenmeyer flasks, was added 1 ml of the yeast suspension (viable cell count of 1.15×10^8/ml), made in deionised water from overnight grown shake culture in YM broth (yeast extract, 0.3%; malt extract, 0.3%; peptone, 0.5%; dextrose, 1.0%; pH 5.0). The inoculated flasks were then plugged with cotton wool, incubated as static cultures at 30°C for 48 h, and analyzed, thereafter, for alcohol content and residual glucose.

Analytical Methods

The analytical methods were performed according to the following procedures. 10 g sugarcane bagasse was dried at 90°C for 24 h, and its cellulose content was analyzed by the method of Crampton and Maynard (1938) after grinding the bagasse to a fine powder. Total sugar concentrations were determined routinely from centrifuged samples

(10,000 rpm; 10 min), using the DNSA reagent method (Miller 1959), while the glucose concentration was determined in the final samples (120 h) by the glucose oxidase method. The nature of the end products of enzymatic hydrolysis was detected by paper chromatography (Hayashida et al. 1988) using butanol, 30 ml: pyridine, 20 ml: water, 15 ml as the solvent system and benzedine, 1g: glacial acetic acid, 200 ml: absolute alcohol, 80 ml as the detection reagent. The alcohol content in fermented samples was estimated by the spectrophotometric method of Caputi et al. (1968).

Data Analysis

All analysis was done in triplicate, and data were expressed in terms of 100% dry matter basis. The results of enzymatic hydrolysis have been expressed in terms of cellulose conversion and glucose yield, where as the results of fermentation have been expressed in terms of ethanol yield.

Cellulose conversion as percentage of the theoretical glucose yield obtained from the equation which involves the transfer of cellulose to sugar $(C_6H_{10}O_5)_n + {}_nH_2O \rightarrow (C_6H_{12}O_6)_n$ was computed by using the following formula,

$$\text{Cellulose conversion } (\%) = [\text{Glucose}]/ (1.11 \times f \times [\text{biomass}]) \times 100 \qquad (1)$$

where [Glucose] is the glucose concentration (g/100ml), [Biomass] is dry biomass concentration used in enzymatic hydrolysis (g/100ml) = 4 g/100ml; f is the cellulose fraction in dry biomass (g/g), and 1.11 is the factor that corresponds to the mass balance of the transfer of cellulose to sugar.

Glucose yield has been expressed in terms of mass of glucose produced/mass of untreated sugarcane bagasse.

Alcohol yield has been expressed in terms of volume of alcohol produced/mass of untreated sugarcane bagasse.

RESULTS AND DISCUSSION

There are several important aspects that should be considered for the development of any bioprocess in SSF. These include selection of suitable micro-organism and substrate, optimization of process parameters, and isolation and purification of the product. In order to achieve high enzyme yield, efforts are made to develop a suitable medium and to work out the favourable environmental conditions for the proper growth and maximum secretion of enzyme. Development of such medium requires the right selection of cheaper and readily available components. A number of substrates have been employed for the cultivation of microorganisms to produce enzymes, however, wheat bran holds the key, and has most commonly been used in various processes (Pandey et al. 2000). We also used wheat bran as the solid substrate in the present study for the cellulase production by *Aspergillus* sp. S_4B_2F. The organism colonized well on the wheat bran, based solid substrate, and exhibited a good growth on the surface after 24h and synthesized all the three components of cellulase complex. The growth and enzyme yields improved gradually, and the maximum enzyme productivity of all the

enzyme components took place after 96h of incubation when the organism had established itself components took place after 96h of incubation when the organism had established itself well in the deeper layers of the solid medium and produced sporulation on the surface of the mycelium. Of the various enzymes, the endoglucanase yield was the highest, followed by exoglucanase and β-glucosidase, which exhibited 57, 50, and 22 IU/g fermented dry matter, respectively (Fig. 1). Further incubation resulted in a slight decline in enzyme yields. The decline after 96h could be attributed to proteases that may start accumulating extracellularly along with cellulases or due to the stationary phase of growth, where it started the production of secondary metabolites, some of which might have inactivated the enzyme(s). The cellulase secretion prior to protease secretion is associated with the substrate utilization by microbes, while protease secretion is associated with the growth of the microbes. A number of earlier studies revealed that *Aspergillus* sp. can produce relatively large quantities of endoglucanase and β-glucosidase, but low levels of exoglucanase (Duarte and Costa-Ferreira 1994; De Vries and Visser 2001). However, the present study has revealed quite high levels of exoglucanase by solid state cultures of a strain of *Aspergillus* sp. Moreover, most of the earlier studies employed a complex mineral salt solution as a moistening agent in the production media involving various lignocellulosic substrates such as grass, corn cob, spruce, wheat straw, sugarcane bagasse, wheat bran, and rice hulls (Mekala et al. 2008; Sohail et al. 2009; Kovacs et al. 2009; Zhao et al. 2009). By contrast, in the present study we have used plain distilled water as the moistening agent for wheat bran, thus making it a cheaper medium for enzyme production. The enzyme yields achieved with this simpler and cheaper medium were also quite high as compared to most of the earlier reports.

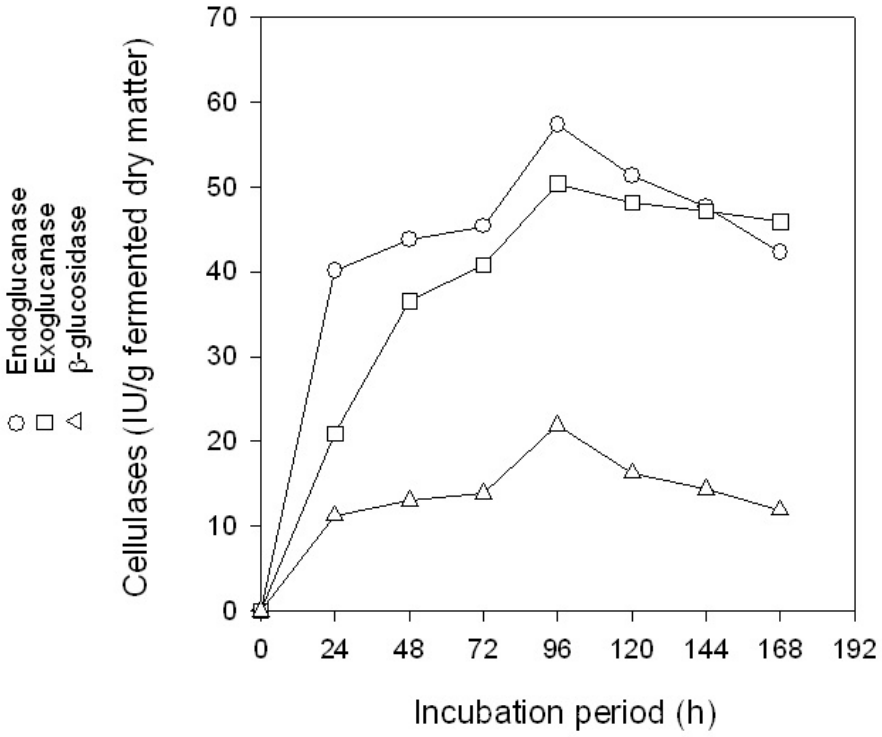

Fig. 1. Time course of cellulase production by solid state cultures of *Aspergillus* sp. S_4B_2F at 30°C

Optimization of Cellulase Production

Commercial wheat bran contains 15.2% cellulose, 23.9% hemicellulose, and 16% protein, in addition to various minerals. However it may not be providing all the nutrients needed by the organism for maximum enzyme production during SSF or some vital nutrients necessary for optimal growth, and product formation may be present at suboptimal levels. Hence, the exogenous addition of various nutrients to the solid medium improves the growth of organism and thus the product yield. Cellulases are inducible and also regulated by catabolite repression in most fungi (Nwodo-Chinedu et al. 2007). The influence of carbon sources on the production of cellulase complex has been investigated by several workers, who have indicated that the enzyme levels vary according to the carbon source used (Jaradat et al. 2008).

In order to achieve high cellulase yields from *Aspergillus* sp. S_4B_2F in the present study, efforts were made to enrich the wheat bran based solid medium by supplementing various carbon and nitrogen sources, as an adequate supply of carbon and nitrogen is critical for optimum growth of organism and its metabolism. The supplementation of various sugars and pretreated lignocellulosic residues in the solid media did not show any overall improvement in the enzyme yields, though the proportions of the three components of cellulase complex exhibited variations with different carbon sources (Fig. 2a). This is probably due to the presence of a blend of complex carbohydrates in the form of cellulose including soluble cellooligosaccharides, hemicelluloses, substituted insoluble xylan, which might be acting as good inducers, and the possible cooperation between them in addition to digestible nitrogen and their in wheat bran (Palmarola-Adrados et al. 2005; Peixoto-Nogueira et al. 2009).

The supplementation of nitrogen sources in the wheat bran had a marked effect on the growth and cellulase production. The maximum enzyme activities were obtained with soyabean meal, which brought about an improvement in all the three cellulase components, including endoglucanase, exoglucanase, and β-glucosidase, revealing 66, 60, and 26 IU/g fermented dry matter. Meat extract and potassium nitrate also brought about an improvement in the levels of β-glucosidase as compared to control, where the productivities of this component were even better than observed in presence of soyabean meal (Fig. 2b).

Moisture is one of the most important parameters in SSF that influences the growth of the organism and thereby enzyme production. Low and high moisture levels of the substrate effect the growth of the microorganism resulting in lower enzyme production (Sivaramakrishnan et al. 2006). Higher moisture level decreases porosity, promotes stickiness, changes wheat bran particles structure, and reduces aeration, which results in lowered oxygen transfer and enhances the growth of organism. By contrast, lower moisture content reduces solubility of the nutrients, which then become inaccessible to organism, also resulting in a lower degree of swelling and higher water tension prevails in the medium (Anto et al. 2006). The substrate-water ratio of 1:1.5 (w/w) was found to be best suited for cellulase synthesis by *Aspergillus* sp. S_4B_2F in the present study (Fig. 2c).

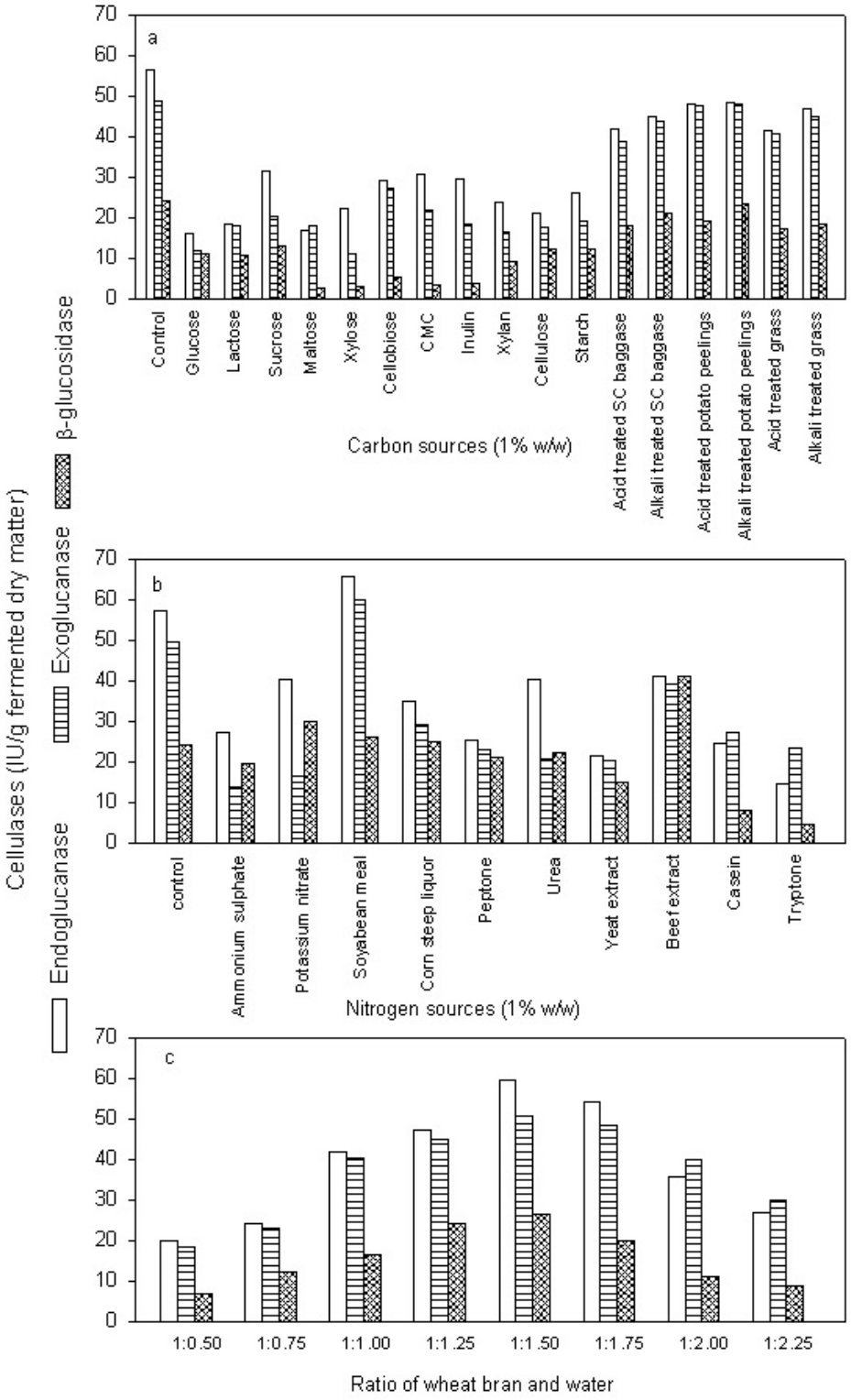

Fig. 2. Effect of (a) exogenous supplementation of carbon sources, (b) exogenous supplementation of nitrogen sources, and (c) moisture level in the basal medium, on cellulase production by solid state cultures of *Aspergillus* sp. S_4B_2F at 30°C

Scale-up of Enzyme Production in Trays

Different types of production vessels have been used for carrying out solid state fermentation, but most of the laboratory studies on the production of enzymes using SSF technique have employed Erlenmeyer flasks and trays (Pandey et al. 2000; Mitchell et al. 2000). In a tray bioreactor a relatively thin layer of substrate is spread over a large horizontal area. There is no forced aeration, although the base of the tray may be perforated and air forced around the tray. Internal temperature may vary with ambient temperature; or the tray may be placed in a temperature-controlled room.

Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial, and commercial scale (Pandey et al. 2000; Mitchell et al. 2000). The scale-up of cellulase production using 200-750 g of wheat bran, moistened with appropriate volumes of water with a final substrate: water ratio of 1:1.5, in trays, as carried out in the present study, revealed promising results exhibiting almost similar yields as achieved in 250 ml Erlenmeyer flasks using 5 g of the substrate (Table 1). This suggested that the solid state cultures of *Aspergillus* sp. S_4B_2F on wheat bran based media hold a great potential for large-scale production of cellulases using tray bioreactors.

Table 1. Cellulase Yields During Scale-up Studies Using Different Quantities of Wheat Bran

Quantity of wheat bran (g)	Enzyme activity (IU/g fermented dry matter)		
	Endoglucanase	Exoglucanase	β-glucosidase
5	57	50	22
200	56	49	23
500	53	44	21
750	51	44	21

Characterization of Crude Cellulase Preparation

The rate of enzymatic hydrolysis of cellulose depends upon various parameters such as temperature, pH, substrate concentration, enzyme concentration, the presence of cofactors, and stabilizing agents. The enzymes with characteristics suitable for the industrially relevant process conditions and applications have to be appropriately selected as per the demand. Moreover, such enzymes have to be used in large volumes, and that too in the form of crude preparations for industrial processing, as the large-scale purification of such enzymes is not feasible. Cellulase complex present in crude enzyme preparation obtained from solid state culture of *Aspergillus* sp. S_4B_2F has been characterized by studying the activity and stability profiles with respect to temperature and pH, along with the various metal ions on enzyme activities. As the lignocellulosic depolymerization is generally carried out at higher temperature, generally ranging from 50-70^{o}C, the thermostable cellulases are of great significance. The increase in assay temperature up to 50^{o}C was accompanied with enhancement of all the enzyme activities, suggesting this temperature to be the optima (Fig 3a). The assay at 60^{o}C brought about a slight decrease, while further increase in the temperature brought about a significant decline in the enzyme activities, suggesting that the enzyme preparation from *Aspergillus* sp. S_4B_2F can be effectively used in a temperature range of 50-60^{o}C.

As the incubation temperature of 30°C also exhibited the relative activities of 24-33% by various enzyme components, the enzyme preparation can also be used for simultaneous saccharification of cellulosic biomass and fermentation of released sugars by *Saccharomyces cerevisiae*, which also converts glucose into alcohol effectively at this temperature, so as to avoid the catabolite repression by the accumulated glucose, a known phenomenon in cellulase synthesis (Lynd et al. 2002).

The pH activity profiles of all the celullases revealed the highest activities at pH 4.0, thus suggesting this to be optimum pH for their action (Fig. 3b). As the cellulases from fungi show catabolite repression during the hydrolysis of cellulose and xylan, there is a need to explore for the enzymes that have compatible pH optima with that of yeast so that the same can be used for simultaneous saccharification and fermentation. As the yeast grows well at pH 4.0, the cellulase preparation from *Aspergillus* sp. S_4B_2F, can be used in presence of yeast for synergistic actions.

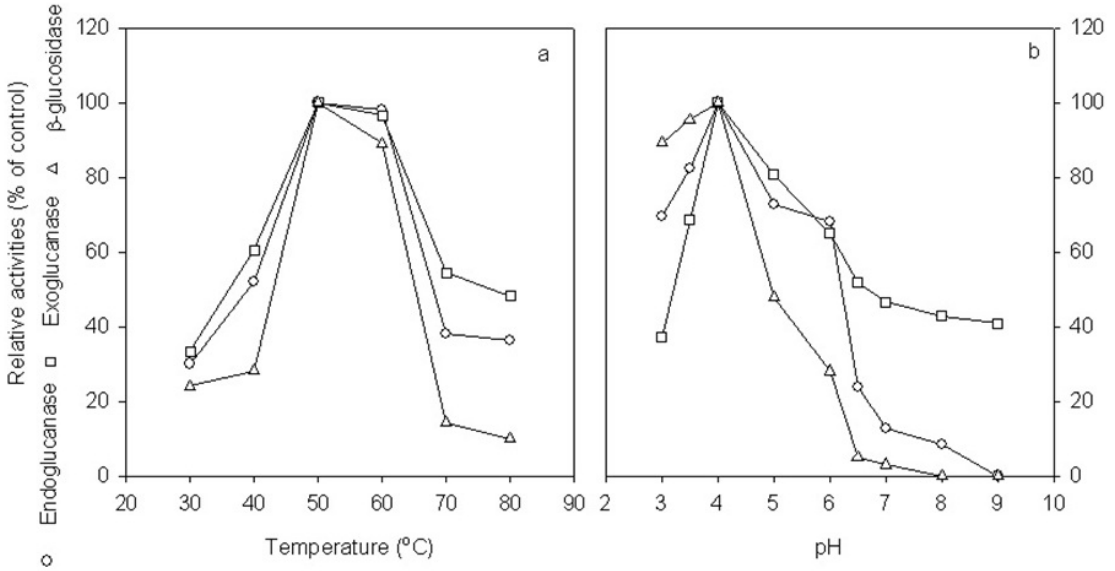

Fig. 3. Temperature (a) and pH (b) versus activity profiles of various components of crude cellulase preparation from solid state cultures of *Aspergillus* sp. S_4B_2F

The utility of an enzyme depends mainly on its operational and storage stability (Fagain 2003), which can be achieved by screening intrinsically stable enzymes, adding stabilizing agents, chemical modification, immobilization, protein engineering, etc. (Sivaramakrishan et al. 2006). As the enzyme system of *Aspergillus* sp. S_4B_2F is intended to be used for the hydrolysis of sugarcane bagasse, which is a source of the substrate for cellulases, the thermostability of various enzyme components was studied in presence of this substrate by supplementing 3% acid-pretreated bagasse in enzyme supernatant, as the thermostability of enzymes is known to be affected by many factors such as the presence of calcium, substrate, and other stabilizers (Reddy et al. 2003). All the cellulases were highly stable at 50°C, as indicated by their thermostability profiles (Fig. 4).

The half-lives of endoglucanase, exoglucanase, and β-glucosidase at this temperature were more than 72 h, as evident from their residual activities of 66, 57, and 84%, respectively, after 72 h of incubation. Exoglucanase was also quite stable at 60 and

70°C, revealing 70 and 28% residual activity after 48 h. Endoglucanase could also tolerate 60 and 70°C for 24 h, where it exhibited 51 and 48% residual activities, respectively. β-glucosidase also revealed a similar trend, but the stability was less than endoglucanase, showing 25 and 13% residual activities at 60 and 70°C. A significantly high thermostability at 50°C even after 72 h suggests the enzyme preparation from *Aspergillus* sp. S_4B_2F can be effectively used for the hydrolysis of cellulosic biomass by carrying out the reaction at this temperature.

The pH stability profiles of various enzyme components are shown in Fig 5. Endoglucanase, exoglucanase, and β-glucosidase were most stable in a pH range of 4.0-5.0, revealing the residual activities of 43, 46, and 56% after 24h of incubation at room temperature.

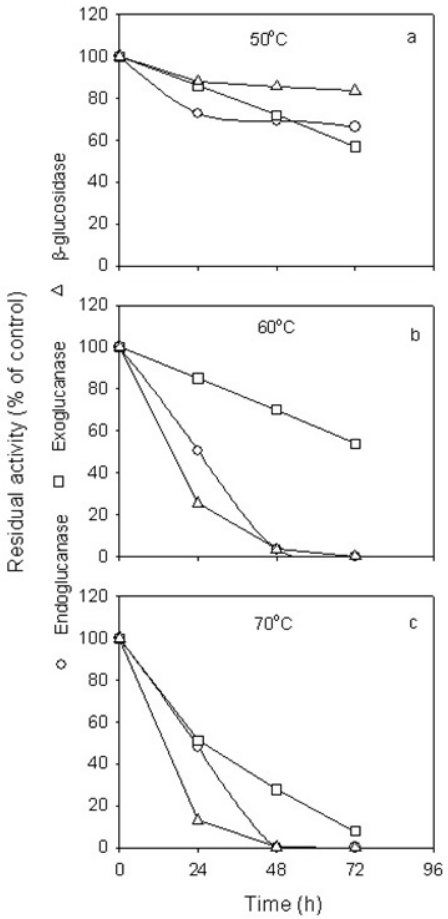

Fig. 4. Thermostability profiles of various components of crude cellulase preparation from solid state cultures of *Aspergillus* sp. S_4B_2F at different temperatures.

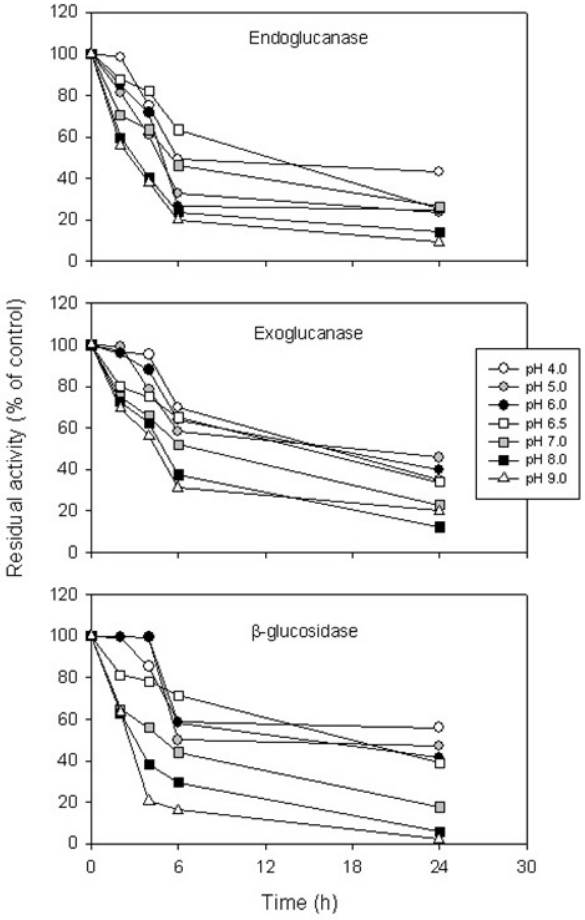

Fig. 5. pH stability profiles of various components of crude cellulase preparation from solid state cultures of *Aspergillus* sp. S_4B_2F at different pH.

Many enzymes require certain metal ions as cofactors for their maximum activity. On the other hand, some metal ions exert an inhibitory effect on the enzyme activity. The cellulase preparation from *Aspergillus* sp. S_4B_2F also requires $CoCl_2$, as evident from the promotion in the activities of various components in its presence (Fig. 6).

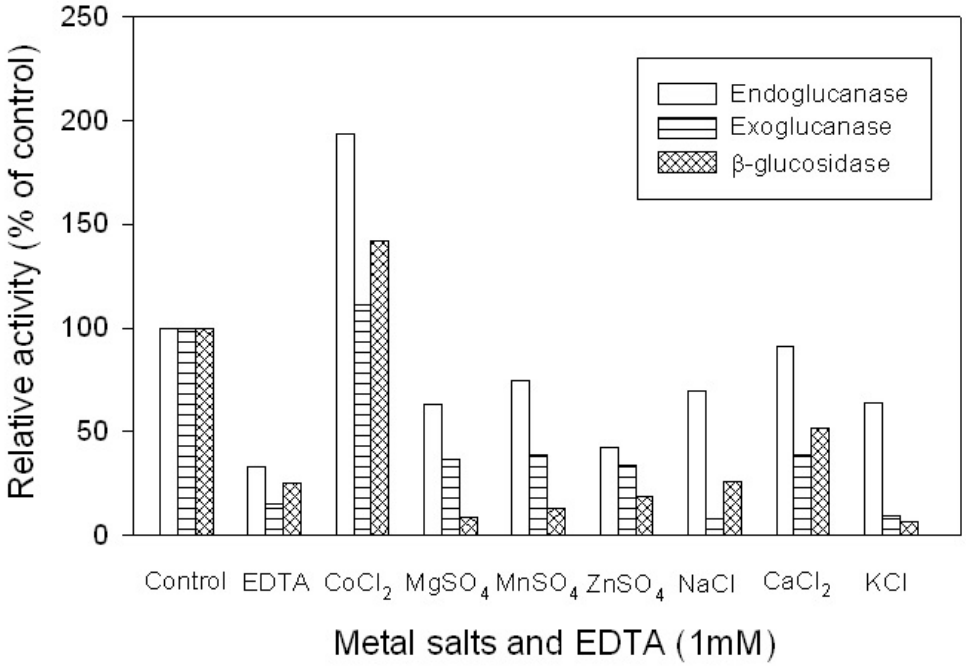

Fig. 6. Effect of various metal salts and EDTA on activities of various components of crude cellulase preparation from solid state cultures of *Aspergillus* sp. S_4B_2F.

Hydrolysis of Sugarcane Bagasse and Fermentation of Glucose

The production of ethanol from any lignocellulosic biomass generally involves four process steps - feedstock pretreatment, enzymatic saccharification, fermentation, and ethanol recovery. Few lignocellulosic residues including sugarcane bagasse, wheat straw, corn stover, spruce, and municipal solid waste have been researched by several workers for enzymatic bioconversion, with commercial or in-house produced cellulases, into glucose employing various pretreatment protocols including acid, alkali, and steam (Li et al. 2007; Rabelo et al. 2009; Kovacs et al. 2009). Following pretreatment, plant cell wall polysaccharides are more susceptible to enzymatic hydrolysis that breaks them into monomeric (single) sugars that can be fermented into ethanol (Lynd et al. 1999). Depending on the type and effectiveness of the pretreatment method, complete hydrolysis takes 24-48 h (Lin and Tanaka 2006). Of the two possible hydrolysis methods, acid and enzymatic hydrolysis, acid hydrolysis is efficient and relatively inexpensive, but it forms compounds that might seriously inhibit the subsequent fermentation (Hu et al. 2008). Alkali pretreatment has regained interest as one of the promising pretreatment technologies being studied. This pretreatment has low formation of fermentation inhibitors, increases pH, and provides a low-cost alternative for lignin solubilization, removing approximately 33% of lignin and 100% of acetyl groups (Wyman et al. 2005). Of the sulphuric acid and sodium hydroxide pretreatments used in the present study with sugarcane bagasse (having a cellulose content of 43.4% oven dry basis), the latter proved to be better as revealed by higher enzymatic bioconversions into sugars as compared to the former (Table 2). Between the two temperature ranges used in each category of pretreatment, the steaming at 121°C was better than 90°C, as indicated by the levels of

total reducing sugars, including glucose, liberated after enzymatic bioconversion in each treatment.

Table 2. Pattern of Sugar Formation during Enzymatic Bioconversion of Alkali and Acid Pretreated Sugarcane Bagasse at 50 °C

Sugarcane bagasse (43.4% cellulose, dry basis)	Reducing sugar concentration after enzymatic hydrolysis* (mg/ml) 0h	3h	6h	24h	48h	72h	96h	Glucose (mg/ml)
Acid pretreatment								
1% at 90°C	0.010	5.00	5.80	6.75	8.90	9.50	10.0	8.01
2% at 90°C	0.011	4.10	4.50	5.20	8.20	8.80	9.10	7.50
1% steamed	0.090	5.30	6.20	7.10	9.50	9.90	10.5	9.00
2% steamed	0.080	4.90	6.10	6.92	8.95	9.10	9.30	7.90
Alkali pretreatment								
1% at 90°C	0.055	4.00	4.75	6.00	9.57	10.6	11.0	10.16
2% at 90°C	0.066	4.25	4.75	6.50	9.20	11.5	12.0	11.23
1% steamed	0.050	4.25	5.50	6.00	10.5	11.9	12.0	11.03
2% steamed	0.040	4.50	6.75	7.00	10.8	12.5	13.0	12.93

The paper chromatography results revealed that the main end products of hydrolysis carried out in the present study were a mixture of glucose and xylose. The presence of xylose in the hydrolyzed mixture is probably due to the hydrolysis of xylan in sugarcane bagasse by xylanases present in the enzyme supernatant of *Aspergillus* sp. S_4B_2F (data not shown). Alkali pretreatment with 2% NaOH in the presence of steam at 121°C resulted in the highest cellulose conversion (67%) as indicated by glucose and alcohol productivities, revealing the yields of 323 mg glucose and 175 µl ethanol/ g dry bagasse, while the yields achieved in similar pretreatment at 90°C, with 58 % cellulose conversion (Table 3) were 281 mg glucose and 153 µl ethanol/ g dry bagasse.

Table 3. Glucose and Alcohol Yields Obtained After Enzymatic Hydrolysis and Fermentation of Alkali and Acid Pretreated Sugarcane Bagasse

Pretreatment Type	Cellulose conversion (%)	Glucose yield (mg/g of bagasse)	Alcohol (µl/ml)	Ethanol yield (µl/g of bagasse)
at 90°C				
1% H_2SO_4	42	200	4.33	108
2% H_2SO_4	39	188	4.05	101
1% NaOH	53	254	5.48	137
2% NaOH	58	281	6.11	153
at 121°C in presence of steam				
1% H_2SO_4	47	225	4.86	122
2% H_2SO_4	41	198	4.27	107
1% NaOH	57	276	5.95	149
2% NaOH	67	323	6.98	175

The yields achieved with 1% NaOH were slightly lower, revealing 57 and 53% conversions at 121°C and 90°C, respectively. Of the two concentrations of H_2SO_4 used in pretreatment, 1% resulted in higher glucose (225 & 200 mg/g bagasse) and ethanol (122 & 108 μl/g bagasse) yields at 121°C and 90°C respectively (Table 3). Silverstein et al. (2007) also studied the effectiveness of sulfuric acid, sodium hydroxide, hydrogen peroxide, and ozone pretreatments for enzymatic conversion of cotton stalks. They found that sodium hydroxide pretreatment resulted in the highest level of delignification (65% with 2% NaOH in 90 min at 121°C) and cellulose conversion (60.8%). Rabelo et al. (2009) evaluated the pretreatment of sugarcane bagasse with calcium hydroxide for enzymatic hydrolysis using commercial cellulase preparation and achieved a maximum glucose productivity of 218 mg/g dry bagasse. Kovacs et al. (2009) used three different steam-pretreated lignocellulosic substrates, namely spruce, wheat straw, and sugarcane bagasse for enzymatic hydrolysis using commercial as well as laboratory produced enzymes and achieved the highest glucose yields of 29, 65, and 55% in case of spruce, wheat straw and sugarcane bagasse respectively. In the present study the glucose yields achieved in alkali pretreated bagasse, thus, appear to be quite significant as compared to the published reports. Moreover, the conversion of released glucose into ethanol makes the present study a novel one.

CONCLUSIONS

Cellulase enzyme complex produced by the solid state cultures of *Aspergillus* sp. S_4B_2F on simple wheat bran based medium have high endoglucanase and exoglucanase activities, along with good levels of β-glucosidase. This enzyme system effectively led to enzymatic conversion of alkali-pretreated cellulose from sugarcane bagasse into glucose, followed by fermentation into ethanol. Although the cellulases from *Aspergillus* sp. S_4B_2F have shown promising results, further extensive laboratory studies are required to optimize the bioconversion and its scale-up trials before any conclusion can be drawn for the commercialization of this process.

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