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EXPERIMENTAL ARTICLES

Production of Bio-Ethanol from Pretreated Agricultural Byproduct Using Enzymatic Hydrolysis and Simultaneous Saccharification[1]

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Abstract—Global warming alerts and threats are on the rise due to the utilization of fossil fuels. Alternative fuel sources like bio-ethanol and biodiesel are being produced to combat against these threats. Bio-ethanol can be produced from a range of substrate. The present study is aimed at the Production of bioethanol from pretreated agricultural substrate using enzymatic hydrolysis and simultaneous saccharification with the addition of purified fungal enzyme. Most cellulosic biomass is not fermentable without appropriate pretreatment methods and so dilute sulfuric acid pretreatment was applied to make the cellulose contained in the waste susceptible to endoglucanase enzyme. A range of acid pretreatment of wheat bran was made in which the sample that was pretreated with 1% dilute sulfuric acid gave maximum yield of ethanol in both methods such as 5.83 g L^{-1} and 5.27 g L^{-1}, respectively. Ethanol produced from renewable and cheap agricultural products (wheat bran) provides reduction in green house gas emission, carbon monoxide, sulfur, and helps to eliminate smog from the environment.

Keywords: wheat bran, bio-ethanol, purification, SDS-PAGE, endoglucanse, enzymatic saccharification and fermentation, simultaneous saccharification and fermentation, *Aspergillus flavus*.

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Emission of green house gases is increasing everyday with the fast depleting oil resources. Utilization of fossil fuels in the form of oil, natural gas, and coal, which modern society relies on for energy contributes significantly to global warming. Alternative fuels made from renewable resources, such as fuel ethanol, provide numerous benefits in terms of environmental protection, economic development, and national energy security. Much research is been poured in finding an alternative fuel through biological ways because of the positive environmental benefits of biofiiels. Potential feed stocks for biofuel production include the cellulosic biomass as well as waste materials, occurring in abundance outside human food chain which can be obtained throughout the year and are relatively inexpensive [1, 2].

Cellulosic materials are renewable natural biological resources and generation of biobased products and bioenergy from such substances is important for the development of humans [3]. Various industries across the world generate huge volumes of cellulosic waste which have an immense potential to be utilized for the production of several bio-products [1]. These dedicated substances provide a low-cost and uniquely sustainable resource for production of many organic fuels and chemicals that can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose of problematic solid wastes, and improve air quality [2]. There are mainly two processes involved in the conversion: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars, and fermentation of the sugars to ethanol [4]. Cellulose can be effectively hydrolyzed and depolymerized in to fermentable sugars by the enzyme cellulase. Cellulase based strategies make the process of biorefmery more economical by means of utilizing cheaper substrates for enzyme synthesis [3]. A number of microorganisms are capable of producing extracellular cellulase enzyme and among which fungi are the widely used candidates for cellulase enzyme production. Currently most commercial cellulases are produced from *Trichoderma* sp. and *Aspergillus* sp. [5]. Cellulase is a term usually used to describe a mixture of cellulolytic enzymes whose synergistic action is required for effective breakdown of substrate to its monomeric units. The action of cellulases involves the concerted action of (i) endoglucanase(s), which randomly attacks the internal, β-1,4-linkages, (ii) cellobiohydrolase, which cleaves off cellobiose units from the nonreducing ends of the glucan, and (iii) β-3-glucosidase, which hydrolyzes cellobiose to glucose [1].

The substrate usually requires a pretreatment process before being subjected to enzymatic breakdown which is aimed at increasing the susceptibility of cellulose to enzyme. Current leading pretreatment technologies include the use of dilute acid, hot water, flow through, ammonia fiber explosion (AFEX), ammonia

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recycle percolation, lime, microwave, steam explosion and "organsolv" which are intensively investigated [6, 7]. The overall performance of cellulase mixture is highly dependent on the residual lignin present along with cellulose. Cellulosic residues such as wheat straw, corn stover, rice straw, corn cobs and other agricultural wastes are the most available renewable resources which store derived energy in their chemical bonds [8].

Wheat bran is a cheap agricultural by-product. It is a rich nutrient which contains adequate amount of nutrients like proteins 1.32%, carbohydrates 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, Mg 0.17%, P 0.35%, K 0.45%, S 0.12%, various amino acids and porosity for oxygen supply. All these nutrients are necessary for production of enzymes as well as cell mass formation [9].

In this work, a study is made on the ethanol production from wheat bran which have been pretreated with dilute sulphuric acid and then subjected to enzymatic hydrolysis and fermentation and simultaneous saccharification and fermentation using purified endoglucanase and *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Organism and inoculum preparation. Fungal strains were isolated from soil of sugarcane field Coimbatore, India by dilution plate method. Culture was screened for endoglucanase enzyme production and fungal strain *Aspergillus* flavus selected for the production of endoglucanase was prepared from 4 days old slant culture.

Cellulosic materials. Wheat bran is used for the production of endoglucanase which is collected from the cattle shop in Coimbatore.

Acid pretreatment. About 100 mL of dilute sulfuric acid was prepared with a concentration range of 0.2, 0.4, 0.6, 0.8, and 1.0% in separate 250 mL Erlenmeyer flasks. The flasks were added with 5 g of wheat bran separately and autoclaved for 30 minutes. The flasks were then neutralized by washing with distilled water and the samples were dried separately for further use.

Endoglucanase enzyme production. *Aspergillus flavus* was cultured on Potato Dextrose Agar Plates incubated at 28°C for seven days for the development of spores. The basal medium used for the growth of endoglucanase production was Czapek-Dox media containing a pH of 5.6. About 200 mL of the basal medium was prepared, autoclaved for 30 minutes and seeded with a spore suspension of *A. flavus*. The flasks were incubated at room temperature for 3 days. At the end of fermentation, the supernatant was harvested by centrifugation at 10.000 rpm for 10 min (4°C) and was used as crude enzyme extract.

Purification of endoglucanase. Crude enzyme preparation obtained after cultivation of the organism under submerged fermentation in static condition was subjected to ammonium sulphate precipitation with 70% saturation. Then fraction was tested for protein content and endoglucanase activity. The fraction which showed significantly high activity were pooled and dialysed. For dialysis the enzyme preparation was filled in dialysis bag and suspended in the same buffer in which the ammonium sulphate precipitated enzyme was dissolved. Dialysed enzyme preparation obtained after ammonium sulphate precipitation was subjected to anion exchange chromatography DEAE-Sephadex G50 column which was pre-equilibrated with phosphate buffer (50 mM, pH 7.0). The column was washed first with equilibration buffer, and then bound proteins were eluted using linear gradient of 0.1–0.4 M NaCl (in phosphate buffer) at a flow rate of 20 mL per hour. The fractions (3.0 mL each) were collected and assayed for endoglucanase activity and those showing high activity were pooled, and used for SDS-PAGE analysis.

Ethanol production was carried out in two steps:

1. Saccharification of cellulosic wastes (wheat bran) by fungal enzymes, and

2. Fermentation of sugar rich hydrolysates obtained from the saccharification of cellulosic wastes by fungal strains.

Enzymatic hydrolysis of pretreated wheat bran. The reaction was carried out in 50 mM phosphate buffer (pH 7) and 5 g of each of pretreated samples were taken in separate 250 mL flasks containing 100 mL of buffer. The flasks were added 1.0 mL of the purified enzyme extract and incubated at room temperature for 3 days.

Simultaneous saccharification and fermentation of pretreated wheat bran. The reaction was carried out in 50 mM Phosphate buffer (pH 7) and 5 g of each of pretreated samples were taken in separate 250 mL flasks containing 100 mL of buffer. The flasks were added 1.0 mL of the purified enzyme extract and then fermentation was carried out as described below.

Fermentation and distillation. *S. cerevisiae* was selected for the fermentation of released sugars. Heavy inoculums of 1 mL of the yeast enriched in Saboraud's Dextrose Broth was transferred to all the flasks containing the released sugars and were kept for fermentation at 28°C for 72 hours on a shaker at 120 rpm [10]. The known volume of fermented mash was distilled. Fermented solution was heated to force the lowest boiling material into the vapor phase. The vapors were passed over the bulb of a thermometer. The vapor was condensed to a liquid in the horizontal condenser that was cooled with a flow of cold water. The distillate was collected in a receiver.

Ethanol estimation. An aliquot volume (1.0 mL) of the sample was made up to 25 mL with distilled water and distilled at 78°C until 3.0 mL of the sample was collected. The distillate (3.0 mL) was collected in a flask containing 25 mL of chromic acid and made up to 50 mL and kept in a boiling water bath at 60°C for

30 minutes. After 30 min, the contents were cooled and the color intensity was measured at 600 nm in spectrophotometer. A calibration curve was plotted using ethanol as a standard [11]. The flasks were estimated for the sugar content every 24 hours by means of DNS method.

Ethanol yield. In separate saccharification and fermentation process, while testing the sample for ethanol, the initial and final residual reducing sugar content of the sample was determined by DNS method. The difference between the initial and final reducing sugar content was interpreted as a reducing sugar utilized (RSU). The ethanol yield was then calculated by the modified formula proposed by Gunasekaran and Kamini, 1991 [12]:

$$\text{Ethanol yield (\%)} = \frac{\text{Ethanol produsedd} \times 100}{\text{Reducing sugar utilized}}.$$

Statistical analysis. All experiments were conducted with triplicates and their mean values represented. Statistical analysis was carried out by One Way ANOVA.

RESULTS AND DISCUSSION

Cellulases are inducible enzymes and their induction and activity depends on the nature of substrate [13, 14]. Major impediments to exploit the commercial potential of cellulases are the yield stability and cost of cellulase production. Therefore, research should also aim at exploiting the commercial potential of existing and new cellulase in nature. Agricultural byproducts (corn stove, wheat straw, rice straw, baggase etc.) rich in lignocellulosic biomass can be exploited as cheap raw material for the industrially important enzymes and chemicals [15]. Although, the raw materials are cheaper, pre-treatment is generally required to improve the utilizability of lignocellulosic materials and the cost is considerable. In the present study, the ethanol production was carried out by using enzymatic hydrolysis and fermentation and simultaneous saccharification (with purified fungal enzymes-endoglucanase) and fermentation (with *S. cerevisiae*) was employed for ethanol production.

Time course of enzyme production plays a very critical role in enzyme synthesis. The *A. flavus* was incubated for 1–6 days (Fig. 1). The production of endoglucanase was increased with increase in the incubation period and found maximum on 3rd day after inoculation. This is in line with the findings of Ali et al., 2008 and 2005 [16, 17] who reported that the enzyme could be harvested at about 72 hours of fermentation when the activity is highest. This phenomenon correlated with our observations.

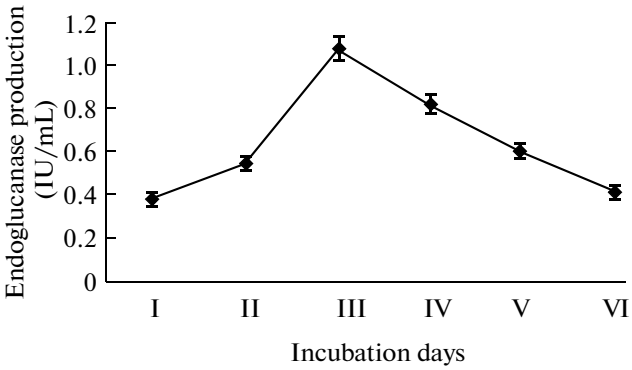

Fig. 1. Effect of incubation days on endoglucanase production. Results are mean of three independent determinations. Bars correspond to standard deviation.

After enzyme production, the enzyme was purified using three steps; 70% ammonium sulphate precipitation, dialysis and SDS-PAGE. To purify the enzyme, *Aspergillus flavus* were incubated in broth culture containing 1% of wheat bran as carbon source to induce the synthesis of the endoglucanase. The enzyme was purified to approximately 2.72 fold with a specific activity of 11.41 U mg^{-1} and a yield of 53% (Table 1 and Figure 2). The purified endoglucanase had 1.9 folds when sephadex chromatography (II step) was used for the purification of CMCase reported by Chen et al., 2004 [18]. However, the CMCase was purified using sephadex G100 and DEAE cellulose chromatography which gave 18.48 folds for *Aspergillus niger* and 17.78 folds for *Aspergillus nidulans* has been reported by Ali et al., 2008 [16]. The difference could be due to the method used to grow the fungus.

Fractions from DEAE-Sephadex G50 column which showed the highest activity were pooled and subjected to SDS-PAGE for determination of molecular weight of the protein. Purified enzyme preparation showed only one band corresponding to molecu-

Table 1. Purification and recovery of endoglucanase from *Aspergillus flavus*

Steps	Endoglucanase production (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	130	31	4.19	1	100
70% Ammonium sulphate precipitation	114	26	4.38	1.04	87.69
Dialysis	95.34	21	4.54	1.08	73.33
Sephadex G-50 Column chromatography	68.5	6.0	11.41	2.72	52.69

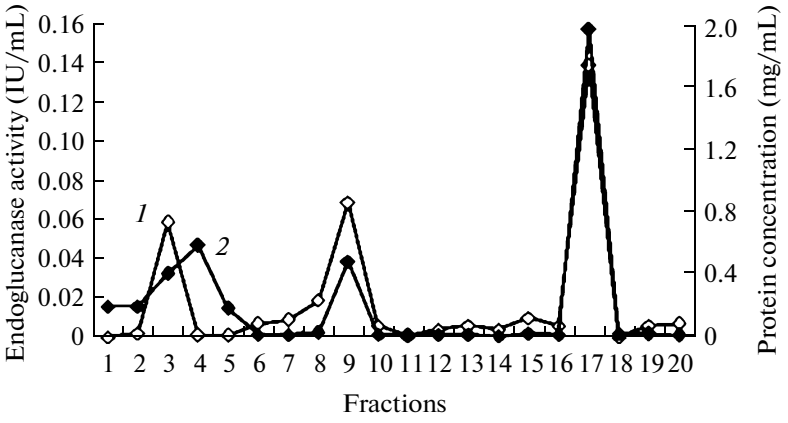

Fig. 2. Elution profile of *A. flavus* by DEAE-Sephadex G-50 column chromatography. *1*—Endoglucanase activity (IU/mL); *2*—protein concentration (mg/mL).

lar weight of approximately 27 kDa (Fig. 3). Different researchers have reported varying molecular weights of cellulases from different organisms. This value is more or less similar to Immanuel et al., 2007 [19] and Sultana et al., 1997[20] for purified cellulase of molecular weight of 36 kDa and 23 kDa for *Aspergillus* species.

Pretreatment is an important tool for practical cellulose conversion processes. It is required to alter the structures of cellulosic biomass to make more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars and to cellulase producing microorganisms. There are several ways to increase the digestibility of cellulose before it is exposed to enzyme or microbial conversion: mechanical, physical chemical or biological pretreatment, as well as the combination of these methods [21].

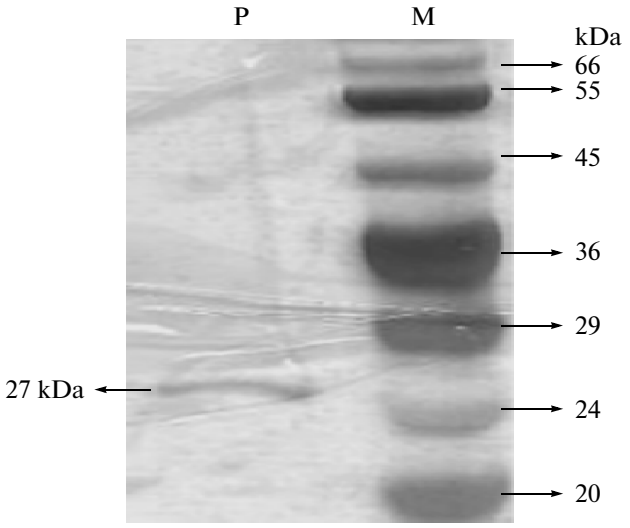

Fig. 3. SDS-PAGE analysis of *A. flavus* strain endoglucanase. M—molecular weight markers and P—purified endoglucanase band of molecular weight approximately 27 kDa.

Acid pretreatment had a greater influence on the sugar release through enzymatic hydrolysis of the wheat bran. An increase in acid severity in terms of concentration resulted in higher sugar releases and the results are presented in the Fig. 4. The figure shows that there was a gradual increase in the release of sugars when the concentration of dilute acid used for pretreatment was increased from 0.2 to 1%. The amount of sugar released with the 0.2% acid treated wheat bran was about 0.295 mg mL^{-1} when 1.0% acid was used which increased to a maximum of 0.65 mg mL^{-1} till the fourth day of fermentation in separate hydrolysis and fermentation. Figure 5 shows the amount of reducing sugar released in simultaneous saccharification and fermentation was found to be 0.523 mg mL^{-1} during the fourth day of fermentation.

Arthe et al., 2008 [1] reported the amount of reducing sugar released with the 0% acid treated cotton waste was about 55 mg mL^{-1} and 295 mg mL^{-1} when 0.5% acid was used which increased to a maximum of 400 mg mL^{-1} gradually when the acid concentration was increased up to 3%.

In this work a study is made on the ethanol production from acid pretreated wheat bran using two methods like enzymatic hydrolysis and fermentation and simultaneous saccharification and fermentation. Both steps are carried out using purified endoglucanasse and *S. cerevisiae*. Ethanol yield in hydrolysates obtained from *A. flavus* was 5.82 g L^{-1} in enzymatic hydrolysis (with purified fungal enzyme endoglucanase) and fermentation with *S. cerevisiae* (Table 2) and 5.27 g L^{-1} in simultaneous saccharification and fermentation (Table 3) during the fourth day of fermentation after that there was a gradual decrease in the ethanol production. There might be increase in saccharification over the period making glucose available to *S. cerevisae* for fermentation [22]. Enzymatic hydrolysis performed separately from fermentation step is known as separation and fermentation [23, 24].

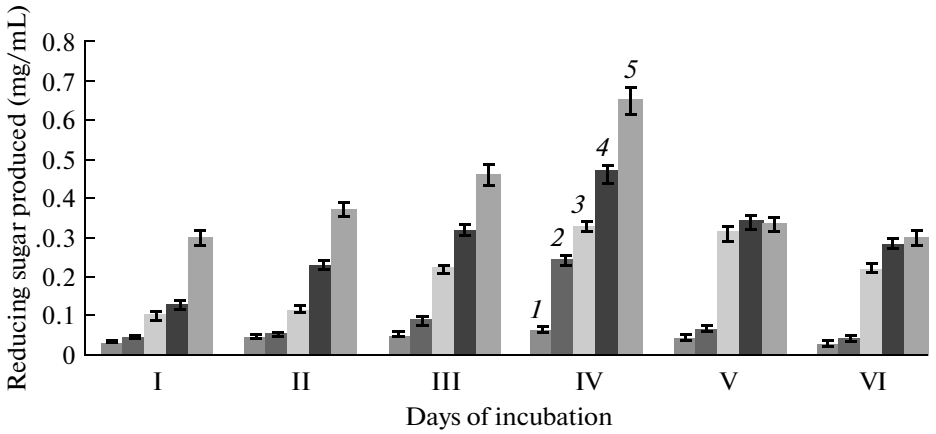

Fig. 4. Evolution of reducing sugar on enzymatic hydrolysis and fermentation. *1*—0.20%, *2*—0.40%, *3*—0.60%, *4*—0.80%, *5*—1.00%, Results are mean of three independent determinations. Bars correspond to standard deviation.

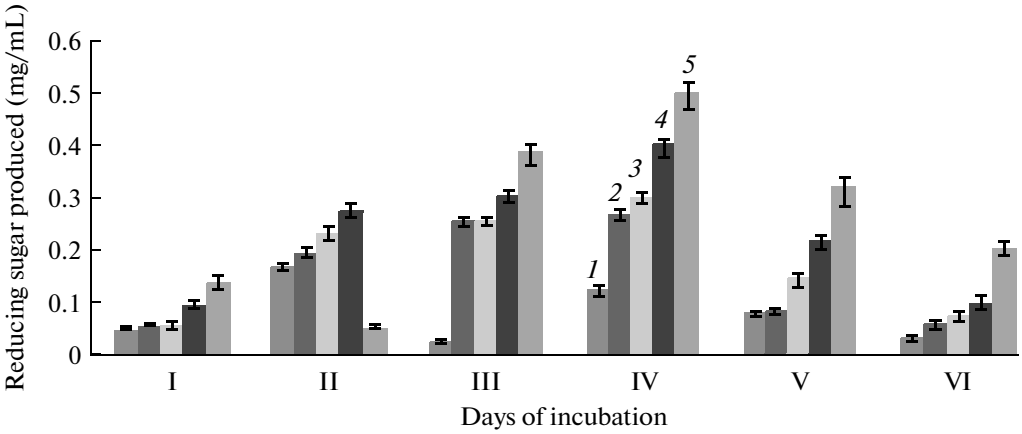

Fig. 5. Evolution of reducing sugar in simultaneous saccarification and fermentation. *1*— 0.20%, *2*—0.40%, *3*—0.60%, *4*—0.80%, *5*—1.00%. Results are mean of three independent determinations. Bars correspond to standard deviation.

Hence, from the study enzymatic hydrolysis was found to be applicable for the production of ethanol which produces 5.83 g L^{-1} from wheat bran when compared with simultaneous saccharification (5.27 g L^{-1}).

Table 2. Ethanol production from wheat bran using enzymatic hydrolysis and fermentation

Incubation days	Pretreated concentration of substrate (%)				
	0.2	0.4	0.6	0.8	1.0
	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})
I	0.50 ± 0.01^{a}	0.52 ± 0.01^{b}	1.01 ± 0.00^{b}	1.64 ± 0.00^{b}	2.82 ± 0.08^{a}
II	1.00 ± 0.00^{b}	0.98 ± 0.01^{c}	1.55 ± 0.00^{d}	2.02 ± 0.00^{c}	3.16 ± 0.00^{c}
III	1.55 ± 0.00^{d}	2.15 ± 0.00^{e}	3.62 ± 0.00^{e}	2.61 ± 0.00^{d}	4.68 ± 0.01^{d}
IV	1.83 ± 0.00^{e}	2.40 ± 0.00^{f}	4.81 ± 0.01^{f}	2.86 ± 0.00^{e}	5.83 ± 0.00^{e}
V	0.63 ± 0.05^{c}	1.22 ± 0.10^{d}	2.28 ± 0.00^{c}	1.64 ± 0.01^{b}	2.91 ± 0.00^{b}
VI	0.50 ± 0.05^{c}	0.49 ± 0.00^{a}	0.63 ± 0.07^{a}	0.76 ± 0.00^{a}	1.26 ± 0.00^{a}

Values are mean ±SD of three samples.

Table 3. Ethanol production from wheat bran using simultaneous saccharification and fermentation

Incubation days	Pretreated concentration of substrate (%)				
	0.2	0.4	0.6	0.8	1.0
	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})	EP (g L^{-1})
I	0.45 ± 0.00^{a}	0.67 ± 0.01^{b}	0.93 ± 0.00^{b}	1.14 ± 0.00^{b}	1.26 ± 0.01^{b}
II	0.76 ± 0.00^{b}	1.01 ± 0.00^{c}	1.39 ± 0.00^{d}	1.67 ± 0.00^{c}	2.48 ± 0.00^{c}
III	1.26 ± 0.01^{d}	1.64 ± 0.01^{e}	2.02 ± 0.01^{e}	2.15 ± 0.08^{d}	3.49 ± 0.00^{e}
IV	1.90 ± 0.01^{d}	3.04 ± 0.00^{f}	3.67 ± 0.05^{f}	4.05 ± 0.00^{e}	5.27 ± 0.01^{f}
V	1.00 ± 0.01^{e}	1.39 ± 0.00^{d}	1.10 ± 0.01^{c}	2.15 ± 0.01^{d}	2.53 ± 0.01^{d}
VI	0.19 ± 0.00^{c}	0.20 ± 0.00^{a}	0.76 ± 0.00^{a}	0.35 ± 0.00^{a}	1.15 ± 0.00^{a}

Values are mean ±SD of three samples. Means followed by a common superscript letter are not signifigantly different at 5 % level by using ANOVA analysis.

Values are mean ±SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using ANOVA analysis.

Krishna et al., 2001 [25] have reported ethanol yield of 2–2.5% (w/v) in 72 hours SSF of lignocellulosic wastes with thermo tolerant yeast at 10% (w/v) initial substrate concentration. Microwave alkali pretreated straw yielded 25.8 gm L^{-1} ethanol with a yield of 57.5% and alkali pretreated straw yielded 23.7 gm L^{-1} with a yield of 0.35 g g^{-1} cellulose under anaerobic conditions. Alcohol content of about 8.9 g L^{-1} was detected in the 72 hours old fermentation broth containing the hydrolyzed sample pretreated with 3% sulphuric acid has been reported by Arthe et al., 2008 [1].

Simultaneous saccharification and fermentation (SSF) was performed by Chang et al., 2000 [26] who reported the ethanol yield from pretreated switch grass, corn stover and pretreated and washed popular wood it was 72, 62 and 73% respectively, whereas from cellulose were 67 to 91% of theoretical. Zayed et al., 1996 [27] used wheat straw for the production of ethanol by SSF reported that ethanol yield was 11.8 g L^{-1} by using *T. viride* and *P. tannophilus*.

Zakpaa et al., 2009 [22] reported the mean ethanol concentration was 0.4326 g L^{-1} for corncobs using cellulase enzyme. Olofsson et al., 2008 [28] reported that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production in SSF. Also the ethanol concentration and other by-products in the broth might not have reached the inhibitory level of the yeast over the period. The highest concentration of 0.642 g L^{-1} was recorded at 24th h.

From our study the enzymatic hydrolysis and fermentation with endoglucanase from *A. flavus* with the addition of *S. cerevisiae* produced high yield of ethanol. So this enzyme may find industrial application in ethanol production. This study indicates a better solution for waste management through the utilization of wheat bran for ethanol production that could be used in the various industrial applications.

Bioethanol can be produced from a range of substrates. Bioconversion offers a cheap and safe method of not only disposing the agricultural residues, but also it has the potential to convert lignocellulosic wastes into usable forms such as reducing sugars that could be used for ethanol production. The use of agriculture wastes is a valuable contribution for ethanol production, in next future as a 2nd generation bioethanol, promoting a sustainable biofuel production and avoiding the depletion of agriculture resources, a determinant strategy for not causing a negative impact on food production. Enzymatic hydrolysis and simultaneous saccharification and fermentation for ethanol production were experimentally investigated for estimating the feasibility of using wheat bran as an alternative substrate for ethanol production. In that, the enzymatic hydrolysis produced large amount of ethanol when compared with simultaneous saccharification using purified endoglucanase.

This study revealed that agricultural waste such as wheat bran produce large amounts of ethanol production instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce ethanol. Overall, the study provides that the cellulosic waste wheat bran has a good potential to be used for production of ethanol production using *Aspergillus flavus*.

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