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Cellulase production through solid-state tray fermentation, and its for bioethanol from sorghum stover

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HIGHLIGHTS

- *Trichoderma reesei* produces high level of endoglucanase when cultivated und SF.
- Process optimizations enhance the cellulase production 3.2-fold in SSF using eesei.
- Alkali treated sorghum stover is hydrolyzed effectively by *in house* cellulase.
- Addition of *A. niger* BGL improves biomass hydrolysis by 174%.
- 80% efficiency in ethanol fermentation achieved with sorghum st r hydrolysat

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ABSTRACT

The p of cellu by *Trichoderma reesei* RUT C-30 under solid-state fermentation (SSF) on wheat br d cel se was o nized employing a two stage statistical design of experiments. Optimization ocess p meters res ed in a 3.2-fold increase in CMCase production to 959.53 IU/gDS. The process w al le in tray fermenters and yielded 457 IU/gDS using the lab conditions and indicati sibility for further improvement. The cellulase could effectively hydrolyze alkali pretreated sorghum r and addition of *Aspergillus niger* β-glucosidase improved the hydrolytic efficiency 174%, indicating potential to use this blend for effective saccharification of sorghum stover biomass. The enzymatic hydrolysate of sorghum stover was fermented to ethanol with ~80% efficiency.

1. Introduction

The e at ion of plant biomass is the most efficient metho lignocellulose breakdown to sugars, though the cost of this op n is still one of the major bottlenecks in commercialization o ond generation alcohol. Cellulase cost reduction remains the top agenda of establishments involved in bioethanol research, and several methods including onsite production, use of cheap raw materials for production of the enzyme and use of enzyme in crude concentrated form without purification are being investigated to further reduce the cost of biomass hydrolyzing enzymes – primarily cellulases. Cellulases are a group of enzymes including endo and exoglucanases and beta glucosidases that act synergistically and are necessary for the efficient hydrolysis of cellulose to soluble oligosaccharides (Beckham et al., 2010; Kubicek et al., 2009).

While cellulases are produced by various microorganisms, commercial sources of the enzyme are mostly the filamentous fungi *Trichoderma reesei* and *Aspergillus species*, mainly due to their high cellulase productivity, safe use in industry and the availability of their whole genome sequences for genetic interventions (Le Crom et al., 2009; Martinez et al., 2008). Both submerged fermentation and solid-state fermentation (SSF) have been used for cellulase production (Singhania et al., 2010). While each of these strategies has their own advantages and disadvantages, SSF is con-

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sidered as less capital intensive, easy to operate and does not require skilled manpower. Another major advantage is the ability to use cheap insoluble lignocellulosic feedstock as substrates/cheap carbon sources for enzyme production (Pandey, 2003).

Sweet sorghum is an ideal food cum bioenergy crop that can produce grain, stalk sugar, forage as well as large amounts of biomass in the order of 10–20 dry tons/ha/3.5 months (Gonsalves, 2006; Rao et al., 2007). Since it require less water compared to sugarcane and since the crop duration is less, cultivation of sorghum offers an interesting opportunity for better utilization of available arable land. With an annual surplus availability of ~1.6 million metric tons (MMT), sorghum biomass in India is a formidable feedstock resource for second generation ethanol and very less work has been done on this feedstock towards its conversion to ethanol (Pandey et al., 2009). Also there is very less information on the enzyme cocktails suitable for optimal sorghum biomass hydrolysis. Hence, the present investigation was undertaken for *in-situ* production of cellulases using *Trichoderma reesei* utilizing cheap raw material under solid-state fermentation and the enzyme was used for hydrolysis of pretreated sorghum stover to generate fermentable sugars and their fermentation to produce bioethanol.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Trichoderma reesei RUT C-30 used for the present study was a kind gift from Prof George Szakach, Technical University of Budapest, Hungary. The fungus was maintained on Potato Dextrose Agar (PDA) at 4 °C for working stocks. For inoculum preparation, 2–5 of sterile saline containing 0.05% Tween 80 was aseptically ad into fully sporulated slants (7 day old culture) and was gen pipetted up and down to dislodge the spores into the liquid. Th spore suspension thus obtained was aspirated int vials, counted under microscope using a haemocyt ter was adjusted to desired count using sterile sali ontaini 0.05% Tween 80. The spore suspension with the d was used as inoculum.

2.2. Enzyme production

Different agricultural wast sidues were uated initially as substrate in the present s y, and wheat bra as selected for production studies wit pplem ation of the added inducer-cellulose. Total of 5 g dr ight the substrate of choice or wheat bran plus cellulose was w d into -ml Erlenmeyer flasks and was moist ith n al medium (Mandels and Weber, 1969 obta the app ate initial moisture content. The flasks e ther erilized by autoclaving. Medium used for the experi h omposition in g/l $(NH_4)_2$ SO_4-1.4, K_2HPO_4-2, Ure $CaCl_2{\cdot}2H_2O$-0.3, $MgSO_4{\cdot}7H_2O$-0.3 g, Peptone -0.75, Yeast Ex -0.25, Tween 80-1.0, $FeSO_4{\cdot}7H_2O$-0.005, $MnSO_4{\cdot}7H_2O$-0.001 $nSO_4{\cdot}7H_2O$-0.0014, $CoCl_2{\cdot}6H_2O$-0.002.The pH of the medium was adjusted to 4.8 with 1 N HCl or 1 N NaOH wherever required. The flasks were inoculated with 1.0 ml of the spore suspension containing approximately 1×10^7 spores/ml or the appropriate volume as per the experiment design. The flasks were incubated at 30 ± 2 °C for the required duration as per the experiment design. Enzyme extraction was performed using 50 mM citrate buffer (pH 4.8). Fifty milliliter of the buffer was added to each flask containing 5 g of moldy bran and was incubated with shaking (200 rpm, 30 °C, 60 min). The slurry was filtered using a nylon mesh to remove debris and the filtrate was centrifuged at 7000 rpm for 10 min at 4 °C to remove fine suspended particles. The supernatant was taken as the crude enzyme, and the enzyme activity of the enzyme was determined.

2.3. Enzyme assays

2.3.1. Carboxymethyl cellulase (CMCase) activity

Endoglucanase (CMCase, endo-1,4-β-D-glucanase; EC 3.2.1.4) activity was assayed as per the IUPAC guidelines (Ghose, 1987) in a total reaction volume of 1.0 ml containing 0.5 ml of diluted enzyme and 0.5 ml of 2.0% (w/v) carboxymethyl cellulose (CMC) solution in citrate buffer (50 mM, pH 4.8). This reaction mixture was incubated at 50 °C for 30 min. Dinitrosalicylic acid (DNS) reagent (3.0 ml) was added to the reaction mixture and boiled for 5 min. Reaction mixture was diluted by adding distilled water and the absorbance was measured at 54 opriate blanks were also included which lacked eithe zyme o strate. Standards prepared with varying glucos ncentration ere run in parallel to calculate the glucose r lease. ase activ was calculated and defined as outlined l hose (1 and s expressed as international units per g dry s bstrat S).

2.3.2. Filter paper activity a

Total cellulase vity w easur using the filter paper assay according PAC (Ghos A rolled Whatman #1 filter paper stri d sion 1.0 × c (~50 mg) was used as substrate. The filter pap rips were saturated with 0.5 ml of Na-citrate b r (0.05 M, p) and 0.5 of an appropriately diluted (in N e buffer −0.0 , pH 4.8) enzyme was added to the tub nd incubated at 50 °C for 60 min. Appropriate controls were als un along h the test. After incubation, 3.0 ml of DNS reag was adde nd boiled for 5 min after which it was diluted appro ly a bsorbance was measured at 540 nm. Glucose released f rent enzyme dilutions was obtained from a simi- processed standard curve. Filter Paper Activity (FPA) was cal- c lowing the concept that 0.37 FPU of enzyme will liberate ng of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs) per gram dry substrate (FPUs/gDS).

2.4. Optimization of cellulase production

Different carbon sources and others parameters were screened in a one factor at a time approach and these were incorporated in the medium for further optimization using a two stage statistical experimental design strategy. In the first stage, a fractional factorial design was used to screen 10 process variables to identify the most significant ones affecting cellulase (measured as CMCase activity) production and in the second stage, a response surface Box Behnken design (Box and Behnken, 1960) was used to optimize the levels of these selected variables.

2.4.1. Screening of variables affecting production

The screening of important variables affecting cellulase production was performed using a Plackett and Burman design (Plackett and Burman, 1946), where 11 variables were evaluated at 2 levels (Table 1). Experiments were performed as per the design and the response (CMCase activity) was recorded. A factorial model was fitted for the main effects using the software Design Expert® (Stat-ease, USA). The effects of experimental variables on CMCase production was computed using the following equation (Eq. (1))

$$\varepsilon = \left(\sum \mu_{+} - \sum \mu_{-}\right)/n \quad (1)$$

where ε is the effect of parameter under study and "μ_+" and "μ_-"are responses (CMCase activity) of trials at the higher and lower levels of each variable respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on CMCase production. Variables

Table 1
Placket and Burman Design matrix for screening of process variables.

Run #	Cellulose (g/g)	Peptone (g/L)	Yeast Extract (g/L)	Moisture (%)	Amm Sulfate (g/L)	Lactose (g/L)	Tween 80 (g/L)	Inoculum size (spores/g)	Time (Days)	pH	Dummy	Enzyme Activity (IU/gDS)
1	0.80	3	5	65	2	20	0.25	2.00E+06	7	5.5	−1	260.59
2	0.2	3	7.5	55	2	20	1	2.00E+06	7	4.8	1	360.95
3	0.80	0	7.5	65	1	20	1	1.00E+07	7	4.8	−1	293.69
5	0.20	0	7.5	55	2	20	0.25	1.00E+07	9	5.5	−1	434.69
6	0.20	0	5	65	1	20	1	2.00E+06	9	5.5	1	237.67
4	0.20	3	5	65	2	0	1	1.00E+07	9	4.8	−1	274.53
7	0.80	0	5	55	2	0	1	1.00E+07	7	5.5	1	502.13
8	0.80	3	5	55	1	20	0.25	1.00E+07	9	4.8	1	547.28
9	0.80	3	7.5	55	1	0	1	2.00E+06	9	5.5		531.88
10	0.20	3	7.5	65	1	0	0.25	1.00E+07	7			52.94
11	0.8	0	7.5	65	2	0	0.25	2.00E+06	9		1	.03
12	0.20	0	5	55	1	0	0.25	2.00E+06	7		−1	55

affecting the production of CMCase most significantly were production.

2.4.2. Box Behnken design (BBD)

The levels of the variables identified as significant by the initial screening test were optimized for enhancing the endoglucanase yield using a response surface Box– Behnken experiment design (Box and Behnken, 1960). The variables selected for optimization, i.e., initial moisture, cellulose concentration, and inoculum size, were coded as X_1, X_2, and X_3, respectively (Table 2). A second-order polynomial (Eq. (2)) was used to represent the response as a function of the tested variables

$$Y = \beta_0 + \sum_{i=1}^{n}\beta_i X_i + \sum_{i=1}^{n}\beta_{ii}X_i^2 + \sum_{i=1}^{n}\beta_{ij}XiX_j$$

where Y is the measured response (CMCase yield), β_0, β_i, and β_{ii} the regression coefficients, and X_1–X_3 are the fa[illegible]er stud[illegible] Regression analysis and estimation of the co[illegible]cients [illegible]ere per[illegible] formed using Design Expert® software (Stat[illegible]e, USA[illegible]e contri-butions of individual parameters a[illegible]d t[illegible]d interaction effects on cellulase produc[illegible] were [illegible]rmined.

2.5. Enzyme evaluation for hydr[illegible]s o[illegible]rghum stover [illegible]d bioethanol production

2.5.1. Raw material, pre[illegible]cessin[illegible] and compos[illegible]on analyses

Sorghum stover [illegible] sam[illegible] were kindly provided by the Indian Institute of Mi[illegible]R[illegible]arch, Hy[illegible]rabad. The samples were air-dried and were mill[illegible] knife [illegible]ill to [illegible]cle size less than 2 mm and were store[illegible]air tig[illegible]containers [illegible]ntil used. Moisture content was estim[illegible]ted [illegible] use u[illegible] an infrared moisture analysis balanc[illegible]ND, Jap[illegible] Mille[illegible] biomass was pretreated by dilute acid [illegible]lkali using p[illegible]y optimized conditions and used a 10%[illegible]loading in e[illegible]r case. In the case of acid pre-treatment, 1.5% H_2[illegible] was used for 60 min at 121 °C in an auto-clave [illegible]in the case[illegible]lkali, 2% NaOH was used for 60 min at 12[illegible] in an autoclave. A[illegible] cooling, the biomass was washed with [illegible] water until the pH became neutral. The wet biomass after solid [illegible]id separati[illegible]using a nylon mesh was air dried and used for e[illegible]matic sac[illegible]rification experiments. Compositional analyses of [illegible]ntre[illegible] and pretreated sorghum stover was carried out based [illegible] NREL protocol (Sluiter et al., 2008a) and Scanning [illegible]tron microscopy of the samples were performed as described [illegible]sly (Kuttiraja et al., 2013).

Table 2
Box Behn[illegible]esign m[illegible] for optimiza[illegible]on of CMCase production by *T. reesei*.

Run No	X[illegible] (g/[illegible]	[illegible]ulum Density (Spores/g)	X_3 Moisture (%)	CMCase (IU/gDS)
1	0.8	3.00E+06	55.0	733.06
2	1.0	3.00E+06	57.5	940.95
3	1.0	3.00E+06	57.5	954.71
4	1.0	3.00E+06	57.5	956.02
5	1.2	3.00E+06	60.0	774.00
6	1.2	5.00E+06	57.5	830.16
7	1.2	1.00E+06	57.5	778.29
8	1.2	3.00E+06	55.0	774.00
9	1.0	3.00E+06	57.5	959.53
10	0.8	1.00E+06	57.5	755.23
11	1.0	5.00E+06	55.0	878.29
12	0.8	3.00E+06	60.0	723.13
13	1.0	5.00E+06	60.0	756.62
14	1.0	1.00E+06	55.0	857.18
15	0.8	5.00E+06	57.5	795.42
16	1.0	1.00E+06	60.0	794.73
17	1.0	3.00E+06	57.5	827.38

2.5.2. Enzymatic hydrolysis and ethanol fermentation

Enzymatic saccharification of biomass was performed at 10% biomass loading by incubating 2 g of pretreated biomass with the crude *T. reesei* cellulase produced under the optimal conditions derived from the experiments above. Cellulase was used at a concentration of 20FPUs/g with or without β-glucosidase (BGL supplementation at a concentration of 50 IU/g or 100 IU/g biomass. The BGL used was from *Aspergillus niger,* produced in the lab as described earlier (Singhania et al., 2011). Hydrolysis was performed in 150 ml screw capped flasks in a reaction volume of 20 ml at 50 °C, and with 100 rpm agitation in a water bath shaker to a maximum duration of 48 h. After hydrolysis, the unhydrolyzed debris was separated by filtration and concentrated by vacuum evaporation. The concentrated filtrate was filter sterilized and was inoculated at 10% v/v using a 12 h old seed culture of *Saccharomyces cerevisiae* cultivated in YPD broth (Himedia, India). Incubation was carried out for 24 h in stopper flasks at room temperature under static condition. Samples (1.0 ml) were withdrawn at 0, 12, and 24 h; were centrifuged for 10 min at 4 °C and supernatant was filtered and analyzed for ethanol by gas chromatography as described previously (Kuttiraja et al., 2013). Total reducing sugars were estimated in hydrolysates by DNS method (Miller, 1959) and glucose estimations were performed by HPLC following NREL protocols (Sluiter et al., 2008b). Sugar concentrations were expressed as mg/g of biomass. Hydrolysis efficiencies were calculated according to the following formula

$$\in = \left(\frac{\textit{concentration of sugar released}}{(\textit{cellulose conc.} \times 1.11) + (\textit{hemicellulose conc.} \times 1.12)}\right) \times 100 \quad (3)$$

where "∈" is the hydrolysis efficiency in percentage and the concentration of sugar released is the total sugar release in the hydrolysate

measured in mg/g of biomass. Cellulose and hemicellulose concentrations are expressed in g/g biomass and 1.11 and 1.12 are the standard conversion factors for calculating the amount of monomeric sugars released on hydrolysis of the respective sugar polymers.

3. Results and discussion

3.1. Selection of carbon sources for CMCase production

Cellulase production by *Trichoderma reesei* RUT C-30 under solid-state fermentation was evaluated using agricultural residues (rice straw (RS), sorghum straw (SS), sugar cane trash (SCT), wheat straw (WS), and wheat bran (WB)) as carbon sources. Among these crude carbon sources, wheat bran could induce the maximum cellulase (CMCase) activity of 298.35 IU/gDS. While the lignocellulosic substrates did induce cellulase activity, the enzyme yields were considerably lower compared to wheat bran as the substrate. Among these substrates, WS supported maximum yield of 80.65 IU/gDS whereas the enzyme yields for RS, SS and SCT were 58.2, 55.75 and 69.7 respectively. Wheat bran is a cheap carbon source routinely used as substrate for SSF and is rich in several nutrients, in addition carbon, and this could be the reason for its better performance since it can enhance cell growth and metabolism. It was selected as the carbon source for all further experiments.

3.2. Optimization of cellulase production

Screening experiments performed following the Placket and Burman design indicated considerable variation in CMCase production ranging from 237.67 IU/gds to 547.28 IU/gds (Table 1). The levels of influence of tested variables on CMCase production were computed using Eq. (1). The factors with highest effect were initial moisture content (negative), cellulose concentration (positive), and inoculum size (positive). Cellulases are induced enzymes and production of the enzyme by *T. reesei* depends on the induction mediated by the lignocellulosic substrate under the conditions. Pure cellulose is a strong inducer of cellulases and apparently a dominant factor of cellulase production (Mischof et al., 2013). Cellulose was supplemented in the production medium to have strong induction of the enzymes and the results indicated that this approach was successful. Increase in moisture content had a negative influence on cellulase production and the results confirmed to our previous observation that *Trichoderma reesei* produce more cellulase at low moisture contents under SSF (Singhania et al., 2007). *T. reesei* is a highly aerobic culture and higher moisture content is reported to reduce oxygen transfer rate through the filling of voids in the substrate with water (Mitchinson et al., 2012). Increase in inoculum concentration within tested range resulted in enhanced activity which might be attributed to higher growth rates achieved initially. The levels of initial moisture content, cellulose concentration and inoculum size was identified as most significant from the screening study (Plackett & Burman Design) and were further optimized following a Box Behnken design (BBD).

The maximum response (959.53 IU/gDS) was obtained in run 9, and in general, runs with middle levels of parameters gave higher enzyme production compared to other combinations (Table 2). Regression coefficients for the design model were determined by multiple regression analysis using the software Design Expert® v8.0 and cellulase production as represented as a function of the independent variables tested through a second-order polynomial equation (Eq. (4))

$$Y = 927.718 + 18.70X_1 + 9.38X_2 - 24.25X_3 - 104.3X_1^2 - 33.64X_2^2 - 72.37X_3^2 + 2.92X_1X_2 + 2.48X_1X_3 - 14.80X_2X_3 \quad (4)$$

where, Y is the predicted response, and X_1, X_2, and X_3 are coded values of cellulose content, inoculation size and moisture content, respectively. The quadratic regression model adopted was suggested significant by the Analysis of Variance (ANOVA) which indicated computed F value of 3.7821and a P value lower than 0.05 (Table 3). X_1^2, X_3^2 were found to be significant model terms based on their low p-values (<0.05). Response surface curves were plotted to view the interaction effects of variables and for identifying the optimal levels of each parameter for obtaining maximum yield (Fig. 1). Increase in cellulose concentration had a positive impact on cellulase production up to a concentration of about 1.0–1.1 g/5 g (20–22% w/w) of total substrate beyond which it decreased (Fig. 1A). Probably this concentration may indicate the threshold maximum attainable with the given biomass growth under the tested SSF conditions. Increasing cellulose concomitantly decreases the wheat bran content in total substrate and this could affect the amount of fungal biomass achieved since wheat bran can be used more easily by the organism. Similar to cellulose concentration, inoculum density had a positive effect till $\sim 3.5 \times 10^6$ spores/g concentration beyond which it diminished gradually. Decrease in enzyme yield at higher spore density may be attributed to decreased growth and biomass accumulation where majority of the resources are used for growth and not for cellulase production. Analysis of the response curve for moisture against inoculum density showed the same trend for inoculum concentration with the optimal concentration remaining between 3.0×10^6 and 4×10^6 spores/g dry substrate. The ideal moisture content was found to be between 55% and 57% (Fig. 1B). The parameters cellulose concentration and moisture content acted totally independently as indicated by the data in Fig. 1C. The optimal cellulose concentration was between 1.0 and 1.1 g/5 g and the moisture content between 55% and 57%. Incidentally, the maximum activity of ~950 IU/gDS was obtained at near middle levels of each of the parameters indicating that the choice of levels have been very appropriate to arrive at the optimal concentration. Many factors such as moisture content, cellulose content, inoculum size, oxygen transfer and concentrations of nutrients are known to influence growth and enzyme production under SSF, and the selection of optimum level for these parameters can lead to increase in enzyme production under SSF. *Trichoderma reesei* produced 298.35 IU/gds SSF on wheat bran in the basal medium and under the optimal combination of parameters the enzyme titers had gone up 3.2-fold higher to 959.53 IU/gDS. This indicated that SSF may be used for cellulase production from plant biomass. In this study, initial moisture content had the largest influence on production of CMCase. Different moisture ranges (37.56–80%) have been used for cultivation of *T. reesei* for production of the enzyme (Kilikian et al., 2014; Singhania et al., 2007). In the current study 57% was found to be optimal for enzyme production. Under the optimal conditions of moisture content, cellulose concentration and inoculum size, the ideal incubation time was found to be 9.0 days. Filter paper activity was determined for the enzyme produced under optimal conditions and it was found to be 22.37 FPUs/gDS.

3.3. Tray fermentation for scaled up cellulase production

Solid-state fermentation on production scales are usually performed in trays and inside climate controlled chambers called 'Koji rooms'. Initial evaluation of the larger scale production of enzymes through SSF using the *T. reesei* culture was attempted in 50 g capacity trays in the Pilot scale SSF facility (Koji Room) of CSIR NIIST with relative humidity and temperature maintained at 80% and 30 ± 2 °C respectively. Fifty grams of total dry substrate containing 20% cellulose was mixed well and moistened with the production medium according to the conditions optimized in flask cultivation. Inoculation was carried out with spore inoculum

Table 3
ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	DF	Mean of Square	F Value	p-value Prob > F
Model	88543.35	9	9838.15	3.78	0.0466
A-Cellulose	2797.89	1	2797.89	1.08	0.3342
B-Inoculum density	704.25	1	704.25	0.27	0.6189
C-Moisture	4706.93	1	4706.93	1.81	0.2205
AB	34.11	1	34.11	0.01	0.9121
AC	24.65	1	24.65	0.01	0.9252
BC	876.75	1	876.75	0.34	0.5797
A^2	45804.39	1	45804.39	17.61	0.0041
B^2	4765.62	1	4765.62	1.83	0.2180
C^2	22052.43	1	22052.43	8.48	0.0226

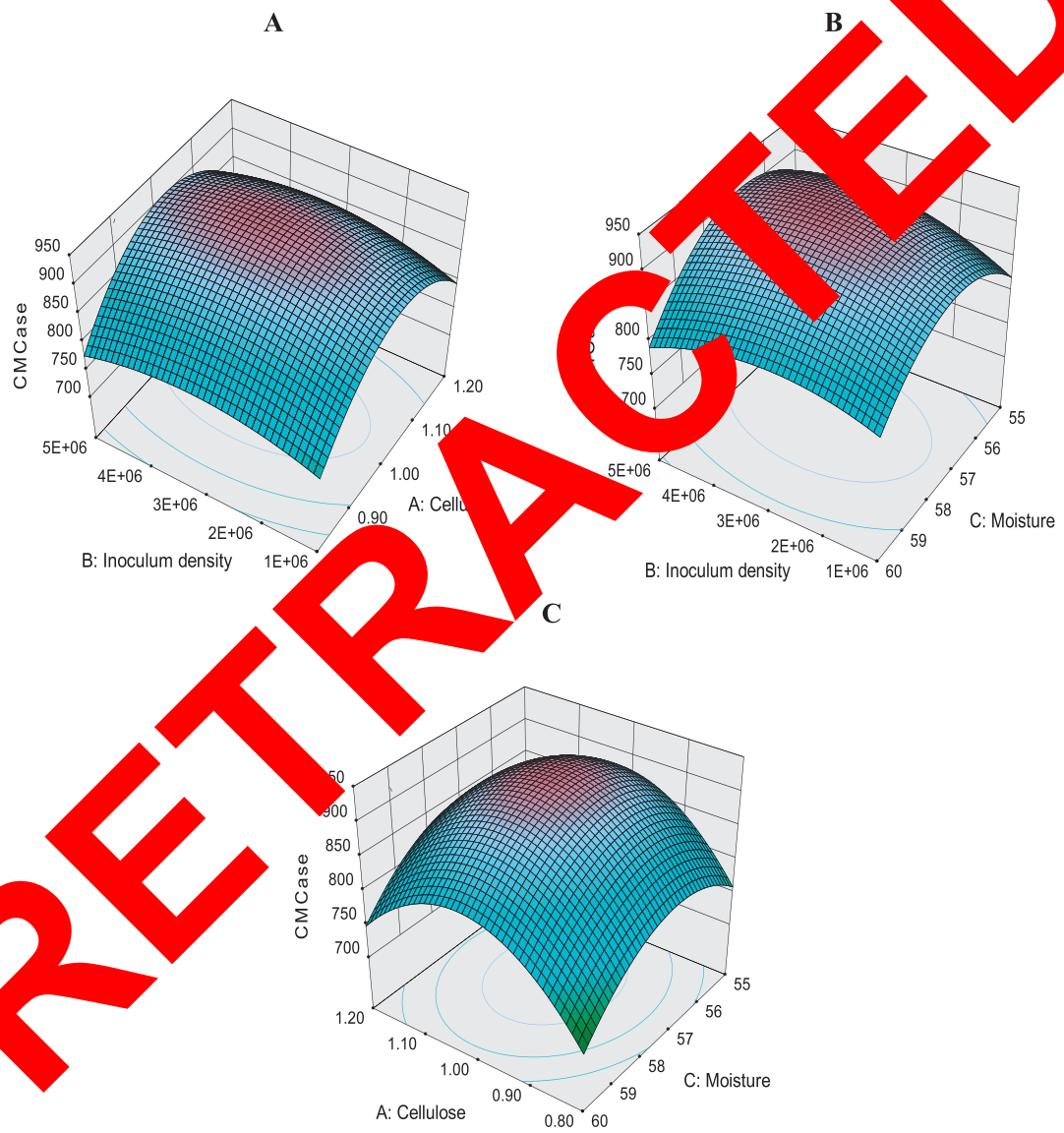


Fig. 1. Response surface plots showing the interaction effects of process variables on production of cellulase by *T. reesei.* A) Interaction of inoculum density and cellulose concentration B) Interaction between inoculum density and moisture content C) Interaction between cellulose and moisture content.

prepared with the optimized concentration and fermentation was carried out till 14 days when mycelial growth was visible and sporulation set in; the conditions when maximum cellulase production was observed in flask fermentation. Enzyme extraction and assay indicated that the yield as almost half (457.27 ± 24.7 IUs/gDS) of what was obtained in flasks, though there was no contamination. It was observed that the trays were warm due to metabolic heat and the mycelial growth was lesser in the centre of trays. It is speculated that the heat and oxygen transfer could be limiting and more studies and optimization in trays have to be performed to obtain the same levels of production as obtained in flasks. It has been demonstrated that there could be very significant temperature gradients with increase in substrate packing height, properties of the substrate, moisture of the atmosphere in

which the trays are incubated etc (Chen, 2013). It is therefore required to perform detailed studies on energy and water balances to determine the appropriate scale up criteria (Figueroa-Montero et al., 2011). Nevertheless, the results seems promising, since the production is still higher than base level and even after performing the experiments in a semi sterile atmosphere, there was no contamination and about 450 IUs/gDS of enzyme was obtained from the system, indicating scope for large scale production of the enzyme using SSF.

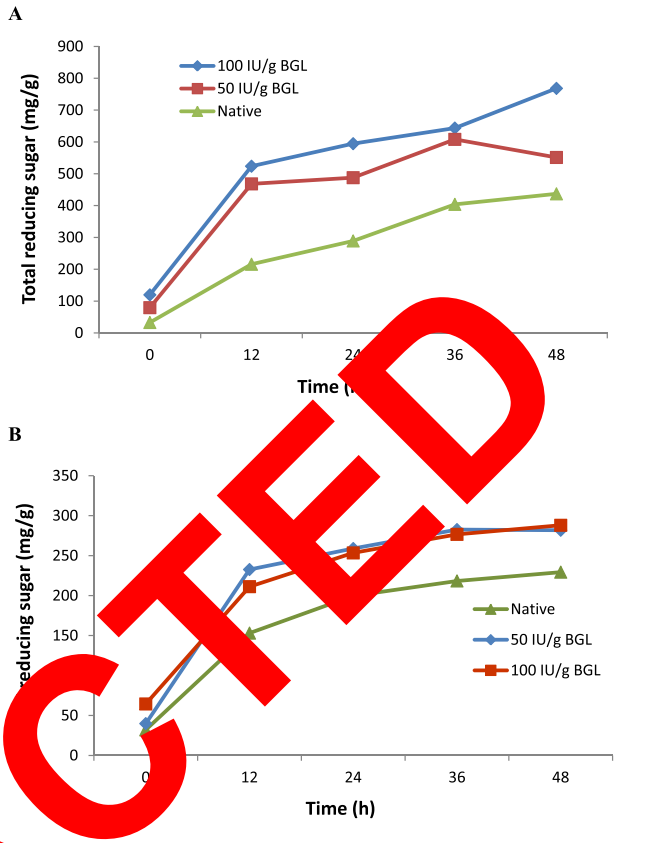
3.4. Hydrolysis of sorghum stover using in-situ produced T. Reesei cellulase and fermentation of the hydrolysate to ethanol

Chemical composition of biomass varies with respect to several parameters and depending on the method of pretreatment. Also the composition of the biomass is essential to estimate the efficiencies of hydrolysis and for calculation of the potential yield of bioethanol. The composition of biomass was analyzed experimentally for the native sample as well as for dilute acid and alkali pretreated sorghum stover. As expected, dilute acid pretreatment removed more of hemicellulose by its break down while alkali pretreatment removed more of lignin (Table 4). While the improvement in cellulose content was similar with all the different pretreatments (From 40% in Native to ~50% in the pretreated samples), the major differences were in the removal of lignin and hemicellulose. While acid pretreatment actually resulted in an increased final percentage of lignin, there was a considerable hemicellulose removal with this treatment. On the contrary, alkali removed more of lignin and the final lignin percentage was 7.36%.

The efficiency of the SSF produced *T. reesei* cellulase for hydrolysis of pretreated sorghum stover was evaluated with and without supplementation of *in-house* produced *Aspergillus* BGL at 50 or 100 IU/g biomass concentration. Cellulase was added at FPUs/g biomass in all the hydrolysis experiments. Fig. 2A & B indicate the sugar yields obtained for hydrolysis employing the cellulase for acid and alkali pretreated sorghum stover respectively. Hydrolytic efficiencies were lower for acid pretreated biomass compared to alkali pretreated in all enzyme combinations attempted. The conversion efficiency was about 25% of theoretical maximum in the case of native unblended *T. reesei* cellulase for acid pretreated SS. With addition of either 50 or 100 IUs of BGL per gram biomass, the hydrolytic efficiency improved to 30% of theoretical maximum. The situation was totally different in the case of alkali pretreated biomass. The native *T. reesei* cellulase had an efficiency of 40% which with supplementation of 50 or 100 IUs/g BGL to the cellulase, efficiencies improved significantly to almost 60% and 82% respectively indicating excellent synergy between the enzymes. Apparently, the type of pretreatment had influenced the hydrolysis efficiencies significantly, since the acid pretreated material was hydrolyzed poorly. This is speculated to be due to the poor removal of lignin in dilute acid pretreatment and its re-deposition on the biomass preventing enzyme access a phenomenon which is already reported (Selig et al., 2007). With a better pretreated sample, the hydrolysis efficiencies were superior and with BGL supplementation it improved to 82%. *T. reesei* is known to produce very less of BGL enzymes and BGL blending

Table 4
Composition analysis of native and pretreated sorghum stover.

	Cellulose	Hemicellulose	Lignin
Native	39.58	20.15	21.72
1.5% Acid	50.4	9.20	25.85
2% NaOH	57.16	27.16	7.36



Hydrolysis of sorghum stover biomass by native and BGL blended *T. reesei* cellulase. A) Enzymatic hydrolysis of acid pretreated SS B) Enzymatic hydrolysis of alkali pretreated SS.

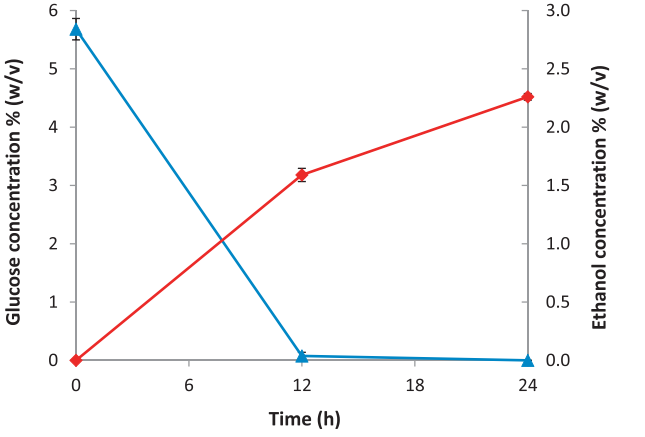


Fig. 3. Bioethanol production using sorghum stover hydrolysate.

is known to improve its hydrolytic potential (Chir et al., 2011; Hu et al., 2011). Apparently, with BGL blending the SSF produced *T. reesei* cellulase is an effective way for improving its hydrolytic efficiency. With the results, it becomes clear that the strategy works well with sorghum biomass as well.

Ethanol production from the enzymatic hydrolysate of alkali pretreated sorghum stover was studied using a lab strain of *Saccharomyces cerevisiae*. The hydrolysate generated using *T. reesei* cellulase supplemented with 100 IU/g of *A niger* BGL contained 56.67 g/L of glucose after concentration. Almost entire glucose was consumed in about 12 h duration and the maximal ethanol yield of 22.2 g/L was obtained in 24 h (Fig. 3) which corresponded to

76.8% conversion efficiency. This proved that the hydrolysate is efficiently fermented by a standard yeast strain.

The current work has addressed onsite production of cellulase and its use for hydrolysis of sorghum stover biomass which is one of the important but under exploited feed stock resources in India. Fermentation process for cellulase production has been demonstrated at pilot scale and the enzyme was successfully used for biomass hydrolysis. Onsite enzyme production reduces the enzyme cost due to the savings on logistics, storage and the stabilization of enzyme and this in turn is expected to reduce the cost of bioethanol from biomass.

4. Conclusions

SSF was used for cellulase production employing *T. reesei* and by optimizing process variables a 3.2-fold increased yield was obtained. The process was demonstrated at pilot scale in tray fermenter system. Supplementation of *A. niger* BGL to the cellulase improved its hydrolytic efficiency 174% indicating synergy between these enzymes. Sorghum stover hydrolysate was efficiently fermented to ethanol. Results indicate the potential of using on-site enzyme production using SSF, and its use for sorghum stover hydrolysis and ethanol production.

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