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Enhanced cellulase producing mutants developed from heterokaryotic *Aspergillus* strain

Baljit Kaur [a], H.S. Oberoi [b], B.S. Chadha [a,*]

[a] Department of Microbiology, Guru Nanak Dev University, Amritsar 143 005, India
[b] Central Institute of Post Harvest Engineering Technology (CIPHET), Ludhiana 141 004, India

HIGHLIGHTS

- Cyclic mutagenesis and rational screening resulted in cellulase hyper-producing mutants.
- Genome and Proteome based profiling for characterization of developed mutants.
- Selected mutant strains were evaluated for cellulase production under shake flask and SSF.
- Saccharification potential of the developed cellulases evaluated using alkali treated rice straw.
- A sequential approach for production of XOS and ethanol from alkali treated rice straw developed.

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ABSTRACT

A heterokaryon 28, derived through protoplast fusion between *Aspergillus nidulans* and *Aspergillus tubingensis* (Dal8), was subjected cyclic mutagenesis followed by selection on increasing levels of 2-deoxy glucose (2-DG) as selection marker. The derived deregulated cellulase hyper producing mutant '64', when compared to fusant 28, produced 9.83, 7.8, 3.2, 4.2 and 19.74 folds higher endoglucanase, β-glucosidase, cellobiohydrolase, FPase and xylanase, respectively, under shake cultures. The sequence analysis of PCR amplified β-glucosidase gene from wild and mutant showed nucleotide deletion/substitution. The mutants showed highly catalytic efficient β-glucosidase as evident from low K_m and high V_{max} values. The expression profiling through zymogram analysis also indicated towards over-expression of cellulases. The up/down regulated expressed proteins observed through SDS–PAGE were identified by Peptide mass fingerprinting The cellulase produced by mutants in conjunction with cellulase free xylanase derived from *Thermomyces lanuginosus* was used for efficient utilization of alkali treated rice straw for obtaining xylo-oligosaccharides and ethanol.

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1. Introduction

Cellulase is a multi-component enzyme comprising of endoglucanase (EC 3.2.1.74), which attack cellulose in amorphous zone and release oligomers, cellobiohydrolase (EC 3.2.1.91), that liberate cellobiose from reducing and non-reducing ends, and β-glucosidase (EC 3.2.1.21), which hydrolyze cellobiose to glucose (Kim et al., 2007) and play a key role in avoiding cellobiose inhibition and thus enhancing the hydrolysis rates of cellulose into glucose (Gao et al., 2012). One of the most important applications of cellulase lies in the bioconversion of lignocellulosic substrate where they are important for bringing out the hydrolysis of pretreated lignocellulosic material to glucose for its subsequent fermentation to ethanol (Xu et al., 2011). In addition cellulase as crude or mono-component enzymes are widely used in textile industry for biostoning, in food industry for clarification of fruit and vegetable juices, improvement in feed digestibility, deinking of waste paper (Sukumaran et al., 2005; Soni et al., 2008). Many fungal strains, *Chrysosporium* sp., *Fusarium* sp., *Sclerotium* sp., *Phanerochaete* sp., *Aspergillus* sp. (Sun and Cheng, 2002) are known to express a wide array of cellulases that differ in their protein composition of complex glycosyl hydrolase mixtures that also correlates with the specific activity of individual enzymes present within the cellulase preparations (Hinz et al., 2009; Chundawat et al., 2011). The production of cellulase by wild type strains is very low, whereas commercialization of these enzymes demands microorganisms with improved activity and better resistance to product inhibition (Cheng et al., 2009). The improvement in the activities and desirable enzyme traits can be obtained through random/site directed mutagenesis and selection. The workers have reported the significant improvement in cellulase

* Corresponding author. Address: Department of Microbiology, Guru Nanak Dev University, Amritsar, Punjab, India. Tel.: +91 183 2258802x3317; fax: +91 183 2258820.
E-mail addresses: baljit_sohal@rediffmail.com (B. Kaur), hari_manu@yahoo.com (H.S. Oberoi), chadhabs@yahoo.com (B.S. Chadha).

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activity in *Acremonium cellulolyticus* (Fang et al., 2009), *Penicillium decumbens* (Liu, 2013) and *Trichoderma citrinoviride* (Chandra et al., 2009). Anti metabolites (2-DG, amphotericin B, benomyl) have been reported for screening and selection of de-repressed hyperproducing mutants (Rubinder et al., 2000; Olejnikova et al., 2010). In addition, genome reshuffling, protoplast fusion and recombinant protein expression technology have also been shown to be great potential for the enhancement of cellulase production (Cherry and Fidantsef, 2003; Savitha et al., 2010; Kaur et al., 2013). In the present work a heterokaryon 28, generated through inter-specific protoplast fusion carried out between *Aspergillus nidulans* and *Aspergillus tubingensis* showing improved cellulase production (Kaur et al., 2013), was taken up for further improvement through cyclic mutagenesis. The selected mutants were characterized at morphological, genomic and proteomic level. Based on rational screening and selection strategies, the deregulated mutants were evaluated for production of cellulases (endoglucanase, β-glucosidase, cellobiohydrolase, FPase) and xylanase under shake flask and solid state fermentations. The cellulases derived from the mutants were also evaluated for the bioconversion of alkali treated rice straw into fermentable sugars and xylo-oligosaccharides (XOS) using a two step approach.

2. Methods

2.1. Microorganism and culture media

All strains were maintained on Yeast Potato Soluble Starch (YPSs) of following composition (% w/v), Starch 1.5, Yeast extract 0.4, KH_2PO_4 0.2, K_2HPO_4 0.23, $MgSO_4.7H_2O$ 0.05, Citric acid 0.057 and Agar 2.0. Cultures were grown for 7 days at 40 °C and then stored at 4 °C.

2.2. Cyclic mutagenesis

In the first step, a spore suspension (1×10^7/ml) of fusant 28, was exposed to ultraviolet (UV) radiation for 45 min, using a Philips germicidal lamp (30 W) emitting primarily at 254 nm, from a distance of 20 cm (Chadha et al., 2005). Treated spores were suitably diluted and plated on YPSs medium containing sodium deoxycholate (0.025% w/v) as a colony growth restrictor. Plates were then incubated in the dark at 40 °C for 4 days. To select for deregulated mutants, the survivors were inoculated on YPSs medium containing 0.5% (w/v) 2-deoxy-D-glucose (2-DG) as a selectable marker. For next cycles of mutagenesis combined chemical and physical treatment was induced in which spore suspension was treated with N-methyl-N^0-nitro-N-nitrosoguanidine(MNNG) (Himedia) at a concentration of 350 μg/ml prepared in 0.5 M/L Tris-buffer (pH 7.0) for 10 min followed by UV treatment for 10 min. The spore suspension was then washed with sodium phosphate buffer (0.05 M; pH 5.5) twice and was then serially diluted and plated on YPSs medium with increasing concentration of 2-DG as selective pressure. The levels of 2-DG in selective medium contained 1.0, 1.5 and 2.0% (w/v) after 2nd, 3rd and 4th cycle of mutation respectively.

2.3. Enzyme production under shake flask culture

The 2-DG resistant mutants were screened for cellulase production employing shake flask cultures in Erlenmeyer flasks (250 ml) containing production medium (50 ml) of following composition (% w/v); Corn cob, 5.0; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 2.4; $MgSO_4$, 0.120; Sodium potassium tartrate, 0.563; urea, 0.4, tween-80, 0.1; $ZnSO_4$, 0.001; $MnSO_4$, 0.001 and $CuSO_4$, 0.001 (pH, 4.0). The culture medium was inoculated with 2 ml spore suspension (10^7 spores/ml) and incubated at 40 °C for 7 days at 150 rpm in orbital shaker. The resultant culture supernatants were centrifuged at 8000*g* for 10 min and used for enzymatic assay.

2.4. Enzyme production under solid substrate culture

The selected 2-DG resistant mutants were also evaluated for cellulase production under solidified medium using sorghum straw (5 g) as carbon source and basal medium (15 ml) of the following composition: KH_2PO_4 0.4%, CH_3COONH_4 0.45%, and $(NH_4)_2SO_4$ 1.3% (pH 7.0). The production medium was inoculated with 2 ml of spore suspension (10^7 spores/ml) and incubated for seven days at 40 °C. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and kept at 40 °C for 1 h under mild shaking. The resultant slurry was filtered through muslin cloth and centrifuged at 8000*g* for 10 min, and the extracts were used for enzymatic assay.

2.5. Estimation of enzyme activities

The endoglucanase (EG) and xylanase activities were determined using carboxymethyl (CM)-cellulose (1% w/v) and birchwood xylan (1% w/v) prepared in 50 mM sodium citrate buffer (pH 6.0). The reaction mixtures (1 ml) containing equal amounts of suitably diluted enzyme and substrate at 50 °C for 10 and 5 min respectively. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS) followed by boiling (Miller, 1959). The color developed was read at 540 nm using Novospec II spectrophotometer (Pharmacia). The amount of released sugars was quantified using glucose and xylose standards respectively. One unit of endoglucanase (CMCase) and xylanase activity was defined as 1 μmol of glucose and xylose equivalent liberated per minute under assay conditions respectively. Total cellulase activity (Fpase) was measured by incubating 1 ml mixture containing a strip of filter paper (Whatman No. 1, 1 × 6 cm) as substrate, 0.5 ml sodium citrate buffer (50 mM, pH 6.0) and 0.5 ml of suitably diluted enzyme at 50 °C for 60 min. The reaction was stopped by adding 3 ml DNS, followed by addition of 20 ml distilled water in each test tube, as described by Wood and Bhat (1988). β-glucosidase and cellobiohydrolase (CBH) were assayed using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) and *p*-nitrophenyl-β-D-lactopyranoside as respective substrate by the micro titer plate method (Parry et al., 2001). A reaction mixture of 100 μl containing 25 μl of enzyme, 25 μl of substrate (10 mM) and sodium acetate buffer (50 mM, pH 5.0) was incubated at 50 °C for 30 min. The reaction was terminated by addition of 100 μl of NaOH-glycine buffer (0.4 M, pH 10.8), and the developed yellow color was read at 405 nm using an ELISA Reader (BioRad). The amount of *p*-nitrophenol released was quantified using the pNP standard. One unit of β-glucosidase and CBH activity was expressed as the amount of enzyme required to release 1 μmol of pNP per minute under assay conditions respectively.

2.6. Kinetic studies

The Michaelis–Menten kinetic parameters (K_m and V_{max}) were determined against *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate, to detect catalytic efficiency of the developed strains, using Lineweaver–Burk plot.

2.7. Molecular characterization

DNA of parental and mutant strains was extracted and amplified for β-glucosidase region using primers *bgl*1F (5′-AGGGGTA ATAGGGAGGGGAG) and *bgl*1R (5′- GTCGATCAGCATACCGGACC).

The PCR products were sequenced (Genei, Bangalore, India) and aligned using Clustal X software.

2.8. SDS–PAGE and activity staining

The culture extracts of the mutants grown under shake flask culture were resolved by SDS–PAGE. Prior to loading on polyacrylamide gel, the protein sample with sample buffer was boiled for 5 min for denaturation and on completion of electrophoresis the gel was stained by using silver staining solutions. For developing zymogram of endoglucanase, enzyme extracts were subjected to 10% SDS–PAGE containing 0.1% CMC polymerized within the gel matrix. Following electrophoresis, the gel was washed once for 20 min in 20% (v/v) isopropanol prepared in phosphate buffer saline (PBS), pH 5.8, followed by three washes (20 min each) in PBS. The gel was incubated at 55 °C in PBS for 1 h and then stained with Congo red (1 mg/ml) for 30 min. Bands corresponding to endoglucanase appeared as clear zone against a dark background after destaining with 1 M NaCl followed by treatment with 10% (v/v) acetic acid solution (Kenneth et al., 2006). The β-glucosidase and CBH activity in gels was detected by developing each zymogram against 10 mM 4-methylumbelliferyl-β-D-glucoside and 4-methylumbelliferyl-β-D lactopyranoside (Sigma) as substrates prepared in sodium acetate buffer (50 mM, pH 5.0) respectively. Upon completion of electrophoresis, the gels were incubated in renaturation buffer containing 20 mM piperazine-N,N-bis (2-ethanesulphonic acid), 2.5% Triton X-100, 2 mM Dithiothreitol (DTT), and 2.5 mM $CaCl_2$ for 1 h at room temperature and held overnight at 4 °C in fresh refolding buffer. Then the gels were washed with distilled water thoroughly and incubated in sodium acetate buffer (50 mM, pH 5.0) for 15 min. and immersed in respective substrates and the β-glucosidase and CBH bands in the gel were detected immediately under UV light using gel documentation system (Gene Genius, Cambridge, UK).

2.9. In gel digestion and peptide mass fingerprinting

For in gel digestion, the visible bands from the stained gels were excised and destained in 500 µl of 50% acetonitrile/50% 100 mM ammonium bicarbonate for overnight. 200 µl acetonitrile was added for 5–10 min while vortexing and repeated until the gels slices were fully dehydrated and turned white. After drying the gel slices for 5–10 min, digestion was carried out using 20 ng/ml solution of modified porcine trypsin (Promega) in 500 mM ammonium bicarbonate. 20 µl of the trypsin solution was added and allowed to rehydrate for 30 min on ice. After rehydration 20–30 µl of 500 mM ammonium bicarbonate solution was added followed by incubating the rehydrated gel slices at 37 °C overnight for digestion. Remove the supernatant in the fresh tube followed by double extraction with 50 µl of 50% acetonitrile/0.1% triflouroacetic acid (TFA) for 15 min. The final pool of peptides was mixed and vacuum evaporated, but not to complete dryness and was then loaded into nano capillary column fitted in Applied Biosystem 4800 MALDI TOF-TOF plus (Biotech Centre, Delhi University, New Delhi).

2.10. Enzymatic hydrolysis and fermentation

The potential of enzyme extracts produced by wild and mutant strains grown under shake flask culture and solid substrate culture were also evaluated for saccharification and fermentation of alkali treated rice straw (1% NaOH) at 15% (w/v) substrate loading rate. Hydrolysis was carried out at 50 °C for 96 h under shaking conditions (160 rpm). The released reducing sugars were quantified using DNS method (Miller, 1959). The resultant hydrolysate was further fermented using a yeast co-culture *Saccharomyces cerevisiae* and *Pichia stipitis* which were activated in glucose yeast extract broth for 48 h at 30 °C and used as inoculum at 4% (v/v) each. The fermentation was carried out at 30 °C for 96 h and produced ethanol was estimated by using spectrophotometric method (Caputi et al., 1968). One ml of fermented sample was taken in 500 ml Pyrex distillation flask containing 30 ml of distilled water. The distillate (20 ml) was collected in a 100 ml volumetric flask containing potassium dichromate (25 ml) solution and flask and was then incubated in a water bath at 62.5 °C for 20 min. The absorbance of the developed color was read at 600 nm using Novaspec II spectrophotometer (Pharmacia). The amount of ethanol produced was quantified using ethanol standard. The resultants products obtained after saccharification and fermentation were also analyzed through High Pressure Liquid Chromatography (HPLC, DIONEX, USA) equipped with a ultimate 3000 RS Pump, and a differential refractive index detector (RI-101, SHODEX). The Aminex column HPX-87P (Bio-Rad) was maintained at 85 °C with water as a mobile phase at a flow rate of 0.6 ml/min^{-1}.

A two step enzymatic process for efficient utilization of hemicellulose and cellulose fractions present in alkali treated rice straw was also developed. In the first step hemicellulose fraction was hydrolyzed by cellulase free xylanase (2800 U/ml) produced by thermophilic strain *Thermomyces lanuginosus* under shake flask culture using corn cob as carbon source (Sonia et al. 2005). For hydrolysis 300 Units of enzyme/g alkali treated rice straw in 50 mM sodium citrate buffer (pH 6.0) was used at substrate loading rate of (15% w/v). The reaction mixture was incubated at 50 °C with rotation (160 rpm) for 96 h. The released reducing sugars were quantified using DNS method and also analyzed through HPLC. The residual cellulose enriched fraction recovered after xylanase treatment was further hydrolyzed with cellulase derived from commercial cellulase complex containing 17.5 FPU (NS22086, Novozymes) supplemented with 35 CBU of β-glucosidase (NS22118, Novozymes) and the developed mutant 'A' containing 10.9 FPU followed by fermentation by *S. cerevisiae*. The saccharified and fermented products were quantified spectrophotometrically as well as analyzed through HPLC.

3. Results and discussion

A fusant 28, derived through protoplast fusion carried between *A. nidulans* and *A. tubingensis* (Dal8) that showed 1.76 and 1.50 folds higher endoglucanase and β-glucosidase production when compared to *A. tubingensis* parental strain (Kaur et al., 2013), was selected as parent strain for developing deregulated hyper cellulase producers through cyclic mutagenesis and rational screening program. Fusant 28 showed resistance to 0.2% 2-DG (w/v) and was used as selectable marker for primary screening of mutants (Chadha et al., 2005).

After first cycle of UV mutagenesis, fifty mutants were randomly picked on YPSs medium containing 0.5% 2-DG (w/v). Among these only eight mutants were 2-DG resistant that were further cultured under submerged (shake flask) and SSF to evaluate their cellulase production ability. The results in Table showed that mutant 25 M produced comparatively higher levels of cellulase when compared to fusant 28 and other selected mutants. Mutant 25 M was further subject to combined chemical (MNNG) and physical (UV) mutagenic treatment and the survivors were picked on YPSs medium containing 1.0% 2-DG. Upon comparing the potential of these mutants for cellulase production, mutant '8' and '30' were found to produce high levels of cellulase under submerged and SSF cultures, respectively. The third cycle of mutagenesis of '8' followed by screening lead to selection of mutants 'O' (shake flask) and 'A' (SSF) that were characterized as partially deregulated and resistant to 1.5% 2-DG. Further mutagenesis of mutant "O" resulted in generation of mutant '64' which was resistant to presence of

2.0% 2-DG and produced high levels of cellulase under shake cultures.

Characterization of mutants: The parent strain '28' obtained through inter-specific fusion (Kaur et al., 2013) produced blackish green colonies on YpSS agar plates. It was observed that mutants generated through UV treatment (first generation) were morphologically similar to parental strain (blackish green), whereas exposure of spores to MNNG in subsequent mutagenesis produced developmental and colored mutants different from wild type as also observed previously (Chadha et al., 2005). The mutants obtained after 2nd and 3rd cycle of mutagenesis were characterized as morphological producing sparsely black sporulation (mutant '30') to developmental non sporulating mutants '24', '23' and 'J' that showed white colonies. Whereas, mutant '31' showed yellow color sporulation and slow growing colonies. The developmental or spore color variation in mutants that has been attributed to defect in cell wall biosynthetic pathway has been reported previously in amylase producer *Thermomyces lanuginosus* strains (Chadha et al., 2005).

The results in Tables 1 and 2, show the genealogy and cellulase producing capabilities of selected mutants under shake flask and SSF, respectively. The mutagenesis and selection approach caused gradual and significant sequential improvement in the cellulase production when compared to parent strain. The fusant (28) was found to produce 1.23, 20.3, 1.07, 0.187 and 24.23 (units/ml) of EG, β-glucosidase, CBH, FPase and xylanase, respectively under shake flask cultures. Whereas, mutant "64" developed after four cycles of mutagenesis produced 12.1, 159.4, 3.52, 0.787 and 478.4 (units/ml) of respective enzyme activities corresponding to 9.83, 7.8, 3.2, 4.2 and 19.4 folds increase in EG, β-glucosidase, CBH, Fpase, and xylanase when compared to the parental strain 28. Interestingly mutants 25 M, 8, O and were partially deregulated and produced high levels of constitutive cellulase in the presence of glucose as sole carbon source (data not shown). Similar approaches of cyclic mutagenesis have been employed for improving cellulase production in *Penicillium strains* by repeated mutagenesis (Dillon et al., 2006; Adsul et al., 2007; Liu, 2013), however the observed levels of improvement in enzyme titres in this study are much higher.

The production of cellulase was also tested under SSF using sorghum straw as carbon source. The mutagenesis and selection resulted in distinct genealogy when compared to those obtained during screening under shake flask cultures (Table 2). The selected mutants in the second and third cycle of mutagenesis i.e., mutants '30' and 'A' respectively were different from those selected from shake flask studies. The fusant '28' produced 125.8, 892.7, 29.8, 8.46 and 1423.6 (units/g dry weight substrate) of EG, β-glucosidase, CBHI, FPase and xylanase, respectively, whereas mutant 'A' produced 243.7, 1114.3, 65, 10.9 and 3476.9 (units/g dry weight substrate) of EG, β-glucosidase, CBHI, FPase and xylanase corresponding to 1.93, 1.24, 2.1, 1.2 and 2.4 folds increase in activity when compared to parent strain (Table 2). Similarly, Xu et al. (2011) also used SSF for the evaluation of cellulase production of the selected mutants of *Trichoderma reesei*.

Besides improving cellulase production capability mutagenesis also resulted in improved catalytic efficiency. To illustrate this point, we analyzed kinetics of β-glucosidase produced by parent and mutant strains. The results in Table 3 based on Line-weaver-Burk plot clearly suggest higher rate of catalysis of β-glucosidase in mutants thus exhibiting significantly lower K_m and higher V_{max} values. These results led us to examine the possible changes in β-glucosidase gene in the mutants. The amplification of *β-glu* using conserved primers, sequencing and analysis of parent and mutants derived from different cycles indicated deletions and substitutions in the nucleotide sequence during the course of cyclic mutagenesis (Fig. 1). For instance hyper-cellulase producing mutant 'O' showed alteration in 17, 479, 481, 482, 485 and 491 base pair when compared to parent strain. Interestingly, developmental mutants '31' and '23' showed higher levels of genetic changes in the aligned sequences. The changes in the nucleotide also led to distinct restriction pattern when the amplified sequences were subjected to fragmentation with *Mbo I* (data not shown). Such approaches have also been employed by various researchers as an important tool for comparative analysis (Savitha et al., 2010; Shafique et al., 2011).

3.1. Proteomic studies

The mutants were further characterized on the basis of protein expression profile. The SDS–PAGE of the parent and selected mutants clearly shows distinct banding pattern in terms of intensity of the protein bands as well as non-expression of some of the proteins which are highlighted in Fig. 2a. In a similar approach, Tian et al. (2009) had observed absence of protein bands in the protein profile of the deletion mutants of cellulase producing *Neurospora crassa.* Activity gel staining (zymogram) analysis was also carried

Table 1
Cellulase production under shake flask cultures by parent and mutant strains using corn cob as a carbon source.

Mutants	Endoglucanase (Units/ml)	β-Glucosidase (Units/ml)	Cellobiohydrolase (Units/ml)	Fpase (Units/ml)	Xylanase (Units/ml)
28 fusant	1.23 ± 0.08	20.3 ± 0.1	1.07 ± 1.0	0.187 ± 0.002	24.23 ± 2.6
25M (I)	2.07 ± 0.01	29.66 ± 0.4	1.94 ± 1.6	0.288 ± 0.014	35.98 ± 1.5
8 (II)	4.81 ± 0.91	107.1 ± 0.05	1.94 ± 2.4	0.587 ± 0.016	196.59 ± 6.7
'O' (III)	11.81 ± 1.7	141.6 ± 4.9	2.52 ± 1.8	0.712 ± 0.011	400.57 ± 8.3
64 (IV)	12.10 ± 1.3	159.48 ± 9.5	3.52 ± 0.96	0.787 ± 0.025	478.45 ± 5.9
Folds increase in activity	9.83	7.8	3.2	4.2	19.74

SE @ 5% level.

Table 2
Cellulase production by parent and mutant strains under solid substrate cultures.

Mutants	Endoglucanase (U/gds)	β-glucosidase (U/gds)	Cellobiohydrolase (U/gds)	Fpase (U/gds)	Xylanase (U/gds)
28 (parent)	125.8 ± 2.9	892.9 ± 6.2	29.8 ± 1.0	8.46 ± 0.002	1423.6 ± 2.6
25M (I)	133.6 ± 2.5	827.8 ± 3.2	36.4 ± 1.6	9.4 ± 0.014	2092.4 ± 4.5
30 (II)	158.6 ± 0.91	1106.3 ± 4.1	50.7 ± 2.4	10.2 ± 0.16	2298.0 ± 4.7
'A' (III)	243.2 ± 1.7	1114.3 ± 4.9	65.0 ± 1.8	10.9 ± 0.011	3476.9 ± 3.3

SE @ 5% level.

Table 3
Enzyme kinetics of β-glucosidase produced by parent and mutant strains.

Mutants	K_m (Mm)	V_{max} (μmol min^{-1} mg^{-1} protein)
28 (parent)	0.22	14.36
25M (I)	0.204	27.43
8 (II)	0.101	48.65
'O' (III)	0.090	49.66

K_m and V_{max} values based on Lineweaver–Burk plot.

out to detect differences in the expression of β-glucosidase and endoglucanase in the crude enzyme extracts (Peterson et al., 2009) produced by parent and mutant strains (Fig. 2b and c). The results in Fig. 2b showed that both parent and mutants produced a 98 kDa β-glucosidase; however they differed in the level of expression. On the other hand multiple endoglucanases were observed where four isoforms were observed in mutant when compared to two isoforms detected in parent strain.

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28(Parent)          -----------ACACATGGCTGAGCGG-TGTCTCTCTTTGACACTGCATGCGGGACTGTG
25M(I)              -----------TACATGCTTTAGCG--TGTCTCTCTTTGA-ACTGCATGCGGGACTGTG
O(III)              --------------AAGGCTTAGCG--TGTCTCTCTTTGACACTGCATGCGGGACTGTG
23(white)           -----------GTGCATGCTT-AGCG--TGTCTCTCTTTGACACTGCATGCGGGACTGTG
31(yellow)          GATTCTAGTTTTTCCCCACTTCTGTAGATGTCTCTCTTTGA-ACTGCATGCGGGACTGTG
                                    *  *    ************* ******************

28(Parent)          AATTGCATGAGTGGGTAGCTTTGCGGAGCACAGCTGCACTGGCATACATCATCGTTGGGT
25M(I)              AATTGCATGAGTGGGTAGCTTTGCGGAG-ACAGCTGCACTGGCATACATCATCGTTGGGT
O(III)              AATTGCATGAGTGGGTAGCTTTGCGGAG-ACAGCTGCACTGGCATACATCATCGTTGGGT
23(White)           AATTGCATGAGTGGGTAGCTTTGCGGAG-ACAGCTGCACTGGCATACATCATCGTTGGGT
31(Yellow)          AATTGCATGAGTGGGTAGCTTTGCGGAG-ACAGCTGCACTGGCATACATCATCGTTGGGT
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28(Parent)          TCCTCAATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGG
25M(I)              TCCTCAATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGG
O(III)              TCCTCAATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGG
23(White)           TCCTCAATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGG
31(Yellow)          TCCTCAATTCGCATGCCGTGGCGGACGGTCACTTTGTGGCGCTCAAACTATTTAATATGG
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28(Parent)          CCCAGCTCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCC
25M(I)              CCCAGCTCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCC
O(III)              CCCAGCTCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCC
23(White)           CCCAGCTCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCC
31(Yellow)          CCCAGCTCCCCTTTCTCTCGCTGTTTTCGTTTCTGTCCTCCCTAAACCTCCAGTCTCTCC
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28(Parent)          ATTGGACAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCT
25M(I)              ATTGGACAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCT
O(III)              ATTGGACAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCT
23(White)           ATTGGACAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCT
31(Yellow)          ATTGGACAGGTGTTGCACGGTTGCTCACCTGGTTTGTTTTGCTCCCCCTTTGGGCGACCT
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28(Parent)          TGCCATCATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAG
25M(I)              TGCCATCATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAG
O(III)              TGCCATCATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAG
23(White)           TGCCATCATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAG
31(Yellow)          TGCCATCATGAGGTTCACTTTGATCGAGGCGGTGGCTCTGACTGCCGTCTCGCTGGCCAG
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28(Parent)          CGCTGTACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTG
25M(I)              CGCTGTACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTG
O(III)              CGCTGTACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTG
23(White)           CGCTGTACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTG
31(Yellow)          CGCTGTACGTGCCGTTACTTTGTCCTGAGAATTGCAATTGTGCTTAATTAGATTCATTTG
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28(Parent)          TTTGTTTCATCATCGCTGACAATGGTCTTTTAATAGGATGAATTGGCCTACTCCCCTCCG
25M(I)              TTTGTTTCATCATCGCTGACAATGGTCTTTTAATAGGATGAATTGGCCTACTCCCCTCCG
O(III)              TTTGTTTCATCATCGCTGACAATGGTCTTTTAATAGGATGAATTGGCCTACTCCCCTCGG
23(White)           TTTGTTTCATCATCGCTGACAATGGTCTTTTAATAGGATGAATTGGCCTACTCCCCTCCG
31(Yellow)          TTTGTTTCATCATCGCTGACAATGGTCTTTTAATAGGATGAATTGGCCTACTCCCCTCCG
                    ************************************************************ *

28(Parent)          TAGGTACGCCCTCCCCCCCCCCC---
25(I)               TAGTTAGCGGGCCAGGGCCACCCCCC
O(III)              GGGGGACGCCCTACCCCCCCC-----
23(White)           -ATTTAGCCCGTGGGGGGGGGG----
31(Yellow)          TAATTACGGGGTGAACC---------
                          *
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Fig. 1. Sequence alignment of amplified β-glucosidase gene of wild and mutant strains.

The over-expressed or unique protein bands were excised from the gel for peptide mass fingerprinting (PMF) analysis. All identified proteins were found to be distinct with high mascot score as shown in Table 4. The protein band designated as '1' (Fig. 2a) was identified as extracellular α-glucosidase belonging to family GH31 and showed close resemblance to *Aspergillus kawachii*. The over-expressed protein bands '6' and '7' in mutant 'O' were identified as alpha-L-arabinofuranosidase and endo -1,4-beta xylanase belonging to family GH43 and GH10, respectively and were closely related to *Aspergillus niger*. Whereas, the unique bands shown as '8' and '9' (Fig. 2a) in the secretome of developmental mutant 31 were identified as superoxide dismutase (SOD) and hypothetical protein which were closely related to *Bacillus subtilis* and *Helicobacter cetorum*, respectively. Superoxide dismutase has been known to produce under oxidative stress and plays an important role in the detoxification of reactive oxygen species (Seyler et al., 2001).

3.2. Enzymatic hydrolysis and fermentation

The cellulases produced by parent and mutant strains under shake flask and SSF were evaluated for their ability to saccharify alkali treated rice straw at 10% loading rate (in terms of available glucan and xylan in pretreated rice straw). The cellulases from mutant '**64**' produced under shake flask culture resulted in 37.8% saccharification where the hydrolysate was analyzed to contain glucose (7.6 mg/ml), xylose (10.3 mg/ml) and arabinose (2.03 mg/ml). Whereas, mutant '**A**' grown under SSF resulted in 70.2% saccharification and contained glucose (29.3 mg/ml), xylose (36.2 mg/ml) and arabinose (8.8 mg/ml) in the hydrolysate that was fermented to ethanol (15 g/l), however, xylose and arabinose remained unutilized during fermentation. Therefore, a two step sequential approach for efficient utilization of hemicellulose and cellulose fractions of alkali treated rice straw for obtaining value

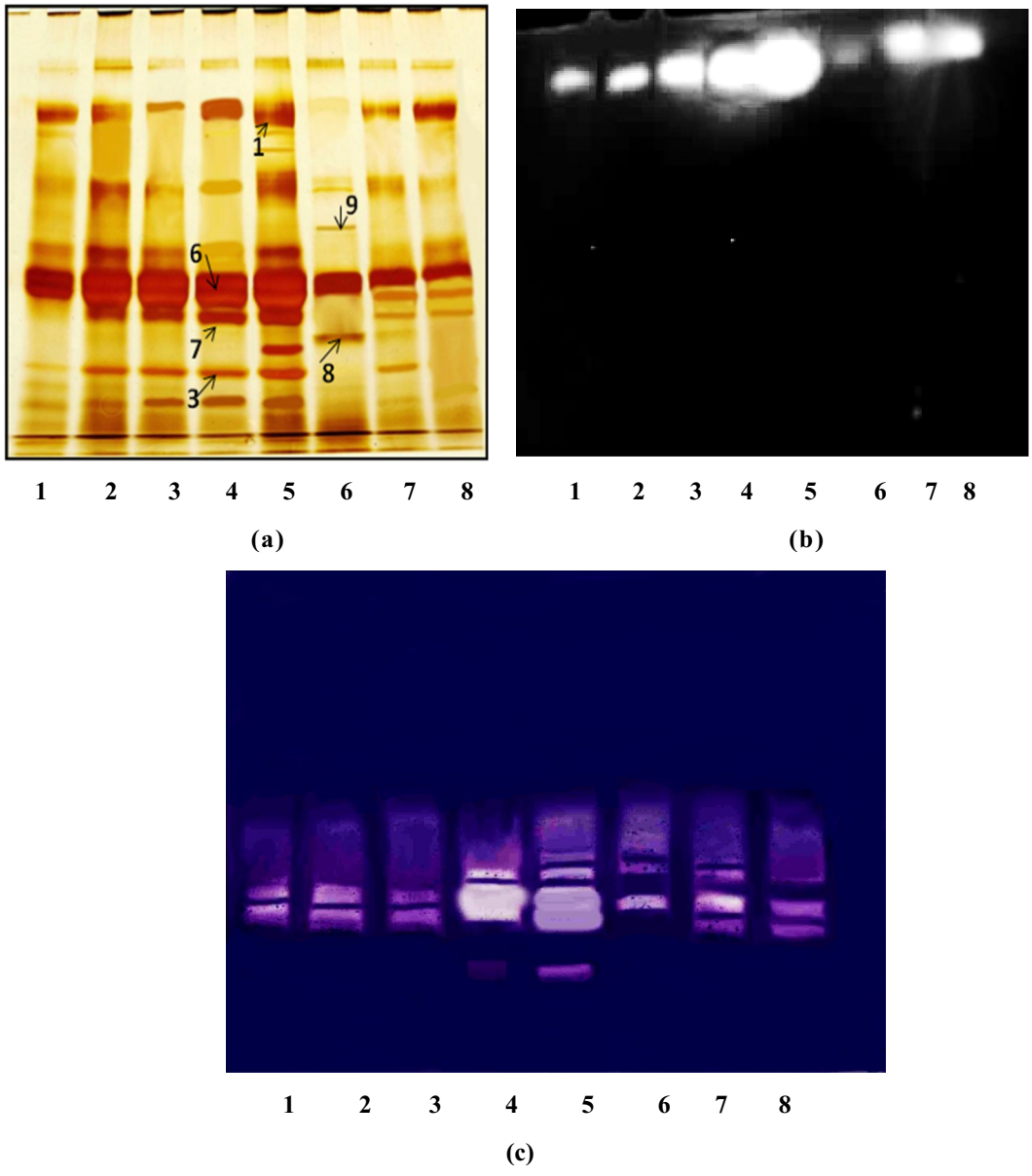

Fig. 2. (a) SDS–PAGE, (b) zymogram showing expression of β-glucosidase and (c) zymogram showing expression of endoglucanases in mutants and parental strain grown under shake flask culture: Lane 1: Dal8; Lane 2: 28 fusant; Lane 3: 25 M; Lane 4: 8; Lane 5: O; Lane 6: 31; Lane 7: J; Lane 8: 23. (Arrows in SDS–PAGE indicate the bands identified through PMF).

Table 4
Peptide mass fingerprinting (LC/MS–MS) of the unique/over expressed protein bands in mutant strains.

Sample	Mascot score	Identified protein	Query matches	Matched peptides	Closest relative (Swiss prot databases)	*E* value
1	133	Extracellular alpha-glucosidase GH31	4	R.NHNELSTIPQEPYR.W R.TLGGGIDLTFYSGPNPADVTR.Q K.SSETDASQEYISLSHGVFLR.N K.FEIPLEYIWTDIDYMHGYR.N	*Aspergillus kawachii* GAA91739.1	$1e^{-19}$
3	566	Xylanase GH11	4	R.VGGTVTTSNHFNAWAK.L K.GTVTSDGSVYDIYTATR.T R.TNAASIQGTATFTQYWSVR.Q K.LGMNLGTHNYQIVATEGYQSSGSSSITVQ.-	*Aspergillus niger* AAK16546.1	$2e^{-32}$
6	535	Alpha-L-arabinofuranosidase GH43	5(3)	K.YLMIVEAIGSTGHR.Y K.SGWTALKDFTDVVSDGK.H K.ANSGATWTEDISHGDLVR.N R.TSQDPTNVNGWSSEQALFTGK.I R.SSMSINDFPGSFGSQYEVILSGAR.N	*Aspergillus niger* XP_001389998.1	$1e^{-21}$
7	416	Endo-1,4-beta-xylanase GH10	7	K.NHITTVMQHYK.G K.LYINDYNLDSASYPK.L K.IYAWDVVNEIFNEDGSLR.D K.ADFGALTPENSMKWDATEPSR.G R.SSSTPLLFDSNYNPKPAYTAIANAL.- K.IYAWDVVNEIFNEDGSLRDSVFYK.V R.GHTLVWHSQLPSWVQAITDKNTLIEVMK.N	*Aspergillus niger* GAA92552.1	$2e^{-22}$
8	158	Superoxide dismutase	3	K.HHNTYVTNLNK.A K.SVEELVADLDSVPENIR.T R.RPDYISAFWNVVNWDEVAR.L	*Bacillus subtilis* ABQ15128.1	$7e^{-10}$
9	71	Hypothetical protein			*Helicobacter cetorum*	

Table 5
Comparative ethanol production from alkali treated rice straw.

Substrate loading rate (g/l)	Enzyme used	Enzyme loading rate (FPU/g)	Ethanol yield (g/l)	Other products	References
100	Commercial *Trichoderma reesei* cellulase	10.6	23.7	–	Zhu et al. (2005)
200	Celluclast + Novozyme 188 + Ultraflol	–	38.0		Watanabe et al. (2012)
100	Accellerase1500 enzyme (Danisco, Rochster, NY)	25	28.6	–	Suriyachai et al. (2013)
150	Cellulase from mutant '**A**'	10.9	15.0	–	This study (Single step)
150	Cellulase (NS22086, Novozyme) + β-glucosidase (NS22118)	17.5 FPU + 35CBU	31.2	XOS (22 g/l)	This study (Two step)
150	Cellulase from mutant '**A**'	10.9	12.0	XOS (22 g/l)	This study (Two step)

added products (xylo-oligosaccharides and ethanol) was developed. In this approach alkali treated rice straw (at substrate loading rate 15% w/v) that constitutes 2.7% xylan (calculated on the basis of presence of 18% hemicellulose in alkali treated rice straw) was selectively hydrolyzed with cellulase free xylanase from *Thermomyces lanuginosus* (Sonia et al., 2005) resulting in the release of 22 (g/l) xylo-oligosaccharides corresponding to xylobiose (12.6 g/l), xylotriose (2.3 g/l), xylotetraose (2.1 g/l) in addition to xylose (3.6 g/l). The XOS are known to have functional food properties with pre-biotic effect (Aachary and Prapulla, 2011) and have been reported to alleviate disease symptoms such as diabetes, arteriosclerosis and colon cancer (Swennen et al., 2006). The residual unhydrolyzed cellulose rich fraction was subjected to saccharification with cellulase derived from commercial cellulase (NS22086, Novozymes) supplemented with β-glucosidase (NS22118, Novozymes) and mutant 'A' resulting in respective release of 6.4% and 2.6% glucose which was subsequently fermented to 31.2 and 12.0 (g/l) ethanol by *S. cerevisae*. The developed process is efficient when compared to those reported for bioconversion of rice straw into ethanol (Zhu et al., 2005; Watanabe et al., 2012; Suriyachai et al., 2013) (Table 5) as in this process value added XOS were also produced in addition to high levels of ethanol at lower enzyme loading rate.

4. Conclusion

The present work demonstrates cyclic mutagenesis, selection and screening in tandem with molecular and proteomic approaches can be a powerful tool for developing improved cellulase producing strains. These developed strains can be used for efficient utilization of agro-residue for production of biofuels and value added products.

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