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High solid saccharification using mild alkali-pretreated rice straw by hyper-cellulolytic fungal strain

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Abstract

Background: The aim of this study was to use traditional mutagenesis to generate hyper-cellulolytic mutants with emphasis on stable, non-spore formers, shorter enzyme producing times and higher saccharification efficiency at high solid loadings. An in-house isolated strain of *Aspergillus terreus (At)* was identified, fingerprinted and mutated. A sequential process of mutation followed by stringent selection generated mutant At_9, which produced optimal cellulase at day 4 instead of day 7, was non-spore former with high stability and grew on a lower pH than parental strain. At_9 cellulases were used successfully at high solid loads [up to 25 % (w/v)] in a modified system at 50 °C with reduced hydrolysis times compared to parent strain.

Conclusion: In current work ultra violet (UV) mutagenesis and intelligent screening design combined with growth on a cheap substrate for enzyme production was demonstrated. With this work we present a single organism enzyme system with substantially lower production time and decreased saccharification time at high solid loads.

Keywords: Mutagenesis, *Aspergillus terreus*, Non-spore former, High solid saccharification

Background

The world has finally woken up to the stark reality of the impeding energy crisis. The thriving economies of developed nations and the potential economics of the developing nations both depend heavily on fossil fuel based energy. The energy crisis is being taken head on by exploiting the potential of various available energy sources around us which can be further converted to energy denser and user friendly sources of fuel. One approach is the use of lignocellulosic wastes instead of food grains as carbon source for ethanol production.

Lignocellulose is one of the most abundant materials available on earth, with immense potential as a substrate for energy generation. It consists of carbohydrate polymers (cellulose and hemicellulose) and lignin is a highly recalcitrant structure and difficult to deconstruct (Krassing 1992) and makes enzymatic hydrolysis a daunting task. There are various enzymes that act synergistically to degrade the complex cellulose polymer. Three sets of enzymes are believed to be the major players in the hydrolysis step: cellobiohydrolases, endoglucanases and β-glucosidases. Endoglucanases (EC 3.2.1.4) randomly cleave the β-1, 4 glycosidic linkages of cellulose; cellobiohydrolases (EC 3.2.1.91) attack cellulose chain ends to produce the constitutive unit of cellulose, cellobiose (a dimer of glucose linked by a β-1, 4 glycosidic bond); and β-glucosidases (EC 3.2.1.21) hydrolyse cellobiose into two molecules of glucose (Marie et al. 2010). It has been observed that efficient individual cellulase activities do not necessarily translate into good hydrolysis on a substrate like lignocellulosic waste, e.g. rice straw or wheat straw. This may be attributed to various other yet un-characterized or partially characterized molecules that help the cellulases to better adhere and attack the complex mesh of lignin, cellulose and hemi-cellulose. Recent work in the field has pointed towards additional chaperon proteins/molecules like Expansion and Swollen (Yennawar et al. 2006; Nevalainen et al. 1980). It is feasible that these swollen in like activities are numerous in

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cellulolytic fungus and may play a major role in improving the hydrolysis by the major cellulases. Thus, it can be argued that many still unknown proteins may be expressed in substantial amounts when grown on the right complex cellulose source, which may in turn make the final hydrolysis step more efficient. A highly purified form of cellulases might degrade a complex lignocllulosic waste like rice straw less efficiently than a crude preparation of the same starting material. Commercial enzyme production involves a mixture of cellulases/hemicellulases from more than one source. Currently, production of cellulases commercially is mostly using a combination of enzymes from *Trichoderma reesei and Aspergillus niger* (Gao et al. 2008). The *Trichoderma* system of cellulases lacks efficient levels of cellobiase thus necessitating the addition of an external source of cellobiase from the *Aspergillus s*ystem adding to the overall cost of enzyme production. To be successful, cellulosic ethanol has to compete with gasoline and first-generation biofuels for cost and ease of production. One approach is to modify/mutate the cellulolytic organism to enable optimal cellulase production. Many strains have been genetically modified/mutagenized in an attempt to cut the cost of enzyme production (Mandels and Andreotti 1978; Saloheimo et al. 2002; Durand et al. 1988; Szengyel et al. 2000) but a commercially viable strain is still not available and the stability of these mutants has been questionable.

High-solids enzymatic hydrolysis is defined as hydrolysis at no free initial liquid leading to concentrated sugar syrups, which in turn give higher concentrations of ethanol in fermentation broth substantially reducing the distillation cost (Wingren et al. 2003). As solid loadings approach 15 % (w/v), the liquid fraction becomes fully absorbed into the biomass leaving little free water. With lower amounts of free water, the apparent viscosity of the mixture increases, and consequently mixing and handling of material become more difficult. Other challenges specific to high-solid enzymatic hydrolysis include long hydrolysis times. Thus it is very important to address these issues for better hydrolysis.

A strain of *At* that grows efficiently on rice straw for cellulase production was isolated, identified and characterized. Further, traditional mutagenesis with intelligent screening/selection design was performed to isolate hyper-cellulolytic mutants. The single culture enzyme system was then tested at high solid loads in a modified system.

Methods

Biomass, chemicals and media

Rice straw used for enzyme production was procured from a local farm, cut into 1–2 cm size with a chaff cutter and passed through 5 mm mesh in a hammer mill. The straw was processed and its composition estimated (Narra et al. 2012). All other reagents were obtained from commercial sources and were of analytical grade. For saccharification studies the rice straw was pre-treated with NaOH as described in previous work (Narra et al. 2012).

Strain isolation, identification and molecular characterization

The screening and isolation of the cellulase producing fungal strain was carried out from various natural sources such as soil samples from decaying waste materials, compost and degraded rice straw, etc. The samples were serially diluted and plated on Mandels and Webers mineral salts medium as described earlier (Narra et al. 2012, 2014). Screening was carried out at different temperatures 40, 45 and 50 °C for 3–5 days and tested for zone of clearance using 0.1 % congo red solution. The selected cellulose degrading microorganisms were purified and maintained on PDA for further experiments. Morphological identification was carried out at IMTECH, Chandigarh and molecular characterization was carried at Merck India Ltd, Bangalore. Sequence data was submitted to the National Centre for Biotechnology Information (NCBI).

Molecular characterization of the isolate

Identification based on conserved ribosomal ribonucleic acid (rRNA) regions

18S rRNA based identification was done using the culture identification service of Merck India Ltd. Briefly, fungal genomic deoxyribonucleic acid was isolated and using consensus primers, the 18S rRNA, internal transcribed spacer (ITS1), 5.8S rRNA, ITS2 and 28S rRNA gene fragment was amplified by high-fidelity polymerase chain reaction (PCR) Polymerase. The PCR product was cloned and sequenced using the forward, reverse and internal primers. Sequence was aligned using combination of NCBI GenBank and ribosomal database project and a distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter) was constructed.

Modified polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP)

The ITS region of the isolate was amplified using universal fungal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). An initial denaturation step of 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 1 min. Amplified products were visualized by 1.5 % agarose gel, stained with 0.5 μg mL^{-1} of ethidium bromide and photographed. For RFLP analysis HhaI restriction enzyme was selected. The reaction mix of 20 μL

contained around 2 μg of PCR product; 1 μL of fast digest HhaI enzyme buffer and water. The mixture was incubated for 5 min at 37 °C, the enzyme was de-activated by phenol chloroform extraction and the product visualized on Agarose gel.

Enzyme production, extraction and concentration

Spores from 7-day-old slant were used to inoculate 5 g rice straw pre-wetted and autoclaved with Mandels and Weber (MW) media as per the optimized culture conditions for *At* (Narra et al. 2012). The spores were suspended in Triton X-100, counted and 10^5 spores (1 mL inoculum size) were inoculated. The flasks were then incubated at 45 °C for 7 days. For mutants, seed inoculum was prepared in potato dextrose broth by transferring the mycelial growth from a 2 days grown agar slant culture and then incubated in an orbital shaker with 150 rpm at 45 °C for 48 h. Seed inoculum of 3 % (v/v) was added to the pre-sterilized mix. The other conditions for enzyme production/optimization with mutants were kept constant. The enzyme preparation was extracted by addition of 0.05 M acetate buffer and filtered using muslin cloth. For ultra-filtration, crude enzyme preparation was pre-clarified by centrifugation at 10,000 rpm for 10 min. The clear supernatant was concentrated using ultrafiltration module (molecular weight cut off 10,000 dalton, Sartorius) fitted with polyethylene sulfonate membrane. The enzyme concentrate was stored at 4 °C until further use.

Mutagenesis and screening

A 7-day-old slant with adequate sporulation was selected and spores were harvested using 1 % Triton X-100. The spore suspension was then exposed to UV radiation for 30 min at distance of 54 cm in a UV chamber. A 30-W germicidal lamp that has about 90 % of its radiation at 2540-2550 A0 UV was used for mutation. Post treatment, the spore suspension was covered with lightproof material for 3 h followed by inoculation into minimal salt media (Narra et al. 2012) with 1 % crystalline cellulose and 0.8 % w/v 2 deoxy-D-glucose (2-DG) and overnight shaking. Next day, mycelia were separated from spores using cotton wool, broken down using glass beads and plated under different dilutions on various screening plates.

The screening media for the mutants contained minimal salt medium, 2 % agar, phosphoric acid-swollen cellulose, i.e. walseth (20–40 % w/v), 0.4 % of sorbose. Listed are the various screening plates used for selection of hyper-cellulolytic mutants: 20 % walseth + glucose (1 %), 20 % walseth + glucose (2 %), 20 % walseth + glycerol (2 %), 20 % walseth + glycerol (3 %), 30 % walseth + glucose (2 %), 40 % walseth + glucose (2 %), 30 % walseth +glycerol (3 %) and 40 % walseth + glycerol (3 %).

Listed below are steps followed for each generation of mutagenesis with slight variations based on the screening plate used in first step of selection. The first-generation cultures were screened on plates with 20 % walseth cellulose and 1 % glucose or 2 % glycerol. Colonies which produced a zone of clearance in the presence of end-product inhibitors and whose zone of clearance was substantial as compared to parent were taken for stability studies. Once the stability of the mutant was established, the above steps were repeated with more stringent selection criteria in form of 30/40 % walseth cellulose and higher concentration of glucose/glycerol. After every step of mutagenesis the selected strains were tested on plate for the same colony to zone ratio by repeated sub-culturing. Once the stability of the strain was established it was taken for the next round of mutagenesis.

Optimization of growth parameters of mutants

Cellulase production was carried out under solid-state fermentation at 45 °C using minimal medium as an initial moistening agent. The parameters optimized for maximum cellulase production were (a) effect of temperature (30–50 °C), (b) pH 4–6, (c) incubation period (4–8 days) and (d) inoculum size (1–4 % v/v).

For temperature optimization, flasks inoculated with appropriate culture were incubated at 30, 35, 40, 45 and 50 °C. For pH optimization production media was prepared and pH adjusted using either 1 M HCl or 1 M NaOH. The flasks were then inoculated and incubated for 7 days. At the end of the incubation period the flasks were harvested and enzyme activity studied. To determine the optimal day for cellulase production, five flasks containing media for enzyme production were inoculated and incubated at the same time. At stipulated time points the flasks were harvested and activity noted. For inoculum size optimization 1, 2, 3 or 4 mL of inoculum additions were studied.

Mild alkali pretreatment of rice straw

Two hundred and fifty grams of dried rice straw was mixed with 5000 mL of 0.5 % NaOH to obtain a solid: liquid ratio of 1:20. Pretreatment experiments were carried out at room temperature (RT) for 24 h. The mixture was filtered through double-layered muslin cloth and the solid residue was neutralized with 1 N HCl. The solid residue was dried at 60 °C constant weight. The pretreated straw was either used immediately for hydrolysis studies or stored at 4 °C in air tight bags.

Saccharification studies

All the saccharification experiments with pre-treated samples were performed using ultrafiltration (UF) concentrated enzymes from parent strain and mutated strain

At_9. Initial experiments with a substrate concentration of 2.5-10 % w/v were performed in 150 mL Erlenmeyer flasks with an enzyme load of 9 FPU g^{-1} substrate at 50ºC, 120 rpm for 4-40 h. For high solid loads (15.0–30.0 % w/v) a 250-mL Oakridge wide mouth bottle (Make-Tarson) was used instead of flask. The system was tested at 15 % substrate load with 9 FPU g^{-1} substrate enzyme load and 50 °C for 40 h at 16 rpm using proper controls to ensure accuracy of the process. This system was compared with other systems such as shake flasks with wet heat and dry heat (50 °C) and at 120 rpm for 40 h. Based on the results from 15 % solid loading further saccharification studies were performed at 15–30 % solid loadings using the enzymes from mutant At_9 and parent strain with an enzyme load of 9 FPU g^{-1} substrate. The influence of substrate concentration (15-30 % w/v), time (8–48 h) and different concentrations of Tween 80 (0.5–3.0 % v/v) on enzymatic hydrolysis were studied. Total volume of the system was maintained at 50 mL (0.05 M citrate buffer, pH 4.8) for all the experimentation. The enzyme was over layered on the substrate and the bottles loaded on the rotating assembly which was pre-set at 50 °C. The saccharification systems were set such that the volume of the material was never exceeded half the total volume of the reactor. Other conditions were kept constant as described earlier (Narra et al. 2012). At the stipulated times, contents were centrifuged at 10,000*g* for 15 min and supernatant analyzed for total reducing sugars. Saccharification efficiency was calculated as mentioned previously (Narra et al. 2012).

Analytical methods

Protein concentration was determined using bovine serum albumin as standard using the method described by Lowry et al. (Lowry et al. 1951). Filter paper activity, endoglucanase and β-glucosidase were measured as described earlier (Narra et al. 2012, 2014). Reducing sugars were determined by the dinitrosalicylicacid method (Miller 1959). The contents of cellulose, hemicelluloses and lignin in untreated and pretreated rice straw were determined according to Goering and Van Soest (1975). The reducing sugar content was multiplied by 0.9 to calculate saccharification yield.

$$\%\ \text{Saccharification} = \text{Total reducing sugars}\left(\text{mg g}^{-1}\right) \times 0.9 \times 100/\text{Carbohydrates in substrate (mg g}^{-1}\text{)}$$

Results and discussion

Isolation and identification of the cellulose degrading fungus

Screening of cellulolytic microorganisms was carried out from soil, compost and degraded rice straw as described earlier (Narra et al. 2012, 2014). The ITS regions are located between the 18S and 28S rRNA genes and the rRNA gene for 5.8S RNA separates the two ITS regions. Ribosomal genes have regions of variability and this sequence variation of ITS regions has led to their use in phylogenic studies of many organisms (White et al. 1990). Using consensus primers, the ~1200 bp; 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene fragment was amplified using high-fidelity PCR Polymerase (Fig. 1). The amplicon was cloned and sequenced. Based on nucleotides homology and phylogenetic analysis the newly isolated fungus was identified as *Aspergillus terreus* (NCBI GenBank Accession Number: KF971363).

Depending on the organism, regions other than the ITS (gene specific; i.e. EF-1 for *Fusarium*) may be required to

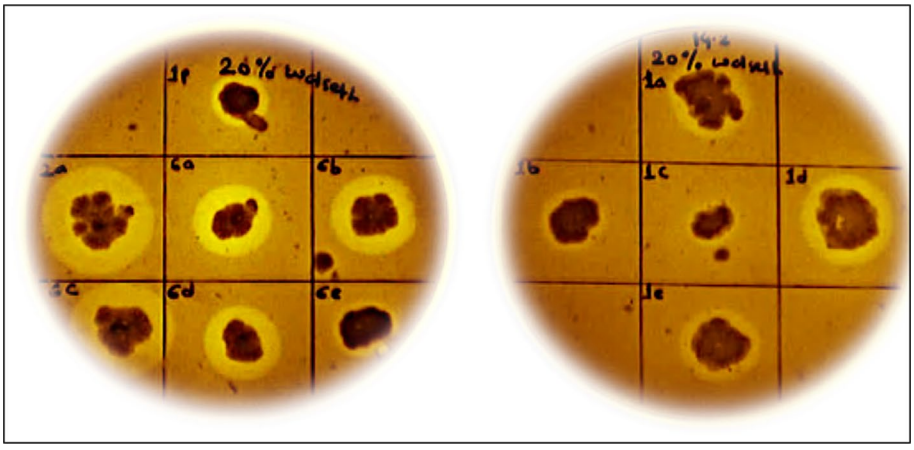

Fig. 1 A representative screening plate used for selection of UV mutants. The suspected hyper-producers were picked from the parent plate and were labeled and placed in sectored 20 % walseth cellulose and sorbose containing plates. The clearing around each colony is the cellulose degradation zone for the associated mutant

confirm identification (Hansen et al. 2008). PCR–RFLP has been used by researchers to differentiate between *Aspergillus* species (Merhendi et al. 2007). Amplification using ITS1 and ITS4 conserved region primers gave an amplicon of 607 bp size which when digested with Hbl1 resulted in three fragments of 96, 179 and 332 bp corresponding to two restriction sites at 333 and 512 bp for *Aspergillus terreus*. The results are in agreement with that of the work done by (Merhendi et al. 2007).

Generation, enrichment and screening of mutant

For UV mutagenesis the 99 % lethality dose for the parental stain was determined as 30 min at a distance of 54 cm. Using this combination of distance and time a series of UV treatments were performed. Hopwood et al. (1985) showed that mutation frequency is higher when the survival rates from UV mutation are between 10 and 0.1 %. Since the aim of this work was to increase the innate capability of the strain to secrete/produce more cellulases, 2-DG, an antimetabolite, was used to screen for catabolite repression-resistant mutants (Montenecourt and Eveleigh 1975). In the presence of 2-DG in the medium only those spores with deregulated or compromised end-product inhibition control would form mycelia and thus can be separated from spores. The addition of sorbose (0.4 %) restricts the growth of mycelia facilitating more number of mutant screening per plate (Chand et al. 2005). The further selection and screening on 20 % (w/v) walseth agar followed by 30 % (w/v) walseth ensured selection of good producers at high substrate concentrations. Screened mutant strains were sub-cultured three–four times on both concentrations of walseth plates to check for reproducibility and stability. After repeated cycles of mutagenesis and selection, mutant At_9 was generated. Two such screening plates are shown in Fig. 2. The mutant gave a larger and clearer zone on walseth agar plates as compared to the parent strain and was also non-spore former. The mutant strain was taken for optimization of media/growth parameters.

Optimization of growth parameters for mutant strains

Optimization of various growth and production parameters for At_9 was performed since its optimal requirements may be different than the parent strain. In experiments for optimal temperature, though a range of temperature were tested, it was observed that just like the parental strain At_9 produced optimal cellulases at 45 °C (data not shown). Thus the selection criteria for mutants were set at 45°C. The mutant also showed a preference to a slightly more acidic pH (pH 4) as compared to the parent. Many fungi acidify lignocellulosic substrates as they grow in them to pH levels that are probably at least partially inhibitory to further growth (Zadrazi and Brunnert 1982). Thus, preference for lower pH may be advantageous to the mutant; also lower pH would mean fewer chances of external contaminants. Inoculum size in terms of number of initial spores/mycelial balls added to the system was found to be 1 mL 5 g^{-1} of substrate for At_9. The parent strain had day 7 as its maximum cellulase production, while At_9 showed its maximum potential on day 4, thus significantly cutting the production time.

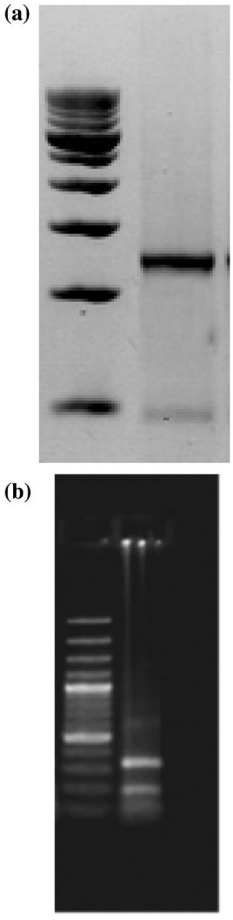

Fig. 2 **a** Analysis of 18S rRNA gene sequence. **b** PCR–RFLP of the amplified region from 18 s r-RNA

The wild strain of *At* performed to its maximum potential at 45 °C temperature, pH 5.5, moisture level 1:7, incubation period 7 days and inoculum size of 1×10^5 spores mL^{-1}. Post optimization At_9 preferred more acidic pH (pH 4) than parent and took 3 days less for cellulase production. Moreover, the enzymes from At_9 had a higher specific activity than the parental strain thus adding to benefits during downstream processing. Table 1 lists the enzyme activity of the mutant post optimization.

Table 1 Specific activity of the mutant strain after final optimization

S. no.	Strain	Enzyme activity (Umg^{-1})					
		Endoglucanase	Fold increase	β-Glucosidase	Fold increase	FP	Fold increase
1	Control	2.50 ± 0.91	–	0.76 ± 0.94	–	0.15 ± 0.82	–
2	At_9	4.45 ± 0.84	1.78	3.39 ± 0.79	4.46	0.26 ± 0.97	1.73

Composition of mild alkali pretreated rice straw

Untreated rice straw contained 40.33 ± 1.53 % cellulose; 28.47 ± 0.58 % hemicellulose and 6.97 ± 0.76 % lignin. After pre-treatment, the solid biomass of rice straw contained 59.87 ± 0.68 % cellulose; 22.17 ± 1.19 % hemicellulose and 3.17 ± 0.55 % lignin. The alkaline pretreatment step led to reduction in lignin (57.96 %) content and increase in cellulose content (48.45 %) in the pre-treated biomass feedstock.

Saccharification studies at high solid loads using parent and mutant enzymes

The maximum solid load that the saccharification system could take without compromising on the hydrolysis efficiency was studied at solid load of 2.5, 5, 7.5 and 10 %. Cellulases from the parent strain could hydrolyze 10.0 % substrate load producing 536 mg g^{-1} reducing sugars with 58.82 % efficiency in 40 h. Enzymes from At_9 performed the same but in shorter time (24–32 h). An enzyme load of 9 FPU g^{-1} substrate was used for both sets of enzymes (Fig. 3a, b).

When higher substrate loads were to be tested a modified system of Okaridge tubes placed on a rotating assembly inside a temperature-controlled hybridization oven was used. This assembly was tested against various controls at 15 % solid loads (Table 2).

The barrels placed in a horizontal position and rotated at 16 rpm provided free fall and thorough mixing of the contents as compared to other systems thus eliminating the problem of efficient mixing at higher substrate loads. Liquefaction of the contents occurred around 8 and 12 h of hydrolysis at the substrate load of 10 and 15 %, respectively, followed by free-fall mixing of the content. As the viscosity was high at 15 % substrate load, the slurry turned into a thick, clay-like paste and remained as a thick paste following 32 h of hydrolysis. Many researchers have tried different mixing mechanisms to get better saccharification yield at high solid loads. Our results are well in agreement with several groups by the use of horizontal orientation of the reactor for high-solid loadings (Jorgensen et al. 2007; Larsen et al. 2008). The efficiency of the mutant enzymes as compared to parent was next tested on the modified barrel system at higher substrate loads (15, 20, 25 and 30 %) at different time intervals

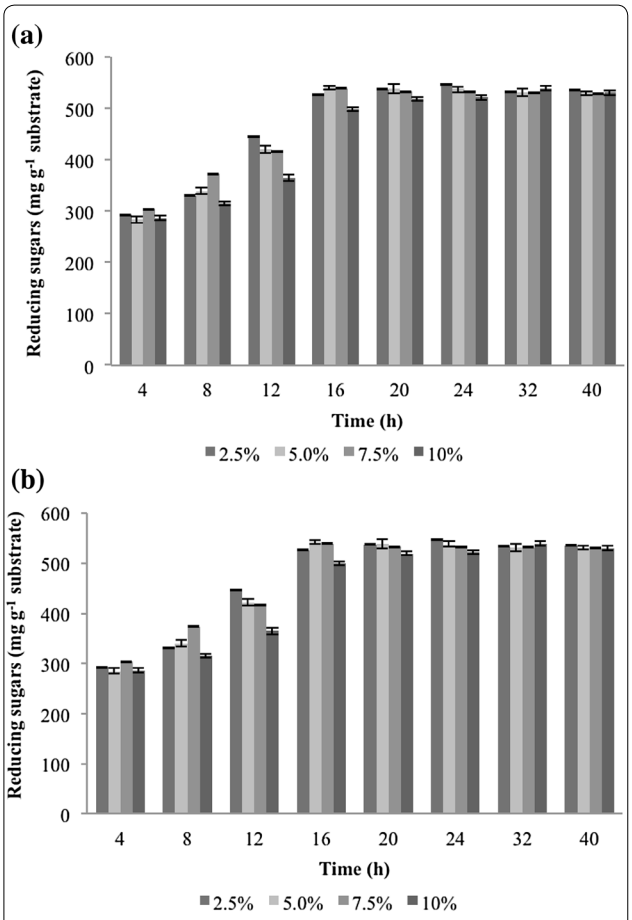

Fig. 3 Effect of substrate concentration (2.5–10 % w/v) on enzymatic hydrolysis of rice straw using parent strain (**a**) and mutant strain At_9 (**b**). Temperature 50 °C, enzyme load 9 FPU g^{-1} substrate, pH 4.8, shaking at 120 rpm, time 8–48 h. Values are means of duplicate experiments

(8–48 h) and at 9 FPU g^{-1} substrate of enzyme load (Fig. 4a, b).

A substrate load up to 25 % could be sustained by the system without a drastic reduction in the saccharification efficiency. Parent strain produced 547 mg g^{-1} substrate reducing sugars in 40 h, whereas mutant strain produced almost the same amount of reducing sugars (540 mg g^{-1} substrate) in 32 h with a saccharification efficiency of 60.03 and 59.29 %. This efficiency was quite comparable

Table 2 Vessel optimization for system working at higher substrate loads with UF concentrated enzymes

Reactor type/condition	Duration (h)		Sugars (mg g^{-1} substrate)		Saccharification (%)	
	Parent	At_9	Parent	At_9	Parent	At_9
Barrel-shaped bottle-rotating assembly (dry heat)	40	32	540 ± 0.51	541 ± 0.37	59.26 ± 0.51	59.37 ± 0.37
Conical flask-shaker water bath (wet heat)	40	32	336 ± 0.37	340 ± 0.52	36.87 ± 0.37	37.31 ± 0.52
Conical flask-incubator shaker (dry heat)	40	32	319 ± 0.28	335 ± 0.39	35.01 ± 0.28	36.76 ± 0.39

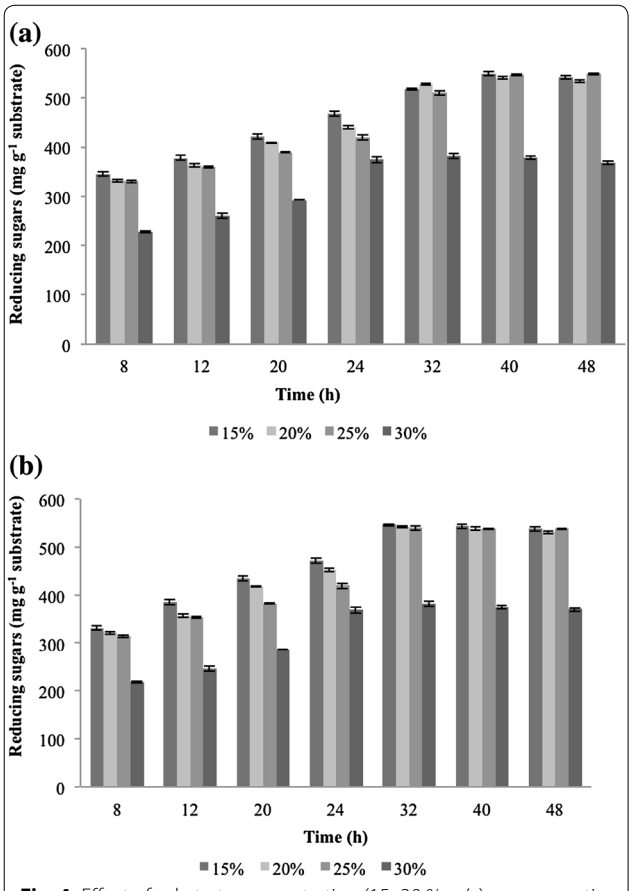

Fig. 4 Effect of substrate concentration (15–30 % w/v) on enzymatic hydrolysis of rice straw using parent strain (**a**), mutant strain At_9 (**b**). Temperature 50 °C, enzyme load 9 FPU g^{-1} substrate, pH 4.8, rotating at 16 rpm, time 8–48 h. Values are means of duplicate experiments

with the efficiency of system (64.09 %) at lower substrate loads (2.5 % w/v). When 30 % load of substrate was tested the efficiency of the system fell to 41.59 and 41.84 % at 9 FPU g^{-1}indicating that the efficiency of conversion decreased at higher substrate concentration. An increase in the enzymatic load from 9 FPU g^{-1}substrate to 15 FPU did not lead to any increase in conversion efficiency (data not shown).

Many researchers have found that substrate concentration limits the saccharification yield because of poor mixing and heat transfer problem owing to the rheological properties of a dense fibrous suspension, which ultimately cause insufficient adsorption of the cellulase to the cellulose (Lowry et al. 1951). Addition of Tween 80 1.5 % (w/v) enhanced the saccharification efficiency by 15.30 and 18.28 %, respectively. Maximum reducing sugars released were 631 and 639 mg g^{-1} substrate from the parent and mutant At_9 at 40 and 32 h, respectively, with 9 FPU g^{-1} substrate enzyme load (Fig. 5). Thus, we were able to further improve the conversion efficiency of the system. Kaar and Holtzapple (1998) similarly have found that Tween 80 increased the rate and extent of hydrolysis.

Thus an improvement in the process was obtained by reducing the time and cost of enzyme production (optimal production at day 4 instead of day 7), downstream processing (mutant produced more concentrated enzymes) and shorter saccharification times (32 vs 40 h) by generating the mutant strain At_9. Saritha et al. (2012) reported that at 10 % solid load pre-treated rice straw yielded a saccharification efficiency of 52.69 % after 72 h. Seung et al. (2013) found that at 15 % (w/v) solid loading of popping pre-treated rice straw yielded a sugar recovery of 0.567 g g^{-1} substrate in 48 h. Pretreated rice straw at low substrate load yielded 26.3 g L^{-1} and 686 mg g^{-1}

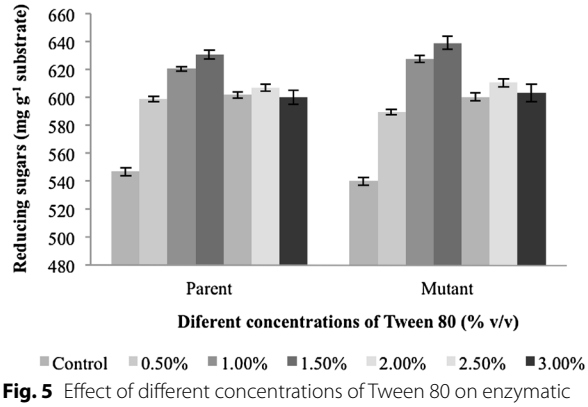

Fig. 5 Effect of different concentrations of Tween 80 on enzymatic hydrolysis of rice straw using mutant strain At_9.Substrate load 25 % (w/v), temperature 50 °C, enzyme load 9 FPU g^{-1} substrate, pH 4.8, rotating at 16 rpm, time 40 h. Values are means of duplicate experiments

substrate of reducing sugars (Sukumaran et al. 2009; Jeya et al. 2009).

Conclusion

In the current work, UV mutagenesis and intelligent screening design combined with growth on a cheap substrate for enzyme production was demonstrated. The mutational scheme generated an efficient cellulase producer, producing highly concentrated enzymes in shorter times. The mutant enzymes worked on higher solid loads with greater efficiency in terms of saccharification time. To the best of author's knowledge this is first report on high solid saccharification on rice straw without external supplementation of β-glucosidase.

Authors' contributions

GD and MN designed and performed the experiments; ARS and DM supervised the complete study, GD and MN wrote the manuscript and interpreted the data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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