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Enhanced cellulase producing mutants developed from heterokaryotic *Aspergillus* strain

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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.

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

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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).