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Short Communication

Purification and characterisation of processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through saccharification of delignified coir pith to glucose

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HIGHLIGHTS

- Purification of a new processive-type endoglucanase and β-glucosidase from *A. ochraceus*.
- Cellulases displayed multiple-substrate specificity, processivity and sequential synergy.
- Offers a novel and cost effective bioprocess for bioconversion of delignified coir pith to glucose.

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ABSTRACT

The study describes purification and characterisation of processive-type endoglucanase and β-glucosidase from *Aspergillus ochraceus* MTCC 1810 through bioconversion of delignified coir pith to fermentable glucose. The purified processive endoglucanase (AS-HT-Celuz A) and β-glucosidase (AS-HT-Celuz B) were found to have molecular mass of ≈78-kDa and 43-kDa respectively with optimum endoglucanase (35.63 U/ml), total cellulase (28.15 FPU/ml) and β-glucosidase (15.19 U/ml) activities at 40 °C/pH 6. The unique feature of AS-HT-Celuz A is the multiple substrate specificity and processivity towards both amorphous and crystalline cellulose. Zymogram indicated both endo and exoglucanase activities residing in different binding sites of a single protein exhibiting sequential synergy with its own β-glucosidase. Accordingly, the identified enzymes could be implemented as synergistic cellulases for complete cellulose saccharification which still considered an unresolved issue in bio-refineries.

1. Introduction

Current fossil fuel consumption profile has forced scientific efforts to explore alternative energy sources, and one among them is lignocellulosic biomass having composed of cellulose (40–60%), hemicelluloses (20–30%) and lignin (15–30%) (Kuhad et al., 1997), an organic carbon rich feed stock for enzymatic saccharification. Cellulases are the main catalytic players of enzymatic hydrolysis and exist as multiple enzyme system comprising endoglucanase (EC3.2.1.4), cellobiohydrolase (avicelase, EC3.2.1.91), and β-D-glucosidase (EC3.2.1.21) that synergistically hydrolyse cellulose into monomeric glucose units (Yi et al., 1999). Their most remarkable applications are in the fermentation industry which demands high yield and stability under extreme bioprocess conditions. However, to make it economically viable, production cost has to be minimised (Banerjee et al., 2010); one way of attaining would be to use less expensive substrates such as lignocellulosic wastes, besides utilising efficient cellulase producers.

Coconut pith or coir pith, a natural renewable resource, is a by product of coir industry having 25% cellulose. Easy availability of coir pith and its possible utility as substrate for saccharification, and the capability of *Aspergillus ochraceus* MTCC 1810 in hyper-cellulase on-site enzyme production would make the saccharification economically feasible. In the present study, we describe the presence of a new type of processive endoglucanase in *A. ochraceus* MTCC 1810 showing sequential synergy with its own β-glucosidase and thus provide complete hydrolytic machinery for the bioconversion of pre-treated biomass to glucose for subsequent bio-ethanol production. Thus the purified industrially potent enzymes, renamed as AS-HT-Celuz A (processive endoglucanase) and AS-HT-Celuz B (β-glucosidase) with compatible biochemical profile

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were employed for studying the micro-structural modifications occurred in the coir pith after enzymatic saccharification.

2. Methods

2.1. Microorganism and culture conditions

The filamentous fungus, *A. ochraceus* MTCC 1810 was selected as the appropriate microorganism from among thirty-seven cellulolytic isolates procured from Microbial Type Culture Collection, IMTECH, Chandigarh (unpublished data). It was inoculated into the shake flasks containing Reese and Mandel's submerged fermentation medium at pH 6 (0.4 g/L $(NH_4)_2SO_4$, 2.2 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 4 mg/L $FeSO_4{\cdot}7H_2O$, 0.04 g/L $CaCl_2{\cdot}2H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$, 2% NaCl, 0.8% Tween 80 and supplemented with 1% pre-treated coir pith, which was chemically delignified according to the optimised conditions (Rojith and Bright Singh, 2012) and grown at 35 °C, 100 rev/min for 5 days. Endoglucanase (CMCase), total cellulase (FPase) and β-glucosidase assay were done according to Ghose (1987).

2.2. Purification of extracellular cellulases from A. ochraceus MTCC 1810

Extracellular proteins were ammonium sulphate precipitated and dialysed using Amicon UF stirred cell (10 kDa, Millipore, USA). Crude protein was then applied on ÄKTA Prime Plus system (GE Healthcare, Uppsala) equipped with C16/40 DEAE Sepharose column (Sigma) equilibrated with 20 mM Tris–HCl buffer (pH 8). The enzyme was eluted by applying linear gradient of NaCl (0–1 M) at a flow rate of 0.5 ml min^{-1}. Active fractions showing cellulase activity were pooled, concentrated by lyophilisation, dialysed and protein was estimated through BCA assay reagent kit (Invitrogen). SDS–PAGE was carried out according to Laemmli (1970) and individual enzyme activity was determined by zymogram analysis (Schwarz et al., 1987)

2.3. Biochemical characterisation of purified enzymes

Optimum pH and temperature was determined in the presence of various buffers with pH range of 2–10 and at temperature range of 4–70 °C for 60 min. Enzymes were pre-incubated with different metal ions (5 mM), 1% of various chemical additives and organic solvents (20% v/v) at room temperature for 60 min. The analysed parameters were shown in Supplementary Tables 1–3. Enzyme stability in terms of residual activity was determined under standard assay conditions.

2.4. Substrate specificity and substrate binding assay

Substrate specificity was measured against various natural and synthetic cellulosic substrates. The assay was prepared in 0.5 M sodium citrate buffer (pH 6.0) at 40 °C for 10 h. Substrate binding assay was performed by incubating the enzymes (50 μg/ml) with 1% insoluble substrates such as avicel and filter paper discs.

2.5. Processivity assay and determination of synergy between purified cellulases

Processivity was determined using whatman No. 1 filter paper as substrate with 50 μg/ml of AS-HT-Celuz A (Zhang et al., 2010). Synergy between the purified cellulases (25 U/ml); AS-HT-Celuz A and AS-HT-Celuz B, were investigated using CMC: cellobiose (1:1) as substrate in 0.05 M citrate buffer at pH 6.0/40 °C for 4 h with single enzyme alone as well as simultaneous and sequential additions of enzymes (Amano, 1997).

2.6. Application of purified enzymes in the saccharification of pre-treated coir pith and end product analysis

Hydrolysis of 12% (w/v) pre-treated coir pith was carried out with sequential enzyme loading of 25 FPU/g – cellulose of AS-HT-Celuz A for initial 12 h and 10 U/g – cellulose of AS-HT-Celuz B for further 12 h, enzyme loading rate selected according to our previous studies (unpublished). Control experiments were also run with single enzyme alone and without enzyme treatment. Saccharification efficiency was calculated (Taniguchi et al., 2005). Residues from the hydrolysis were subjected for thin-layer chromatography and gas chromatography. FT-IR, XRD and SEM were adopted to analyse the micro-structural changes after the enzymatic saccharification.

3. Results and discussion

3.1. Purification of multi-component cellulase from A. ochraceus MTCC 1810 through bioconversion of delignified coir pith

Purification steps of cellulase from *A. ochraceus* are summarised in the Table 1. Purified proteins appeared as single prominent peaks (Supplementary Fig. S1) and most of the endoglucanase and total cellulase activity (fractions 53–89) corresponded to the molecular mass of ≈78 kDa (Fig. 1a, lane 2). The overlapped enzyme activities indicated the presence of processive cellulase capable for degradation of amorphous and crystalline cellulose, implying that both enzyme active sites were residing in the single protein, noticed for the first time in *A. ochraceus* MTCC 1810. Meanwhile, similar bifunctional cellulases from various bacteria and fungi have been reported (Han et al., 1995). Zymogram analysis also agreed with the protein profile of endoglucanase and exoglucanase on CMC-congo red and 4-MUC gel respectively (Fig. 1b, lane 2 and 3). While most of the β-glucosidase activity was concentrated on the fractions 36–61 with an apparent molecular mass of 43 kDa (Fig. 1, lane 2), its zymogram profile showed a black precipitate due to the hydrolysis of esculin to esculitin (Fig. 1b, lane 1).

3.2. Biochemical characterisation of purified cellulases

Both enzymes showed a similar pH stability profile from pH 3 to 8 after 10 h of incubation (Supplementary Fig. S2a and b). Based on the optimum activity at pH 6.0, it showed a decreasing profile up to 50% at above pH 9.0 and at below pH 3.0. Optimum temperature was observed at 40 °C with significant enzyme activity in the range of 4–60 °C and a complete inactivation profile at 70 °C (Supplementary Fig. S2c and d). Thus the two purified cellulases demonstrated the industrial potency in terms of its compatible temperature and pH stability. Endoglucanase (70–98%) and total cellulase (67–86%) activities were found enhanced by the metal ions in the order of $Na^+ > K^+ > Mg^{2+} > Cu^{2+} > Fe^{3+} > Co^{2+}$ (Supplementary Table 1), while Hg^{2+}completely inhibited the enzyme activities. Non-ionic surfactants such as tween 20, tween 80 and triton X-100 were found to enhance the enzyme activities, whereas SDS, EDTA and β-mercapto ethanol partially inhibited the same (Supplementary Table 2). Both enzymes showed higher organic solvent stability in the presence of 20% v/v ethanol (Supplementary Table 3). Annamalai et al. (2013) reported an organic solvent (25%, v/v ethanol) stable alkaline cellulase from *Bacillus halodurans* CAS 1 strain as well. This is the first report on *Aspergillus* derived cellulases with higher stability in (20%v/v) ethanol much useful in industrial bio-ethanol production.

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