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Synergistic action of recombinant accessory hemicellulolytic and pectinolytic enzymes to *Trichoderma reesei* cellulase on rice straw degradation

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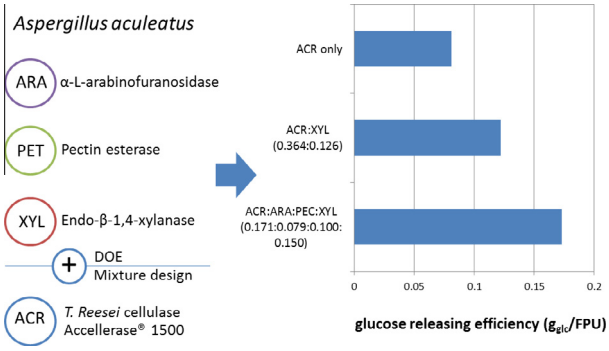
HIGHLIGHTS

- Synergy of accessory hemicellulase and pectinase to core cellulase was shown.
- A varying degree of synergism of ARA, PEC, XYL to ACR cellulase was found.
- Removal of ara side chain by arabinofuranosidase enhanced cellulase activity.
- Synergy of pectin esterase to cellulase mixture was firstly reported.
- The quaternary mixture showed 214% glc obtained/FPU compared to ACR alone.

GRAPHICAL ABSTRACT



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ABSTRACT

Synergism between core cellulases and accessory hydrolytic/non-hydrolytic enzymes is the basis of efficient hydrolysis of lignocelluloses. In this study, the synergistic action of three recombinant accessory enzymes, namely GH62 α-L-arabinofuranosidase (ARA), CE8 pectin esterase (PET), and GH10 endo-1,4-beta-xylanase (XYL) from *Aspergillus aculeatus* expressed in *Pichia pastoris* to a commercial *Trichoderma reesei* cellulase (Accellerase® 1500; ACR) on hydrolysis of alkaline pretreated rice straw was studied using a mixture design approach. Applying the full cubic model, the optimal ratio of quaternary enzyme mixture was predicted to be ACR:ARA:PET:XYL of 0.171:0.079:0.100:0.150, which showed a glucose releasing efficiency of 0.173 g_{glc}/FPU, higher than the binary ACR:XYL mixture (0.122 g_{glc}/FPU) and ACR alone (0.081 g_{glc}/FPU) leading to a 47.3% increase in glucose yield compared with that from ACR at the same cellulase dosage. The result demonstrates the varying degree of synergism of accessory enzymes to cellulases useful for developing tailor-made enzyme systems for bio-industry.

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1. Introduction

Over the past decade, lignocellulosic plant biomass has attracted attention as a renewable resource for production of biofuels and commodity chemicals. As fossil resources become scarcer and the use of their derivatives generate environmental concern on

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global warming, biorefinery is considered as a more sustainable platform industry with competitive advantages in the long term (Cherubini, 2010). Extensive research has been conducted to understand the complex natural bioconversion of lignocelluloses, particularly on degradation of recalcitrant plant biomass. Insights into biomass decomposition by means of intricate enzymatic processes may lead to the development of efficient enzyme systems for saccharification and modification of lignocellulosic materials in bioindustries.

Plant cell wall is a lignocellulosic material composed of mainly three different types of polysaccharide polymers, i.e. cellulose, hemicellulose and pectin. Cellulose is a linear homopolymer of D-glucose formed into highly organized microfibers, which are intimately associated with an intricate network of hemicellulose, a branched heteropolymer of various pentoses, hexoses, and sugar acids. Pectin, a galacturonic acid containing polysaccharide forms a gel layer between cellulose microfibers. The polysaccharide microstructure is covered by lignin, a complex heteropolymer of phenolic substance, which acts as a protective shield. Lignocelluloses are highly recalcitrant to biological degradation, and thus lignocellulolytic microorganisms employ a variety of cellulolytic, hemicellulolytic, and lignolytic enzymes that act specifically and synergistically to degrade plant biomass (Himmel et al., 2007). The core of cellulase contains three types of enzymes including endoglucanase (EG, endo-1,4-D-glucanohydrolase: EC3.2.1.4), which randomly cleaves internal bonds in the cellulose fiber creating free chains ends, exoglucanase or cellobiohydrolase (CBH, 1,4-β-exoglucanase-glucan cellobiohydrolase: EC 3.2.1.91), which sequentially cleaves cellobiose units from free chain ends, and β-glucosidase (EC 3.2.1.21), which releases glucose units from cellobiose (Howard et al., 2003). A wide variety of hemicellulolytic enzymes attack the heterogenous hemicellulose structures, including several endo-/exo-acting hydrolytic enzymes that degrade the main polysaccharide chain and a range of accessory enzymes attacking the "decorated" branches of hemicelluloses, e.g., α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, and β-mannanase (Gottschalk et al., 2010). The embedded pectin matrix is degraded by various hydrolytic and non-hydrolytic pectinases (Willats et al., 2001).

Many cellulolytic and hemicellulolytic enzymes from aerobic or anaerobic bacteria and fungi display synergistic action (Bayer et al., 2004; Zhang and Lynd, 2006). Different mechanisms have been described to explain the synergy, including: (i) enhancement of upstream enzymes by downstream enzymes acting on smaller substrates e.g. alleviation of cellobiohydrolase inhibition by a β-D-glucosidase which cleaves its inhibitor, cellobiose to glucose, (ii) the endo/exo effect, where endocellulases create new, free cellodextrin chain ends for cellobiohydrolases; (iii) synergism between exocellulases attacking reducing and non-reducing ends; (iv) cooperative action of cellulolytic and hemicellulolytic enzymes which increase accessibility to each other's respective target substrates, and (v) physical alteration of the substrates e.g. loosening of the crystallised region of cellulose by auxiliary proteins and non-hydrolytic enzymes e.g. expansins and lytic polysaccharide monoxygenases (Bunterngsook et al., 2015; Leggio et al., 2015). This synergistic enzyme action forms the basis of efficient lignocellulose degradation in nature and could be applicable for the development of efficient lignocellulolytic enzyme systems for biotechnological application.

It is well accepted that different lignocellulosic biomasses pretreated using different methods require empirically determined combinations of different enzymes for optimal digestibility in biotechnological applications. Identification of the key enzymes and optimization of their relative ratios can thus reduce enzyme usage without sacrificing the rate or yield from substrate hydrolysis. In the present study, the synergistic action of different types of accessory enzymes including an α-L-arabonifuranosidase (GH62; ARA) which acts on removing the arabinosyl side chain from the main-chain of hemicellulose, a pectinesterase (CE8; PET) which functions cooperatively with other pectinolytic enzymes on pectin degradation by cleaving the methyl group from the pectin structure, and an endo-1,4-beta-xylanase (GH10; XYL) which attacks the internal glycosidic bond in main-chain xylan and other xylan-containing hemicellulosic polymers from *Aspergillus aculeatus* BCC17849 to a *Trichoderma reesei* cellulase (Accellerase® 1500; ACR) on hydrolysis of alkaline pretreated rice straw was studied using a systematic experimental design approach. The work showed strong synergistic actions of carbohydrate processing enzymes with different specificities on hydrolysis of lignocelluloses. The findings provide a basis for formulation of active enzyme mixtures for efficient biomass saccharification and modification.

2. Methods

2.1. Materials

Rice straw was collected from a local field in Pathum Thani province, Thailand. The biomass was physically processed using a cutting mill (Retsch SM 2000, Hann, Germany) and sieved to particles of diameter 250–420 μm 0.21–0.35 mesh). The biomass was pretreated with 10% (w/v) NaOH at 80 °C for 90 min at the solid/liquid ratio of 1:3, washed with water until neutral pH was obtained, and air-dried before use as the substrate for enzymatic hydrolysis. *A. aculeatus* BCC17849 was obtained from the BIOTEC Culture Collection, Thailand (www.biotec.or.th/bcc) and maintained on potato dextrose agar (PDA). Polysaccharides used as substrates in enzymatic activity analysis including carboxymethylcellulose sodium salt (CMC) (Cat. No. 21101) was purchased from Fluka while beechwood xylan (Cat No. X4252), oat-spelt xylan (Cat. No. X0627), and pectin from citrus peel (Cat No. P9135) were obtained from Sigma–Aldrich. The alkali-pretreated rice straw contained 78.36% cellulose, 10.88% hemicellulose, 9.86% lignin and 1.25% ash according to the standard NREL analysis method (Sluiter et al., 2008).

2.2. Culture preparation and total RNA extraction

A. aculeatus BCC17849 was grown aerobically in submerged liquid culture in 250 ml conical flasks. The culture was prepared from the culture on PDA by plunging 4 agar pieces each covered with a profuse mycelia mat using a cock borer number 2 into 50 ml of WS medium (3% (w/v) wheat bran and 1% (w/v) soy bean). The inoculated culture was incubated at 25 °C for 5–7 d with no shaking. The mycelia were harvested by filtration on sterile gauze, and ground up to fine powder in liquid nitrogen until a powdery consistency was achieved. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc., Ohio, USA) following the manufacturer's instruction. The quality and integrity of RNA was determined by gel electrophoresis in 1% agarose containing 3.5% formaldehyde.

2.3. cDNA synthesis

The first-strand cDNA of BCC18949 was synthesized using the total RNA as a template. The 20 μl reaction contained 0.5 μg of oligo$(dT)_{18}$, 1 μl of 10 mM dNTPs and 10 pg-5 μg of total RNA. The reaction was incubated at 65 °C for 5 min and kept on ice for 1 min. The reaction mixture was then supplemented with 4 μl of 5× First-Strand buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT and 1 μl of SuperScriptIII RT (Invitrogen, Carlsbad, CA, USA) and incu-

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