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Identification of novel bacterial expansins and their synergistic actions on cellulose degradation

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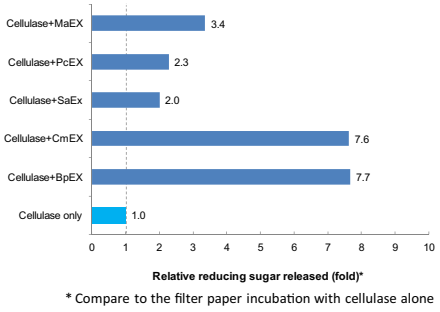
HIGHLIGHTS

- Bioinformatics pipeline was developed for annotation of putative expansins.
- A number of putative bacterial expansins were identified from public databases.
- The LDL motif conserved among entire bacterial expansin family was identified.
- Five identified expansins showed varying degree of synergy on filter paper hydrolysis.
- Optimal enzyme mixture showed strong synergism on pretreated rice straw degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Novel expansins, non-catalytic proteins which induce weakening of the rigid cellulose structure, have been identified in this study. A pipeline of bioinformatics was implemented for sequence and structure-based prediction of putative bacterial expansin-like group × family from NR databases. All putative expansins had no detectable activity against cellulosic and hemicellulosic substrates but showed varying degrees of synergy (2.0–7.6 folds) with the commercial *Trichoderma reesei* cellulase (Celluclast™ 1.5 L) on degradation of filter paper in order of BpEX ≈ CmEX > MaEX > PcEX > SaEX. A mixture design with full cubic model predicted optimal formulation comprising Celluclast™: CmEX from *Clavibacter michiganensis* = 72.4%: 27.6%, with no synergy of β-glucosidase on degradation of alkaline pretreated rice straw. Under these conditions, the reducing sugar yield was 163.6% compared with the reaction containing cellulase alone. This work demonstrated the potential benefit of novel bacterial expansins on enhancing cellulose degradation efficiency in lignocellulosic biomass degradation.

1. Introduction

Lignocellulosic biomass is the most abundant and sustainable renewable carbon source for biofuel, biochemical and biomaterial production (Lynd, 1996). Lignocellulose is the major structural component of plant cell wall. The three major components of

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lignocellulose, namely cellulose, hemicellulose, and lignin, are tightly packed into a rigid structure (Howard et al., 2003; Van Dyk and Pletschke, 2012). Lignocellulose is recalcitrant to enzymatic degradation because of the crystalline structure of cellulose (Park et al., 2010), the high degree of polymerization of polysaccharide (Merino and Cherry, 2007), and the presence of lignin and hemicellulose which restrict enzyme accessibility to the cellulose fiber (Grabber et al., 2008). Complete saccharification of lignocellulosic biomass requires a combination of cellulolytic and hemicellulolytic enzymes which act in a synergistic manner (Himmel et al., 2007). Cellulolytic enzymes can be classified according to their modes of action as endoglucanase, exoglucanase, or β-glucosidase (Arantes and Saddler, 2010; Davies and Henrissat, 1995; Van Dyk and Pletschke, 2012). Endoglucanases randomly hydrolyze intramolecular β-1,4-glucosidic bonds in cellulose (Liu et al., 2009). Exoglucanases hydrolyze cellulose from the reducing and non-reducing ends, producing either cellobiose or glucose as products (Davies and Henrissat, 1995), while β-glucosidases cleave cellobiose into glucose residues. Endo-xylanase and β-xylosidase are the major hemicellulolytic enzymes responsible for hydrolysis of xylan, which is the main component of hemicellulose. Other enzymes capable of acting cooperatively to degrade the heterogeneous hemicellulose structure include acetyl xylan esterase, α-arabinofuranosidase and mannanase (Kosugi et al., 2002; Van Dyk and Pletschke, 2012).

In order for plants to grow and develop, plants employ non-hydrolytic proteins called expansins, which loosen the network of cellulosic packaging in the plant cell wall (Arantes and Saddler, 2010; Cosgrove, 2000a). This property of expansins improves the cooperative action of cellulases and hemicellulases on cellulosic degradation. Expansins are approximately 25 kDa proteins consisting of two domains, D1 and D2, separated by a linker sequence (Kerff et al., 2008). Expansins can be classified into four families, namely α-expansin (EXPA), β-expansin (EXPB), α-like expansin (EXLA), and β-like expansin (EXLB). Recently, expansin-like proteins have been found in several fungi and bacteria, e.g. swollenin from *Trichoderma reesei* and BsEXLX from *Bacillus subtilis*. However, the direct relationship of these non-plant proteins to the four plant expansin families is unclear, and so they are grouped together in the expansin-like family X (EXLX) (Kende et al., 2004). Expansin proteins from different families share 20–40% amino acid identity, and the highest degree of conservation is in domain 1. This domain shows structural and sequence similarity to the catalytic domain found in glycosyl hydrolase family 45 (GH45). Expansin domain 1 lacks a critical Asp residue conserved in GH45 hydrolases, and thus possesses no plant cell wall polysaccharide hydrolysis activity (Yennawar et al., 2006). A cellulose binding domain (CBD) is found in domain 2, which is responsible for substrate binding (Sampedro and Cosgrove, 2005). Nevertheless, the actions of these expansin-like proteins from bacteria and fungi have not been investigated in details.

The loosening action of expansin has been proposed as a non-enzymatic mechanism for disrupting the non-covalent bond between the matrix polysaccharide and the surface of cellulose microfibrils (Cosgrove, 2000a; McQueen-Mason and Cosgrove, 1994). The action of expansins causes the glucan chains within the microfibrils to be more accessible to cellulolytic enzymes. However, this mechanism of expansin during enzymatic hydrolysis of cellulose has not been elucidated. Several studies have demonstrated that expansins can enhance the efficiency of cellulose hydrolysis. For example, Zea h protein purified from fresh postharvest corn stover showed synergistic interaction with cellulase and increased degradation of filter paper, even though it did not have any detectable cellulase activity by itself (Han and Chen, 2007). An expansin-like protein named swollenin isolated from *T. reesei* was shown to disrupt and swell cotton fiber (Saloheimo et al., 2002). In addition, Kim and coworker demonstrated that BsEXLX1, the first bacterial expansin-like protein described from *B. subtilis* enhanced the enzymatic activity of cellulase for hydrolysis of filter paper by 240% (Kim et al., 2009). These results represent the potential use of expansins to increase lignocellulose hydrolysis efficiency for biotechnological application. Identifying novel expansins from bacteria sources and utilization of these expansins in a synergistic actions with existing cellulase will be beneficial to important industries such as bioethanol and pulp and paper production.

In this study, several computational tools were employed to search for novel bacterial expansin-like proteins with low sequence homology to known plant expansins from public databases. The function of five putative expansins on enhancing activity of *T. reesei* cellulases was demonstrated. A biomass degrading enzyme system comprising cellulase and expansin was then optimized for degradation of alkaline pretreated rice straw using a mixture design method. The work provides an important basis for increasing efficiency of lignocellulose degrading enzyme systems for biotechnological applications.

2. Methods

2.1. Biomass preparation

Rice straw was obtained from a local farm in Ayutthaya province, Thailand. The biomass was physically processed using a SM2000 cutting mill (Retsch, Haan, Germany) and sieved through a 0.5 mm mesh. The pretreatment of the rice straw was performed using 10% (w/v) NaOH (with a liquid/solid ratio of 3) and treated at 80 °C for 90 min in an autoclave. The pretreated biomass was washed with distilled water until its pH decreased to 7 and then dried at 60 °C. The chemical compositions of the alkaline pretreated rice straw were 68.7% cellulose, 9.4% hemicellulose, 4.4% lignin according to the standard TAPPI methods: α-cellulose (T203 om-83); Klason lignin (T222 om-83); and pentosans (T223 hm-84).

2.2. Bacterial strains and plasmids

Expression vector pET28a (+) and pET32a (+) (Novagen, Darmstadt, Germany) were used for expression of putative expansin genes. *Escherichia coli* DH5α was used as the host strain for DNA cloning. *E. coli* Rosetta™ (DE3) pLysS (Novagen, Darmstadt, Germany) was used as an expression strain. Bacteria were grown at 37 °C in Luria–Bertani (LB) medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl) or on 1.5% agar plates supplemented with appropriate antibiotics.

2.3. Bioinformatics search for bacterial expansins

The overall pipeline for identifying expansin candidates consisted of sequence annotation followed by an additional structure level prediction. Bacterial expansin candidates were initially screened by performing PSI-BLAST (Position Specific Iterative-BLAST) (Altschul et al., 1997) against NR (non-redundant protein database) using default parameters. The known bacterial expansin amino-acid sequence from *B. subtilis* YoaJ (PDB: 2BH0) was used as a query to identify sequences with putative expansin domains. The *E*-value cutoff and the number of iterations were defined as 0.003 and 5, respectively. MEME (Multiple EM for Motif Elicitation) (Bailey et al., 2009) was then applied to find conserved motifs of 21 homolog sequences that represented bacterial species of EXLX. The patterns of identified motifs were subsequently used as input for searching sequences containing the EXLX conserved motifs in NR database.