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Heterologous expression and production of *Trichoderma reesei* cellobiohydrolase II in *Pichia pastoris* and the application in the enzymatic hydrolysis of corn stover and rice straw

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ABSTRACT

The cellobiohydrolase II (CBH II) gene *cbh2* from *Trichoderma reesei* was cloned and its codons were optimized in accordance with the codon usage frequencies of the host *Pichia pastoris*. The AOX1 strong promoter inducible by methanol was employed to efficiently express the foreign gene *cbh2* in *P. pastoris*. It was found that 5.84 ± 0.42 U cm^{-3} CBH II was obtained at 96 h using the synthetic *cbh2* gene whose codons were optimized, 2.02-fold higher than using the native *cbh2* gene (2.89 ± 0.32 U cm^{-3}), indicating that the codon optimization strategy was an effective approach to enhance the heterologous expression of CBH II in *P. pastoris*. The product of recombinant *P. pastoris* CBH II had an approximate molecular weight 58 kDa. Its optimal pH and temperature were 5.0 and 50 °C, respectively. The recombinant CBH II was used to enhance the yields of the enzymatic hydrolysis of the corn stover and rice straw pretreated with sodium hydroxide by improving the exo-exo-synergism between CBH II and CBH I in *T. reesei* cellulase. The yields 94.7% and 83.3% were achieved in the enzymatic hydrolysis of corn stover and rice straw pretreated by sodium hydroxide, respectively.

1. Introduction

During the bioprocesses of converting lignocelluloses which are one of the most abundant renewable resources on the earth to reducing sugars that could be used as feedstock for a variety of value-added chemicals such as fuels, cellulase with a complete composition plays a very important role, presenting a hopeful alternative for conventional chemical processes. *Trichoderma reesei*, a mesophilic soft-rot ascomycete fungus that is widely used as the biotechnological workhorse of the genus, is a household name in both academic and industry-driven studies into lignocelluloses degrading enzymes and their applications [1–3]. A complete mixture of cellulase from *T. reesei* is composed of cellobiohydrolases (CBH: EC 3.2.1.91), exoglucanases which release cellobiose as main product from crystalline cellulose; endoglucanases (EG: EC 3.2.1.4), preferably attack amorphous cellulose and some short chain oligomers; and β-glucosidases (EC 3.2.1.21), which hydrolyze cellooligosaccharides and cellobiose into glucose [4–6].

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Synergisms exist between the components of *T. reesei* cellulase. The best known is the synergism between β-glucosidases and cellobiohydrolases. Many studies pivoted the β-glucosidases to enhance that synergism, diminishing the inhibitory effect of cellobiose accumulation in enzymatic hydrolysis on cellobiohydrolases (and endoglucanases) caused by the deficiency of *T. reesei* cellulase in the β-glucosidase content [5–8]. Nonetheless the exo-exo-synergism between cellobiohydrolase I (CBH I) coded by *cbh1* gene and the cellobiohydrolase II (CBH II) coded by *cbh2* gene in *T. reesei* cellulase is of importance because CBH I mainly acts on reducing ends and CBH II preferably acts on non-reducing ends, preparing a more readily hydrolysable substrate for each other [4,9]. In *T. reesei*, CBH II has higher specific activity toward microcrystalline cellulose than CBH I, but the latter one has 50–60% of the total secreted protein of *T. reesei* due to the fact that the promoter of *cbh1* gene is regarded as a strong promoter [10]. In contrast, CBH II only accounts for 10–15% of total protein [10], thus rendering it deficient in CBH II that is essential to an excellent exo-exo-synergism. So improving CBH II is important to a great performance of *T. reesei* cellulase in enzymatic hydrolysis of lignocelluloses and the deficiency of CBH II is one of the limiting factors of *T. reesei* cellulase needed to be overcome using different strategies.

Producing *T. reesei* CBH II in *Pichia pastoris* is a desirable approach because *P. pastoris* is an excellent expression system for expressing heterologous protein in secreted forms with many advantages among which the most important one is its perfect protein processing mechanism including signal peptide cleavage, protein folding, and post-translational modification inside the cell, and secretion ability into medium with normal function [11]. Owing to the large difference of codon usage preference in different species and some other reasons such as unstable mRNA of exotic gene, heterologous gene expressions always result in low extracellular proteins production, especially for those proteins with active functions. Codon optimization in accordance with the codon usage preference of host cell, therefore, is a promising and effective technique to increase heterologous protein expression level. This has been proven by some reports [11–13], although they had differentiated outcomes.

Corn stover and rice straw, main agricultural residues in northern and southern China respectively, could be made use of as renewable feedstock for biorefinery. A great amount of corn stover and rice straw is always set on fire every year in China, giving rise to severe environmental problems. Producing lignocellulosic ethanol or other value-added products from corn stover or other agricultural residues, therefore, is feasible and has many advantages in China [14]. Thus corn stover and rice straw were used as substrate of enzymatic hydrolysis to compare the performance of the cellulolytic enzyme mixture. Pretreatment is required to smash the recalcitrant structure of lignocelluloses naturally formed before enzymatic hydrolysis in order to increase the enzymatic digestibility and to facilitate subsequent conversion. Sodium hydroxide pretreatment, carried out at lower temperature and pressure than acid hydrolysis and steam explosion, can substantially increase the lignin removal and enhance the accessibility and digestibility of cellulose [7,15]. So it was used in this work for the pretreatment of corn stover and rice straw.

In this work, the codons of *T. reesei cbh2* gene were optimized according to the codon usage preference of *P. pastoris*. Then the synthetic *cbh2* gene with codons optimized and the native *T. reesei cbh2* gene were put into the *P. pastoris* expression vector pPIC9K containing the α-Factor secretion signal, respectively. The former one was used for the efficient expression in *P. pastoris* GS115 and the latter one was used as control. The enzymatic properties of recombinant CBH II were investigated. And the recombinant CBH II was added to the cellulolytic enzymes mixture so as to enhance exo-exo-synergism between CBH I and CBII, thereby improving the enzymatic hydrolysis yields of corn stover and rice straw.

2. Material and methods

2.1. Microorganisms, plasmids and mediums

Escherichia coli strain DH5α was used for plasmid manipulation and propagation throughout the work as described by Sambrook et al. [16]. *T. reesei* ZU-02 [4,8,17] provided total mRNA containing the mRNA of the *cbh2* gene used as the template of reverse transcriptase polymerase chain reaction (RT-PCR) for producing cDNA, which was subsequently used as the template of native *cbh2* gene cloning. *P. pastoris* GS115 was the host of *cbh2* gene expression. All the microorganisms above were from the strain collection of Key Laboratory of Biomass Chemical Engineering of Ministry of Education, Zhejiang University.

The pMD18-T cloning vector (Takara, Otsu, Japan) was used for TA cloning. The plasmid pPIC9K, harboring α-factor signal peptide gene and the methanol-inducible alcohol oxidase gene (AOX1) that is a strong promoter controlling recombinant gene expression, was employed as expression vector of *cbh2* gene in *P. pastoris* GS115 [18].

Luria–Bertani (LB) medium for *E. coli* cultivation had the following composition: 10 kg m^{-3} tryptone, 5 kg m^{-3} yeast extract, 10 kg m^{-3} NaCl, pH 7.0. The potato dextrose agar (PDA) slants used for short term *T. reesei* ZU-02 culture preservation were composed of 200 kg m^{-3} potato, 20 kg m^{-3} dextrose and 20 kg m^{-3} agar. The seed medium for *T. reesei* was as follows: 15 kg m^{-3} glucose, 20 kg m^{-3} yeast extract, 2.5 kg m^{-3} $(NH_4)_2SO_4$, kg m^{-3} KH_2PO_4, 0.8 kg m^{-3} $MgSO_4$, kg m^{-3} $CaC1_2$, 0.005 kg m^{-3} $FeSO_4 \cdot 7H_2O$, 0.0016 kg m^{-3} $MnSO_4 \cdot H_2O$, 0.0014 kg m^{-3} $ZnSO_4 \cdot 7H_2O$, 0.0037 kg m^{-3} $CoCl_2 \cdot 6H_2O$. The ingredients of YPD medium for *P. pastoris* GS115 cultivation were as follows: 10 kg m^{-3} yeast extract, 20 kg m^{-3} peptone, 20 kg m^{-3} glucose. MD medium used for screening transformation consisted of 3.4 kg m^{-3} yeast nitrogen base (YNB) without amino acids, 4×10^{-4} kg m^{-3} biotin, and 20 kg m^{-3} glucose. The composition of BMGY medium for seed preparation of *P. pastoris* transformants was as follows: 10 kg m^{-3} glycerol, 10 kg m^{-3} yeast extract, 20 kg m^{-3} peptone, 3.4 kg m^{-3} YNB, 10 kg m^{-3} $(NH_4)_2SO_4$ and 4×10^{-4} kg m^{-3} biotin in pH 6.0 0.1 mol L^{-1} potassium phosphate buffer. The BMMY medium for recombinant gene induction and fermentation was comprised of 5 kg m^{-3}