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Cocktail production of an endo-β-xylanase and a β-glucosidase from *Trichoderma reesei* QM 9414 in *Escherichia coli*

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ABSTRACT

The enzymes used for biomass decomposition comprise a major cost in the production of biofuels from lignocellulosic feedstocks. Engineering of key enzymes *de novo* in heterologous hosts provides one strategy for the rational improvement of enzyme cocktails. Until recently, *Escherichia coli* has remained the most commonly used host for recombinant protein expression. Nevertheless, to our knowledge, there are few reports describing the co-expression of biomass degrading enzymes in *E. coli*. In this study, bicistronic and dual-promoter constructs based on pET30a were built for the co-expression of an endo-β-xylanase gene (xyn) and a β-glucosidase gene (bgl) from *Trichoderma reesei* QM 9414 in *E. coli*. The internal ribosome binding site used in the bicistronic constructs was originally found in pET30a. In the dual-promoter constructs described here, a pET30a-derived BioBrick base vector was built for the standard assembly of two targeted genes. Compared with monocistronic constructs, the crude enzyme expressed from a bicistronic construct (xyn located upstream of bgl) and a dual-promoter construct (xyn located upstream of bgl) offered the comparable activity of two recombinant proteins. Our results indicated that the common commercial vectors, such as pET30a, could be modified and optimized for a particular co-expression strategy in *E. coli*.

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1. Introduction

The cost of enzymes for converting biomass materials to fermentable sugars is a major impediment to the development of a practical lignocellulosic ethanol industry [1,2]. Current commercial preparations, mainly derived from fermentation of the filamentous fungus *Trichoderma reesei*, contain more than 80 proteins [3]. Beyond a few of the better-characterized cellulases and hemicellulases, the roles of most of these proteins in lignocellulose deconstruction are poorly understood [4,5]. This causes difficulty in the development of more efficient enzyme cocktails with the partially defined complex mixtures [4]. Furthermore, although *T. reesei* has already undergone extensive strain improvement, it is difficult to achieve a protein yield of more than 100 mg/l using existing methods [1]. Co-production of the core enzymes *de novo* in heterologous hosts is one strategy to reduce the costs of enzymes [1,5]. This would permit elimination of nonessential enzymes, and focus on rational improvement of key enzymes required for efficient lignocellulose deconstruction.

Until the present, *Escherichia coli* remains the most commonly used host for recombinant protein expression because of its rapid growth, low production cost, high yields and ease of genome modifications [6]. Numerous biomass degrading enzymes have been successfully expressed in *E. coli* [7,8]. However, to our knowledge, there are few reports describing the co-expression of biomass degrading enzymes in *E. coli*. Usually, co-expression can be conducted using either single or multiple plasmids in *E. coli*. In the case of a single plasmid, this can be either poly-cistronic (*i.e.* having a single promoter for multiple genes that are transcribed in the same mRNA), or, alternatively, the plasmid can contain multiple genes, each controlled by a separate promoter (transcribed each in a distinct mRNA) [6]. Standard biological parts, such as BioBrick parts, which include promoter, RBS, and protein coding sequence, among others, are flanked by XbaI and SpeI restriction sites on their 5′ and 3′ ends, respectively [9]. At present, a BioBrick base vector used to provide and propagate BioBrick parts has already been created, which could be applied in a co-expression strategy [10].

In the present study, we constructed bicistronic and dual-promoter vectors for co-expression of an endo-β-xylanase gene (Uniprot accession no. P36217) and a β-glucosidase gene (Uniprot accession no. O93785) from *T. reesei* QM 9414 in *E. coli*. From

Abbreviations: xyn, endo-β-xylanase gene; bgl, β-glucosidase gene; XYN, endo-β-xylanase; BGL, β-glucosidase; *T. reesei*, *Trichoderma reesei*; *E. coli*, *Escherichia coli*; IPTG, isopropyl-β-D-thio-galactopyranoside; RBS, ribosome binding site; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OE-PCR, overlap extension PCR.

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the perspective of the best well-studied enzyme systems, β-glucosidase and endo-1,4-β-xylanase are key enzymes to catalyze the degradation of cellulose to glucose and hemicellulose to pentose monomers [1]. β-glucosidases could break down cellobiose and other short cello-oligomers hydrolyzed by endoglucanases and cellobiohydrolases to glucose [2,11]. Endo-1,4-β-xylanase catalyzing the hydrolysis of internal β-1,4-D-xylose units, is the crucial enzyme for xylan depolymerization [12].

The expression vectors tested in bicistronic and dual-promoter constructs were both based on the T7 promoter (pET30a) for transcriptional regulation in combination with an *E. coli* strain harboring the DE3 prophage. In bicistronic constructs, the internal RBS that originated from pET30a was introduced upstream of the second gene for co-expression of two genes. In dual-promoter constructs, a pET30a-derived BioBrick base vector was built for the standard assembly of two genes. The expression statuses of XYN and BGL in both co-expression strategies will be discussed. Moreover, because gene location affects enzyme expression significantly in the co-expression process, its effect will also be investigated in the work reported here [13,14].

2. Material and methods

2.1. Strains, plasmids and culture media

E. coli BL21 (DE3) (Novagen, USA) used as expression host was cultivated in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% sodium chloride (w/v)) at 37 °C, supplemented with 50 μg/ml kanamycin. *T. reesei* QM9414 (ATCC) was cultivated in the cellulose inducing medium (0.3% peptone, 0.2% $(NH_4)_2SO_4$, 0.05% yeast extract, 0.4% KH_2SO_4, 0.03% $CaCl_2{\cdot}2H_2O$, 0.03% $MgSO_4{\cdot}7H_2O$, 0.02% TWeen-80, 2% Avicel) at 30 °C. The pET30a expression vector was purchased from Novagen (USA).

2.2. Engineering of pET30a for bicistronic operons

In order to co-express two targeted genes in a bicistronic operon, the RBS and its surrounding sequence originating from pET30a (AATAATTTTGTTTAACTTTAAGAAGGAGATATACAT, 36 nt) were introduced upstream of the second gene.

2.3. Construction of the pET30a-derived BioBrick base vector

A pET30a-derived BioBrick base vector with the EcoRI and XbaI recognition sites upstream of T7 promoter and the SpeI and PstI recognition sites downstream of T7 terminator was constructed as follows: Firstly, the EcoRI recognition site of pET30a was deleted by PCR using primers 1 and 2 (Table 1); secondly, the sequence (originated from pET30a, SphI → NdeI) containing EcoRI and XbaI sites upstream of T7 promoter and no XbaI site downstream of T7 promoter was prepared by gene synthesis (Sangon, China), which was subcloned into EcoRI-deleted pET30a using primers 3 and 4 (Table 1); thirdly, the sequence containing SpeI and PstI sites was prepared by OE-PCR using primers 5–8 (Table 1), which was then ligated into EcoRI-deleted pET30a (containing EcoRI and XbaI sites upstream of T7 promoter) to create the BioBrick base vector. The pET30a plasmid was used as PCR template in the first and third processes.

2.4. Construction of the co-expression vectors

The xyn and bgl genes were amplified from *T. reesei* genomic DNA by OE-PCR using primers 9–12 and 13–14, respectively. The pxyn (xyn monocistronic expression vector, Fig. 1) and pbgl (bgl monocistronic expression vector, Fig. 1) were created by ligating the PCR products digested with NdeI and EcoRI into pET30a, respectively. The bgl gene, along with an internal RBS sequence and a 6× Histag, was amplified from pbgl using primers 15 and 16 (Table 1). The PCR product was digested with EcoRI and HindIII and ligated into pxyn digested with the same enzymes to create pxyn-rbs-bgl (bicistronic expression vector, Fig. 1). The bicistronic expression vector pbgl-rbs-xyn (Fig. 1) was constructed similarly with pxyn-rbs-bgl using primers 17 and 18 (Table 1). The xyn and bgl genes were ligated into pET30a-drived BioBrick base vector using primers 9, 18 and 13, 16, respectively. The dual-promoter vectors (pxyn-T7pr-bgl and pbgl-T7pr-xyn, Fig. 1) were constructed as described in Fig. 2.

Table 1
Primers used in the work.

Name	Sequence (5′ → 3′)[a]
Primer 1	GGAATTC CATATGCACCATCAT (NdeI)
Primer 2	CCC AAGCTTGTCGACGGAGCTCCAATTCGGA (HindIII)
Primer 3	ACAT GCATGC AAGGAGATGGCGCCCAAC (SphI)
Primer 4	GGAATTC CATATG TATATCTCCTTCTTAAAGT (NdeI)
Primer 5	CACC GCTGAGC AATAACTAGCATAA (Bpu1102I)
Primer 6	CTGCAGCGGCCGCTACTAGTAATCCGGATATAGTTC
Primer 7	TACTAGTAGCGGCCGCTGCAGTGGCGAATGGGACG
Primer 8	GGCC CACTACGTGAACCATC (DraIII)
Primer 9	GGAATTCCATATGCAGACGATTCAGCCC (NdeI)
Primer 10	AAGTTGATGACCTTGTTCTTGGTGCCGGGCTGCC
Primer 11	CGGCACCAAGAACAAGGTCATCAACTTCTCGGGCA
Primer 12	CCGGAATTCTTAATGGTGGTGATGATGGTGGCTGACGGTGATGGAA (EcoRI)
Primer 13	GGAATTCCATATGTTGCCCAAGGACTTTCAGTGGGGGTTCGCCA-CGGCTGCCTACCAGATCGAGGGCGCCGTC (NdeI)
Primer 14	CCGGAATTCTCAATGGTGGTGATGATGGTGCGCCGCCGCAATCAGCT (EcoRI)
Primer 15	CCGGAATTCAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT ATGTTGCCCAAGGACTTTCAG (EcoRI)
Primer 16	CCCAAGCTTTTAATGGTGGTGATGATGGTGGCTGACGGTGATGGAA (HindIII)
Primer 17	CCGGAATTCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG CAGACGATTCAGCCC (EcoRI)
Primer 18	ATAAGAATAAGCTTTCAATGGTGGTGATGATGGTGCGCCGCCGCAA-TCAGCT (HindIII)

[a] Restriction sites were underlined.

2.5. Protein expression

The constructed vectors 1–6 (Fig. 1) were transformed into *E. coli* BL21 (DE3) competent cells using a heat pulse at 42 °C. *E. coli* BL21 (DE3) strains containing recombinant vectors were grown in 100 ml LB medium (kanamycin: 50 μg/ml) at 37 °C until the optical cell density at 600 nm (OD600) reached 0.6–0.8, and the growth continued at 16 °C overnight after 0.1 mM IPTG was added.

2.6. Protein harvest and purification

After cultivation, cells were harvested by centrifugation (3000 × *g* for 15 min), washed with PBS buffer and resuspended in 50 mM Tris–HCl (pH 8.0). Cells were disrupted by sonication and cell debris and the insoluble fraction was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C. All purifications were performed using ÄKTA Purifier FPLC purification system and facilitated with the His Trap™ HP column (GE Healthcare, Sweden). Protein concentration was determined by the method of Bradford [15]. Protein expression was analyzed by 15% SDS-PAGE. Gel band intensity was determined using a Gel-Pro Analyzer 6.0 (Media Cybernetics, Bethesda, MD), and the total synthesized XYN/BGL was calculated as the value of XYN/BGL band intensity divided by the total intensity of each lane, as described previously [16].

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