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Effect of mRNA secondary structure on expression level of catalytic domain of XynI from *Caldicellulosiruptor saccharolyticus* DSM 8903 in *E. coli*

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Abstract: This study describes the cloning and expression of catalytic domain of XynI from *Caldicellulosiruptor saccharolyticus* in *Escherichia coli*. The 996bp gene encoding xylanase was cloned using the plasmid pET22b(+) and expressed in *E.coli* BL21 Codon Plus (DE3) RIPL. The gene was expressed as 35.8 kD protein in soluble form, but the expression level was rather low. MFOLD analysis of the sequence between the ribosomal binding site and the 5'-end codons of the gene showed that the start codon AUG was trapped in the mRNA secondary structure. Cloning the gene using pET28a(+) allowed expression of the protein to a level of 35% as compared to about 4% when expressed using pET22b(+). pET28a(+), having his-tag before the start codon, would prevent strong secondary structure formation thus allowing higher expression level.

Keywords: Xylanase, *Caldicellulosiruptor saccharolyticus*, MFOLD, rare codons, pET22b(+), pET28a(+)
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INTRODUCTION

Hemicellulose is one of the important polymers in plant biomass that needs hemicellulases for its degradation. Xylanases are amongst the most studied hemicellulases because they can degrade hemicelluloses in the most effective way[1]. Xylanases are produced by a variety of fungal and bacterial species[2]. A wide range of mesophilic xylanases have been cloned and expressed in *E. coli*[3]. Nevertheless, xylanases from extreme thermophiles have been a major attraction for most of the industrial applications. It has been shown previously that *Thermotoga maritima*, *Caldicellulosiruptor saccharolyticus*, *Thermoascus aurantiacus* and *Thermotoga petrophila* RKU1 are the few examples of thermophilic bacteria whose wide range of enzymes are being cloned and expressed in *E. coli* for commercial applications[4].

Xylanases find potential applications in bleaching of paper and pulp. In addition, they have also been used in baking and poultry feed industry[5]. These enzymes are also necessary in the biofuel industry where they are used in combination with the cellulases and β-glucosidases for efficient hydrolysis of plant biomass[6]. There is an ardent need to produce recombinant xylanases with high expression levels to meet the demands of industries.

Expression level of proteins in *E. coli* is affected by a number of factors[7]. Codon bias at N-terminus of genes is one of the most important factors that may increase or decrease the expression of recombinant proteins. Some studies suggest that rare codons increase translational efficiency by slowing ribosomal elongation rate during initiation[8,9], while others suggest that the mRNA structure rather than codon rarity is responsible for enhanced gene expression[10].

This paper describes cloning and expression of catalytic domain of XynI from *Caldicellulosiruptor saccharolyticus* DSM 8903 using two different vectors to look for enhanced expression.

MATERIALS AND METHODS

Bacterial strains and vectors

E.coli DH5α and *E.coli* BL21 Codon Plus (DE3) RIPL strains were used for cloning and expression of the xylanase respectively. Recombinant plasmid was constructed by ligating the gene of interest in the plasmid pET22b(+) and pET28a(+) (Novagen, Maidson, USA). Chromosomal DNA of *Caldicellulosiruptor saccha-rolyticus* DSM 8903 (kindly provided by Prof. D. B. Wilson, Cornell University, Ithaca, NY) was used as a source for the xylanase gene.

Cloning and expression

Sequence of catalytic domain of Xylanase I (Csac_0696) was retrieved from GenBank accession no. AF005382.1. The forward and the reverse primers 5'-GCATATGTCCCTTTGTGAAAAGTACAA-3' and 5'-GTCGAGTTACTCTTCTGGCACAAC-3' containing *NdeI* and *Hind III* restriction sites, respectively, used for PCR amplification of the 996bp gene were designed using the softwares NEBcutter[11], Primer 3.0[12] and Oligocalc[13]. PCR reaction was run for 30 cycles with denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 2 min using *Taq* DNA polymerase. Final extension was done at 72°C for 20 min. PCR product was purified using gel DNA

recovery kit (Vivantis Technologies, Malaysia) according to the recommended protocol.

Purified PCR product and pET22b(+) vector were double digested with *Nde*I and *Hind*III. The restricted products were purified and ligated. Competent cells of *E. coli* DH5α were transformed with ligation mixture and positive colonies were confirmed by restriction analysis of the extracted plasmid. The correctness of sequence of the insert was checked by performing DNA sequencing on Beckman Coulter CEQ™ 8000 Genetic Analysis System. The plasmid from positive constructs was purified and used to transform competent cells of *E. coli* BL21 CodonPlus (RIPL). The transformed cells were cultivated in 400 ml LB medium at 37°C up to an OD_{600} of 0.7-0.8 and then induced with 0.5 mM IPTG. After incubation for another 6 h and cells were harvested by centrifugation. After resuspension in 0.05 M phosphate buffer (pH 6.0) the cells were lysed by exposing these to 20 cycles, each of 30 sec sonication at amplitude 60 followed by interval of 60 sec, using a UP400S ultraschall processor (Dr. Hielscher GmbH, Teltow, Germany). The sonicated cells were centrifuged at 6,000 rpm for 20 min. The protein contents of the *E. coli* cells, the soluble and insoluble fractions of the lysed cells were analyzed on SDS-PAGE by the method of Laemmli[14].

Analysis for Rare Codons and mRNA Secondary Structure

In order to determine the number of rare codons downstream the start site ATG, an online Rare Codon Calculator (RaCC) (http://nihserver.mbi.ucla.edu/RACC/) tool was used. Possibility of mRNA secondary structure formation in the 5' end of the gene sequence was checked by determining the free energy values for the nucleotide sequence of first 37 nucleotides starting from ribosomal binding site using Mfold web-server[15].

Cloning and Expression in pET28a(+)

pET28a(+) vector was double digested with *Nde*I and *Hind*III and ligated with the previously restricted and purified PCR product. Competent cells of *E. coli* DH5α were transformed with ligation mixture and positive colonies were confirmed by restriction analysis of the extracted plasmid. The correctness of sequence of insert was checked by performing DNA sequencing on Beckman Coulter CEQ™ 8000 Genetic Analysis System. The plasmid from positive constructs was purified and used to transform competent cells of *E. coli* BL21 CodonPlus (RIPL). The transformed cells were cultivated in 400 ml LB medium at 37°C up to an OD_{600} of 0.7-0.8 and then induced with 0.5 mM IPTG. After incubation for another 6 h and cells were harvested by centrifugation. The culture was also grown in an auto-induction medium with lactose as the inducer[16]. The cultivated cells after suspension in 0.05 M phosphate buffer were lysed by ultrasonication through 20 cycles, each of 30 sec sonication at amplitude 60 followed by interval of 60 sec, using a UP400S ultraschall processor (Dr. Hielscher GmbH, Teltow, Germany). The cells were processed for the release of the expressed enzyme, which was then analyzed by SDS-PAGE as described above.

RESULTS & DISCUSSION

Cloning and expression in pET22(+)

Catalytic domain of XynI of 996bp was amplified, restricted and ligated into the expression plasmid pET22b(+). Analysis of the recombinant plasmid isolated from the colonies after transformation by colony PCR and restriction analysis confirmed presence of the insert. The correctness of the sequence of the gene insert was also confirmed by DNA sequencing. SDS-PAGE analysis of total proteins of *E. coli* BL21 Codon Plus (DE3) RIPL cells transformed with recombinant vector containing xylanase 10B gene showed successful expression of 35.8 kD xylanase (Figure 1).

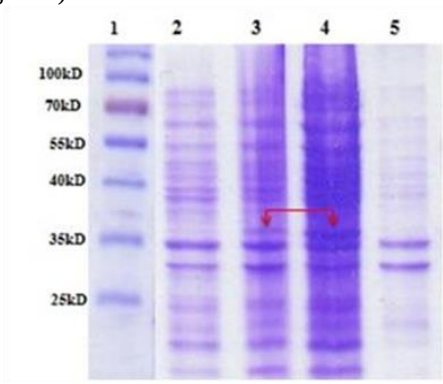

Figure 1: SDS-PAGE showing the protein profile in *E. coli* cells expressing catalytic domain of XynI cloned in pET22b(+) vector. lane 1: protein markers; lane 2. un-induced cells; lane 3: induced cell proteins; lane 4: soluble fraction of the lysed cells; lane 5: insoluble fraction of the lysed cells. Arrows indicate the enzyme bands.

Densitometric analysis of SDS-PAGE using Syngene Gene Tool Software revealed the expression level to be only 4% in total cell protein. Analysis of soluble and insoluble fractions obtained from ultrasonic disintegration of transformed cells showed the enzyme to be present in soluble fraction.

Obtaining high expression level of proteins in *E.coli* is always a hit and trial matter. Although various methods have been described so far in order to obtain over-expression of proteins, still there is no universal method to solve this problem. Co-expression of other genes and changing the vector, host, culturing parameters of host and/or gene sequences may increase the expression of proteins[17].

Other factors like *lac* operator and *lac* repressor genes in vectors have previously been reported to affect the expression level of proteins[18]. Nevertheless, the only significant difference between pET22b(+)-XynI and pET28a(+)-XynI constructs is the initial codons present downstream ribosome binding site.

Rare codon analysis for cloning in pET22b(+) versus pET28a(+)

Rare codons (AGG, AGA, CGA, CTA, ATA and CCC) are known to increase the expression level of proteins in *E.coli* [19]. Analysis of first 12 codons downstream ATG showed absence of any rare codon for both pET22b(+)-XynI and pET28a(+)-XynI constructs. This shows that higher expression of XynI in pET28a(+) vector is not due to rare codons.

MFOLD analysis for cloning in pET22b(+) versus pET28a(+)

mRNA secondary structure around the region downstream of the initiation codon has been reported to have an important impact on protein expression level [20,21]. It has been recently reported that it is mRNA folding rather than the rare codons that affects the expression level of protein[10]. MFOLD analysis sequence between the ribosome binding site and the twelve 5' end bases of the enzyme gene showed that the start codon AUG was trapped in secondary structure. Very low ΔG value (Δ=-6.70) of highly trapped structure revealed the possibility that the translation machinery may not access the start codon thereby hindering the rate of protein synthesis. This could be the reason for low expression level of target protein.

Mfold analysis showed that if the gene is cloned in pET28a(+) vector using same restriction sites i.e. *Nde*I and *Hind*IIII, start codon will be more easily available to the translation machinery in the possible secondary structure of mRNA (Figure 2). ΔG value obtained for this structure was -4.70.

Cloning and expression in pET28a(+)

Catalytic domain of XynI was also cloned using the expression plasmid pET28a(+) and the correctness of the sequence of the gene insert was also confirmed by DNA sequencing. SDS-PAGE analysis of the total proteins of *E. coli* cells transformed with this recombinant vector showed successful expression of 35.8 kD xylanase (Figure 3).

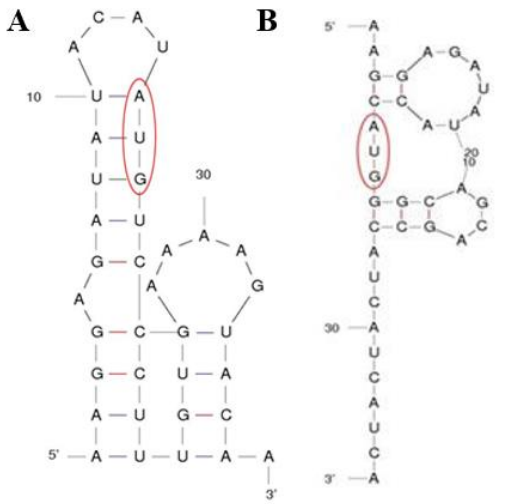

Figure 2: Possible secondary structures of mRNA for catalytic domain of XynI cloned in pET22b(+) (A) and pET28a(+) (B). Their ΔG values are = -6.70 and -4.70, respectively. Start codon (AUG) is encircled.

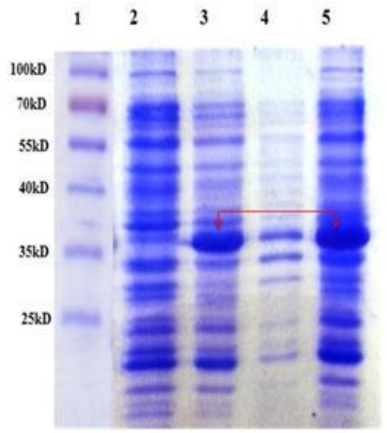

Figure 3: SDS-PAGE showing the protein profile in *E. coli* cells expressing catalytic domain of XynI cloned in pET28a(+) vector. lane 1: protein markers; lane 2. un-induced cells; lane 3: induced cell proteins; lane 4: insoluble fraction of the lysed cells; lane 5: soluble fraction of the lysed cells. Arrows indicate the enzyme bands.

Densitometric analysis of SDS-PAGE using Syngene Gene Tool Software revealed the expression level to be 35% in total cell protein. Analysis of soluble and insoluble fractions obtained from ultrasonic disintegration of transformed cells showed that most of the enzyme expressed was present in the soluble fraction of the cell lysate.

Current study reports the enhancement of expression of a xylanase (XynI) which can be highly beneficial enzyme for various industries. It has been shown that correct mRNA folding has a great impact on the expression of recombinant proteins in *E.coli*. Using the pET28a(+) vector, which did not allow the formation of strong secondary structure formation in the 5' region of gene, resulted in increased expression level to 35% of total cell proteins.

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