Subcloning of *Fusarium oxysporum* Endoglucanase Gene into pET39b(+) vector and Expression in *Escherichia coli*

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**Abstract**— Endoglucanase (EGI) is one of the key components of complex multienzyme system collectively known as cellulase, which degrade cellulose to glucose. Cellulases have been used in various industrial applications; however, there is demand for efficient and effective Cellulases with wide range of applications. Thus, protein engineering to create modified variants of these Cellulases for biotechnological and other applications will be highly desirable. A precondition for such an approach is a recombinant expression system. The endoglucanase gene has been cloned and expressed in *Escherichia coli* using pET-28a (+) expression vector but the recombinant endoglucanase were expressed as inclusion body. In this study, to express the recombinant endoglucanase in a soluble form, a DsbA (disulphide oxidoreductase) tag was placed before the gene using pET-39b(+) vector to obtain soluble and active endoglucanase. Thus the targeted soluble and active endoglucanase is expected to be more efficient and posses improved characteristics that will facilitates it wider industrial applications.

**Keywords**—Cellulases, Endoglucanase, Inclusion body, Overexpression, Soluble expression

I. INTRODUCTION

CELLULASE is a group of enzymes that degrade cellulosic materials and belong to the O-glycoside hydrolases (EC 3.2.1.x), hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety [1]. Endo-1, 4-beta-glucanases (EC 3.2.1.4), or endoglucanases, can degrade the beta-1, 4-linkages of cellulose, the most abundant and renewable source of energy on earth. [2]. Endoglucanases is one of the key components of cellulase, which has been known and used in various industries. In the textile industry and laundry for stain removal, fabric softening, depilling, color [3], biopolishing, biofinishing and smooth surfacing of cotton fabrics [4],[5], food and feed [6],[7], Pulp and paper [8], baking, alcohol from biomass, and waste treatment[9],[10].

*Fusarium oxysporum* is an ubiquitous, complex soilborne fungal species consisting of saprophytic and pathogenic isolates. Pathogenic isolates of *F. oxysporum* are based on host plant specificity and further onto pathogenic races based on host cultivar specificity. Many forms of *Fusarium oxysporum* have been identified, most of which cause severe wilt diseases to many economically important crops [11]. *Fusarium oxysporum* is one of the most potent fungi [12] for the degradation of the cellulolytic materials and the genus *Fusarium* is indeed widely being used in industrial applications such as detergents [13] and food [14] due to their ability to produce extracellular cellulolytic enzymes.

Among many expression systems available for heterologous expression of recombinant proteins, *Escherichia coli* (E. coli) is the most attractive system due to various reasons [8]. *Escherichia coli* remain the most predominant expression host for major production of recombinant cellulases to date [16], [17], and [18].

II MATERIALS AND METHODS

A. Strains, Growth media and Vector

*E. coli* BL21 (DE3) was used as the expression host for egl, which is available in One Shot® BL21 (DE3) Kit (Invitrogen). Other E.coli strains used were TOP10, which is available in Zero Blunt ® TOPO® PCR Cloning Kit (Invitrogen), and expression vector pET39b (+) was from Novagen. Luria-Bertani (Merck KGaA, Darmstadt, Germany) medium [1%tryptone, 0.5%yeast extract, and 1% sodium chloride (w/v)] supplemented with 50mg/ml kanamycin was used for cultivation.

B. Construction of Expression Vector

The recombinant *Escherichia coli* containing the WTegl-pET 28a plasmid constructs from Associate Prof. Dr Hamzah's Molecular and Genetic lab at International Islamic University Malaysia (IIUM). (Unpublished results) was grown and plated on selective plate (LB/Kan). A single
colony was isolated, extracted and analyzed by agarose gel electrophoresis. New set of primers were designed and the amplified gene purified and ligated into Zero-Blunt TOPO. Cloning into pET-39b (+) was achieved by using a forward primer (5'-CCATGGATGCAGACC-3') and a reverse primer (5'-ACTAGTGCTCAGCCCTTAC-3') having restriction sites SpeI and NcoI. The PCR fragments were purified using the QIAquick PCR Gel Extraction Kit (Qiagen, Hamburg, Germany). The purified PCR fragments were digested with SpeI and NcoI endonucleases (NEB, USA) and then ligated into the SpeI and NcoI sites of the pET-39b (+) vector. The resultant plasmids were transformed into E. coli strain BL21DE3. The correct insertion was confirmed by DNA sequencing, restriction digestion and colony PCR.

C. Protein Expression and Purification

The recombinant strains were grown in LB medium supplemented with kanamycin (50 mg/ml), at 37°C until OD 0.6, induced with 0.005 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG) and grown for an additional 24h at 20°C. The bacterial cells were collected by centrifugation at 6000g for 20 min, suspended in bugbuster solution in accordance to the pET system manual (Novagen). The lysate was then centrifuged at 16,000g for 20 min to remove the insoluble cell debris. Each of the culture samples was tested for the expression of protein on sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis (SDS-PAGE). The recombinant proteins from the crude cell lysate were concentrated and purified by column chromatography using Ni-NTA resin and the proteins were eluted with an elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM imidazole). The column fractions were identified in each purification step by 12% SDS-PAGE.

D. Enzyme Assay

EG, activity was determined by measuring the amount of reducing sugars released from CM-cellulose by the DNS-method using glucose as standard [5]-[7]. Reaction mixtures contained 40 μl enzyme solutions, 50 μl 2% CMC and 10 μl 0.5mM citrate phosphate buffer (pH 4.8). After incubation at 50°C for 30 minutes, the reaction was terminated by the addition of 100-μl dinitrosalicylic acid (DNS) reagent. The mixture was boiled for 10 minutes, cooled under tap water, and the absorbance was noted at 550 nm against blank. One unit of enzyme activity was defined as the amount of enzyme that produced one μmol reducing sugar equivalents per min under the assay conditions.

III. RESULTS AND DISCUSSION

The targeted gene was successfully sub-cloned into pET 39b (+) vector and transformed into E.coli (BL21 DE3) as shown in figure 1, with the cut at the desired size of 1248bp and 6100bp for the gene and vector respectively. This was also confirmed by sequencing analysis (result not shown).

Protein expression and purification study of the crude and the purified endoglucanase are shown in figures II and III, respectively. The size of the expressed gene is approximately 66kDa, which is lower than the expected calculated size of about 68kDa from the Expacy molecular Wt/pl compute.

Fig. 1 Gel electrophoresis from restriction enzyme analysis. Lane1: M, standard molecular marker; 2, 3 and 4 digested pET39b(+) -wt EG I plasmid

Fig. 2 SDS-PAGE of EG1 from crude lysate, protein marker (lane 1)
IV. CONCLUSION

The production of endoglucanase using pET39b(+) in E. coli (BL21DE3) was carried out in this study. The expression at lower temperature and inducer concentration appears to be soluble though the actual size of the protein is not very clear. This may be as a result of low protein expression however, retransformation, optimization of the process conditions, application of different purification and detection techniques may improve the discrepancies in order to meet standards for industrial applications.

REFERENCES