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Production of truncated peptide (cellobiohydrolase Cel6A) by *Trichoderma reesei* expressed in *Escherichia coli*

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Enzymatic cellulose hydrolysis is an important step for the production of second-generation biofuels. The filamentous fungus *Trichoderma reesei* is among the most important organisms for obtaining cellulolytic enzymes. The Cel6A (CBH II) cellulase from *T. ressei* plays an important role in cellulose hydrolysis and acts on the non-reducing end of cellulose, in contrast to Cel7B (CBH I), which acts on the reducing end of cellulose thus releasing cellobiose. Therefore, Cel6A deficiency becomes a limiting factor in cellulose saccharification. This work attempted to use codon optimization to enhance Cel6A expression in *Escherichia coli*. A plasmid expression vector, pUCITD04, was designed; this vector contains: the *cel6a* gene, regulatory regions (the promoter and terminator T7 sequences), the OmpT signal peptide that allows the secretion of proteins into the culture medium, and a 6His tail to allow purification of the protein by affinity chromatography. The protein expression experiment using a strain of *E. coli* transformed with pUCITD04 resulted in a 31 kDa polypeptide being secreted into the culture medium that did not possess enzymatic activity, meanwhile, the control strain transformed with the empty plasmid did not secrete any protein fragments, indicating that a truncated Cel6A was being produced by the experimental strain. This phenomenon has been reported during the production of recombinant cellulases in *E. coli.* In this research, we discuss probable causes of this phenomenon, as well as the drawbacks in the production of cellulases by *E. coli,* directing efforts to elucidate the causes of the production of truncated cellulases by this bacterial factory.

Key words: Gene construct, recombinant cellobiohydrolase, *Escherichia coli*.

INTRODUCTION

Lignocellulose materials are mainly composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are sugar-rich fractions of interest for use in fermentation processes, as many microorganisms can

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use these sugars for growth and the production of various compounds, such as ethanol, food additives, organic acids, enzymes, pigments, and drugs (Robak and Balcerek, 2020). Cellulose is the most important glucose reservoir in the world; however, its industrial utilization is limited by its polymerization degree and crystallinity index, as well as its association with hemicellulose and lignin polymers. Particularly, the recalcitrant lignin compound can reduce the efficacy of lignocellulosic feedstocks. To resolve these limit, it is necessary to subject these materials to pre-treatment procedures (Zoghlami and Paës, 2019; Meneses et al., 2020).

The hydrolysis of cellulose may be achieved via chemical or enzymatic procedures. Specifically, the enzymatic procedure requires a consortium of cellulolytic enzymes, including endoglucanases, cellobiohydrolases, and ß-glucosidases (Østby et al., 2020). This consortium is produced by numerous microbial groups, with *Trichoderma reesei* highlighted as a principal producer of cellulolytic enzymes (Runajak et al., 2020). These enzymes are key to developing a viable biorefinery process, which requires the cost-effective production of fermentable sugars from lignocellulosic biomass. Supplementing these various enzymes to optimize the ratio of cellulase components in the enzyme cocktail is an important strategy to obtaining an efficient cellulose hydrolysis; however, implementing this strategy, requires obtaining sufficient amounts of individual cellulase proteins (Fubao et al., 2016). Due to this requirement, research efforts have been oriented towards the development of recombinant procedures such as recombinant enzyme production, particularly recombinant enzymes expressed on prokaryotic systems, such as *Escherichia coli*, as this is the most widely used host and presents rapid and elevated expression levels (Parisutham and Sung, 2012; Rosano and Ceccarelli, 2014; Demain and Vaishnav, 2016). This recombinant system has been widely demonstrated to be useful for expressing non-glycosylated proteins; additionally, the machinery that performs the transcription, translation, and protein folding of this system is known (Wruck et al., 2017). Moreover, the genome can be easily modified, the promoter control is not complex, and the number of plasmids copies can easily be altered (Virolle et al., 2020). This system is able to accumulate up to 80% of its dry weight in recombinant proteins and survive at various environmental conditions (Demain and Vaishnav, 2016; Kent and Dixon, 2019). However, heterologous proteins, which are frequently expressed intracellularly in *Escherichia coli*, require an expensive separation process that includes cell lysis and target protein purification (Zhou et al., 2018). On the other hand, overexpressed proteins often form inclusion bodies or aggregates in the cytoplasmic space, thus requiring complicated and costly pretreating processes to obtain biologically active proteins and resulting in low active protein yields (Choi et al., 2006; Cui et al., 2016; King-Batsios et al., 2018). The likelihood of incorrect folding increases with the routine uses of strong promoters and elevated inducer concentrations, which can result in product yields that exceed 50% of the total cell proteins (Sandomenico et al., 2020). One solution to this problem may be the extracellular production of heterologous proteins, which, in most cases, facilitates further processing as well as provides *in vivo* folding and stability, thus allowing the production of soluble and biologically active proteins at a reduced cost (Mergulhao et al., 2005; Clark and Pazdernik, 2016). Although transfer of proteins to the periplasm is an approach used to facilitate the recovery of recombinant proteins, this method can also increase the rate of protein degradation and the accumulation of secretion precursors, which induces the heat-shock stress response and leads to increased proteolysis (Sandomenico et al., 2020). Full knowledge of the target protein enables the choice of an appropriate method of protein production and facilitates the design of the signal peptide needed to transfer the protein to the periplasmic space (Kleiner-Grote et al., 2018). Given the aforementioned, the aim of this work was to demonstrate that the expression of an optimized gene codifying the production of cellobiohydrolase Cel6A recombinant enzyme results in protein production and transfer to the periplasm.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growing conditions

The *E. coli* strains Top10F´ and BL21 (DE3) were acquired from Invitrogen and Novagen, respectively, while the pUCITD04 expression vector was derived from a pUCIDT cloning plasmid engineered to express the codon optimized *cel6a* gene from *T. ressei*, AmpR (Table 1). Luria-Bertani culture medium was used to spread the strains, while M9 culture medium was used in the recombinant protein production assays (Miller, 1972). The cells were cultured in a liquid medium with vigorous agitation at a temperature of 37°C while cell growth was monitored via measurements of the absorbance at 600 nm. The recombinant strains were selected via the addition of 50 mg/mL kanamycin, sold by SIGMA-ALDRICH.

Design of the gene encoding the synthesis of β-cellobiohydrolase Cel6A

The gene used to encode the synthesis of the recombinant Cel6A was designed using the sequence encoding the synthesis of *T. reesei* Cel6A (XM 006962518.1) as a target; this sequence was obtained from the NCBI database and was optimized for its recognition by *E. coli*. The designed synthetic construct contains the T7 promoter, a lac operating region, a ribosome binding site, an OmpT signal peptide, the optimized coding sequence of *T. reesei* Cel6A, six codons for 6His tail synthesis, and the T7 transcriptional terminator. This construct was synthetized by Integrated DNA Technologies (IDT) Inc.

Table 1. Relevant strains and plasmids utilized in the current study.

Strain/plasmid	Genotype: Relevant characteristics	Source
***E. coli* Strains**		
E. coli Top10F'	F'[lacIq Tn10(tetR)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 λ-	Invitrogen
E. coli BL21 (DE3)	*E. coli* str. B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)	Novagen
Plasmid		
pUCITD04	Expression vector derivative of pUCIDT cloning plasmid, expressing the codon optimized Cel6A gene from *T. ressei*, AmpR.	This study

Native and synthetic β-cellobiohydrolase Cel6A structure

Three-dimensional structures of the native and synthetic Cel6A enzymes were constructed using Raptor X structures tool (Morten et al., 2012) and visualized with Discovery Studio software (Dassault, 2017).

Cloning of synthetic cellobiohydrolase cel6a gene and transformation of E. coli

The construct was cloned in the pUCIDT KanR plasmid by the Integrated DNA Technologies company (IDT). This plasmid was named pUCITD04. The synthetic plasmid contains the *Bam*HI and *Hind*III restriction sites for gene subcloning. This sequence was verified by the Synthesis and Sequencing Unit of the Biotechnology Institute of Autonomous National University of Mexico (UNAM). Insertion of the *cel6a* gene in the pUCITD04 plasmid was verified by restriction analysis using *Bam*HI and *Hin*dIII endonucleases and electrophoresis on agarose gel stained with EtBr.

Molecular biology techniques

Preparation of $CaCl_2$ competent cells, transformation tests, and plasmidic DNA extraction from *E. coli* were performed using the Sambrook techniques (Sambrook and Green, 2012). The *E. coli* BL21 (DE3) strain transformed with the pUCITD04 plasmid was used to produce the recombinant protein. Insertion of the plasmid with the synthetic *cel6a* gene in the *E. coli* BL21DE3 strain was verified by extraction of plasmidic DNA from the transformed strain and subsequent restriction analysis with *Bam*HI and *Hind*III enzymes. The transformed strain was inoculated on 50 mL of M9 culture medium supplemented with kanamycin and incubated at 37°C for the time necessary to reach an optical density (OD600) of 0.5.

SDS-PAGE analysis

The production of recombinant Cel6A enzyme was induced via the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the samples were incubated for 4 h at 37°C. During the incubation, a 1 mL sample was taken every 30 min and centrifuged, after which the supernatant was used to obtain protein via precipitation with a methanol:chloroform:water solution in a 4:1:3 V/V ratio. The precipitated proteins were resuspended in 100 µL of phosphate-buffer solution and 10 µL of the sample was analyzed by SDS-PAGE, applying a voltage of 100 V for 90 min.

Enzymatic activity determination

To determine enzymatic activity, 250 µL of enzyme extract was incubated with 750 µL of 0.1 M acetate buffer (pH 4.8) and 1% microgranular cellulose (as a substrate) for 1 h at 50°C (Montoya et al., 2015). Next, the samples were centrifuged for 5 min at 13,000 RPM, after which 500 µL of the supernatant was taken and the reducing sugars were determined via the DNS method (Miller, 1959).

RESULTS AND DISCUSSION

Design of cel6a gene

The sequence encoding synthesis of the enzyme Cel6A (XM 006962518.1) that is produced by *T. reesei* was obtained from the NCBI database and then optimized to be recognized and synthesized by *E. coli*. This protein (Figure 1) has a length of 471 amino acids, a homology of 100% with respect to the Cel6A protein, and a homology of 74% with respect to the gene sequence encoding the synthesis of Cel6A that is produced by *T. reseei*.

Modeling of native and synthetic structures of β-cellobiohydrolase Cel6A

According to Raptor X portal, the protein structures of the cellulose-binding domains (Figure 2) demonstrate minor differences due to the signal peptide added to the *cel6a* synthetic gene (Figure 2A) being slightly longer than the signal peptide of native Cel6A (Figure 2B). The catalytic domains of the native and synthetic Cel6A (Figures 3A and 3B) do not show any visual differences between their tertiary structures. The cellulose-binding domain is located between amino acids Methionine-1 and Glycine-

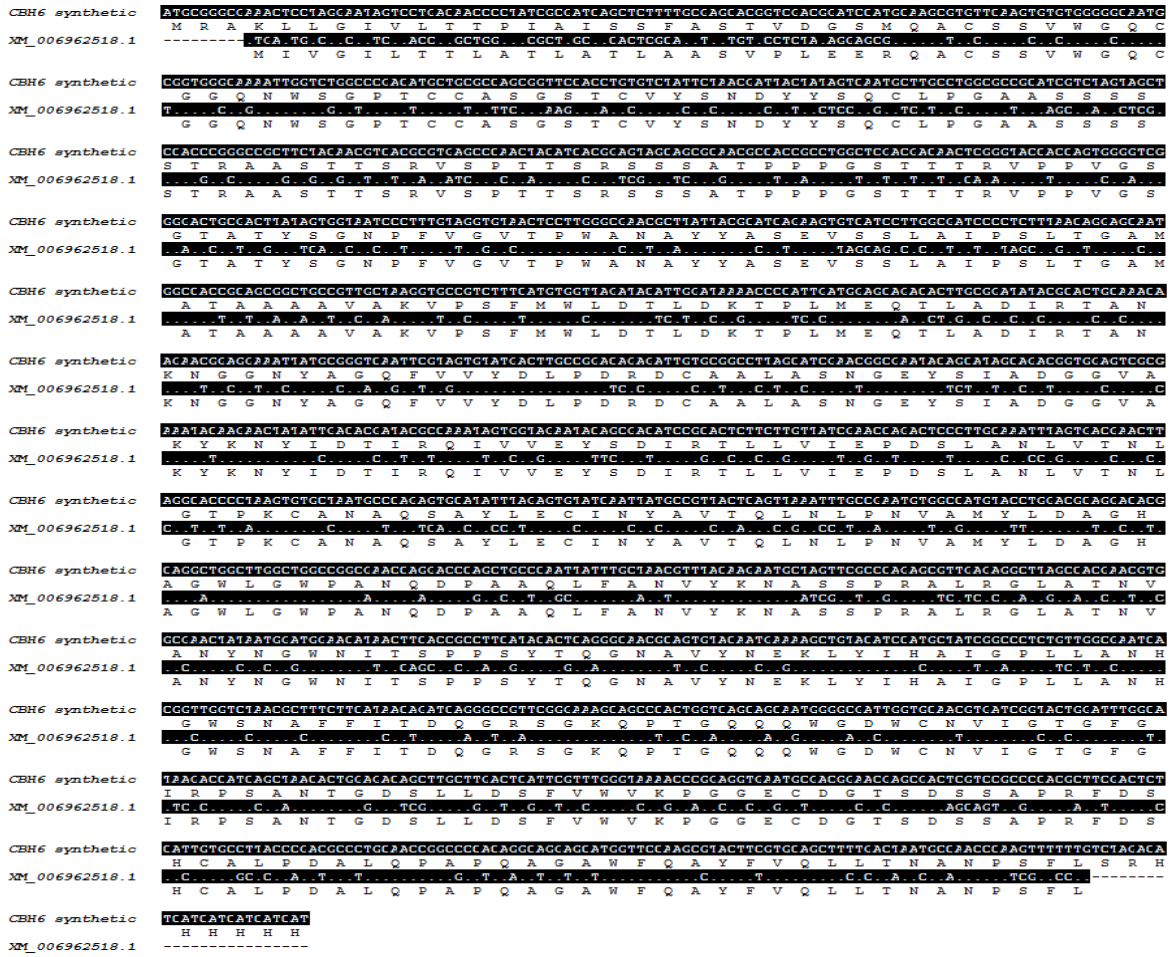

Figure 1. Alignment of the synthetic Cel6A sequence and the XM_006962518.1 sequence corresponding to the *Trichoderma ressei* native Cel6A. The nucleotides of both sequences are highlighted by a black shadow, identical nucleotides between both sequences are shown with dots, the OmpT signal peptide added to the synthetic sequence is framed with a solid line box, and the signal peptide of the native Cel6A from *T. ressei* is framed with a discontinuous line box, while the gaps (dashed lines) present at the beginning and ends of the amino acid sequences represent non-aligned sequences

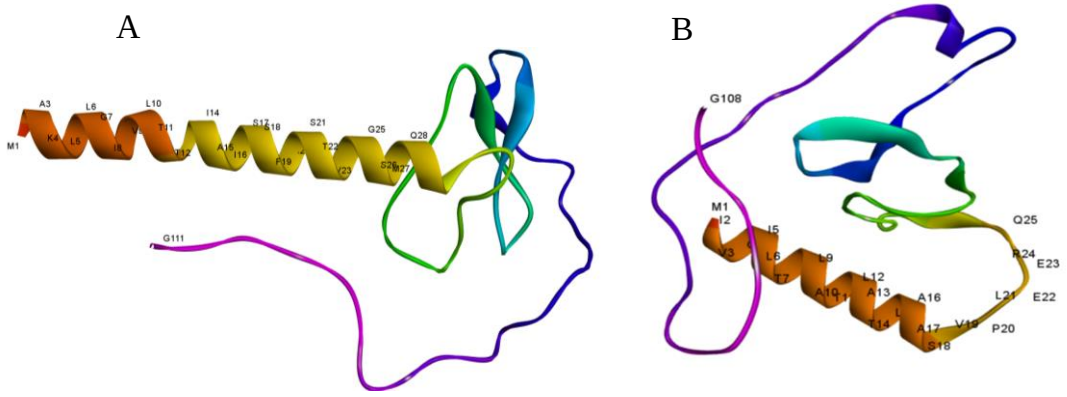

Figure 2. Comparison of the cellulose binding domain (CBD) of the native (A) and synthetic (B) Cel6A enzymes. The CBDs of both enzymes are between the amino acids Methionine-1 and Glycine-108.

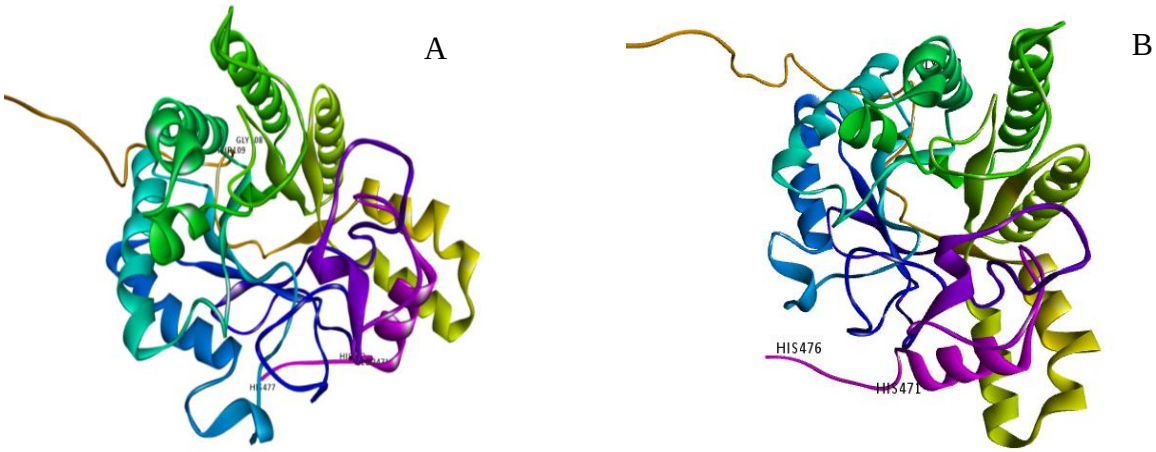

Figure 3. Comparison of the catalytic domain (CD) of the native (A) and synthetic (B) Cel6A enzymes. The CDs of the native and synthetic Cel6A enzymes are between the amino acids Threonine-109 and Leucine-471.

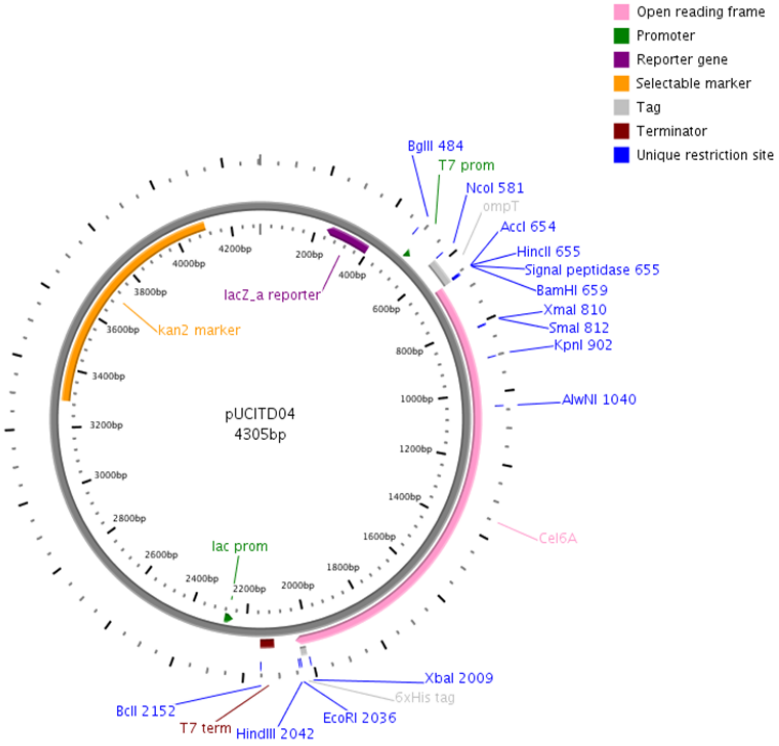

Figure 4. pUCITD04 plasmid map, showing the positions of the *cel6a* gene, the T7 promoter and T7 terminator regulatory zones, the OmpT signal peptide, the 6His tag, and the kanamycin marker.

108, while the catalytic domain is located between Threonine-109 and Leucine-471. The native protein has a molecular weight of approximately 50 kDa and is composed of 471 amino acids, while the synthetic protein has a molecular weight of 50.5 kDa and is composed of 477 amino acids.

The differences between native and recombinant enzymes are attributed to the addition of the six-histidine tail. The designed plasmid, pUCITD04, was utilized as a vector for the production of Cel6A recombinant enzyme in the *E. coli* BL21 (DE3) strain (Figure 4). Plasmid DNA extracted from transformed *E. coli* BL21 (DE3) cells

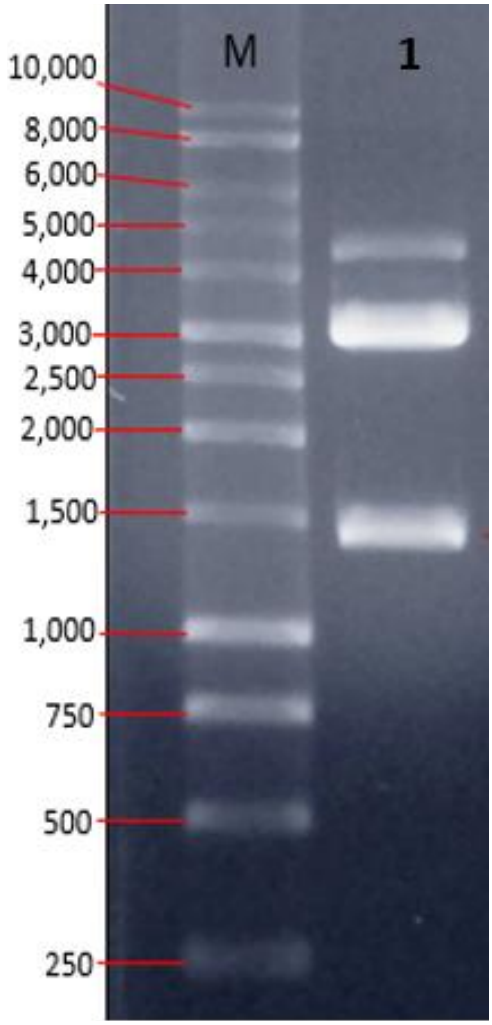

Figure 5. Restriction assay of the pUCITD04 plasmid. Lane M: DNA ladder (1Kb); Lane 1: restriction products from pUCITD04 hydrolysis with *Bam*HI and *Hind*III enzymes. The restriction mixture samples were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

resistant to kanamycin was subjected to a restriction analysis using hydrolysis with *Bam*HI and *Hin*dIII enzymes. Results of the restriction analysis showed that the 2705 bp pUCITD04 plasmid contained a 1446 bp fragment, which corresponds to the cel6a synthetic gene (Figure 5), indicating that the synthesized gene and plasmid were adequately constructed.

The designed and constructed plasmid contains the *cel6a* gene flanked by *Bam*HI and *Hind*III restriction enzyme sites, T7 promoter and T7 terminator regulatory regions, the OmpT signal peptide necessary for protein secretion into the culture medium, and a 6His Tag introduced to favour the purification of the enzyme via affinity chromatography (Freudl, 2018).

Expression and secretion of synthetic CBH Cel6A enzymes

Results from the expression and secretion of the natural and synthetic Cel6A enzymes show that synthetic Cel6A protein was not found in the cell extract. However, when using M9 culture medium with isopropyl-β-D-1-thiogalactopyranoside (IPTG) added as an inducer, results showed that the transformed BL21DE3 strain produced a 31 kDa peptide (Figure 6) that the native strain did not produce. The phenomenon of expression of truncated cellulases has been reported during protein production by recombinant *E. coli* cells (Liu et al., 2018), with prior observations indicating that periplasmic Cel6A is prone to proteolytic truncation in LK111 and K514 *E. coli* strains. The fractions obtained in ion-exchange columns were analyzed by zymogram analysis resulting in two carboxymethyl cellulase (CMScase) bands at 57 and 47 kDa. The larger of these bands corresponds to the full portion of the Cel6A protein, while the smaller band corresponds with proteolytic cleavage near the linker. In other research, an additional truncated CD with higher specific activity on soluble substrates was discovered, however, this enzyme was also found to be prone to proteolytic cleavage (Nakamura et al., 2020). *E. coli* BL21 (DE3) lacks the OmpT signal peptide and Lon proteases and produces large quantities of biomass with important effects on the production of recombinant proteins. While *E. coli* BL21 (DE3) is a genetically modified strain laking the Lon and OmpT proteases (Table 1), it may nonetheless contain low quantities of other proteases, such as DegP, Plp, HtrA, and ClpB, which degraded aggregated protein and, consequently, may impede Cel6A production (Gottesman, 1996; Laskowska et al., 1996; Langen et al., 2001; Jiang et al., 2002).

Protein expression using *E. coli* is the procedure most frequently used in bacterial expression as it is a well characterized procedure that is easy to genetically manipulate. However, the expression of cellulases in *E. coli* has encountered numerous problems, such as degradation of linker sequences in multi-domain cellulases, the formation of inclusion bodies, incorrect transportation across the outer membrane, and decreased specific activity of the cellulases (Choi et al., 2006). In contrast, the protein over-production system in *E. coli*, which is attributed to the RNA polymerase expression system of bacteriophage T7, is limited or incorrectly expressed in the BL21 (DE3) strain. The incorrect expression of Cel6A may be attributed to a toxicity problem caused by the pUCITD04 plasmid (Miroux and Walker, 1996). In our laboratory, the ß-glucosidase, endoglucanase, and xylose reductase

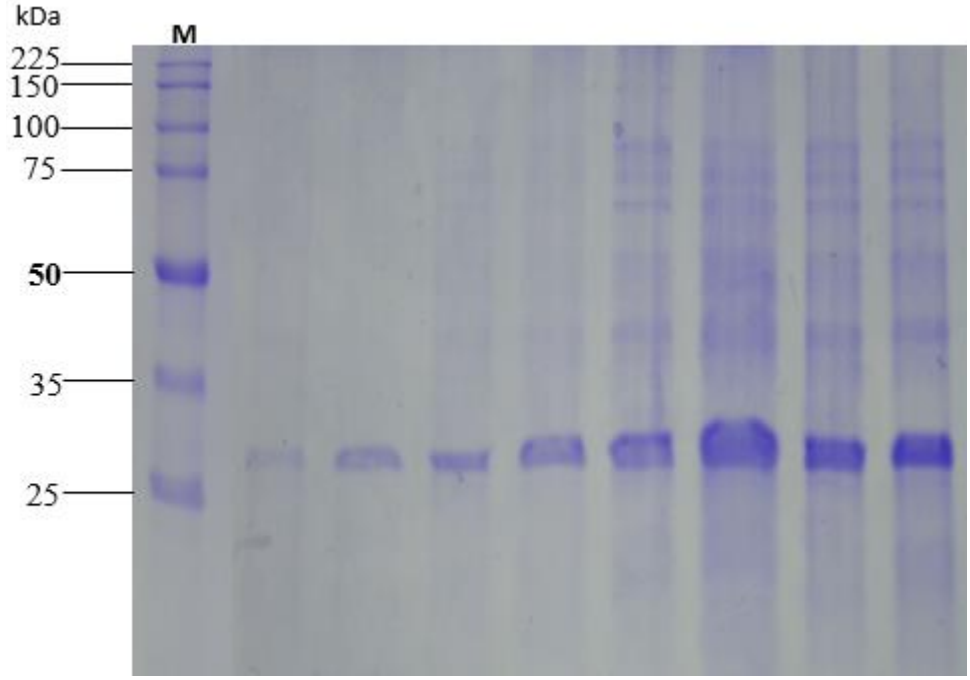

Figure 6. SDS-PAGE analysis of protein secreted into the culture medium by E. coli BL21 (DE3) transformed with the pUCITD04 plasmid. Lane M: molecular marker; lanes 1 to 8: secreted protein in samples taken at 30 min intervals. The secreted proteins were precipitated.

enzymes were expressed using similar conditions that the used for Cel6A recombinant enzyme, however, this protein is structurally more complex, and probably this is the reason that makes it difficult to produce. However, it has been observed that proteolytic cleavage between catalytic and cellulose-binding domains of some ß-glucanases occurs near the linker, and many modular-type-ß-glucanases contain two conserved cysteine residues near their cellulose-binding domains (Kont et al., 2016; Nakamura et al., 2020). The results observed suggest the possibility of exploring several alternatives, including the use of different carbon sources and galactose inducers to produce recombinant Cel6A, experimenting with new microbial vectors to achieve production of Cel6A recombinant enzyme, or production in a cell-free system to reduce complications relating to plasmid toxicity (Kigawa et al., 2004; Robak and Balcerek, 2020).

Conclusion

The vector pUCITD04 does not allow the production of Cel6A enzyme in the BL21 (DE3) *E. coli* strain; however, it does produce a 31 kDa periplasmic polypeptide that must belong to a fraction of Cel6A, although it lacks catalytic and cellulose-binding domains. Results demonstrate the necessity of exploring the use of new microbial vectors. Additionally, to prevent possible plasmid toxicity, it is important to investigate the use of cell-free systems to bypass potential residual proteolytic activity and the complications related to it.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Choi J, Chang KK, Lee S (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chemical Engineering Science 61(3):876-885.

Clark PD, Nanette JP (2016). Chapter 10 - Recombinant Proteins, Editor(s): David P. Clark, Nanette J. Pazdernik, Biotechnology

(Second Edition), Academic Cell pp. 335-363, ISBN 9780123850157, https://doi.org/10.1016/B978-0-12-385015-7.00010-7.
Cui Y, Meng Y, Zhang J, Cheng B, Yin H, Gao C, Xu P, Yang C (2016). Efficient secretory expression of recombinant proteins in *Escherichia coli* with a novel actinomycete signal peptide. Protein Expression and Purification 129:69-74.
Dassault S (2017). BIOVIA, Discovery Studio**.** Modeling Environment, Release 2017. San Diego: Dassault Systèmes 2016.
Demain A, Vaishnav P (2016). Production of Recombinant Enzymes. Reference Module in Food Science, Elsevier: Doi.org/10.1016/B978-0-08-100596-5.03023-7.
Freudl R (2018). Signal peptides for recombinant protein secretion in bacterial expression systems. Microbial Cell Factories 17(1):52. https://doi.org/10.1186/s12934-018-0901-3
Fubao FS, Xiaoqin Z, Jiapeng H, Yanjun T, Liang W, Haiyan S, Xiang L, Jinguang H (2016). Industrially relevant hydrolyzability and fermentability of sugarcane bagasse improved effectively by glycerol organosolv pretreatment. Biotechnology for Biofuels 9:59 DOI 10.1186/s13068-016-0472-7.
Gottesman S (1996). Proteases and their targets in Escherichia coli. Annual Review of Genetics 30:465-506. https://doi.org/10.1146/annurev.genet.30.1.465
Jiang X, Oohira K, Iwasaki Y, Nakano H, Ichihara S, Yamane T (2002). Reduction of protein degradation by use of protease-deficient mutants in cell-free protein synthesis system of *Escherichia coli*. Journal of Bioscience and Bioengineering 93(2):151-156. doi: 10.1263/jbb.93.151. PMID: 16233180
Kent R, Dixon N (2019). Contemporary Tools for Regulating Gene Expression in Bacteria. Trends in Biotechnology 38(3):316-333. doi.org/10.1016/j.tibtech.2019.09.007
Kigawa T, Yakubi T, Matsuda N, Nakajima R, Tanaka A and Yokoyama S (2004). Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression. Journal of Structural and Functional Genomics 5:63-68. doi: 10.1023/B:JSFG.0000029204.57846.7d
King-Batsios E, Pujol-García A, Tamargo-Santos B, Marrero-Trujillo G, Fontanies-Hernández M, Blain-Torres K and Sierra-González V (2018). Influencia del medio de cultivo sobre la cinética de crecimiento y expresión de la proteína recombinante Rv2626c de Mycobacterium tuberculosis H37Rv expresada en Streptomyces lividans TK24. Vaccimonitor 27(3):84-92.
Kleiner-Grote GRM, Risse JM, Friehs K (2018). Secretion of recombinant proteins from *E. coli*. Engineering in Life Sciences 18:532-550.
Kont R, Kari J, Borch K, Westh P, Väljamäe P (2016). Inter-domain Synergism Is Required for Efficient Feeding of Cellulose Chain into Active Site of Cellobiohydrolase Cel7A. Journal of Biological Chemistry 291(50):26013-26023.
Langen GR, Harper JR, Silhavy TJ, Howard SP (2001). Absence of the outer membrane phospholipase A suppresses the temperature-sensitive phenotype of Escherichia coli degP mutants and induces the Cpx and sigma(E) extracytoplasmic stress responses. Journal of Bacteriology 183(18):5230-5238. doi:10.1128/jb.183.18.5230-5238.2001
Liu Y, Liu S, Dong S, Li R, Feng, Cui Q (2018). Determination of the native features of the exoglucanase Cel48S from *Clostridium thermocellum*. Biotechnology for Biofuels 11(6).
Meneses D, Montes de Oca-VG, Vega BJ, Rojas-Álvarez M, Corrales-Castillo J, Murillo-Araya L (2020). Pretreatment methods of lignocellulosic wastes into value-added products: recent advances and possibilities. Biomass Conversion and Biorefinery. Doi.org/10.1007/s13399-020-00722-0
Mergulhao F, Summers D, Monteiro G (2005). Recombinant protein secretion in Escherichia coli. Biotechnology Advances 23(3):177-202. https://doi.org/10.1016/j.biotechadv.2004.11.003
Miller GL (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31(3):426-428.
Miller JH (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miroux B, Walker J (1996). Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. Journal of Molecular Biology 260(3):289-298.
Montoya S, Sánchez J, Levin L (2015). Production of lignocellulolytic enzymes from three white-rot fungi by solid-state fermentation and mathematical modeling. African Journal of Biotechnology 14(15):1304-1317.
Morten K, Haipeng W, Sheng, Jian P, Zhiyong W, Hui L, Jinbo X (2012) Template-based protein structure modeling using the RaptorX web server. Nature Protocols 7(8):1511-1522. doi: 10.1038/nprot.2012.085.
Nakamura A, Ishiwata D, Visootsat A, Uchiyama T, Mizutani K, Kaneko S, Murata T, Igarashi K, Iino R (2020). Domain architecture divergence leads to functionaldivergence in binding and catalytic domains of bacterialand fungal cellobiohydrolases. Journal of Biological Chemistry 295(43):14606-14617. Doi.org/10.1074/jbc.RA120.014792
Østby H, Hansen LD, Horn SJ (2020). Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives. Journal of Industrial Microbiology and Biotechnology 47(9-10):623-657.
Parisutham V, Sung KL (2012). Heterologous Expression and Extracellular Secretion of Cellulases in Recombinant Microbes. Bioethanol. Intechopen, London pp. 239-252.
Robak K, Balcerek M (2020). Current state-of-the-art in ethanol production from lignocellulosic feedstocks, Microbiological Research 240:226534. doi: https://doi.org/10.1016/j.micres.2020.126534
Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in microbial systems. Frontiers in Microbiology 5:341. DOI:10.3389/fmicb.2014.00341
Runajak R, Santi C, Wawat R, Malinee S, Prapakorn T, Somkiat P (2020). Analysis of Microbial Consortia with High Cellulolytic Activities for Cassava Pulp Degradation. E3S Web of Conferences 141, 03005, doi: 10.1051/e3sconf/202014103005 RI²C 2019.
Sambrook J, Green M (2012). Molecular cloning: a laboratory manual. In Molecular cloning: a laboratory manual (pp. 1890-1890).ISBN 978-1-936113-41-5 (cloth) – ISBN 978-1-936113-42-2 (pbk.)
Sandomenico A, Sivaccumar JP, Ruvo M (2020). Evolution of Escherichia coli expression system in producing antibody recombinant fragments, International Journal of Molecular Sciences 21(17):6324. DOI: 10.3390/ijms21176324
Virolle C, Goldlust K, Djermount S, Bigot S, Lasterlin C (2020). Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. Genes 11(11):1239.
Wruck F, Katranidis A, Nierhaus K, Büldt G, Hegner M (2017). Translation and folding of single proteins in real time. Proceedings of the National Academy of Sciences 114(22):E4399-E4407.
Zhou Y, Lu Z, Wang X, Selvaraj J, Zhang G (2018). Genetic engineering modification and fermentation optimization for extracellular production of recombinant proteins using *Escherichia coli*. Applied Microbiology and Biotechnology 102(4):1545-1556.
Zoghlami A, Paës G (2019). Lignocellulosic Biomass: Understanding Recalcitrance and Predicting Hydrolysis. Frontiers in Chemistry 7(874):1-11.