



# Cloning and Expression of *Ruminococcus flavefaciens* Cellulase-encoding Gene in *E. coli* as Feed Additive for Poultry

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**Abstract.** Carbohydrate-metabolizing enzymes are crucial in improving feed digestibility and efficiency in the poultry industry. Among these, cellulase, which breaks down cellulose, is particularly valued across various industries, especially in animal feed. However, producing cellulase in its native host, such as *Ruminococcus flavefaciens*, is often challenging due to complex cultivation requirements and limited yields. In this study, the cellulase-encoding gene from *Ruminococcus flavefaciens* was synthesized and expressed in *Escherichia coli*. The gene was codon-optimized and cloned into pET15b vector suitable for the *E. coli* expression system. The results showed a protein band at approximately 38 kDa, matching the expected size of the cellulase enzyme produced in *E. coli*. The optimized codon usage for *E. coli* significantly enhanced the soluble fraction of cellulase, which is crucial for large-scale production. In summary, the recombinant *Ruminococcus flavefaciens* cellulase-encoding gene was successfully expressed in *E. coli*. Although the expression of cellulase in *E. coli* was successful, further optimization is needed to increase yields and assess enzymatic activity.

**Keywords:** Carbohydrate-Metabolizing Enzyme, Poultry Industry, *Ruminococcus flavefaciens* Cellulase, Recombinant Protein, Codon Optimization, *E. coli* Expression System

## 1 Introduction

Sustainable poultry production can markedly contribute to achieving food security and economic well-being in most developing countries, such as Indonesia. Poultry production in a modern system is well-known to be more effective and sustainable

compared to other livestock, particularly in terms of the utilization of resources and the impacts on the environment per unit of production output [1–4]. It has previously been reported that broiler chickens consume 27–28 kg of dry matter feed to produce one kilogram of protein [5], emitting approximately 2.37 kg CO<sub>2</sub>-eq/kg carcass weight, including the effect of land use change [6]. Feeding is considered the most essential factor that has a significant impact on the economic sustainability of the poultry industry, accounting for up to 70% of the total production expenditures [1]. Importantly, more than 60% of the raw materials utilized in poultry diets directly compete with human food ingredients, such as wheat, maize and soybeans [5]. Therefore, there has been increasing interest in the utilization of local feedstuffs derived from agricultural and agroindustry by-products as alternative components in poultry feed, potentially reducing production costs and competition with human food ingredients. Nonetheless, the utilization of local feedstuffs is limited owing to anti-nutrient factors, high fibre content and low protein content affecting nutrient availability and digestibility [7]. In addition, non-starch polysaccharides (NSPs) and lignin are the main components of plant cell walls, comprising up to 20% of plant-based poultry diets [8, 9]. These components mostly remain undigested along the gastrointestinal tract (GIT) since poultry lacks endogenous NSP-hydrolyzing enzymes [9].

Carbohydrate-metabolizing enzymes have widely been applied as feed additives in the poultry industry. They are biocatalysts which are capable of hydrolyzing and converting polysaccharide substrates for various industrial applications [10]. Among these enzymes, cellulase is one of the most important enzymes, as it breaks down cellulose, a major component of plant cell walls, into simpler molecules, such as glucose and cellobiose, which can be utilized as energy resources for animal growth and production. Cellulase is a member of the hydrolase family and is classified into three enzymes, including endo-glucanase, exo-cellulase (cellobiohydrolase) and  $\beta$ -glucosidase [11–13].

Cellulases are naturally generated by a variety of microorganisms, including fungi, bacteria and protozoans that are involved in the process of cellulose decomposition in natural ecosystems [14]. Nevertheless, the production of cellulase in its native microorganisms, such as *Ruminococcus flavefaciens* [15–17], is typically challenging and inefficient for industrial purposes owing to complicated cultivation requirements and limited yields. In addition, cellulase production in indigenous hosts frequently needs specific substrates to induce enzyme expression. These substrates, primarily cellulose and its derivatives, induce cellulase gene expression as previously reported [15, 18], complicating the process of industrial cellulase production.

In contrast, heterologous expression systems, such as the *Escherichia coli* expression system, provide a more efficient alternative for cellulase production. *E. coli* is a well-established host system that has commonly been utilized for the industrial production of various recombinant proteins due to several merits, such as easy to cultivate, fast growth, simple genetic manipulation, and inexpensive cultivation [19]. This study aimed to clone and express the *Ruminococcus flavefaciens* cellulase-encoding gene (celE) in the *E. coli* expression system. The celE gene was cloned into a T7 promoter-based vector (pET15b), with its expression regulated by the T7 Lac operon. His tag (6 $\times$ His) was inserted upstream of the celE gene to enable protein detection.

The recombinant cellulase was successfully expressed in *E. coli* and confirmed by SDS-PAGE, with protein bands visualized using Western blotting. Although cellulase expression in *E. coli* was successful, further optimization is necessary to achieve greater yields and evaluate the enzymatic activity. The successful production of cellulase in *E. coli* offers major prospects for the poultry industry, as it could enhance feed digestibility by degrading cellulose in plant-derived feed components.

## 2 Materials and Methods

### 2.1 Culture Medium

Luria-Bertani (LB) medium containing 100 µg/mL of ampicillin was used as a basic medium to culture the transformed *E. coli*. LB medium contained 10 g/L of sodium chloride, 5 g/L of yeast extract and 10 g/L of tryptone. To prepare LB-agar plates, 15 g/L of agar powder was added. Terrific Broth (TB) was used to culture the transformed *E. coli* for protein expression as described in the references [20, 21] with slight modification. The TB medium consisted of 12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K<sub>2</sub>HPO<sub>4</sub> and 2.2 g/L KH<sub>2</sub>PO<sub>4</sub> with the pH adjusted to 7.0. In addition, The TB medium was supplemented with 1.25 mM MgSO<sub>4</sub>, 25 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05% glucose, 0.2% lactose monohydrate and 100 µg/mL of ampicillin. Glucose and lactose were added as additives of auto-induction within the culture medium as reported by Studier [22].

### 2.2 Codon Optimization and Cloning the *CelE* Gene into the Expression Vector

The *Ruminococcus flavefaciens* cellulase gene (GenBank Acc.: L03800.1) was codon-optimized to enhance gene expression and the translation efficiency in *E. coli*, resulting in the improvement of Codon Adaptation Index (CAI) of 0.96 and the GC content of 41.2% [23]. The optimized codon was subsequently integrated into a T7 promoter-based vector (pET15b), with its expression regulated by the T7 Lac operon, by Gene Universal Inc. His tag (6×His) and thrombin cleavage site were inserted upstream of the *celE* gene.

### 2.3 Transformation of pET15b-*celE* into *E. coli*

The pET15b-*celE* plasmid was transformed into Shuffle T7 Express Competent *E. coli* (NEB, Inc.) according to the manufacturer's protocol. A total of 2 µL of plasmid DNA was added to the competent cells and then mixed by carefully flicking the tube five times. The mixture of plasmid DNA and competent cells was placed on ice for 30 minutes, followed by heat shock in a 42°C water bath for 30 seconds. After a five-minute incubation on ice, 950 µL of SOC medium (NEB, inc.) was added, and the mixture was incubated at 30°C for 60 minutes with shaking at 250 rpm. Finally, 100

$\mu\text{L}$  of the mixture was spread onto LB-agar plates containing 100  $\mu\text{g}/\text{mL}$  of ampicillin and incubated overnight at 30°C.

#### 2.4 Expression of Recombinant Cellulase in *E. coli*

The TB medium that was supplemented with 1.25 mM  $\text{MgSO}_4$ , 25 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.05% glucose, 0.2% lactose monohydrate and 100  $\mu\text{g}/\text{mL}$  of ampicillin was used as a basic medium for cellulase expression in *E. coli*. A single colony of the transformed *E. coli* was selected and inoculated into 5 mL of the supplemented TB medium. The culture was performed using the reaction tube and incubated at 30°C for 72 hours. The cell pellet was harvested by centrifugation at 10,000 rpm for 30 minutes at 4°C. The cell pellet was then rinsed three times using phosphate-buffered saline (PBS) pH 7.4. Finally, the cell pellet was stored at -20°C until further use.

#### 2.5 Sonication

The pellet of bacterial cells was thawed at room temperature and suspended in 10 mL of PBS (pH 7.4). The cell suspension was sonicated to lyse bacterial cells following the protocol described by Alimuddin et al. [24]. After sonication, the lysate was centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant containing the soluble fraction was transferred to a new tube. The remaining pellet, which represents the insoluble fraction, was resuspended in 10 mL of PBS (pH 7.4).

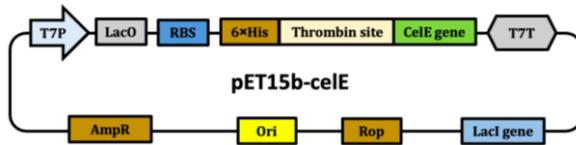
#### 2.6 Protein Analysis By SDS-PAGE

The soluble and insoluble fractions were analyzed by 12.5% SDS-PAGE under reducing conditions. In brief, a total of 14  $\mu\text{L}$  of each sample was mixed with 4  $\mu\text{L}$  of 5 $\times$  sample buffer and 2  $\mu\text{L}$  of 100 mM dithiothreitol (DTT) as a reducing agent. The mixture was boiled at 100°C for 3 minutes before loading into the SDS-PAGE gel for electrophoresis. The protein bands were visualized by Coomassie brilliant blue (CBB) staining and Western blot using Anti-His tag mAb-horseradish peroxidase (HRP) conjugate (MBL Co. Ltd.).

### 3 Results and Discussion

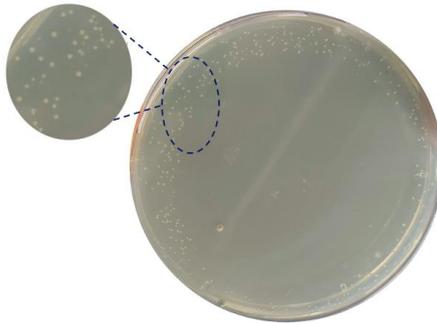
#### 3.1 Cloning and Transformation pET15b-ceIE into *E. coli*

The cellulase gene, ceIE, from *Ruminococcus flavefaciens*, was codon-optimized for efficient expression in *E. coli* before integrating into the expression vector. The ceIE gene, consisting of 963 bp, was inserted into the pET15b plasmid using a double digestion technique with restriction endonuclease enzymes, NdeI at the N-terminal and XhoI at the C-terminal. These two enzymes generated the complementary sticky ends that facilitate the precise insertion and ligation of the gene into the vector. The plasmid map is presented in Fig. 1.



**Fig. 1.** The plasmid map of pET15b-celE used for the *E. coli* expression system. The celE gene was fused in-frame with a 6×His and a thrombin cleavage site and was inserted downstream of a ribosome binding site (RBS). The expression of cellulase is regulated by the T7 Lac operon that can be induced by lactose or its derivatives.

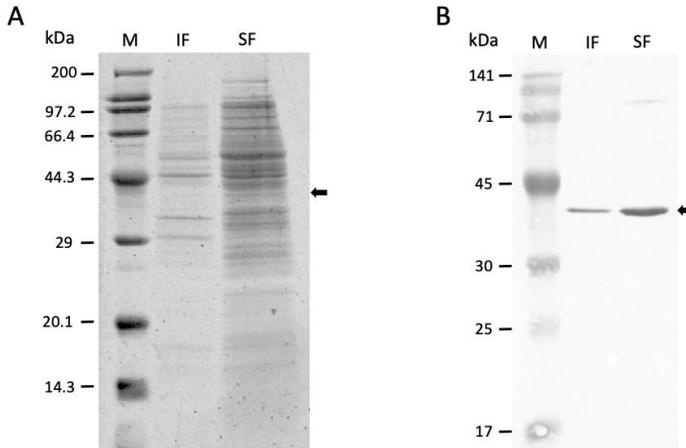
The cellulase gene, celE, from *Ruminococcus flavefaciens*, was codon-optimized for efficient expression in *E. coli* before integrating into the expression vector. The celE gene, consisting of 963 bp, was inserted into the pET15b plasmid using a double digestion technique with restriction endonuclease enzymes, NdeI at the N-terminal and XhoI at the C-terminal. These two enzymes generated the complementary sticky ends that facilitate the precise insertion and ligation of the gene into the vector. The plasmid map is presented in Fig. 1.



**Fig.2.** The grown colonies of the transformed *E. coli* on the LB-agar plate containing ampicillin.

### 3.2 Expression of the Recombinant Cellulase in *E. coli*

The recombinant cellulase (celE) of *Ruminococcus flavefaciens* was successfully produced using the *E. coli* expression system. In this study, the expression of the recombinant cellulase was induced by supplementing glucose and lactose into the culture medium, followed by the incubation at 30°C for 72 h. The auto-induction approach aimed to promote soluble expression of the recombinant cellulase as glucose and lactose provide a slower rate of induction that contributes to enhance protein solubility [22, 25]. The recombinant cellulase expression was shown in Fig. 3.



**Fig. 3.** Expression of the recombinant cellulase in the *E. coli* expression system. A. CBB staining of the insoluble fraction (IF) and soluble fraction (SF). M indicates protein markers. The expected protein band is shown by an arrow. B. Western blotting analysis of the insoluble fraction (IF) and soluble fraction (SF). M indicates protein markers. The target protein band is shown by an arrow.

The results demonstrated that a relatively thin band (around 38 kDa) was observed in the soluble fraction sample under reducing SDS-PAGE conditions, corresponding to the target protein as shown by the CBB staining result (Fig. 3A). The target proteins were also confirmed by Western blotting, suggesting that the clear bands were detected at approximately 38 kDa, as indicated in Fig. 3B. As shown in Fig. 3B, the protein band observed in the soluble fraction was thicker than that in the insoluble fraction, highlighting that the soluble cellulase was expressed at a higher level compared to the insoluble fraction or inclusion bodies. The highly expressed soluble fraction is likely due to the effect of the optimized codons that improve expression efficiency in *E. coli*. Liu et al. [26] reported that codon optimization markedly increased the expression of 37-kDa immature laminin receptor protein (iLRP) in *E. coli*. The frequency of codon usage in the DNA sequence of a target gene is favourably linked with the corresponding tRNA in a given species. Thus, the amount of amino acids which are available for protein elongation during translation is determined by the tRNA concentration, contributing to protein synthesis efficiency. In contrast, rare codon usage tends to diminish the rate of protein translation and may even lead to translation errors [27, 28].

The successful production of the recombinant cellulase in the *E. coli* expression system, facilitated by optimized codon usage, can contribute to large-scale cellulase production, enhancing its availability and utility as a feed additive in the poultry industry. Zulkarnain et al. [29] reported that sago palm waste with cellulase enzyme supplemented to 0.75 g/kg led to the highest value of in vitro dry matter digestibility and in vitro organic matter digestibility. Additionally, cellulase supplementation resulted in a significant decrease in crude fibre and cellulose content. Another carbohydrate-hydrolyzing enzyme, metagenome-derived xylanase expressed in *E. coli*, has

also been reported to enhance poultry feed utilization [10]. Cellulase supplementation can reduce crude fibre and cellulose content in poultry feed, enabling the utilization of local feedstuffs derived from agricultural and agroindustrial waste more efficiently, ultimately leading to a reduction in feed costs.

## 4 Conclusion

In conclusion, the successful expression of the recombinant cellulase from *Ruminococcus flavefaciens* in the *E. coli* expression system emphasized the importance of codon optimization that can aid in improving protein expression efficiency. The high level of soluble expression obtained in this work suggested that appropriate conditions and the selected expression system are suitable and convenient for the efficient and scalable production of cellulase. This advancement possesses great potential for providing feed additives in the poultry industry, particularly through cellulase supplementation for increasing feed digestibility and reducing dependence on high-priced feed components in the poultry industry. The evaluation of the enzymatic activity and the performance of the recombinant cellulase in practical feed applications still requires more studies in the future.

**Disclosure of Interests.** The authors have no competing interests to declare that are relevant to the content of this article.

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