

**EXPRESSION OF BETA GLUCOSIDASE MINED FROM METAGENOMIC  
DNA DATA OF BACTERIA IN VIETNAMESE GOATS' RUMEN  
IN *Escherichia coli* SYSTEM**

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**ABSTRACT**

Screening and expression of new  $\beta$ -glucosidase genes (*bgc*) have attracted much attention because of their valuable application in a wide range of industrial areas such as bioethanol production, food and animal feed processing, paper making, and biotechnological processes. Previously, we mined a new *bgc* gene coding for its mature enzyme (BGC) from metagenomic DNA data of bacteria in Vietnamese goats' rumen. Based on the NCBI database, the BGC sequence was found to be the highest similarity with  $\beta$ -glucosidase of *Bacteroidales bacterium* (60.52%). The BGC enzyme was previously annotated to have two domains GH3 and GH31 and highly expressed in *E. coli*. The aim of this work was to experimentally confirm the predicted gene by expressing and assessing the activity of recombinant BGC from *E. coli* strains of BL21, Rosetta 1, C43, and SoluBL21. Furthermore, the expression level and solubility of the BGC were also investigated by varying fermentation conditions such as temperature, medium components, and IPTG concentration. The activity of the crude enzyme was identified through substrates including esculin and p-Nitrophenyl- $\beta$ -D-glucopyranoside (pNPG). The new BGC could be used as potential material for further enzyme characterization.

**Keywords:** Bacterial DNA metagenome,  $\beta$ -glucosidase, *Escherichia coli*, Glycosyl hydrolase family 3, Glycosyl hydrolase family 31, Vietnamese native goats' rumen.

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## INTRODUCTION

Beta glucosidase (EC.3.2.1.21) (BGC) is an important enzyme in combination with endoglucanase and exoglucanase to hydrolyze cellulose into glucose subunits. The activity of BGC is pivotal to the rate of saccharification due to negative feedback of cellobiose accumulation that affects endo- and exoglucanase activity (Ahmed et al., 2017). In fact, BGCs are used in many industrial areas such as biofuel, textile, paper, food and animal feed industries (Ahmed et al., 2017; Chen et al., 2017; Raza et al., 2020; Zhang & Zhang, 2013). Although fungal BGCs are used mostly in commerce, this enzyme production from fungi faces several drawbacks. For example, *Trichoderma reesei* has been widely used to produce cellulases but the yield of BGC is very poor. Besides, most of the produced BGCs attach to the fungal cell wall, leading to a low enzyme secretion into medium which causes a tedious enzyme extraction. Another commercial fungal BGC source comes from *Aspergillus niger*. However, this enzyme is affected deleteriously by high glucose concentration (Srivastava et al., 2019). In contrast, although bacterial strains secrete BGCs with a low level, enzyme production from them attracts great interest because of their fast growth rate and ability to produce enzyme with distinct properties (Srivastava et al., 2019). For example, *Bacillus subtilis*, *Acidothermus cellulolyticus* have been reported as potential producers of more thermostable BGCs than fungi although they are slow producers (Bagudo et al., 2014; Li et al., 2018). BGCs from *Thermoanaerobacterium thermosaccharolyticum* were also characterized by their tolerance of high glucose concentration (Pei et al., 2012). However, there are still some limitations towards BGCs such as hydrolysis rate, final product inhibition, and thermal instability those are bottlenecks for efficient conversion of cellulose (Srivastava et al., 2019). Therefore, screening and characterization of new BGC enzymes from a wide range of organisms are still being continued.

Metagenomic approaches enabled researchers to clone experimentally genes into culturable bacteria to characterize them (Ariaeenejad et al., 2020). Digestive tracts from termite, goat or ruminants show rapid and efficient lignocellulose degrading environments owing to living symbiotic microbiome. These sources could be utilized for discovering new lignocellulose degrading enzymes including BGCs.

BGCs have been classified on the basis of two methods. The first one is based on substrate specificity. In this method, BGCs are divided into three groups: (1) aryl- $\beta$ -glucosidase, which splits aryl-glucosides; (2) cellobiases, which converse cellobiose into glucose; and (3) glucosidases, which express specific activity against a wide range of substrates. The second method based on both nucleotide sequence identity and enzyme structural similarity is widely accepted. Accordingly, BGCs are grouped mainly in Glycosyl hydrolase family 1 (GH1 family) and Glycosyl hydrolase family 3 (GH3 family) as in Carbohydrate active enZYme database (CaZy), with less presence in GH families 5, 9, 16, 30, and 116 (Ahmed et al., 2017; Ariaeenejad et al., 2020). The GH1 family is almost derived from archaea, plants, and mammals. While the GH3 family originated mostly in bacteria, mold, and yeast (Srivastava et al., 2019). In further information, BGCs belong to GH3 family and some BGCs from GH1 family show strong inhibition in low glucose concentration. While, most of the characterized glucose tolerant BGCs belong to GH1 family except for one GH3 BGC from *Mucor circinelloides* (Srivastava et al., 2019).

In our project MetagenLig (No. NĐT.50.GER/18), we have analyzed and identified a new  $\beta$ -glucosidase gene (*bgc*) from metagenomic DNA data of bacteria extracted from Vietnamese native goats' rumen. The gene was 3723 bp in length coding for a mature BGC enzyme of ~137 kDa. In terms of amino acid sequence, this protein has a 61.21% similar sequence with GH3 family 3 C-terminal domain-containing

protein (MBR4478618.1) and was annotated to be BGC from *Bacteroidales bacterium* using BLAST (MBE6253225.1) (60.52% in sequence similarity). In addition to GH3 domain, this enzyme was also predicted to have another domain GH31 that could be  $\alpha$ -glucosidase. To verify it, in this study we report results about cloning and expression of *bgc* in *E. coli* strains of BL21, Rossetta 1, C43, and SoluBL21. Next, we changed the enzyme expression conditions such as temperature, medium components, and IPTG concentration to improve the enzyme solubility. Then, the crude BGC activity was identified using substrates esculin and pNPG. The recombinant BGCs could be potentially utilized as materials for further enzyme characterization.

## MATERIALS AND METHODS

### Materials

The DE3 strains of *E. coli* BL21, Rosetta 1, C43, and SoluBL21 harboring plasmid pET22\_BGC\_GH3-31 were used to express recombinant BGCs. Esculin (117830050, Acros) and ammonium iron (III) citrate (Sigma) were used to rapidly detect the presence of BGCs in the crude lysates. Cellulase (C9748, Sigma) was used as a positive control. To quantify BGC activity, p-nitrophenyl- $\beta$ -D-glucopyranoside (pNPG, N7006, Sigma) and p-nitrophenol of spectrophotometric grade (pNP, 1048, Sigma) were used as substrate and standard, respectively. Restriction enzymes (*Nco*I, *Xho*I), DNA marker (1 kb) and protein marker were purchased from Fermentas (Germany). Other analytical-grade chemicals were from Merck, Prolabo, etc.

### Methods

#### *Vector construction and enzyme expression in E. coli strains*

The *bgc* gene mined from the metagenomic DNA data of bacteria in goats' rumen was 3725 bp in length. This gene encoded a mature 137 kDa BGC enzyme with two annotated domains GH3 and GH31. The expression level of the *bgc* gene was

previously predicted to be ~4.28 g enzyme per litre in *E. coli* using an online software Periscope (<http://lightning.med.monash.edu/periscope/>). The gene codons were also optimized for a suitable expression in *E. coli*, then subcloned in pET22b(+) at *Nco*I and *Xho*I restriction sites (named pET22\_BGC\_GH3-31). DNA sequencing analysis of the gene in the obtained plasmid was conducted by Genscript Co. (USA). Besides, the presence of *bgc* in the plasmid was also checked by restriction digestion.

Plasmids pET22\_BGC\_GH3-31 were sequentially transformed into *E. coli* (DE3) strains of BL21, Rosetta 1, C43, and SoluBL21. Bacterial expression was performed as a standard protocol. *E. coli* strains were grown in Luria-Bertani medium (0.5% yeast extract, 1% peptone, 1% NaCl) containing ampicillin of 100  $\mu$ g/ml (LBamp) at 37°C, 200 rpm for 16 hours. Then, the culture was inoculated into 10 ml LBamp (initial OD<sub>600</sub> ~ 0.1) and grown until the OD<sub>600</sub> reached 0.6–0.8. A suitable expression condition of recombinant BGC was investigated by conducting induction at different temperatures (20–37 °C), IPTG concentrations (0.05–2 mM), and media such as LB, TB (1.2% peptone, 2.4% yeast extract, 72 mM K<sub>2</sub>HPO<sub>4</sub>, 17 mM KH<sub>2</sub>PO<sub>4</sub>, 0.4% glycerol), modified TB (1.2% peptone, 2.4% yeast extract, 72 mM K<sub>2</sub>HPO<sub>4</sub>, 17 mM KH<sub>2</sub>PO<sub>4</sub>, 0.24% glucose), SB (3.2% peptone, 2% yeast extract, 0.5% NaCl), and PE (1% yeast extract, 2% peptone). Cells were harvested after 4-hour-induction by centrifugation at 5000 rpm for 5 minutes. Cell pellets were re-suspended in PBS 50 mM (pH 7) until OD<sub>600</sub> ~ 10. Then, 500  $\mu$ L cells suspension was subjected for cell disruption by sonication until clarified (3 s on, 3 s off, 5 min), followed by centrifugation at 12,000 rpm for 10 minutes to separate the supernatant from cell debris. The expression level of BGCs was checked on SDS-PAGE 12.6% according to Laemmli (1970). Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

### Enzyme activity and protein concentration assays

The protein concentrations were quantified by the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA). The BGC percentage in the total lysate was estimated using an online software ImageLab (BioRad).

The qualitative activity of recombinant BGCs towards esculin was rapidly evaluated according to Veena et al. (2011). In detail, 50  $\mu$ L of enzyme solutions were dropped into a 0.5 cm diameter well on LB agar plates containing 0.3% esculin and 0.02% ferric ammonium citrate. The plates were then incubated at 37 °C for 16 hours. Cellulase (Sigma) of 0.05 U was used as the positive control. While PBS 1x (pH 7) and sonicated cells from induced *E. coli* harboring pET22b(+) were both used as negative controls. Enzymatic activity of BGCs was identified by appeared darkening or black zones.

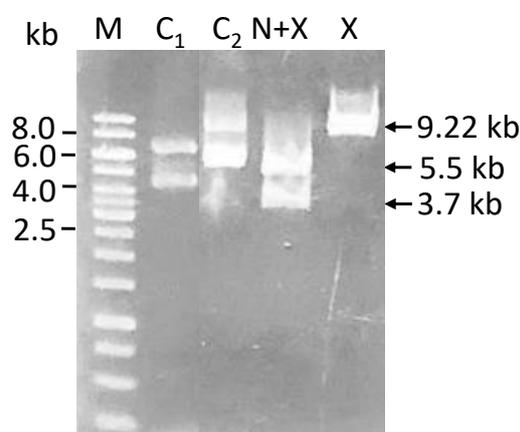
The quantitative activity of recombinant BGCs was determined using pNPG (Sigma) according to Fang et al. (2014). Briefly, 20  $\mu$ L of a properly diluted enzyme solution were added into 180  $\mu$ L 5 mM pNPG solution. The enzymatic reactions were allowed to take place at 37 °C for 30 min. Then, the reactions were terminated by adding 800  $\mu$ L NaCO<sub>3</sub> 0.2 M. The amount of released *p*-nitrophenol was measured at 410 nm. The content of *p*-nitrophenol was calculated according to the *p*-nitrophenol standard curve. The concentration range of the *p*-nitrophenol standard curve was from 0–0.2  $\mu$ M. One unit of enzyme activity was defined as the amount of enzyme required to release one  $\mu$ mol of *p*-nitrophenol per minute under the reaction conditions. Each measurement was performed in triplicate.

## RESULTS AND DISCUSSION

### Enzyme expression in *E. Coli*

As previously reported, the *bgc* gene was codon optimized, synthesized and cloned into pET22b(+) at *Nco*I and *Xho*I sites. Then, DNA sequencing analysis was conducted by Genescript Co. (USA). In this study, the *bgc*

size in pET22b(+) was further checked by restriction enzymes. Fig. 1 indicated that the treated plasmids by *Nco*I and *Xho*I produced two separate DNA bands. One corresponded to pET22b(+) (~5.5 kb) and the another corresponded to *bgc* gene (~3.7 kb) (Lane N+X). In addition, the treated plasmids by *Xho*I only gave a single band of ~9.22 kb that corresponded to a total length of pET22b(+) and *bgc* (Lane X). In conclusion, the *bgc* gene was inserted in the recombinant plasmid, named pET22\_BGC\_GH3-31 (Fig. 1).

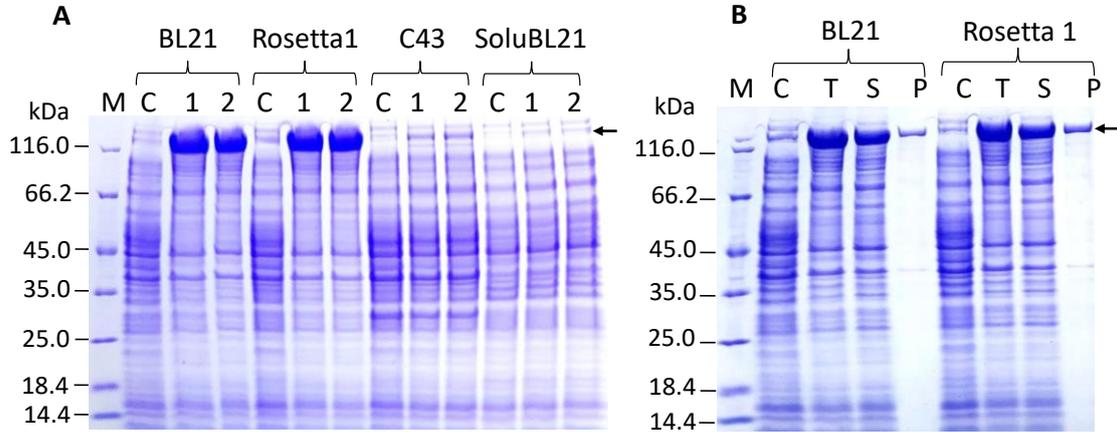


**Figure 1.** Analysis of *bgc* gene in pET22\_BGC\_GH3-31 by restriction enzymes; Lane M: 1 kb DNA marker (Fermentas); Lanes C<sub>1</sub> and C<sub>2</sub>: plasmids pET22b(+) and pET22\_BGC\_GH3-31, respectively; Lane N+X: plasmid pET22\_BGC\_GH3-31 was treated with *Nco*I and *Xho*I; Lane X: plasmid pET22\_BGC\_GH3-31 was treated with *Xho*I only

For enzyme expression, plasmid pET22\_BGC\_GH3-31 were sequently transformed into BL21, Rosetta1, C43, and SoluBL21. Then, enzyme expression was induced by 0.5 mM IPTG at 30 °C for 4 hours. SDS-PAGE analysis in Figure 2A showed that BGC enzymes were strongly expressed in *E. coli* BL21 and Rosetta 1 (Clones 1 and 2, marked as an arrow). The enzyme band was apparent with a molecular weight of ~137 kDa. This was suitable to the calculated value of 136.51 kDa based on the 1241 amino acids and 6 histidine residues. In contrast, very faint

BGC bands were visualized in SoluBL21 or C43 (Fig. 2A). This implied that BGCs were hardly expressed in these hosts. For solubility,

we found that BGCs were produced as both soluble and insoluble forms in BL21 and Rosetta 1 at 30 °C (Lanes S and P; Fig. 2B).



**Figure 2.** Analysis of BGC expression in *E. coli* strains by SDS-PAGE. (A) total cell lysate; (B) protein phases of the cell lysis; Lanes 1 and 2: total cell lysis of two recombinant clones bearing pET22\_BGC\_GH3-31; Lane C: uninduced recombinant strains; Lane M: protein molecular weight marker (Fermentas, SM0431); Lanes T, S, P: represent total cell lysate, supernatant, and pellet proteins, respectively

In terms of protein expression, *E. coli* hosts are known not to effectively express proteins having too big molecular sizes (> 500 amino acids) or too small ones (< 80 amino acids). In our study, BL21 and Rosetta 1 might be more suitable for BGC expression than C43 and SoluBL21. Although BGCs have a long amino acid sequence that contained two annotated domains GH3 and GH31, they were well expressed in BL21 and Rosetta 1. BL21 is known to be suitable for nontoxic protein expression at a high level. This strain has been modified to be deficient in two genes coding for Lon and OmpT proteases. As a result, recombinant proteins avoid from being cleaved after being synthesized (Rosano & Ceccarelli, 2014). While Rosetta is a BL21-derived strain that has been modified to enhance the expression of animal proteins with rare codons. Two strains were suitable for high level expression of BGCs. While C43 is used for specific purposes including expression of toxic or membrane proteins, SoluBL21 is used to express animal proteins in soluble form. The expression level of BGCs in BL21 and

Rosetta 1 was in good agreement with the theoretical prediction using the online software Periscope. For enzyme solubility, only some BGCs were produced as the insoluble form in BL21 and Rosetta 1. It was possible that several BGCs were over-produced at 30 °C. At this temperature, the host cells seemed to lack specific chaperones that help the protein to coil properly, resulting in inclusion bodies (Yin et al., 2007). This limitation may be overcome by changing the expression temperature.

### Enzyme activity

The ability of BGCs to hydrolyze esculin is an important characteristic for their identification. Principally, BGCs hydrolyze esculin into esculetin and glucose. Then, the ferric ions in the medium will be reduced to get brown or black due to iron production. As shown in Fig. 3, the dark brown zones developed strongly in the total protein and the soluble fractions derived from BL21 and Rosetta 1. This implied a clear activity from the recombinant BGCs in both strains. Whereas,

this enzyme showed weak blackening zones in C43, and SoluBL21. This observation was in good agreement with BGC expression level from 4 strains in Figure.

2A. With lighly higher cell density in Rosetta 1, we chose this strain for further experiment.

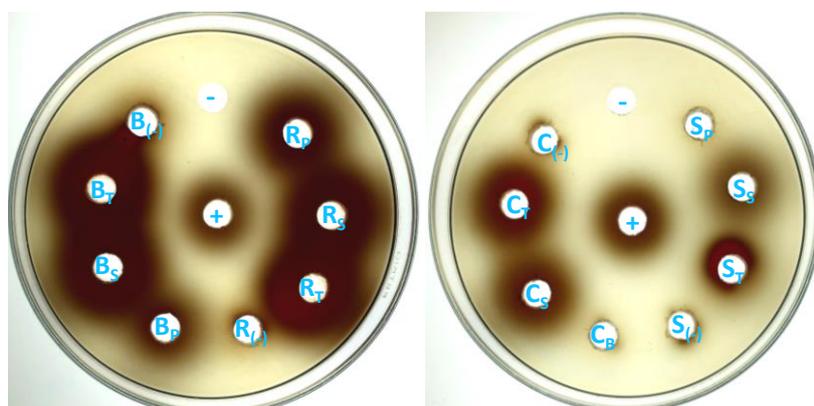


Figure 3. Rapid determination of BGCs expressed in *E. coli* strains toward esculin; B, R, C, S represent *E. coli* BL21, Rosetta 1, C43, and SoluBL21, respectively; (+): positive control of 0.05 U cellulase (Sigma); (-): negative control of PBS 50 mM, pH 7

Using Bradford assay, the concentration of total protein from Rosetta 1 was 0.85 mg/ml. Using ImageLab (BioRad), BGCs were estimated to be ~32.6%. To determine quantitatively the BGC activity from crude extract, we used pNPG as a substrate for reaction at 37 °C for 30 minutes. We found that the activity of BGC was 0.28 U/mg. With the same substrates of esculin or pNPG, other BGCs were also identified such as highly glucose-tolerant and halotolerant BGC from sheep rumen metagenomic data (Ariaeenejad et al., 2020), recombinant BGC from *Bacillus licheniformis* (Chen et al., 2017) and thermophilic bacterium *Caldicellulosiruptor caccharolyticus* (Hong et al., 2009), BGC producing bacteria (Veena et al., 2011). The activity of recombinant BGC in this study was not as high as other reported BGC enzymes. It might come from the un-optimized condition of the enzyme reaction. However, our result experimentally confirmed that the predicted enzyme was BGC.

#### Effect of temperature, culture medium and induction concentration on BGC expression

Until now, great efforts have been made to improve BGC production and its activity for

further utilization. For example, genetic manipulation, screening high BGC producing strains or thermo-stable enzyme mutants, co-culturing *T. reesei* with some other fungi, using recombinant DNA technology, which has been done to produce an ideal amount of saccharifying enzyme containing BGCs. Finding out suitable fermentation conditions is one of the very important steps towards profitable enzyme production. In this research, we focused on varying temperature, medium and IPTG concentrations for more soluble and better expression of BGCs in Rosetta 1.

It is known that temperature is always a crucial factor that affects protein quality. Low temperature may improve protein solubility but limits bacterial growth rate (Sahdev et al., 2008). While high temperature is suitable for bacterial growth but many recombinant proteins are produced as inclusion bodies. In this experiment, we induced the BGC expression at 20 °C, 25 °C, 30 °C and 37 °C. The result in Figure 4 showed that BGCs were expressed very well at all the tested temperatures (Lanes T). However, the enzymes were only produced as soluble forms at 20 °C

and 25 °C. Among these temperatures, cell density at 25 °C was higher than at 20 °C (2.83 and 2.25, respectively). In terms of solubility, the higher temperature was (30–37 °C), the less

soluble enzyme expressed (Lanes S) but the more inclusion bodies produced (Lanes P). From the results above, 25 °C was suitable for BGC expression.

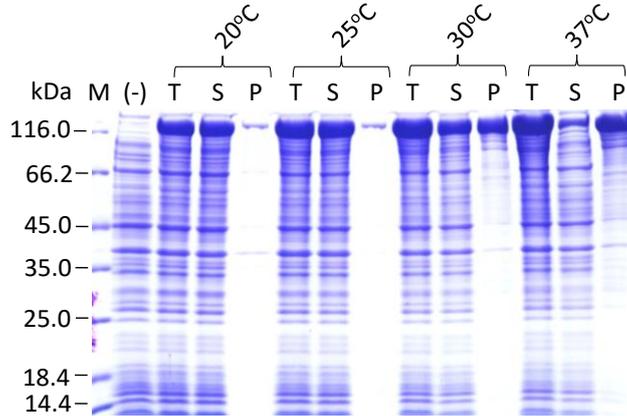


Figure 4. SDS-PAGE analysis of BGC expression at different temperatures; Lane M: protein molecular weight marker (Fermentas, SM0431); Lane (-): Total proteins of Rosetta 1 baring pET22b(+) induced by IPTG at 30°C; Lanes T, S, and P represent total cell lysate, supernatant, and pellet proteins, respectively of Rosetta 1 strain harboring pET22\_BGC\_GH3-31

Culture medium has a significant impact on protein expression as well as cell biomass. Different recombinant protein will have their best expression level in a certainly suitable medium. To efficiently express BGC in Rosetta 1, we induced strains using different

nutritious media. As shown in Figure 5A, BGCs were most strongly produced in TB and modified TB. Furthermore, the cell density was the highest in modified TB (Fig. 5B). This result indicated that BGCs were best expressed in modified TB.

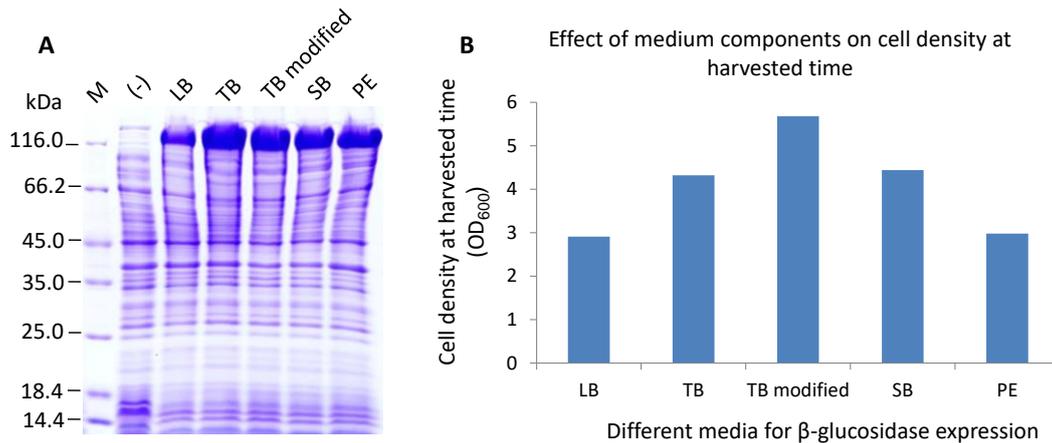
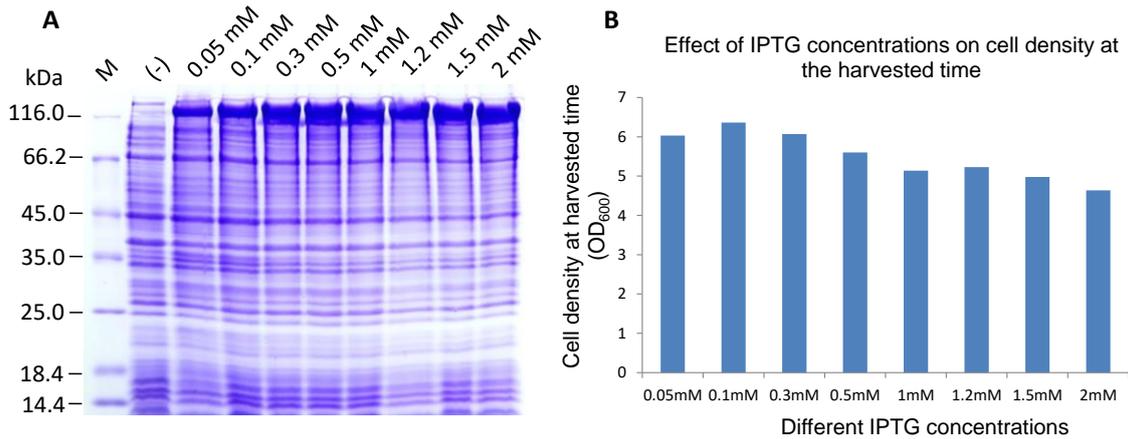


Figure 5. SDS-PAGE analysis of BGC expression in different media. (A) Total protein, and cell density at the harvested time (B); Lane M: protein molecular weight marker (Fermentas, SM0431); Lane (-): negative strain of Rosetta 1 baring pET22b(+) induced by IPTG at 30 °C; Lanes LB, TB, TB modified, SB, and PE represent various media were used to induce for BGC production in Rosetta 1 harboring pET22\_BGC\_GH3-31

In the next experiment, the effect of IPTG concentration on BGC expression was investigated. We found that the enzyme expression was IPTG dose-dependent. Low (0.05–0.1 mM) or high IPTG concentration (1.2–2 mM) induced low amount of BGCs. On the other hand, 0.3 mM, 0.5 mM and 1 mM

IPTG could produce a higher amount of enzyme (Fig. 6A). In addition, the higher the IPTG concentration was, the lower the cell density recorded (Fig. 6B). Remarkably, 0.3 mM IPTG could induce both higher BGC amount and cell density. Therefore, this IPTG concentration was suitable for BGC production.



**Figure 6.** SDS-PAGE analysis of BGC expression at different IPTG concentrations. (A) Total protein, and cell density at the harvested time (B); Lane M: protein molecular weight marker (Fermentas, SM0431); Lane (-): Rosetta 1 baring pET22b(+) induced by 0.3 mM IPTG at 25 °C; Lanes 0.05–2 mM represent various IPTG concentrations used for BGC production in Rosetta 1 harboring pET22\_BGC\_GH3-31

After all, the host strain was induced for BGC expression under the suitable parameters. We found that BGCs could be strongly expressed in Rosetta 1 at 25 °C with 0.3 mM IPTG in the modified TB medium. Interestingly, the enzyme was produced as soluble form and cell density increased to 1.8 fold (OD<sub>600</sub> = 3.81 and 6.86, respectively). The total protein concentration was 1.23 mg/ml (Bradford assay) of which the amount of BGCs accounted for 30.7% (ImageLab). The pNPG assay showed the BGC activity was 7.89 U/mg (28 folds increased in the activity).

To optimize the expression of recombinant proteins in the host strain, it is necessary to find out suitable expression conditions. A high concentration of salt, peptone, yeast extract could improve recombinant protein amount. Various media such as LB, TB, 2YT have been used to optimize protein expression (Joseph et al., 2015). Cell density could be increased by

trying medium formulations, increasing the amount of dissolved oxygen, and avoiding foaming (Rosano & Ceccarelli, 2014). It is also possible to examine other factors such as cell density at the time of induction, inducer concentration, etc. (Joseph et al., 2015). For protein solubility, the lowering temperature might increase soluble protein formation. Our result showed the increased BGC expression in soluble form and higher cell density compared to the initial fermentation condition at 30 °C. The annotated enzyme mined from goats' rumen DNA metagenomic data was experimentally confirmed to be BGCs with GH3 domain. This result is fundamental for enzyme purification to characterize them, including the testing activity of GH31 domain.

## CONCLUSION

The *bgc* derived from goats' rumen bacteria was expressed successfully in *E. coli* BL21 and Rosetta 1 as BGC enzymes with a

predicted molecular weight of ~137 kDa. At 30 °C and 0.5 mM IPTG, the enzymes were produced as both soluble and insoluble forms. Accordingly, the soluble fraction showed its GH3 activity through darkening zones on the esculin plate and 0.28 U/mg on the pNPG substrate. Furthermore, enzyme solubility was improved well in Rosetta 1 by inducing enzyme expression at 25 °C, 0.3 mM IPTG using a modified TB medium. At this condition, the BGCs were almost in soluble form and showed their 28 folds increased activity on pNPG (7.89 U/mg). The recombinant BGC is potential material for further enzyme characterization.

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