

## SELECTION OF OPTIMAL CULTURE CONDITIONS FOR EXPRESSION OF RECOMBINANT BETA-GLUCOSIDASE IN *ESCHERICHIA COLI*

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### SUMMARY

Beta-glucosidase (BGL) is an enzyme involved in the hydrolysis of cellulose and plays an important role in many biological processes. This enzyme is widely available in animals, plants, and microorganisms. Expression of the recombinant enzyme in *Escherichia coli* is considered a suitable choice for high production via a fast growth rate and feasible manipulation. In this study, we optimized some conditions for the high soluble production of recombinant beta-glucosidase whose gene sequence was mined from the DNA metagenome of Cuc Phuong humus surrounding white rot fungi. The gene *bgl* was cloned in pET 22b(+) and expressed in the *E. coli* Rosetta 1 strain. Production of the recombinant BGL was examined at temperature ranges from 18 to 37°C. The recombinant BGL was obtained as a soluble form at low temperatures, and the optimal temperature was 25°C. In comparison to LB, TB, SB, PE media, mTB rich medium in presence of glucose produced the most BGL. Besides, the results assessing the inducer condition showed that the best IPTG concentration for producing the BGL was 0.3 mM IPTG. Furthermore, some fermentation conditions affecting the level of BGL production were assessed as the induction point and harvesting time. The BGL production was the most suitable when the cell at mid-log phase with an OD<sub>600</sub> 1 and harvesting time after 4 hours of induction. Importantly, the recombinant BGL had good activity on the esculin substrate plate. Based on the selected conditions, the recombinant BGL could be produced high amount to facilitate for future purification and characterization of the recombinant BGL.

**Keywords:** *Escherichia coli*, esculin substrate, fermentation conditions, recombinant BGL enzyme.

### INTRODUCTION

Beta-glucosidase (EC 3.2.1.21) is an enzyme that catalyzes the hydrolysis of beta-glucosidic linkages in a wide range of di- and oligo glucosaccharides and glycol conjugates. The enzyme is known to be broadly distributed in animals, plants, and microorganisms. Beta-glucosidases play important roles with various

potential applications in many fundamental biological and biotechnological processes (Ahmed *et al.*, 2017; Czjzek *et al.*, 2000; Srivastava *et al.*, 2019). They can be applied to the production of ethanol from agricultural biomass. Besides, they can be used for the synthesis of useful glucosides and releasing aromatic compounds from fruits in the flavour industry and for removing printing ink from

recycled paper (Dhake, Patil, 2005). In addition, the enzymes can be useful in enhancing isoflavone during fermentation of soymilk (Hu *et al.*, 2007; Otieno, Shah, 2007) and in the bioconversion of phenolic anti-oxidants (McCue, Shetty, 2003).

*Escherichia coli* is the most common expression system for recombinant protein production because of its high growth rate, easy genetic manipulation, and simple large-scale production of recombinant proteins (Maldonado *et al.*, 2007; Zhang *et al.*, 2010). Almost all proteins and enzymes can be produced in *E. coli*. However, the level of expression and formation of heterologous proteins are still major difficulty. The overall productivity of a recombinant protein depends on both the optical density of the cell and the specific product yield. Culture conditions during growth are related to gene expression and significant influence on the recombinant protein yield (Jana, Deb, 2005; Shojaosadati *et al.*, 2008). Therefore, it is essential to optimize culture fermentation conditions to maximize the recombinant protein production. Here, we present the analysis of the expression conditions for beta-glucosidase (BGL) which was expressed in *E. coli* Rosseta 1. We optimized culture temperature for obtaining soluble recombinant beta-glucosidase at high level. We further selected media components and inducer concentration. Besides, we also examined induce time and harvesting time for improving production of the recombinant BGL.

## MATERIAL AND METHODS

Expression strain *E. coli* Rosetta 1 was used for recombinant protein expression. Vector PET22b-BGL harbouring a gene encoding beta-glucosidase constructed by our research group was used to express recombinant beta-glucosidase. This BGL gene sequence was mined from the metagenome DNA data (data not shown) of bacterial community from humus surrounding white rot fungi *Trametes versicolor* at Cuc Phuong national park.

Medium components were purchased from

BD (Germany). Other chemicals for analysis of protein, coomassie stain, were purchased from Merck (Germany), protein marker from Fermentas (Germany), enzyme and esculin from Sigma (USA).

### Expression of gene encoding beta-glucosidase

The vector pET22b-BGL was transformed into *E. coli* Rosetta 1 by heat shock (Sambrook *et al.*, 1989). The recombinant strain was inoculated in Luria-Bertani (LB) liquid medium supplemented with 100 µg/ml ampicillin (LBA) and grown overnight with shaking 200 rpm at 37°C. Then, the cell culture was transferred into fresh LBA medium at an optical density (OD<sub>600</sub>) about 0.1 and continually incubated at 37°C with shaking at 200 rpm to reach an OD<sub>600</sub> about 0.6. The culture was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) (William Studier *et al.*, 1990) and fermented at temperature ranges from 18 to 37°C with shaking at 200 rpm for 4 hours. After fermentation, the cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in buffer 20 mM Tris-HCl, pH = 8 to final OD<sub>600</sub> of 10. Expression of the recombinant BGL was estimated by SDS-PAGE at 12.6%.

After choosing the optimal fermentation temperature, to obtain the highest heterologous protein production, expression of the recombinant BGL was continually assessed on other parameters such as medium components, including LB (0.5% yeast extract, 1% peptone, 1% NaCl), 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), mTB (0.4% glycerol instead of 0.24% glucose), SB (3.2% peptone, 2% yeast extract, 0.5% NaCl), and PE (1% yeast extract, 2% peptone). In addition, IPTG inducer concentration and induction time point as well as harvesting time were also examined.

### Extraction of recombinant protein from *E. coli*

The recombinant cells harvested from the fermentation culture were resuspended in buffer 20 mM Tris HCl, pH=8 to an OD of 10. The cells were disrupted by sonication with Amplitude for

10 minutes. After sonication, total soluble proteins were separated from the pellet by centrifugation at 12,000 g at 4°C for 10 minutes. The pellet was resuspended in an equivalent volume of buffer (20 mM Tris HCl, pH=8). Proteins from soluble and insoluble fractions were checked by SDS-PAGE at 12.6% (Laemmli, 1970).

### Determination of beta-glucosidase activity

Activity of beta-glucosidase was performed according to Veena *et al.* (2011) with a minor modification. Briefly, beta-glucosidase activity was screened on an LB ampicilin agar plate using 0.3% esculin substrate and 0.02% ferric ammonium citrate. Fifty microliters of protein extraction fraction of cell disruption were dropped onto the substrate plate. The plates were subsequently incubated overnight at 37°C. The activity of BGL was determined by estimation of esculin degradation with dark brown halo. The diameter of the brown halo was measured in millimetres.

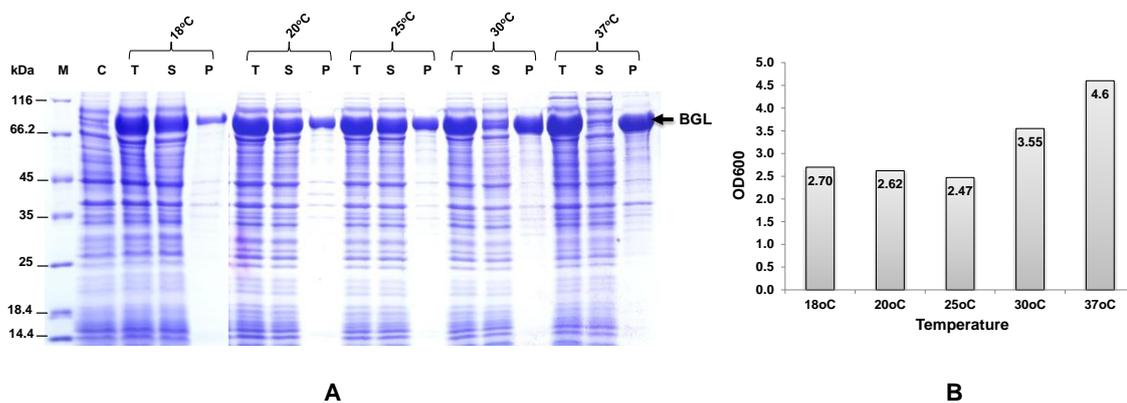
### RESULTS AND DISCUSSION

To obtain higher production of BGL with a

molecular weight of about 91 kDa, we had to optimize growth temperature, components of culture, IPTG inducer concentration, induction time, and harvesting time, which have major impacts on the recombinant protein expression (Overton, 2014; Quy *et al.*, 2015; Seyfi *et al.* 2019).

### Effect of culture temperature on expression of BGL

Culture temperature is one of the important factors that have an effect on *E. coli* cell growth and the expression of foreign proteins in *E. coli*. *E. coli*'s standard growth temperature is 37 °C, and recombinant proteins are normally synthesized at a high level at that temperature. However, in several cases, fermentation conditions at the high temperature leads to the production recombinant protein in insoluble form. To prevent the formation of inclusion bodies and correct folding of the recombinant protein, reduction of fermentation temperature is one of the common research questions (Dragosits *et al.*, 2011; Schein, Noteborn, 1988; Seyfi *et al.*, 2019; Vera *et al.*, 2007).



**Figure 1.** Effect of temperature on the cell growth and production of the recombinant BGL. The recombinant strain overexpressing BGL was fermented in LBA medium with the induction of 0.5 mM IPTG at different temperatures. After 4 hours of induction, the cell growth was determined. The cells were harvested and disrupted by sonication. Soluble and insoluble protein fractions were separated by centrifugation. Protein samples were separated on SDS-PAGE and detected with Coomassie Blue. (A) SDS-PAGE; (B). Harvesting cell density. (M) protein marker (Fermentas #SM0431), (C) control sample, (T, S, P) total, soluble, insoluble protein, respectively.

As expected, in comparison of temperature conditions, optical density showed a higher

growth rate at a higher temperature and, in all conditions, the recombinant BGL produced has a

molecular weight of approximately 91 kDa as estimated (Fig. 1). The level of the soluble recombinant protein production at 25°C was the highest and lower at 20 and 18°C. Yields of the soluble form were decreased at 30°C and then not obtained at 37°C. Fast accumulation of the recombinant BGL may increase the capacity to form inclusion bodies. When induced at 25°C, the rate of the recombinant BGL production was slower, so the lower concentration could prevent the aggregation. Thus, the culture at 25°C was used to obtain soluble recombinant protein production and to avoid inclusion body formation. This result was in agreement with other findings that lowering temperature can produce recombinant protein in soluble form (Dragosits *et al.*, 2011; Schein, Noteborn, 1988; Seyfi *et al.*, 2019).

#### **Effect of medium components on expression of BGL**

Components of the culture medium play an important role in increasing the production of recombinant proteins. Five media were examined, including LB, TB, modified TB, SB, and PE. Compared to standard LB medium, the others all increased the growth of the recombinant strain overexpressing BGL. Especially, modified TB containing glucose instead of glycerol in TB got the biggest growth, up to 2.4 folds as obtained from LB medium. Importantly, the recombinant BGL produced from the cell in the modified TB was also high that similar to TB, SB, PE (Fig. 2). This indicates that carbon source in the culture medium is an important factor for the product due to its impact on final cell density (Seyfi *et al.*, 2019). Thus, the selected medium for expression of the recombinant BGL was modified TB medium.

#### **Effect of IPTG inducer concentration on expression of BGL**

The gene encoding BGL was cloned in the pET 22b(+) vector under the control of T7 promoter system. Thus, IPTG was used to induce production of the recombinant protein, and the amount of IPTG can affect the yield of recombinant proteins. However, IPTG is a toxic

chemical and harmful to host cells at high concentration, leading to a lower yield of recombinant protein (Gutiérrez-González *et al.*, 2019; Villaverde, Carrió, 2003). In addition, the price of this chemical is high. Therefore, optimal IPTG concentration should be investigated for heterologous proteins. BGL expression was examined with IPTG at various concentrations from 0.05 to 2 mM. Our results showed that OD values at harvesting times were gradient increased for IPTG concentration from 0.05 to 0.3 mM and after that it was significantly reduced from 0.5 mM IPTG. It is worth noting that amount of recombinant BGL was also achieved by incline rise from 0.05 to 0.3 mM IPTG and then balancing at the subsequent concentrations (Fig. 3). Taken together, the suitable IPTG concentration chosen was 0.3 mM for further analysis.

#### **Effect of cell density on BGL expression at induction time**

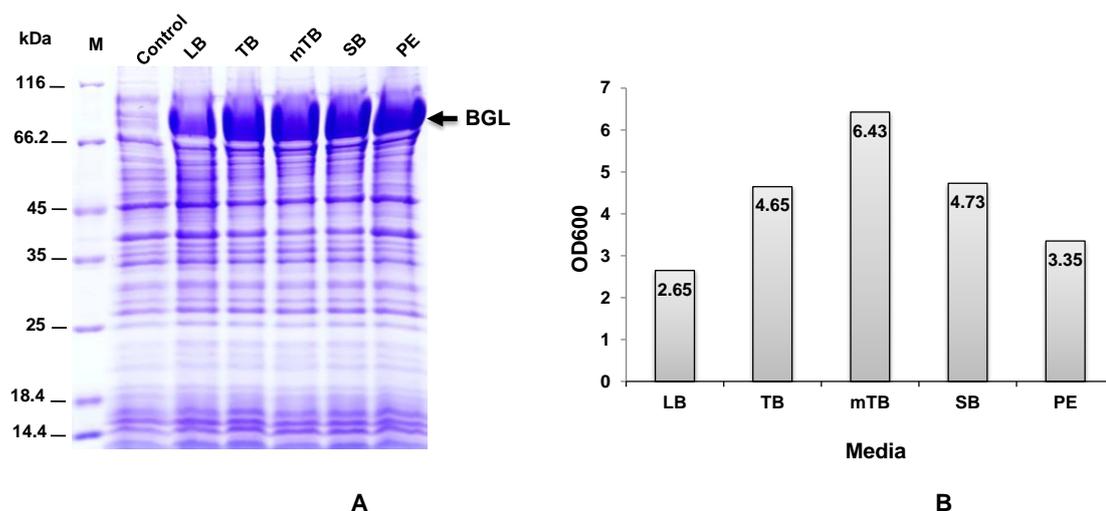
In addition to the inducer IPTG concentration, induction time is also very important for the high production of recombinant protein because it depends on the cell growth cycle (Babaeipour *et al.*, 2013). To determine optimal optical density for induction of the recombinant cell overexpressing the BGL, we measured the induction OD from 0.4 to 4 as initiate, mid and late log phase of cell growth. The result showed final cell density was raised and correlated to increasing cell density of induction time at an OD of 1 and maintained. The BGL was produced at its highest level when induced with IPTG at an OD of 1. Thus, maximum concentration and productivity of the recombinant BGL were obtained with induction at an OD of 1. The results indicated that the optimal induction suitable for production of the recombinant BGL was at the mid-log phase (Fig. 4).

#### **Determination of optimal harvesting time for the BGL production**

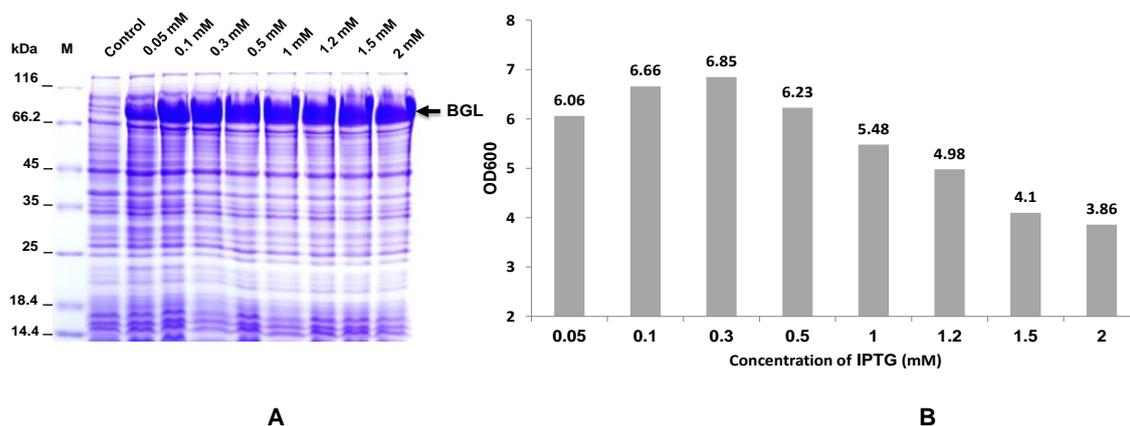
Post-induction duration strongly affects the concentration and overall productivity of the recombinant proteins expressed in *E. coli*. This is

affected by several factors, such as inducer concentration, induction time, and the response of the cell for the recombinant protein, as well as the solubility and characteristics of the recombinant protein. To select the proper post-induction duration for expression of the

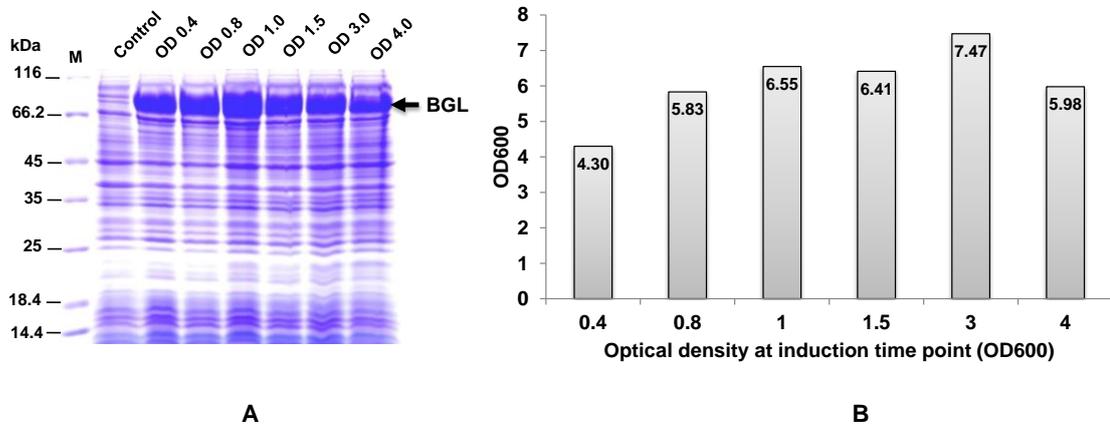
recombinant BGL, fermentation samples were harvested at a range of 1–6 hours and 22 hours after induction. The OD and protein analysis showed that the best productivity of the recombinant BGL that could be obtained was 4 hours after induction (Fig. 5).



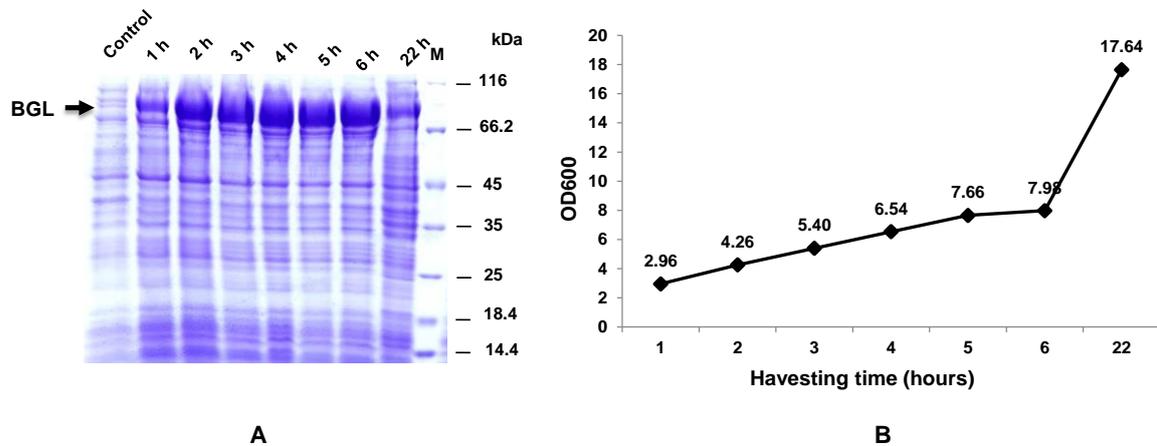
**Figure 2.** Effect of media components on the cell growth and production of the recombinant BGL. The strains were fermented in media with 0.5 mM IPTG at 25 °C for 4 hours of induction. Cell growth was determined and protein samples were separated on SDS-PAGE and detected with Coomassie Blue. (A) SDS-PAGE; (B). Harvesting cell density. (M) protein marker (Fermentas #SM0431).



**Figure 3.** Effect of IPTG concentration on the cell growth and production of the recombinant BGL. The strains were fermented in modified TB medium with induction of various IPTG inducer concentrations at 25 °C. After 4 hours of induction, the cell growth was determined. Protein samples were separated on SDS-PAGE and detected with Coomassie Blue. (A) SDS-PAGE; (B). Harvesting cell density. Control sample, IPTG concentrations from 0.05 to 2 mM, respectively. (M) protein marker (Fermentas #SM0431).



**Figure 4.** Effect of cell density at induction time on the cell growth and production of the recombinant BGL. The recombinant strain overexpressing BGL was fermented in modified TB medium with 0.3 mM IPTG inducer concentrations at different induction times at 25 °C. After 4 hours of induction, the cell growth was determined. Protein samples were separated on SDS-PAGE and detected with Coomassie Blue. (A) SDS-PAGE; (B) Harvesting cell density. Control sample, samples induced when optical density values ranged from 0.4 to 4, respectively. (M) protein marker (Fermentas #SM0431).



**Figure 5.** Determination of optimal harvesting time for production of the recombinant BGL. The recombinant strain overexpressing BGL was fermented in modified TB medium with 0.3 mM IPTG inducer concentrations at an OD<sub>600</sub> of 1 at 25 °C. Samples were harvested at different times. Cell growth was determined and protein samples were separated on SDS-PAGE and detected with Coomassie Blue. (A) SDS-PAGE; (B) Harvesting cell density. Control sample, harvesting times from 1 hour to 22 hours. (M) protein marker (Fermentas #SM0431).

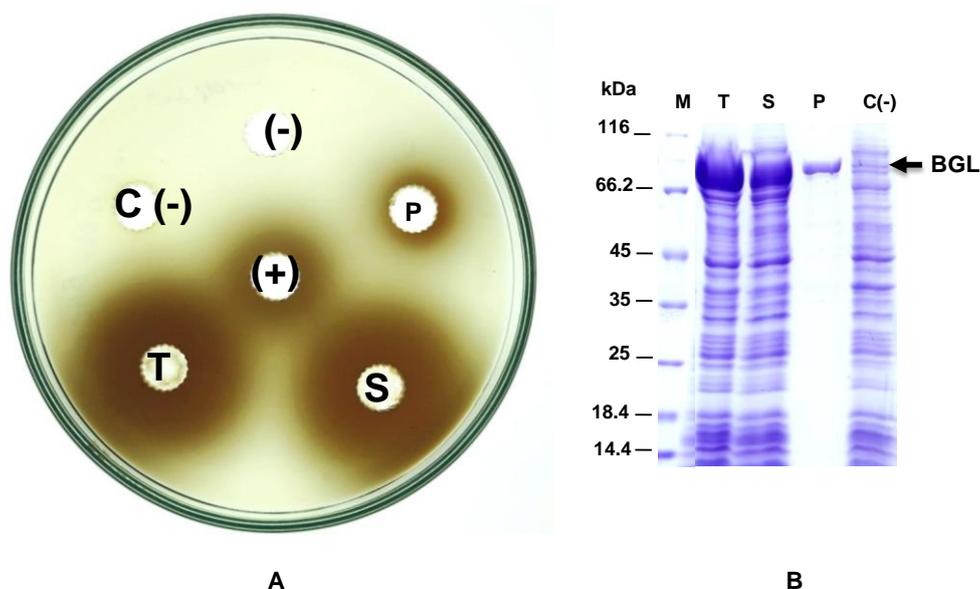
### Estimation of the recombinant beta-glucosidase activity using esculin substrate

Beta-glucosidase activity was evaluated on LB medium using esculin substrate (Veena *et al.*, 2011). Beta-glucosidase cleaves esculin to generate esculetin and glucose. Then the

product esculetin reduces the ferric ions in the medium to produce iron that results in the brown colour. Extraction of the recombinant BGL revealed beta-glucosidase activity. The halos were visualized at an overnight incubation. Figure 6 shows the brown halo sizes for samples with different levels of beta-

glucosidase activity. Total extraction and fraction of the soluble BGL form had the strongest activity compared with insoluble form and also negative control samples. The diameter of the degradation halo by the soluble beta-glucosidase was about 4 cm (Fig.

6). This indicated that the recombinant BGL was successfully expressed in soluble form and showed clear activity. This result had the potential for purification of the recombinant BGL and analysis of the characteristics of the recombinant enzyme.



**Figure 6.** Estimation of the recombinant BGL activity on LB agar plate using esculin substrate. (A): LB esculin plate, T, S, P: Total, soluble, insoluble samples, respectively, C-: control samples from the strain with pET22b(+); (-) Buffer; (+) standard enzyme (0.05U cellulase). (B): SDS-PAGE. T, S, P: Total, soluble, insoluble samples, respectively, C-: control samples from the strain with pET22b(+); (M) protein marker (Fermentas #SM0431), BGL: Beta-glucosidase.

## CONCLUSION

In conclusion, we have optimized some conditions for fermentation of the recombinant beta-glucosidase in recombinant *E. coli* Rosseta 1. The selected parameters for expression were 25°C, mTB medium, 0.3 mM IPTG inducer, induction time when cell density was an OD<sub>600</sub> of 1, and harvesting time after 4 hours of induction. The recombinant beta-glucosidase obtained had activity on the esculin substrate plate.

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