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Research article

Effect of supplemented sugar in lysogeny broth medium on growth of *Escherichia coli* BL21(DE3) and recombinant protein production

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Abstract

Importance of the work: *Escherichia coli* BL21(DE3) is among the powerful hosts for recombinant protein expression. Varying approaches have been explored to improve the production of large amounts of recombinant protein.

Objectives: To examine the effect of single sugar addition on the growth of *E. coli* BL21(DE3) and the production ability of recombinant green fluorescence protein (GFP) as a model for this study.

Materials & Methods: Bacteria were cultured in Luria-Bertani broth (LB) media with supplemental sugars as additional carbon sources (2–10 g/L). Cell growth was monitored by measuring the optical density at 600 nm. Following the inducement of recombinant GFP expression by adding isopropyl β-d-1-thiogalactopyranoside, the content of soluble GFP was quantitated using two distinct methods: fluorescence detection and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Results: The effect of sugar on the growth of *E. coli* BL21(DE3) could be classified into four groups of positive, negative, positive/negative and neutral effects, with a similar trend when pET-11a was included in the bacterial cells. Then, the expression of GFP under the optimum condition was determined in LB with the addition of the positive effect sugars (xylose or maltose). The addition of xylose or maltose in LB media significantly ($p < 0.05$) increased both cell growth by 2.0–2.5 fold and the production of recombinant GFP by 1.5–2.0 fold than those of the control and other sugars.

Main finding: This study presented a proof of concept to improve the recombinant protein production in *E. coli* BL21(DE3) by adding a carbon source into traditional culture media without using engineered cells.

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Introduction

The process for recombinant protein production from recombinant DNA that generally uses microorganisms as a host is useful for biotechnological applications, such as the production of protein used in the chemical and pharmaceutical industries (*Mattanovich* et al., 2012; Overton, 2014). *Escherichia coli* BL21(DE3) is the most widely bacterium used to host the expression and production of recombinant proteins (Liu et al., 2013; Rosano et al., 2019). The pET vector is a powerful and widely used system for expressing recombinant proteins in *E. coli* (Dubendorff and Studier, 1991; Mierendorf et al., 1998). The gene of interest is normally cloned in the multiple cloning site in the pET vector under the control of the strong bacteriophage T7 transcription and translation regulatory system.

Normally, *E. coli* BL21(DE3) can easily be cultured on Luria-Bertani broth (LB) and auto-induction media, with the cells grown in LB media using peptone and yeast extract as the nutrients, and gene expression is induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG), which turns the gene on by binding to the *lac* repressor (Marbach et al., 2012). Auto-induction media are made from a well-balanced mixture of carbon substrates of glucose, glycerol, and lactose (Studier, 2005). Glucose is the preferred carbon source and catabolic repressor of the *lac* promoter in the early stages of the culture, whereas glycerol could be used as a supporting carbon source during the recombinant protein production stage and lactose induces the expression by endogenously metabolizing to allolactose, which turns on the gene by binding to the *lac* repressor (Studier, 2005). Using auto-induction media commonly results in higher levels of functional target protein compared with conventional and manual induction with IPTG (Studier, 2005).

Several approaches have been used to obtain large amounts of recombinant protein production in *E. coli*, including the selection of an appropriate host, vector, promoter, inducer, medium and induction temperature (Liu et al., 2013; Rosano and Ceccarelli, 2014; Kaur et al., 2018; Wang et al., 2019). Other approaches include testing various combinations of hosts and vectors to maximize protein production ability (Khushoo et al., 2005), the improvement of pET expression plasmid design (Shilling et al., 2020) and the co-expression of two distinct proteins (Vincentelli and Romier, 2013). One simple approach for increasing recombinant protein expression is the addition of a carbon source into the media. Several reports have described media optimization to obtain higher yields of recombinant protein produced from *E. coli* (Ye et al., 2010; Chen et al., 2014; Deng et al., 2014; Mansey et al., 2014). Glucose is widely used as a carbon source in microorganism fermentation because it is a simple sugar easily consumed by cells (Kazan et al., 1995; Robbins Jr. and Taylor, 1989). Approaches have been reported for using mixed sugars in cultured media, normally auto-induction media; however, the hierarchy of sugar usage is problematic, as is carbon catabolic repression, which limits the efficient growth of bacteria cells (Hua et al., 2004; Zhou et al., 2013; Aidelberg et al., 2014; Beisel and Afroz, 2016). In contrast, limited research has demonstrated the use of single sugars as a carbon source, which is easier and cheaper (Kaur et al., 2018; Wang et al., 2019).

Therefore, the current study examined the effect of sugar on *E. coli* BL21(DE3) growth and the expression level of the target recombinant protein. LB medium was chosen to avoid carbon catabolite repression from the mixture of sugars in auto-induction media. Recombinant green fluorescence protein (GFP)—which is easy to detect—was used as a representative recombinant protein. Observing the effect of sugar on recombinant protein expression in *E. coli* BL21(DE3) could provide useful knowledge to enhance universal recombinant protein production in other systems.

Materials and Methods

Cell and culture media

E. coli BL21(DE3) was purchased from ECOS™ Competent Cells (Yeastern Biotech Co., Ltd; Taiwan). The LB medium was prepared by mixing 10 g/L peptone (Condalab; Spain), 5 g/L yeast extract (Condalab; Spain) and 10 g/L sodium chloride (Merck; USA). Chemical reagents and sugars—D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, D-(-)-fructose, D-(+)-xylose, L-(+)-arabinose, α-lactose monohydrate, maltose monohydrate, and sucrose—were purchased from Sigma-Aldrich (USA) or Tokyo Chemical Industry Co. Ltd (Japan). All reagents including media and sugars were sterilized using autoclaving at 121°C for 15 min before use. The synthesized recombinant plasmid of *gfp*-pET-11a that encodes for the recombinant green fluorescence protein (GFP) was purchased from GenScript (USA).

Preparation of competent E. coli BL21(DE3)

The method used to prepare competent *E. coli* BL21(DE3) was adapted from the protocol reported by Nakata et al. (1997). Briefly, glycerol stock of *E. coli* BL21(DE3) was streaked on an LB agar plate and incubated at 37 °C overnight. A single colony of *E. coli* BL21(DE3) was inoculated in 5 mL of LB medium and shaken overnight at 200 revolutions per minute (rpm) and 37 °C. Then, the resulting culture (1% volume per volume; v/v) was inoculated in 250 mL of LB medium and incubated with shaking at 37 °C and 200 rpm until the optical density at wavelength 600 nm (OD_{600}) reached 0.3–0.4. The cultured *E. coli* BL21(DE3) was stopped by transferring into an ice-cold screw cap tube and storing on ice for 10 min. The cell was harvested using centrifugation at 4,000 rpm at 4 °C for 10 min. The cell paste was washed using suspension in a solution of ice-cold 80 mM $MgCl_2$ with 20 mM $CaCl_2$ before it was collected using centrifugation at 4,000 rpm at 4 °C for 10 min. Next, the cell was suspended in an ice-cold 0.1 M $CaCl_2$ solution. Glycerol was added into *E. coli* BL21(DE3) to obtain the final concentration of 15% (v/v). The competent cells were aliquoted into microfuge tubes (100 µL each) and stored at -80 °C pending use.

Culture of E. coli BL21(DE3) on lysogeny broth medium with sugar addition

In the absence of recombinant plasmid, a single colony of competent *E. coli* BL21(DE3) cells on LB agar was inoculated into 10 mL of LB medium and incubated with shaking at 200 rpm and 37 °C overnight to prepare the starter culture. Next, 1% (v/v) of starter culture was inoculated into 200 ml of LB medium (in a 1 L Erlenmeyer shake flask) and cultured by incubating with shaking at 37 °C and 200 rpm until the OD_{600} reached 1.0. Then, the culture was divided into 10 mL medium samples in 50 mL screw cap tubes to avoid bias from the originating cells. In each tube, sugars—glucose, galactose, lactose, fructose, arabinose, maltose, mannose, sucrose and xylose— were individually added at final concentrations of 2 g/L, 4 g/L, 6 g/L, 8 g/L and 10 g/L. Medium with no sugar added was set as a control. Then, the cultured bacteria were continuously incubated with shaking at 200 rpm and 37 °C. The growth curve of *E. coli* BL21(DE3) for each condition was monitored by measuring OD_{600} until the cell entered the stationary phase, at which point the OD_{600} values were compared to observe how the addition of sugar had affected cell growth ability.

Expression of recombinant green fluorescence protein in E. coli BL21(DE3)

The recombinant *gfp*-pET-11a plasmid was inserted into *E. coli* BL21(DE3) using the heat-shock transformation method. The transformed cells were spread on an LB agar plate containing ampicillin (100 µg/mL) as the selective antibiotic and incubated overnight at 37 °C. The single colony was cultured using a similar protocol as described in the previous section; however, 50 µg/mL of ampicillin was included in LB media. In addition to the sugar supplement, IPTG (final concentration of 1 mM) was added into the culture when OD_{600} reached 1.0 to induce the recombinant protein expression. Cells were continuously cultured at 37°C overnight. The final OD_{600} was recorded before the cells were harvested using centrifugation at 5,000 rpm at 4 °C for 15 min. The cell paste was suspended in 50 mM Tris-HCl pH 7.0 before it was lysed using ultrasonication. Cell debris was removed using centrifugation at 15,000 rpm for 30 min at 4 °C. Cell lysate in the supernatant was used to determine the content of soluble protein for each condition. The total milligrams of protein were determined based on Bradford's assay (Bradford, 1976). The amount of soluble GFP was measured based two distinct approaches. In the first approach, the fluorescence signal from GFP was measured using an excitation wavelength of 485 nm with an emission wavelength of 535 nm in a Spark™ 10M (Tecan; Switzerland) microplate reader. Samples were diluted 10 times in 50 mM Tris-HCl pH 7.0 to discard the self-inner filter effect (Panigrahi and Mishra, 2019). In the second approach, the amount of GFP was quantitated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide) analysis by measuring the band intensity of GFP (~27 kDa in size) using the ImageJ software (Schneider et al., 2012).

Statistical analysis

All data was measured in three biological replications and the results were reported as average values ± SD. Statistical significance was tested using an independent t test in the Statistical Package for Social Sciences (SPSS; IBM Corp.; Armonk, NY, USA). The test results were considered significant at $p < 0.05$.

Results and Discussion

Effect of sugar on E. coli BL21(DE3) growth on LB broth medium

The effect of sugar addition on the growth of *E. coli* BL21(DE3) culture was initially screened by monitoring the growth curve of *E. coli* BL21(DE3) cultured in LB medium supplemented with 10 g/L of sugar. The structures of the sugars used in this experiment—hexoses (glucose, galactose, mannose, fructose), pentoses (xylose and arabinose) and disaccharides (lactose, maltose, and sucrose)—are illustrated in Fig. 1. The control was performed using the basal medium component without sugar addition. The OD_{600} of the culture was monitored for 50 hr; the growth curve is shown in Fig 2. The growth ability of *E. coli* BL21(DE3) in LB media supplemented with glucose, galactose, fructose, arabinose or lactose was slightly less than the control, whereas additions of mannose and sucrose resulted in a similar growth curve to that of the control. Surprisingly, the addition of maltose and xylose potentially increased cell growth compared with the basal condition. The OD_{600} for the stationary phase increased to about 6 and 8 in the presence of maltose and xylose, respectively, compared to OD_{600} of approximately 4 for the basal medium.

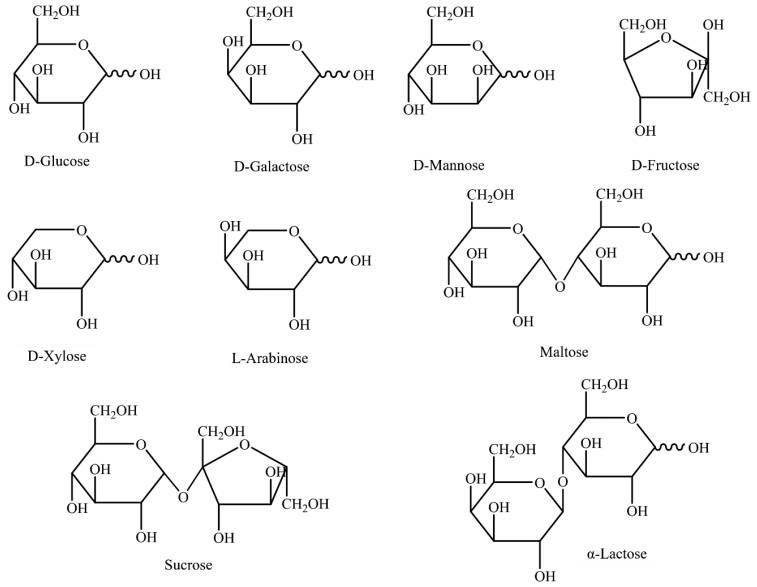

Fig. 1 Chemical structures of sugars used in the current study

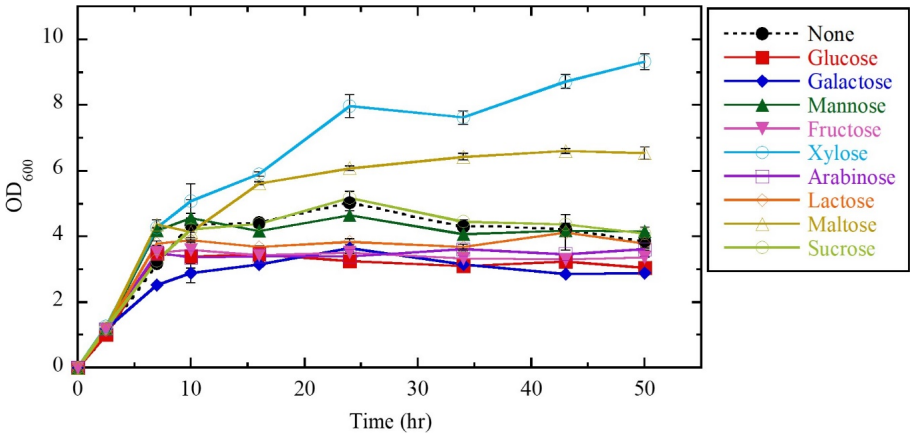

Fig. 2 Growth curves of *Escherichia coli* BL21 (DE3) in LB media with 10 g/L of supplemental sugars, where OD_{600} is the optical density at 600 nm and error bars indicate ± SD

Then, the concentrations of added sugars in LB media were varied at 2 g/L, 4 g/L, 6 g/L, 8 g/L or 10 g/L to study the effect of sugar concentration on the growth of *E. coli* BL21(DE3). Based on the growth curves in Fig. 2, the growth of *E. coli* BL21(DE3) in LB medium for each of the added sugars reached the stationary phase at 24 hr. Thus, cells were incubated with shaking for 24 hr before OD_{600} was recorded to determine cell growth. The results were plotted in a bar graph (Fig. 3A). The data revealed that the sugars affecting the growth of *E. coli* BL21(DE3) could be classified into four groups. First, xylose and maltose had a positive effect. Cells grew significantly when the concentration of xylose was increased, yielding OD_{600} values of 8–9, while maltose increased the OD_{600} to 6–7 which was significantly higher than that for the control. Second, glucose, fructose, mannose, arabinose and lactose had both positive and negative effects. Adding these sugars in culture at low concentrations increased the growth of *E. coli*; however, adding them at high concentrations resulted in reduced cell growth. This phenomenon has been reported elsewhere (Kazan et al., 1995; Mansey et al., 2014). Third, galactose had only a negative effect, reducing the growth of *E. coli* when the concentration was increased. Last, sucrose had a neutral effect in that it did not alter cell growth ability. These different effects might have been due to different metabolic pathways within the cells when the distinct sugars were added as a supplemental carbon source. For example, the negative effect might have been caused by the carbon catabolic repression mechanism whereby the carbon source represses the activity of other central metabolic pathways (Stülke and Hillen, 1999; Görke and Stülke, 2008).

Effect of sugar on growth of E. coli BL21(DE3) with pET-11a plasmid on LB broth medium

To achieve recombinant protein expression, the recombinant plasmid must be incorporated into a bacterium cell. The effect of added sugars was confirmed in *E. coli* BL21(DE3) with pET-11a plasmid, which was used as an expression vector containing the *lac* operator. The vector pET-11a was transformed into the *E. coli* BL21(DE3) prior to culturing in LB media with various concentration of added sugars. The OD_{600} was recorded after the cell had been cultured at 37 °C for 24 hr (stationary phase). The trend of cell growth in the presence of the pET-11a vector was similar to that of the sole bacterial cell; however, the overall OD_{600} decreased (Fig. 3B). Adding xylose and maltose had a significantly more positive effect on *E. coli* BL21(DE3) growth compared to the control and the other added sugars. Arabinose and lactose seem to increase the growth of *E. coli* BL21(DE3); however, the OD_{600} values were less than those of xylose and maltose. The rest of the sugars had neutral or negative effects on cell growth. Thus, transformed plasmids might affect the overall growth of *E. coli*; however, they did not alter the effect of sugar on cell growth. The addition of sugars, especially xylose or maltose, for the improvement of recombinant protein expression should be further studied.

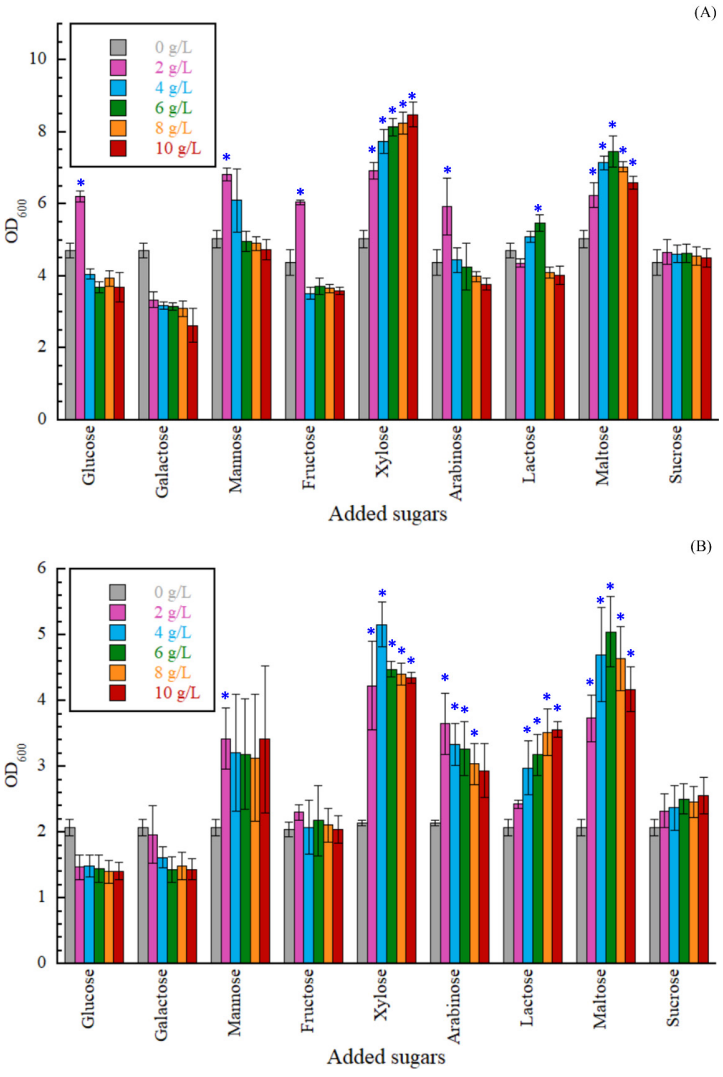

Fig. 3 Optical density at 600 nm (OD_{600}) values of cell growth in various final concentrations (0–10 g/L) of supplemental sugars in lysogeny broth media recorded after 24 hr of cultivation at 37 °C: (A) absence of plasmid; (B) presence of pET-11a vector, where asterisks (*) indicate histograms (values) that significantly ($p < 0.05$) increased compared to the control, histograms without asterisk indicate non-significantly ($p > 0.05$) difference from the control; error bars indicate ± SD

Conditions for expression of recombinant green fluorescence protein in E. coli BL21(DE3)

Before testing the effect of sugar on recombinant protein expression, the conditions were tuned for recombinant GFP expression. The plasmid of *gfp*-pET-11a was transformed into *E. coli* BL21(DE3). Then, *E. coli* BL21(DE3) + *gfp*-pET-11a was cultured following the protocol described in the Materials and Methods section above. After protein expression had been induced by adding IPTG, the cultivation temperature was switched to 37 °C, 25 °C or 16 °C, and the cultures were continuously shaken for 24 hr. Uninduced cells without the addition of IPTG were used as a negative control. The OD_{600} values of bacterial cultures at different cultivation temperatures are shown in Fig. 4A, with *E. coli* BL21(DE3) + *gfp*-pET-11a having greater growth ability at 25 °C than at 37 °C and 16 °C. The protein content shown in the SDS-PAGE analysis (Fig. 4B) revealed that GFP can be expressed in soluble form when induced by adding IPTG in all cultivation temperatures. Based on these data, the best conditions for expression of recombinant GFP in LB medium were IPTG induction with a cultivation temperature of 25 °C. Thus, this condition was used to study the effect of added sugar as described in the next section.

Effect of xylose and maltose for recombinant green fluorescence protein production in E. coli BL21(DE3)

The amounts were monitored of recombinant GFP production in *E. coli* BL21(DE3) in the presence of glucose (as the control), xylose or maltose in LB media. Following a similar trend to that described in the previous section, the OD_{600} values obtained after overnight cultivation significantly increased with the addition of xylose or maltose; however, no significant effect was observed with the addition of glucose (Fig. 5A). The amount of GFP expression for each condition was determined by two approaches, namely the detection of GFP based on fluorescence measurement and the protein band intensity measured using SDS-PAGE analysis (see Materials and Methods section). The values were normalized based on the total milligrams of protein calculated using Bradford assay. The results of the two methods were similar in that LB supplemented with xylose or maltose significantly increase the production of recombinant GFP in *E. coli* BL 21(DE3), as shown in Figs. 5B–5C. The results showed that 2 g/L of xylose and 4–10 g/L of maltose produced the highest levels of both cell growth ability and GFP production. The amount of recombinant GFP was increased about 1.5-fold and 2.5-fold higher than that of the control when fluorescence intensity and

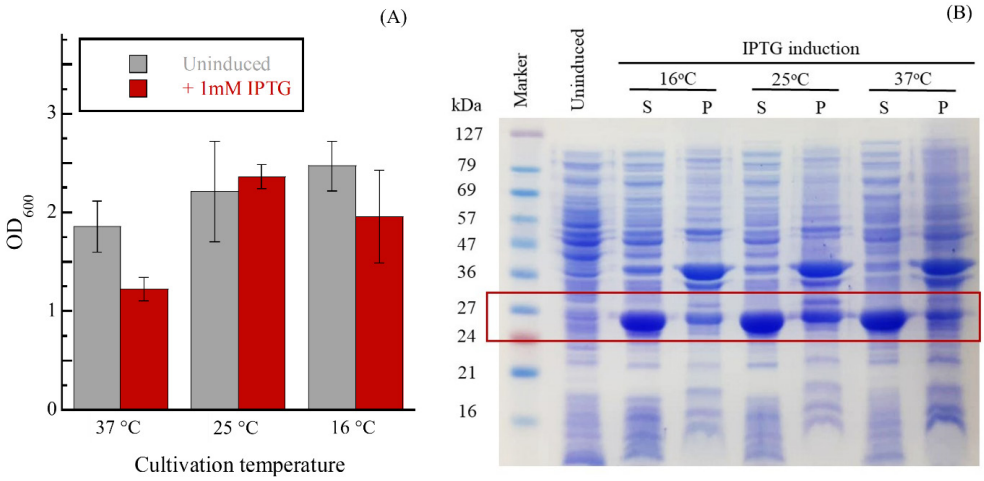

Fig. 4 (A) Optical density at 600 nm (OD_{600}) of cell growth measured following cultivation for 24 hr after 1 mM IPTG was added to induce protein expression at various cultivation temperatures and error bars indicate ± SD; (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of protein content (20 ug in each lane) with GFP bands in red box, where markers were AccuProtein Chroma molecular weight markers (Enzmart Biotech, Thailand), uninduced denotes soluble part of cell without induction and soluble protein (S) and pellet (P) parts of each sample were analyzed

band intensity were used, respectively. The different values might have resulted from error detection in each method. Noticeably, with a high concentration of xylose, the production of GFP dropped compared to 2 g/L of xylose. This might have been due to the repression of the T7 promoter, as has been reported for high concentrations of xylose weakly repressing protein expression from the *lacZ* promoter (Ammar et al., 2018). Thus, based on the current results, it is recommended that the addition of either 2 g/L of xylose or 4 g/L of maltose is sufficient to improve GFP production under the control of the T7 promoter. Measurement of the protein content using SDS-PAGE analysis (Fig. 5D) revealed an intense band of GFP when xylose or maltose were applied; however, LB supplemented with glucose did not efficiently increase cell growth or recombinant protein production compared to glucose with no sugar addition. Thus, all these results indicated that the addition of xylose or maltose (2–10 g/L) into LB media could improve recombinant GFP production under the control of the *lac* operator.

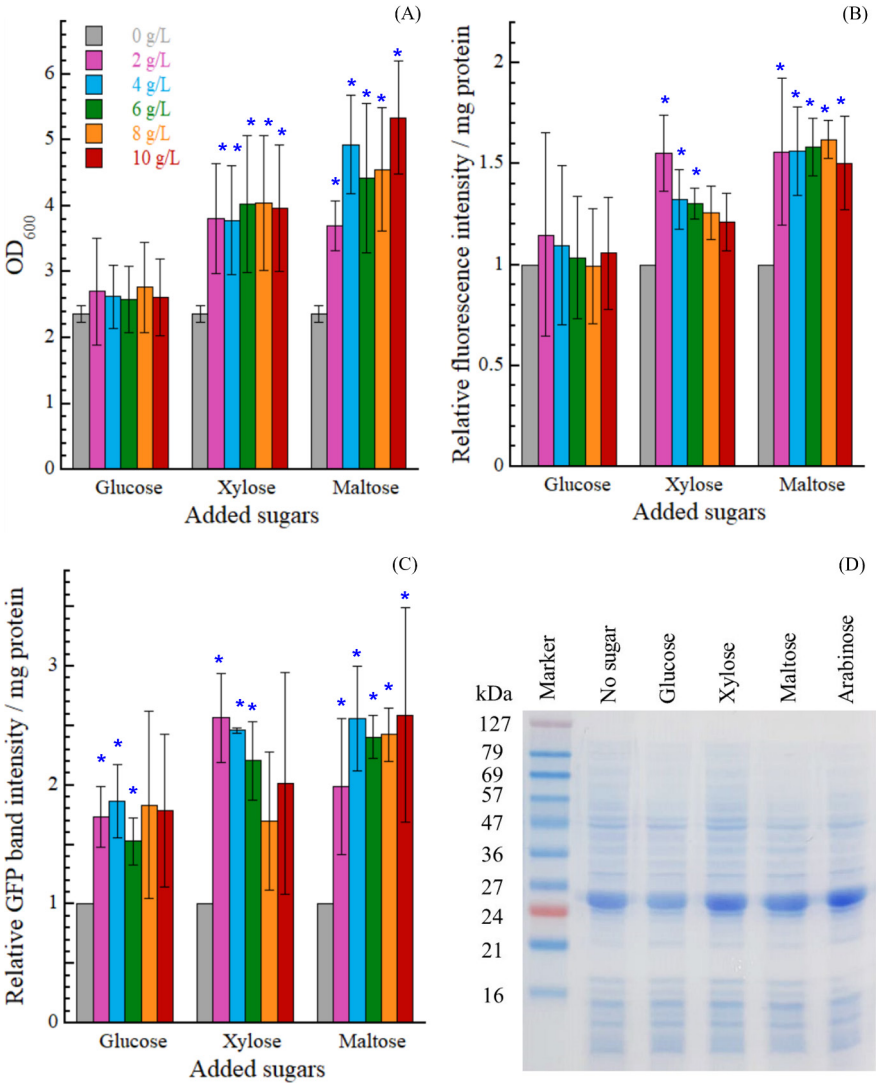

Fig. 5 (A) Optical density at 600 nm (OD_{600}); (B) relative fluorescence intensity; (C) relative green fluorescence protein band intensity obtained from the growth of *E. coli* BL21(DE3)+*gfp*-pET-11a in lysogeny broth media supplemented with various concentrations (0–10 g/L) of glucose, xylose or maltose, where asterisks (*) indicate histograms (values) significantly ($p < 0.05$) increased compared to control, histograms without asterisk indicate non-significantly ($p > 0.05$) difference from the control; error bars indicate ± SD; (D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of protein content with addition of 10 g/L of glucose, xylose or maltose compared to no sugar (negative control) and protein marker (Enzmart Biotech, Thailand)

Perspective of recombinant protein production with sugar supplement

The results indicated that many types of sugar could serve as a carbon source to increase the growth ability of *E. coli* BL21(DE3), especially when the sugar concentration was low, such as the growth of *E. coli* in 2 g/L of glucose (Fig. 2A). Glucose is the most common monosaccharide and it is found in various substances, such as nutrients and cellulose in biomass (Corma et al., 2007), which makes it attractive as a carbon source. However, the current results demonstrated there was no significant effect on *E. coli* growth on LB media from the addition of glucose or various other sugars. However, surprisingly, the addition of xylose or maltose in the LB media significantly increased the growth ability of *E. coli* BL21(DE3) and improved recombinant GFP production. Because these processes are proportionally dependent, this finding suggested that the higher yield of recombinant protein might be caused by the larger amount of *E. coli*. Generally, bacterial cells (including *E. coli*) can metabolize both xylose and maltose as sole carbon sources (Boos and Shuman, 1998; Zhao et al., 2020). In the case of the xylose metabolic pathway, uptake to the cell occurred via a xylose transporter encoded from *xylA*. Next, D-xylose was initially converted to D-xylulose using xylose isomerase encoded from *xylF*; then, xylulokinase catalyzed the conversion of D-xylulose to D-xylulose-5-phosphate, which was then directed to the pentose phosphate pathway (Lawlis et al., 1984; Kuhad et al., 2011; Zhao et al., 2020). Maltose was transported to the *E. coli* cell via the maltodextrin/maltose transport proteins encoded by its maltose operon (*mal*) (Boos and Shuman, 1998). Then, maltase or α-glucosidase could catalyze the hydrolysis of maltose to two molecules of glucose, which were further metabolized by the central metabolic pathway (Olusanya and Olutiola, 1986). Several studies have demonstrated that the expression of genes in metabolic pathways in bacteria can be regulated by different carbon sources (Yao et al., 2011; Beisel and Afroz, 2016). Based on these findings, it could be proposed that the addition of xylose or maltose in the media of *E. coli* growth might promote the expression of proteins in both transportation and metabolism.

Other studies have described the use of supplemental sugar in culture media for improving protein production in *E. coli* and various papers have described the metabolic engineering of *E. coli* to efficiently use xylose as the main carbon source (Liu et al., 2018; Rossoni et al., 2018; Fujiwara et al., 2020). Xylose was considered the most attractive sugar because it is the second-most abundant sugar in nature and can be extracted from hemicellulose in the lignocellulosic biomass (Saha, 2003; Kuhad et al., 2011). Using sugar from biomass as a carbon source supplement for recombinant protein production could produce high-cost protein from biological waste in an economically feasible manner. Since only one globular protein, GFP, under the control of the T7 promoter of the pET expression plasmids was used as a model in the current study, there may be different effects from single sugar addition for other recombinant protein production, especially for membrane proteins. Thus, this simple strategy could be one option to obtain higher amounts of recombinant protein for research and industry without using an engineered cell. It could be useful for the production of other recombinant proteins in an economically feasible manner.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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