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EXTRACELLULAR β-GLUCOSIDASE PRODUCTION FROM *bglp15.2* GENE CARRYING INULINASE SIGNAL PEPTIDE IN *Saccharomyces cerevisiae* BY4741

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Abstract. *One of the important enzymes in cellulase complex is β-glucosidase. In this research, adding signal peptide of inulinase gene from Kluyveromyces marxianus, cloning, and expressing of bglp15.2 gene in S. cerevisiae BY4741 had been done. Gene of bglp15.2 encoding β-glucosidase has 90% identity to nucleotide sequence of Shewanella frigidimarina NCIMB 400 bacteria. Adding nucleotide sequence of signal peptide was aimed to secrete β-glucosidase and had been done with PCR (Polymerase Chain Reaction) method. The addition of nucleotide sequence of signal peptide in bglp15.2 gene had been done succesfully that indicated from nucleotide sequencing result and the increment of amplicon band size in electroferogram of the last addition PCR step. The bglp15.2 and bglp15.2INU gene (the bglp15.2 gene that has signal peptide nucleotide sequence) were cloned in Escherichia coli DH5α using pGEM-T-Easy vector and pBEVY-GL shuttle vector. The pBEVY-GL shuttle vector was used for transforming S. cerevisiae BY4741 with bglp15.2 and bglp15.2INU. The recombinant S. cerevisiae BY4741 carrying bglp15.2INU gene and growing in 48 hours had extracellularly β-glucosidase enzyme activity of 0,0178 U/ml and the intracellularly activity was 0,0181 U/ml. The β-glucosidase enzyme without signal peptide was not secreted. With K. marxianus inulinase signal peptide, about 50% Bglp15.2INU protein could be secreted. The protein molecular weight of secreted Bglp15.2INU was 44 kDa in SDS-PAGE result.*

Keywords : *cloning, expression, signal peptide, β-glucosidase, extracellular, enzyme activity.*

How to Cite
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INTRODUCTION

Lignocellulosic biomasses obtained from agricultural byproduct and agroindustry waste, such as bagasse, corn straw, rice straw and wheat, as well as wood chips, are abundant in

nature and can not be used as food, so the lignocellulosic biomasses can be used in ethanol production replacing starch and molasses (Hasunuma and Kondo, 2012; Treebupachatsakul et al., 2015). In general, lignocellulosic biomass comprises of 40% to 50% cellulose, 25% to 30% hemicellulose, 15% to 20% lignin and other components (Menon and Rao, 2012). Enzymatic hydrolysis of cellulose requires three types of cellulase enzymes, namely endo-β-1,4-glucanase (EG, E C 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91 and EC 3.2.1.176), and β-glucosidase (BGL, EC 3.2.1.21). β-glucosidase plays an important role in the degradation of cellulose because it eliminates cellobiose that inhibits enzymatic reaction catalyzed by endo-β-1,4-glucanase and cellobiohydrolase (Gao et al., 2014). *Bglp15.1* gene encoding β-glucosidase enzymes had been isolated from metagenome of Kawio Islands, North Sulawesi sea water and sediment samples. *bglp15.1* gene sequence has 90% similarity with the β-glucosidase nucleotide sequences of the *Shewanella frigidimarina* NCIMB 400 (Acc .: CP000447.1) bacteria. Protein β-glucosidase encoded from *bglp15.1* gene has a size of 52 kDa and has 93% similarity with the amino acid sequence of β-glucosidase *S. frigidimarina* NCIMB 400 bacteria. *bglp15.1* enzyme has β-glucosidase enzyme activity of 1,605 U / ml at a temperature of 55 ° C and pH 7 (Erawijantari, 2014).

The effective method of ethanol fermentation from cellulosic biomass is SSF *(Simultaneous Saccharification and Fermentation).* However, to increase ethanol production by 90% to 100%, requires the addition of extracellular β-glucosidase enzymes into the system. The addition of extracellular β-glucosidase enzyme can increases the production costs. This problem can be overcome by expressing β-glucosidase gene into *S. cerevisiae* so that the enzyme production, saccharification and ethanol fermentation can take place in one step (Hasunuma and Kondo, 2012; Lee et al., 2013). In this research, cloning and expression of genes encoding β-glucosidase *bglp15.1* of thermophilic bacteria from sea of Kawio Islands, North Sulawesi on *S. cerevisiae* BY4741 had been done to obtain *S. cerevisiae* BY4741 cells capable of expressing the protein β-glucosidase *bglp15.1* extracellularly. Since extracellular protein secretion in *S. cerevisiae* requires a signal peptide (Schekman et al., 1982), a signal peptide is added to the *bglp15.1* gene. Tang et al. (2013) reported that the highest enzyme activity of β-glucosidase *Saccharomycopsis fibuligera* in *S. cerevisiae* obtained from β-glucosidase protein secreted using inulinase *(inu1) Kluyveromyces marxianus* signal peptides and low enzyme activity was obtained from β-glucosidase protein that used the prepro-α-mating factor signal peptide. *S. fibuligera* β-glucosidase secreted in *S. cerevisiae* by using native, invertase (*suc2*), and acid phosphatase (*pho5*) signal peptide have lower enzyme activity than those secreted by using *K. marxianus* inulinase (*inu1*) signal peptide. Chung et al. (1995) reported that the secretion of human lipokortin-1 (37 kDa) in *S. cerevisiae* using *K. marxianus* inulinase signal peptide reaches 95%, while the secretion of these proteins by using the prepro-α-mating factor *(mfα1)* signal peptide only account for less than 10%. Similarly, most of the human interleukin-2 protein (17 kDa) can be secreted by using *K. marxianus* inulinase signal peptide. *K. marxianus* inulinase signal peptide can be used to secrete the protein human α-antitrypsin (52 kDa) up to 70% and inulinase protein by 70% to 90% in *S. cerevisiae* (Kang et al., 1996; Chung et al., 1995). Because of its high ability to secrete recombinant proteins in *S.*

cerevisiae, the *K. marxianus* inulinase signal peptide was choosen to secrete the protein β-glucosidase *(bglp15.1)*.

MATERIALS AND METHODS

Materials

The initial sample used in this study was *Escherichia coli* BL21 carrying pET-32b-*bglp15.1* plasmid from previous result of Erawijantari (2014). The cloning vector was pGEM-T-Easy from Promega. Host cell used to perform pGEM-T-Easy carrying *bglp15.2* and *bglp15.2INU* genes was *E. coli* DH5α. The expression vector was pBEVY-GL shuttle vector located within the *E. coli* TOP10 cell and host cell used to express the *bglp15.2* and *bglp15.2INU* gene was *Saccharomyces cerevisiae* BY4741. The primers used to add the nucleotide sequence of the *K. marxianus* inulinase gene signal peptide in *bglp15.1* gene were InuFm1, InuFm2, InuFm3, InuFm4, InuFm5, InuFm6, and InuRm. Those six pairs of primer produced the *bglp15.2INU* gene. The primers used to produce the *bglp15.2* gene (without the signal peptide) were *bglp15.1* Fm and *bglp15.1* Rm. These primer pair used to add the restriction side of *Sac*I and *Eco*RI to the *bglp15.2INU* and *bglp15.2* genes. The nucleotide sequence of primers were showed in table 1.

Table 1. Primer used to produce *bglp15.2* and *bglp15.2* gene

Primer name	Nucleotide sequens 5'→3'	Direction	Tm (°C)	Long (nt)	Addition/Function
bglp15.1Fm	TCCGAGCTCATGGGGAGT TGCTAC	*Forward*	70,8	24	TCCGAGCTC (restriction site of *Sac*I)
bglp15.1Rm	CCGGAATTC**CTA**CGCCCA TTCAAAATTATC	*Reverse*	69,1	30	CCGGAATTC (restriction site of *Eco*RI) **CTA** (**Kodon stop)**
InuFm1	GTGCTTCAGTGATCAATT ACAAGAGAATGGGGAGT TGCTAC	*Forward*	73,2	41	GTGCTTCAGTGATCAATT ACAAGAGA (26 sekuens nt sinyal peptida)
InuFm2	ATTGGCAGGAGTCAGTG CTTCAGTGATCAATTACA AGAGAATG	*Forward*	74	43	ATTGGCAGGAGTCA (14 sekuens nt sinyal peptida)
InuFm3	TCTTGCTTCCATTGGCAG GAGTCAGTGCTTC	*Forward*	73,5	31	TCTTGCTTCC (10 sekuens nt sinyal peptida)
InuFm4	GCATACTCCCTCTTGCTT CCATTGGCAGGAG	*Forward*	73,2	31	GCATACTCCC (10 sekuens nt sinyal peptida)
InuFm5	ATGAAGTTCGCATACTCC CTCTTGCTTCCATTG	*Forward*	72,1	33	ATGAAGTTC (9 sekuens nt sinyal peptida)
InuFm6	TCCGAGCTCATGAAGTTC GCATACTCCCTCTTG	*Forward*	73,3	33	TCCGAGCTC (restriction site of *Sac*I)
InuRm	CCGGAATTC**CTA**CGCCCA TTCAAAATTATCC	*Reverse*	70,2	31	CCGGAATTC (restriction site of *Eco*RI) **CTA** (**Kodon stop)**

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Modification of *bglp15.1* Gene to Produce *bglp15.2INU* and *bglp15.2* Gene

The addition of signal peptide nucleotide sequences to the *bglp15.1* gene to produce the *bglp15.2INU* gene was performed by six times gradually PCR method. The DNA template used in each signal peptide addition step was the PCR result of the previous step which has been purified by using ATP ™ Gel / PCR Fragment DNA Extraction Kit from ATP Biotech, Inc. The fragment from sixth step PCR was ligated into the pGEM-T-Easy vector and used to transform the *Escherichia coli* DH5α host cell. Production of *E. coli* DH5α competent cell was performed using CCMB80 method and its transformation was performed by heat shock method (Hanahan et al., 1991). Selection of positive transformant was performed on LB / Ampicilin / IPTG / X-Gal selection media. Confirmation of successful transformation was done by colony PCR method (Bergkessel and Guthrie, 2013). The pGEM-T-Easy vector carrying *bglp15.2INU* and *bglp15.2* were isolated from positive transformant using ATP® Plasmid Mini Kit from ATP Biotech, Inc.. The nucleotide sequence of *bglp15.2INU* and *bglp15.2* gene were verified by sequencing.

Construction and Subcloning of pBEVY-GL Vector Carrying *bglp15.2INU* and *bglp15.2* Genes

The pGEM-T-Easy plasmid carrying *bglp15.2INU* and *bglp15.2* genes and the pBEVY-GL vector were cut by *Sac*I and *Eco*RI restriction enzymes. Restriction reaction with *Sac*I enzyme was carried out at 37° C for 1 hour and the inactivation was carried out at 65° C for 5 minutes. The result of the restriction reaction with *Sac*I was cut with *Eco*RI. The restriction reaction with the *Eco*RI enzyme was carried out at 37°C for 1 hour and the inactivation was done at 80°C for 5 minutes. The results of restriction with *Sac*I and *Eco*RI were visualized by 1% agarose gel electrophoresis for 25 min at 100 Volt by using TAE 1X buffer. The fragment of *bglp15.2INU*, *bglp15.2*, and pBEVY-GL vector were purified from agarose gel. The *bglp15.2INU* and *bglp15.2* fragments were ligated to pBEVY-GL vector by using T4 DNA ligase. The recombinant vectors were subcloned to *E. coli* DH5α. Plasmid of pBEVY-GL carrying *bglp15.2INU* and *bglp15.2* gene were isolated from positive transformant that confirmed by colony PCR. These plasmids were used to transform *S. cerevisiae* BY4741. The schematic diagram of pBEVY-GL carrying *bglp15.2INU* and *bglp15.2* was showed in figure 1.

***S. cerevisiae* BY4741 Transformation**

Competent cell production of *S. cerevisiae* BY4741 was performed using LiAc / PEG method without single strand carrier DNA by Gietz and Woods (2002). One transformation reaction requires 100 µl of competent cell suspension. The transformation of *S. cerevisiae* BY4741 was done by Gietz and Woods (2002). Competent cell pellets of *S. cerevisiae* BY4741 were mixed with 240 µl PEG 4000 50%, 36 µl lithium acetate 1 M, 1 µl pBEVY-GL plasmid carrying *bglp15.2* and *bglp15.2INU* gene, and 83 µl of sterile water. This mixture was homogenized and incubated at 30°C for 30 minutes. The transformation was carried out by using heat shock method at 42°C for 25 minutes. A total of 100 µl of the transformation result was grown on a minimal SC medium without the leucine amino acid and incubated at 30°C for 3 days.

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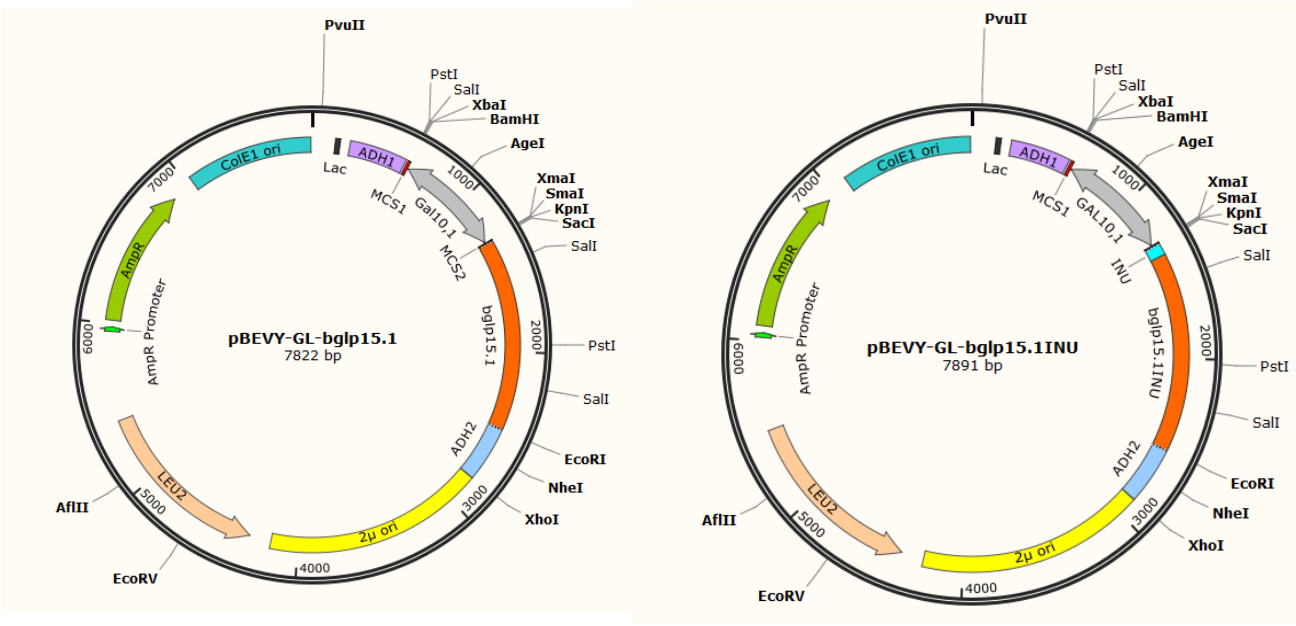

Figure 1. Schematic diagram of pBEVY-GL carrying β-glucosidase gene with and without signal peptide.

Expression of *bglp15.2INU* and *bglp15.2* Gene by *S. cerevisiae* BY4741

Colony grown on minimal SC medium without leucine amino acid was inoculated into 15 ml SC liquid medium without leucine amino acid at 30°C with agitation of 200 rpm for 24 hours and taken several mililter so that the initial concentration (OD600) of expression induction culture was 0.4. The cell suspension was centrifuged at 1,500 x *g* for 5 min in 4°C to obtain cell pellets (Invitrogen, 2008). Cell pellets were dissolved in 1 ml of induction medium (minimal SC medium without leucine amino acid made by replacing 2% dextrose with 2% galactose) and inoculated into 50 ml of induction medium. Induction cultures were incubated until 72 hours at 30°C with agitation of 200 rpm. Induced cell culture was centrifuged at 1,500 x *g* for 5 min in 4°C. Supernatant was used as a sample of extracellular proteins. The pellet was resuspended in 5 ml of sterile water and 500 µl pellet suspension was transferred into the microtube. The pellet suspension was centrifuged at a rate of 14,000 rpm (11,000 x *g*) for 30 seconds. The supernatant was removed and the pellet was broken down (Invitrogen, 2008; Gupta, et al., 2003). The breakdown of cell samples was performed using Ausubel method, et al. (2003) that used breaker buffer and acid wash glass beads. The collected solution was centrifuged at a rate of 10,000 rpm (12,000*g*) for 60 min at 4°C. The supernatant was taken and used as an intracellular protein sample for the next step.

β-Glucosidase Enzyme Activity

The β-glucosidase enzyme activity was performed on cell samples and culture supernatant of *S. cerevisiae* BY4741 carrying the *bglp15.2* and *bglp15.2INU* genes. A 10 µl protein solution that was added in 50 mM phosphate buffer and mixed with 2 mM p-NPG incubated in 55°C for 5 min. To stop the reaction, 600 µl of Na_2CO_3 was added to the reaction solution and measured at λ 410 nm. The molar coefficient of p-NPG (p-nitrophenyl β-D-glucopyranoside) at λ 410 nm is 18.3 mM-1cm-1 (Sigma, 1997).

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SDS-PAGE Analysis

The protein expression was performed on cell samples and culture supernatant *S. cerevisiae* BY4741 carrying the *bglp15.2* and *bglp15.2INU* genes using SDS-PAGE. The concentration of gel stacking was 5% while concentration of gel separating was 10%. A total of 15 µl samples were inserted into the hole in the gel. SDS-PAGE process was done at 100 Volts for 2 hours. Staining was done on Coomassie Staining Solution at least 4 hours. Destaining was done for 4-8 hours by replacing it several times. The last destaining was done for 24 hours (Sambrook, et al., 2001).

RESULTS AND DISCUSSION

β-Glucosidase Enzyme Activity of *Bglp15.2INU* and *Bglp15.2*

The *K. marxianus* inulinase signal peptide was succesfully introduced to *bglp15.1* gene to produce *bglp15.2INU* gene. The β-glucosidase enzyme activity was performed on culture supernatant and cell protein extract samples of *S. cerevisiae* BY4741 carrying *bglp15.2* and *bglp15.2INU* genes. Enzyme activity of culture supernatant that was done by using p-NPG (4-nitrophenyl β-D-glucopyranocide) as substrate was performed to determine the ability of cells to produce extracellular β-glucosidase enzymes. Artificial substrate of p-NPG was usually used to determine the β-glucosidase enzyme activity (Singhania et al., 2012). Cell protein extract samples were used to determine intracellular β-glucosidase enzyme activity. The enzyme activity was conducted at 55°C and pH 7 that was the optimum condition of β-glucosidase enzyme activity reported by Erawijantari (2014). The culture supernatant of *S. cerevisiae* BY4741 carrying the *bglp15.2INU* gene grown on a minimal SC medium without the leucine amino acid for 48 hours had enzyme activity of 0.0178 U/ml. The value of enzyme activity from culture grown for 72 hours was aprroximately 2 times lower than that for 48 hour. The culture supernatant *S. cerevisiae* BY4741 carrying the *bglp15.2* gene (without the signal peptide) had no enzyme activity (0 U/ml). The absence of enzyme activity in culture supernatant of *S. cerevisiae* BY4741 carrying the *bglp15.2* gene (without signal peptide) was estimated because *bglp15.2* protein was not secreted. This showed that the inulinase signal peptide (INU1) *K. marxianus* played an important role in secretion of *bglp15.2* protein.

The *K. marxianus* inulinase signal peptide comprised of 23 amino acids and had three cutting areas recognized by peptidase signal in the endoplasmic reticulum (ER) and one other cutting area 6^{th}, 7^{th}, or 8^{th} amino acids downstream from the ER peptidase signal region and recognized by ycsF endoproteinase (product of the *kex2* gene) (Chung et al., 1995; Kang et al., 1996). Three cutting areas recognized by the endoplasmic reticulum peptidase signal were in the order of the S-A-S-V (Serine-Alanine-Serine-Valine) amino acid. The cutting area identified by the ycsF endoproteinase was K-R (Lysine-Arginine) amino acid sequence which played a role in peptide signaling in mature Golgi before transported out of the cell. These cutting areas made the *K. marxianus* inulinase signal peptide had an important role in the secretion of *bglp15.2INU* protein in *S. cerevisiae* cells that required for transport across the ER membrane and Golgi body before being transported out of the cell surface (Chung et al., 1995; Kang et al. , 1996; Rakestraw et al., 2009; Fitzgerald and Glick, 2014).

Cell extract of *S. cerevisiae* BY4741 carrying the *bglp15.2INU* gene grown on a

minimum SC medium without the leucine amino acid for 48 hours (OD_{600} 3) had an intracellular activity of 0.0181 U/ml. The decline also occurred in intracellular activity at 72 hours growth. Cell extract of *S. cerevisiae* BY4741 carrying the *bglp15.2* gene had intracellular enzyme activity of 0.0079 U/ml after 10 hours induction (OD_{600} 1.5). In this study, the high enzyme value of *bglp15.2INU* β-glucosidase enzyme was achieved at 48 hours and there was retaining β-glucosidase enzyme in *S. cerevisiae* BY4741 cells. The diagram of extracellular and intracellular *bglp15.2INU* enzyme activity in 48 hours growth was showed in figure 2. The 48 hours and 72 hours of culture growth time were used to observe the secretion of recombinant proteins in *S. cerevisiae* using *K. marxianus* inulinase signal peptide. The secretion of recombinant proteins in *S. cerevisiae* performed by using *K. marxianus* inulinase signal peptide showed high secretion at 48 hours of growth. The secretion of recombinant β-glucosidase protein without signal peptide was observed in 10 hours induction time because the highest expression of the *lux* gene on *S. cerevisiae* by inducing GAL promoter on the pBEVY-GL vector was achieved at the 10th hour. The gene expression vector and promoter used in this research was same with *lux* gene research (Chung et al., 1995; Kang et al. 1996; Tang et al., 2013; Hong et al., 2015; Gupta et al., 2003). Growth of cells performed on the batch system at some point will stop which can be caused by the reduced substrate required by the cell or due to the accumulation of autotoxic products that inhibited cell growth. *S. cerevisiae* cells grown on the medium contain fermentable carbon sources of cells, such as glucose and galactose, undergo a transition from the exponential phase to the diauxic phase at 2 days growth (48 hours). Cells entered the post-diauxic phase at the 3 days growth (72 hours). Exponential phase occured at first day of growth time (24 hours). Growth of cells was slow down in the diauxic phase and in the post-diauxic phase. In the transition of the exponential phase to the diauxik phase, the genes encoding the process of translation and ribosomal proteins began to undergo repression. The repression caused the amount of rRNA, tRNA, and ribosomal protein mRNA were decreased so that the translation process was disrupted. The amount of rRNA and tRNA reached 95% of the total RNA present in the cell and the number of ribosomal protein mRNAs reaches 20% of the total mRNA in the cell. The decline started in the diauxic phase. The physiological conditions of these cells might affect the translational process that was also required to produce an active heterologous enzyme (Washburne et al., 1993; Stahl et al., 2004; Stanbury et al., 1995; Buchholz et al., 2012).

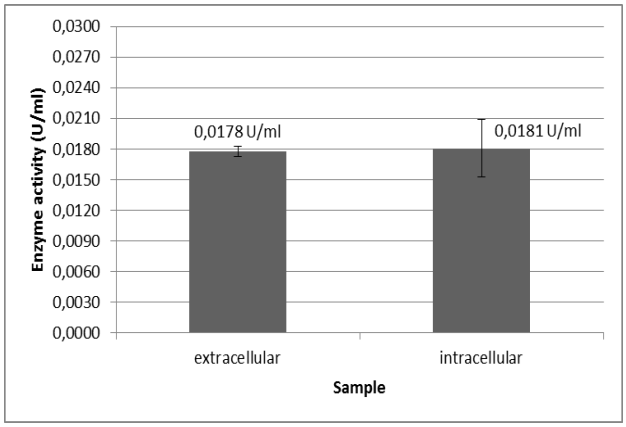

Figure 2. Diagram of enzyme activity from 48 hours grown culture.

The *bglp15.2* enzyme was not entirely secreted by *S. cerevisiae* BY4741. This was indicated by the presence of β-glucosidase enzyme activity in supernatant cultures and cell extracts sample of recombinant *S. cerevisiae* BY4741 carrying *bglp15.2INU* gene. Treebupachatsakul et al. (2015) reported

that the *Aspergillus aculeatus* β-glucosidase enzyme (AaBGL1) had enzyme activity in supernatant culture and extract cells. The activity of the β-glucosidase AaBGL1 enzyme on the extract cell was higher than the enzyme activity in the culture supernatant. Extracellular heterologous protein secretion in eukaryotic cells was influenced by the type of signal peptide used. The varied results in secreting the *Saccharomycopsis fibuligera* β-glucosidase enzyme (SF-BGLN1) were reported by Tang et al. (2013). The secretion of SF-BGLN1 protein reached the highest secretion by using native signal peptide and inulinase *K. marxianus*, whereas other petide signals such as *mfα*, *suc2*, *pho5* gene signal peptides had lower secretion than the two signal peptides. The more hydrophobic amino acids present in the signal peptide, the higher secretion of recombinant proteins (Yarimizu et al., 2015).

The efficiency of signal peptide cutting was not only influenced by the signal peptide type, but also the overall protein precursor structure (Chung et al., 2005). The folding of proteins was influenced by the use of codons in the translation process. The use of different codons between organisms caused heterologous proteins (enzymes) in host cells had a folding error that caused reduced enzyme activity (Buchholz et al., 2012). Yu et al. (2015) reported that the use of codons affected the rate of elongation in the translation process. This was because the number of tRNAs that recognized the preferred codon was more than the tRNAs that recognized non-preferred codon. Therefore, the preferred codon will be translated more quickly by the ribosome than the non-preferred codon. This difference in elongation rate in the translational process affected co-translational protein folding and protein activity. The other factors affecting heterologous protein secretion in *S. cerevisiae* was promoter strength (Tang et al., 2013). The value of *bglp15.2INU* β-glucosidase enzyme activity that was expressed using a GAL1 promoter was lower than the value of β-glucosidase enzyme activity reported by Tang et al. (2013). The *Aspergillus niger* Nip35 (AN-BGL1) β-glucosidase enzyme that was expressed on *S. cerevisiae* 102-ΔTPI using a native signal peptide had the highest enzyme activity of 0.85 U/ml. AN-BGL1 β-glucosidase expression was performed using a TPI1 promoter.

SDS-PAGE of *Bglp15.2INU* and *Bglp15.2* Protein

The result of SDS-PAGE protein *bglp15.2* and *bglp15.2INU* was showed in figure 3. The weight of *bglp15.2* protein molecule without signal peptide, based on *in silico* prediction, was 44 kDa. *S. cerevisiae* BY4741 cells carrying the *bglp15.2* gene without a signal peptide expressed *bglp15.2* proteins intracellularly that was shown by a protein band between the bands marker of 40 - 50 kDa in intracellular BGL samples (cell extracts) and no protein band found in the extracellular BGL sample. *bglp15.2* protein without signal peptide in this study was used for control of β-glucosidase protein secretion. *bglp15.2INU* protein was detected in culture supernatant (extracellular) samples and cell extracts samples (intracellular) of INU48 and INU72. *bglp15.2INU* protein that still carried signal peptide and retained in cell was predicted to had a molecular weight of 47 kDa (figure 3). The existence of protein band in extracellular INU48 and INU72 samples but no protein band in extracellular BGL sample was in line with the result of extracellular β-glucosidase enzyme activity that showed enzyme activity in supernatant of INU48 and INU72 samples but no enzyme activity in supernatant of BGL sample.

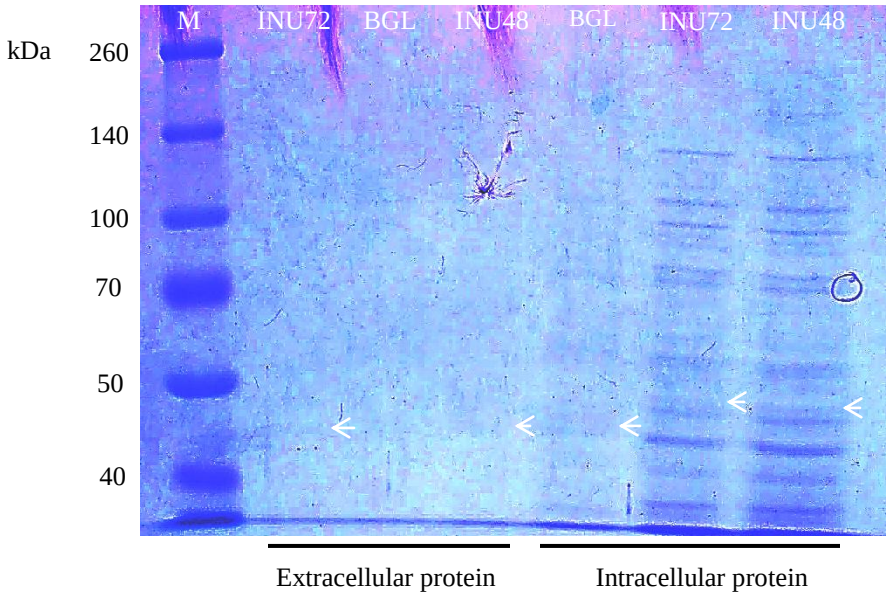

Figure 3. Electropherogram of total protein. (M) Marker. (BGL) *bglp15.2* without signal peptide. (INU48), (INU72) *bglp15.2INU* with signal peptide from *S. cerevisiae* BY4741 recombinant grown in 48 hours and 72 hours. *bglp15.2* Protein was showed in whitte arrow.

The absence of protein band in BGL culture supernatant samples indicated that Bglp15.2 protein which has no signal peptide was not secreted. Saloheimo et al. (2002) reported that the *Trichoderma reseei* (BGLII) β-glucosidase protein, which has no signal peptide, was not secreted in the culture medium. The Bglp15.2INU protein band on the INU48 and INU72 intracellular samples was larger than the Bglp15.2INU protein band in the extracellular samples of INU48 and INU72. This indicated that the signal peptide in Bglp15.2INU protein retaining in the cell did not cut, so the Bglp15.2INU protein was not extracellularly expressed. Unexcepted Bglp15.2INU protein may be due to the unsuccessful translocation into the lumen of the endoplasmic reticulum (ER) and the presence of folding proteins before entering the RE. In the translational phase of the co-translocation, the signal peptide binds to the Sec61 translocon and the translation remained in place so that the protein chain extended further into the RE lumen. At this stage, the protein chain could form a loop or non-loop position against the RE lumen through translocons caused by the affinity and stability of the signal peptide bond with the Sec61 binding side of the translocon. An increasingly long protein chain in a non-loop position will fail translocation (Hedge and Kang, 2008). The β-glucosidase *bglp15.2INU* protein was detected in the culture supernatant samples of INU48 and INU72 indicated that the signal peptide played a role in the protein secretion. Lee et al. (2017) reported that the β-glucosidase protein *Saccharomycopsis fibuligera* (SfBGL1) having native and *mfα* signal peptides was detected in the culture supernatant. From this research, *bglp15.2INU* protein could be secreted by using *K. marxianus* signal peptide though was not entirely secreted. Increasing the expression of heterologous protein in *S. cerevisiae* could be

done by optimizing codon, using other signal peptides that could be used to express heterologous proteins in *S. cerevisiae*, such as signal peptides from prepro-α-factor mating, phosphatase acid (PHO5), invertase (SUC2), and using other promoters, such as GPD promoters, PGK1, HXT7, ADH2, GAL10.

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