

Characteristics of Glucose Oxidase Gene (GGOx) from *Aspergillus niger* IPBCC 08.610

Popi Asri Kurniatin¹, Laksmi Ambarsari¹, Annisa Dhiya Athiyyah Khanza¹, Inda Setyawati¹, Djarot Sasongko Hami Seno¹, Waras Nurcholis^{1,2*}

¹Department of Biochemistry, Faculty of Mathematics and Natural Sciences, IPB University, Bogor, Indonesia

²Tropical Biopharmaca Research Center (Trop-BRC), IPB University, Bogor, Indonesia

*Corresponding author: wnurcholis@apps.ipb.ac.id

Received: May 2020; Revision: August 2019; Accepted: April 2020; Available online: May 2020

Abstract

Glucose oxidase is used in various industries for the development of enzymatic fuel cell. Based on prior studies, this compound is sourced from the local isolates of *Aspergillus niger* IPBCC 08.610, although investigations on the encoding gene have not been conducted. The purpose of this research, therefore, is to identify and characterized the gene responsible for encoding glucose oxidase, in the aspect of sequence, length, and restriction patterns. This experiment involved the amplification of genomic DNA using specific primers for gene recognition, which was followed by the restriction technique with *EcoRI* and *PstI* endonucleases. Furthermore, the gene is inserted into vector pGEM[®]-T-Easy and transformed into competent *Eschericia. coli* DH5a cells, in an attempt to perform sequencing. The glucose oxidase gene from *A. niger* IPBCC 08.610 was confirmed to possess a size of 1848 bp, and a GC content of 57.8%, with a possibility of restriction into two fragments of size 908 bp and 980 bp, using the *EcoRI* restriction.

Keywords: *Aspergillus niger*, cloning, glucose oxidase gene, restriction endonuclease, sequencing.

DOI: 10.15408/jkv.v6i1.9440

1. INTRODUCTION

Glucose oxidase is an enzyme with the propensity to catalyze the oxidation reaction of β -D-glucose to form D-glucono- δ -lactone and H_2O_2 (Bankar *et al.*, 2009). This compound is widely used in the technology of food, beverage, pharmaceuticals, and enzymatic fuel cell (EFC), as well as possible application in renewable energy and biosensor devices, including blood glucose sensors (Bhatti and Saleem 2009). In addition, glucose oxidase has successfully been purified from several mold sources, e.g., *Aspergillus* and *Penicillium*, while a research by Fiedurek *et al.*, (1986) reported *A. niger* as the highest enzyme activity producer, compared to *P. paxii* and *P. Purpurogenum*.

Aspergillus niger is an anaerobic filamentous mold, which is easily isolated from soil, water, and agricultural products (Gandjar *et al.*, 2000), with the capacity to live in temperatures between 30-37 °C and 88% relative humidity (Anwar, 2006). Furthermore,

the tropical climate present in Indonesia is known to facilitate the ease of growth, therefore alleviating possible procurement difficulties. The IPBCC 08.610 is one of the local isolates, which is identified as an IPB Culture Collection isolated from *Dryobalanops* plants in Tarakan, North Kalimantan. These forms are able to produce glucose oxidase with a Km value of 39-46 mM (Triana, 2012; Abdullah, 2013; Desi, 2015), while a range of 33-100 mM was reported for the Km-commercial variety. In addition, there is a potential for the glucose oxidase produced by local isolates to compete with the commercially available forms, hence the need to conduct further exploration.

According to Yamaguchi *et al.*, (2007), the glucose oxidase produced by *A. niger* ATCC 9029 is encoded by a 1818 bp gene, which is possibly restricted by several endonuclease, including *SallI*, *EcoRI*, and *PstI* (Frederick *et al.*, 1990). These generally tend to encode certain proteins within cells, as a

research using large plasmids showed the possibility for the carried gene to generate numerous copies. Thus, a number of identical copies ought to be initially created prior to application in sustainable research, conducted through a process known as the cloning technique (Koolman and Roehm, 2005).

Based on literature review, studies on the glucose oxidase gene obtained from *A. niger* IPBCC 08.610 have not been conducted, which increased interest in this investigation. This research involves the extraction and characterization of glucose oxidase gene from *A. niger* IPBCC 08.610 isolate, in terms of sequence, base length, and restriction pattern. Also, scientific information related to the base length and the restriction pattern is elucidated, to provide basic information for further studies in the areas of mutation to increase enzyme availability.

2. MATERIALS AND METHODS

Growth of *A. niger*

The growing media consisted of $(\text{NH}_4)_2\text{HPO}_4$ 0.4 g/L, KH_2PO_4 0.2 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g/L, peptone 10 g/L, and 70 g/L sucrose, using the modified version of Desi (2015). This was subsequently sterilized in an autoclave at 121 °C for 15 minutes and chilled, followed by the introduction of one transfer loop of a single colony spore of *A. niger*. Subsequently, the medium was incubated at room temperature at a speed of 130 rpm for over 16 hours, and then the grown mycelia were filtered using sterile gauze.

Genomic DNA Isolation of *A. niger*

The genomic DNA Isolation of *A. niger* was performed using the Plant Genomic DNA Extraction Kit, using the procedure of Tiangen (2012), where a total of 100 mg mycelia was crushed by means of liquid nitrogen or quartz sand. Furthermore, 0.1% β -mercaptoethanol was introduced to a total of 700 μL of GP-1 buffer and heated at 65 °C, which was then added to the mycelia powder. Using a vortex, this mixture was homogenized for 15 seconds, and incubated for 20 minutes at 65 °C, followed by the addition of 700 L chloroform, and centrifugation at 12000 rpm for 5 minutes. Subsequently, the supernatant was transferred to a new micro tube, to which 700 μL of GP-2 buffer was added, and the mixture homogenized by flipping the tube several times. Therefore, all mixtures were

transferred to the spin column CB3 installed in the collection tube, followed by centrifugation at 12000 rpm for 30 seconds, and then the filtrate was discarded. A total of 500 μL of GD buffer was placed in the spin column CB3 and centrifuged at 12000 rpm for 30 seconds, and then the filtrate was discarded. This procedure was also performed with 600 μL of PW buffer, and the entire process was repeated 2 times. Therefore, the spin column CB3 was centrifuged at 12000 rpm for 2 minutes, and reserved at room temperature for the next 20 minutes. This was then placed in a new micro tube, followed by the direct addition of 150 μL TE buffer to the membrane, and incubation for 10 minutes at room temperature. Subsequently, the tube was centrifuged for 2 minutes at 12000 rpm.

DNA Qualitative Test

Qualitative DNA test was performed using 1% (w/v) agarose gel electrophoresis in accordance with the procedure by Sambrook and Russell (2001). Furthermore, 0.3 g of agarose powder was dissolved in 30 mL of 0.5x TBE buffer, followed by the addition of 1.5 μL of ethidium bromide (10 mg/mL). This mixture was poured into a mold, allowed to condense, and then transferred into an electrophoresis tub filled with 0.5x TBE buffer, and 5 μL of sample was mixed with 1 μL of 6x loading dye. Therefore, 1 μL of DNA Ladder 1kb was used as a marker, by mixing with 1 μL loading dye and 4 μL ddH₂O. The electrophoresis device was run at 80 volts for 90 minutes, and the results were observed, while the quantitative DNA test involved NanoDrop spectrophotometer analysis (Desjardins and Conklin, 2011). The blank used was the TE buffer, where a total of 2 μL was dripped in the optical hole of the NanoDrop spectrophotometer, which was then closed, and the "blank" menu in the computer program was subsequently selected. Furthermore, the optical hole was opened and the remaining blanks were cleaned, followed by the insertion of 2 μL DNA sample. The spectrophotometer was closed again, and the "measure" menu in the computer program was selected, where the measurement results appear in the form of DNA concentration (ng/ μL) and purity (the ratio of $A_{260/280}$ and $A_{260/230}$).

Optimization of DNA Amplification with The Polymerase Chain Reactions Technique

Amplification was conducted with GODF1 and GODR1 as primers, using the polymerase chain reactions (PCR) technique, according to Guo *et al.*, (2010). The PCR reaction mixture consisted of 1 μ L primary GODF1 (10 pmol/ μ L) and 1 μ L of primary GODR1 (10 pmol/ μ L), 12.5 μ L of 2x Taq Plus MasterMix, 7 μ L of ddH₂O and 3.5 μ L of mold DNA (28.25 ng/ μ L). This process was performed for 33 cycles, with initial denaturation at 94 °C for 90 seconds, denaturation at 94 °C for 45 seconds, primary annealing at 53 °C for 50 seconds, extension at 72 °C for 110 seconds, and the final extension at 72 °C for 5 minutes. In addition, the entire procedure was repeated by changing the primary annealing temperature to 50, 48, and 45 °C, respectively, followed by the analysis of amplicon base length, using 1% agarose gel electrophoresis technique.

Thermo Scientific 2012a, Thermo Scientific 2012b PstI and EcoRI endonuclease were used to evaluate hydrolysis, which involved the introduction of 18 μ L ddH₂O, 3 μ L enzyme buffer, and 10 μ L amplicon (0.3 μ g) into a 0.2 mL micro tube. Subsequently, 2 μ L of endonuclease was inserted, and then the mixture was homogenized and incubated for 8 hours at 37 °C. Therefore, restriction patterns were observed using 1% agarose gel electrophoresis technique.

Cloning of Glucose Oxidase Gene in the pGEM[®]-T Easy Vector Sample Preparation (Macherey-Nagel, 2014).

The 1% (w/v) agarose gel containing the amplification was cut over the UV transilluminator, followed by the dissolution of 0.68 g gel in 1.2 mL of NT1 buffer, which was then incubated at 50 °C to allow for liquefaction. Therefore, all mixtures were transferred to a spin column that had been previously attached to the collection tube, and centrifuged at 11000 g for 30 seconds, and the filtrate was then discarded. A total of 700 μ L of NT3 buffer was placed in a spin column, centrifuged at 11000 g for 30 seconds, and the filtrate discarded. This procedure was repeated 2 times, followed by centrifugation at 11000 g for 1 minute, and the filtrate was discarded. A total of 30 μ L NE buffer was incorporated in the spin column, incubated at room temperature for 3 minutes, and subsequently

centrifuged at 11000 g for 1 minute. In addition, the results of purification were observed using 1% (w/v) agarose electrophoresis.

Gene Ligation in the pGEM[®]-T Easy Vector (Promega, 2015).

A total of 3 μ L sample was placed in a 0.2 mL micro tube, and 1 μ L of vector was added, alongside 1 μ L ligase buffer and 1 μ L T4 ligase. This mixture was incubated at 4 °C for one night.

Transformation to *E. coli* DH5 α (Promega, 2015).

A total of 5 μ L ligation outcome was mixed with 50 μ L of competent *E. coli* DH5 α cells in a micro tube, and incubated in an ice bath for 30 minutes. Therefore, heat shock treatment was introduced to the mixture at 42 °C for 50 seconds, followed by incubation in an ice bath for 10 minutes. A total of 400 μ L growing media was placed in a micro tube, and incubated for 1.5 hours at 37 °C in a shaking incubator, from which 100 μ L was poured on the selection media (culture 1/10). Therefore, the remaining mixture was centrifuged at 8000 g for 5 minutes, followed by the homogenization of pellets with little supernatant, which was then poured on the selection media (culture 9/10). Subsequently, both cultures were incubated at 37 °C for one night, and the presence of white colonies indicates a positive outcome.

Amplification of Positive Transformant Colonies (PPBBI, Personal Communication)

Using a toothpick, a single positive transformant colony was transferred to a micro tube containing 10 μ L of nuclease free water (NFW), and then a lysis program was used to lyse the solution on a PCR machine (Appendix 3). Furthermore, 4.7 μ L of KAPPA2G Fast ReadyMix PCR Kit, 0.15 μ L of GODF1 primer, and 0.15 μ L of GODR1 primer were added, which was followed by the amplification process conducted for 30 cycles with denaturation at 94 °C for 15 seconds. The primary annealing at 45 °C was then performed for 15 seconds, extension at 72 °C for 15 seconds, and the final extension at 72 °C for 5 minutes. Therefore, the ammonium base length was analyzed using the 1% (w/v) agarose gel electrophoresis technique.

Nitrogen Bases Sequencing of Glucose Oxidase Gene

Sample preparation was carried out by Thermo Scientific method (2014). A total of 4 mL *E. coli* suspension was centrifuged at a speed of 13000 rpm for 1 minute, and the pellets obtained were subsequently resuspended with 250 μ L each of resuspension and lysis solution. Therefore, the mixture was homogenized to achieve a became slightly thick consistency, followed by the addition of 350 μ L neutralization solution, and another phase of homogenization to achieve slight turbidity. This mixture was centrifuged for 5 minutes, and the supernatant formed was transferred to the spin column for a 1-minute re-centrifugation. In addition, the filtrate was then discarded, followed by the insertion of 500 μ L wash solution, and for 1 minute. This stage was repeated twice, and the filtrate discarded, which was followed by spin column centrifugation for 1 minute, and then the transfer of the mixture to a new micro tube where 30 μ L of elution buffer was added. This was then incubated for 2 minutes at room temperature, and centrifuged for the next 2 minutes. Furthermore, the results of plasmid isolation were observed using 1% (w/v) agarose electrophoresis technique.

Nitrogen Base Sequencing.

The isolated plasmids were sent to First Base Company, Malaysia, for nitrogen base sequencing, which was conducted using four primers, including GODF1, GODR1, SP6, and T7Promoter.

Analysis of Nitrogen Base Sequences.

The results of nitrogen base sequencing were analyzed using the ChromasPro software, in order to obtain a consensus sequence. This was identified at the homology level, using the glucose oxidase gene sequence obtained from *A. Niger*, published in GeneBank with the BLASTN software, as stipulated on the website, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

3. RESULTS AND DISCUSSION

Genomic DNA of *A. niger*

Isolation is a key stage in DNA studies, which involves amplification, cloning, characterization, and mutation. The methods adopted ought to be in accordance with the physical properties of the sample tissue, and

success is qualitatively and quantitatively observed using agarose electrophoresis, and spectrophotometric techniques, respectively (Fatchiyah *et al.*, 2011).

The size of the isolated genomic DNA is possibly determined using 1% (w/v) agarose electrophoresis, based on the principle instigating the faster movement of shorter fragments than long counterparts on the agarose gel media charged by electric fields (Campbell and Farrell, 2012). Furthermore, the isolated DNA band is recognized above the marker band, which indicates the presence of large DNA quantities, estimated to be over 10 kbp (Figure 1), although Jin *et al.*, (2004) reported 13 kbp. The results obtained from a quality test using 1% (w/v) agarose technique showed an intact genomic DNA in *A. Niger*, despite the presence of impurities.

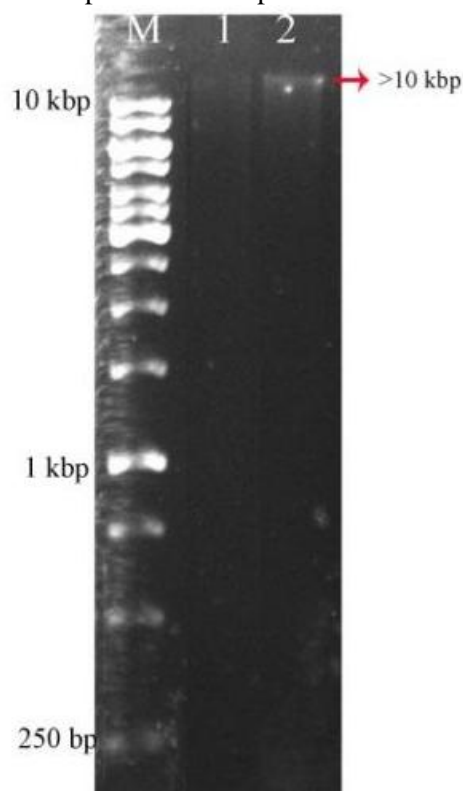


Figure 1. Electropherogram of genomic DNA isolated from *A. niger* IPBCC 08.610: DNA marker (M), the result of isolation using quartz sand (1), liquid nitrogen (2)

The quantity of isolated nucleic acids is determined using a NanoDrop spectrophotometer, based on the absorbance ratio at wavelengths of 230, 260, and 280 nm. Furthermore, values of A_{260/280} and A_{260/230} shows DNA purity against protein and RNA contamination, as well as

polysaccharide and phenol contamination, respectively, and a good range is attained between 1.8-2.0 and 1.5-2.1 (Kundu *et al.*, 2011; Kheyroodin and Ghazvinian, 2011). These results indicate the higher propensity for impurities present to be derived from polysaccharides, phenols, and RNA, with relatively smaller quantities of protein (Table 1). In addition, both values denote the potential for isolated DNA to contain polysaccharides, phenols, and RNA molecules, alongside relative purity from protein, hence the quantity of nucleic acid produced is very small, compared to other isolation methods. This is due to the use of an unsuitable kit, which was originally used to isolate fungi DNA.

Both bands were observed to display smear fragments on the bottom, although the intensity was clearer in the lane obtained using liquid nitrogen (Figure 1). This also indicates the presence of contaminants in the form of polysaccharides and phenolics (Pharmawati, 2009), while the thickness observed demonstrates the superiority of liquid nitrogen over quartz sand, due to the ability to reduce sample temperature, as well as the subsequent deactivation of cellular enzymes and other compounds with the capacity to damage DNA. This also produces a smooth scour, and is thus expected to increase the concentration of extracted DNA (Arif *et al.*, 2010).

A higher quantity of nucleic acid is obtained through the use of quartz sandstone (Table 1), although liquid nitrogen produces better quality, hence the results are better for PCR analysis techniques.

Table 1. The isolation results of genomic DNA of *A. niger* IPBCC 08.610

Sample	[Nucleic acid] (ng/ μ L)	A _{260/280}	A _{260/230}
Using quartz sand	30.65	2.89	0.09
Using liquid nitrogen	28.25	3.10	0.08

Amplification Optimum Conditions of Glucose Oxidase Gene

Amplification is one of the ways to determine the presence of glucose oxidase genes, attained by the multiplication process, which is conducted on the target DNA fragments through the polymerase chain

reaction (PCR) technique. The optimum conditions are determined using the appropriate PCR program, with a technique that involves three main stages, including denaturation (separation of DNA double strand), primary annealing, and strand extension (Sambrook and Russell, 2001).

The temperature adopted in the primary annealing stage is critical for amplification (Sambrook and Russell 2001), hence the need to perform optimization. This requires segregation into four different temperatures (53, 50, 48, and 45 °C), where 53 °C was used as the primary melting temperature (T_m). Generally, values lesser by 3-5 °C are selected as the optimal primer annealing temperature (Sambrook and Russell, 2001), which is the basis for variation selection.

The amplification tends to only show one alongside different band thickness (Figure 2), where the general appearance on the agarose gel of electrophoresis are directly proportional to sample quantity. Furthermore, different levels indicate variations in the quantity of the resulting amplicon, as a higher primary annealing temperature leads to thinner amplicon bands as observed in Figure 2. This occurs because of the enhanced tendency of obstruction towards achieving the desired target, subsequently leading to the production of smaller amplicons (Sambrook and Russell, 2001). Meanwhile, the thickest single band was obtained at 45 °C, which is indicative of higher PCR program optimization, making this the best condition.

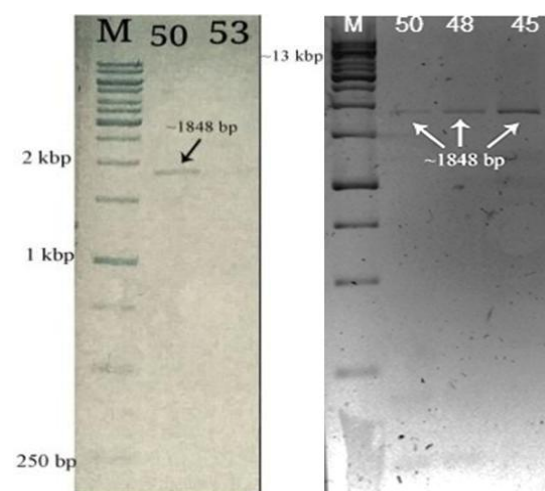


Figure 2. Electropherogram of the glucose oxidase gene amplicon optimization: DNA marker (M), amplicon temperature at 53 °C (53), 50 °C (50), 48 °C (48), and 45 °C (45).

The presence of one band in the PCR sample shows the specificity of primer used in the amplification of target gene region with the desired amplicon size, estimated at 1848 bp. This is not so different from the value obtained for glucose oxidase gene obtained from *A. niger* Z-25 and ATCC 9029, of about 1818 bp (Yamaguchi *et al.*, 2007; Guo *et al.*, 2010). This indicates the presence of one glucose oxidase gene region in *A. niger* IPBCC 08.610.

Restriction Pattern of Glucose Oxidase Gene

Using the *EcoRI* enzyme, the glucose oxidase gene restriction pattern produced two bands with different base lengths (980 bp and 908 bp) (Figure 3). The results of endonuclease hydrolysis with *PstI* generated a single band of 1848 bp (Figure 3), which indicates the possibility of cutting glucose oxidase gene from *A. niger* IPBCC 08.610 with *EcoRI* and not *PstI*.

The use of endonuclease is one way to ensure the truth of successfully obtained genes, while gene digestion demonstrates the intrinsic characteristics (Campbell, 2006). The principle involves the cutting of DNA into fragments in a specific recognition sequence, using type II endonuclease (Nelson and Cox, 2012), including *EcoRI* and *PstI*.

EcoRI is used to cut the glucose oxidase gene obtained from *A. niger* IPBCC 08,610 into two thin fragments, hence the low intensity of the resulting band indicates a reduced size of the tested DNA. This also shows a successful restriction process (Tan *et al.*, 2011), as the size of the output is estimated at 987 bp and 923 bp (Figure 3). Meanwhile, the recognition sequence of this enzyme is 5'-GAATTC-3', where the cutting site exists between G and A bases (Nelson and Cox, 2012), indicating the presence of GAATTC in the gene. The result of *EcoRI* restriction is similar to the digested glucose oxidase gene of ATCC 9029 (1116 bp and 907 bp) (Frederick *et al.*, 1990) and *A. niger* Z-25 (949 bp and 869 bp).

The digestion of glucose oxidase gene by *PstI* tends to only produce a single thick band, which indicates an unsuccessful process. In addition, the enzyme recognition sequence is 5'-CTGCAG-3', with cutting site between A and G bases (Nelson and Cox, 2012), demonstrating the absence of CTGCAG in the gene. The results of digestion are consistent

with the digestion of glucose oxidase gene obtained from *A. niger* Z-25, although different from the the ATCC 9029 sourced type, which is possibly cut into two parts, measuring 1085 bp and 938 bp (Frederick *et al.*, 1990). Conversely, the characteristics of the glucose oxidase gene extracted from *A. Niger* IPBCC 08,610 are relatively similar with a closer kinship to *A. niger* Z-25, compared to ATCC 9029.

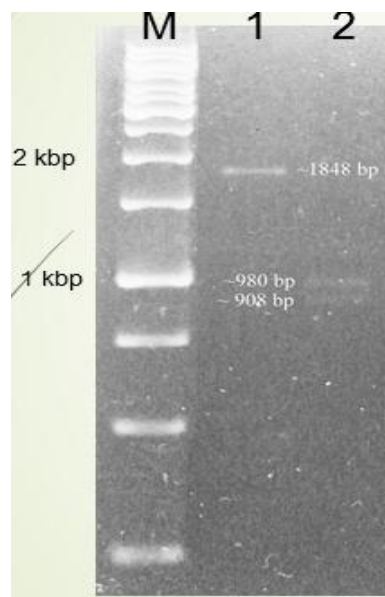


Figure 3. Electropherogram of endonucleases hydrolysis: DNA markers (M), hydrolysis with *EcoRI* (1), and *PstI* (2)

Transformant colonies of *E. coli* DH5 α

The success of pGEM[®]-T Easy transformation on *E. coli* DH5 α is observed from the growth of colonies on the selection media. Meanwhile, a productive ligation is seen from the development of a white colony, which four were identified in 1/10 culture and six in 9/10 (Figure 4). This ligation is verified using the colony PCR method, as the amplification of five positive single transformant colonies from the 9/10 culture showed the presence of only one amplicon band in colonies number 2.3 and 5 (Figure 5), which confirms the occurrence of ligation between pGEM[®]-T Easy and glucose oxidase gene.

Cloning refers to the production of a number of identical organisms originating from a single colony (Voet *et al.*, 2013), which is usually conducted on bacterial and yeast cells. This technique utilizes the intrinsic

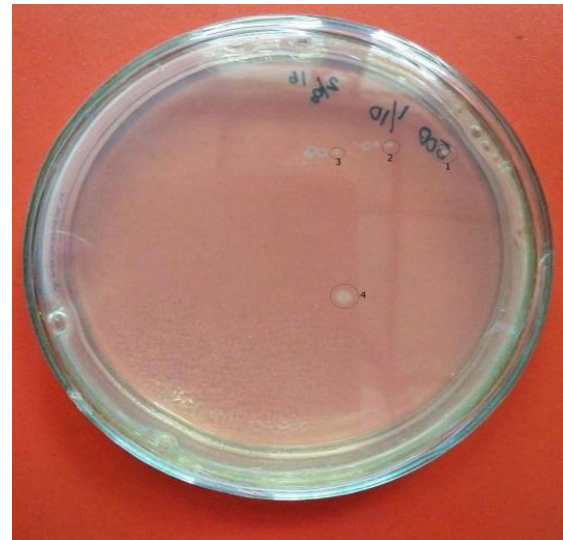
ability to retrieve and replicate target DNA (Koolman and Roehm, 2005), through a process aimed at obtaining large amounts of glucose oxidase genes.

Cloning is initiated by inserting the target gene in a vector with the ability to replicate independently, and the linear pGEM[®]-T Easy plasmid was selected for this study. This unit measured 3015 bp, and is often used for PCR products due to the presence of one thymine base at both ends of the restriction result. Furthermore, it also has regions of β -galactosidase encoding gene as well as ampicillin resistance (Appendix 6), and the intrinsic characteristics facilitate the recombinant identification process by selecting blue/white colonies in media containing ampicillin (Promega 2015). This process, therefore, leads to the production of a recombinant plasmid.

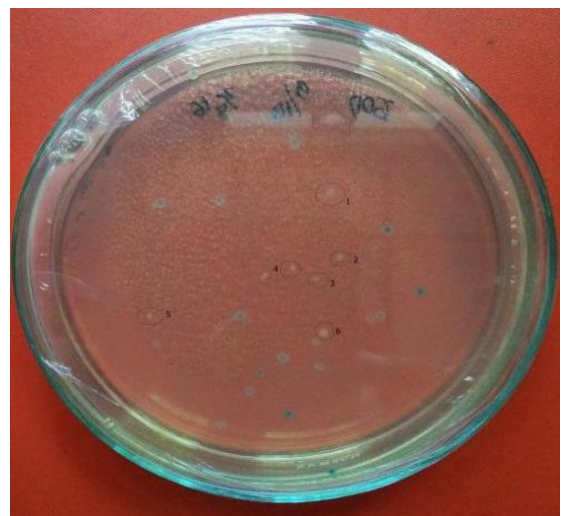
The resulting plasmid is then transferred to the host cell, in order to initiate DNA replication (transformation) into competent *E. coli* DH5 α cells via the heat shock method. In addition, the main principle requires an increase in temperature from 0 °C to 42 °C, which enhances the non-selective nature of the competent cell membrane towards foreign molecules, thus making it easier for recombinant plasmids entry to the host cell (Tarigan, 2008). Conversely, the *E. coli* bacterium is often selected for the purpose in the transformation process, due to the ease of proliferation, numerous associated vectors, and the availability of rapid DNA transfer techniques. (Nelson and Cox, 2012; Reece *et al.*, 2012). This is then grown on a selection media to ensure a successful process.

The results of transformation on the LA media containing ampicillin, IPTG, and X-gal shows the presence of two types of growing colonies (white and blue) (Figure 4). These indicate the capacity for plasmids to enter the host cell due to the production of ampicillin resistance properties conveyed by the pGEM[®]-T Easy plasmid. Specifically, the white colonies contain recombinant plasmids, while the blue type comprise of pGEM[®]-T Easy plasmid, as the resulting blue coloration is a breakdown product of X-gal (5-Bromo-4-chloro-3-indolyl-b-D-galactoside) into b-D-galactose and 5-Bromo-4-chloro-3-hydroxindole. Conversely, the entry point of the target DNA fragment (insert) is situated in the region of the β -galactosidase encoding

gene (Appendix 6), leading to interference and the subsequent inhibition of protein production. Therefore, the colony is unable to break down the X-gal, as there were no changes in the colors observed to blue (white) (Voet *et al.* 2013).



(a)



(b)

Figure 4. The transformation results of pGEM[®]-T Easy on *E. coli* DH5 α (a) 1/10 Culture (b) 9/10 Culture

It is, therefore, necessary to be ensured that the white colonies formed contain the glucose oxidase gene, using the PCR colony technique. This is not much different from PCR in general, although more reliable and highly efficient compared to plasmid amplification, because the bacterial colonies are directly used as template for amplification (Riyadi *et al.*, 2011). However, there is need

for the initial cell lyses through the provision of heat at different temperatures, and the colony PCR results showed the presence of glucose oxidase gene in only three of five white colonies (Figure 5). This possibly occurred because the target DNA is connected inversely to the vector, which leads to the inability for positive (white) colonies amplification to produce a band during electrophoresis (Riyadi *et al.*, 2011).

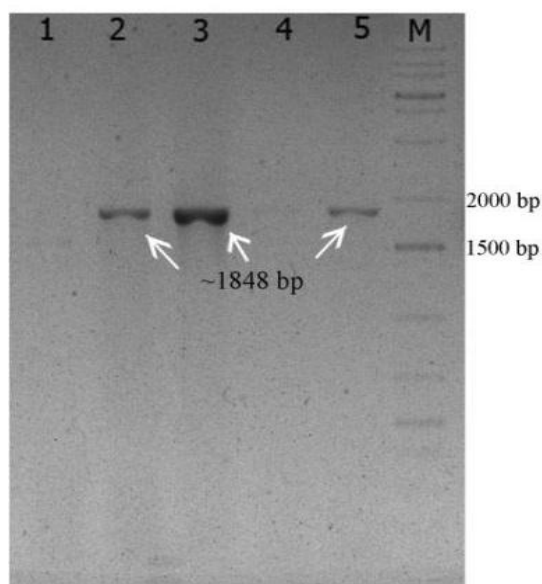


Figure 5 Electrophoregram of positive transformant colonies PCR results: DNA markers (M), positive colonies 1 (1), 2 (2), 3 (3), 4 (4), and 5 (5)

Nitrogen Base sequence of the Glucose Oxidase Gene

The nitrogen base sequence of the glucose oxidase gene collected from *A. niger* IPBCC 08.610 (Appendix 4) was obtained after an alignment with the sequencing results for four different primers (GODF1, GODR1, T7Promoter, and SP6). Furthermore, the measure of length was 1860 bp, with a GC content of 57.8%, and a characteristic high degree of similarity with other *A. niger* glucose oxidase genes published on GeneBank, by up to 90-96% (Table 2). This, therefore, indicates the occurrence of successful amplification.

The sequencing of nitrogen bases is a process used to determine the DNA sequence in a fragment (Campbell, 2006), which establishes the exact size. This technique is also conducted to ascertain orientation accuracy of genes that have been successfully inserted in the cloning vector.

Base sequencing was performed using four different primers, where GODF1 and GODR1 served as specific primers for the glucose oxidase gene, while SP6 and T7Promoter were universal for sequencing the insertion fragment bases in the pGEM®-T Easy vector (Promega 2015). These were adopted to obtain a complete nitrogen base sequence, and also to determine orientation accuracy.

The nitrogen base sequencing results confirmed the presence of correct insertion orientation. This was shown by the sequence suitability of the primary gene-specific form (GODF1) against the initial sequencing outcome, as the genes size is similar to the estimated base length of the amplicon, using 1% (w/v) agarose electrophoresis technique. Therefore, the pGEM®-T Easy vector insertion fragment was the previously obtained glucose oxidase gene amplicon, while the guanine and cytosine base content (% GC) from this local isolate was similar to *A. niger* Z-25 and ATCC 9029 at 57.54% and 56.18%, respectively.

The BLAST (basic local alignment search tool) analysis was performed to identify the similarities and kinship of nucleotide sequences between test samples and the GeneBank database, as observed from the bit score, E-value, and % Ident parameters (Xiong 2006). Thus, a higher bit score and % Ident values alongside lower E-value was indicative of higher nucleotide sequence homology level (Claviere and Notredame 2003). The results of BLAST analysis (Table 2) showed highest similarity between the glucose oxidase gene obtained from *A. niger* IPBCC 08.610 and Z-25 (access number FJ979866.1), and less with ATCC 9029 (access number X16061.1). These outcome as well as the endonuclease restriction previously conducted confirm the presence of greater similarity between IPBCC 08610 isolate and Z-25, compared to ATCC 9029.

Nucleotide sequencing was conducted using dye terminator, which is a developed dideoxy (Sanger) method. This required the use of dideoxynucleosides (ddNTP), characterized by different fluorescent dyes, therefore, increasing the ease of performing base sequencing on a single one test tube. Furthermore, primers used at the amplification stage are also adopted, in order to ensure easy and quick procedure (Graham and Hill 2001)

Table 2. BLASTN analysis result of glucose oxidase gene from *A. niger* IPBCC 08.610 and other strains

No. Accession	E-value	%Ident (%)
FJ979866.1	0.0	96
KC333175.1	0.0	96
DQ661005.1	0.0	96
DQ836361.1	0.0	96
KT176097.1	0.0	91
J05242.1	0.0	91
X16061.1	0.0	91
AF234246.2	0.0	90
KJ774107.1	0.0	90

4. CONCLUSION

PCR technique was used to collect the glucose oxidase gene extracted from *A. niger* IPBCC 08.610 isolate, using primers stipulated in previous studies. In addition, the recorded base length was around 1848 bp, and *EcoRI* endonuclease was used to achieve a cut into two fragments, measuring 980 bp and 908 bp, respectively. A total of three *E. coli* DH5 α transformant colonies containing the glucose oxidase gene were identified. Moreover, it is necessary to optimize *A. niger* genomic DNA isolation with other methods, in order to reduce RNA and polysaccharide contamination, and to also carry out recombinant plasmid characterization with endonuclease and sequencing of nitrogen bases to ensure gene amplification.

ACKNOWLEDGMENTS

This research was funded by the Higher Education Leading Basic Research of Bogor Agricultural Institute with Contract Number 1760/IT3.11/PN/2018.

REFERENCES

- Abdullah. 2013. Pemurnian parsial dan karakterisasi glukosa oksidase dari isolat *Aspergillus niger* (IPBCC.08.610) [skripsi]. Bogor (ID): IPB.
- Anwar YAS. 2006. Produksi dan karakterisasi enzim tanin asli hidrolase dari *Aspergillus niger* [tesis]. Bogor: IPB.
- Arif IA, Bankir MA, Khan HA, Ahamea A, Al Farhan AH, Al Homaidan AA, Al Sadoon M, Bahkali AH, Shobrak M. 2010. A simple method for DNA extraction from mature date palm leaves: impact of sand grinding and composition of lysis buffer. *Int J Mol Sci.* 11 (9): 3149-3157
- Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. 2009. Glucose oxidase – An overview. *Biotechnol Adv.* 27: 489-501.
- Bhatti HN, Saleem N. 2009. Characterization of glucose oxidase from *Penicillium notatum*. *Food Technol Biotechnol.* 47: 331-335.
- Campbell PN. 2006. *Oxford Dictionary of Biochemistry and Molecular Biology*. Revised Edition. Cammack R, Attwood TK, Parish JH, Smith AD, Stirling JL, Vella F, editor. New York (US): Oxford Univ Pr.
- Campbell MK, Farrell SO. 2012. *Biochemistry*. 2nd Ed. Belmont (US): Cengage Learning.
- Claverie JM, Notredame C. 2003. *Bioinformatics for Dummies*. Indianapolis (US): Wiley.
- Desi ES. 2015. Pemurnian dan karakterisasi glukosa oksidase dari isolat *Aspergillus niger* (IPBCC.08610) [skripsi]. Bogor (ID): IPB.
- Desjardins PR, Conklin DS. 2011. Microvolume Quantitation of Nucleic Acids. *Curr Protoc Mol Biol.* 93: A.3J.1–A.3J.16.
- Fatchiyah, Arumningtyas EL, Widyarti S, Rahayu S. 2011. *Biologi Molekular: Prinsip Dasar Analisis*. Jakarta (ID): Erlangga.
- Fiedurek J, Rogalski J, Ilczuk Z, Leonowicz A. 1986. Screening and mutagenesis of moulds for the improvement of glucose oxidase production. *Enzyme Microb Technol.* 8:734-736.
- Frederick KR, Tung J, Emerick RS, Masiarz FR, Chamberlain SH, Vasavada S, Rosenberg S. 1990. Glucose oxidase from *Aspergillus niger*-Cloning, gene sequence, secretion from *Saccharomyces cerevisiae*, and kinetic analysis of a yeast-derived enzyme. *J Biol Chem.* 265 (7):3793-3802.
- Gandjar I, Samson RA, Tweel-Vermeulen K, Oetari A, Santoso I. 2000. *Pengenalan Kapang Tropik Umum*. Jakarta (ID): Yayasan Obor Indonesia.
- Graham CA, Hill AJM. 2001. *Methods in Molecular Biology: DNA Sequencing*

- Protocols*. 2nd Ed. New Jersey (US): Humana Pr.
- Guo Y, Lu F, Zhao H, Tang Y, Lu Z. 2010. Cloning and heterologous expression of glucose oxidase gene from *Aspergillus niger* Z-25 in *Pichia pastoris*. *Appl Biochem Biotechnol*. 162:498-509.
- Jin J, Lee YK, Wickes BL. 2004. Simple chemical extraction methods for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol*. 42(9): 4293-4296.
- Kheyrodin H, Ghazvinian K. 2011. DNA purification and isolation of genomic DNA from bacterial species by plasmid purification system. *Afr J Agricul Res*. 7: 433-442.
- Koolman J, Roehm KH. 2005. *Color Atlas of Biochemistry*. 2nd Ed. Stuttgart (DE): Georg Thieme.
- Kundu A, Sarkar D, Bhattacharjee A, Topdar N, Sinha MK, Mahapatra BS. 2011. A simple ethanol wash of tissue homogenates recovers high-quality genomic DNA from *Corchorus* species characterized by highly acidic and proteinaceous mucilages. *Electron J Biotechnol*. 14: 1-7.
- [Macherey-Nagel]. 2014. *PCR Clean Up Gel Extraction: NucleoSpin® Gel and PCR Clean-up*. Düren (DE): Macherey-Nagel.
- Melzak KA, Sherwood CS, Turner RFB, Haynes CA. 1996. Driving forces for DNA adsorption to silica in percholate solutions. *J Colloid Interface Sci*. 181: 635-644.
- Nelson DL, Cox MM. 2012. *Lehninger Principles of Biochemistry*. 6th Ed. New York (US): WH Freeman.
- Pharmawati M. 2009. Optimalisasi Ekstraksi DNA dan PCR-RAPD pada *Grevillea* spp. (Proteaceae). *J. Biologi* 13 (1):12-16.
- [Promega]. 2015. *pGEM®-T and pGEM®-T Easy Vector Systems*. Madison (US): Promega.
- Reece JB, Urry LA, Cain ML, Wasserman SA, Minorsky PV, Jackson RB. 2012. *Campbell's Biology*. 9th Ed. New York (US) : Benjamin Cummings.
- Riyadi S, Maheswari RRA, Sudarwanto M, Fransiska RZ, Ali M. 2011. Kloning gen penyandi antigen *HbsAg100* dalam rangka produksi protein rekombinan sebagai model imunogen untuk menghasilkan antibodi. *J Peternakan Ind*. 13(3): 241-248.
- Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd Ed. New York (US): CSHL Pr.
- Tan H, Tie M, Zhang L, Zhu Y, Li X. 2011. The effect of three different grinding methods in DNA extraction of cowpe (*Vigna unguiculata* L. Walp). *Afr J Biotechnol*. 12(16): 1946-1951.
- Tarigan D. 2008. Isolasi dan kloning fragmen gen penyandi aminocyclopropane carboxylic synthase (ACS) dari daun tanaman karet (*Hevea brasiliensis*) [skripsi]. Bogor (ID): IPB.
- [Thermo Scientific]. 2012a. *Product Information: EcoRI*. Vilnius (LT): Thermo Scientific.
- [Thermo Scientific]. 2012b. *Product Information: PstI*. Vilnius (LT): Thermo Scientific.
- [Thermo Scientific]. 2014. *Product Information: GeneJET Plasmid Miniprep Kit*. Vilnius (LT): Thermo Scientific.
- [Tiangen]. 2012. *Plant Genomic DNA Kit*. Beijing (CN): Tiangen.
- Triana R. 2013. Pemurnian dan karakterisasi enzim glukosa oksidase dari isolat lokal *Aspergillus niger* (IPBCC.08610) [skripsi]. Bogor (ID): IPB.
- Xiong J. 2006. *Essential Bioinformatics*. Cambridge (UK): Cambridge Univ. Pr.
- Yamaguchi M, Tahara Y, Nakano A, Taniyama T. 2007. Secretory and continuous expression of *Aspergillus niger* glucose oxidase gene in *Pichia pastoris*. *Prot Express Purif*. 55: 273-278.