Isolation of Metallothionein (MT) Gene from Phytoremediation Agent, *Eleusine indica*

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Abstract. Belulang grass (*Eleusine indica*) is a plant in the Poaceae family that is commonly found in the coastal area of Dumai, Riau Province. *Eleusine indica* is characterized by narrow leaves, concave stems that can reach up to 95 cm high and strong roots. *E. indica* is known to be very tolerant of its environment, including the environment contaminated with heavy metals. The ability of *E. indica* as a phytoremediation agent in absorbing heavy metals has been widely known as the role of metallothionein (MT) protein. MT is believed to have a function in the metal metabolism and detoxification process through the metal chelating interaction between the cysteine amino acid residues. This unique function prompted the interest to isolate the MT gene from *E. indica*. This method involves the isolation of genomic DNA from *E. indica* followed by the process of amplification of the MT gene using specific primers, namely MTFS and MTRS by polymerase chain reaction (PCR) technology. The success of the MT gene isolation process from *E. indica* was evidenced by the presence of a single band size of around 172 bp via the visualization process on 1% agarose gel. Furthermore, the results of the PCR product are purified for the purpose of sequencing activity. The results of sequencing analysis of the 172 bp fragment showed 99.31% identical similarity with the complete metallothionein gene from *E. indica* (DQ082855.1) by using the BLASTN tool, NCBI website.

1. Introduction

A goosegrass (*Eleusine indica*) is a type of C4 plant that is known as Belulang grass by Indonesian people. This plant is commonly found on the soil such as in coastal areas and plantations. Dumai coastal is one of Riau’s areas that has full sunlight intensity whilst is the main factor to support the growth of *E. indica*. *E. indica* can grow quickly and lush in this area. Bandar Bakau is one of Dumai’s coastal locations that have the kinds of these grasses. The characteristics of *E. indica* are narrow leaves, strong roots, and concave stems that can reach up to 95 cm high [1]. *E. indica* reported as one of many plants that very tolerant with its environment. For instance, the place was contaminated by heavy metals such as Cd, Pb, ZN, Cr, Mn and Cu [2].

Based on the research, most of the coastal areas of Dumai city have been contaminated with heavy metals as an effect of anthropological activities from settlements, oil palm plantation, oils factories, and the shipping sector [3]. The heavy metals pollution in coastal areas automatically gives a bad impact on aquatic organisms such as fishes, planktons, plants and other microorganisms [4]. These pollutions have been founding both in seawater and sediment for years ago now. A number of gastropods and...
bivalves are known to contain heavy metals such as Pb, Cd and Hg over the threshold of organism had [5].

_E. indica_ has the potential as a phytoremediation agent. A word of phytoremediation refers to a remediation approach using a plant that is used to remove and extract any pollutants, including heavy metals [6]. Recently, some researchers provide data about _E. indica_ protein’s role in the metal metabolism, namely Metallothionein (MT) protein. In addition, the MT gene could be detoxified the metal pollutions through its metal chelating interaction [7]. This protein has 30 percent cysteine from the total of amino acids and a molecule heavy of about 7 kDa [8]. These processes involve the character of cysteine amino acid residues in MT protein composition. Due to this, its roots and leaves can store plenty of heavy metals content without interfering with growth and development. The MT genes could be found in other organisms like bacteria, fungi, plants, and animals [9].

There are found four types of MT genes, namely MT1, MT2, MT3 and MT4 based on the cysteine motif at its terminals of amino acid and carboxyl [10]. The molecular interactions between some cysteine and heavy metals (Zn, Cu, and Cd) make it easy in protein folding and its structure more stable. The ability of MT protein as a heavy metal bioindicator in marine and terrestrial environments already tested in sea crabs and great tits (_Parus major_) [11]. They are known to be poisoned and the population is decreasing due to Cd accumulation in their kidneys. This unique function prompted the interest of the researcher in isolating the MT gene from _E. indica_ as a step to understand the details of its roles as a phytoremediation agent.

2. Methodology
This study used an experimental method using _E. indica_ plants obtained from Bandar Bakau Dumai and then processed at the Marine Microbiology Laboratory, Faculty of Fisheries and Marine Science, University of Riau.

2.1. Isolation of genomic DNA (_E. indica_)
The protocol used is a modified CTAB in a mini preparation [12]. _E. indica_ leaves were weighed around 100 mg and placed in a mortar. Then, it was added 0.5 ml of CTAB buffer (already heated in 65°C) and 0.01 g PVP. The sample was crushed until it become a smooth porridge and transferred it into the 1.5 ml centrifuge tube after it was already mixed with CTAB buffer up to 1 ml. The lysis process was occurred when the microtube was incubated in a waterbath (65°C, 90 minutes) and showed the two layers (solid and liquid). Next, the liquid phase (supernatant) was moved to a new microtube and poured with 500 µl cold isopropanol (-20°C, 30 minutes). Furthermore, it was centrifuged at 12,000 rpm in 10 minutes whilst the liquid phase was removed and the tube was reversed for drying the pellet at the bottom of it. Repeat this step but the buffer was used 70% alcohol (200 µl). Finally, the pellet was added 50 µl TE buffer (stored at -20°C). The DNA extract was run using an electrophoresis tools (agarose gel 0.8%, 50 V at 30 minutes).

2.2. Amplification process of the MT gene
The total of 25 µl PCR mix consists of 1 µg DNA genome, 10X PCR buffer (Promega, the US), 10 mM dNTP mix, 25 mM MgCl2, 2.5 U Taq DNA polymerase, 10 PM MTFS and MTRS [13]. The negative control, ddH2O water was used to replace the DNA genome. The PCR machine was set at 95°C, 3 minutes (pra-denaturing) and followed about 40 cycles at 95°C, 1 minute (denaturing); 56.7°C, 1 minute (anneling); 72°C, 45 sec (extension); ended at 72°C, 10 minutes (post-extension). The PCR results were analyzed by agarose gel electrophoresis (0.8%, 50 V at 30 minutes).

2.3. Sequencing Analysis
DNA samples were sent to the sequencer company in Malaysia, First BASE. The sequences were got continues to the steps of bioinformatic analyses. Some software was used such as Bioedit, ClustalW, and BLASTN.
3. Results and Discussion

DNA isolation is a crucial starting step in molecular analysis to be successful. The method of CTAB buffer is effective to extract DNA genome from plants in a good quality based on the appearances of DNA bands on an agarose gel electrophoresis (Figure 1).

![Figure 1. The DNA genome of *E. indica* in the agarose gel electrophoresis (0.8 %). S1 and S2: samples; L: 1 kb Ladder](image)

Both DNA extraction (S1 and S2) are located at the upper of threshold of 1kb ladder (10,000 bp). The breaking process of the cell wall was done physically by using CTAB liquids [14]. These liquids help to release DNA and prevent the degradation of DNA. Some components inside CTAB liquids such as PVP, β-mercaptoethanol, EDTA and others would be removed some phenolic compounds inside plant tissues that affected the DNA’s quality and PCR analyses [15]. The mixture of chloroform/isoamyl alcohol (24:1) is used to stable and binding the polysaccharide and protein complexes that are produced before the adding process of isopropanol. If the isolation process can’t produce good quality and quantity of DNA, an amplification process through the PCR technique will not get the best results. The spectrophotometer tool was used to measure the DNA concentration and DNA purity (Table 1).

| Sample | DNA concentration (µg/mL) | DNA purity (A260/A280) |
|--------|--------------------------|------------------------|
| S1     | 2.80                     | 1.82                   |
| S2     | 2.47                     | 1.93                   |

Based on the measurement results, the concentration of sample 1 (S1) is higher than sample 2 (S2). There are two factors that could be influenced the value of DNA concentration, the incubation of temperature and time. The highest temperature will destroy DNA, but the lowest temperature will make the membrane and tissue cells can’t break. The combination of temperature and time incubation
will release the best result. The incubation temperature and time that was applied in this study is about 65°C for 90 minutes. However, the purification step must be considered to get the expected results.

The standard of DNA purity is a range ratio between A260 and A280. Where is the good result must provide value about 1.8 – 2.0. Both samples have the data at that’s range. If the ratio value is under 1.8, the DNA isolation was contaminated by phenol and buffers. In contrast, if the ratio value is above 2, the sample was contaminated by protein and other metabolites. The concentration and purification of DNA could be caused by technical factors during the process of DNA extraction.

![Figure 2. The eiMT gene of E. indica in the agarose gel electrophoresis (1%). S1 and S2: samples; C-: negative control; M: DNA marker 100 bp](image)

The molecule analysis by using PCR technique was applied to get the target gene (eiMT) from E. indica. This method is able to increase the copy number of DNA from thousands to millions. A pair of primers that were used for this research is based on the last research, namely MTFS, and MTRS [16]. The primers were designed according to the conserved sequences from 5’ to 3’ for a target size of eiMT at around 172 bp (Figure 2). The PCR results have thick fragments that indicate to the highest concentration of the genome DNA. The use of negative control that illustrates an empty condition indicates the absence of contaminations while the working process. The eiMT sequences were provided by First BASE Laboratories Sdn Bhd (1st BASE). These sequences were continued with a statistical estimation through the BLASTN tools at the GenBank website.

The principle of this software is based on the algorithm heuristic to align the samples’ nucleotides with the GenBank data. The BLASTN analysis from the eiMT gene’s samples shows a similarity of about 99.31% to the metallothionein from E. indica (DQ082855.1). The overlap regions between the two samples could be seen in the symbol * (Figure 3). These results provide E-value at 4e-66 and score around 263 bits (Table 2). Referring to statistical analysis, the lowest level for E-value means the highest value of analysis. In addition, a high score indicates a high identity to the entire region (length) of referenced gene.

**Table 2.** The comparative analysis of the eiMT samples and reference through BLASTN tools.

| Gen | Identity | Alignment | E-value | Score-value | Organism | ID Sequence | Reference |
|-----|----------|-----------|---------|-------------|----------|-------------|-----------|
| eiMT | 99.31%   | 144/145   | 4e-66   | 263 bits    | E. indica| DQ082855.1  | [13]      |
Figure 3. The diagram shows the overlap area of the ciMT gene between samples and reference (DQ082855.1).

Based on the sequence analysis and some studies, the type of MT1 of E. indica has the cysteine form of Cys-Xaa-Cys in both the N- and C-terminus. This pattern was reported in other plants such as barley [17], wheat [18], and pea [19]. The MT genes have varies functions, one of them is heavy metal tolerant. Festuca rubra cv. Merlin has shown heavy metals tolerance that referred to phytoremediation agent. This ability believed has potency as good as in MT1 from E. indica.

4. Conclusion
The success of the MT gene isolation process from E. indica was evidenced by the presence of a single band size of around 170 bp via the visualization process on 1% agarose gel. Furthermore, the results of the PCR product are purified for the purpose of sequencing activity. The results of sequencing analysis of the 170 bp fragment showed 99.31% identical similarity with the complete metallothionein gene from E. indica (DQ082855.1) by using the BLASTN tool, NCBI website.

References
[1] Septiani D, Hastuti E D and Darmanti S 2019 Bulletin of Anatomy and Physiology 4(1) 1-7
[2] Krezel A and Maret W 2017 International Journal of Molecular Sciences 18 1237-1257
[3] Farma R Fadilah R Sari N K, Taer E and Deraman M 2018 Journal of Physics: Conference Series 1120(1) 012017
[4] Nayar S, Goh B P L and Chou L M 2004 Ecotoxicology and environmental safety 59(3) 349-369
[5] Allah A A, Wanas M Q S and Thompson S N 1997 Journal of Molluscan Studies 63(1) 79-86
[6] Lee L J and Ngim J 2000 Pest Management Science 56 336-339
[7] Tarasava K, Loebus J and Freisinger E 2016 International journal of molecular sciences 17(3) 371-382
[8] Vašák M 2005 Journal of Trace Elements in Medicine and Biology 19(1) 13-17
[9] Peresia P and Rini D S 2018 Journal of Visuals of Science 1208(1) 012039
[10] Leszczyszyz O I, Imam H T, and Blindauer C A 2013 Metallomics 5(9) 1146-1169
[11] Vanparys C, Dauwe T, Van Campenhout K, Bervoets L, De Coen W, Blust R and Eens M 2008 Science of the Total Environment 401(1-3) 184-193.
[12] Ardiana D W 2009 Buletin Teknik Pertanian 14(1) 12-16
[13] Sidiq N M, Roslina M Y, Che Radziah C M Z and Ismanizan I 2010 Sains Malaysiana 39(6) 927-933
[14] Surzycki S 2000 Springer-Verlag Berlin Heidelberg New York
[15] Jose J and Usha R 2000 Plant Molecular Biology Reporter 18 349-355
[16] Sidik N M Yazid R M Dahlan D Z M Othman B A and Ismail I 2019 Australian Journal of Crop Science 13(4) 599-604
[17] Okumura N, Nishizawa N K, Umehara Y, Mori S 1991 Plant Molecular Biology 17(3) 531-533
[18] Snowden K C and Gardner R C 1993 Plant Physiology 103(3) 855-861
[19] Marta E I, Gatehouse L N, Gatehouse J A, Robinson N J and Croy R R 1990 FEBS letters 262(1) 29-32