Targeted discovery of glycoside hydrolases from enzymes reservoir of digestive gland *Achatina fulica* through functional metagenomic strategies

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**Abstract.** Digestive gland *Achatina fulica* is source of bioprospecting of glycoside hydrolases enzymes for many biotechnological and industrial processes. Nevertheless, there is limitation to discover novel enzymes. The functional-based approach analyzes the metagenomic library based on the genomic function of an organism used to look for new enzyme-producing genes. The aim of this study was to determine the chances of obtaining the novel genes for glycoside hydrolases from enzymes reservoir of digestive gland *A. fulica* through functional metagenomic method. The results showed that the total RNA concentration isolated from the digestive gland of *A. fulica* was 2,343.2 ng/μL. A total of 2 μL of total RNA has been used to construct the metagenomic library, so there are $4.69 \times 10^{10} - 1.87 \times 10^{11}$ transcribed molecules from $1.17 \times 10^9 - 4.68 \times 10^9$ genes. This is a great number of chances to acquire the novel glycoside hydrolase genes.

1. **Introduction**

The sequencing of genes and genomes from cultured microbes used media in the laboratory, only had a chance of getting 0.1% of the entire microorganism community environment. In contrast to metagenomics, which is the study of all genetic material that comes directly from environmental samples without the need to be grown first. Metagenomic strategies have been used to explore novel genes from various environments and ecosystems [1]. Functional metagenomics has the added advantage of not requiring information about preexisting gene sequences [2]. In addition, functional metagenomics is an effective method, because gene discovery is based on the expression of these genes with real activity [3].

Digestive gland *A. fulica* is the right source for exploration of new enzymes with functional metagenomics methods, especially glycoside hydrolases (GH), because these herbivores contain a large ecological scope of microorganisms in their digestive system and are able to rapidly hydrolyze cellulose and hemicelluloses of various plant species. This enzyme comes from the microbiota in the digestive gland of *A. fulica* [4,5]. The digestive gland of *A. fulica* is a rich reservoir of glycoside hydrolase and is a potential source of novel enzymes. This study was to determine the opportunity of obtaining the novel genes for glycoside hydrolases from enzymes reservoir of digestive gland *A. fulica* through functional metagenomic strategies.
2. Materials and methods

2.1. Animal and tissue collections
The sample of this research is digestive gland *A. fulica* (snail). *A. fulica* was obtained from Blitar, East Java, Indonesia. Digestive gland samples include the crop, salivary glands, and intestinal tissue. *A. fulica* was choosed weight 150-200 g.

2.2. Sample collection
Five snails (*A. fulica*) were caught randomly, then cleaned with sterile distilled water and taken to the laboratory. *A. fulica* was placed in a closed vessel and anesthetized with chloroform steam. *A. fulica* that has been given anesthesia is placed on a surgical tray and its shell is broken. The sample used was the digestive gland of *A. fulica* and the materials in it. The sample was mashed using a mortar until smooth while continuing to add liquid nitrogen periodically to keep the sample tissue cool and continued in the RNA isolation process.

2.3. RNA isolation
RNA isolation was processed according to the PureZolTM kit protocol ((Bio-Rad, US) RNA Isolation Reagent Instruction Manual.

2.4. Synthesis of cDNA
Synthesized first strand cDNA according to the SMARTTM cDNA Library Synthesis Kit (BD Bioscience, US) protocol using the LD (long distance) PCR protocol.

2.5. Construction of cDNA libraries
The cDNA library was constructed using the SMARTTM cDNA Library Construction Kit (BD Bioscience, US).

2.6. Screening of recombinant clones of glycoside hydrolase genes
Recombinant clone screening was performed using the method used by Kurniawati *et al*. [6].

3. Result and discussion
The metagenomic strategy through the metagenomic functional approach is a strategy for constructing gene libraries from mRNA sources. RNA samples were isolated from the *A. fulica* digestive gland. One picogram (pg) of mRNA is equivalent to $10^6$ transcribed molecules from the range of 25,000 - 30,000 genes [7]. Thus, the higher the mRNA concentration, the more diverse the metagenomic library diversification, so that the greater the chance of obtaining novel genes with a metagenomic functional approach.

The total RNA concentration isolated from the digestive gland of *A. fulica* was 2,343.2 ng / µL. mRNA makes up about 1-4% of the total RNA in the cytoplasm of eukaryotic cells. If 2 µL of total RNA is used to construct the metagenomic library, then there are 4,686.4 ng of total RNA or about 46.86 - 187.46 ng mRNA. In 1 pg mRNA equivalent to $10^6$ molecules, there are $4.69 \times 10^9 - 1.87 \times 10^{11}$ transcribed molecules. This amount provides a great opportunity to acquire the novel 1,3-β-glucanase gene.

Total RNA was isolated from the digestive system of *A. fulica* which includes crop, salivary gland, and intestines. Cardoso *et al*. [4] reported that *A. fulica* crop and intestines are enzyme reservoirs, which release various enzymes of glycoside hydrolases with specific activity, such as cellulases, glucans, xylanases, mannanases, arabinofuranosidase, galacturonase, glucosidase, and galactosidase. However, in this research, the total RNA isolated from the 100 µL *A. fulica* crop fluid sample only obtained a low concentration of total RNA, namely 0.7 ng/µL. As a sample for the purpose of metagenomic library construction, the total RNA concentration isolated from the crop fluid was too low. Efforts to increase the RNA concentration were carried out by giving pre-treatment of *A. fulica*, which is induced by foods
rich in β-glucan (Table 1), which is the substrate for inducing the expression of the target gene, so that the amount of mRNA increases.

Table 1. Observation results of *A. fulica* condition after induction used foods with high GH concentration.

| No. | Type of food   | *A. fulica* condition after induction                      |
|-----|---------------|------------------------------------------------------------|
| 1.  | Papaya leaves | Snail did not consume papaya leaves                        |
| 2.  | Oyster mushrooms | Snail did not consume papaya leaves and 3 snails died       |
| 3.  | Mustard leaves | Snail in good condition                                    |

Three types of food are used as inducers, including papaya leaves, oyster mushrooms and mustard leaves. The results showed that the *A. fulica* group which was given papaya leaves and oyster mushrooms did not consume the food that had been provided, so there were 3 snails died. While the *A. fulica* group that was given mustard leaves showed different conditions, then this group was used as the sample for the reprocessing of total RNA isolation.

The low concentration of total RNA isolated from the crop fluid sample was used as an empirical basis to increase the sample portion for total RNA isolation. Next, the sample is taken from the digestive gland, intestine, and crop fluid, which is then the source of the mixture of these three types of samples called the digestive system. A total of 100 mg of the digestive system that has been mashed together with liquid nitrogen is put in a tube and 1 ml of purezole reagent is added. The reaction results show that there are 3 layers in the tube as shown in Figure 1.

![Figure 1. Water phase and organic phase layer in the RNA isolation process.](image)

The top layer (Figure 1) is the colorless water layer, the second layer is white, and the lowest layer is the organic phase layer with red color. RNA and DNA are found in the top layer, while the second and lowest layers are layers with protein components. DNA is eliminated by addition of DNase. The measurement results using nanodrop obtained total RNA 2343.2 ng / μL from 100 mg of digestive gland samples (Table 2), using Thermo Scientific Nanodrop 2000.

Table 2. Total RNA concentrations isolated from crop fluids and digestive glands.

| No. | Source of RNA     | Mass/volume tissue | RNA concentration |
|-----|-------------------|--------------------|-------------------|
| 1.  | Crop fluids       | 100 μl             | 0.7 ng/μL         |
| 2.  | Digestive gland   | 100 mg             | 2343,2 ng/μL      |

Isolation and purification of total RNA in high quantity and quality is an important step in the construction of the cDNA library [8]. Inadequate RNA quality can affect the stability of RNA. Meanwhile, the small quantity of RNA will have an impact on the success rate of the RT-PCR process, which results in low cDNA concentrations. RNA with good quality and high quantity is expected to produce full-length cDNA from the target gene.
The isolated RNA was tested by electrophoresis using agarose gel 1.1% at a potential difference of 110 V for 30 minutes. The results of electrophoresis of RNA isolates from the digestive gland of *A. fulica* showed a long smear between 0.5 kb - 2 kb (data not shown). These smear data indicate that the RNA obtained varies in size. High intensity smears are located between 0.65 kb to 1.2 kb. The high intensity of the smear shows the integrity of the RNA obtained, because of the guanidine thiocyanate buffer found in the purezol reagent. The guanidine thiocyanate buffer can change the tertiary structure of the RNase, thereby deactivating the ability of RNase to degrade RNA [9]. With the inactivity of RNase, most of the RNA will be obtained intact and not degraded.

The RNA purity test was carried out by measuring the absorbance ratio at OD$_{260}$ and OD$_{280}$ wavelengths using nanodrop (Thermo Scientific Nanodrop 2000). The OD$_{260}$/OD$_{280}$ ratio as an indicator of protein contaminant levels [10]. The OD$_{260}$/OD$_{280}$ ratio of the total RNA isolates obtained was 1.95 and the OD$_{260}$/OD$_{230}$ ratio of the total RNA isolates was 1.69. This ratio value indicates no protein contaminants [7]. As a comparison, the results of measuring the OD ratio using the same isolation kit (PureZOL RNA Isolation Reagent) were tested by Franҫa *et al.* [10]. RNA that has been isolated can be stored at -20°C for 1 month or -70°C for 1 year. Several research articles reporting the process of RNA isolation are listed in Table 3.

### Table 3. The OD ratio of isolated RNA from several RNA sources.

| No. | Samples                          | OD$_{260}$/OD$_{280}$ | OD$_{260}$/OD$_{230}$ | References |
|-----|----------------------------------|-----------------------|----------------------|------------|
| 1.  | Jaringan tumbuhan *Populus hopeiensis* | 1.944                | 2.019                | [11]       |
| 2.  | Human liver                      | 1.890                | -                    | [8]        |
| 3.  | Biofilm *Staphylococcus epidermidis* | 1.700                | 0.630                | [10]       |

The metagenomic library construction carried out in this study was the metagenomic expression library construction. The difference between the metagenomic library and the metagenomic expression library lies in the type of vector used. The metagenomic library uses cloning vectors, while the metagenomic expression library uses expression vectors.

The use of cloning vectors will require a sub-clone stage to an expression vector, which involves a longer stage, including the transformation process or expression vector transfection that has carried the target gene to the appropriate host. The long stage in the construction of the metagenomic library can be simplified by the construction of the metagenomic expression library. In the metagenomic expression library, recombinant E. coli colonies can directly express the target gene while still using the initial vector. An outline of the differences between the metagenomic library strategy and the metagenomic expression library is shown in Figure 2.

The recombinant plaque encoding glycoside hydrolase gene from the metagenomic library of *A. fulica* digestive expression was carried out using an activity-based approach of the target gene expression product. A glycoside hydrolase activity-based approach was carried out using the Congo Red staining method [12-14] in LB agar containing IPTG and laminarin substrate [5,15,16]. Some results of recombinant plaque screening based on target gene activity are presented in Figure 3.

The approach to metagenomic expression literature is limited by the following: differences in codon usage, signal transcription and translation, protein folding, and post-translational modification, which do not provide heterologous expression effects which in the host system E. coli cannot always facilitate heterologous expression. On the other hand, this approach is able to develop a completely new gene discovery in a new family that is not found by conventional screening based on sequence homology of known genes [1].
Figure 2. The difference in the process of metagenomic library strategy and metagenomic expression library strategy to obtain novel genes. A. The process of metagenomic library construction (modified Li et al., [17]). B. The process of metagenomic expression library construction.

Figure 3. Some results of recombinant plaque [λTriplEx2-glucoside hydrolase] using laminarin substrate stained with Congo Red with a magnification of 400X.

4. Conclusion
Two μL of total RNA with high concentration 2,343.2 ng/μL which transcribed from 1.17. 10^9 - 4.68. 10^9 genes are a great number of opportunities to obtain the novel genes of glycoside hydrolases from digestive gland A. fulica through functional metagenomic strategies.

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