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Research paper

Metagenomic DNA Library: Exploration of Novel Genes Encoding Glycoside Hydrolases

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Abstract

Metagenomic is a potential approach to explore novel genes from a reservoir of genes. Metagenomic cDNA library as part of metagenomic approach be able to explore abundant quantity of microbiota genes. Metagenomic library collection was screened by functional and sequence analysis. Functional analysis can be done through induction genes expression substrat. Positive clones can be screened by expression of interest genes. Data of sequence analysis is very important in the process of product identification.

Keywords: *Metagenomic, cDNA Library, Glycoside Hydrolase, Digestive Gland, Achatina fulica.*

1. Introduction

Glycoside hydrolase are produced by bacteria, fungi, and also found in animals such as mollusks, sea shells, termites, sea urchins, and blue mussel [1]. *Achatina fulica* is a species of mollusk that feeds on plants as a source of nutrients, including producing digestive gland to degrade food sources [2]. There are epithelial and microbiota cells in digestive gland (gantrointestinal) *Achatina fulica* that produce glycosides hydrolase enzyme [3]. Glycoside hydrolase enzymes such as cellulase, hemicellulase, glucanase, glucanhidrolase, chitinase, xylase, amylase, maltase are classified into 113 family [4].
Metagenomic analysis of digestive gland of *Achatina fulica* through a sequence-based approach has managed to see the diversity of microbial species and bacterial associations present in digestive glands of *Achatina fulica*. The result of microbiota analysis found the dominant bacteria phylum such as Proteobacteria, Bacteriodetes and Firmicutes, and also found the existence of virus, fungi and archaea. The analysis through a functional-based approach on digestive gland *Achatina fulica* is divided into analysis on eukaryotic and prokaryotic cells. The epithelial cells of *Achatina fulica* and fungi in this case fall into the category of eukaryotic cells. In the DNA of eukaryotic cells there are introns and DNA exons. The process of DNA transcription can produce pre-mRNA which then becomes a variety of mRNA or mix-mRNA [5].
The search for a new gene that encoding the glycoside hydrolase enzyme from digestive gland of *Achatina fulica* through metatranscriptomic cDNA library is performed by reverse transcriptase of mRNA into cDNA. The cDNA is used to construct the library, search for glycoside glycoside hydrolase encoding gene, expression and characterization of recombinant glycoside hydrolase enzyme obtained.

2. Genes Exploration Using the Metagenomics cDNA Approach

Metagenomics is a method that aims to collect and characterize genetic material directly from the environment or habitat. The metagenomics method differs from the characterization and isolation of individuals from a colony. Metagenomics offers a unique opportunity to study an organism that cannot be cultured (unculturable) [1] on an artificial medium. The opportunity to open access into the source of new microbial genes, especially unculturable microbes. It is estimated that unculturable microbes reach 99% of the population of microbes in the nature [2].
Metagenomics is the applications method of genomics technology to unculturable microbiota from its environment ([6]; [7]) and reservoir genes (gut, gastrointestinal [8], rumen [9], etc) with making genomic libraries, followed by analyzing the genetic information contained therein. The metagenomics approach incorporates several fields of science, such as genetics, microbiology, and bioinformatics [10]. The metagenomics approach generally consists of three stages: (1) Sampling and microbial nucleic acid extraction, (2) microbial genome and gene enrichment, (3) sequence analysis of genes trageting (sequence analysis of amino acids) [8].
The aims of construction of the metagenomics library is collecting genes from microbes that encode a particular enzyme. There are three methods that can be done in the construction of the metagenomics library: (1) genes cloning randomly (shotgun cloning), (2) using the Polymerase Chain Reaction (PCR) machine, and (3) combination of both methods (gene cloning randomly and PCR).

Table 1: Novel genes from metagenomic library

No	Source of metagenomics	Estimate library	Interest genes	Reference
1.	Soil metagenome	7,500 clones fosmid	1 gene β-1,4-endoglucosidase	[7]

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2.	Soil metagenome	1,700 plasmid clones	1 gene endoglucanase	[11]
3.	Forest soil, dung of elephant, cow rumen, and rotted tree	1 – 4 . 10^4 phages	5 genes β-1,4-glucanases and 2 genes β-glucosidase	[12]
4.	Green-waste compost switchgrass	225 Mbp, 800 genes	Glycoside Hydrolase (GH5 and GH9)	[13]
5.	Neocallimastix patriciarum	10^6 titers	B-1,3-1,4-glucanase	[14]
6.	Soil microbes and rumen fluid	29,006 fosmid clones	4 genes exocelluloses	[15]
7.	Rumen of cattle	20,160 clones	β-glucosidase	[16]
8.	Metagenome	24,000 clones plasmid	1 gene endoglucanase	[17]
9.	Sugarcane bagasse	120,000 clones fosmid	1 gene endoglucanase and 1 gene endoxylanase	[18]
10.	The bovine rumen metagenomic	38.400 clones fosmid	1 gene cellulase	[19]

The most common of cloning the gene is shotgun cloning. This method begins with the isolation of DNA from microbes, then the obtained DNA is cut off by endonuclease restriction enzyme. Each piece of DNA produced is inserted into the plasmid DNA. The clones obtained are transformed into the bacteria *E. coli*, through the transformation technique. The result of transformation is known as metagenomics library [10].

Based on the desired objectives of the metagenomics approach, there are two methods of analysis performed, ie function-based and sequence-based. Function-based analysis is performed by constructing genomic DNA clones and then screening or screening of genomic DNA clones based on the desired phenotypic expression. Genomic DNA clones were tested for the presence and absence of certain enzyme activity. The predominance of a function-based analysis would be to get the entire functional gene encoding and expressing from an expected function [20].

3. Glycoside hydrolase from Digestive Gland *Achatina fulica*

Achatina fulica produces enzymes in its digestive gland to degrade food. Digestive gland of *Achatina fulica* produces mixture of glycoside hydrolases enzymes and other types of hydrolases. The glycoside hydrolases enzymes which produced digestive gland of *Achatina fulica* were mixture of several enzymes such as β-1,3-glucanase, β-1,6-glucanase, β-1,4-glucanhidrolase, endo-β-1,4-glucanase, chitinase, xylase, cellulase, lichenase, inulase, hemicellulase, amylase, maltase and sucrase [2].

One of the glycoside hydrolase from digestive fluid of *Achatina fulica* namely endo-β-1,4-glucanase (AfEG22) has been successfully purified and characterized. The hydrolytic activity of carboxy methyl cellulose (CMC) from pure endo- β-1,4-glucanase enzyme is 12.3 U/mg protein. The mass of endo-β-1,4-glucanase molecules is 22081 Da determined using MALDI-TOF. The N-terminal amino acid sequence is EQRCTNQGGILKYYNT, which has no significant homology on the BLAST database. The temperature and pH for hydrolytic activity against CMC is 50°C and pH 4.0. The endo-β-1,4-glucanase enzyme is stable at pH between 3.0 and 12.0 incubated at 4°C for 3 hours or 37°C for 1 hour. The enzyme activity is more than 80% between pH 4.5 and pH 7.0 after incubation at 50°C for 1 hour. Enzyme endo- β-1,4-glucanase is an enzyme of digestive fluid of *Achatina fulica* which has high stability [21].

Lignocellulose is composed of cellulose, hemicellulose and lignin. Among the three, cellulose is the most abundant organic molecule compared to the others [22]. Cellulose is a polysaccharide compound composed of β-D-glycopyranose units incorporated through a 1,4-glycosidic bond. Cellulose is a major component of plant cell walls [23]. Between cellulose and lignin are linked by hemicellulose. The largest component of hemicellulose is xylan. The difference between cellulose and hemicellulose is cellulose more soluble in acid and difficult to soluble in alkali, but hemicellulose is more soluble in alkali and difficult to dissolve in acids.

Cellulose can be degraded by cellulase enzyme groups. There are three types of functional cellulase enzymes that can hydrolyze cellulose into sugars. First, 1,4-β-glucan cellobiohydrolase (exo-glucanase; EC 3.2.1.91) degrades the cellobiose unit at the tip of the cellulose polymer. Second, endo- β-1,4-glucanase (endo-glucanase; EC 3.2.1.4) randomly degrades the internal of the β-1,4-glycosidic bond into cello-oligosaccharides of various lengths. Third, 1,4-β-glucosidase (EC 3.2.1.21) intersects cello-oligosaccharides into glucose through a saccharification process [22] [24].

Cellulase is classified into 14 glycoside hydrolase families (GHF), among others, GHF 5, 6.7, 8, 9, 10,12, 26, 44, 45, 48, 51, 61 and 74. There are five families reported in animals GHF5, GHF6, GHF9, GHF10 and GHF45. The most widely found GHF9 is dispersed with various animal cellulase types [23].

Laminarinase or β-1,3-glucanase is a group of hemicellulase digestive enzymes that exhibit high activity in hydrolyzing β-1,3-glycosidic bonds in β-1,3-glucan such as laminarin. β-1,3-glucanase belonging to the GHF 16 group found in decapod gastrointestinal tissue namely *Gecarcoidea natalis* and *Cherax destructor*. This enzyme is capable of degrading cellulose and hemicellulose [24].

4. Glycoside Hydrolase from Digestive Gland *Achatina fulica*

The digestion process is performed by using endonuclease restriction enzyme type II by breaks the phosphodiester bonds which connect between nucleotides with a single strand of DNA [25]. Restriction enzymes are enzymes produced by bacteria to defend themselves from foreign DNA entering in bacterial cells. Restriction enzyme recognizes 4-8 pairs of specific bases in a DNA strand. Each restriction enzyme has a distinct and specific cutting site. Selection of endonucleases based on cutting frequency, buffer suitability, denatured ability, and the type of cutting tip produced [26]. Several examples of restriction enzymes are presented in Table 2.

Table 2: Retriction enzymes and specific cutting site

Enzyme	Specific cutting site
Tetranucleotide (4 bases)	
*Mbo*I, *Dpn*I, *Sau*3AI	/GATC
*Msp*I, *Hpd*II	C/CCGG
*Alu*I	AG/CT
*Hae*III	GG/CC
*Ta*lI	ACGT/
SfilA	ATTA/C
SfilB	GCCT/C
Hexanucleotide (6 bases)	
*Bgl*II	A/GATCT
*Cla*I	AT/CGAT
*Pvu*I	CAG/CTG
*Kpn*I	GGTAC/C
*Bam*HI	G/GATCC

There are two types of cutting tip which produced by endonuclease restriction enzymes, namely the cohesive end (sticky ends) and the blunt ends. Restriction enzymes that produce cohesive ends include: Sau3A1, BamHI, and EcoRI. Restriction enzymes that produce produce blunt ends are HaeIII and AluI. The choice of

restriction enzymes should be adjusted to the prepared vector so as to eliminate the step to modify the ends of the DNA fragments to be used for the ligation process [26].

5. Confirmation of transduction results containing cDNA insert

The final stage in cloning techniques is the selection of cells containing recombinant plasmids. This selection is generally done by using LB media containing antibiotics. Cells that do not contain plasmids will not proliferate well on LB-antibiotics, whereas plasmid containing cells can multiply well on the media. Antibiotics added to the LB medium depend on the type of plasmid used. In general, the plasmid used contains a gene encoding resistance to ampicillin, so the media used is the LB-ampicillin medium. Selection using the LB-ampicillin medium can distinguish between cells contain plasmids and cells without plasmids [27].

The problem is separating the cell containing plasmid without insert, ie the self-ligated vector, so that the circular form can also enter into the competent cells. Solving this problem can be done by selecting a plasmid using a second antibiotic or using blue-white screening. The method chosen depends on the type of plasmid used. Selection methods with second antibiotics are applied to plasmids that have at least 2 genes that encode resistance to antibiotics. In the second selection method with antibiotics, cells that do not contain recombinant pasmids will continue to grow, but cells containing recombinant plasmids will die. For selection using blue-white screening, a medium containing X-gal (5 bromo-4-chloro-3-indolyl-β-D-galactopiranosida), a molecule similar to galactose and IPGT (isopropyl-triogalactosida) inducer β-galactosidase enzyme. If cells containing plasmids without inserts are grown on the media, the Lacz gene is expressed and β-galactosidase enzyme will be produced. This enzyme will break down the X-gal and produce a blue compound. If a cell containing a recombinant plasmid is grown on the medium, the Lacz gene will not be expressed and the β-galactosidase enzyme is not formed. Therefore, it can be concluded that selection using blue white screening will provide blue for cells that do not contain recombinant plasmids and white color for cells containing recombinant plasmids [28].

6. Sequencing of DNA Target

Determination of DNA or RNA sequences encoding the formation of proteins and RNAs is known by sequencing techniques. Two DNA sequencing methods developed in the 1970s were the Maxam-Gilbert method based on chemical base destruction, while the Sanger method was based on inhibition of nucleotide synthesis due to competition between dNTP and ddNTP [29].

In the Maxam-Gilbert method, the DNA to be sequenced is characterized by P, then the DNA is denatured and divided into four test tubes. Each tube is reacted with materials that specifically weaken the bonds between one or two of the four bases present in DNA. Subsequently piperidine will be added which will break the DNA chain at the base site attenuated the bond. This will result in a collection of fragments marked with radioactive length depending on the distance between the location of the base removed with the tip of a molecule marked with radioactivity. DNA sequences can be read from the separation of fragments formed on polyacrylamide gel [30].

The most widely used sequencing method is the modified Sanger method to improve the sensitivity and effectiveness of the analysis. The modification lies in the use of dNTPs marked with fluoroesence substances. The reaction of each base with the fluorescent dye will give a different fluorescence signal, so that the four bases can be distinguished. Therefore, with this method, each sample only needs to be reacted once (no separation is necessary between one base and another) and the result can be well separated by polyacrylamide gel [28].

7. Conclusion

Digestive gland *Achatina fulica* is a potencial enzymes reservoir. Exploration by metagenomic expression library from digestive gland *Achatina fulica* have a great opportunity to find novel genes that encoding glycoside hydrolases.

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