Research Article

Constructing and Screening Beta-Glucanase Activity of Metagenomic cDNA Expression Library of Digestive Gland of *Achatina fulica*

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ABSTRACT.
Digestive juice of *Achatinafulica* shows hydrolase activities which are useful as an antibiofilm. Insidethe digestive system of snail many microorganisms live together as a normal flora.In this study, a metagenomicbased-activity technique was carried out to construct the metagenomic expression library of digestive gland of*Achatinafulica.* The metagenomic library was screened for the recombinant harbouring genes encoding beta-glucanase again the specific substrate of laminarin.The metagenomic cloning was carried out by using λTriplEx2 as vector and *E.coli* XL1-Blue as host. The longer than 0.5 cDNAs ligated to vector and the total ligation solution were 1000 µL.Circularization of each recombinant phage inserted the target sequence were conducted in *E.coli* BM25.8, to become recombinant phagemids. Recombinant phagemids screening were carried out by using restriction method to analyse the DNA insert.We produced $2.8x10^8$ plaques and $1.1x10^{10}$ library amplification titer. Seventeen *hallo* plaques from 100 µL of the ligation solution positive screening with laminarin substrate.Eight recombinant phagemids were randomly picked and analysed using restriction method showing different fragment. A high quantity cDNA library from digestive gland *Achatinafulica* was successfully constructed.

INTRODUCTION

The group of glycoside hydrolase enzymes such as cellulase, hemicellulase and lignase enzymes have been used for various needs, including bioenergy processing, utilization of health, pulp and paper industry, textile industry, and detergent industry [1]. Cellulase and hemicellulase enzymes are great interest in research and commercial fields of bioenergy because they are potentially to be developed in applications through biotechnology [2]. The cellulase function is degrade or hydrolyze cellulose into glucose [3]. Glucose can be fermented into ethanol as a substitute for fossil fuels[3][4]. A number of studies have paid more attention to the development of new and more stable, high-efficiency cellulases and hemicellulases such as the endo-β-1,4-glucanase enzyme[5][6], β-1,3-1,4-glucanase [7]; and β-1,3-glucanase [8] for bioenergy development.The use of the β-1,3-1,4-glucanase and β-1,4-glucanase enzymes in the poultry feed industry is needed to increase the nutritional value of feed. Barley as the base material of predominantly poultry feed with β-1,4-glucan component and small part β-1,3-glucan. The use of a mixture of both the β-1,3-1,4-glucanase and β-1,4-glucanase enzymes as a poultry feed supplement, is more effective in hydrolyzing β-glucans in poultry feed [9].

Glycoside hydrolase enzymes are produced by bacteria and fungi, also found in animals such as mollusks, termites, sea shells, sea urchins, blue mussel, *Ciona intestinalis* and *Corbicula japonica*[5]. *Achatinafulica* is a species of mollusks that lives in the tropics and subtropics, and feeds on plants as a source of nutrients, including producing digestive glands to degrade food sources. The digestive gland of *Achatinafulica* produces a mixture of hydrolase enzymes. The hydrolase enzyme digestive gland *Achatinafulica* is a mixture of several enzymes such as glucanase, chitinase, xylanase, cellulase, lichenase, hemicellulase, amylase, maltase, sucrase*etc*[10].An enzyme consortium from digestive gland *Achatinafulica* effectively hydrolyze the extracellular matrix polymer in the fungal biofilms especially *Candida albicans*. This enzyme consortium was developed as an anti-fungal drug to eradicate Candida biofilms for all types of pathology of candidiasis. Candida biofilms are difficult penetrated by antifungal drugs. Main component of the extra cell biofilm matrix *Candida albicans* can be hydrolysed using glycoside hydrolase enzyme in digestive fluid *Achatinafulica*[11]. The search for a new gene encoding the glycoside hydrolase enzyme from digestive gland of *Achatinafulica* through metagenomics cDNA expression library is performed by reverse transcriptase of mRNA into cDNA. Metagenomics is a method to collect and characterize genetic material directly from the environment, habitat, gland, and other source of metagenomics. 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)[12] 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 [13]. Based on the facts, very importance to do constructing and screening β-glucanase activity from digestive gland *A. fulica*.

Experimental Method

Samples were digestive system (salivary glands, crop, and esophagus) of *Achatinafulica*. Sampleswere crushedby mortar and keep cool by adding liquid nitrogen periodically. RNA isolation was processed according to the PureZol™(Bio-Rad, US) Instruction Manual. The quality of RNA was known from the comparison of absorbance (A) at 230 nm, 260 nm, and 280 nm, A_{260}/ A_{230} and A_{260}/A_{280}.The first strand of cDNA synthesis in accordance with the protocol SMART™ cDNA Library Construction Kit (BD Bioscience, US) and double-strand cDNA synthesis protocols based LD (long distance) PCR. Amplification was done by following a program of pre-denaturation (60°C for 2 minutes, followed 45-60°C for 15 minutes and 94°C for 2 minutes), PCR process was performed by denaturation (94°C for 15 s), anneling(55-65°C for 30 s), and extention(68-72°C for 30 s), and post-PCR (68°C for 5 minutes)with 20 cycles, and then followed by Proteinase K Digestion process to stop the activity of DNA polymerase. The PCR product was purified usingQiaquick PCR Purification Kit (Qiagen, Germany) procedure. The digestion of cDNA usedSfiI enzyme. Fractionation of cDNA size was performed using Chroma SPIN-400. Sixteen fractions were collected in separate tubes. The each fraction was taken for electrophoresis in 1.1% agarose at 150 V for 3 minutes. The peak fraction was visualized under UV.The fractions with three peak largest fractions were mixed into eppendorf tubes and followed ligation process the cDNA into λTriplEx2 vector to construct cDNA library.

Constructing of cDNA library

cDNA and λTriplEx2 vector were ligated by a ratio of 3: 2; 1: 1; and 2: 3 (with initials L1, L2, and L3 respectively).λ phage in vitro packaging of each ligation (L1, L2, and L3) to produce unamplified cDNA library in accordance with protocols MaxPlax™ Packaging Extract (Epicenter, US) and XL1-Blue as the host.

Screening of clone recombinant of β-Glucanasegenes

Recombinant Phage Screening was performed on the activity of the enzymes β-Glucanase (a specific enzyme against *Candida albican* biofilm) using Congo Red method and laminarin substrate. Recombinant phage with positive activity of β-glucanase will show the halo around the phage recombinant (plaque). Recombinant phage with positive halo converted to plasmid (pTriplEx2) with SMART cDNA Library Construction Kid User Manual method. The host used was *E. coli* with a BM 25.8 strain. The result of the conversion process was then performed recombinant plasmid isolation. The plasmid isolation process follows the GeneJET Plasmid Miniprep Kit method (Fermentas, Lithuania). PCR DNA insert was performed based on the GoTaq Green Master Mix (Promega, US) product protocol. Amplification was carried out by pre-denaturation program 94°C for 5 minutes, then PCR process was done with denaturation 94°C for 30 s, anealing 58°C for 60 s, extension 72°C for 30 s and post PCR at temperature 72°C for 7 minute with 35 cycles. The PCR product was purified following the Qiaquick PCR Purification Kit (Qiagen, Germany) procedure.Restriction analysis of DNA insert using HindIII enzyme based on GoTaq Green Master Mix (Promega) procedure. The treated DNA insert was then electrophoresed at 1.1% agarose, 110 V for 30 min. DNA inserts with electrophoretic bands from different restriction results were estimated to have different sequence sequences.

Result and Discusion

Total RNA was isolated from digestive gland *Achatinafulica*(crop and intestinal). Cardoso *et al* (2012) reported that the crop and intestinal *Achatinafulica*as glycoside hydrolase reservoir. The RNA isolation process followed the PureZOL RNA Isolation Reagent Bio-Rad procedure.The isolated RNA was tested with electrophoresis using a 1.1% agarose gel at a potential difference of 110 V for 30 min. The results of RNA isolation electrophoresis from digestive gland *Achatinafulica* showed a long smear more than 0.5 kb. This data smear shows that the RNA obtained in varying sizes (Figure 2).The RNA purity test was performed by measuring the absorbance ratio using nanodrop at A_{260} and A_{280} (A_{260}/A_{280}) wavelengths as an indicator of the level of protein contaminants. The RNA purity test was also based on the ratio of A_{260}/ A_{230} as an indicator of polysaccharide contaminants, phenols and chaotropic salts. The A_{260}/A_{280} ratio of RNA obtained was 1.95 with the purity criteria when the A_{260}/A_{280} ratio was in the range 2.0 + 0.1. This ratio value shows without protein contaminants. The OD_{260} / OD_{230} ratio is 1.69 with the purity criteria when the A_{260}/A_{230} ratio is in the range 2.0-2.4. The value of this ratio indicates that there were still contaminants of proteins, polysaccharides, phenols and salts. This contaminant may be due to the remainder of the guanidine or β-mercaptoetanol buffer carried over during the RNA isolation process. As a comparison was the measurement of A ratio by using the same isolation kit (PureZOL RNA Isolation Reagent) [14].

Table 1. Ratio of Absorbance result of RNA isolation.

No.	Absorbance ratio	França *et al.* (2011)	*Achatina fulica*
1.	A_{260}/A_{280}	1,70	1,95
2.	A_{260}/A_{230}	0,63	1,69

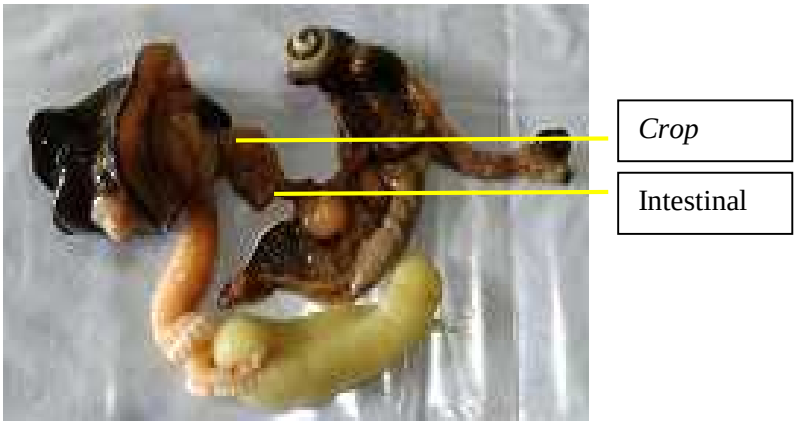

Figure 1. Anatomy of *Achatina fulica*.

Synthesis of cDNA with RT-PCR using Long-Distance Polymerase Chain Reaction (LD-PCR) method was used to produce double strand cDNA fragments after the first strand synthesis of cDNA from reverse transcriptation mRNA. LD-PCR was an ideal method for research with the initial material is total RNA and the number of samples was limited. Stages in RT-PCR reverse transcription of mRNA to obtain cDNA. cDNA was then molded to the PCR process. In RT-PCR techniques require reverse transcriptase enzymes (DNA polymerase) that can synthesize a complementary cDNA with mRNA. At SMART™ cDNA Library Construction Kit used mesophilic viral reverse transcriptase (RTase) by moloney murine leu virus (MMLV) under the name SMARTScribe MMLV Reverse Transcriptase. MMLV was highly processive and capable of synthesizing cDNA up to 10 kb. The RTase enzyme MMLV has an RNase H activity that will cause RNA degradation in RNA-cDNA hybrids. Thus it can be believed that the RT-PCR product with the SMART™ cDNA Library Construction Kit is a cDNA.Electrophoresis results of the LD-PCR RNA samples from digestive glands *Achatina fulica* produced a long smear, distributed from 250 bp to 1300 bp, and visible a clear band and two obscure bands. All three bands are located at 1100 bp, 900 bp and 700 bp (data not shown).

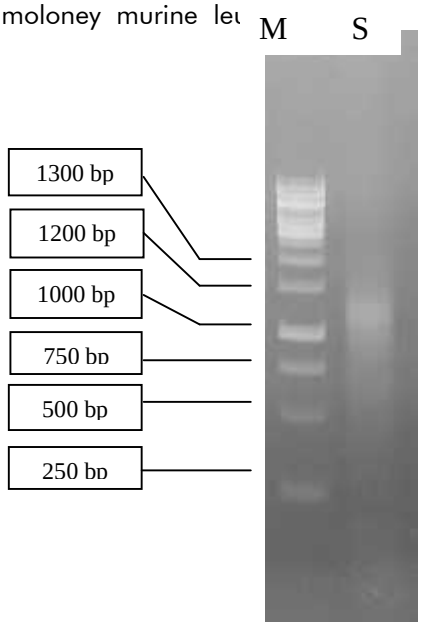

Figure 2. Total RNA electrophoresis with agarose 1.1%.

Primers used in LD-PCR were CDS III primers and SMART IV Oligonucleotide. Primary CDS III (3 'PCR primer) was a modified primer of oligo (dT), determining the first strand synthesis reaction. SMART IV Oligo serves as an acceptor template. When reverse transcriptase reaches the 5 'end, the enzyme transferase activity adds some additional nucleotides, especially deoxycytidine. SMART IV oligo has a sequence of oligo G at the 3 'end in pairs with deoxycytidine, resulting in an extension of the template. SMART IV oligo then serves as a SMART anchor on the next LD-PCR amplification process. Only a cDNA strand that has an SMART anchor can act as a template and experience exponential amplification. Incomplete cDNA and cDNA transcribed from poly A RNA without SMART anchor will not be amplified. DNA genomic and nonpolyadenilate RNA contamination is also eliminated. This selective amplification allows to make cDNA libraries of the sample by the number of nanograms, either total RNA or poly A RNA with a high percentage of clones. The result of LD PCR with SMART cDNA library construction kit is a cDNA derived from eukaryotic organisms.The digestion process with SfiI restriction enzyme on cDNA in 5 minutes, 10 minutes and 2 hours (based on the SMART cDNA Library Construction Kit manual) shows the same band size (Figure 3). The same band size digestion process with SfiI restriction anzyme indicates that SfiI did not intersect the internal cDNA samples. SfiI intersects the cDNA extension area that was outside the target cDNA region. SfiI did not bypass the internal cDNA, it means that the cDNA was obtained intact so that it has a great chance to get the whole gene.

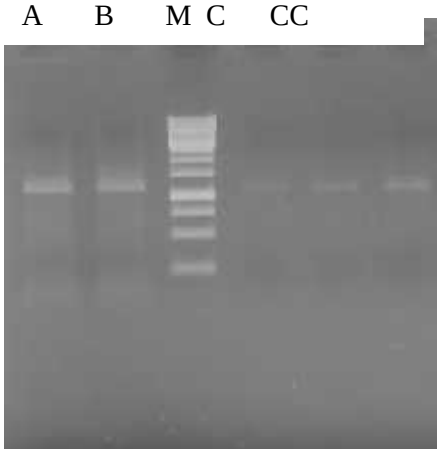

Figure 3. Electrophoresis of optimization time of cDNA digestive gland restriction of *Achatina fulica* with Sfi I. A. Samples of restriction for 5 min; B. Retrieve samples for 10 minutes; M. Marker, C. Samples of retreat for 2 hours.

Fragments of cDNA smaller than 500 bp will be eliminated in the fractionation process using CHROMA SPIN-400 columns to prevent libraries with very short insert size or non-recombinant clones. The electrophoresis profiles of the digestive gland fractionation cDNA digestive gland of *Achatina fulica* were shown in Figure 4. The cDNA was separated into 16 fractions and tested by fractionation using gel electrophoresis 1.1% at a potential difference of 100 V for 15 min.Profile of fractional cDNA visible bands on fractions 8 to 16 which has a band size of 1100 bp (Figure 5). The three fractions with the largest cDNA size were then selected and then combined. The largest cDNA size was chosen because it was thought that the cDNA was not degraded so it is expected to be expressed during the screening process. The fractionation result turns out that the fraction of 8-16 shows the same cDNA size, so that the fractions of 8-16 are subsequently recombined for cDNA ligation process in the lambda vector.There are 3 cDNA / vector comparisons to obtain the best cDNA libraries, ie cDNA: vectors with a ratio of 2:3 (L1); 1:1 (L2); and 3:2 (L3). Ligation efficiency calculation results with various cDNA comparisons: vectors are presented in the following table (Table 1).

Table 2. Data of efficiency ligation.

No	Initials	cDNA : Vektor	Plak (Pfu/ml)
1.	L1	3 : 2	$9 . 10^7$
2.	L2	1 : 1	$15 . 10^7$
3.	L3	2 : 3	$28 . 10^7$

Results on Lambda Vectors the cDNA and vector ratios of ligation reactions were important factors that determine the efficiency of the transformation and determine the number of independent clones of libraries. The optimal ratio of cDNAs and vectors in ligation reactions should be determined empirically for cDNA /

vector combinations. Optimal ligation was obtained in the ligation process with a cDNA ratio: the vector is 2: 3 with the number of libraries obtained is 28. 10^7. This number more than the minimum standard was above 1. 10^6.

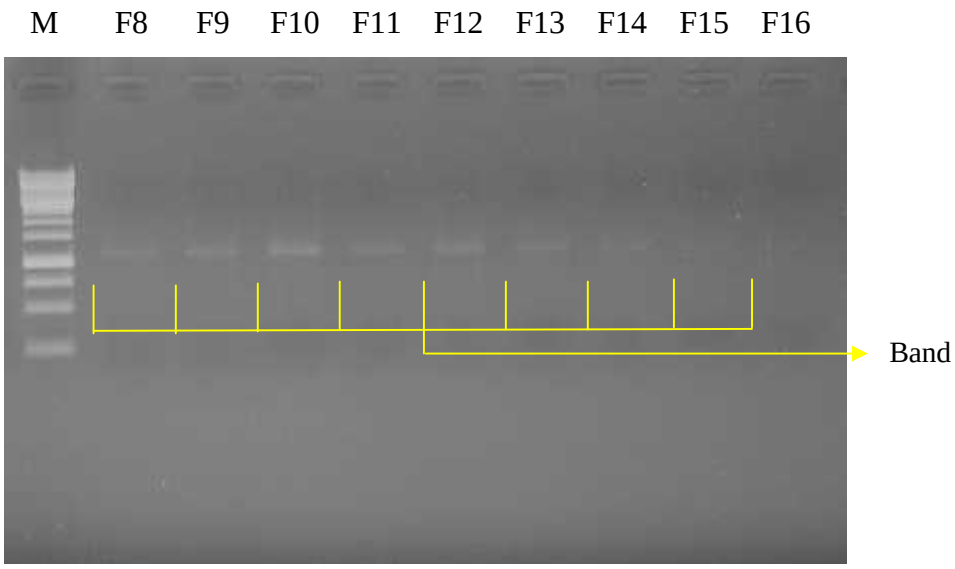

Figure 4.Electrophoresis profile of cDNA digestive gland *Achatina fulica.* M: Marker; F8: Fraksi 8; F9: Fraksi 9; F10: Fraksi 10; F11: Fraksi 11; F12: Fraksi 12; F13: Fraksi 13; F14: Fraksi 14; F15: Fraksi 15; dan F16: fraksi 16

Lambda lisat packaging libraries with 2:3 ligation ratios are further amplified by 1: 1.000.000 dilution. The plaque titering result was 1.1. 10^{10} pfu / ml. These results indicate the successful construction of cDNA libraries from digestive glands of *Achatina fulica* (Figure 5).

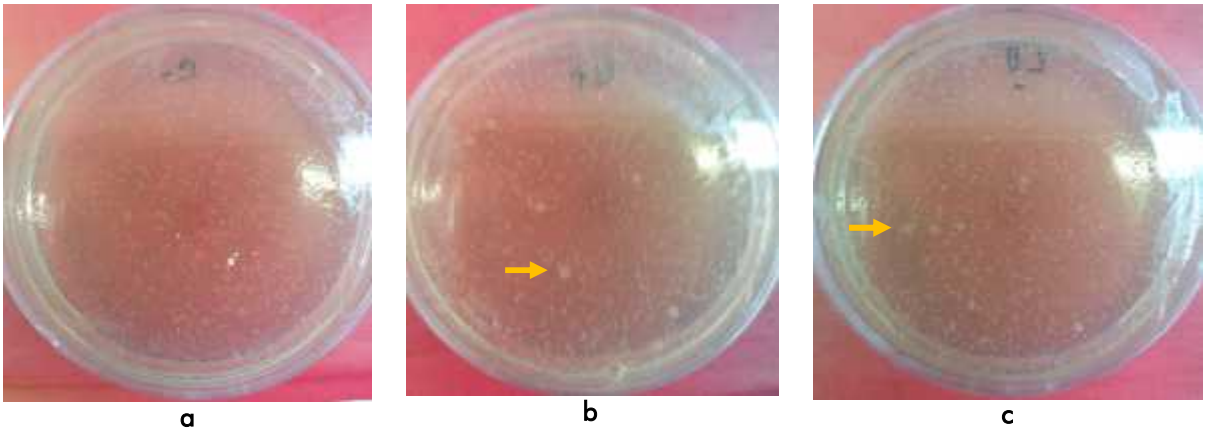

Figure 5. Recombinant clones, further: a. Negative control (*E. coli*); b. Positive control (recombinant plaque); c. Recombinant plaque of cDNA ligation:vector = 3:2 (: plaque)

Sreening of recombinant plaque of β-Glucanase encoding gene was done using Congo Red staining method with laminarin substrate. Recombinant plaques that express glucanase especially 1,3-β-Glucanase or laminarinase will show a white *halo* around the recombinant plaque. The results of screening of recombinant plaque with laminarin substrate are showed Figure 6.

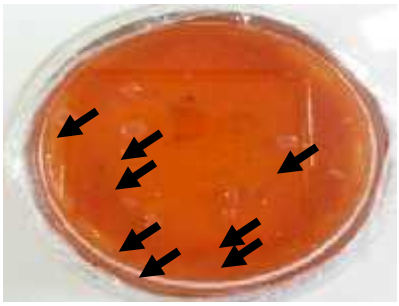

Figure 6. Eukaryotic metagenomic expression library of digestive gland of *Achatinafulica*; (→: recombinant plagues)

Recombinant plaques with positive expression and*hallo* around the plaques were isolated and then converted from lambda phage to phagemid in vivo. The eight randomly selected clones are clone 1, clone 3, clone 6, clone 8, clone 10, clone 11, clone 14 and clone 17.

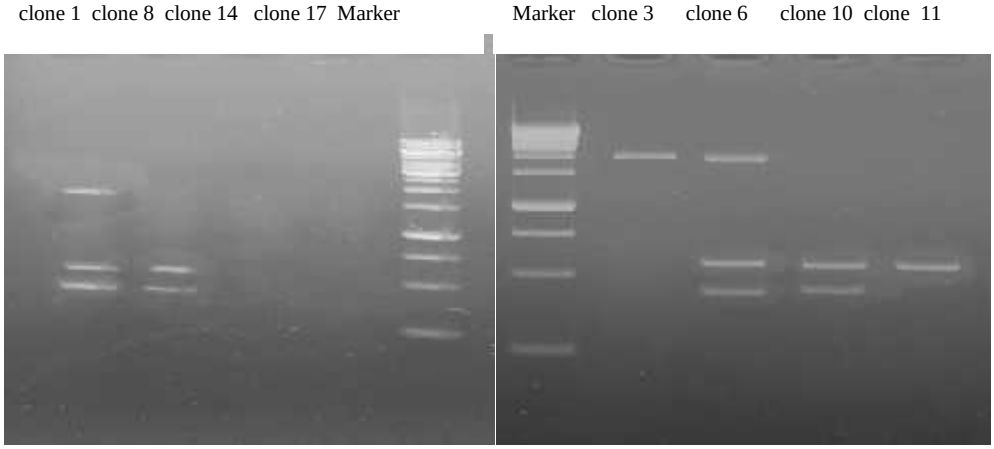

Figure 7. Restriction analysis of DNA insert clone 1, 3, 6, 8, 10, 11, 14 dan 17.

The phagemid circularization occurs when the recombinant phage is tranduced in host bacteria *E. coli* 25.8. This process was called Cre-recombinase. In the Cre-recombinase process, a lisogenic host was required because it did not undergo lysis at the transduction process to be recombinant phagemid clones. The recombinant plasmid isolation process was continued restriction analysis. Restriction analysis with HindIII on cloned DNA insert as shown in Figure 7. Clone 1 and clone 6 wereshowing the same 3 bands from the restriction analysis. Clone 8 and clone 10 were also predicted the same DNA insert. Clone 3 and clone 11 produce 1 band only but at different sizes. Clone 14 and 17 did not see any band. Perhaps clone 14 and 17 havelow concentrations of DNA amplicon.

Conclusion

Metagenomic cDNA cloning technique was carried out to constract the metagenomic expression library of digestive gland of *Achatina fulica*. The expression library have 28.10^7 plaques and $1.1\ 10^{10}$ library amplification titer. Positive screening from the expression library with laminarin substrate are seventeen *halo* plaques. Eight recombinant plasmids were randomly picked and analyse using restriction method showing different fragment. The work demonstrates successfully cloned using metagenomics expression library and identification of novel β-glucanase in discovering potential novel biocatalists.

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