Faculty of Resource Science and Technology

Extraction and Characterization of Cellulase Enzyme from *Teredinidae* Marine Wood Borer

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Bachelor of Science with Honours
(Resource Biotechnology)
2018

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This project is submitted in partial fulfilment of the requirements for the

Degree of Bachelor Science with Honours

(Resource Biotechnology)

Faculty of Resource Science and Technology

University Malaysia Sarawak

2018

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ACKNOWLEDGEMENT

Bismillahirrahmanirrahim. First and foremost, thanks to Allah the Almighty for His blessing and give me the strength and good health that enable me to complete this project. I would like to submit my heartiest gratitude to my respected supervisor, Associate Prof. Dr Mohd Hasnain Md Hussain for his sincere guidance and advice throughout this project. I also would like to extend my deepest thanks to my co-supervisor, Associate Prof. Dr Khairul Adha A. Rahim, lab assistants, postgraduate student of Proteomic Laboratory; Nur Ezzati Hamdin, Students of Aquatic Programme; Fauzan and Gabriel, and the villagers of Kg. Asajaya Laut for their help throughout the sampling process.

Besides, I also would like to express my special thanks to all the postgraduate students in the Proteomic Laboratory especially Nikson Chong for the invaluable guidance, advice and encouragement which really help me a lot in completing this project. I'm also deeply indebted to the postgraduate students in Molecular Genetic Laboratory, Genetic Engineering Laboratory, Biochemistry Laboratory, Microbiology Laboratory, Microbiology Laboratory 2, and Virology Laboratory for their guidance and allow me to use the chemicals and instruments in order to complete this project.

I humbly extend my thanks to all my laboratory mates and concerned friends who give their support either directly or indirectly throughout this project. Last but not least, I revere the patronage and moral support extended with love, by my beloved parents, Mohd Nazri Bin Othman and Noor Hayati Binti Zakaria, and all my family members whose passionate encouragement and endless support made it possible for me to complete this project.

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LIST OF ABBREVIATIONS

°C	degree Calcius
kDa	kilodaltons
bp	base pairs
mL	mililiter
µL	microliter
rpm	revolutions per minutes
M	Molarity
U	Units
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CMC	carboxymethylcellulose
CBB	Coomassive Brilliant Blue
DNS	dinitrosalicyclic acid
DTT	Dithiothreitol
EtBr	Ethidium Bromide
EL	External Laboratory
FPA	Filter Paper Activity
FPU	Filter Paper Unit

GS	Glucose Standard
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulfonyl Fluoride
RC	Reducing Carbohydrates
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBE	Tris-Borate Electrophoresis
TCA	Trichloroacetic Acid
TEMED	Tetramethylenediamine

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Extraction and Characterization of Cellulase Enzyme from *Teredinidae* Marine Wood Borers

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Abstract

Teredinidae or called as shipworms is one of the biological diversity that live in the marine environment. This mollusks enters submerged timbers when it is very small and grows rapidly inside the wood. The shipworms utilize wood as their nutritional source. This wood fragments scraped from the anterior end of the tunnel and enter digestive tract. The activity of the cellulase enzyme from the *Teredinidae* need to be determined in understanding the digestive activity of this marine wood borer. This study were initiated to extract the cellulase enzyme from the intact tissue of the *Teredinidae* and characterized their cellulase activity in digesting the woods. Besides, the isolation and determining the availability of the cellulolytic bacteria of the *Teredinidae* marine wood borer were done in better understanding of the mechanism of wood digestion in marine wood borer. An approach by using the CMC agar plate and Congo red staining were carried out in order to isolate the cellulolytic bacteria from the shipworms. Besides, the Bradford Assay and SDS-PAGE analysis showed that the protein extracted from the guts of the shipworms are more concentrated compared to the protein extracted from the gills of the shipworms. Furthermore, three cellulolytic bacteria were successfully isolated from gills of the shipworms and the amplified 16S rRNA gene of those cellulolytic bacteria produced the PCR reaction product of 1500 bp. The sequencing results that were alignment with BLAST showed that those cellulolytic bacteria were *Streptomyces sp.*, *Azospirillium sp.* and *Cellulosimicrobium sp.* The enzymes extracted from the *Streptomyces sp.*, gills and guts of the shipworms also showed the cellulase activity when tested with Filter Paper Activity and Native-PAGE. The molecular weight of the cellulase extracted from both gills and guts of shipworms were determined approximately 48 kDa by using non-denaturing SDS-PAGE.

Keywords: *Teredinidae*, cellulase enzyme, cellulolytic bacteria, Filter Paper Activity, Native-PAGE

Abstrak

Teredinidae atau dikenali sebagai temilok merupakan salah satu kepelbagaian biologi yang hidup di persekitaran laut. Pada usia yang sangat kecil, moluska ini memasuki kayu yang terendam dan membesar dengan cepat di dalam kayu. Temilok menggunakan kayu sebagai sumber nutrisi mereka. Serpihan kayu ini dikikis dari hujung anterior terowong dan seterusnya memasuki saluran pencernaan. Oleh itu, aktiviti enzim selulase dari temilok perlu ditentukan dalam usaha memahami proses pencernaan temilok. Kajian ini dilakukan bermula dengan usaha untuk mengekstrak enzim selulase dari temilok dan kemudian menentukan ciri-ciri aktiviti enzim selulase dalam proses pencernaan kayu. Selain itu, proses untuk mendapatkan bakteria yang menghasilkan enzim selulase daripada temilok dan menetukan ciri-ciri enzim selulase dalam bakteria tersubut juga dilakukan bagi memberikan pemahaman yang lebih baik mengenai proses pencernaan kayu dalam temilok. Pendekatan dengan menggunakan kaedah plat agar yang mengandungi CMC telah dijalankan dalam menentukan aktiviti enzim selulase daripada bakteria selulosa dalam temilok. Selain itu, ujian Bradford dan SDS-PAGE yang dilakukan telah menunjukkan bahawa unsur protein yang diekstrak daripada usus temilok lebih tinggi berbanding dengan protein yang telah diekstrak daripada insang temilok. Tambahan lagi, tiga bakteria selulosa telah berjaya diasingkan dari insang temilok dan amplikasi gen rRNA 16S bakteria selulosa tersebut telah menghasilkan saiz kira-kira 1500 bp. Keputusan penjujukan melalui BLAST menunjukkan bakteria selulosa tersebut ialah Streptomyces sp., Azospirillium sp. dan Cellulosimicrobium sp. Enzim-enzim yang telah diekstrak dari Streptomyces sp., insang dan usus temilok juga menunjukkan aktiviti enzim selulase apabila diuji dengan Aktiviti Kertas Turas dan Native-PAGE. Berat molekul selulase yang telah diekstrak dari kedua-dua insang dan usus temilok telah ditentukan iaitu kira-kira 48 kDa melalui kaedah 'non-denaturing-SDS-PAGE'.

Kata Kunci: temilok, enzim selulase, bakteria selulosa, Aktiviti Kertas Turas, Native-PAGE

CHAPTER 1

Introduction

The wood and woody plant materials that available in the marine environment were contributed to the coastal erosion riparian transport and human activities. Up to 50% of the dry mass of this materials is made of cellulose and this is potentially rich of carbon and energy source for the marine organisms (Xu & Distel, 2004). One of the marine organisms are marine-wood boring bivalve called shipworms from the family *Teredinidae*. *Teredinidae* are capable of normal growth and reproduction with wood as their sole source of energy (Gallager *et al.*, 1981).

The shipworms are ecologically and economically important which acts as the principle agents in mineralizing wood in marine environments (Didziulis, 2011). Besides, it also costly nuisance species which cause the extensive damages to wooden piers, vessels, fishing equipment and other human structures (Distel, 2003). The mechanisms by which the shipworms digest wood are remain largely unknown. It has been argued that the symbiotic microorganisms in the digestive system are the sole source of cellulolytic enzymes in cellulose consuming animals such as ruminants. However, it have been challenge by the discovery of the nuclear encoded cellulase gene in termite, which is one of the cellulose consuming animals (Watanabe & Tokuda, 2001). Nonetheless, anatomical considerations suggest that the cellulose mechanisms in the shipworms differ from those observed in terrestrial cellulose consumers such as termites, wood-eating roaches and ruminants (Distel, 2003).

The unique characteristics and digestive tract structure of the *Teredinidae* makes the scientists wants to investigate the mechanism and activity of cellulase enzymes in the process of wood digestion by the shipworms until they can used the components from the wood as their sources of food.

This study was initiated to isolate the cellulolytic bacteria from gills and guts. Then, the cellulase enzyme extracted from those bacteria will compared with the crude cellulase enzymes extracted from the gills and guts of the shipworms through the Filter Paper Activity and Non-denaturing SDS-PAGE. The molecular weight of the cellulase enzymes extracted were determined by the Native-PAGE.

The hypothesis of this study were that there are cellulolytic bacteria in the *Teredinidae* marine wood borer that help them in digestion of the cellulose components of wood and make it as their sole sources of food and those cellulase extracted from the shipworms was solely from those cellulolytic bacteria.

The objectives of this study were to:

1) Extract the cellulase enzymes from the *Teredinidae* marine wood borers.
2) Isolate the cellulolytic bacteria from the *Teredinidae* marine wood borer
3) Determine the cellulase activity from the crude cellulase extracted from the *Teredinidae* and the symbiotic bacteria in *Teredinidae*.

CHAPTER 2

LITERATURE REVIEW

2.1 Marine Wood Borer

Marine wood borer or termites of the sea such as shipworms, gribbles and pill bugs are the ecological interest that are responsible for wood deterioration in marine water ecosystem. Marine wood borers have been classified into three groups of taxa which are the Crustacean, Amphipod and Bivalvia. The taxa of the Bivalvia consists of the family of *Teredinidae* (used in this study) and the *Pholadidae*. Fourteen genera of the *Teredinidae* are known but only two are commonly found in Malaysian marine and brackish water which are *Teredo* and *Bankia* (Singh & Sasekumar, 1994). The invertebrates of the marine borer will burrow into and damage the wood that are exposed to the marine environment. Deterioration of wood that are exposed to the sea are faster and more severe than those exposed to the atmospheric environment. Besides, the warm tropical water are more effective to the marine borers than the temperate waters (Roszaini & Salmiah, 2015).

2.2 Family of *Teredinidae*

The *Teredinidae*, commonly called as shipworms is a marine wood-boring bivalves that are responsible in damaging the wooden structures that found in estuarine and marine habitats worldwide (Sipe *et al.,* 2000). Teredinids are classified as an obligate woodborers that are utilize the wood both as a means of shelter and as a source of nutrient (Distel, 2003). This mollusc can damage the marine wooden structures such as ships, piers and boats. The shipworms used it valves which is also called as shells to break down the wood and create the burrow and result in a tunnel with a calcareous lining. As they burrow, they ingest and digest

the excavated wood particles with the aid of enzymes produced by heterotrophic bacterial endosymbiosis located in specialized cells in the shipworm's gills (Distel *et al.*, 2017).

The physical appearance of shipworms is soft and wormlike body but not categorized as worm. It has shells but not used as protection like the other animal such as snails and turtle, but its shell are used for the drilling and boring the woods. According to Michael (1961), parts of its body have become greatly modified in adaptation to its peculiar mode of life and it is differs greatly in appearance from such familiar bivalves as clams and oysters although shipworms is a bivalve mollusc. The edges of its shells are equipped with rows of teeth that make them efficient for boring the woods. The figure of the shipworms can be referred in the Appendix A.

2.3 Structure and Composition of Wood

Woods consists of three main components which are strong polymer chains called cellulose that will aligned to form micro fibrils, hemicellulose and lignin. The cellulose that occur in wood has crystalline regions and amorphous components. The hemicelluloses also involved in amorphous content, meanwhile the role of pectin which is complex polysaccharide is to cross the link of the other components of the cell wall and stabilise them (Malyon, 2011).

Cellulose is the most important chemical entity in wood and classified among the high polymers (Anderson, 1958). Glucose is the monomer form of the cellulose and it occur as anhydroglucose. Meanwhile, the hemicellulose contain different types of monomers which gives the complexity and non-uniformity of the wood. The lignin made the greater portion of the wood by surrounding the fibers and cements together. Thus, it gives strength to the walls of the fibers (Anderson, 1958).

2.4 Cellulase Enzyme

Cellulase is an enzyme made up of protein that are capable to break down the cellulose molecules into the monosaccharide (simple sugars). Cellulase is categorized as the hydrolysis enzyme that are produced mainly by fungi, bacteria, protozoans and termites. There are also cellulase produced by other types of organisms such as plants, molluscs and animals with the help of symbiotic bacteria in the ruminating chambers of herbivore. However, the endogenous genes have also be found in many invertebrates such as nematodes, insects and molluscs (Watanabe & Tokuda, 2001). These findings contradict previously held notions that cellulose can only be degraded by the microorganisms.

There are three main types of enzymes found in cellulase system that can degrade the crystalline cellulose which are endo-1-ß-D-glucanase, exocellobiohydrolase and ß-glucosidase (Wood & Bhat, 1988). These three components have different mode activity. The endo-1-ß-D-glucanase are randomly scission of cellulose chains yielding glucose and cello-oligo saccharides. The exocellobiohydrolase attack on the non-reducing end of cellulase with the cellobiose as the primary structure and the ß-glucosidase hydrolyse the cellobiose to the glucose (Food And Agriculture Organization, n.d.). The cellulase are widely used in various of the industries such as pulp and paper industry, textile industry, food processing industry and bioethanol industry (Kuhad *et al.*, 2011).

2.5 Host-Symbiotic Interaction in Cellulose Consuming Animals

The symbiosis between bacteria and the host of the mostly cellulose consuming animals are considered as mutualism. There are two important enzymes that the bacteria produce for the host which are cellulose enzymes and the nitrogenase enzyme (Microbe Wiki, 2012). Cellulase enzyme are important in degrading the cellulose into sugar. The cellulose is the primary carbohydrate that are found in wood. Meanwhile the nitrogenase are responsible in converting the nitrogen into ammonia. This nitrogen fixation is a usable source of nitrogen for both the bacteria and shipworms.

According to Distel (2003), many terrestrial cellulotrophic animals such as insects and ruminants have the symbiotic microbial populations that have the ability to degrade the cellulose. These symbiotic microorganism were observed in the digestive tract of the cellulose consuming animals. These microbes have been shown to aid the host in digestion process of the cellulose.

2.6 Cellulolytic Bacteria of Teredinidae Marine Wood Borers

The cellulolytic bacteria was defined as the ability of the bacteria to hydrolyse the cellulose. Therefore, this type of the bacteria secretes the cellulase enzyme and made it capable to hydrolyse the cellulose and form reducing sugars or glucose. According to Sipe *et al.* (2000), cellulolytic nitrogen-fixing bacteria reside in the distinct structures of the gills provide the host with the necessary enzymes for survival on a diet of wood cellulose.

The bacteria that were reported lived in the shipworm is a gamma-proteobacterium which categorized as gram-negative rod with single polar flagellum that are used for mobility. This organism can be found in fresh water streams. It can grows optimally at 30-35°C. This