UNIVERSITI PUTRA MALAYSIA

DEGRADATION OF SHRIMP WASTE USING CHITINASE PRODUCED BY TRICHODERMA VIRENS UKM1 THROUGH MEDIA SELECTION

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By

TEOH LAY SIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master

2008

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEGRADATION OF SHRIMP WASTE USING CHITINASE PRODUCED BY TRICHODERMA VIRENS UKM1 THROUGH MEDIA SELECTION

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TEOH LAY SIN

12th September 2007

Chairman: **Associate Professor Suraini Abd Aziz, PhD**

Faculty: **Biotechnology and Bimolecular Sciences**

The current increase in the amount of seafood wastes produced by the shrimp industry has led to the finding of new methods for shrimp waste disposal or its recycle. Chitinase-producing fungi is investigated to be used as a biocontrol agents in particular their potential for waste degradation of shrimps and crab shells from fishing industry. Locally isolated *Trichoderma virens* UKM1 expressing chitinases enzyme was used in this study. Three types of media were selected namely Medium 4, Medium 5 and *Trichoderm*a minimal medium (TMM) for the degradation study. The substrate used was colloidal chitin as control substrate, ground and unground sun-dried shrimp shell wastes. Scanning Electron Microscopy (SEM) studies showed penetration of fungus mycelium into the colloidal chitin as compared to ground and unground sun-dried shrimp shells.

Trichoderma virens UKM1 in TMM produced the less biomass and highest chitinase enzyme and in Medium 4 the protein concentration is the highest if compared with other media used. The results obtained suggested that colloidal chitin was the best carbon source for modelling the degradation of chitin materials. Stereo microscope studies showed that the mechanism of chitinous materials degradation by the chitinase enzyme is via sequential degradation. Shrimp wastes were further analysed for end products in the crude media using High Performance Liquid Chromatography (HPLC). Results showed that the *Trichoderma virens* UKM1 secretes a significant amount of exochitinase compared to endochitinase by the identification of monomeric *N*- acetyl-glucosamine (NAG) from the chitinous substrate. *Trichoderma virens* UKM1 produced more NAG using TMM with colloidal chitin as the substrate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN KE ATAS PENGURAIAN SISA PEMBUANGAN KULIT UDANG OLEH KITINASE DARIPADA TRICHODERMA VIRENS UKM1 MELALUI PEMILIHAN MEDIA

Oleh

TEOH LAY SIN

12hb September 2007

Pengerusi: **Profesor Madya Suraini Abd Aziz, PhD**

Fakulti: **Bioteknologi dan Sains Biomolekul**

Dewasa ini, peningkatan pembuangan sisa makanan laut industri udang membawa kepada kajian untuk mencari kaedah baru bagi pelupusan dan penggunaan semula sisa kulit udang. Oleh itu, penghasilan enzim kitinase oleh fungi telah dikaji secara meluas sebagai agen kawalan biologi. Selain daripada itu, enzim kitinase berupaya dan berpotensi tinggi untuk penggunaan sisa kitin dalam pengguraian kulit udang dan kulit ketam di dalam industri perikanan. Pencilan tempatan *Trichoderma virens* UKM1 tempatan digunakan dalam kajian ini. Tiga jenis media telah dipilih iaitu Medium 4, Medium 5 dan *Trichoderma* Medium Minimal (TMM) untuk kajian penguraian kitin. Substrat yang digunakan ialah kitin koloidal sebagai substrat kawalan, kulit udang yang dikeringkan dengan menggunakan cahaya matahari (dikisar atau tidak dikisar). Analisa

Imbasan Elektron Mikroskop (SEM) menunjukkan penetrasi misellium fungi ke dalam kitin koloidal berbanding dengan kulit udang kering (dikisar atau tidak dikisar). *Trichoderma virens* UKM1 juga menghasilkan biosisa yang sedikit and kepekatan enzim kitinase yang paling tinggi jika dibandingkan dengan media lain. Malahan, fungus dalam Medium 4 menunjukan kepekatan protin yang paling tinggi. Keputusan yang diperolehi mencadangkan kitin kolloidal adalah substrat terbaik untuk digunakan dalam permodelan proses degradasi bahan-bahan kitin. Kajian menggunakan stereo mikroskop menunjukkan fungi mendegradasi bahan-bahan kitin secara lapisan demi lapisan, di mana ia ditunjukkan oleh penipisan lapisan kitin. Sisa kulit udang seterusnya dianalisa menggunakan HPLC untuk mengkaji kehadiran produk akhir dalam media asal. Hasil menunjukkan *Trichoderma virens* UKM1 merembes lebih banyak enzim eksokitinase berbanding dengan enzim endokitinase melalui petanda monomerik *N*-asetil-glukosamin (NAG) daripada substrat kitinos. *Trichoderma virens* UKM1 menghasilkan lebih banyak NAG di dalam media TMM dengan kitin koloidal sebagai substrat.

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I certify that an Examination Committee has met on 17th October 2006 to conduct the final examination of Teoh Lay Sin on her Master of Science thesis entitled "Degradation Studies of Shrimp Waste During Chitinase Enzyme Production Using *Trichoderma virens* UKM1 Through Media Selection" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the degree of Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisor Committee were as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that is has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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TEOH LAY SIN

Date: 10th December 2007

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LIST OF ABBREVIATIONS

GlcNAc	*N*-acetyl-D-glucosamine
NAG	*N*-acetylglucosamine
TMM	*Trichoderma* minimal medium
pH	Hydrogen potential
%	Percentage
dp	degree of polymerization
NH_2	Amonnium
Pb^{2+}	Lead ions
Cu^{2+}	Copper ions
Cd^{2+}	Cadmium ions
g	gram
kg	kilogram
PDA	Potato Dextrose Agar
^oC	Celsius
L	Liter
$NaNO_3$	Sodium nitrate
K_2HPO_4	Potassium hydrogen phosphate
KCL	Potassium chloride
$MgSO_4.7H_2O$	Magnesium sulfate 7-hydrate
$FeSO_4$	Iron sulfate
Min	Minute

KH_2PO_4	Potassium di-hydrogen phosphate
$CaCl_2.6H_2O$	Calcium chloride-6 anhydrous
HCL	hydrochloric acid
ml	Milliliter
NaOH	Sodium hydroxide
No.	Number
sp.	Species
SEM	Scanning Electron Microscope
Na_2CO_3	Sodium carbonate
$CuSO_4.5H_2O$	Copper 5-hydrate sulfate
nm	Nanometer
BSA	Bovine Serum Albumin
DNS	3,5-Dinitrosalicylic acid
w/v	weight/ volume
M	Molar
rpm	Revolution per minute
µmol	Micromole
HPLC	High Performance Liquid Chromatography

CHAPTER 1

INTRODUCTION

The advancement in biotechnology especially in the area of genetic, protein engineering, developments in bioinformatics and the availability of sequence data, had opened a new era in enzyme applications in many industrial processes (Dahiya *et al.,* 2006). Chitin, derived from the Greek word 'envelop', was discovered in 1811 in mushrooms by Professor Henri Braconnot. It is a linear β 1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc) and constitutes more than half of the total organic matter in chitinous structures. It is the second most abundant natural biopolymer derived from exoskeletons of crustaceans and also from cell walls of fungi and insects. Crustaceans, mollusks, insects and fungi produce about 100 billion tones of chitin every year. Chitin produced in enormous amounts in the biosphere, chitin wastes are cheaper and can be easily recovered from the seafood industry (Wang *et al.,* 2006). Its annual production and steady state amount is in the order of 10^{10} to 10^{11} tons and is degraded by most species alive today as their structural component (Gooday, 1990 and 1990a). Some pre-treated chitin is used as a substrate for microbial chitinase production (Wang *et al.,* 1995, 1997; Wang and Chang, 1997). Chemically, chitin is linear polysaccharides consisting of *N*-acetyl-D-glucosamine and D-glucosamine units which are present in different ratios in the polymers. Glucosamine is a natural amine sugar extracted from the

chitin in the sea shrimp and crab shells. In combination with chondroitin sulphate, it can build blocks for cartilage, up regulate chondrocytes and reduce the extent of cartilage degradation. Because of its therapeutic effect in osteoarthritis, glucosamine can be used as foods supplement (Punín *et al.,* 2006).

Chitinase (EC 3.2.11.14) is enzyme capable of hydrolyzing insoluble chitin to its oligo and monomeric components, like *N*-acetyl-D-glucosamine. In numerous bacteria, fungi, insects, plants and animals, chitinase are involved in natural protection mechanism (Yuli *et al.*, 2004). In nature, this enzyme is widely distributed and plays an important role in the degradation of chitin, for example degradation of fungal cell walls (Inbar and Chet, 1991). Chitinases are involved in many stages of fungal development, including production and germination of spores, hyphal elongation and ramification (Souza *et al.*, 2003). Chitinases are used effectively as a biocontrol agent against many phytopathogenic fungi for fungal protoplasts generation. Chitinases are effectively used in the bioconversion of shellfish to single cell protein and other value added products, and thus acts as an effective method in the disposal of chitinous waste (Felse and Panda, 1999). Chitin-degrading enzymes (chitinases) can be classified into two major categories which are endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.29) (Dahiya *et. al.,* 2006).

Trichoderma sp. is one of the most widely used organism for the production of chitinases in many studies. This fungus can produced large amounts of chitinases that is active over a wide range of environmental conditions. *Trichoderma* sp. chitinase is also

active against a wide range of phytopathogens (Tronsmo and Harman, 1992). However, *Trichoderma harzianum* may produce seven individual chitinases: two *N*-acetyl-glucosaminidases (102 and 73 kDa) (Haran *et. al.,* 1995), four endochitinases (52, 42, 33 and 31 kDa) (Harman *et. al.,* 1993; Haran *et. al.,* 1995 and De La Cruz *et. al.,* 1992) and one chitobiosidase (40 kDa) (Harman *et. al.,* 1993). It should be emphasized that chitinases of *Trichoderma harzianum* are substantially more active and effective against a wide range of fungal than chitinolytic enzymes from plants and other microorganisms (Li, 2006). Felse and Panda (2000) reported that two kinds of *Trichoderma* used in the production of chitinases in the bioreactor studies which will produced the highest enzyme activity if compared with other microbial used and Haran *et. al.* (1993) also reported that transformed *Trichoderma harzianum* will increased constitutive chitinase activity.

Chitinous fungi always produce chitinases during exponential growth. Examples include *Mucor* (Humphreys and Gooday 1984c; Gooday *et al.,* 1986 and Rast *et al.,* 1991), *Neurospora crassa* and *Candida albicans*. As well as soluble chitinases activities in zygomycetes there also membrane-bound chitinases requiring phospholipids for activity and having properties in common with chitin synthase activities (Humphreys and Gooday 1984c). Possible roles for these soluble and membrane-bound chitinases were studied by Gooday *et al.,* 1986 and Rast *et al.,* 1991. That include the maturation of chitin microfibrils, apical growth, branching, spore germination and cell separation in yeasts. From the study, all chitin-containing organisms have the ability to produce

chitinolytic enzymes. In some cases, such as in arthropod moulting, its role is obvious (Gooday, 1995).

The morphology is an important observation parameter during the growth of fungi. Depending on the culture conditions and the genotype of the strain, the growth form of filamentous fungi in submerged culture varies between two extremes, the pelleted and the filamentous, each form having its own characteristics, which can effect the process productivities by influencing the mass transfer rates (Papagianni *et al.*, 1999).

The important of this project are to discover the chitinolytic enzyme which can use for environmental friendly and produced the products which from the degradation of the shrimp shell wastes. The objectives of this research are:

1) To study the morphological of the degradation of the shrimp shells by *Trichoderma virens* UKM1
2) To study the physiological characteristics of the degradation of the shrimp shells by *Trichoderma virens* UKM1
3) To study the degradation of shrimp waste into NAG during chitinase enzyme production using High Performance Liquid Chromatography (HPLC) analysis

CHAPTER 2

LITERATURE REVIEW

2.1 Chitin and Chitosan

2.1.1 Chemistry/ Molecular Structure

Chitin is the most abundant natural biopolymer derived from exoskeletons of crustaceans (crab and shrimp) and also from cell walls of many fungi and insects. It is a cationic amino polysaccharide, essentially composed of *N*-acetyl-D-glucosamine (GlcNAc) residues linked β 1,4-. Chitin is the most under exploited biomass resource available on earth. Approximately 75% of the total weight of the shellfish, such as shrimp, crabs and krill, is considered waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Dahiya *et al.,* 2006). The occurrence of chitin in various organisms is given in Table 2.1 (Tharanathan and Kittur, 2003).