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UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF CHITINASE BY *TRICHODERMA VIRENS* UKM1 FROM COLLOIDAL CHITIN AND SHRIMP WASTE

CHRISTINE CHERYL FERNANDEZ

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By

CHRISTINE CHERYL FERNANDEZ

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

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For Allah the Almighty

and for my parents…

for this gift called LIFE…

For my dearest jaan…

the reason for the multitude of colours in my LIFE…

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

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CHRISTINE CHERYL FERNANDEZ
(MARIAM AISHA FATIMA)

October 2007

Chairman: Associate Professor Suraini Abdul Aziz, PhD

Institute: Institute of Bioscience

Shrimp waste being the main waste from marine industry is a source of surface pollution in coastal areas consisting of mainly protein, calcium carbonate and chitin. Chitin, the second most abundant biopolymer is a β-(1,4)-linked N-acetyl-D-glucosamine (GluNac) heterogeneous polymer that has versatile biological and agrochemical applications. Chitinase a glycosyl hydrolase is produced constitutively as isozymes in fungus for *de novo* chitin metabolism. Chitin chains are converted into chitooligosaccharides and GluNac reducing sugars by chitinase with specific modes of action at the reducing ends. In this study, shrimp waste was pretreated with chemical and physicochemical methods to determine the best pretreatment before fermentation with a locally isolated fungus, *Trichoderma virens* UKM1. Experiments in shake flasks and 2 L stirred tank reactor (STR) demonstrated sun dried ground shrimp waste as the best pretreatment, 1 x 10^6 spores/mL as the best total spore concentration and fermentation pH control at pH 6.0 as the most effective for chitinase production. Subsequent optimisation in 2 L STR showed that fermentation at 200 rpm and 0.33 vvm gave the highest chitinase productivity of 4.1 U/L/h and 5.97 U/L/h, respectively. Microbial chitin bioconversion employing optimal

conditions in medium with colloidal chitin and medium with sun dried ground shrimp waste as the sole carbon source showed an increase of 7.25 fold and 1.57 fold in chitinase activity, respectively from shake flasks culture to 2 L STR. The respiration rate (Qo_2X) during the highest chitinase productivity was 3.864 mg of DO g^{-1} of fungal biomass h^{-1} while the specific respiration rate (Qo_2) was 20.337 mg of DO g^{-1} of fungal biomass h^{-1} and the maximum specific growth rate, μ_{max} was 0.0078 h^{-1} with the corresponding doubling time, t_d of 88.85 hours. Concentration and partial purification of crude chitinase showed that ammonium sulphate precipitation at 80% saturation gave highest chitinase activity in line with the results of enzymatic chitin bioconversion from DNS chitinase assay and HPLC analysis.

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

PENGHASILAN KITINASE OLEH *TRICHODERMA VIRENS* UKM1 DARIPADA KITIN KOLOID DAN SISA UDANG

Oleh

CHRISTINE CHERYL FERNANDEZ
(MARIAM AISHA FATIMA)

Oktober 2007

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

Institut: **Institut Biosains**

Sisa udang ialah sisa utama dari industri marin yang merupakan punca pencemaran permukaan di kawasan persisiran pantai. Ia terdiri daripada sebahagian besarnya protein, kalsium karbonat dan kitin. Kitin, biopolimer kedua terbanyak terdiri daripada polimer heterogenus N-asetil-glukosamin (GluNac) dengan β-(1,4) ikatan glikosidik yang mempunyai ciri-ciri biologi dan kegunaan serbaguna agrokimia. Kitinase merupakan glikosil hidrolase yang dihasilkan secara konstitutif sebagai isozim oleh kulat untuk metabolime *de novo* kitin. Rantai kitin ditukar kepada gula penurun kito-oligosakarida dan GluNac oleh kitinase melalui mekanisme spesifik di hujung penurun rantai tersebut. Dalam kajian ini, sisa udang telah dirawat terlebih dahulu dengan kaedah kimia dan fisiokimia untuk mengenal pasti prarawatan yang terbaik sebelum fermentasi dengan kulat pencilan tempatan iaitu *Trichoderma virens* UKM1. Eksperimen di dalam kelalang goncangan dan 2 L reaktor tangki pengaduk (STR) menunjukkan bahawa sisa udang kisar yang dikeringkan di bawah cahaya matahari merupakan prarawatan yang terbaik. Kepekatan spora keseluruhan terbaik adalah 1 x 10^6 spora/mL dan fermentasi dengan pH terkawal pada pH 6.0 adalah paling efektif untuk penghasilan kitinase. Pengoptimuman di dalam 2 L STR

menunjukkan fermentasi pada 200 psm dan 0.33 vvm memberikan hasil kitinase tertinggi iaitu masing-masing sebanyak 4.1 U/L/h dan 5.97 U/L/h. Biopenukaran kitin oleh mikrob menggunakan keadaan optimum untuk medium dengan kitin koloid dan sisa udang kisar yang dikeringkan di bawah cahaya matahari sebagai punca karbon tunggal menunjukkan peningkatan aktiviti kitinase masing-masing sebanyak 7.25 ganda dan 1.57 ganda daripada fermentasi kelalang goncangan ke 2 L STR. Kadar respirasi (Qo_2X) semasa penghasilan kitinase tertinggi ialah 3.864 mg DO g^{-1} biomas kulat jam^{-1} manakala kadar respirasi spesifik (Qo_2) ialah 20.337 mg DO g^{-1} biomas kulat jam^{-1}. Kadar pertumbuhan spesifik maksimum, μ_{max} ialah 0.0078 jam^{-1} dengan masa penggandaan, t_d selama 88.85 jam. Pemekatan dan penulenan separa campuran kitinase menunjukkan bahawa pemendakkan amonium sulfat dengan 80% ketepuan menghasilkan aktiviti kitinase tertinggi bersamaan dengan keputusan analisis DNS dan HPLC biopenukaran kitin secara berenzim.

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In the name of Allah, the Most Gracious, the Most Merciful

"Take time to work, it is the price of success
Take time to think, it is the source of power
Take time to read, it is the fountain of wisdom
Take time to pray, it is the foundation of everything"

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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.

Members of the Supervisory Committee were as follows:

Suraini Abdul Aziz, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd. Ali Hassan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Madihah Salleh, PhD
Lecturer
Faculty of Science
Universiti Teknologi Malaysia
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 February 2008

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 it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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CHRISTINE CHERYL FERNANDEZ

Date: 5 December 2007

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LIST OF ABBREVIATIONS

AG	NaOH treated ground shrimp waste
AUG	NaOH treated unground shrimp waste
B	Baffle width
BSA	Bovine serum albumin
CC	Colloidal chitin
CCRBB	Colloidal chitin treated with Remazol Brilliant Blue dye
C_E	Saturated dissolved oxygen concentration
C_L	Actual dissolved oxygen concentration
C_o	Initial dissolved oxygen concentration
D_i	Impeller diameter
DNS	Dinitrosalicyclic acid
DO	Dissolved oxygen
DOT	Dissolved oxygen transfer
D_t	Vessel diameter
EG	Cellobiase treated ground shrimp waste
EUG	Cellobiase treated unground shrimp waste
Glu	Glucosamine
GluNac	N-acetyl-D-glucosamine
h	Hour
H^+	Hydrogen ion
H_i	Impeller height from sparger
H_L	Liquid height
HPLC	High pressure liquid chromatography
k_La	Volumetric mass transfer coefficient
M4	Optimised medium 4
M4CCpH	Optimised medium 4 with colloidal chitin with pH 6.0 control
M4SDGpH	Optimised medium 4 with sun dried ground shrimp waste with pH 6.0 control
M5	Medium 5 or optimised medium 4 without peptone and yeast extract
M5CCpH	Medium 5 with colloidal chitin with pH 6.0 control
M5SDGpH	Medium 5 with sun dried ground shrimp waste with pH 6.0 control

N	Impeller speed in seconds
NAG	N-acetyl-D-glucosamine
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate or respiration rate
PDA	Potato dextrose agar
Qo_2	Oxygen uptake rate or respiration rate
Qo_2X	Specific oxygen uptake rate or specific respiration rate
rpm	Revolutions per minute
RSG	Raw ground shrimp waste
RSM	Response surface methodology
RSUG	raw unground shrimp waste
S	Impeller spacing
SDG	Sun dried ground shrimp waste
SDUG	Sun dried unground shrimp waste
sf	Shake flask
sp.	Species (singular)
spp.	Species (plural)
STR	Stirred tank reactor or stirred tank bioreactor
t_L	Time corresponding to C_L
t_o	Initial time
U	Unit of enzyme activity
UDP	Uridino di-phospho
v/v	Volume per volume
Vtip	Impeller tip speed
vvm	Volume of air per minute per volume of solution
w/v	Weight per volume
W_i	Impeller height

CHAPTER 1

INTRODUCTION

1.1 Introduction

Shrimps have been a popular raw material for the burgeoning marine and food industry contributing to increasing marine waste. Shrimp waste which is rich in organic compounds is an abundant source of chitin, a natural polymer of N-acetyl-D-glucosamine (GluNac), a reducing sugar. Essentially, shrimp waste constitutes 45 – 60% of the whole shrimp in the form of the head and body carapace and only 25% is recovered as meat (Sachindra and Mahendrakar, 2005 and Coward-Kelly *et al.*, 2006). More importantly Tharanathan and Kittur, (2003) cited that of the organic weight of shrimp cuticle 69.5% on average is chitin.

Chitin and chitinolytic materials are abundant renewable natural resources obtained from marine invertebrates, insects, fungi, yeast and algae. Chitin occurs in nature as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in cell walls of fungi. Although 22 to 44% of fungal cell wall comprises of chitin, its amount in terms of chitin production is negligible in comparison to marine sources (Patil *et al.*, 2000). It is abundantly derived mainly from crustacean waste, the shrimp and crab (Rinaudo, 2006). Almost 10% of the global landings of aquatic products consist of organisms rich in chitinous material (10-55% on dry weight basis). These include shrimps, crabs, squids, oysters, and cuttlefish. It was estimated that the worldwide recovery of chitin from the processing of marine invertebrates alone was 37, 300 tonnes in 1991 (Shaikh and Deshpande,

1993). Approximately 75% of the total weight of shellfish are considered waste. Out of this, 20 – 58% of the dry weight are chitin (Dahiya *et al.*, 2006). Chitin is a polymer of unbranched chains of β-l,4-linked sugar (N-acetyl-D-glucosamine) residues, whereas chitosan, the deacetylated form of chitin, contains glucosamine residues. In fact, chitin is the second most abundant natural biopolymer in the world, behind only cellulose. It is also the most abundant naturally occurring polysaccharide that contains amino sugars. This abundance, combined with the specific chemistry, bioversatility and biocompatibility of chitin and its next best derivative chitosan, make for the array of its potential applications. Owing to its abundant and cheap resource and biocompatibility, chitin has the potential for bioconversion to simpler molecules of N-acetyl-D-glucosamine monomers and chitooligosaccharides by means of enzyme catalyzed reactions or chemical procedures with the ease in production coming from the former (Kumar, 2000, Tharanathan and Kittur, 2003, Rinaudo, 2006).

In Malaysia, aquaculture industry has been one of the emerging industries promoted by the government. Shrimps and prawns are alone harvested to an astounding total of 99, 377 tonnes locally in 2003 (FAOSTAT, 2005). Recent statistical database showed that the import quantity for crustaceans in Malaysia for 2004 alone was 368, 800 tonnes (FAOSTAT, 2006). One of the main issues that need to be resolved is the by-products or waste generated by the shrimp industry. Normally, the shrimp waste would be discarded as mere kitchen waste or some lucrative industries would employ it for conversion to chitosan and chitin through chemical means which involved heavy usage of acid and alkaline in the chemical treatment, creating additional environmental issues. Due to the annual mass volume of shrimps and prawns harvest,

it is only feasible to utilise the waste that is derived from the industry to address environmental issues and to produce industrial viable products using low cost substrates via environmentally friendly processes.

Preliminary work has been done in 2004 on aquaculture waste (especially shrimp waste) processing enzymes, mainly on chitinases in order to develop an environmentally-friendly system for converting shrimp waste into useful industrial specialty chemical products via biotechnological means by shake flask culture using a locally isolated fungus. A number of significant studies have been performed on chitinolytic enzymes from *Trichoderma* spp. especially on *Trichoderma harzianum* in which some seven individual chitinases have been elucidated (De La Cruz *et al.*, 1992 and Gokul *et al.*, 2000, Duo-Chuan, 2006). All the studies reported that chitinase production in fungal batch fermentation was carried out in laboratory scale shaker flask and their potential in shellfish waste biodegradation was modestly studied. From most of the bioreactor studies, an investigation utilised shrimp waste as a supplementary carbon source in a rich medium for chitinase production from *Verticillium lecanni* and another attempted *Trichoderma harzianum* as their fungus of choice with chitin flakes as the chitinase inducer in a defined salt medium for chitinase production in a 1 L stirred tank reactor (Felse and Panda, 2000b, Liu *et al.*, 2003).

Therefore, the main objective of this research is to increase the production of chitinase by *Trichoderma virens* UKM1, a locally isolated fungus in a 2 L stirred tank reactor (STR) from colloidal chitin and shrimp waste using the optimised conditions previously obtained in prior preliminary studies. At the same time to

identify the different methods of shrimp waste pretreatments that are the best for producing chitinolytic enzymes from *Trichoderma virens* UKM1. After obtaining the optimal parameters from the 2 L STR, further microbial and enzymatic shrimp waste bioconversion shall be expounded with colloidal chitin as the reference substrate. This is to study the concentration of end products of shrimp waste bioconversion which are GluNac, reducing sugars and proteins that may be extrapolated to conclude the significance of this entire study.

Thus, the objectives of this study are as follows:

1. To determine the production of chitinase by *Trichoderma virens* UKM1 using various pretreatments of shrimp waste.
2. To optimise the 2 L stirred tank reactor variables for chitinase production by *Trichoderma virens* UKM1 from colloidal chitin as reference substrate.
3. To compare the microbial and enzymatic chitin bioconversion of colloidal chitin and pretreated shrimp waste.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Rapid increase in the world population has led to the search for alternative forms of protein sources. Consumers being more educated and health conscious prefer organic sources of protein in the forms of seafood rather than the more recent forms being offered via biotechnology in single cell proteins, which spurred minimal interest. Entrepreneurs have seen much potential in the burgeoning marine industry to fulfil this nascent demand (Zeller and Pauly, 2005). Apart from fishes, crustaceans and molluscs are the major raw materials for the marine industry. Shrimps and prawns being one of the more popular of these are alone harvested to an astounding total of 99, 377 tonnes locally in 2003 (FAOSTAT, 2005). Recent statistical database showed that the import quantity for crustaceans in Malaysia for 2004 alone was 368, 800 tonnes (FAOSTAT, 2006).

2.1.1 Shrimp Waste

Shrimps come in a myriad of varieties according to its origins from the different continents. Generally, in the biological hierarchy they come under the phylum arthropoda, class crustacea, and subclass malacostrae, however, they differ in their order henceforth according to its fishing origins (Dore and Frimodt, 1987).