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PRODUCTION OF CHITINASE BY A LOCALLY ISOLATED FUNGUS

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By

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SPECIALLY DEDICATED TO:

MY SUPERVISOR AND CO-SUPERVISOR

MY WIFE AND MY DAUGHTER

MY BELOVED PARENTS

AND FAMILY MEMBERS

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

PRODUCTION OF CHITINASE BY A LOCALLY ISOLATED FUNGUS

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Chitin and chitinolytic enzymes are gaining importance for their biotechnological applications. Particularly, chitinases which have been widely used in agriculture to control plant pathogens. Chitinases and chitooligomers produced by enzymatic hydrolysis of chitin have also been used in human health care products. The success in employing chitinases for different applications depends on the supply of highly active preparations at reasonable cost. In this study, isolation and screening of fungus were carried out. *Trichoderma* sp. was selected due to the ability to produce chitinase enzyme with biggest clear zone formation on Chitinase Detection Agar (CDA) for qualitative confirmation. The slides were viewed under light microscope to determine the isolated fungi morphology. *S*hake flask fermentation using *Trichoderma* sp. was carried out to produce chitinase enzyme. Different types of media were investigated to find the best medium for chitinase enzyme production using colloidal chitin as the reference substrate. It was observed that the highest chitinase activities (0.70 U/mL) were obtained using Kawachi Medium at day 4 of fermentation. Kawachi Medium was selected as the basal medium for induction studies. Several types of inducers that included chitin

colloidal and direct chitin, N-acetylglucosamine, glucosamine hydrochloride and chitosan oligosaccharides were used to investigate the feasibility of different inducers for enhancing chitinase enzyme production. The results indicated that all the tested inducers supported chitinase production by *Trichoderma* sp. It was suggested that the level of chitinase production were regulated or induced by the type of media and inducers used. In addition, it showed that colloidal chitin was the second best inducer after NAG. For economic considerations, colloidal chitin was chosen as the inducer for further study. The effect of different concentration of the inducer selected was studied on chitinase enzyme production. Low colloidal chitin concentration at 0.3% (w/v) gave the highest chitinase activity of 0.72 U/mL. Additionally, raw shrimp waste was also employed to induce chitinase enzyme production. Raw shrimp waste that was subjected to different pre-treatments including sun-dried, acid treated, alkali treated and enzyme treated were used to find cheaper alternative substrates for chitinase enzyme production. Sun dried and ground raw shrimp waste gave the highest chitinase enzyme production. The pretreated raw shrimp waste were also analysed for their composition include protein, moisture and ash content using proximate analysis.

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

PENGHASILAN KITINASE OLEH FUNGUS PENCILAN TEMPATAN

Oleh

ZULKARAMI BERAHIM

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Enzim kitin dan enzim kitinolitik menjadi semakin penting bagi penggunaannya dalam aplikasi bioteknologi. Enzim kitinase digunakan meluas di dalam bidang pertanian untuk mengawal patogen tumbuhan. Kitinase dan kitooligomer yang dihasilkan daripada hidrolisis kitin juga digunakan dalam produk penjagaan kesihatan manusia. Kejayaan menggunakan kitinase untuk pelbagai kegunaan bergantung kepada bekalan bahan dan harga yang berpatutan. Di dalam kajian ini, fungus telah diasingkan dan dipemencilkan *Trichoderma* sp. telah dipilih berdasarkan keupayaannya membentuk zon jernih yang besar pada 'Chitinase Detection Agar' (CDA) bagi pengesahan secara kualitatif. Penghasilan enzim kitinase dengan menggunakan fungus *Trichoderma* sp. dilakukan melalui kaedah fermentasi kelalang goncang. Pelbagai jenis media telah dikaji untuk mencari media terbaik bagi penghasilan enzim kitinase dengan menggunakan koloidal kitin sebagai substrat rujukan. Daripada pemerhatian, enzim kitinase tertinggi dihasilkan dengan menggunakan Medium Kawachi iaitu 0.70 U/mL pada hari ke-empat fermentasi. Medium Kawachi telah dipilih sebagai media asas untuk kajian induksi. Beberapa jenis bahan induksi yang termasuk ialah kitin, koloidal kitin, N-acetilglukosamin (NAG),

glucosamine hydrochlorik dan kitosan oligosakarida telah digunakan untuk mengenalpasti keupayaan induksi yang berbeza bagi merangsang penghasilan enzim kitinase. Keputusan menunjukkan semua bahan induksi yang dikaji menyumbang kepada penghasilan enzim kitinase oleh *Trichoderma* sp. Ini mencadangkan tahap penghasilan enzim kitinase dikawal atau dirangsang oleh jenis media dan juga induksi yang digunakan. Hasil kajian menunjukan koloidal kitin adalah induksi yang kedua terbaik selepas NAG. Di atas pertimbangan ekonomi, koloidal kitin telah dipilih sebagai bahan induksi untuk kajian seterusnya. Kesan kepekatan induksi yang terpilih seterusnya dikaji untuk penghasilan enzim kitinase. Kepekatan koloidal kitin yang rendah iaitu pada 0.3% (w/v) dapat menghasilkan aktiviti kitinase yang tertinggi sebanyak 0.72 U/mL. Seterusnya, hasil buangan udang mentah juga digunakan untuk merangsang penghasilan enzim kitinase. Hasil buangan udang mentah diberi pelbagai rawatan termasuk pengeringan di bawah cahaya matahari, rawatan dengan asid, rawatan dengan alkali dan rawatan dengan enzim digunakan untuk mencari substrat alternatif yang murah bagi penghasilan enzim kitinase. Bahan buangan udang yang dikeringkan di bawah cahaya matahari serta dikisar memberikan keputusan aktiviti kitinase yang tertinggi. Semua bahan buangan udang yang telah dirawat juga dianalisis untuk kandungan protein, kelembapan dan abu melalui kaedah analisis anggaran.

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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 Supervisory Committee were as follow:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

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ZULKARAMI BERAHIM

Date: 26 March 2008

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CC	Colloidal chitin
GHCL	Glucosamine hydrochloride
CO	Chitosan oligosaccharide
DNS	Dinitrosalicylic acid
CC-RBB	Colloidal chitin stain with Remazol Brilliant Blue
U	Unit of enzyme activity
pH	Hydrogen potential
CDA	Chitinase Detection Agar
PDA	Potato Dextrose Agar
TMM	*Trichoderma* Minimal Medium
$CaCl_2 . 6H_2O$	Calcium chloride hexahydrates
g	Gram
mg/ml	Milligram per milliliter
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	diPotassium hydrogen phosphate
L	Liter
M	Molarity
mg	Milligram
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrates
min	minute
ml	Milliliter
N	Normality
NAG	*N*-acetylglucosamine
$NaNO_3$	Sodium nitrate
Na_2SO_3	Sodium sulfide
rpm	rotation per minute

CHAPTER 1

INTRODUCTION

Chitin, a poly-β-1, 4-*N*-acetylglucosamine, is an abundant renewable natural resource obtained from marine invertebrates, insects, fungi, yeast and algae. Almost 10% of the global landings of aquatic products consist of organism rich in chitinase material (10-55% on dry weight basis). These include shrimps, crabs, squids, oyster, and cuttlefish. More than 80,000 metric tons of chitin is obtained per year from the marine waste. Chitin comprises 22 to 44% of cell wall of fungi. In comparison with marine sources, chitin production from fungal waste is negligible (Subasinghe, 1995). But the mucoraceous fungi, which are known to contain chitosan –a deacetylated form of chitin– can be used for its commercial production. For chitosanases and chitinolytic enzyme, these enzyme systems catalyse the hydrolytic depolymerisation of chitosan (Reetarani *et al.,* 1999).

Chitinase are widely distributed in nature and play an important role in the degradation of chitin. This enzyme is found in microorganisms such as fungi, bacteria and plants. In fungi, chitinase are involved in many stages of its development, including production and germination of spore, hyphal elongation and ramification. It was also found in human serum and protozoa for the protection against fungal infections and penetration of mosquito peritrophic membrane by the malaria parasite, respectively (Souza *et al*., 2003).

Chitinase have received increased attention due to their potential application in biocontrol of other chitin containing phytopathogenic organisms such as insects and other phytopathogenic fungus. In this respect, chitinase producing fungi have been intensively studied as biocontrol agents and the role of chitinase and other hydrolytic enzymes in antagonism has been suggested (Souza *et al*., 2003). In addition to the potential applications as inhibitors and biopesticides, chitinases have been used for the production of single cell protein for animal and aquaculture feed, for the isolation of fungal protoplasts, preparation of bioactive chito-oligosaccharides and phytopathogen inhibition (Patil *et al.,* 2000).

The genus *Trichoderma* currently consist of more than 40 known taxa (Kubicek *et al*., 2001). The investigation on the production of chitinase (EC 3.2.1.14) as lytic enzyme by *Trichoderma* species has been reported by Harman *et al.*, (1992). High level of secretion and diversity of the produced enzymes with various substrate specificities promotes wide application of enzyme preparations from *Trichoderma* sp. in different fields of biotechnology (Markov *et al.*, 2004).

The objectives of this study are:-

1. To screen and isolate chitinase-producing fungi
2. To select the suitable medium for chitinase enzyme production
3. To induce chitinase enzyme production using different types of inducers
4. To produce chitinase enzyme using the best inducer obtained

CHAPTER 2

LITERATURE REVIEW

2.1 Microorganisms

2.1.1 *Trichoderma* sp.

Fungi in genus *Trichoderma* sp. are the most promising biocontrol agents against plant pathogenic fungi. Specific strains have the ability to control a range of pathogens under a variety of environmental conditions. Moreover, they may be rhizosphere competent, which allows them to colonise and protect plant roots. Their biological activity can be increased by genetic manipulation. Among the mechanisms proposed is mycoparasitism. It is presumed that this complex process requires the production of enzyme that digest the fungal cell wall. *Trichoderma* sp. is known to be efficient producers of polysaccharide lyses, proteases and lipases, all of which may be involved in cell wall degradation (Lorito *et al.*, 1993).

Biological control of soil-borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proven harmful to the environment. In recent years several investigators have suggested that chitinase-producing fungi, e.g. species of *Trichoderma* sp., can be an effective biological control agent against fungal pathogens. The main mechanism involved in the antagonism of *Trichoderma* sp.

pathogenic fungi appears to be the release of lytic enzymes, including chitinases (Ulhoa and Peberdy, 1991).

2.2 Chitin

2.2.1 Properties of chitin

Chitin is an example of highly basic polysaccharides. Their unique properties include polyxylate formation, ability to form films, chelate metal ions and optical structural characteristics. Like cellulose, it naturally functions as structural polysaccharide, but differs from cellulose in the properties. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohol in conjugation with aqueous solutions of minerals acid and dimethylacetamide containing 5% lithium chloride. The nitrogen content in chitin varies between 5 to 8% depending on the extent of deacetylation (Yalpani *et al*., 1992).

Acetic anhydride can fully acetylate chitin. Linear aliphatic *N*-acetyl groups above propionyl, permit rapid acetylation of hydroxyl groups. Highly benzoylated chitin is soluble in benzyl alcohol, dimethyl sulfoxide, formic acid and dichloroacetic acid. The *N*-hexanol, *N*-decanoyl and *N*-dodecanoyl derivatives have been obtained in metthanesulfonic acid (Muzarelli, 1973).

According to Yalpani *et al*., (1992), cellulose is a homopolymer, while chitin is heteropolymer. An important parameter to closely examine is the degree of *N*-

acetylation in chitin. The ratio has striking effect on chitin solubility and solution properties. Chitosan, the universally accepted non-toxic *N*-deacetylated derivatives of chitin, where chitin is *N*-deacetylated to such an extent, that is becomes soluble in diluted aqueous acetic and formic acids. The average molecular weight of chitin has been determined by light scattering. Viscometry is a simple and rapid method of determination of molecular weight, and constant α and K in the Mark-Houwink equation have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution.

Both cellulose and chitin are highly crystalline, intractable materials and only a limited number of solvents are known which are applicable as reaction solvents. Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve chitin in an appropriate solvent system to impart functionally. For each solvent system, polymer concentration, pH, counterion concentration and temperature effects on the solution viscosity must be known. The comparative data from solvent to solvent are not available. As a general rule, researchers dissolve the maximum amount of polymer in a given solvent that still retained homogeneity and then regenerated it in the required from. A coagulant is required for polymer regeneration of solidification. The nature of the polymer coagulant is also highly dependent on the solvent and solution properties as well as the polymer used (Muzarelli, 1973).

2.2.2 Chitin structure

Chitin is the most widespread amino polysaccharide in nature and is estimated annually to be produced almost as much as cellulose. It is mainly found in antraphode exoskeletons, fungal cell walls or nematode eggshells. However, derivatives of chitin oligomers have also been implicated as morphogenetic factors in the communications between leguminous plants and rhizobium and even in vertebrates, where they may be important during early stages of embryogenesis (Merzendorfer and Zimoch, 2003). Chitin is composed largely of alternating N-acetylglucosamine residues, which where linked by β-(1-4) glycosidic bonds. Since hydrolysis of chitin by chitinase treatment leads to release of glucosamine in addititon to N- acetylglucosamine, it was concluded that it might be the significant portion of polymer. Chitin polymer tends to form microfibrils (also referred as rod or crystallites) of ~3 nm in diameter that are stabilized by hydrogen bonds formed between the amine and carbonyl groups. Chitin micro fibrils of peritropic matrices may exceed 0.5μm in length and frequently associate in bundles containing parallel groups of 10 or more single microfibrils. X- ray diffraction analysis suggested that chitin is a polymeric substance that occurs in three different crystalline modifications, termed α, β and γ. They mainly differ in the degree of hydration, in size of the unit cell and in the number of chitin chains per unit cell In the α form, all chains exhibit an anti-parallel orientation; in the β form the chains are arranged in a parallel manner: in the γ form sets of two parallel strands alternate with single anti parallel strands (Merzendorfer and Zimoch, 2003).

The anti parallel arrangement of chitin molecules in α form allows tight packaging into chitin microfibrils, consisting ~20 single chitin chains that are stabilized by a high number of hydrogen bonds formed between the molecules. This arrangement may contribute significantly to the physicochemical properties of cuticle such as strength and stability (Merzendorfer and Zimoch, 2003). In contrast, the packaging tightness and numbers of inter-chain hydrogen bonds of the β and γ chains are reduced, resulting an increased in the number of hydrogen bonds with water. The high degree of hydration and reduced packaging tightness resulted in more flexible and soft chitinous structures, as are found in peritropic matrices. Structure of chitin is shown in Figure 2.1.

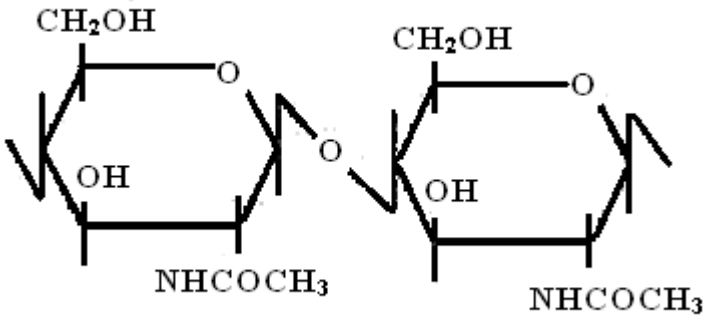

Figure 2.1: Structure of chitin

2.3 Chitosan

Chitosan is a modified carbohydrate polymer derived from the chitin component of the shells of crustacean, such as shrimp, crab and cuttlefish. Chitosan is produced from shrimp waste by a multistage process. First the salt is washed out and the material is shredded. Then the protein, which makes up 30% of the waste, is removed in a hot bath.