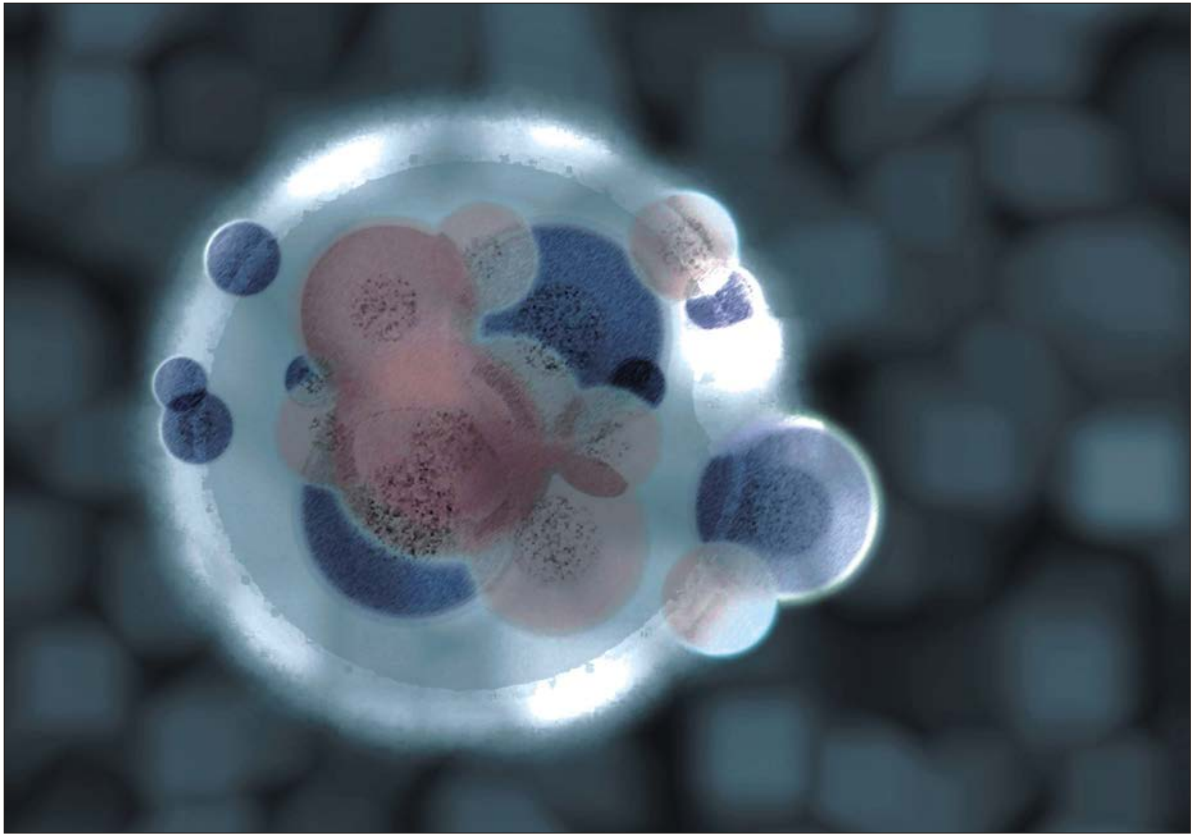

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 8 (1): 52-59, 2008
ISSN 1727-3048
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Microbial Degradation of Chitin Materials by *Trichoderma virens* UKM1

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Abstract: The current increase in amount of seafood wastes produced by the shrimp industry has lead to the finding of new methods for shrimp waste disposal or waste reused. For this respect, chitinase-producing fungi have been extensively studied as biocontrol agents. Locally isolated *Trichoderma viren* UKM1 was used in this study. From preliminary study, commercialized *Trichoderma* Minimal Medium (TMM) was selected for the degradation study. The substrates used were colloidal chitin as control substrate, sun dried ground and unground shrimp shells. Scanning Electron Microscopy (SEM) studies showed penetration of fungus mycelium into the colloidal chitin as compare to sun dried ground and unground. This observation suggested that colloidal chitin was the best carbon source for modeling the degradation of chitin materials. Stereo microscope studies suggested that the fungus removed (degraded) the chitinous materials layer by layer as indicated by the significant reduction in shell thickness. Shrimp shells were further evaluated for end products in the crude medium using High Performance Liquid Chromatography (HPLC). A simple, rapid, selective and specific HPLC method was developed to quantify glucosamine indirectly using the value of total N-acetyl-glucosamine (NAG) produced which the production of chitooligomer was used as marker. 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. The highest specific enzyme activity obtained using colloidal chitin was 14.59 U mg^{-1}. Percentage of residual chitooligomer in impure chitinases samples was 86%.

Key words: Degradation, *Trichoderma virens* UKM1, chitooligomer, chitinase, HPLC

INTRODUCTION

Chitin is a white substance which most abundant natural amino polysaccharide and occurs mainly in the exoskeleton of crustaceans (crabs, lobsters, shrimps, etc.), insects, worms and fungus or mushrooms (Mislovičová *et al*., 2000; Zhu *et al*., 2005). It is a polysaccharide consisting of units N-Acetyl glucosamine and its highly crystalline structure accounts for its poor solubility (Ekblad and Näsholm, 1996). Chitin has chemical structures very similar to that of cellulose. Chitosan is the N-deacetylated derivative of chitin, although this N-deacetylation is almost never complete (Zhu *et al*., 2005). Chitin and chitosan are recommended as suitable functional materials, because these natural polymers have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties etc (Kumar, 2000).

Glucosamine, a natural component of glycoproteins found in connective tissues and gastrointestinal mucosal membranes, has therapeutic potential for the treatment of a variety of diseases, including arthritis, inflammatory bowel disease and general inflammatory damage (Zhu *et al*., 2005). Chitin is hydrolyzed by endochitinases mainly to N-acetylglucosamine oligomers and chitobiose. In addition, exochitinases hydrolyse chitobiose and chitin oligomer to N-acetylglucosamine (NAG) (Studer *et al*., 1992; Ren *et al*., 2000; Wang *et al*., 2001; Suginta *et al*., 2005).

Early on, the limitations of using chromatography were highlighted because of the physical-chemical properties of glucosamine. Although gas chromatography

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is very sensitive and has a high specificity, it involves a derivatisation procedure of the hydrolysis products into volatile components which is time-consuming and requires specialized lab skills. If used with an ultraviolet detector, HPLC also involves the problem of derivatisation. Nevertheless, HPLC combined with Refractive Index (RI) detection the only on-line detection method that is available for general use in chromatography, shows great potential. RI detection is non-selective; registers all substances with an RI that differs from that of the mobile phase. They are generally less sensitive than UV detectors, but can be extremely useful for detecting those compounds that are non-ionic, do not absorb in UV and do not fluorescence (Punín Crespo *et al*., 2006).

The objectives of this study are to investigate the microbial degradation of chitin materials by *T. virens* UKM1 and to develop a simple, rapid and selective and specific method in order to quantify glucosamine indirectly using the value of total monomeric N-acetyl-glucosamine (NAG) produced divided by the total production of chitooligomer.

MATERIALS AND METHODS

Microorganism: The locally isolated fungus, *Trichoderma virens* UKM1 was supplied by research collaborator from Universiti Kebangsaan Malaysia (UKM). The culture was maintained at 4°C on Potato Dextrose Agar (PDA) plate.

Preparation of chitin substrates: Chitin flakes was prepared by milling chitin from shrimp shells. Three different treatment of chitin flakes were carried out, which were colloidal chitin, sun dried ground and unground shrimp shells which is straight forward dried under the sun. Colloidal chitin was prepared by adding 10 g of chitin flakes from shrimp shells into 100 mL of concentrated HCl (37% w/v) and stirred with glass rod for 10 min. The chitin was precipitated by addition of 1 L of deionized water and stirred using magnetic stirrer for an hour. The precipitated chitin was stored in the fridge at 4°C overnight. The pellet was washed with deionised water. NaOH (2 M) was used to adjust the pH to 6 or 6.5 and filtered with No. 4 Whatman paper and the pellet was oven dried at 40-50°C (Gómez Ramírez *et al*., 2004).

Culture conditions: The medium for enzyme production was *Trichoderma* Minimal Medium (TMM) contained 2.0 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $MgSO_4$. $7H_2O$, 0.3 g L^{-1} $CaCl_2.6H_2O$ (Deane *et al*., 1998). This medium was adjusted to pH 5.5 and supplemented with 0.5% (w/v) of chitin substrates. Erlenmeyer flasks (250 mL) containing 100 mL of the medium were inoculated with suspension of spores (1×10^7) and incubated at 30°C on a rotary shaker at 120 rpm. All experiments were done in duplicate.

Morphological studies: The morphology of the fungus was observed under the JEOL JSM 6400 Scanning Electron Microscope (Philip Model XL30 ESEM, Netherlands) and stereo microscope studies by measuring the thickness of the shrimp shells using micrometer (0-25/ 0.01 mm, PEACOCK) during the degradation period.

Chitinolytic enzyme assay: One milliliter (mL) of the enzyme sample and 1 mL of a 10% (w/v) suspension of colloidal chitin, in 0.2 M sodium phosphate buffer (pH 6.5-7), were incubated at 50°C for 1 h. The reaction was stopped by adding 1 mL of 1% NaOH, followed by boiling for 5 min. Samples tubes were then centrifuged at 7000 rpm and the reducing sugars produced were determined in the supernatants. Briefly, 1 mL of supernatant and 1 mL of 1% 3,5-dinitrosalicylic acid (DNS) (dissolved in 30% sodium potassium tartrate in 2 M NaOH) were mixed and incubated for 5 min in a boiling water bath. Thereafter, their absorbance at 535 nm was recorded. The chitinase activity were interpolated in a standard curve prepared with a series of dilutions (0-10 µmol mL^{-1}) of N-acetyl-D-glucosamine (NAG) and DNS. The chitinase activity in U mL^{-1} was defined as the amount of the enzyme required to produce 1 µmol of NAG in 1 h (Gómez Ramírez *et al*., 2004).

Protein determination: Protein concentration was determined using the method of Lowry *et al*. (1951).

HPLC analysis: Twenty micro liter of sample was injected into the HPLC system containing a Jasco PU-1580 Intelligent HPLC pump, a Jasco DG-1580-54 4-line degasser and two detectors connected in series: a Jasco RI-1530 Intelligent RI detector and Jasco UV-1570 Intelligent uv/Vis detector. The HPLC separation was performed on a Purospher® STAR NH_2 column (Merck), using a mixture of acetonitrile and water (70:30) as the mobile phase and was run at a flow rate of 0.8 mL min^{-1} as described by Il'ina and Colleques in 2004. The absorption of the solution was measured using RI detector. The standard used in the HPLC system are chitotriose (trimer), chitobiose (dimmer) and NAG (monomer) for the chitooligomer (Donzelli *et al*., 2003). All experiments were done in duplicate.

RESULTS AND DISCUSSION

SEM analysis: Observation using SEM was executed to visualize effect of *T. virens* UKM1 on shrimp shells. The micrographs of SEM for three different substrates (colloidal chitin, sun dried ground and unground shrimp shells) were shown in Fig. 1a-c, respectively. Fig. 1a

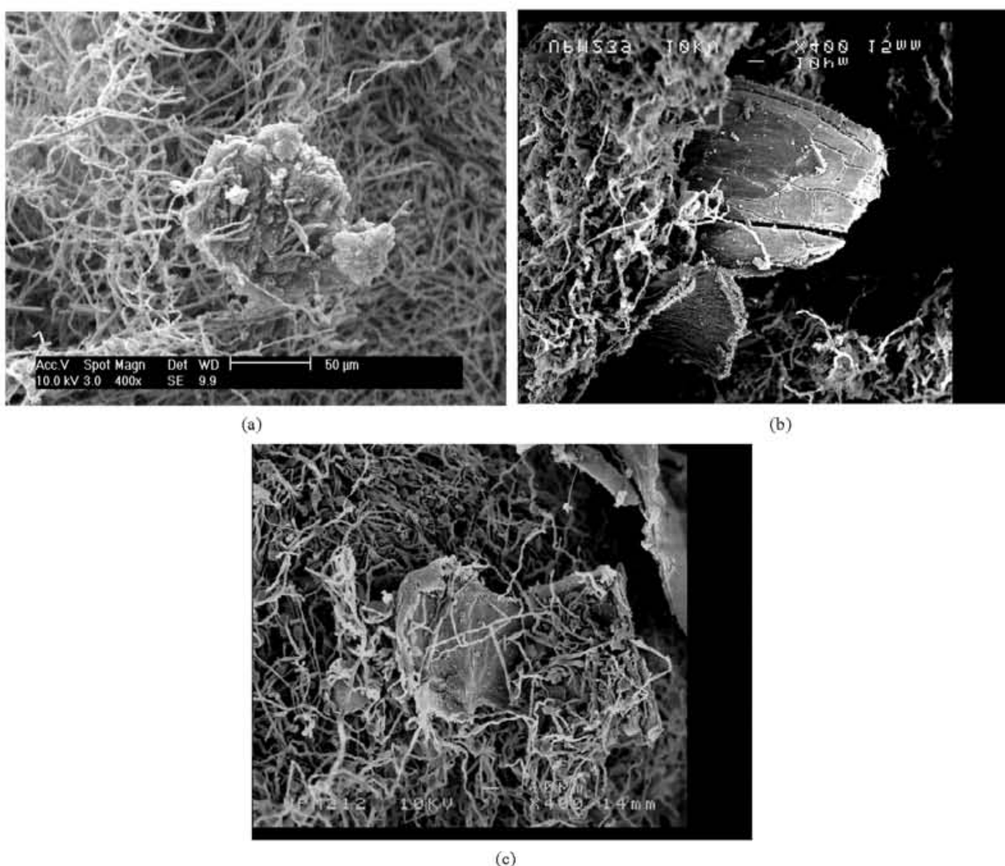

Fig. 1: SEM micrographs showing the growth of *T. virens* UKM1 at day 5, (a) Colloidal chitin; (b) Sun dried ground shrimp shell (c) Sun dried unground shrimp shell

shows that maximum hyphal penetration observed was on day 5. The carbon source used was colloidal chitin which is chitin flakes treated with hydrochloric acid which is responsible in breaking β-1-4 linkages of chitin polysaccharide (Gómez Ramirez *et al*., 2004). Observation for first 5 days of fermentation showed gradual penetration of fungal hyphae into the colloidal chitin with low biomass concentration in comparison to using sun dried ground (Fig. 1b) and unground (Fig. 1c) shrimp shells.

Surface degradation study by stereo microscope: Figure 2 showed the morphology of *T. virens* UKM1 under stereo microscope. The diameter of the shrimp shells was unchanged but became less opaque from day 1 till day 8. Measurement of shrimp shell thickness is important to quantify the extent of degradation imposed by *T. virens* UKM1. The thickness of the shrimp shells decreased consistently from day 1 (0.36 mm) till day 8 (day 8, 0.12 mm).

Anatomy study of shrimp shells shown that the cuticle of shrimp is divided into four layers and an underlying epidermis (Cep). The outer layer (Cu1) is called the epicuticle. The epicuticle lacks chitin but contains varying levels of calcium. It has been shown in arthropods and particularly in some crustaceans, the shrimp shell is subdivided into two additional layers. The next layer (Cu2) is the exocuticle, which contains chitin and calcium. Its distinguishing component was melanin-like pigment. The next layer (Cu3) is the endocuticle and it contains chitin and calcium. It differs from the exoculticle most notable in having a much higher proportion of calcium. The inner-layer (Cu4) is the membranous or uncalcified layer. As its name implies, its major components is not calcium, but chitin (Fig. 3). During the degradation of chitin, the medium's pH become more alkaline due to released of calcium content originated from the shrimp shells (Thomas and Donald, 1988). From the observation, it was shown and suggested that the fungus degrade the shrimp shells layer by layer and not by diameter.

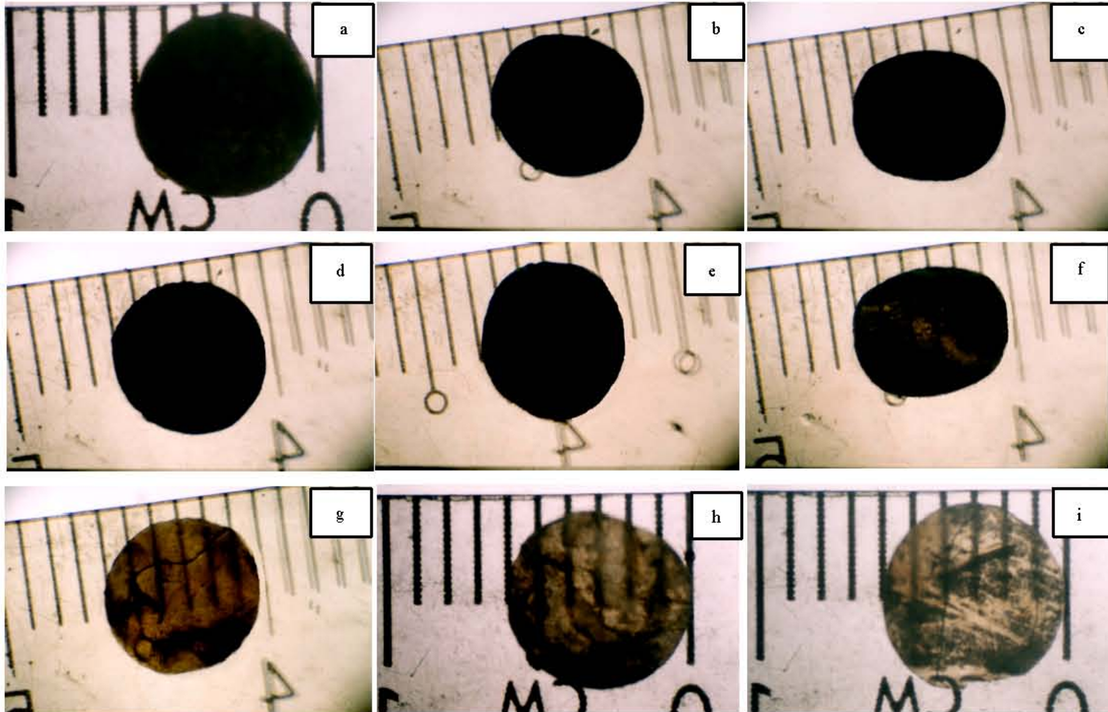

Fig. 2: Photographs of *T. virens* UKM1 observed under stereo microscope, (a)control; (b) day 1; (c) day 2; (d) day 3; (e) day 4; (f) day 5; (g) day 6, (h) day 7 and (i) day 8

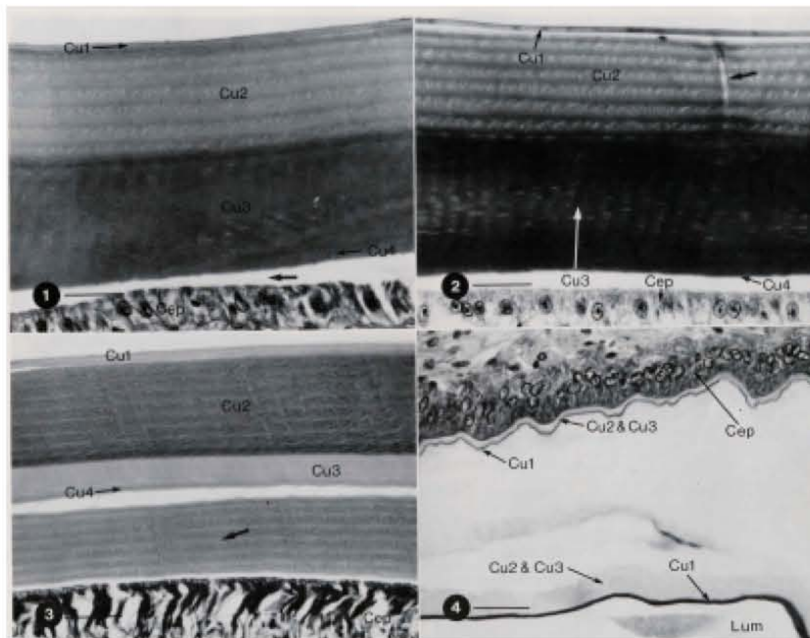

Fig. 3: High magnification of cephalothoracic exoskeletal cuticle taken during intermolt stage (Source: Thomas and Donald, 1988)

Effect on the types of substrates: Chemically defined media are usually preferred in laboratory research since they permit one to determine the specific requirements for growth and product formation by systematically adding or eliminating chemical species from the formation, with minimal complicated medium interactions and reproducible culture condition (Zhang and Greasham, 1999). TMM was the minimal medium for *Trichoderma* sp. that contain chitin as the sole carbon source (Deane *et al*., 1998).

The production of extracellular chitinolytic enzymes was monitored during growth of *T. virens* UKM1 in different chitin substrates medium (Fig. 4). The production of chitinolytic enzymes increased rapidly, with two and three peaks in activity detected at 8 days. Several types of enzyme activities can be involved in the degradation of chitin. Endochitinase cuts at random locations within long chitin chains, producing oligomers of varying length and exochitinase releases the monomer, N-acetylglucosamine from the non-reducing end of the chain (Havukkala *et al*., 1993). The highest specific activity produced by colloidal chitin was 14.59 U mg^{-1}. By using a treatment of acid on chitin which causes the lipid and protein components to be removed from the chitin and this process would enable substrate accessibility for enzyme hydrolysis (Deane *et al*., 1998). Gómez Ramírez *et al*. (2004) have stated that by utilizing hydrochloric acid, the polysaccharide molecules were being induced to separate in the colloidal chitin. This allowed the carbon source from the colloidal chitin to be easily utilized by the fungus.

Wang and Hwang (2001) reported that maximum enzyme chitinase yield (U mL^{-1}) was obtained using colloidal chitin as the substrate for *B. alvei* (1.34 U mL^{-1}), *B. sphaericus* (1.29 U mL^{-1}), *B. cereus* (1.34 U mL^{-1}) and *P. aeruginosa* (0.68 U mL^{-1}). Therefore, from this study *T. virens* UKM1 produced more enzyme activity in comparison to amount reported above. Most bacteria isolates express maximum chitinase concentration/activity on the third day of fermentation, while fungi and actinomycetes express maximum chitinase around the sixth day of fermentation. In all orgaisms, addition of carbon sources other than chitin reduced chitinase production but supported growth. Chitinase is produced as an inducible enzyme using chitin or its degradation products as inducers. In most cases, chitin concentration in the range of 1-1.5% found to be most suitable for chitinase production. Among the carbon sources studied for all organisms, colloidal chitin was found to be the best carbon source (Felse and Panda, 2000). It is known that an ideal substrate concentration in any fermentation process resulted in higher conversion efficiencies and optimum substrate utilization (Madhavan and Ashok, 1996).

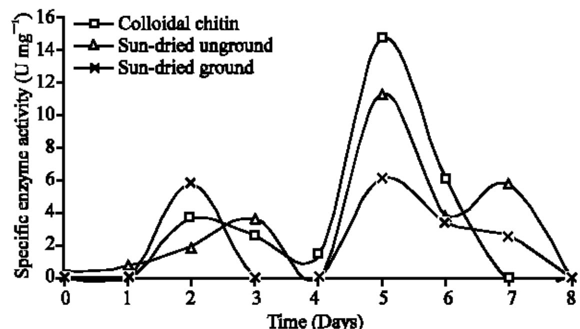

Fig. 4: Production of extracellular chitinolytic activity of *T. virens* UKM1 grown in a medium with three different chitin substrates. The temperature was controlled at 30°C and the pH, initially adjusted to 5.5. Specific activity is expressed as U mg^{-1} of protein

Felse and Panda (2000) reported that *Trichoderma* sp. with colloidal chitin at concentration range to 0.8-1.4 kg m^{-3}, giving maximum chitinase activity after 5 days of fermentation. After optimisation, chitinase activity increased from initial value of 0.054 U to a final value of 0.197 U. In this study, results shown in Fig. 4 indicated that the chitinase activity was 0.136 U. Takayanagi *et al*. (1991) reported that the optimal carbon source for chitinase production by *Bacillus licheniformis* X-7u was by using 0.3% of colloidal chitin. Park *et al*. (1997) used 0.4% colloidal chitin as a major carbon source to enhance the chitinase production of *Enterobacter* sp. Yabuki *et al*. (1986) indicated that 1.0% chitin was the optimal carbon source for chitinase production by *Aeromonas hydrophilia* and that production decreased with an increase chitin concentration. Treated (colloidal) chitin was more efficient than chitin in the form of powder or flakes (crystalline chitin was a less active due its insolubility) (Andronopoulou and Vorgias, 2004).

Analysis of chitooligomer in crude chitinase enzyme: The percentage of glucosamine was obtained by the total NAG produced divided by the total production of chitooligomer at day 8 (last day production) which was measured by HPLC. The hydrolysis products from colloidal chitin were analysed using HPLC by measuring the percentage of residual chitooligomer in crude enzyme obtained. Percentage of residual chitiooligomer in three medium for colloidal chitin (Fig. 5a), sun dried ground (Fig. 5b) and sun dried unground shrimp shells (Fig. 5c) was monitored during the growth of *T. virens* UKM1 for the duration of 8 days. No degradation occurred on day 0, which signifies no NAG production. Henceforth,

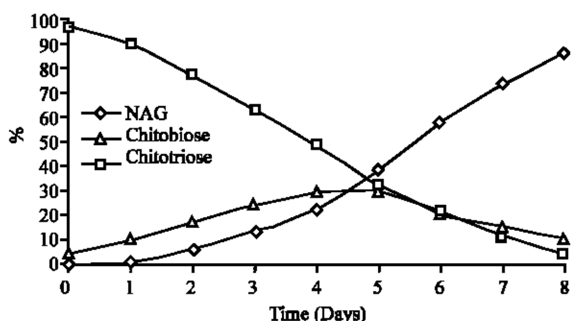

Fig. 5a: Percentage of residual chitooligomer of *T. virens* UKM1 in the medium for colloidal chitin. The temperature was controlled at 30°C and the pH, initially adjusted to 5.5

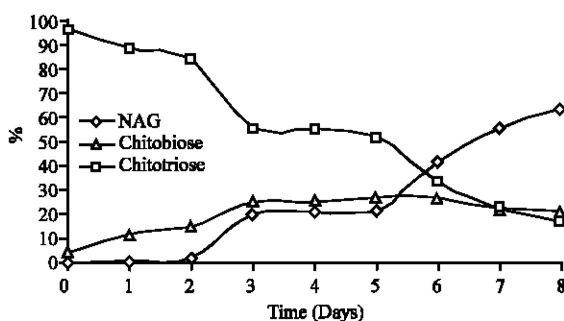

Fig. 5b: Percentage of residual chitooligomer of *T. virens* UKM1 in the medium for sun dried ground shrimp shells. The temperature was controlled at 30°C and the pH, initially adjusted to 5.5

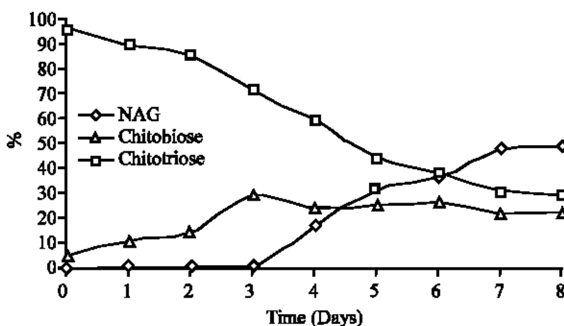

Fig. 5c: Percentage of residual chitooligomer of *T. virens* UKM1 in the medium for sun dried unground shrimp shells. The temperature was controlled at 30°C and the pH, initially adjusted to 5.5

NAG production gradually increased, chitotriose decreased and the process of degradation increased every day till day 8. Chitin sources as the main carbon source was provided in the medium for the fungus to produce NAG. Higher NAG concentration produced during the fermentation indicated better media formulation. Colloidal chitin produced the highest NAG (86%) followed by sun dried ground (63%) and sun dried unground (49%) at day 8.

Deane *et al.* (1998) reported that the hydrolysis products from swollen chitin generated by *T. harzianum* chitinolytic enzymes over a period of time were analyzed using HPLC. The only hydrolysis product detected after HPLC analysis was the monomer of NAG and its release is in accordance to time. Usage of different medium containing different types of substrates (sun dried ground, sun dried unground and colloidal chitin) were attempted for enhancement of chitinase production. It was found that TMM with colloidal chitin produced the highest percentage of residual chitinase in crude enzyme due to simple medium composition. Fungus favors easily degradable carbon source such as colloidal chitin as compared to the raw shrimp shells and other complex media due to complex chemical composition. Wang *et al.* (2006) and Dahiya *et al.* (2005) reported that colloidal chitin hydrolysed by acid produced lower yields of oligosaccharide and a large amount of monomeric N-acetylglucosamine.

Chitin can be degraded by two enzyme systems which are an endochitinase (EC 3.2.1.14) and an exochitinase (EC 3.2.1.30). The degradation occurs in two consecutive steps: first the hydrolysis by endochitinase to oligomers (mainly dimers) followed by their degradation to free NAG by exochitinase (Dahiya *et al*., 2006). From Fig. 5a it was observed that TMM (colloidal chitin) produced the highest percentage of NAG, which is in agreement with the production of exochitinase by *Trichoderma* sp.

A study by Donzelli *et al.* (2003), Deane *et al.* (1998) and Bolar *et al.* (2001) reported that *Trichoderma* having high ratio of exochitinase to endochitinase activity release almost exclusively monomeric NAG from chitin. Endochitinases cleave chitin randomly generating soluble low molecular weight NAG multimers, such as chitobiose, chitotriose and chitotetraose. Chitobiosidases cleave chitin to release NAG dimmers (chitobiose) one at a time from the non-reducing end of chitin chain. β-N-acetyl-hexosaminidases hydrolyse chitobiose, chitotriose and chitotetraose from the non-reducing end of N-acetylchitooligosaccharides resulting in the release of N-acetyl-glucosamine (Wang *et al*., 2001). Horn *et al.* (2006) reported that the chitinases from *Serratia* is unlikely to produce monomers directly from chitin. Instead, they are produced when chitotriose is degraded to chitobiose and NAG. Generally, enzymatic degradation of polysaccharides occurs from one of the chain ends (exo-mechanism) or from a random point along the polymer chain (endo-mechanism). Each of these two

mechanisms can occur in combination with the processive mode of action, meaning that the substrate is not released after successful cleavage but slides through the active site for the next cleavage event to occur (Horn *et al*., 2006).

CONCLUSION

Trichoderma virens UKM1 that growth in the medium with colloidal chitin as carbon source shown the best medium to degrade shrimp waste with the highest percentage of NAG (86%) produced which was indirectly converted to glucosamine using chitooligomer as marker. Further study should be done on purified chitinase enzyme from *T. virens* UKM1 for better understanding of chitin degradation.

ACKNOWLEDGMENT

The authors wish to thank National Biotechnology Directorate (BIOTEK) from the Ministry of Science, Technology and Innovation Malaysia for research funding.

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