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# Production of Fungal Chitosan by Enzymatic Method and Applications in Plant Tissue Culture and Tissue Engineering: 11 Years of Our Progress, Present Situation and Future Prospects

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## 1. Introduction

Chitin is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine units linked with  $\beta$ -(1-4)-glycosidic bond, here *N*-acetyl-D-glucosamine units are predominant in that polymer chain. The deacetylated form of chitin refers to chitosan. Chitin usually refers to a copolymer with a degree of acetylation (DA) of more than 40% [i.e., number of *N*-acetyl-D-glucosamine more than 40% and number of D-glucosamine less than 60%] and insoluble in dilute acids. The name chitosan is used for a copolymer with less than 40% DA [i.e., more than 60% DD (degree of deacetylation), number of *N*-acetyl-D-glucosamine less than 40% and number of D-glucosamine more than 60%] that, in most cases, will be soluble in dilute acid (Nwe & Stevens, 2008). Chitin and chitosan are found as supporting materials in many aquatic organisms (shells of shrimps and crabs and bone plates of squids and cuttlefishes), in many insects (mosquitoes, cockroaches, honey bees, silkworms, *Drosophila melanogaster*, *Extatosoma tiaratum* and *Sipylodea sipylus*), in terrestrial crustaceans (*Armadillidium vulgare*, *Porcellio scaber*), in nematode, in mushrooms (*Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, *Trametes versicolor*, *Armillaria mellea*, *Pleurotus ostreatus*, *Pleurotus sajo-caju* and *Pleurotus eryngii*) and in some of microorganisms (yeast, fungus, and algae) (Carlberg, 1982; Nemtsev et al., 2004; Veronico et al., 2001; Paulino et al., 2006; Moussian et al., 2005; Tauber, 2005; Hild et al., 2008; Anantaraman & Ravindranath, 1976; Pochanavanich & Suntornasuk, 2002; Mario et al., 2008; Yen & Mau, 2007 cited in Nwe et al., 2010).

Nowadays, commercially, chitins and chitosans are produced from biowastes obtained from aquatic organisms. The production of those products in industrial scale for medical and agriculture applications appears in inconsistent physico-chemical characteristics of products because of seasonal and variable supply of raw materials, as well as variability and difficulties of process conditions (Ashford et al., 1977 cited in White et al., 1979). Moreover, in 1996, main argument on the production of chitosan from shells of shrimps was the present of shrimp antigen in the final product of chitosan (Tan et al., 1996, *Proceedings of 2<sup>nd</sup> Asia Pacific Chitin and Chitosan Symposium*, Bangkok, pp 50-57). These problems on production of chitosan may be circumvented by extracting chitosan from fungal mycelia.

The production of chitosan from fungal mycelia has a lot of advantages over crustacean chitosans such as the degree of acetylation, molecular weight, viscosity and charge distribution of the fungal chitosan are more stable than that of crustacean chitosans; the production of chitosan by fungus in a bioreactor at a technical scale offers also additional opportunities to obtain identical material throughout the year and to obtain chitosans with a radioactive label or with specific changes in its polymeric composition; and the fungal chitosan is free of heavy metal contents such as nickel, copper (Tan et al., 1996, Arcidiacono & Kaplan, 1992, Nwe & Stevens, 2002a). Moreover the production of chitosan from fungal mycelia give medium-low molecular weight chitosans ( $1-12 \times 10^4$  Da), whereas the molecular weight of chitosans obtained from crustacean sources is high (about  $1.5 \times 10^6$ Da) (Nwe & Stevens, 2002). Chitosan with a medium-low molecular weight has been used as a powder in cholesterol absorption (Ikeda et al., 1993) and as thread or membrane in many medical-technical applications. For these reasons, there is an increasing interest in the production of fungal chitosan. However, so far, the extraction of high yield pure chitosan production from fungal cell wall material has not been accomplished upto 2001 (Stevens, 2001).

In our research, we have investigated the bond between chitosan and glucan in fungal cell wall and developed a powerful enzymatic method for the production of excellent quality chitosan from the fungal mycelia in a high yield. In this process a heat-stable alpha-amylase is used to cleave the covalent link between chitosan and glucan in cell wall of *Gongronella butleri* (Nwe & Stevens, 2002 & 2002a; Nwe et al., 2008). In 2006, the effect of shrimp and fungal chitosan on the growth and development of orchid plant meristemic tissue in culture was investigated in liquid and on solid medium (Nge et al., 2006). In recent years, the scaffold and membrane were prepared using the fungal chitosan and their mechanical and biological properties were evaluated together with the scaffolds and membranes of chitosans obtained from crustacean sources to use as a tissue regeneration template (Nwe et al., 2009; Nwe et al., 2010a). Among the tested chitosans in here, fungal chitosan showed as an excellent growth stimulator to apply in agriculture sector and as an excellent scaffolding material to construct a biodegradable tissue regeneration template. In this chapter, we summarize all our research contributions on last 11 years in 4 sections: (1) Investigation of a method to produce high quality and quantity of fungal chitosan and study the bond between chitosan and glucan in the cell wall of fungi, *Gongronella butleri* and *Absidia coerulea*, (2) Optimization of fermentation conditions for production of high yield chitosan by solid substrate fermentation, (3) Application of fungal chitosan in plant tissue culture, and (4) Application of fungal chitosan in tissue engineering.

## **2. Investigation of a method to produce high quality and quantity of fungal chitosan and study the bond between chitosan and glucan in the cell wall of fungi, *Gongronella butleri* and *Absidia coerulea***

### **2.1 Growth of fungus and extraction of chitosan by traditional method**

Chitosan is a substantial component of cell wall of certain fungi, particularly those belonging to the class Zygomycetes (Bartniki-Garcia, 1968). Tan et al., 1996 evaluated the yield of chitosan from several Zygomycetes fungi including *Absidia*, *Gongronella*, *Mucor* and *Rhizopus* and concluded that *G.butleri* gave the highest yield of chitosan. At the same time, Crestini et al., 1996 reported that the yield of chitosan produced from *Lentinus edodes* grown in solid state fermentation, 6.18 g/kg was higher than that in submerged fermentation, 0.12

g/l. In 1998, fungus *Gongronella butleri* was selected to produce chitosan in our research. Firstly, a comparison was made between the yield of chitosan from fungal mycelia grown in solid substrate fermentation (SSF) and in submerged fermentation (SMF) using various nitrogen sources. The Termamyl assayed extraction method was not discovered yet at that time. The chitosan was extracted using vacuum filtration and  $\beta$ -glucanase treatment method. It was observed that the yield of chitosan obtained from fungal mycelia grown in SSF (3.7 g chitosan/kg of solid substrate) was higher than that in SMF (0.6 g chitosan/L of fermentation medium) due to the low amount of mycelia produced in SMF (Nwe et al., 2002). Based on the results obtained from our research and Crestini et al., 1996, solid substrate fermentation was selected as the best fermentation method to produce chitosan by fungus *Gongronella butleri* (Figure 1).



Fig. 1. Fungus *Gongronella butleri* USDB 0201 was grown on sweet potato pieces in a tray-type solid substrate fermentor. Sweet potato pieces were used as solid support and as carbon source. The dried fungal mycelia were used to extract chitosan.

The history of the development of chitosan extraction procedure by enzymatic method started with the work of Mr. Su Ching Tan from the National University of Singapore, Singapore. In his method, mycelia were treated with 1 M NaOH and the resultant alkaline insoluble material (AIM) was treated with 0.35 M acetic acid at 25°C for 2 h (Tan et al., 1996). In our research the extraction of chitosan from fungal mycelia was started according to the method described by Tan et al., 1996 with minor modification (Figure 2). The yield of chitosan extracted from fungal mycelia grown in solid substrate fermentation was 2-3 g/100 g of mycelia. An effective chitosan extraction procedure is essential for an economical production of fungal chitosan.

Several methods have been developed for the extraction of chitosan from the fungal mycelia (Table 1). Most methods used 1 M NaOH to remove protein and other cell wall materials



and then the chitosan was extracted with 2 % acetic acid. The yield of chitosan produced from the fungal mycelia treated in this way is very low. The extraction procedure for high yield production of pure chitosan from the fungal cell wall material has not yet been accomplished up to 2001 (Stevens, 2001).



Fig. 2. Extraction of chitosan from mycelia of fungus *G.butleri* grown in solid substrate fermentation

Reference	Strain	Deproteination (NaOH)			Acetic acid extraction			Chitosan yield
		Conc:	Temp:	Time	Conc:	Temp:	Time	
Jaworska and Konieczna, 2001	<i>Absidia orchidis</i> <sup>a</sup>	1M	121°C	10 min	1%	-	-	1.84 g/L
Crestini et al., 1996	<i>Lentinus Edodes</i> <sup>b</sup>	1M	121°C	15 min	2%	95°C	14h	6.18 g/kg of wheat straw
Crestini et al., 1996	<i>Lentinus Edodes</i> <sup>b</sup>	1M	121°C	15 min	2%	95°C	14h	120 mg/L
Tan et al., 1996	<i>G. butleri</i> <sup>b</sup>	1M	121°C	15 min	2%	25°C	1 h	467 mg/L
Muzzarelli et al., 1994	<i>A.Coenocia</i> <sup>a</sup>	25%	boil	3 h	0.1%	25°C	16h	1,800 mg/L
Rane and Hoover, 1993	<i>G.butleri</i> <sup>b</sup>	1M	121°C	15 min	2%	95°C	12h	230 mg/L
Rane and Hoover, 1993	<i>A.Coenocia</i> <sup>a</sup>	1M	121°C	15 min	2%	95°C	12h	480 mg/L
Arcidiacono and Kaplan, 1992	<i>M. rouxi</i> <sup>a</sup>	1 M	121°C	15 min	2%	95°C	-	250 mg/L
Hang, 1990	<i>E. oryzae</i> <sup>b</sup>	2%	121°C	15 min	2%	95°C	12h	700 mg/L

<sup>a</sup>Submerged fermentation, <sup>b</sup>Solid state/substrate fermentation

Table 1. Extraction of chitosan from fungal mycelia by different methods

Glucose joined through  $\alpha$ -1,3-,  $\alpha$ -1,4-,  $\alpha$ -1,6-,  $\beta$ -1,3-,  $\beta$ -1,4- or  $\beta$ -1,6-linkages is called glucan (Carbonero et al., 2005; Schmid et al., 2001; Hochstenbach et al., 1998; Wessels et al., 1990; Wolski et al., 2005; Marchessault & Deslandes, 1979 cited in Nwe et al., 2008). Chitin/chitosan and glucan are the main fungal skeletal polysaccharides. In the fungal cell

wall, chitin/chitosan occurs in two forms, as free aminoglucoside and covalently bonded to  $\beta$ -glucan (Bartnicki-Garcia, 1968; Gooday, 1995; Robson, 1999; Wessels et al., 1990). In 1990, Wessels et al. proposed that initially chitin and  $\beta$ -glucan chains accumulate individually in the fungal cell wall and thereafter form the interpolymer linkage. The formation of the chitin/chitosan-glucan complex chains results in a rigid cross-linked network in the cell wall (Gooday, 1995; Robson, 1999) and causes a considerable problem for the extraction of intact chitosan and glucan. It does not break down easily under mild extraction condition (Muzzarelli et al., 1980). Under the above mentioned conditions only free chitosan, that is chitosan unbounded to other cell wall components is extracted (Nwe & Stevens, 2002). Chitosan bounded to insoluble cell wall components will not be extracted (Figure 2 and 3).

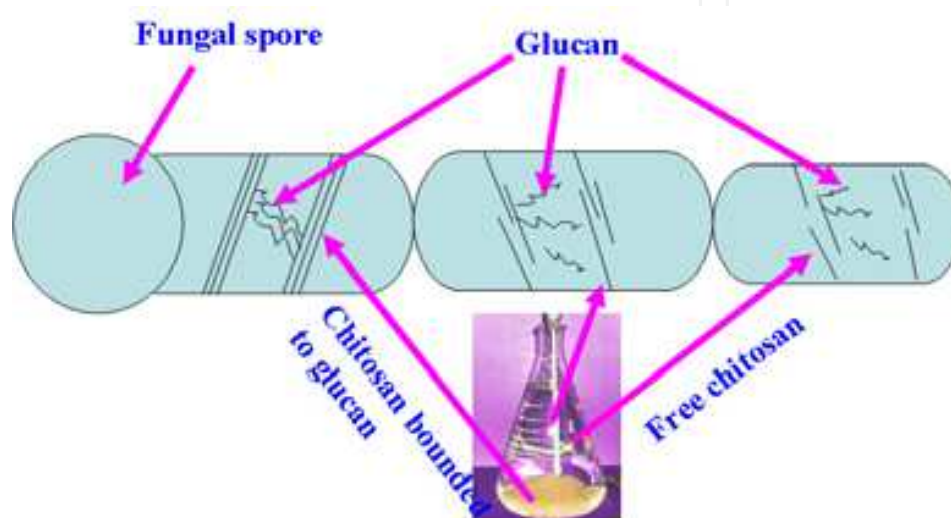


Fig. 3. A proposed model for the synthesis of chitosan (straight line) and glucan (wave line) in fungal hyphae

To extract the high quality and quantity of chitosan and glucan from cell wall of fungi, the bond between chitosan and glucan in cell wall of fungi must be investigated. Most of the researchers are trying to find the linkage between the chitosan and glucan in the fungal cell wall by digestion with glucanase, chitinase and amylase. In 1979, Sietsma and Wessels reported that 90% of  $\beta$ -glucan obtained from the chitin-glucan complex by digestion with (1-3)- $\beta$ -glucanase and *N*-acetyl-glucosamine, lysine and/or citrulline were identified as products after digestion with chitinase. Therefore they proposed that the bridge linking the glucan chain with the chitin contains lysine, citrulline, glucose and *N*-acetyl-glucosamine. Similar evidence was obtained by Gopal et al., 1984 for the degradation of chitin-glucan complex by (1,3)- $\beta$ - and (1,6)- $\beta$ -glucanase, and subsequently by chitinase. Carbohydrate expressed as glucose and *N*-acetyl-glucosamine monomers was detectable in equivalent amounts in the hydrolysate. The residue after chitinase treatment was further treated with  $\alpha$ -amylase but additional release of glucose could not be detected colorimetrically. Surarit et al., 1988 suggested a glycosidic linkage between position 6 of *N*-acetyl-glucosamine in chitin and position 1 of glucose in  $\beta$ -(1-6)-glucan in the cell wall of *Candida albicans*. In 1990, Wessels et al., proposed that a direct link between free amino groups in the glucosaminoglycan and the reducing end of the glucan chains forming the inter-polymer linkages in the chitin-glucan complex. Up to 1992, no clear evidence of the identity of the chemical link between the chitosan and glucan polymer chains had been uncovered (Roberts, 1992). After 1995, Kollar et


al., 1995 and Fontaine et al. 2000 digested the cell wall of *Saccharomyces cerevisiae* and *Aspergillus fumigatus* with 1% SDS and 1 M NaOH respectively and the insoluble fractions were digested with (1,3)- $\beta$ -endoglucanase and chitinase. The soluble fractions were analyzed. Based on their results, they concluded that the terminal reducing residue of a chitin chain is attached to the non-reducing end of a  $\beta$ -(1,3)-glucan chain by a  $\beta$ -1,4 linkage. An insoluble residue was remained at the end of both extraction processes.

Importantly, cell wall matrix must be broken down far enough in order to study the linkage between the chitosan and glucan in the fungal cell wall and to extract total chitosan (i.e., free chitosan plus chitosan bounded to glucan). In 1994, Muzzarelli et al., reported that the chitosan-glucan complex can be split by 25 % NaOH (Table 1). Therefore further treatment of cell wall matrix was investigated.

## 2.2 Decomposition of fungal cell wall materials by acid and alkaline treatment

In order to decompose the AIM matrix into molecular level of chitosan-glucan complex, chitosan and glucan, the cell wall matrix has to be weakened and the chitosan from the complex has to be soluble in acetic acid. Thereafter the covalent bond between the chitosan and glucan has to be split by enzymatic hydrolysis. These steps are critical factors for the decomposition of fungal cell wall and for the extraction of high yield chitosan. To investigate the condition for the maximum solubility of chitosan in acetic acid, the duration and temperature of the acetic acid extraction step were varied (Figure 2). It was found that

1 M NaOH,  
45°C for 13 h



Treatment	Temperature (°C)	NaOH concentration (M)	Time (h)	Decomposition of AIM in acetic acid
1	79	1.77	13.50	-
2	46	1.77	13.50	-
3	79	5.08	4.93	-
4	46	5.08	4.93	-
5	95	6.75	13.50	-
6	63	6.75	13.50	-
7	30	6.75	13.50	-
8	79	8.41	22.07	+
9	46	8.41	22.07	+
10	79	11.73	13.50	++++
11	46	11.73	13.50	++++

11 M NaOH,  
45°C for 13 h




Table 2. Effect of NaOH treatment conditions on decomposition of the AIM in 0.35M acetic acid at 95°C for 5h (Reproduced from Nwe et al., 2008a, International Journal of Biological Macromolecules, 43, 2-7)

the yield of chitosan increased to 3.14 g/100g of mycelia when the AIM was treated with 0.35 M acetic acid at 95°C for 5 h (Nwe et al., 2008a). However the AIM matrix did not break down at that condition (Figure 2, Table 2). The condition of acetic acid treatment at 95°C for 5h was selected and the NaOH treatment step was carried out with a randomized experimental design (Figure 2 and Table 2).

An experimental condition to break the rigid cell wall matrix into a suspension form was investigated without degradation of extracted chitosan during acid and alkaline treatment (Nwe et al., 2008a). The best decomposition condition was the mycelia treated with 11 M NaOH at 45°C for 13 h and then the AIM was treated with 0.35 M acetic acid at 95°C for 5 h. In this situation, if the chitin present in cell wall of fungus, probably all chitin will be deacetylated to chitosan using 11 M NaOH instead of 1 M NaOH (Nwe & Stevens, 2002 and 2002a). Upto now it is not clear yet the formation of chitin and chitosan in cell wall of fungi. Here it appeared very difficult to isolate pure chitosan from that suspended solution (Table 2, Figure 4). In order to isolate pure chitosan, the AIM suspended solution was treated under different conditions.

### 2.3 Isolation of chitosan from the AIM suspended solution

In the process of fungal mycelia treated with 11 M NaOH, nearly all proteins, soluble glucan, other polysaccharides, DNA and RNA presented in the fungal hyphae might wash out from alkaline insoluble materials (AIM) of the fungal hyphae during washing step of the AIM with distilled water upto neutral pH. Here it was assumed that chitosan, insoluble glucan and chitosan-glucan complex might remain in the AIM material (Nwe & Stevens, 2002a). These polymers suspended in 0.35M acetic acid during treatment of the AIM with 0.35M acetic acid at 95°C for 5 h (Table 2, Figure 4).

pH of solution	Solubility of chitosan and glucan		
	CTS	GLU	CTS-GLU
4	Soluble	Soluble	Clear solution
		Insoluble	<b>Turbid suspension</b>
9	Insoluble	Soluble	<b>Precipitate</b>
		Insoluble	<b>Precipitate</b>



Fig. 4. Nature of chitosan (CTS), glucan (GLU), and chitosan-glucan complex (CTS-GLU) in 0.35 M acetic acid at pH 4 and 9

According to the polycationic nature of chitosans, chitosans dissolve in organic acid at pH 4 and they precipitate at pH 9. Most of the glucans are insoluble at pH 4 and 9. At pH 4, the AIM components in 0.35 M acetic acid form a stable suspension and the turbid components precipitate at pH 9 (Figure 4, Nwe & Stevens, 2002a). As a hypothesis it is assumed that chitosan and glucan are bound to each other and show suspension/precipitation behavior



according to the physico-chemical nature of the chitosan component (Nwe & Stevens, 2002a). The chitosan component of the material is heavily protonated at pH 4 and supports the complex to stay in suspension (Nwe & Stevens, 2002a). At pH 9, chitosan lost its charge and does not longer sustain the complex to remain in suspension and the whole complex precipitate. To confirm this assumption, the turbidity of the AIM suspended solution was measured at pH 4 and the suspension was twice-centrifuged and then turbidity of the suspension was measured again. It was found that the turbidity of the suspended solution decreased from more than 1000 NTU (above detection limit) to about 600 NTU after twice centrifugation (Nwe & Stevens, 2002a). This observation confirmed that the turbid material in the suspended solution is linked to the chitosan chains. In order to isolate chitosan from the suspended solution, the suspended solution was treated with glucanase, promozyme and amylase (Nwe & Stevens, 2002a).

It was found that the turbidity of the suspended solution reduced to >300 NTU when the AIM suspended solution was treated with glucanase or promozyme (Table 3). Most glucans in the fungal cell wall synthesize as linear form with  $\beta$ -1,3 linkages and as branched chain with  $\beta$ -1,6 linkages (Herrera, 1991). Here it can be assumed that glucanase and promozyme could not be cleaved the glucan component from the complex when the glucan chains were stable in the suspension as a triple helix (Case II, III, and IV, Figure 5). However the turbidity of the solution decreased sharply from >1000 to <30 NTU when the suspension was treated with Termamyl Type LS, Termamyl type L or  $\alpha$ -amylase (Table 3). These enzymes cleave the  $\alpha$ -(1,4) glycosidic bond. The turbidity of suspension decreased to 10 NTU at the optimum conditions of Termamyl Type LS: pH 4.5, 4% (v/v) enzyme, at 65 °C, 225 rpm for 3 h (Table 4, Nwe & Stevens, 2002a). The maximum shaking speed for the water bath in laboratory of Bioprocess Technology, Asian Institute of Technology, Thailand used was 200 rpm. Therefore shaking speed, 200 rpm was used in the later experiments.

Enzyme	Enzyme % (v/v)	Temp (°C)	RPM	Time (h)	Turbidity
Glucanase ( $\beta$ -1,3 )	5	25	100	2	>300 NTU
Promozyme ( $\alpha$ -1,6)	5	50	175	2	>500 NTU
Diazyme ( $\alpha$ -1,4)	5	50	100	2	<30 NTU
Termamyl Type LS ( $\alpha$ -1,4)	5	50	175	2	<30 NTU
Termamyl Type L ( $\alpha$ -1,4)	5	50	175	2	<30 NTU
$\alpha$ -amylase ( $\alpha$ -1,4)	5	50	175	2	<30 NTU

Table 3. Turbidity of chitosan solutions after treatment of the AIM suspended solutions with glucanase, promozyme and amylase

After Termamyl treatment, a large amount of precipitate and clear supernatant was obtained (Figure 6). This precipitate is supposed to be glucan, freed from the chitosan-glucan complex. After removal of this precipitate by centrifugation (1600g, 15min), the supernatant was collected. After adjusting the pH of supernatant to 9, a heavy precipitate

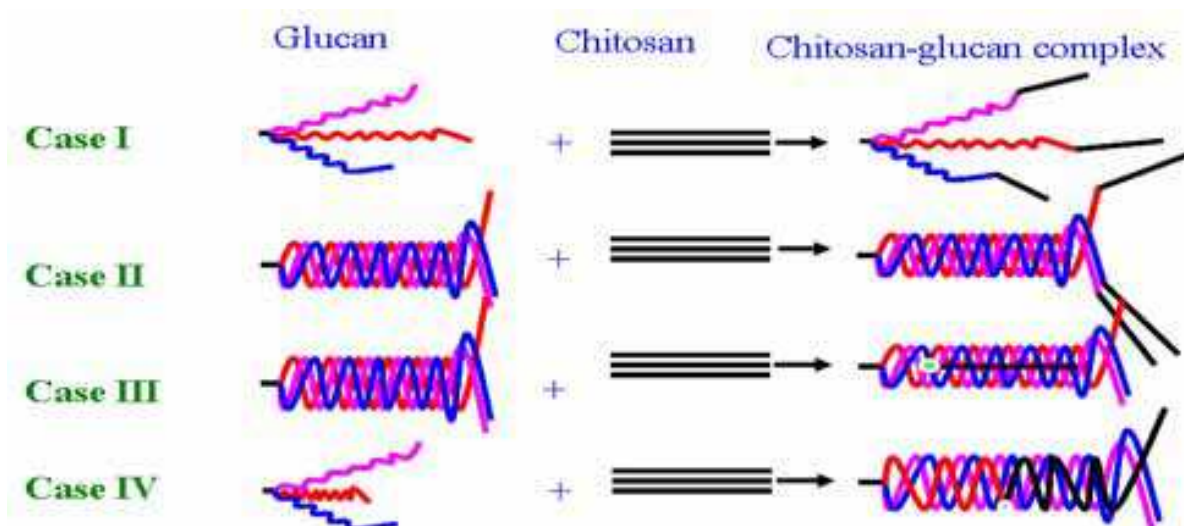


Fig. 5. Proposed model for the formation of chitosan-glucan complex in cell wall of fungus *Gongronella butleri*

Parameter to be optimized	Experimental conditions					Conditions for variable parameter				
	pH	Enz (%)	Time (h)	Temp (°C)	rpm	A	B	C	D	E
pH	V	5	3	65	200	3.5	4	4.5	5	-
Enzyme volume (% v/v)	4.5	V	3	65	200	1	2	3	4	5
Time (h)	4.5	4	V	65	200	0.5	1	1.5	2	3
Temperature (°C)	4.5	4	3	V	200	45	55	65	75	85
Shaking (rpm)	4.5	4	3	65	V	125	150	175	200	225

Parameter to be optimized	Turbidity of chitosan after enzymatic treatment at specified condition (NTU)				
	A	B	C	D	E
pH	139 ± 3	169 ± 3	31 ± 3	197 ± 5	
Enzyme volume	455 ± 5	307 ± 16	53 ± 2	15 ± 3	31 ± 3
Time	35 ± 1	30 ± 1	32 ± 1	26 ± 2	15 ± 3
Temperature	313 ± 6	71 ± 3	15 ± 3	25 ± 0	58 ± 3
Shaking	62 ± 1	23 ± 0	17 ± 2	15 ± 3	10 ± 0

Table 4. Turbidity of chitosan solutions after treatment of the AIM suspended solutions with Termamyl, type LS under different experimental conditions (Reproduced from Nwe & Stevens, 2002a, Biotechnology Letters, 24, 1461-1464).

was obtained. This material easily can be redissolved in 0.35 M acetic acid and is apparently glucan-free chitosan (Figure 4). The Termamyl treatment is obviously highly efficient to separate the chitosan from the glucan and offers possibilities for the isolation of purified chitosan. Based on these observations, it was proposed that the bond between the two polysaccharides, chitosan and glucan is linked by  $\alpha$ -(1-4)-glycosidic bond (Nwe & Stevens, 2002a).

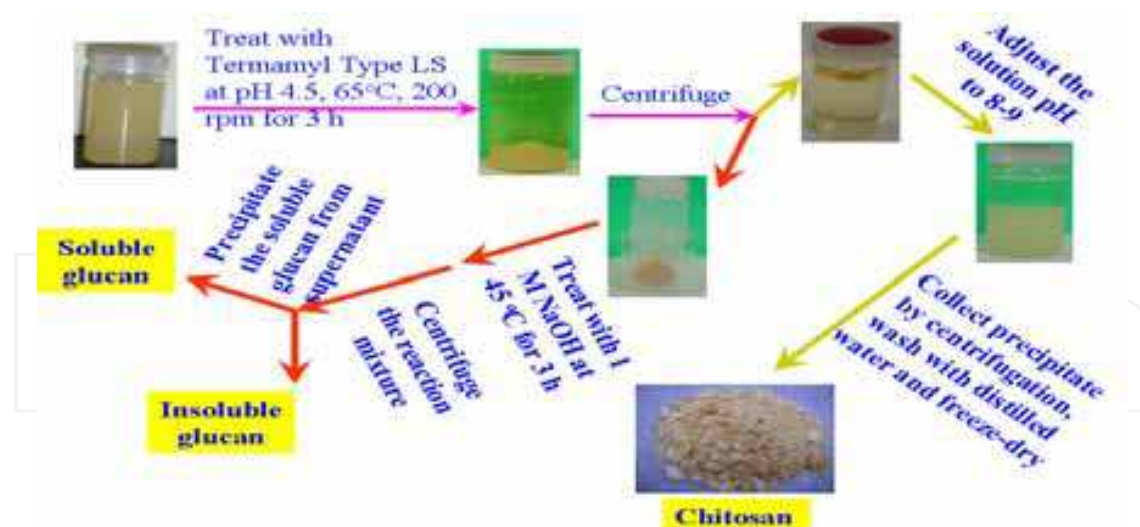


Fig. 6. Extraction of chitosan and glucan from the AIM suspended in 0.35M acetic acid by treatment with Termamyl Type LS (Nwe & Stevens, 2002 and Nwe et al., 2008)

#### 2.4 Confirmation the bond between the chitosan and glucan

To confirm the bond between the chitosan and glucan, the suspension was centrifuged and filtered with GF/C filter paper and then the turbidity of the filtrate was recorded as 39 NTU (Figure 7). In this process the glucan unbounded to chitosan and glucan bounded to chitosan larger than 1.2  $\mu\text{m}$  might remain on the GF/C filter paper and these materials smaller than 1.2  $\mu\text{m}$  might pass through the GF/C filter paper (Nwe et al., 2008). The resultant filtrate was treated with  $\beta$ -glucanase, the turbidity of solution decreased to 32 NTU. This result suggested that at least a part of the turbidity is caused by glucan bounded to the chitosan. After glucanase treatment, the reaction mixture was adjusted to pH 8-9 to precipitate the chitosan. The precipitate was washed with distilled water upto neutral pH and dried and then weighted (Figure 7 and Table 5, product **A**). In order to observe the purity of product A, the product A was redissolved again in dilute acetic acid and the solution A was digested with Termamyl Type LS at optimal conditions (Nwe et al., 2008). Chitosan, product B was purified from the solution B after treatment with Termamyl Type LS enzyme. The concentration of reducing end in the chitosan solution A and B was measured (Figure 7, Table 6). The results of product A and B was compared with the yield of product C, which extracted from the AIM suspension treated with Termamyl Type LS directly (Figure 6 and 7 and Table 5).

Although IR spectrum of product A was very similar to that of shrimp chitosan (Figure 8), product A, 8.2 g composed with 5 g glucan (3.2 g chitosan, product B). This result clearly showed that  $\beta$ -glucanase did not completely remove glucan from the product A and once the bond between chitosan and glucan was broken by Termamyl, Type LS, this glucan precipitated in chitosan solution (Figure 7). In case Termamyl was directly applied to the AIM suspended in acetic acid, chitosan, 5.9 g was obtained (Table 5). This was 2.7 g of chitosan more than the yield of product B. Apparently this chitosan was part of a cell wall component that did not pass through the GF/C filter paper in absence of Termamyl treatment (Figure 7 and Table 5).

Moreover the amount of reducing ends in the chitosan solution B was about 40 times higher than that in the chitosan solution A (Figure 7, Table 6). The enzyme is effective in the



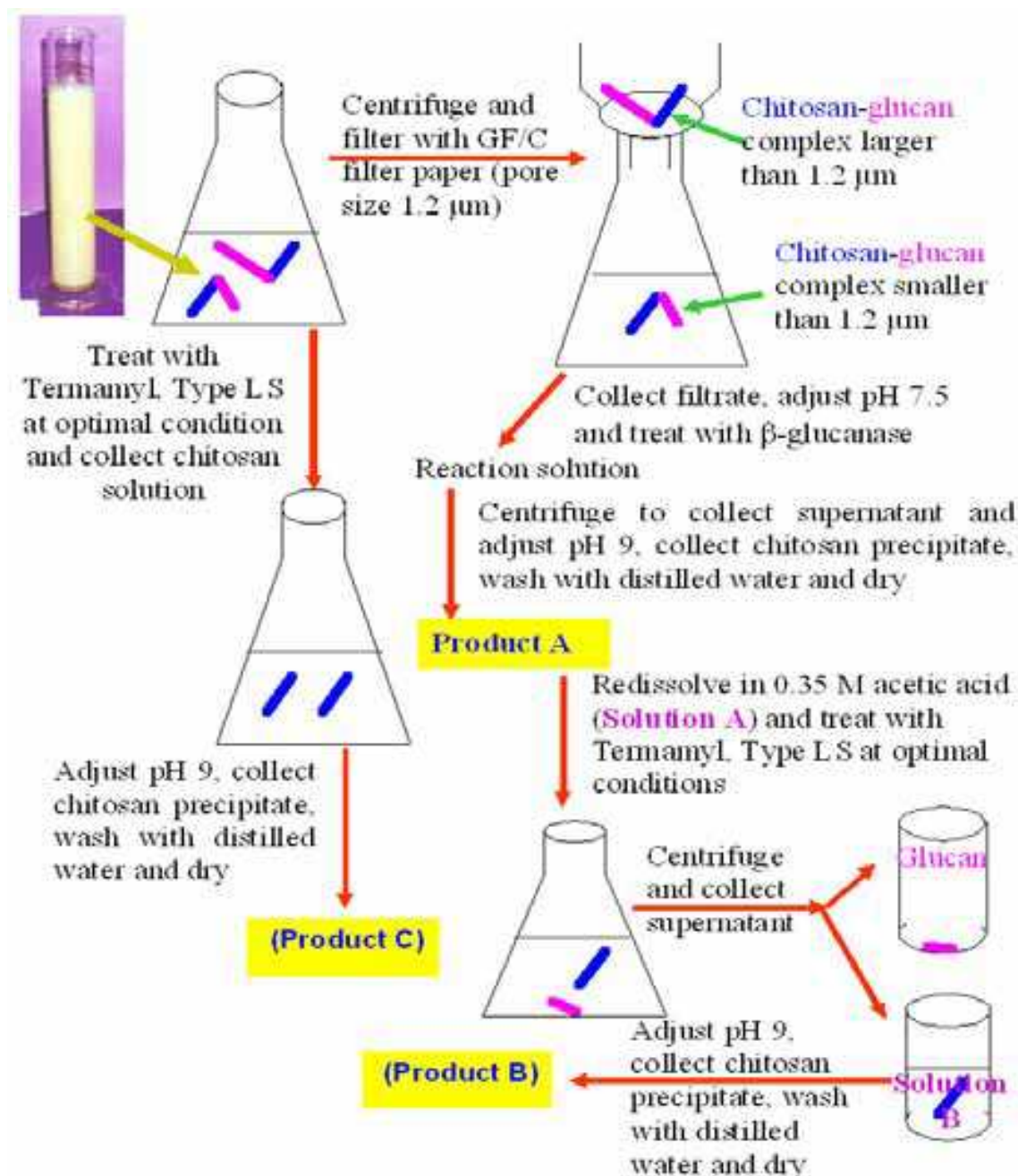


Fig. 7. Extraction of chitosan from the AIM suspended in 0.35M acetic acid by different methods (Nwe et al., 2002, 2002b & 2008, Nwe & Stevens, 2002)

hydrolysis of  $\alpha$ -(1-4)-glycosidic linkages, leading to a sharp increase in 1-glucose end groups in the solution (Nwe et al., 2008). Since Termanyl does not split the  $\beta$ -glycosidic bonds in chitosan, the increase must originate from hydrolysis of 1-4 bonds between chitosan and glucan and on  $\alpha$ -1-4 bonds within the glucan molecule. Based on the results presented in this study it is obvious that reducing end of chitosan chain and non-reducing end of glucan chain are bound by  $\alpha$ -(1-4)-glycosidic bond and that probably glucan has also internal  $\alpha$ -(1-4)-glycosidic bond, as concluded from the overwhelming increase in the concentration of reducing ends. This evidence is one of the confirmations of  $\alpha$ -(1-4)-glycosidic linkages between chitosan and glucan in cell wall of fungus *G. Butleri* (Nwe et al., 2008). Pure  $\alpha$ -amylase (Sigma)



was used to confirm the above results. This enzyme gave same result as Termamyl, Type LS enzyme (Nwe & Stevens, 2002a). Here the application of Termamyl, Type LS in production of fungal chitosan are (1) glucan is removed from the extracted chitosan solution more effectively than by filtering with GF/C filter paper (2) high purity of fungal chitosan is obtained (3) less time consuming and (4) status of enzyme is food grade (Nwe et al., 2001).

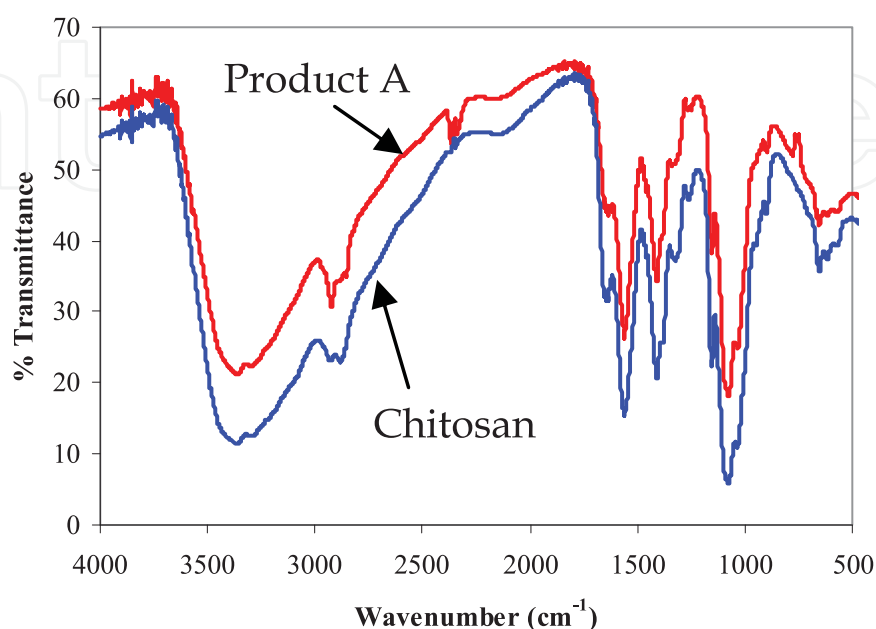


Fig. 8. IR-spectra of chitosans from shells of shrimps and from cell wall of *G. butleri* grown on solid substrate supplied with mineral solution, basic medium (product A)

Growth condition	Biomass (g/kg sweet potato, w.b)	Number average molecular weight (kDa)	Yield of chitosan (g chitosan/100 g of mycelia)		
			Product A	Product B	Product C
Sweet potato	30.1 ± 3.2	-	-	-	2.29 ± 0.49
Basic medium	24.6 ± 2.0	32 ± 3	8.2 ± 0.4	3.2 ± 1.3	5.91 ± 0.11
Basic medium & peptone	28.5 ± 1.3	33 ± 3	10.5 ± 0.7	6.4 ± 1.0	-
Basic medium & (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	19.8 ± 3.2	36 ± 1	11.7 ± 2.0	4.8 ± 2.4	-
Basic medium & NaNO <sub>3</sub>	25.6 ± 0.5	32 ± 3	10.2 ± 0.7	5.8 ± 1.0	-
Basic medium & urea	29.3 ± 1.8	33 ± 1	12.7 ± 1.1	7.8 ± 0.2	-

Table 5. Biomass (dry weight) and chitosan yield obtained by solid substrate fermentation with different nitrogen sources (Basic medium: Sweet potato pieces impregnated with mineral solution) (Nwe & Stevens, 2002a & 2002b, Nwe et al., 2002 & 2008)

Condition	Reducing ends concentration (mg/ml)
Before Termamyl treatment (A)	0.043 ± 0.001
After Termamyl treatment (B)	1.85 ± 0.085

Table 6. The concentration of reducing ends in the chitosan solution A and B (Reproduced from Nwe et al., 2008, Enzyme and Microbial Technology, 42, 242-251)

For further confirmation, the extracted chitosan and glucan fractions from chitosan-glucan complex (Figure 6) were analyzed by IR and  $^{13}\text{C}$ -NMR spectroscopy and elementary analysis (Nwe et al., 2008). The IR spectra of alkaline soluble and insoluble glucan, and chitosan showed the peak at  $1550\text{ cm}^{-1}$ , amide II band (Figure 9A). This observation indicated that some chitosan polymers might be entrapped in the glucan triple helices and heat stable  $\alpha$ -amylase enzyme could not cleave the chitosan from the glucan polymers (case III and IV in figure 5). In the  $^{13}\text{C}$ -NMR spectrum of fungal chitosan (Figure 9B), the

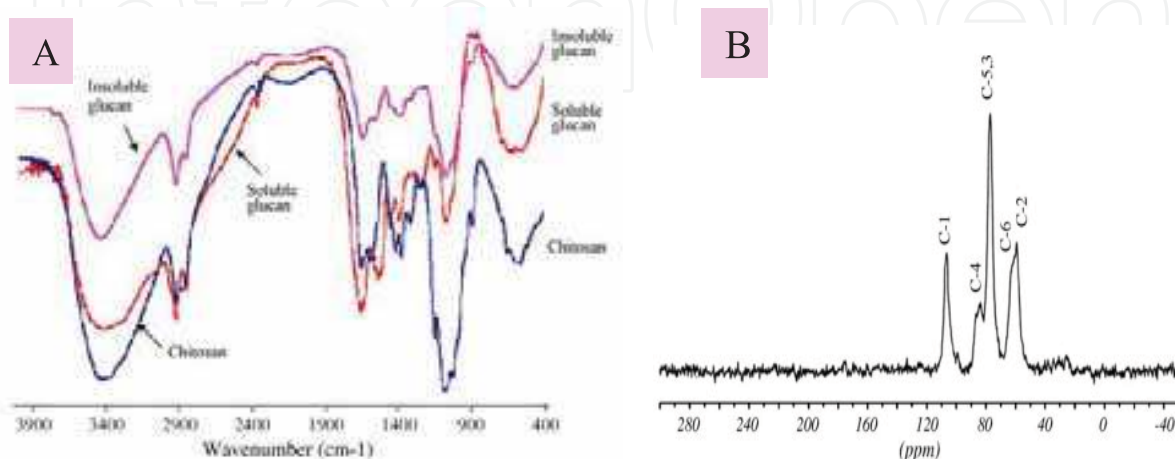


Fig. 9. (A) IR spectra of fungal chitosan and alkaline soluble and insoluble glucan (B)  $^{13}\text{C}$ -NMR spectrum of purified fungal chitosan (Reproduced from Nwe et al., 2008, *Enzyme and Microbial Technology*, 42, 242-251)

chemical shift on the fungal chitosan spectrum at approximately  $\delta 68$  was absent. This result suggested that there is no chain branching in the major monomer units in the fungal chitosan molecules and lack of the free non-reducing end in the major monomer units of the polymers. The analytical result from determination of nitrogen content in the fungal chitosan (8%) strongly proved that 95% purified chitosan was obtained from Termamyl treatment. Taken together, these investigations proved that chitosan and glucan in the cell wall of fungus *G.butleri* are linked by  $\alpha$ -(1-4)-glycosidic bond (Figure 10, Nwe & Stevens, 2002a, Nwe et al., 2008).

After proved the bond between chitosan and glucan by Termamyl treatment, chitosan was extracted from the fungal mycelia grown on solid substrate fermentation medium supplied with urea by method I and II (Table 7). It was found that the amount of chitosan extracted by method II leads to a four times higher than that by method I (Table 7). The similar result has been reported from the fungal mycelia grown in the basic medium (Nwe & Stevens, 2002). These results confirmed that total chitosan in the mycelia of fungus *G. Butleri* is extracted using method II and free chitosan from the fungal mycelia is extracted using method I.

By applying these two methods, method I and II, the bond between chitosan and glucan in cell wall of fungus *Absidia coerulea* ATCC 14076 was investigated. Fungus *Absidia coerulea* was grown in submerged fermentation medium (Figure 11) and free chitosan and total chitosan (free chitosan plus chitosan bounded to glucan) were extracted from the fungal mycelia using method I and II (Table 7). The free chitosan, 6.5 g/100 g of mycelia and total chitosan 8-9 g/100g of mycelia were isolated from fungal mycelia of *A.coerulea* (Nwe et al., 2008a).

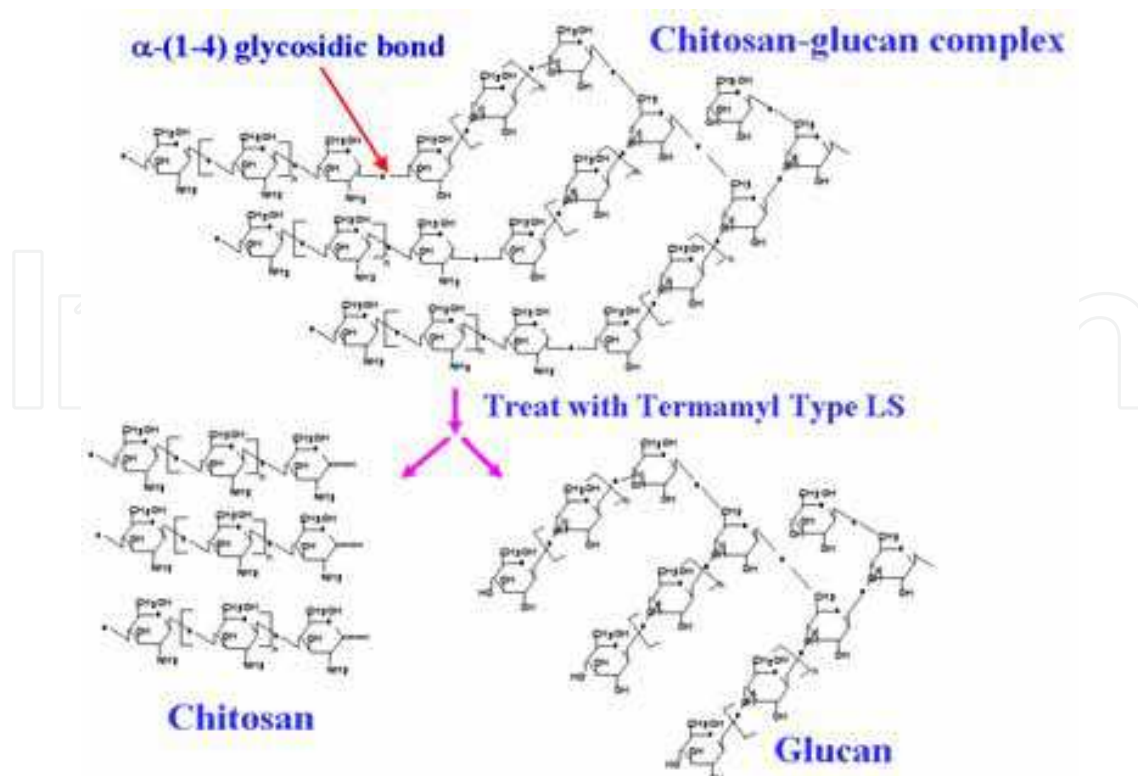


Fig. 10. Possible molecular structure of chitosan and glucan obtained from the treatment of chitosan-glucan complex by Termamyl, Type LS (Reproduced from Nwe et al., 2008, *Enzyme and Microbial Technology*, 42, 242-251)

Chitosan extraction Method	Yield of chitosan (g/ 100 g mycelia)	Number average molecular weight (kDa)
Method I	1.74	35
Method II	8.09	55

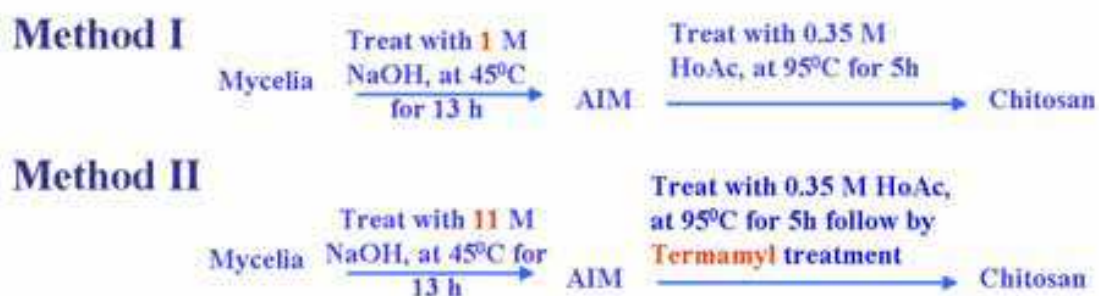


Table 7. Yield and number average molecular weight of chitosan extracted from fungal mycelia using method I and II (Nwe et al., 2008a)

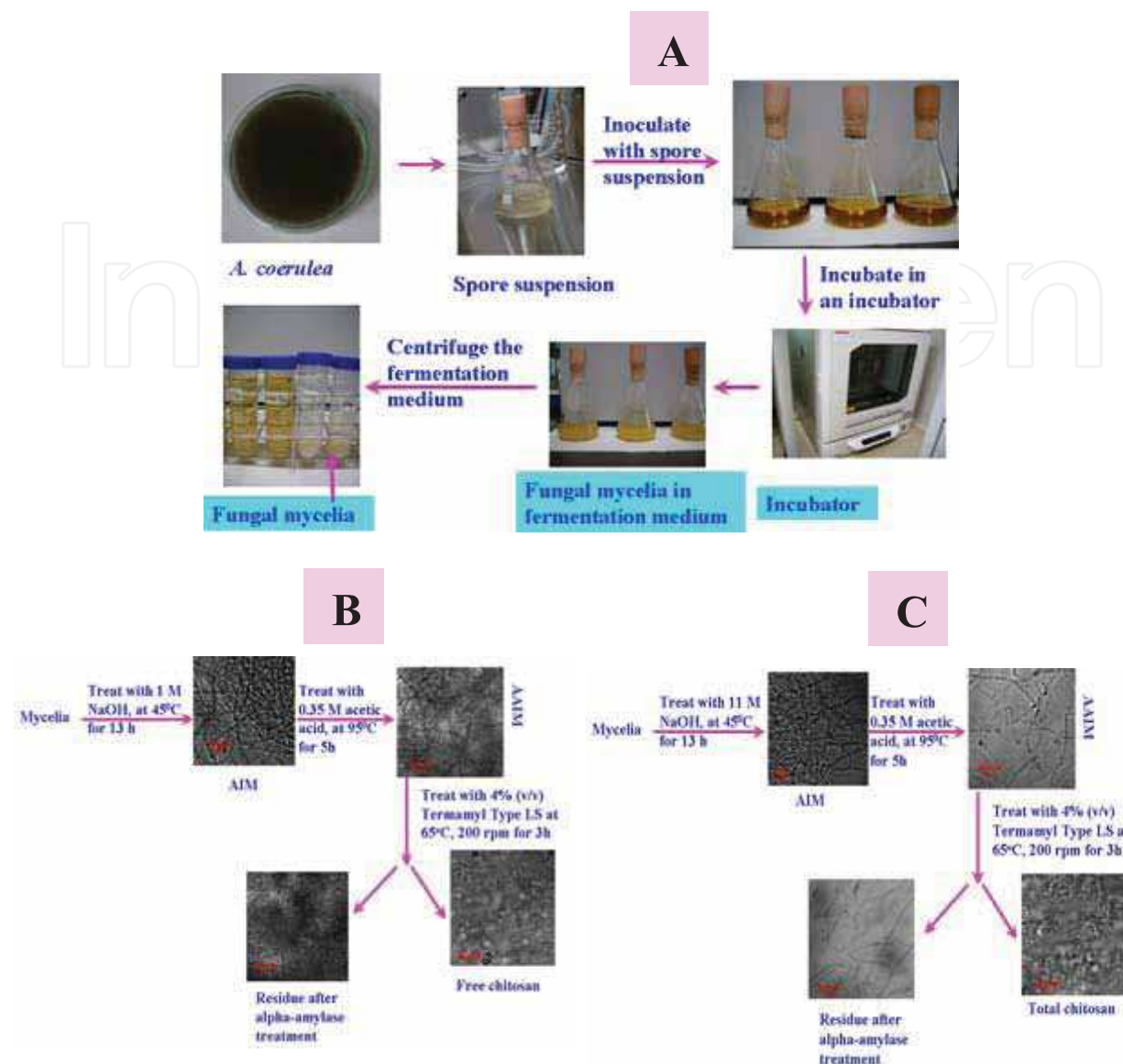


Fig. 11. Cultivation of fungus *Absidia coerulea* in submerged fermentation medium (A) and Laser scanning micrographs of fungal cell wall materials obtained from treatment of fungal mycelia with NaOH solution (1M NaOH, B and 11 M NaOH, C) for extraction of free chitosan and total chitosan (Nwe et al., 2008a)

In this research free chitosans and total chitosans have been isolated from fungi, *G. butleri* and *A. coerulea*. These observations indicated that fungi, *G. butleri* and *A. coerulea* separately synthesize chitosan and glucan in their cell wall and these two polymers are linked together afterwards to synthesize the chitosan–glucan complex (Nwe et al., 2008a). These observations agreed with finding of Sietsma et al., 1996. Moreover it can be assumed that the chitosan-glucan complex may be growing polymer since the number average molecular weight (42 kDa) and yield of chitosan product C, 5.9g/100g of mycelia gave higher result than that of chitosan product B (32 kDa and 3.2 g/100g of mycelia). These two results are compared with number average molecular weight of free chitosan, 35 kDa (Table 7 and Nwe & Stevens, 2002 & 2002b). These evidences confirmed that the bond between chitosan and glucan chains in fungi, *A.coerulea* and *G. butleri* is  $\alpha$ -(1,4)-glycosidic bond.



#### 4. Optimization of fermentation conditions for production of chitosan in a high yield

The quality and quantity of chitosan extracted from the fungal mycelia depend on the fungal strain: *Absidia*, *Gongronella*, *Rhizopus*, etc; fermentation type: solid substrate/state fermentation and submerged fermentation (batch fermentation, fed batch fermentation and continuous fermentation); fermentation medium composition: carbon source and concentration, nitrogen source and concentration, and metal ions and their concentration; fermentation conditions: inoculum size, harvesting time, fermentation temperature; and chitosan extraction procedure (Arcidiacono & Kaplan, 1992; Crestini et al., 1996; Rane & Hoover, 1993; Nwe & Stevens, 2002, 2002b, & 2006; Tan et al., 1996; Jaworska & Konieczna, 2001; Nwe et al., 2002 & 2008a). Among the different fermentation methods, SSF has shown as the best fermentation method for the production of chitosan from fungal mycelia (Nwe et al., 2002; Crestini et al., 1996). Therefore it was decided to design a solid substrate fermentor for the growth of fungus *Gongronella butleri* USDB 0201. Most researchers have been designed various types of solid substrate/state fermentors (Durand et al., 1996; Laukevics et al., 1984). Among them, tray type fermentor is practical for the production of chitosan from fungal mycelia. Based on these published solid state/substrate fermentor designs, a tray-type solid substrate fermentor was designed to use in this research (Figure 1, Nwe & Stevens, 2002).

The fermentor was designed containing 3 trays of perforated aluminium with small holes (a tray volume of about 9.6 liters and a tray-working volume of about 1.6 liters). Each tray mounted on a steamer pot and the top tray was covered by an aluminium lid (fermentor cover). The steamer pot has two holes in the wall connected with a pipe for humidified air inlet (upper pipe) and to take out condense water after completion of the sterilization (lower pipe). The air outlet pipe is mounted on the fermentor cover. Two PVC (polyvinylchloride) tubes with the diameter of 2.5 cm and length 5.5 cm were fixed vertically on each of the perforated trays to pass the humidified air through the mycelia layer. Because the mycelia layer is covering all the holes in the aluminium trays after 3 days of growth, the inlet air can pass through these PVC tubes to the next compartment above. The fermentor was inoculated while applying a laminar flow of sterilized air and then wrapped with sterilized tape on the vim between the trays to prevent contamination and to avoid leakage of the inlet air. An air pump was used to pump fresh air and pass through the air-filter. Sterilized air was first humidified in heated and sterilized water and then cooled to reach the desired inlet air temperature. The capacity of each tray for solid substrate was 850 g of peeled raw sweet potato. The cost of fermenter is low. The materials are widely available. It does not need extensive maintenance since it is very simple. Taking into consideration all these factors, this fermentor is the most suitable for production of fungal chitosan.

Sweet potato was selected to use as a cheap solid carbon source for the growth of fungus *G. butleri* USDB 0201 in small-scale fermentation. The fungus *G. butleri* USDB 0201 was grown on sterilized sweet potato pieces under sterilized and humidified air supply in the solid substrate fermentor for 7 days. The mycelium is formed on the surface and does not penetrate into the sweet potato. Therefore it is easy to separate the mycelia from the substrate at the end of the fermentation (Nwe & Stevens, 2002). Mycelia were detached from the solid substrate by flotation in water. The detached mycelia were washed with water for several times to remove remaining sweet potato pieces. Finally, the mycelia were dried to assay for the amounts of mycelia and to extract chitosan.

Firstly, fungus *G. butleri* was grown in SSF using only sweet potato without additional mineral solution for 7 days (Nwe & Stevens, 2002b). The amount of mycelia yield was  $30.1 \pm 3.2$  g/kg of solid substrate. Chitosan was extracted from dried mycelia using method II (Table 7). The production yield of chitosan extracted from fungus grown on sweet potato pieces without further supplementation was low, only 0.69 g per kg sweet potato. Sweet potato contains  $2.63 \pm 0.47$  elementary N/kg (Nwe et al., 2006). Therefore, this endogenous nitrogen is probably not available for chitosan production because it is part of existing sweet potato macromolecules (protein, DNA). The solid substrate was treated with a mineral solution as first step to improve the fermentation conditions for a better yield of mycelia and chitosan. In this treatment 1kg potato pieces was sterilized in the presence of 1L mineral solution. After sterilization, free fluid was decanted and spores of fungus *G. butleri* was inoculated and incubated in the fermentor for 0 - 8 days (Nwe & Stevens, 2002). It was observed that the amount of chitosan did not increase after 6 days of fermentation. However the production yield of mycelia increased up to 7-8 days of fermentation. The maximum production yield of chitosan, 1.54 g per kg of solid substrate was obtained from the mycelia grown on solid substrate for 7 days (Figure 12). According to these results, the best time to harvest the mycelia from solid substrate fermentation medium was 7 days of fermentation.

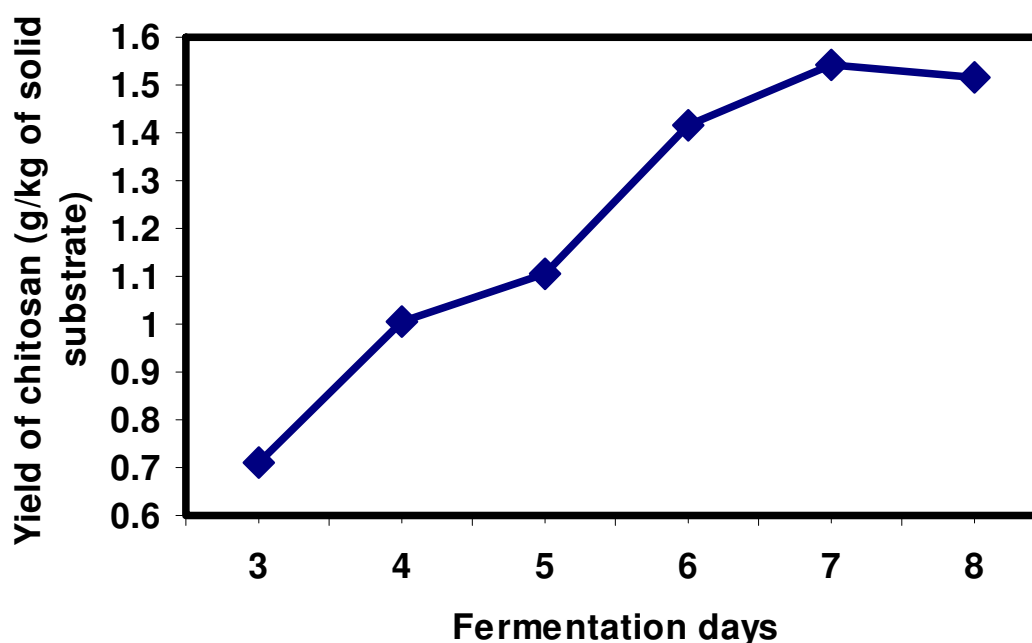


Fig. 12. Production yield of chitosan from mycelia of fungus *Gongronella butleri* USDB 0201 grown in solid substrate fermentation medium for 3–8 days (Nwe & Stevens, 2002)

The chitosan polymer has a high nitrogen content, 8% (Nwe et al., 2008). Sufficient amount of nitrogen should be available during synthesis of chitosan in the cell wall of fungus (Nwe et al., 2006). Therefore *G. butleri* USDB 0201 was grown on peeled sweet potato pieces supplied with mineral solution and different nitrogen sources: peptone, ammonium sulfate, sodium nitrate and urea (Nwe et al., 2002). It was found that the yield of mycelia and chitosan obtained from fungus grown on solid substrate fermentation medium supplied with urea was higher than that from fungus grown on solid substrate fermentation medium supplied with other nitrogen sources (Table 5). Thereafter solid substrate fermentation media were supplied with different amounts of urea and fungus was grown on these media

to produce chitosan. The yields of chitosans obtained from mycelia of fungus grown on 1 kg of solid substrate supplied with 7.2 or 14.3 g urea,  $3.59 \pm 0.29$  and  $4.31 \pm 0.65$  g respectively was not significant difference. The best fermentation medium to grow fungal mycelia was the solid substrate supplied with 7.2 g urea for 1 kg of solid substrate at pH 4.5 (Nwe & Stevens, 2004).

After that, conditions for solid substrate fermentation was optimized to improve yield of chitosan from fungus mycelia grown in solid substrate fermentation. Five fermentation parameters were varied to study the yield of chitosan and growth of mycelia on the sweet potato pieces supplied with 7.2 g urea for 1 kg of solid substrate at pH 4.5 (Nwe & Stevens, 2006). Those parameters are inoculum size ( $0.15 - 24 \times 10^8$  spores/kg SS), inlet airflow rate ( $0.4-2.3 \text{ l min}^{-1} (\text{kgDM})^{-1}$ ), humidity of inlet air (70 - 95 %), solid substrate moisture content (53 - 80 %) and outside fermentor temperature (26-35°C) (Table 8). The chitosan yield was

No	Parameter to be optimized	Conditions for variable parameters					Experimental conditions for the other parameters				
		A	B	C	D	E	1	2	3	4	5
1	Inoculum size ( $\times 10^8$ spores/kg of SS)	0.15	1.5	10	18	24	V	1.2	95	80	26
2	Inlet air flow rate ( $\text{L min}^{-1} \text{kg DM}^{-1}$ )	0.4	0.9	1.2	1.5	2.3	0.15*	V	95	80	26
3	Humidity of inlet air (% Humidity)	70	90	95	-	-	0.15	1.2*	V	80	26
4	Solid substrate moisture content (%)	80	73	53	-	-	0.15	1.2	95*	V	26
5	Outside fermentor temperature (°C)	26	29	35	-	-	0.15	1.2	95	80*	V

V, Variable conditions in that experiment;

\*Selected condition, as concluded from the previous experiment

No	Parameter to be optimized	Production yield of chitosan (g/kg of solid substrate)				
		A	B	C	D	E
1	Inoculum size ( $\times 10^8$ spores/kg of SS)	$3.28 \pm 0.22$	$3.48 \pm 0.15$	$3.52 \pm 0.14$	$3.59 \pm 0.29$	$3.38 \pm 0.23$
2	Inlet air flow rate ( $\text{L min}^{-1} \text{kg DM}^{-1}$ )	$3.42 \pm 0.44$	$3.21 \pm 0.14$	$3.28 \pm 0.22$	$3.41 \pm 0.26$	$3.74 \pm 0.19$
3	Humidity of inlet air (% Humidity)	$4.06 \pm 0.04$	$3.95 \pm 0.27$	$3.28 \pm 0.22$	-	-
4	Solid substrate moisture content (%)	$3.28 \pm 0.22$	$2.74 \pm 0.12$	$1.65 \pm 0.22$	-	-
5	Outside fermentor temperature (°C)	$3.28 \pm 0.22$	$4.60 \pm 0.16$	$0.00 \pm 0.00$	-	-

Table 8. (A) Experimental conditions for growth of fungus *G.butleri* USDB 0201 in the solid substrate fermentation and (B) production yield of chitosan from the fungal mycelia obtained from each experimental condition (Reproduced from Nwe & Stevens, 2006, Journal of Chitin and Chitosan, 11, 11-15)

increased to maximal 4.6 g per kg sweet potato under the fermentation conditions of inoculum size,  $1.5 \times 10^7$  spores/kg solid substrate; inlet air flow rate,  $1.2 \text{ l min}^{-1} (\text{kgDM})^{-1}$ ; humidity of inlet air, 95 %; outside fermentor temperature, 29°C; and moisture content of the solid substrate 80 % (Nwe & Stevens, 2006). Based on these results, it is concluded that fermentation medium compositions, fermentation conditions, harvesting time of mycelia and chitosan extraction procedure are critical factors to obtain high yield of chitosan from the fungal mycelia.

The characteristics of fungal chitosan have been determined and reported (Nwe & Stevens, 2002 & 200b; Nwe & Stevens, 2006; Nwe et al., 2006a). All chitosans have the degree of deacetylation 87 ~ 90 % (measured by UV spectrophotometry method and HPLC method) and the number average molecular weight 20-70 kDa (GPC method). The nitrogen content in the chitosan sample is 7.91% (measured by Micro-Kjeldahl method), moisture and ash content are 8.59% and 0.73% respectively (AOAC method, 1991). The viscosity of 1% (w/v) chitosan solution is 13.2 cps (measured by Brookfield viscometer). The turbidity of chitosan solution is 10-20 NTU (measured by Turbidity meter). Fungal chitosan is soluble in dilute organic acids such as 4% citric acid, 25 % formic acid, 1% lactic acid and 1 % acetic acid but insoluble in 0.2 % benzoic acid, 0.2 % cinnamic acid and 6.3 % oxalic acid. It is soluble in 0.6 % HCl and 96% H<sub>2</sub>SO<sub>4</sub> but insoluble in 3% HCl and 5% H<sub>2</sub>SO<sub>4</sub> solution (Nwe et al., 2001). The fungal chitosan has 95% purity, which has been confirmed by elementary analysis, IR, <sup>13</sup>C-NMR spectroscopy and UV spectroscopy (Nwe et al., 2008). In a number of applications of chitosan in agriculture and medical sectors, chitosan with low molecular weight and DA is more powerful than chitosan with high molecular weight and low DA (Nwe & Stevens, 2008). According to these properties, fungal chitosan is proposed to use in agriculture, food, cosmetic and medical sectors. In our research, the fungal chitosan has been applied to stimulate the growth of orchid tissue in solid and liquid tissue culture media (Nge et al., 2006) and to prepare scaffold and membrane for tissue regeneration template (Nwe et al., 2009 & Nwe et al., 2010a).

## 5. Application of fungal chitosan in plant tissue culture

In agriculture, chitosan has been used in seed, leaf, fruit and vegetable coating; as fertilizer to control release of agrochemical; to increase plant product; to stimulate the immunity of plants; to protect plants against microorganisms; and to stimulate plant growth (Devlieghere et al., 2004; Sukwattanasinitt et al., 2001; Wanichpongpan et al., 2001; Chandkrachang, 2002; Nwe et al., 2004; Hadwiger et al., 2002; Pospieszny et al., 1991; Struszczyk & Pospieszny, 1997; Bautista-Baños et al., 2003 cited in Nge et al., 2006). However the effect of chitosan depend on its molecular weight and degree of acetylation. The effect of chito-oligosaccharide (CTS-O, Kitto Life Co. Ltd., Korea) has been tested in the paddy field (Nwe et al., 2004). The Myanmar rice variety Manaw Thukha was used as seed sample and experimental paddy field was in Thanlyin, Yangon, Myanmar. The CTS-O, 1.5 g was dissolved in 100 ml of 1% acetic acid and the solution was used as CTS-O stock solution for making dilutions into different dosage. The rice seeds were soaked with water for 1 day and cleaned with water to remove the sand and mud. The cleaned seed was soaked in 500 times diluted CTS-O solution for 1 day. The CTS-O coated seed was taken out from the solution and put in a bamboo sieve and then covered with a wet gunny bag for 2 days. The germinated seeds were planted in the field after puddling. In week 4, the rice plants were sprayed with 1000 times diluted CTS-O solution. In week 7, urea, one bag (50 kg) per acre was applied. The 1000 times diluted chitosan solution was sprayed again to the paddy field in week 9. Plants were harvested in week 18. Non chitosan treated rice seeds served as control. It was found that the leaves of the CTS-O supplied plants were greener than those of control plants. After one month, the CTS-O supplied plant had a length of 22.5 inches, compared to 15 inches for the control. After three months, the CTS-O supplied plants had 8 crops and the control plants had 4 crops (Figure 13). The rice yield from CTS-O supplemented field was 1.6 times higher per acre than control field (Nwe et al., 2004).



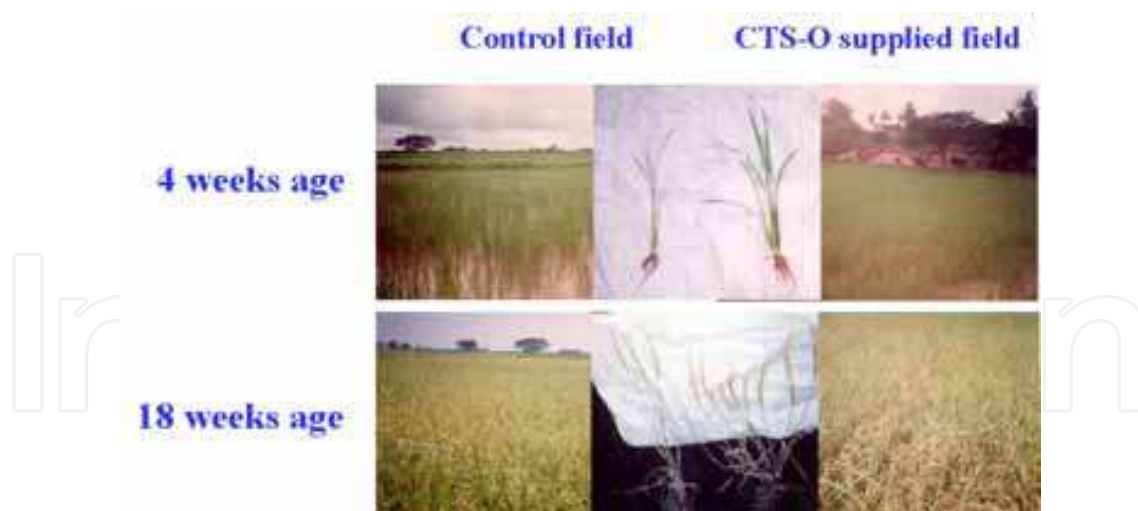


Fig. 13. Effect of chito-oligosaccharide on yield of rice

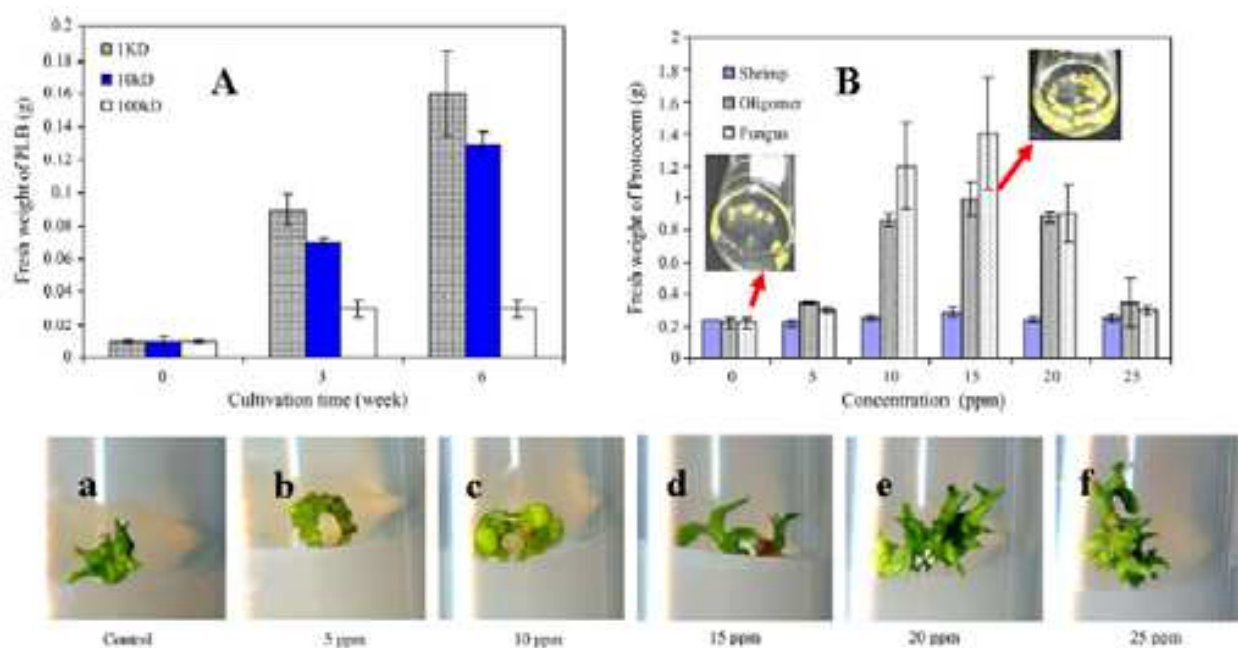


Fig. 14. (A) Effect of molecular weight of chitosan on growth of orchid protocorm-like bodies (PLB's) in VW liquid medium supplemented with 15 ppm of shrimp chitosan with molecular weights of 1 kDa (chito-oligosaccharide), 10 kDa and 100 kDa. (B) Effect of various concentrations of shrimp chitosan (10 kDa), shrimp chito-oligosaccharide (1kDa), and fungal chitosan (10 kDa) on the propagation of total fresh weight of PLB's after six weeks cultivation in liquid VW medium. (a-f) Effect of various concentrations of fungal chitosan (10 kDa) on the production of plantlets by the fractionated PLB's explant of *Dendrobium phalaenopsis* cultivated on VW agar media at 12 weeks. (Reproduced from Nge et al., 2006, Plant Science, 170, 1185-1190)

In addition, the effect of chitosan on chili plants and orchid plants had been studied in Thailand. It was found that the application of chitosan improved the yield of plant products (unpublished data). The effect of molecular weight of chitosan on germination rate of mung bean, yard long bean and soy bean seeds has been studied. The beans showed a higher

germination rate in the presence of chitosan with a molecular weight of 40 kDa (Nwe et al., 2006b). Furthermore the effect of different concentration of fungal chitosan on green gram bean production was studied at Government Technological College, Kyaukse, Myanmar. The field trial was completed in 10 weeks and non-chitosan treated field served as control. The results showed that the number of pod per plant (30 pods/plant), number of seed per pod (12 seeds/pod) and production yield (3500 kg/ha) increased by soaking the seeds with 20 ppm and by spraying the field with 100 ppm chitosan solution when compared with other fields supplied with 0, 50, 150 and 200 ppm chitosan solution (Han et al., 2006). In order to confirm these results, chito-oligosaccharide, fungal chitosan (10 kDa) and shrimp chitosan (10<sup>6</sup> Da) were used to propagate the plantlet from orchid protocorm in liquid and on solid tissue culture medium (Nge et al., 2006). The highest number of plantlets was observed in the presence of 20 ppm using either 10 kDa fungal chitosan or 1 kDa chito-oligosaccharide. High molecular weight chitosan (100 kDa) had no stimulating effect. The 10 kDa fungal chitosan was more effective compared with 1 kDa oligomer. The data in tissue culture and in the field proved that low molecular weight chitosan in the dosage of 15–100 ppm to plant results in a considerable positive effect on plant growth (Figure 14).

## 6. Application of fungal chitosan in tissue engineering

Pure chitosan is non-toxic, polar compound and bioadhesive and bioactive polymer; it has free of antigenic effects, biocompatible and biodegradable properties, and antimicrobial and hemostatic activities; and it can accelerate tissue regeneration and wound healing (Vandevord et al., 2002; Onishi et al., 1994; Kim et al., 2001; Maslova & Krasavtsev, 1998; Shigemasa et al., 1998; Tokura et al., 1994 cited in Nwe & Stevens, 2008 & 2008a). It has been used to prepare a variety of forms such as powders, hydrogels, fibers, membranes, beads and porous scaffolds that have been tested in many medical and biological applications (Tamura et al., 2006; Madihally & Matthew, 1999 cited in Nwe & Stevens, 2008 & 2008a). For tissue engineering applications, chitosan scaffolds have been prepared by the freeze-drying and freeze-gelation method and by a 3-axis robotic arm dispensing system and chitosan membranes have been prepared by solvent-casting method (Gravel et al., 2006; Ng et al., 2004; Chupa et al., 2000; Huang et al., 2005; Vandevord et al., 2002; Madihally & Matthew, 1999; Thein-Han & Kitiyanant, 2006 cited in Nwe & Stevens, 2008a).

In our research, scaffolds were prepared using fungal chitosan by the freeze-drying method and membranes of fungal chitosan were prepared using a solvent-casting method. The mechanical and biological properties of the fungal chitosan scaffolds and membranes were evaluated and compared with those of scaffolds and membranes prepared using chitosans obtained from shells of shrimps and crabs and bone plates of squids (MW 10<sup>5</sup>-10<sup>6</sup> Da and DA 10-20%) (Nwe et al., 2009 & 2010a). It was observed that the fungal chitosan scaffold had excellent mechanical and biological properties than shrimp, crab and squid chitosan scaffolds (Figure 15). However the fungal chitosan membrane had lower mechanical properties and higher degradation rate than membranes of shrimp and squid chitosan (Nwe et al., 2010a).

For wound therapy, nowadays artificial skin is available, but it is very expensive. The biopolymers such as chitosan, gelatin, alginate and bacterial cellulose are cheap and can be obtained easily. Aim of our present study is to develop an artificial skin using biopolymers that are low in production costs and in more than one beneficial way for wound healing. In which, there needs to be solved the problems in lowering the mechanical and water holding properties and cellular activities of the fungal chitosan membrane.

A	Parameters	Chitosans from various sources				
		Shells of Shrimp SHCTS 10 <sup>6</sup> Da	Shells of Crabs CRCTS 10 <sup>5</sup> Da	Bone plates of squids SQCTS 10 <sup>5</sup> Da	Fungal mycelia FCTS 10 <sup>4</sup> Da	
	Pore size of chitosan scaffold (μm)	64 ± 20	77 ± 22	54 ± 17	84 ± 21	
	Porosity of water absorbed scaffold (%)	88 ± 4	90 ± 3	96 ± 5	97 ± 1	
	Amount of absorbed water in scaffold (g/g of scaffold)	36 ± 4	43 ± 3	46 ± 0	53 ± 2	
	Fibroblast cell no. (x 10 <sup>5</sup> cell / cm <sup>3</sup> of scaffold)	0 day	0.38 ± 0.02	0.35 ± 0.04	0.33 ± 0.07	0.37 ± 0.02
		14 days	1.3 ± 0.2	2.1 ± 0.3	1.9 ± 0.3	1.8 ± 0.2
	Degradation of scaffold (%)	2 ± 0.8	5 ± 0.7	5 ± 0.5	11 ± 1.1	
	Tensile strength	Force (cN)	141 ± 37	88 ± 09	151 ± 31	209 ± 20
		Elongation (%)	15.1 ± 4.4	20.3 ± 4.2	5.4 ± 1.7	7.2 ± 1.5

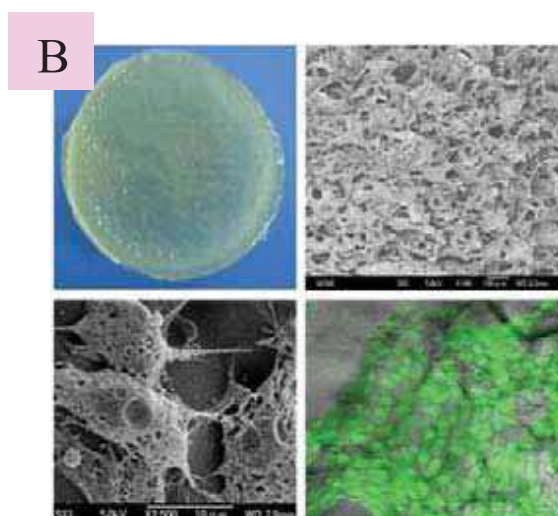


Fig. 15. (A) Characteristics of scaffolds prepared using chitosan from shells of shrimps and crabs, bone plates of squids and fungal mycelia, (B) Photo and SEM micrograph of FCTS scaffold and SEM micrograph and confocal laser microscopic image of fibroblast NIH/3T3 cells on FCTS scaffold (Reproduced from Nwe et al., 2009, Materials, 2, 374-398)

To solve these problems, alginate, bacterial cellulose (BC) and gelatin were selected based on available material and published data and the membranes were prepared. The water absorption properties and cellular activities on these membranes were examined to select the best material for the preparation of a novel membrane by incorporation with fungal chitosan for tissue regeneration template (Nwe et al., 2010b). The cells on alginate membrane crosslinked with Ca<sup>2+</sup> (AGM\_Ca) showed a spherical morphology and the NIH/3T3 cells grown on the BC membrane (BC\_M) and membrane of glutaraldehyde (GTA) crosslinked gelatin (GTA\_GM) had a polygonal morphology. The proliferation rate of fibroblasts on the GTA\_GM was faster than that on the BC\_M (Figure 16). However



cytotoxic effects of GTA were observed on the GTA\_GMs prepared with molar ratio higher than 0.033, except pork skin gelatin membrane with a molar ratio, 0.033, which showed the cytotoxic effects on fibroblast cells. In which the similar amounts of water absorbed in the AGM\_Ca and BC\_M that was lower than the amount of water absorbed in GTA\_GM.

Parameters		AGM_Ca	BC_M	GTA_GM_6
Thickness of the dried membrane (µm)		20	5	85
Area of membrane before and after water absorption (mm <sup>2</sup> )	Before	10	10	10
	After	10.7 ± 1.2	11.4 ± 1.2	24.6 ± 3.6
Dry weight of membrane (mg/cm <sup>2</sup> )		6.45 ± 1.9	1.05 ± 0.07	12.35 ± 0.78
Amount of absorbed water (g/g of membrane)		2.5 ± 0.6	3.1 ± 0.5	15.9 ± 1.1

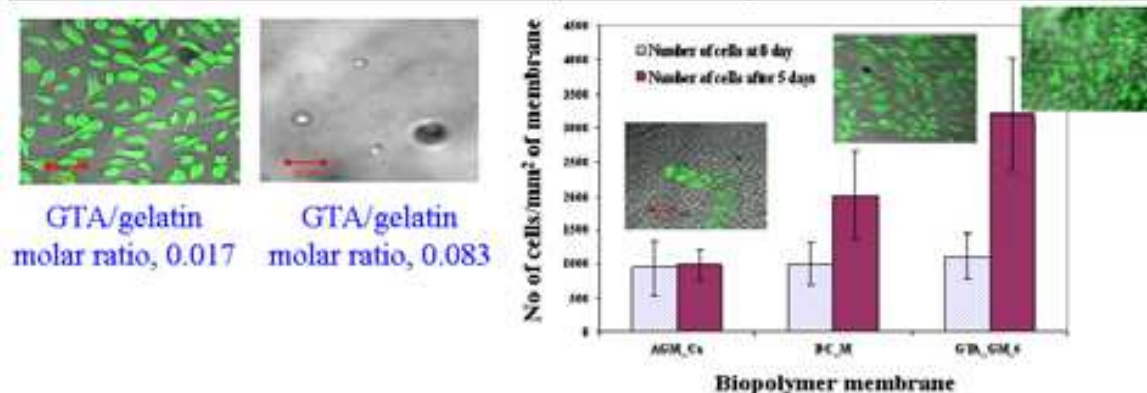


Fig. 16. Characteristics of membranes of alginate crosslinked with Ca<sup>2+</sup>, bacterial cellulose and glutaraldehyde crosslinked gelatin and attachment, morphology and proliferation of fibroblast NIH/3T3 cells on these membranes (Reproduced from Nwe et al., 2010b, Process Biochemistry, 45, 457-466)

## 7. Present situation and future prospects

We are working on fungal chitosan production and its application in agricultural and medical sectors enters to 12 years. In this research, fungal mycelia production and enzymatic chitosan extraction method have been developed to obtain high yield fungal chitosan in very easy way. This new enzymatic method is proposed to apply in large-scale production of low molecular weight chitosan from fungal source. The resultant chitosan has been tested in plant tissue culture for agriculture applications and has been tested as matrix for tissue regeneration template. The data obtained from this research point out that the selection of chitosan is one of the important factors to apply chitosan in agriculture and medical sectors. Among the tested chitosans in here, fungal chitosan shows as an excellent plant growth



stimulator to apply in agriculture sector and as an excellent scaffolding material to construct a tissue regeneration template. For further research it needs to study the effect of chitosan and its degradation products in plant and animal systems. At that moment,  $^{13}\text{C}$  labeled chitosan has been synthesized by fungus *A.coerulea* in submerged fermentation to study metabolic pathway of chitosan and its degradation pathway (Nwe et al., 2010c).

For application of fungal chitosan in tissue engineering, there need to be improve mechanical properties and water holding capacity of chitosan membranes. In recent years, significant progress has been made on the characterization of biopolymers for tissue regeneration template, gelatin has been found to be the material that can enhance the mechanical and water holding properties of membrane. Taking together, it is expected that there will be reach to the final goal on the development of a double-layered biodegradable scaffold for skin regeneration template by constructing fungal chitosan and gelatin composite membrane/scaffold in near future.

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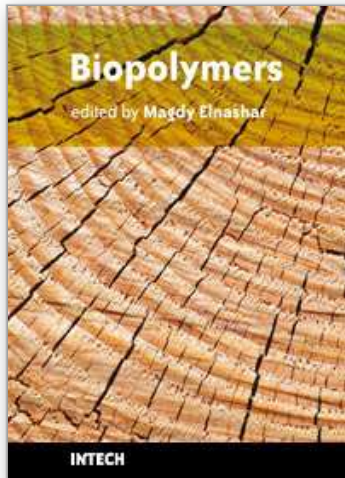


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