KMITL Sci. Tech. J. Vol. 7 No. S2 Nov. 2007

CHITIN OLIGOMERS PRODUCTION FROM FUNGAL MYCELIUM CULTIVATING ON CASSAVA STARCH MEDIUM

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ABSTRACT

The morphologies and growths of *Monascus* sp. KB20M1, *Mucor rouxii* and *Rhizopus oligosporus* were studied on MYS medium containing cassava starch as a C-source and the maximum dry mycelium weights were produced by the condition of 250 rpm of rotary shaker at 30^{o}C for 7 days, which gave the yields of 11.24, 8.08 and 8.15 g/l, respectively. The analysis of FT-IR spectrophotometer on fungal mycelium of the three-species showed the composition of chitin-chitosan by the detection of amide I group (1640-1660 cm.$^{-1}$) and hydroxyl group (2920-2930 cm.$^{-1}$). The chitin oligomers production was performed on fungal mycelium hydrolysis by biological method (chitinase) and chemical method (phosphoric acid). The retrieved product of chitin oligomers were varied due to the hydrolysis conditions and time-period. The O1 and O2 samples from enzymatic digestion and O3 sample from the chemical digestion were selected for further study of biological activity on *Phalaenopsis* growth. It was found that chitin oligomers enhanced the survival and rooting of *Phalaenopsis. Phalaenopsis* which was immersed in the O2 sample at the concentration of 1000-time dilution for 3 hr presented the maximal growth ($p<0.01$). The survival and rooting was found at 100% and the average length of rooting was about 94.00 mm.

KEYWORDS: chitin oligomers, chitooligomers, fungal mycelium, *Phalaenopsis*

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1. INTRODUCTION

Chitin, the second most abundant polymer in nature, is a co-polymer of N-acetyl-glucosamine and N-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer (Figure 1). When the number of N-acetyl-glucosamine units is higher than 50%, the biopolymer is termed chitin. Conversely, when the number of N-glucosamine units is higher, the term chitosan is used. Chitosan has been the better researched version of the biopolymer because of its ready solubility in dilute acids rendering chitosan more accessible for utilization and chemical reactions. Chitin is a major structural component in the shells or cuticles of arthropods, including crustaceans and insects, as well as nematodes, mollusks, and worms. In animal exoskeletons, chitin fibers are embedded in a matrix of proteins and colored by lipid-containing proteins [1]. Within shellfish, this orderly array is reinforced by crystalline deposits of calcium salts. The cell walls of many filamentous fungi and of a few algae contain chitin [2-4].

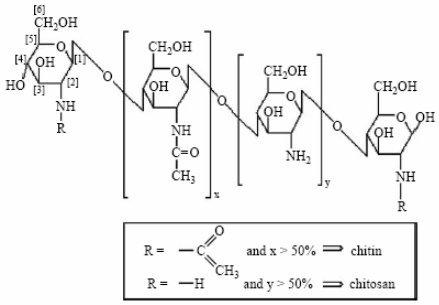

Figure 1 The structures and components of chitin and chitosan

Today, the production, chemistry and applications of chitin and chitosan are well known. Commercially, chitin and chitosan are obtained from shellfish sources such as crabs and shrimps. It is likely that future sources of chitin and chitosan will come from biotechnology innovations, especially when medical applications are the focus. This effort continues and, where biomedical applications are concerned, have become more streamlined and concentrated, a consequence of the specialist nature of applying materials to biomedical scenarios. Especially, the chitooligomers present the effecting bioactive activity.

Chitooligomers (e.g., chitobiose (G2), chitotriose (G3), chitotetraose (G4), chitopentaose (G5), chitohexaose (G6), chitoheptaose (G7)) (Figure 2) are increasingly employed in medical applications due to their interesting biological activities, such as, antibacterial [5], lysozyme inducing [6], and immunostimulating [7]. Higher chitooligomers (chitohexaose and chitoheptaose) show antitumor activity against mice sarcoma 180 [8], and they also elicit chitinase activity in plants, thus triggering defense mechanisms against fungal pathogens [9]. Recently, chitotetraose was found to have strong stimulating activity towards natural killer cells [10]. Chitopentaose is an important building block for NOD factor synthesis [11].

Chitooligosaccharides can be obtained by chemical or enzymatic methods from chitin or chitosan. Enzymatic cleavage of chitin employing chitinase [12], hydrolysis of chitosan with chitinase followed by chemical *N*-acetylation of the hydrolysates [13], as well as lysozymic hydrolysis of partially *N*-acetylated chitosans [14], were shown to be useful in preparation of various *N*-acylchitooligosaccharides. However, all these degradation methods mostly afford

mixtures of lower chitooligomers, whereas the yield of higher chitooligomers is very low, or they are not produced at all.

However, Krairak and Budda [15] successfully isolated the chitinolytic bacteria that produced the higher chitooligomers. In this study, the chitin from fungal mycelium were examined for the maximum chitooligomers production by acid digestion and enzymatic hydrolysis. The retrieved chitooligomers were testing for the bioactive activity on survival and rooting of *Phalaenopsis*

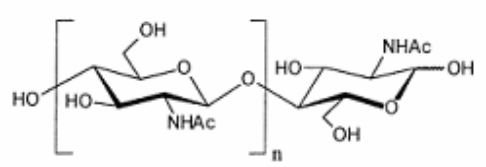

Figure 2 The structures and composition of chitooligomers. N-acetyl glucosamine (GlcNAc, n = 1; G1), Chitobiose (n = 2; G2), Chitotriose (n = 3; G3), Chitotetraose (n = 4; G4), Chitopentaose (n = 5; G5), Chitohexaose (n = 6; G6), Chitoheptaose (n = 7; G7) and Chitooctaose (n = 8; G8)

2. MATERIALS AND METHODS

2.1 Microorganisms and culture condition

Monascus sp.KB20M1[16], *Mucor rouxii* and *Rhizopus oligosporus* were received from Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The culture condition was carried out on MYS medium (composed of 2.0% starch, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract) and incubated on 250-rpm rotary shaker at 30^{o}C.

2.2 Chitooligomers production

The acid digestion of fungal mycelium chitin with phosphoric acid was performed by mixing chitin (1 g) with 85% phosphoric acid (20 ml). After stirring for a given time and temperature, digested sample was centrifuged and then the retrieved supernatant was neutralized. In case of enzymatic hydrolysis, chitin (1 g) was mixed with 50 mM borate buffer pH 8.0 (15 ml) and crude chitinase (5 ml) [17]. The enzymatic hydrolysis was carried out by gently mixing at given time and temperature. The amount of chitooligomers in hydrolyzed sample was analyzed by HPLC.

2.3 Effect of chitooligomers on survival and rooting of *Phalaenopsis* growth

Phalaenopsis orchid protocorms three months of age were obtained from the Plant Tissue Culture Laboratory, Chatuchak Weekend Market, Bangkok, Thailand. Shoot segments with one meristematic auxillary buds (6–15 mm) were washed with soap and tap water three times to remove the non-attached tissue. After that the shoot segments were rinsed several times in distilled water and air-dried for 30 min. Each *Phalaenopsis* was immersed in the retrieved chitooligomers suspension at various given time and concentration and then cultured in a pot, triplically. The survival and rooting of *Phalaenopsis* was observed for 10 weeks.

2.4 Chemical reagents

N-acetyl glucosamine (G1), Chitobiose (G2), Chitotriose (G3), Chitotetraose (G4), Chitopentaose (G5) and Chitohexaose (G6) were purchased from Nakalai company (Japan). Chitin powder was purchased from Siambionet (local company, Thailand)

2.5 Analysis

Fungal mycelium dry weight was analyzed by drying at 80°C for 48 hr and then grounded to 0.5 mm diameter. The chitin from fungal mycelium was analyzed by FTIR (Spetrum GX, PerkinElmer, USA). The chitooligomers from acid treatment and enzymatic treatment were analyzed by HPLC [17].

3. RESULTS AND DISCUSSION

3.1 Mycelium production

The growth of *Monascus* sp. KB20M1 showed the maximal mycelium weight on 3-day and 4-day of cultivation (Figure 3). By the FTIR analysis, these mycelium state presented the composition of chitin (data not shown). However, both *M. rouxii* and *R. oligosporus* showed the slower growth than *Monascus* sp. KB20M1. Therefore, the further study on chitooligomers production would be emphasized on acid and enzymatic treatment on chitin from *Monascus* sp. KB20M1 mycelium.

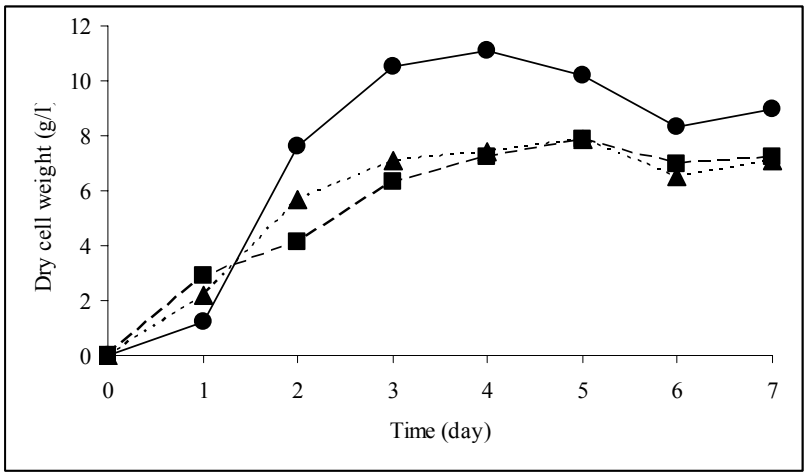

Figure 3 The cultivation of *Monascus* sp. KB20M1 (●), *M. rouxii* (■) and *R. oligosporus* (▲) on rotary shaker speed of 250-rpm at 30°C in MYS medium

3.2 Chitooligomers production

The chitooligomers production were performed by acid digestion with phosphoric acid (Table 1) and enzymatic hydrolysis with chitinase (Table 2). It was found that the acid treatment on chitin form *Monascus* mycelium gave a various chitooligomers due to the random digestion by phosphoric acid. At 40°C, the lower chitooligomers were observed with small amount of higher chitooligomers. However, at 60°C and 80°C, only the lower chitooligomers was found. The resulted showed that the more digestion time and temperature, the more amounts of lower chitooligomers were found. Jia and Shen [18] also found that the lower oligomers were received by the digestion of chitosan with phosphoric acid at higher temperature and more time. The hydrolysis of chitin by acid treatment was random resulting in various types of chitooligomers. The long chain oligomers would be further hydrolyzed into the short chain oligomers or monomer, finally, when the time was lengthened. On the other hand, the chitin hydrolysis with chitinase showed the limited cleavage and retrieved the specific chitooligomers. The more digestion period, the higher concentration of chitooligomers was received. The amount of lower chitooligomers were mainly G1 and G3. However, the higher chitooligomers of G5 and G6 were also found with very small amount. The retrieved chitooligomers should be related to the source and type of chitinase enzyme.

Three samples of the retrieved chitooligomers were selected. O1 (the enzymatic hydrolysis for 2 days) composed of G3 (76 %), O2 (the enzymatic hydrolysis for 10 days) composed of G1 and G3 about 51% and 41%, respectively, and O3 (acid digestion 8 hr at 80°C) composed of G1 (90%) would be examined for the further study on the effect of survival and rooting of *Phalaenopsis*.

3.3 Effect of chitooligomers on *Phalaenopsis* growth

Table 3 and 4 shows the % survival and rooting of *Phalaenopsis* at 10 weeks of plantation. The result of O1 showed that the higher concentration (10x dilution) and more immersion period (3 hr, 6 hr and 9 hr), the lower % survival and rooting, respectively. When O1 was diluted to 100x and 1000x), the %survival and rooting was increased. However, at 9 hr of immersion period, the % survival and rooting was low due to the amount of water penetrated into *Phalaenopsis*. O2 and O3 also showed the same result. It was concluded that the optimal concentration and immersion period related to the survival percentage and rooting of *Phalaenopsis*. However, the effect of concentration and immersion period was significant to the rooting. This might be related to the % survival of *Phalaenopsis*.

Table 1 The chitooligomers production by acid treatment with phosphoric acid at 40°C, 60°C and 80°C for 2, 4, 6 and 8 hr, respectively (concentration unit = mM)

Product	Temperature 40°C				60°C				80°C			
	Time (hr)				Time (hr)				Time (hr)			
	2	4	6	8	2	4	6	8	2	4	6	8
G1	0.302	0.295	0.060	-	0.048	-	0.054	0.064	0.050	0.050	0.238	0.068
G2	0.257	0.255	–	0.255	-	-	-	0.003	-	-	-	-
G3	-	-	0.018	0.386	-	-	-	-	-	-	-	-
G4	-	-	0.219	0.219	-	-	-	-	-	-	0.009	-
G5	-	-	-	-	0.003	-	0.004	0.002	0.005	0.005	0.003	0.007
G6	-	-	-	-	-	-	-	-	-	-	-	-

Table 2 The chitooligomers production by enzymatic treatment with chitinase at 25°C for 2, 4, 6, 8, 10, 12 and 14 day, respectively (concentration unit = mM)

Product	Time (day)						
	2	4	6	8	10	12	14
G1	1.927	7.038	7.085	12.378	9.254	8.033	10.229
G2	0.648	0.773	1.085	0.538	1.017	1.178	1.444
G3	8.663	10.448	10.022	8.154	7.385	6.466	5.666
G4	0.162	0.436	0.316	0.159	0.101	0.110	0.130
G5	-	-	0.040	-	-	0.228	0.301
G6	-	-	-	0.097	-	0.087	0.097

Table 3 % Survival of *Phalaenopsis* by immersion in O1, O2 and O3 sample at given time and dilution concentration. The result was observed at 10 weeks of plantation in comparison to the immersion in distilled water (control).

Dilution (Time)	Control*	O1 (hr)			O2 (hr)			O3 (hr)		
		3	6	9	3	6	9	3	6	9
0	60.00	66.67	100.00	33.33	66.67	100.00	33.33	66.67	100.00	33.33
10		0	0	0	0	33.33	66.67	66.67	33.33	33.33
50		100.00	66.67	33.33	33.33	100.00	100.00	100.00	66.67	0
100		100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1000		100.00	100.00	33.33	100.00	100.00	100.00	100.00	100.00	66.67

* Immersion in distilled water

Table 4 % Rooting of *Phalaenopsis* by immersion in O1, O2 and O3 sample at given time and dilution concentration. The result was observed at 10 weeks of plantation in comparison to the immersion in distilled water (control).

Dilution (Time)	Control*	O1 (hr)			O2 (hr)			O3 (hr)		
		3	6	9	3	6	9	3	6	9
0	33.33	66.67	66.67	33.33	66.67	66.67	33.33	66.67	66.67	33.33
10		0	0	0	0	0	33.33	66.67	33.33	33.33
50		100.00	100.00	33.33	33.33	100.00	66.67	100.00	33.33	0
100		33.33	100.00	100.00	66.67	100.00	66.67	100.00	100.00	66.67
1000		100.00	100.00	33.33	100.00	100.00	100.00	100.00	66.67	100.00

* Immersion in distilled water

4. CONCLUSIONS

Monascus sp. KB20M1 gave the maximal mycelium weight with chitin component in mycelium at 3-day and 4-day of cultivation. The chitooligomers production by enzymatic treatment gave the higher amount of lower and higher chitooligomers due to the specificity and type of chitinase. The random cleavage of acid hydrolysis by phosphoric acid resulted in the various chitooligomers. By using higher temperature and lengthen period, the retrieved chitooligomers was shifted to monomer (G1). The concentrations of O1, O2 and O3 at 1000x dilution and immersion period for 3 to 6 hr showed the maximal % survival and rooting of *Phalaenopsis*. Further study will be emphasized on the effect of G1 and G3, directly.

5. ACKNOWLEDGMENTS

This research has been financially supported by the Subproject: Graduate Study and Research in Agricultural Biotechnology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang under the sponsorship of the Asian Development Bank Loan and the Thai Government.

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