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Chitin Degradation by the Resting Cells of Chitinolytic Microorganism

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ABSTRACT

The chitinolytic microorganisms, isolates 18 and 19, were selected from soil samples around a region of frozen shrimp factory in Chonburi province. These isolates had high potential for using chitin as a carbon source from the chitin broth, which was composed of 1.0% chitin, 1.0% NH_4NO_3 and 3.0% yeast extract. The cultures were grown at 200 rpm, 30°C and initial pH of 6.8-7.0 for 7 days. It was found that the intact cells of isolates 18 and 19 had the chitinolytic activity of 19.51% and 20.31%, respectively. This study, the microorganisms were induced to transform the intact cells into the resting cells by freeze-thaw and ultrasonic method. The results showed that the resting cells which differentiated from the isolate 18 could be prepared by either 7 times of freeze-thaw or by 50% and 80% of sonicated amplitude for 35s and 15s, respectively. Additionally, the resting cells from these three methods had the chitinolytic activity about 31.01%, 25.06% and 25.01%, respectively. The preparation of the resting cells of isolate 19 was achieved by means of either 8 times of freeze-thaw or by 50% and 80% of sonicated amplitude for 70s and 35s, and yielded 24.78, 25.31 and 24.61% of chitinolytic activity, respectively.

Key word: chitinolytic microorganism, chitin degradation, resting cell, ultrasonic, freeze-thaw

INTRODUCTION

Since chitin and chitosan were found, their applications have been increasing dramatically. Many researches have been focused on their functions and biological properties. Recently, the oligomers of chitin and chitosan have been of interest since the various bioactivities of these compounds have been published. Chitin is a β (1 → 4)-linked 2-acetamido-D-glucopyranan. N-Deacetylation of chitin with either enzyme (Araki and Ito, 1988) or alkaline solution (Chang *et al*., 1997) produces chitosan, which is a cationic biopolymer. The non-toxic, biodegradable and biocompatible properties of chitin and chitosan provide much potential for many food, pharmaceutical, and biotechnological applications (Li *et al*., 1992; Muzzarelli, 1993; Muzzarelli, 1996). Incomplete digestion of either chitin or chitosan produced oligosaccharides consisting of β (1 → 4)-linked N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose; GlcNAc) and glucosamine (2-amino-2-deoxy-D-glucose; GlcN). N-acetyl-chitooligosaccharides (NACOs) and chitooligosaccharides (COs) have various biological properties. Hexa-N-acetyl-

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chitohexaose, $GlcNAc_6$, has immunopotent and antitumor functions (Tokoro *et al*., 1988; Tokoro *et al*., 1989; Kobayashi *et al*., 1990). Both NACOs and COs inhibit the growth of fungi and phytopathogens (Kendra and Hadwiger, 1984) and elicit the plant defense mechanisms by activating the production of pisatin, chitinase, and phytoalexin (Hirano and Nagao, 1989; Rody *et al*., 1987). These oligosaccharides affect the mitogenic response and chemotactic activities of animal cells (Inui *et al*., 1995). Both GlcNAc and GlcN also have a property to ameliorate osteoarthritis but GlcNAc has flavor taste (Sashiwa *et al*., 2002). There have been rapid advances in the understanding of the biological functions and various novel preparation methods of NACOs production.

The production of chitooligosaccharides by chemical hydrolysis showed the major products of monosaccharide and small amount of oligosaccharides due to the reaction-in-series mechanism (Chang *et al*., 1997). The ratio $GlcNAc_2$ to $GlcNAc_6$ related to acid concentration and reaction temperature. It was shown that parallel formation of oligosaccharides was occurred during hydrolysis and was similar to the specific splitting of short polymer chain (Wang *et al*., 1995). In case of the enzymatic hydrolysis, the end product of chitooligomers depended on the enzyme specificity. This method provides the specific product but a slow reaction rate. On the other hand, biological digestion of chitin by intact cells gave higher performance than enzymatic digestion of chitin. However, digested products could be consumed by the intact cell. Therefore, the biotransformation of intact cells into resting cells for chitin digestion was studied. The resting cells deserved the enzymatic digestion ability without consumption of digested product. This report focuses on resting cell preparation, chitin digestion activity and accumulation of reducing sugar, which is the product of their digestion.

MATERIAL AND METHODS

Microorganism

The isolates 18 and 19 were selected from composed shrimp waste around the frozen shrimp factory in Chonburi province (Krairak and Budda, 2002). These isolates were examined for chitin digestion and production of reducing sugar by intact cells and resting cells, respectively.

Medium

The colloidal chitin slant (colloidal chitin, 1.0%; peptone, 1.0% and yeast extract, 0.5%) was used as stock culture. Nutrient broth (NB), composed of 0.5% peptone and 0.3% meat extract, was used for cell propagation. The chitin medium composing of 1.0% chitin powder, 1.0% NH_4NO_3 and 0.1% yeast extract was used for chitinolytic experiment.

Culture conditions

One loop of isolates 18 and 19 in stock culture, was transferred to the chitin medium. The cultivation was grown at 200 rpm and 30°C.

Resting cell preparation

One loop of isolates 18 and 19 was transferred to 250-ml flask which contained 100 ml of NB medium, and then cultured at 30°C and 300 rpm until the mid-log phase. Then, cell mass was collected by centrifugation at 10,000 rpm and washed twice with sterile distilled water. Cell mass was resuspended with distilled water. The 10-ml of cell suspension was treated for the resting cell by freeze-thaw and ultrasonic methods. By freeze-thaw method, cell suspension was frozen at -80°C for 30 min and then thawed at room temperature until it became liquid and number of repeat process as indication. For ultrasonic method, cell suspension was exposed to various amplitudes of 20-kHz ultrasound for various lengths of times.

Analysis

Cell growth was evaluated by absorbance of 600 nm and cultured on NB. The resting cells retrieved from freeze-thaw method and ultrasound method, was identified by the lethal dye staining and colony forming on nutrient agar (NB with 1.5% agar). Colorless-staining cell indicates cell viability due to the permeability cell membrane protected the diffusion of lethal dye into the cell. The cell division caused the colony forming on agar medium due to the functional of cell wall, which indicated the intact cell. Therefore, the resting cells presented the colorless-staining cell (permeable function) with non-colony forming (no cell division) on agar medium. The percentage of chitinolytic activity was examined by the ratio between the amount of chitin residue at 7-day of cultivation and the amount of initial chitin in chitin medium. The amount of chitin was received by filtration on filter paper and then drying at 80°C for 24-48 hr. The high chitinolytic activity referred to the more ability of chitin digestion. Reducing sugar determination was performed by Nelson-Somogyi method by using N-acetyl glucosamine as a standard agent (Nelson, 1944).

RESULTS AND DISCUSSIONS

Microorganism characteristics

The soil samples from frozen shrimp company were screened for chitinolytic microorganisms by cultured on colloidal chitin agar. The chitinolytic microorganisms, which were produced clear zones, were selected and examined the cell morphologies. The isolates 18 and 19 were selected due to the large clear zone. The cell properties and chitinolysis in chitin medium are shown in Table 1.

Microbial growth on chitin broth

Figure 1 showed the microbial growth and reducting sugar accumulation of isolates 18 and 19 cultured on chitin broth grown at 30°C, 200 rpm for 7 days. From Figure 1, isolate 19 yielded the maximum cell number at 3-day of cultivation, whereas the cell number of isolate 18 peaked at 4-day of cultivation. The reducing sugar of isolate 19 reached at the maximum reducing sugar concentration at 2-day of cultivation and then gradually declined as a result of an increase in cell number, and the digestion products were more consumed. This similar profile of reducing sugar accumulation was also obtained from isolate 18. However, the reducing sugar concentration was higher in isolate 19 than that of isolate 18 during the course of cultivation.

The results showed that the intact cell had the ability of chitin digestion and produced the oligomers of chitin and then some of these digested products were consumed by intact cell, too. The maximum reducing sugar accumulation by isolates 18 and 19 were about 15 and 20 mg/ml and then, finally, reduced to 15 and 10 mg/ml, respectively.

Microbial growth curve on NB medium

The growth curve profiles of isolates 18 and 19 were examined on NB medium grown at 200-rpm and at 30°C. The cell number was estimated by optical density at 600 nm and on viable cell count on NB medium. Figure 2

Table 1 The characteristic properties of isolates 18 and 19.

Isolate	pH		Chitinolytic activity	Cell characteristics	
	Initial	Final	(%)	Size (nm)	Shape
18	6.8	6.9	19.51	0.6	coccus (G+)
19	6.8	7.1	20.31	0.3	coccus (G-)

demonstrated the growth curve of isolates 18 and 19. It was found that the maximum cell concentration of both isolates was reached by 13 hr. However, the mid-log phase status was reached within 7 hr of cultivation. At the mid-log phase, cell wall is very susceptible to destruction. Thus, the mid-log phase cell status would be applied for the further experiment for making resting cells by disability of cell division.

The resting cells preparation from isolates 18 and 19 by freeze-thaw method

The 10-ml of cell suspension in cryotube was frozen at -80°C for 30 min and then thawed at

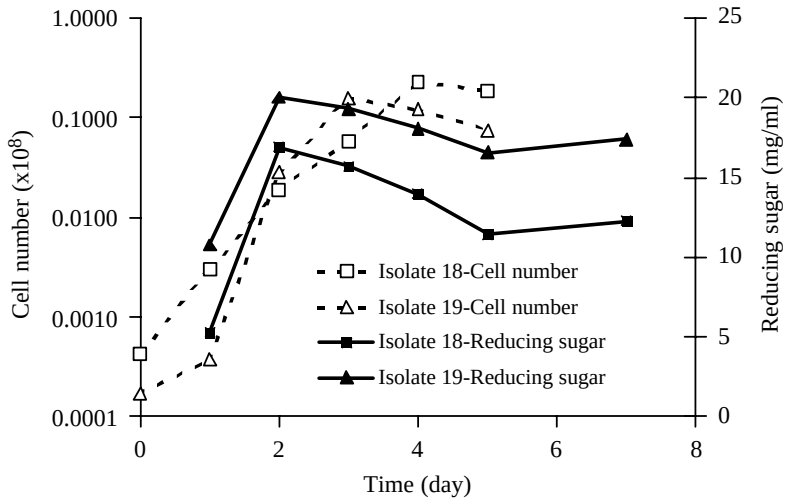

Figure 1 Cell number and Reducing sugar accumulation of isolates 18 and 19 during 7-day of time-course cultivation.

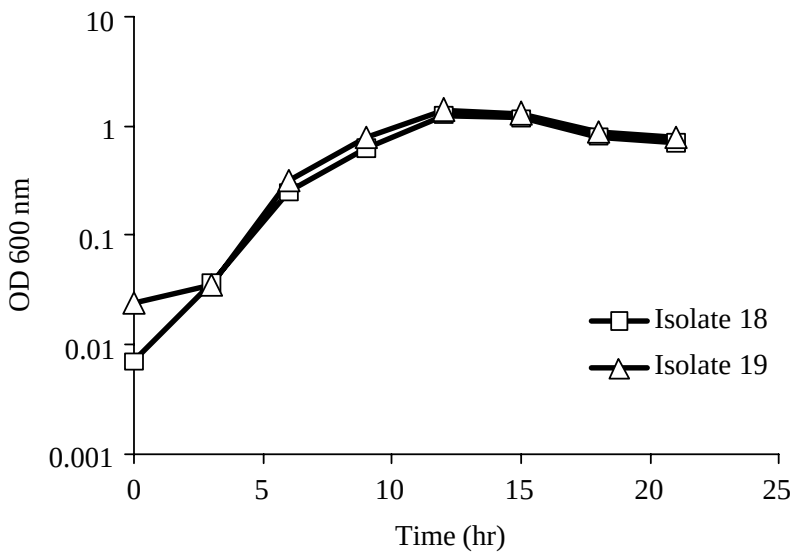

Figure 2 The growth curves of isolates 18 and 19 on NB.

room temperature until it became liquid. The treated cells were examined for resting cells property, which showed colorless-staining of lethal dye and non-colony forming on agar medium. It was indicated that the intact cells of isolates 18 and 19 were transformed into the resting cell by 7 times and 8 times of freeze-thaw method, respectively. (Table 2)

The resting cells preparation from isolates 18 and 19 by ultrasonic method

With ultrasonic method, the exposing time was related to the amplitude of 20 kHz of ultrasonic. For the isolate 18, the 35s and 15s of 50% and 80% amplitudes could transformed the intact cells to the resting cells but, the cell of isolate 19 was resistant to ultrasonic application. The effective amplitudes and exposing time were 50% and 80% for 70s and 35s, respectively (Table 3). The low and high amplitudes (50% and 80%) were chosen for the further studies as the time-period of ultrasonic exposure that might cause the different level of cell wall disability (such as injured cell or non-living cell), even though both methods gave the same property of resting cells, which had the permeability and non-colony forming function.

Chitinolytic activity of resting cells preparation by freeze-thaw method

The resting cells of isolates 18 and 19 prepared by 7-time and 8-time of freeze-thaw, were examined for the chitinolytic activity in chitin suspension. The 10-ml of resting cell suspension was inoculated and then incubated at 30°C and 200 rpm. Figure 3A showed the chitinolysis by resting cell of isolate 18. The level of reducing sugar accumulation was increased and reached the maximum concentration within 3-day of incubation and then sharply decreased. A decrease in reducing sugar could be the assimilated by the recovery cell. The alteration of cell property from resting cell to intact cell might occur due to the damaged level of cell wall.

In case of chitinolysis by resting cells of isolate 19, the level of reducing sugar was gradually increased until 5-day of incubation and then reduced. This indicated that recovery cells appeared and consumed the reducing sugar after 6-day of

Table 2 Effect of freeze-thaw on cell viable of isolates 18 and 19 in NB medium.

Number of freeze-thaw	Growth level	
	Isolate 18	Isolate 19
1	+++	+++
2	+++	+++
3	+++	+++
4	++	+++
5	++	++
6	+	++
7	-	+
8	-	-
9	-	-
10	-	-
11	-	-

Remark;
+++, high growth level; ++, moderate growth level; +, low growth level; -, no growth

incubation (Figure 3B).

When comparing the reducing sugar production of the intact cells and resting cells, it was found that the chitinolysis by intact cells showed the low reducing sugar accumulation because the viable cells consumed the digested products resulting in cell number increase. The accumulation of reducing sugar was higher by

Table 3 The growth level of isolates 18 and 19 after exposing to various amplitudes of 20-kHz ultrasonic method and the growth level was examined on nutrient agar.

Time (sec)	Isolate 18				Isolate 19			
	Amplitude of 20 kHz ultrasonic				Amplitude of 20 kHz ultrasonic			
	30%	50%	60%	80%	30%	50%	60%	80%
5	+++	+++	+++	+	+++	+++	+++	+++
10	+++	+++	+++	+	+++	+++	+++	+++
15	+++	+++	+++	-	+++	+++	+++	+++
20	+++	++	++	-	+++	+++	+++	++
25	+++	++	+	-	+++	+++	+++	++
30	+++	+	-	-	+++	+++	+++	+
35	+++	-	-	-	+++	+++	++	-
40	+++	-	-	-	+++	+++	++	-
45	+++	-	-	-	+++	++	++	-
50	++	-	-	-	++	++	+	-
55	++	-	-	-	++	++	+	-
60	++	-	-	-	++	+	-	-
65	n/a	n/a	n/a	n/a	++	+	-	-
70	n/a	n/a	n/a	n/a	++	-	-	-
75	n/a	n/a	n/a	n/a	++	-	-	-

Remark;
+++, high growth level; ++, moderate growth level; +, low growth level; -, no growth and n/a, not testing

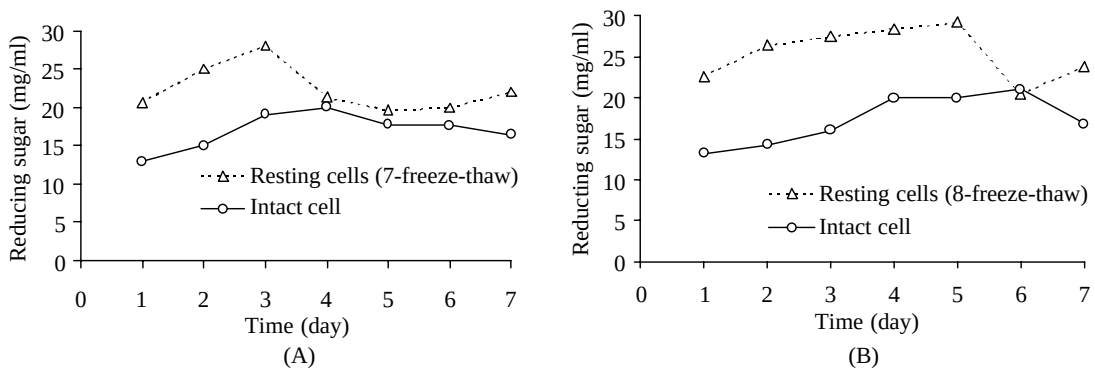

Figure 3 The chitinolytic profile of the resting cells of isolates 18 (A) and 19 (B) in chitin suspension. These cells were prepared by 7-time and 8-time of freeze-thaw, respectively.

resting cells than that by intact cells. However, the reducing sugar concentration was declined at the late incubation as a result of cell recovery.

These results assumed that the destructive level of cell wall for resting cell preparation was related to the capability of cell recovery. Even if, there was no growth in the early detection, the longer the incubation period resulted in the more cell recovery.

Chitinolyitic activity of resting cells preparation by ultrasonic method

The resting cell of isolate 18 prepared by 50% and 80% of sonicated amplitude for 35s and 15s, and isolate 19 prepared by 50% and 80% of sonicated amplitude for 70s and 35s, respectively, were examined for the chitinolysis in chitin suspension. The 10-ml of resting cells suspension was inoculated and then incubated in 200-rpm of rotary shaking speed at 30°C. Figure 4A showed the chitinolysis by resting cells of isolate 18. The level of reducing sugar accumulation was increased and reached the maximum concentration within 4-day of incubation and then gradually decreased due to the assimilation by recovery cells.

In case of chitinolysis by resting cells of isolate 19, the level of reducing sugar was gradually increased within 4-day of incubation and then reduced. This indicated that recovery cells appeared and consumed the reducing sugar after 6-day of incubation (Figure 4B). However, at the final incubation, the reducing sugar was increased due to the high chitinolytic activity of recovery cells

The chitinolytic activity of intact cells (isolates 18 and 19) in comparison to the resting cells (isolates 18 and 19), which were prepared by one freeze-thaw and two ultrasonic methods, was shown in Figure 5. The chitinolytic activity was evaluated after 7 days of incubation by subtraction the initial to final chitin dry weight. It was found that the resting cells of isolate 18 which was prepared by 7 times of freeze-thaw and by 50 and 80% of sonicated amplitude for 35s and 15s provided the chitinolytic activity of 31.01, 25.06 and 25.01%, respectively, whereas, the resting cells of isolate 19 which was prepared by 8 times of freeze-thaw and by 50 and 80% of sonicated amplitude for 70s and 35s yielded 24.78, 25.31 and 24.61% of chitinolytic activities, respectively.

CONCLUSION

The resting cells of isolates 18 and 19 had higher efficiency of chitinolytic activity (31.01% and 25.31%, respectively) than that of the intact cells (20.31 and 19.51%, respectively). It was

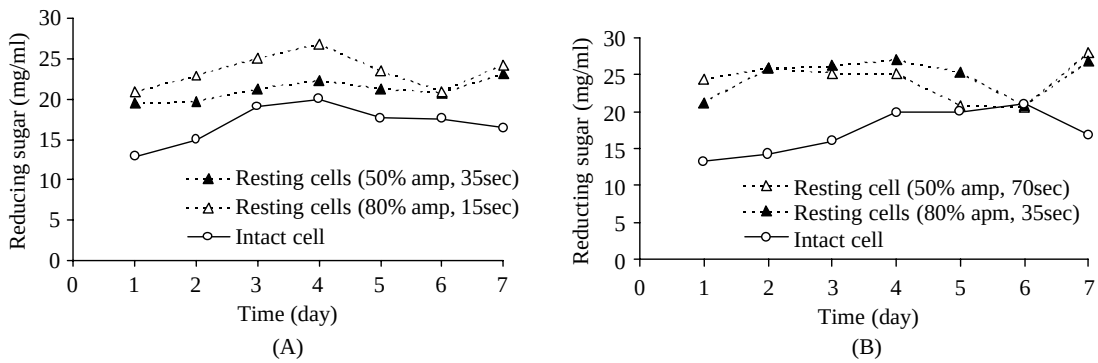

Figure 4 The chitinolytic profile of the resting cells of isolates 18 (A) and 19 (B) in chitin suspension there resting cells were prepared by 50 and 80% of sonicated amplitude for 35s and 15s, and by 50% and 80% of sonicated amplitude for 70s and 35s, respectively.

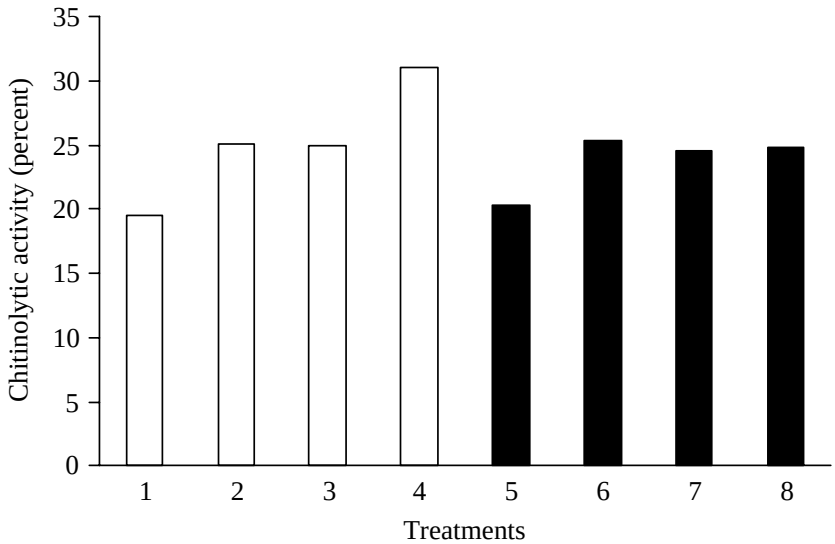

Figure 5 The chitinolytic activity of intact cells and resting cells on chitin suspension (percents).
Treatment 1: Intact cells of isolate 18 (control)
Treatment 2: Resting cells of isolate 18 by 50% sonicated amplitude with 35s
Treatment 3: Resting cells of isolate 18 by 80% sonicated amplitude with 15s
Treatment 4: Resting cells of isolate 18 by 7-time freeze-thaw
Treatment 5: Intact cells of isolate 19 (control)
Treatment 6: Resting cells of isolate 19 by 50% sonicated amplitude with 70s
Treatment 7: Resting cells of isolate 19 by 80% sonicated amplitude with 35s
Treatment 8: Resting cells of Isolate 19 by 8-time freeze-thaw

found that 2-day of cultivation resulted in high levels of reducing sugar, which then reduced gradually due to the consumption by recovery cells. Thus, for maintaining the level of reducing sugar accumulation, the digestion of chitin by resting cells would be introduced. The resting cells preparation by freeze-thaw and ultrasonic method could destroy the cell wall function, which is related to the cell division. The resting cells would be identified by both cell staining technique and non-colony forming on agar medium. An intact cells still has a permeability, which prevents cell from the entering of lethal compound such as lethal dyes and colony forming on agar medium. It was assumed that the preparation method by ultrasonic extended the period of cell recovery longer than the one of freeze-thaw method. Moreover, the higher chitinolytic activity by resting cells was probable to the increment of cell number of recovery cells.

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