CHAPTER IV

DISCUSSION

Screening of Crystalline Chitin Degrading Bacteria

Crystalline chitin degrading bacteria were isolated from soil of Thailand. Shells of prawns were buried underground in the target soil, which may have chitinase producing bacteria, such as musk in the bottom of prawn field, moisture soil around the river bank and soil which has prawn and crab shell waste. After one week, the decomposed chitin and soil was picked up and resuspended in distilled water. This suspension was spread on colloidal chitin minimum medium and incubated at 37°C. The colonies, which can produce chitinase by showing visible clear zone, were isolated and determined chitinase activity.

All colonies with clear zone were cultured on colloidal chitin minimum medium and assayed chitinase activity by using colorimetric method. Because bacteria were isolated from varied places, they had different characters such as sizes and colors of colonies, optimum condition for growth, enzyme production and amount of enzyme in each day. In order to reduce this effect, %PC/CC was used to determin high crystalline chitin degrading chitinase activity instead of activity when only powdered chitin was used as substrate.

%PC/CC is the percentage ratio of the activity of chitinase when powdered chitin was used as substrate over the activity when colloidal chitin was used as substrate. After calculating the %PC/CC, chitinase-producing bacteria were divided

into five groups. Group 1 is the group of bacteria that can not digest powdered chitin. Group 2 to 5 are the groups of bacterium that have %PC/CC less than 10% to over 40%. From this result, it presented that not all bacteria can digest high crystalline chitin although they can digest colloidal chitin and the bacteria that have high activity on colloidal chitin may not have high activity on high crystalline chitin.

Bacteria in group 5, the highest activity group, were cultured on colloidal chitin and assayed activity again to ensure the result. This second assay found that MC10 and MC11 lost activity. So the three highest %PC/CC were strains PP5, PP8 and MC27. After multiple assay, PP8, which known after identified that is *Bacillus circulans*, was selected to study further due to the producible results.

Enzyme production of *Bacillus circulans* PP8

B. circulans PP8 was cultured on colloidal chitin and powdered chitin minimum medium and enzymes activity assay at every 12 hours. From enzyme production profiles (Figure 22 and 23) show the different pattern of enzymes production in both medium. In colloidal chitin minimum medium, it shows 2 chitinase activity, which was detected at 12 hours (ChiA) and 24 hours (ChiB) and slowly dropped when colloidal chitin and powdered chitin was used as substrate for assay respectively. After 60 hours, activity with chitosan was detected. In powdered chitin minimum medium, chitinase activities were detected at the same times as in colloidal chitin minimum medium, but activity with chitosan was detected earlier, at 24 hours. This result shows that the enzyme production of *B. circulans* PP8 depends on substrate induction. When induced with high crystalline substrate, the activity of

chitosanase came earlier and higher. This suggested that *B.circulans* PP8 prefer to produce chitosanase for the degrading of high cystalline degrading. This result also suggested that *B. circulans* PP8 may produce 2 difference chitinase to digest amorphous and crystalline chitin , ChiA and ChiB, respectively.

Chitosanase is the enzyme that can digest chitosan at 3 point of glycosidic bond, which are between two glucosamine units, N-acetyl glucosamine - glucosamine, and glucosamine - N-acetylglucosamine. It can not digest the bond between two N-acetylglucosamine units, therefore chitosanase can not hydrolyzed chitin. From the result of enzyme production, it had the activity of chitosanase although it is induced with chitin and its chitosanase production was higher when induced with high crystalline chitin. Thus, *B. circulans* PP8 may also produce deacetylase to convert chitin to chitosan.

In this experiment, we may hypothesize that *B. circulans* PP8 has a pattern of enzyme production by firstly, producing two chitinases, one for amorphous substrate degrading and another for crystalline substrate degrading. Then, chitin deacetylase was produced to convert chitin oligomer to chitosan and finally chitosanase was produced to hydrolyze chitosan substrate into small sugar units.

Characterization of crude enzymes

A. Optimum pH

Two crude chitinases were collected at 12 hours (ChiA) and 24 hours (ChiB) and crude chitosanase was collected at 84 hours from colloidal chitin minimum medium and incubated with colloidal chitin, powdered chitin, and chitosan for pH range 3-10. The highest chitinase activity of ChiA was detected when incubate with colloidal chitin in McIlvain buffer pH 7. ChiB has the highest chitinase activity when incubated with powdered chitin in McIlvain buffer pH 6. Chitosanase had highest activity when incubated with chitosan in McIlvain buffer pH 7. Optimum pH of these three crude enzymes was compared with purified chitinase (Table 5) and chitosanase (Table 6) from the other organisms. Optimum pH of ChiA was observed closely to *Burkhoderia cepacia* and *Enterobacter* sp. G-1. Optimum pH of ChiB was similar to *Streptomyces* sp. J13-3 and optimum pH of chitosanase was nearly with *Bacillus* sp. CK-4.

B. Optimum temperature

The effect of temperature on enzyme activities was determined at temperature 30- 70 °C. From the temperature profiles (Figure 21), ChiA and ChiB had different optimum temperature, which are 50° and 30°C, respectively. The highest chitosanase activity was detected when incubated with chitosan at 60°C. Table 4 and 5 show the comparison of optimum temperature with the other chitinases and chitosanases.

C. Estimate molecular weight

On SDS-PAGE chitinase activities stained gel, there were 4 major bands presented after staining. This result suggested that Bacillus circulans PP8 produced at

Table 5. Comparison of the Characteristics of Chitinase from several Microorganisms

Species	Molecular weight	Optimum pH	Optimum temp(°C)	Reference
Burkhoderia cepacia				
Crude chitinase	at least 47,500	7.0	40	
Aeromonas sp. 10S-24				
ChitinaseI	12,000	4.0	50	Ueda, *et* al., 1995
Chitinase III	115,000	4.0	50	Tsujibo, *et* al., 1992
Alteromonas sp. StinO-7	70,000	8.0	50	
Enterobacter sp. G-1:				
ChitinaseA	60,000	7.0	40	Park, *et* al.,1996
Streptomyces spJ13-3	31,000	6.0	45	Okazaki, *et* al.,1993
Streptomyces olivaeoviridis	47,000	7.3	45-55	Blaak, *et* al., 1996
Streptomyces thermoviolaceus				
OPC-520	30,000	4.0	60	Tsujibo, *et* al., 1993

Table 6 Comparison of the characterization of Chitosanase from several Microorganisms

Species	Molecular weight	Opt pH	Opt temp.	Reference
Bacillus sp. CK4	29,000 +/-2,000	6.5	60	Yong, *et al.*, 2001
Bacillus cereus S1	45,000	6.0	60	Kurakarem, *et al.*, 2000
Bacillus sp. R-4	31,000	5.6	40	Tominag, 1975
Streptomyces sp. NO.6	29,000-26,000	6.0	60	Price and Stock, 1975
Martsuebacter chitosanolabidus 3000	34,000	4.0	30-40	Park, *et al.*, 1999
Bacillus sp. NO 7-M	41,000	6.0	50	Yasushi, 1995
Streptomyces griseus	35,000	8.0	37	Akiro, 1998
Aspergillus sp. Y2K	25,000	6.5	65-70	Chi yu, *et al.*, 2000

least 3 major chitinase, which have molecular weight approximately 200, 55, and 42 kDa. The comparison of chitinase molecular weight was presented in Table 4.

Detection of transformant containing chitinase gene

Transformants containing chitinase gene were screened on colloidal chitin medium with amplicilin, X-gal, and IPTG. The colonies that produced visible clear zone on plate indicated that they might have chitinase gene. Two colonies from 1,800 colonies presented positive result. To confirm this clear zone came from recombinant plasmid which containing chitinase gene, recombinant plasmid was extracted and retransformed into the other hosts, which also presented visible clear zone after transformation. This suggested that two recombinant plasmid contain chitinase gene. To further confirm that recombinant plasmid of two clones were not the same, they were cut with *Pst*I and partially map with other restriction enzymes to determined insert fragments. Clone 847 and 1691 show different insert fragments and restriction pattern, which are approximately 6 and 7 kb respectively. Moreover the clear zone of them is also different. Clone 847 produces visible clear zone around colony whereas clone 1691 produces a little clear zone under its colony.

Estimate molecular weight of chitinase from recombinant clones

Chitinase activities stained were done after SDS-PAGE. Clone 847 presents one major chitinase band approximately 55 kDa, which is the same as in *Bacillus circulans* PP8. Clone 1691 was not determined due to its low activity.

Detection of chitinase activity from recombinant clones by colrimetric method

From the result in Table 4, clone 847 and clone 1691 had chitinase activity with both colloidal chitin and powdered chitin. Crude chitinase from clone 847 digested colloidal chitin as well as powdered chitin, while chitinase from clone 1691 digested powdered chitin better than colloidal chitin. These seem to chitinase from clone 1691 was specific with powdered chitin like ChiB from *Bacillus circulans* PP8. However, there was low chitinase activity from both recombinant clones when compared with chitinase from *Bacillus circulans* PP8. Thus, chitinase overproduction is nescessary for the future studies.