CHAPTER III

RESULT

1. Screening of Crystalline Chitin Degrading Bacteria

1.1 Isolation of chitinase producing bacteria

Chitinase producing bacteria were isolated from four soil sources, Thailand. Fifty from two hundred colonies showed visible clear zone on colloidal chitin minimum medium. Most of chitinase producing bacteria were isolated from Mahachai soil, 32 colonies. Others were: one colony with clear zone from Pattaya soil, seven colonies from Chachengsao, and ten colonies from PP Islands. All of them were grown for chitinase production and activity assay.

1.2 Screening for high crystalline chitin degrading chitinase

Chitinase activity was assayed by colorimetric method. According to the % PC/CC and ability to digest crystalline chitin, we can divide them into 5 groups (Figure 15). Group 1 is the members that cannot digest powder chitin. Group 2, 3, and 4 are the members that have %PC/CC less than 10%, 10-29%, and 30-39% respectively. Group 5, has the highest %PC/CC, which is over 40%, has 8 members (Figure 16). The average of %PC/CC ± SD of them are 42.19 ± 6.9, 47.2 ±2.9, and 34.13± 4.4 respectively. Since the activity of PP5 fluctuated from previous assay, strain PP8 was selected for futher study.

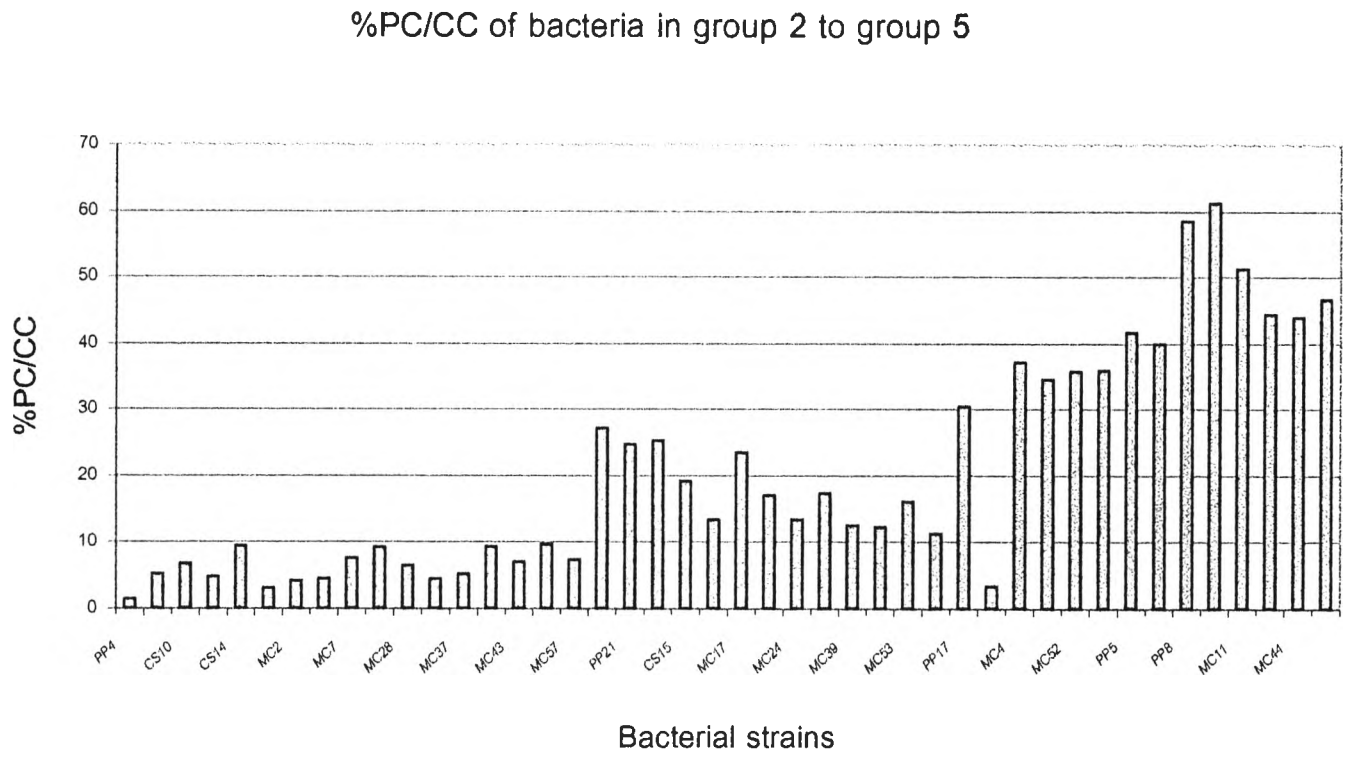

Figure 15. %PC/CC of chitinase producing bacteria from group 2 to group 5. The members in group 2 has %PC/CC less than 10%. Other, 10-29% in group3, 30-39% in group4, and over 40% in group5.

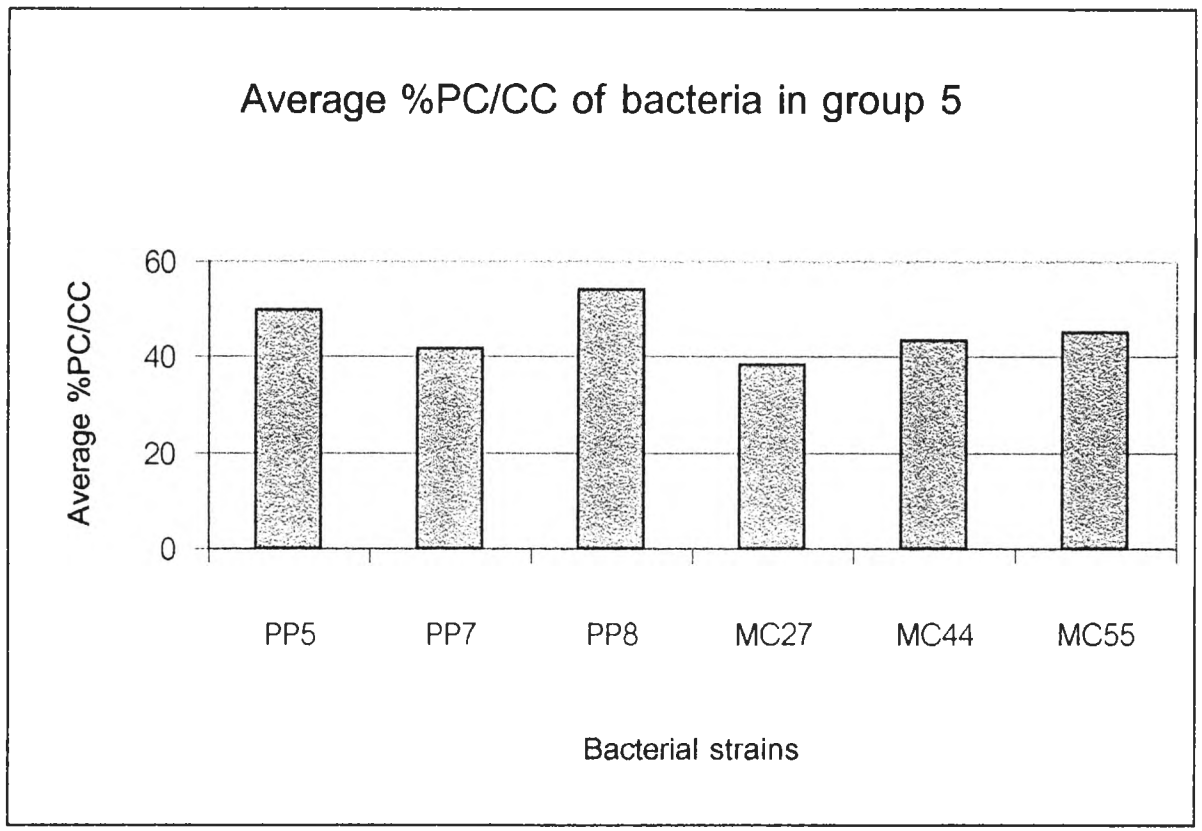

Figure 16. Average %PC/CC of members in group5. PP8 has the highest average %PC/CC, 54%.

1.3 Identification of bacteria strain PP8

Bacteria strain PP8 (Figure 17) was identified by using biochemical method and found that it is *Bacillus circulans* PP8 (Appendix A).

2. Characterization of Crystalline Chitin Degrading Enzyme of *Bacillus circulans* PP8

2.1 Enzyme Production

Bacillus circulans PP8 was cultured on 2 culture medium; colloidal chitin minimum medium and powdered chitin minimum medium. Crude enzymes were collected every 12 hours and assayed for chitin-degrading activities with 3 substrates; colloidal chitin, powdered chitin, and chitosan. The enzyme production profiled is shown in Figure 18 and 19, respectively.

In colloidal chitin minimum medium, activity with colloidal chitin was detected at 12 hours and then slowly dropped. Powdered chitin degrading activity was detected at 24 hours and vanished at 48 hours. After 60 hours chitosanase was detected and peaked at 84 hours.

In powdered chitin minimum medium, chitinase activity with colloidal chitin and powder chitin was the same as in colloidal chitin minimum medium, but chitosanase activity was detected earlier, at 24 hour.

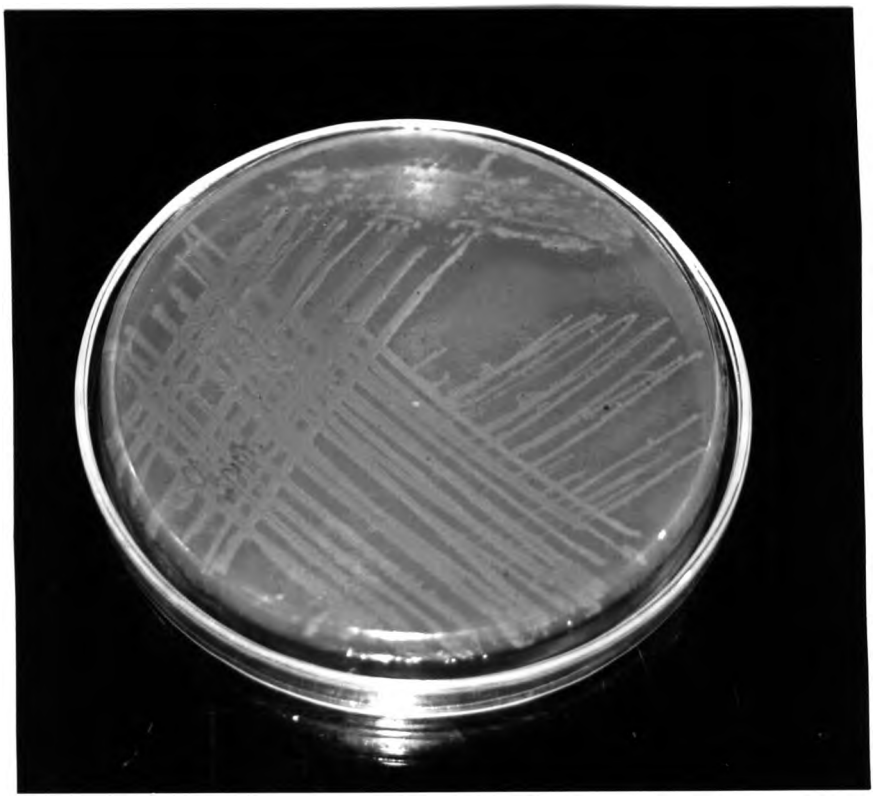

Figure 17. *Bacillus circulans* PP8 on colloidal chitin minimum medium plates.

Bacillus circulans PP8 was grown on minimum medium supplemented with 0.2% colloidal chitin. The culture was grown for 3 days at 37 °C

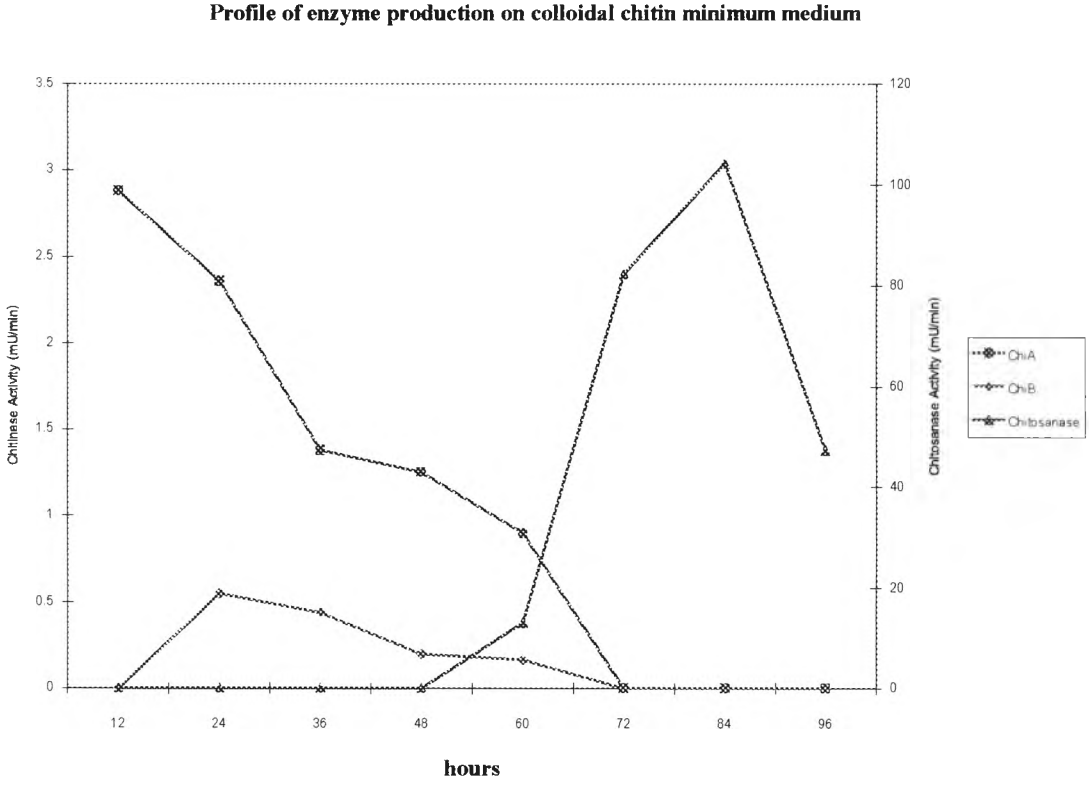

Figure 18. The profile of enzyme production from *Bacillus circulans* PP8 when cultured in colloidal chitin minimum medium. ChiA and ChiB presented chitinase activity at 12 and 24 hours before slowly dropped. Chitosanase activity was detected after 60 hours.

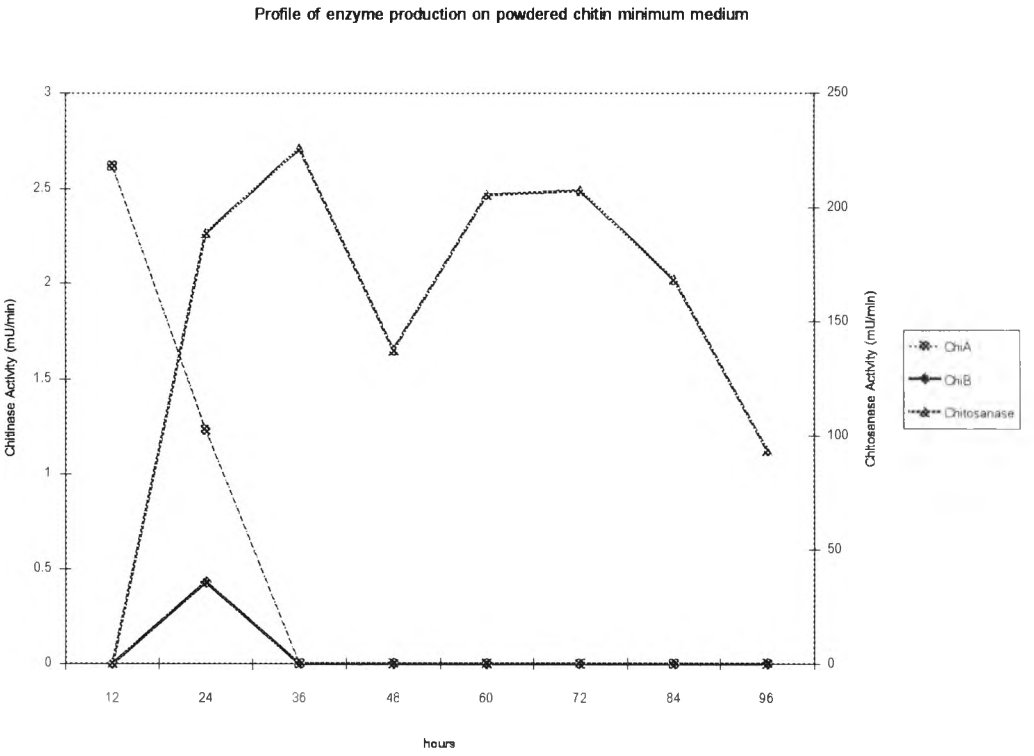

Figure 19. Profile of enzyme production from *Bacillus circulans* when cultured on powdered chitin minimum medium. ChiA and ChiB show a little chitinase activity. On the other hand, chitosanase activity came earlier. And much more

2.2 Optimum pH

From the profile of enzyme production in colloidal chitin minum medium, there were 3 activities with 3 substrates at the different time. Thus crude enzymes were collected at 12 hours (ChiA), 24 hours (ChiB), and 84 hours (chitosanase).

ChiA, ChiB, and chitosanase were incubated with colloidal chitin, powdered chitin, and chitosan respectively in pH range 3-10. After assayed activity by colorimetric method, optimum pH of ChiA, ChiB, and chitosanase are 7, 6, and 7 (Figure 20).

2.3 Optimum Temperature

ChiA, ChiB, and chitosanase were incubated with colloidal chitin, powdered chitin, and chitosan respectively in temperature ranges 30-70°C. Optimum temperature of ChiA, ChiB, and chitosanase were 50, 30, and 60 °C (Figure21).

2.4 Estimate molecular weight of chitinase from *Bacillus circulans* PP8

Bacillus circulans PP8 was grown in colloidal chitin minimum medium and collected crude enzyme at 12, 24, and 84 hours for polyacrylamide gel electrophoresis. Crude enzyme at 12 hours showed chitinase activity with molecular weight between 42 to 50 kDa, whereas 42 and 55 kDa at 24 hours crude enzyme

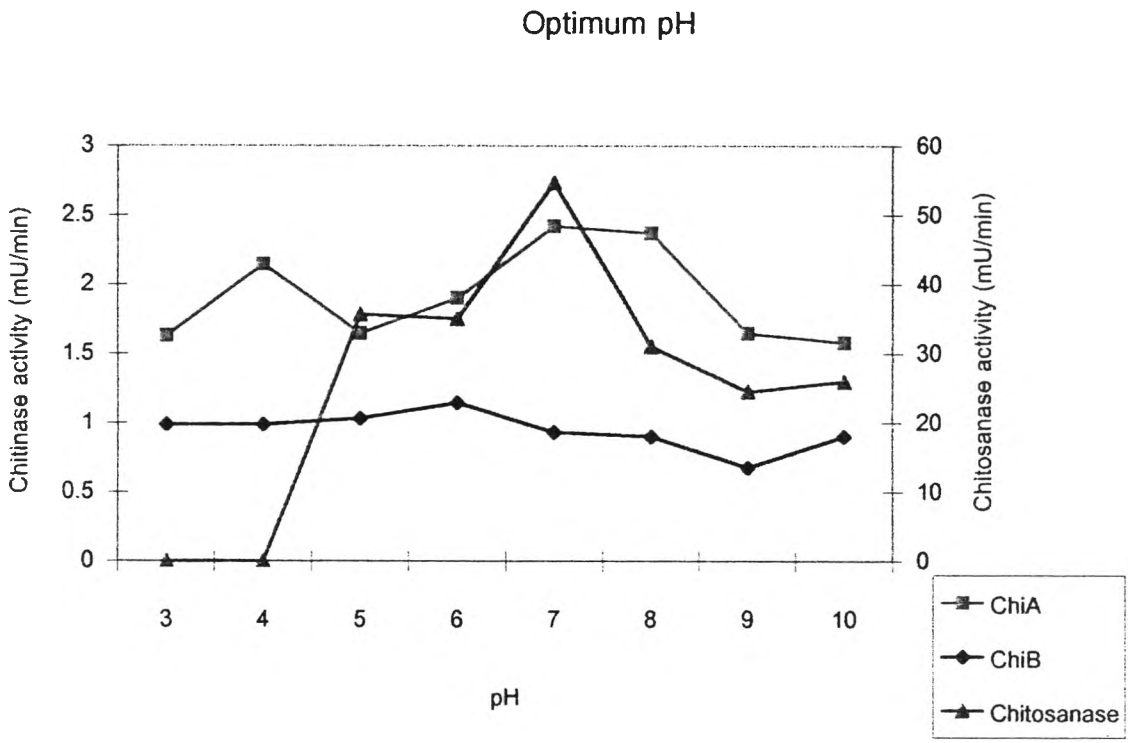

Figure 20. Effect of pH on enzyme activities. Enzyme activities were measured at pH range from 2-10, using colloidal chitin , powdered chitin and chitosan as substrate.

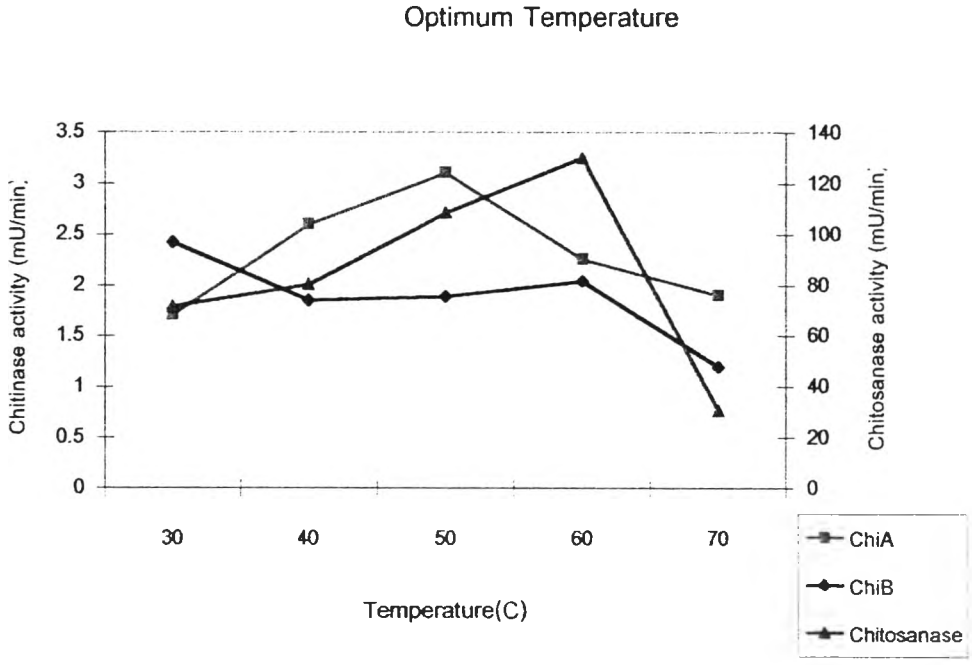

Figure 21. Effect of temperature on enzyme actitvity. Enzymes activities were measure at 30,40,50,60, and 70 ^{o} C.in buffer by using colloidal chitin, powdered chitin, and chitosan as substrate.

(figure 22). Crude enzyme at 84 hours represented chitinase activity at least 4 bands, which are approximately 200, 116, 55, and 42 kDa (Figure 22 and 25).

3. Cloning

DNA fragments sizes 2-9 kb was cloned by shot gun cloning into *E.coli* DH5α. Transformants were screened on colloidal chitin medium with 100mg/ml ampicillin, 20 mg/ml X-gal, and 25mg/ml IPTG. From 1,800 colonies, 2 recombinant clones; Clone 847 (Figure 23) and Clone 1691 were found .

4. Determination of recombinant plasmid

Two recombinant plasmid were cut with *Pst*I to analyze the insert sizes. Clone 847 has approximately 6 kb insert size whereas a 7 kb was found in clone 1691 (Figure 24).

5. Detection of chitinase gene by SDS-PAGE.

After refolding the protein, chitinase activity from clone 847 was detected at 55 kDa, which is also present in *Bacillus circulans* PP8 crude enzyme (Figure 25). Clone 1691 produced a little clear zone on colloidal chitin medium and can not detected chitinase activity band on gel.

6. Detection of chitinase activity by colorimetric method

Clone 847 and 1691 were grown on colloidal chitin minimum medium

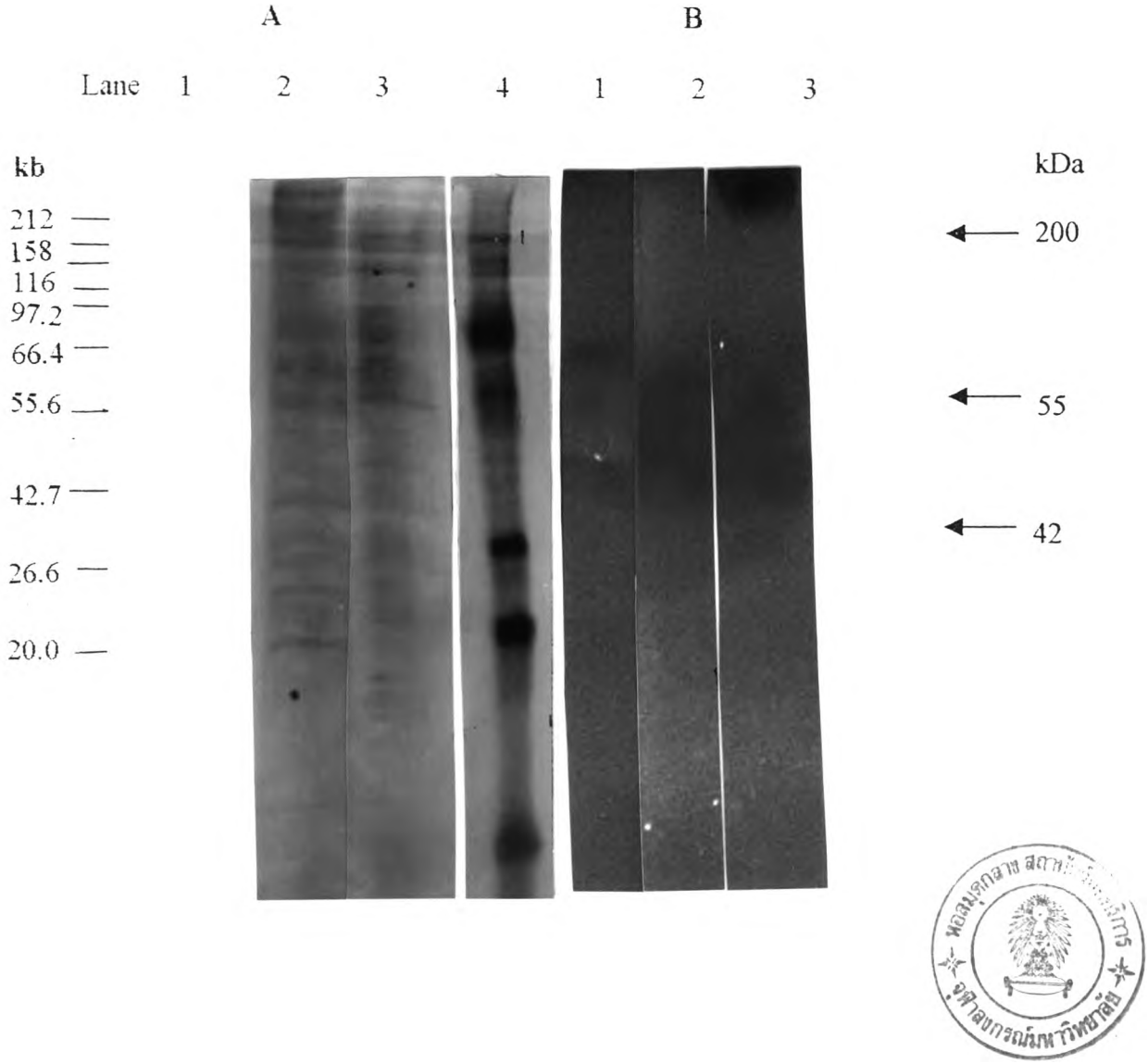

Figure 22. SDS-PAGE of Chitinase activity from *Bacillus circulans* PP8

Panel A: Protein stain

Lane 1	protein mareker
Lane 2	crude enzyme at 12 hours
Lane 3	crude enzyme at 24 hours
Lane 4	crude enzyme at 84 hours

Panel B: Activity stain

Lane 1	crude enzyme at 12 hours, 42-50 kDa
Lane 2	crude enzyme at 24 hours, 42 and 50 kDa
Lane 3	crude enzyme at 84 hours, 200, 55, and 42 kDa

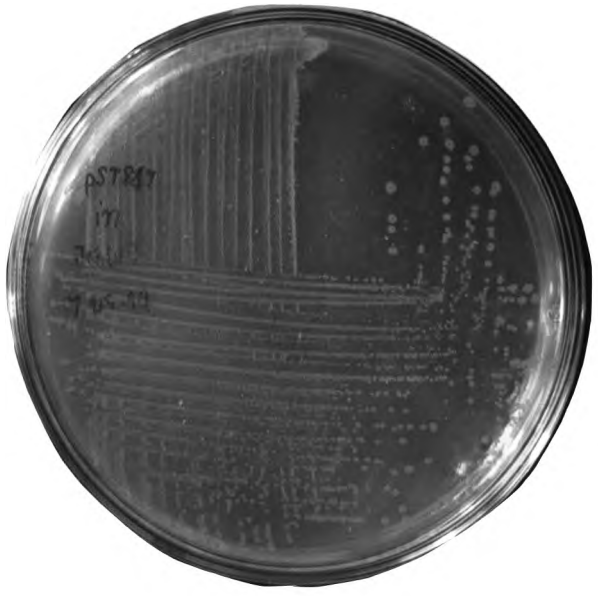

Figure 23. Clone 847 on colloidal chitin medium

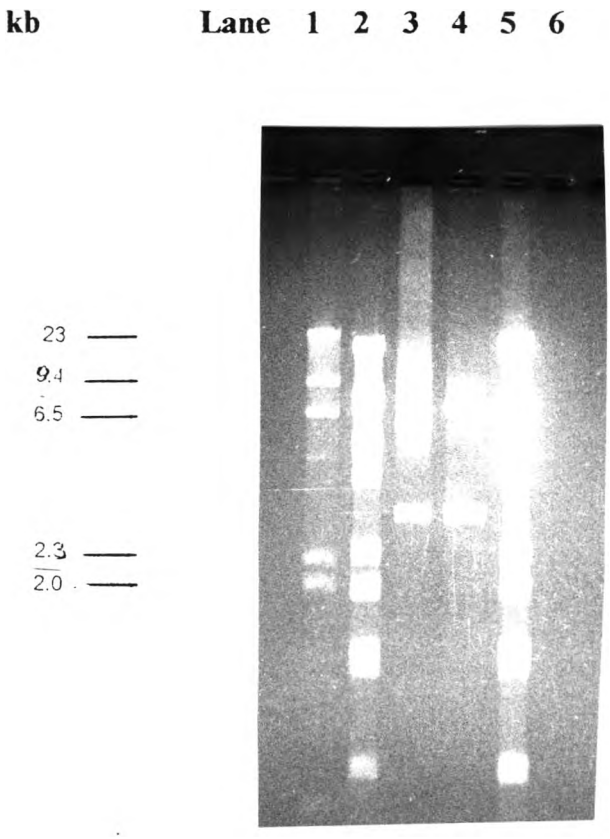

Figure 24. Determination of insert fragments. Recombinant plasmid were cut with *Pst* I to analyze the insert fragments. When cut with *Pst*I, clone 847 has approximately 6 kb and clone 1691 has approximately 7 kb in size of insert fragment.

Lane 1	Lambda/*Hind*III
Lane 2	Lambda/*BstEII*
Lane 3	p847/*PstI*
Lane 4	p1691/*PstI*
Lane 5	Lambda/*BstEII*

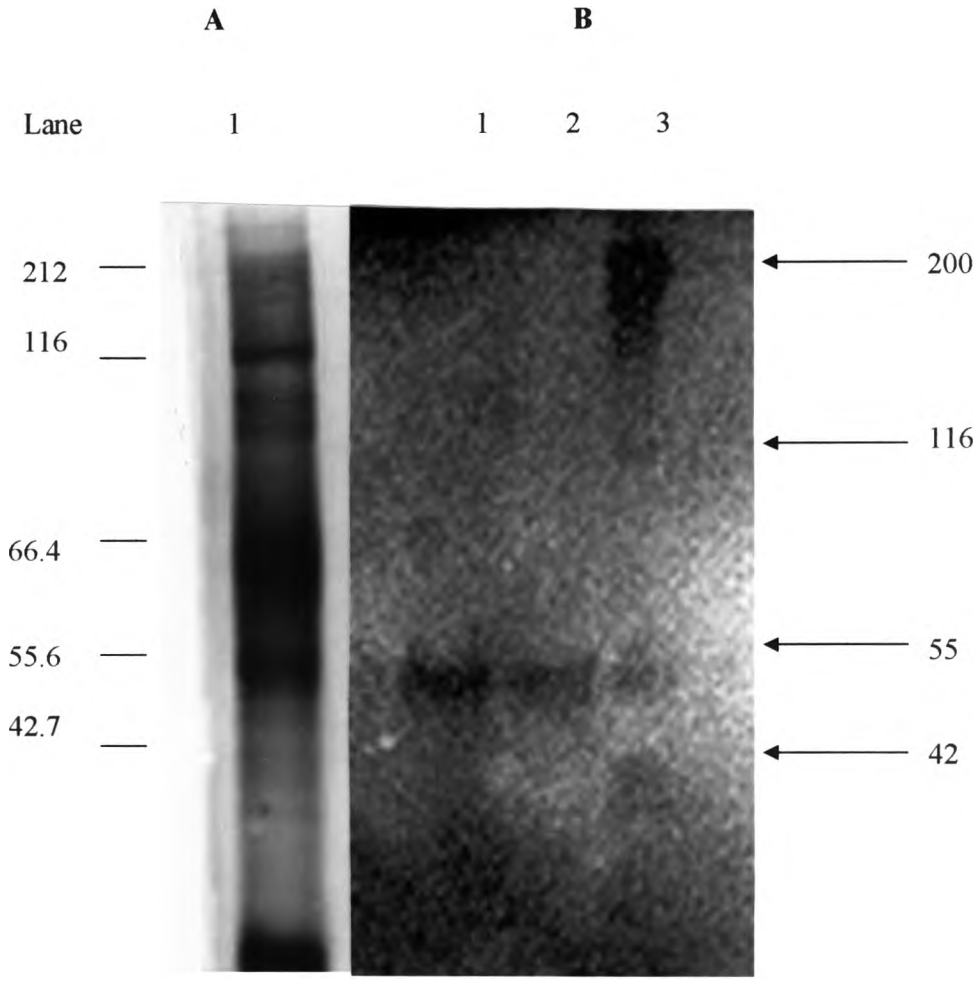

Figure 25. SDS-PAGE of chitinase activity from clone 847.

Panel A: Protein stain

Lane 1 marker

Panel B: Activity stain

Lane 1 activity bands from clone 847 (load 30 µl)

Lane 2 activity bands from clone 847 (load 20 µl)

Lane 3 activity bands of crude chitinase from *Bacillus circulans* PP8

for one week for chitinase assay. Crude enzymes were incubated with two substrates, colloidal chitin and powdered chitin. Chitinase activities with colloidal and powdered chitin of clone 847 were 23.02 and 21.42 mU, respectively. Chitinase activity with colloidal and powdered chitin of clone 1691 were 21.68 and 25.68 mU respectively (Table 4).

Table 4. chitinase activities with colloidal chitin and powdered chitin of recombinant clones.

Bacterial strains	Chitinase activity with colloidal chitin (mU/h)	Chitinase activity with powdered chitin (mU/h)
Clone 847	23.02	21.42
Clone 1691	21.68	25.68