CHAPTER IV

DISCUSSION

Screening, isolation and identification of a thermotolerant bacterium producing chitinase.

A thermotolerant bacterium, SK-1, which has chitinolytic activity was isolated from soil by conventional plate assay which is simple and easy to perform. Thus, this method was selected for screening of chitinolytic producers from numbers of bacteria isolates. SK-1 can grow in LB medium at 30-60 °C, with optimum growth at 45-50 °C indicating that it is a thermotolerant bacteria, as thermotolerant or facultative thermophilic bacteria have been defined to have a maximum growth temperature (T_{max}) of 55 °C (Wiegel and Ljungdahl, 1986). Morphological and physiological characteristics of strain SK-1, clearly demonstrated that SK-1 belong *Bacillus licheniformis*. This classification is consistent with the result from 16S rRNA sequence comparison. From these results, the SK-1 was identified and classified as *Bacillus licheniformis*. *B. licheniformis* has been shown to produce extracellular chitinolytic enzyme (Takayanagi et al., 1991; Trachuk et al., 1996 and Tantimavanich et al., 1997) and is one of the most efficient chitinolytic producers among *Bacillus* species (Cody, 1989 and Clark, 1956). Our results on quantitative chitinolytic enzyme measurement showed that *B. licheniformis* SK-1 was one of the strongest chitinolytic producer.

Enzyme production

B. licheniformis SK-1 produced chitinolytic enzyme continuously during exponential phase of growth, when grown in 0.02% CCMM. The highest level of chitinolytic enzyme was found on the second day of cultivation. Moreover, when bacterium entered the stationary phase, the activity of chitinase in the culture medium remains constant. This suggests that chitinolytic enzyme activity from *B. licheniformis* SK-1 was stable through out the cultivation period. SK-1 could produce higher amount of chitinolytic enzyme in 0.02% CCMM at 50 °C. Chitinolytic enzyme

production was investigated at cultivation temperatures of 30, 40, 50 °C. It was found that the bacterium yielded maximum chitinolytic activity, 134 mU/ml at 50 °C. Production of chitinolytic enzymes from *B. licheniformis* was studied in various cultivation medium. It was found that the culture medium with colloidal chitin yielded maximum chitinolytic activity, 74 mU/ml, where only 21 mU/ml was found in the LB medium. Although the chitinolytic enzyme could be produced constitutively by *B. licheniformis* SK-1, the addition of chitin to the culture media greatly enhanced the enzyme production. This suggested chitinase gene in *B. licheniformis* SK-1 is an inducible gene and can be induced with the presence of chitin in the culture medium. This result was similar to chitinase production in *Serratia macescens* (Vyas et al., 1989), the high chitinolytic activity was produced in the medium when it contained *S. rolfsii* cell wall as the carbon source. Deane et al., (1998) also found that in mycoparasitic strain, *Trichoderma harzianum*, the chitinolytic enzyme was produced when cultured in the medium with the presence of chitin. The bacterium can produce higher amount of chitinolytic enzyme when the amount of colloidal chitin was increased. Chitinolytic enzyme production was studied at colloidal chitin concentrations of 0.02%, 0.04%, 0.08%, 0.16% and 0.2% (w/v) in CCMM. It was found that the culture medium with 0.08% CCMM yielded maximum chitinolytic activity, 184 mU/ml. Further increase of the amount of colloidal chitin to 0.16 or 0.2 (w/v) did not enhance the production of chitinolytic enzymes.

Characterization of crude enzyme

The crude enzyme from *B. licheniformis* SK-1 gave maximum activity at temperature 60-70 °C. The activity of the crude enzyme exhibited a broad pH range between 5-10, with optimum activity at pH 5.0-6.0. The crude enzyme has the highest hydrolytic activity on regenerated chitin, followed by colloidal chitin, powdered chitin, 45% deacetylated chitin, 80% chitosan and flaked chitin. The relative hydrolytic activity on crystalline chitin were 22% and 17% of the activity observed on amorphous and soluble chitin, respectively. The result suggest that crude enzyme contains endochitinase. The crude enzyme was able to hydrolyzed chitosan, but the activity drops when the percent deacetylation of the substrate increase. This suggests that the crude enzyme was able to hydrolyze only β-1,4 glycosidic bond between N-

acetylglucosamine not glucosamine residues. Chitinolytic activity in crude enzyme in the cultured medium after second day, showed that at least 8 protein bands with chitinolytic activity with molecular weight of 72, 70, 66, 60, 58, 38.5, 30 and 20 kDa, respectively, on 10% SDS-PAGE. There are two products, monomer (N-acetylglucosamine) and dimer (N,N'-diacetylglucosamine), when colloidal chitin was completely hydrolyzed with crude enzyme. It suggests that crude enzyme contains both chitinase and chitobiase activity. Most bacterial chitinolytic enzymes produce a mixture of monomer and dimer. Crude enzyme *B. licheniformis* SK-1 produced 75% of monomer and 20% dimer from β-chitin within 6 days (Pichyangkura et al., 2002).

Characterization of partial purified enzyme

The crude enzyme was partially purified by colloidal chitin affinity absorption and by DEAE cellulose column chromatography. The partial purified enzyme was analyzed on the 7.5% native PAGE. A single protein band was found. The isoelectric point (pI) of partial purified chitinase was 4.62. The result was similar with pI of chitinase from *A. hydrophila* subsp. *anaerogenes* A52, 4.6 (Yebuki et al., 1986), and the chitinase M and S from *Bacillus* strain MH-1, pI values of 4.8 and 4.7, respectively (Sakai et al., 1998). The molecular weight of the partial purified chitinase from *B. licheniformis* SK-1, was estimate by SDS-PAGE. The partial purified chitinase showed a major band with molecular weight 72 kDa and 2 minor bands with molecular weight 70 and 58 kDa. The result was similar to that of the molecular mass of chitinase from *B. licheniformis* TP-1 (Tantimavanich et al., 1998)

The partial purified chitinase showed optimum activity at 2 pHs: 6 and 8, which was similar to the chitinase from *B. licheniformis* 6839 (Trachuk et al., 1996) using colloidal chitin as substrate. The optimum pH observed in a number of chitinases seems to be dependent on the substrate used. For analytical purposes, the soluble substrates glycol chitin and N-acetyl chitooligosaccharides were used instead of chitin. The optimum pH of chitinase toward glycol chitin was observed at a slightly alkaline pH or at two pHs compared with the short substrates such as the N-acetyl chitooligosaccharides and their derivatives. For example, the chitinases from the silkworm (Koda et al., 1997) and plant yam (Tsukamoto et al., 1984) showed two

optimum pH values such as 4 and 8-10 toward glycol chitin. However, these chitinases show only one optimum pH I the acidic pH range, such as pH 4-6 toward the N-acetyl chitooligosaccharides. This fact may be due to the chitin-binding ability of chitinase or to the existence of another chitin binding domain. The chitinase with a high chitin-binding ability would show two optimum pHs in the reaction with glycol chitin. However, certain yam chitinase shows only one optimum pH at 4 even during the reaction with glycol chitin. Therefore, the two optimum pHs do not necessarily come from the substrate glycol chitin.

The optimum temperature for the enzyme activity was found to be 55 °C at pH 6.0. Whereas chitinase from *S. marcescens* (Robert and Cabib, 1982), *Xanthomonas* sp. (Sakka et al., 1998), and *Aeromonas* sp. (Yabuki et al., 1986) were optimally active at acidic pH 4.0. *B. licehniformis* SK-1 enzyme was stable over the pH range of 6-8 at 4 °C for 5 day.

The K_m and V_{max} for colloidal chitin of the partial purified chitinase which determined from the Lineweaver-Burk plot was 0.023% (w/v, 0.23 mg/ml) and 7.03 U/mg, respectively. This value was comparable to that of many chitinase previously reported. Chitinases reported in each bacteria seemed to have very different from this study. We found that the K_m of partial purified chitinase from DEAE peak2 was less than other bacterial chitinase. The K_m value implies the dissociation constant of an enzyme-substrate complex; therefore, the smaller the K_m value, the stronger the affinity toward the substrate. The parameter V_{max}, which measures the maximum velocity, indicates the rate constant for the reaction from the enzyme-substrate complex to enzyme and product. The kinetic parameter values for long substrates such as chitin, colloidal chitin, CM-chitin (carboxymethyl chitin) and glycol chitin differ from those for short substrates such as N-acetyl chitooligosaccharide. Perhaps the long substrates are the real substrates for chitinases.

Cloning and characterization of crude enzyme from *chi66*

One chitinase gene, *chi66* gene, was cloned by PCR. The PCR product, 2.0 kb fragment, harboring *chi66* gene was cloned into pGEM-Teasy resulting in pSKChi66. pSKChi66 was nucleotide sequenced. One open reading frame of 1,797 bp encoding for a 598 amino acid protein, corresponding to a protein with molecular weight of 66

kDa was found. The data obtained from the deduced amino acid sequence comparison revealed that *chi66* is similar to ChiA of *B. subtilis* and Chi of *B. licheniformis* TP-1, 84% homology. Chi66 showed optimum activity at 2 pHs: 5 and 9, which was similar to the chitinase from *B. licheniformis* 6839 (Trachuk et al., 1996), using colloidal chitin as substrate. The optimum temperature was observed at 60-70 °C. The molecular mass determination of the cloned chitinase on SDS-PAGE followed by activity staining showed 3 bands with chitinase activity at 70, 65 and 58 kDa, which was similar to that of *B. licheniformis* SK-1. The 65 and 58 kDa chitinase activity bands are probably the proteolytic products of the 70 kDa protein. When the cloned enzyme was used to hydrolyze colloidal chitin, $(GlcNAc)_2$ was the major product.