

## Characteristics of Thermostable Chitinase Enzymes of *Bacillus licheniformis* Isolated from Red Palm Weevil Gut

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**Abstract:** *Bacillus licheniformis* strains (A2 and A35) were isolated from Red Palm Weavils' gut (*Rhynchophorus ferrugineus*). The bacteria were identified by 16sDNA. *B. licheniformis* strains were grown in media containing 0.5% chitin at a temperature of 50°C and a pH of 7.0. The highest production of A2 was observed on day four while A35 was observed on day six. The chitinases were purified through ammonium sulfate fractionation, dialysis, heat treatment for 3 hr and chromatographed onto DEAE Sepharose CL-6B. The optimum temperature and pH of purified chitinase were found at 70°C and pH 5.

**Key words:** Red Palm Weevil, *Rhynchophorus ferrugineus*, gut microbiota, *Bacillus licheniformis*, thermostable chitinase

### INTRODUCTION

Chitin is insoluble polysaccharide composed of linear chains of  $\beta$ -1,4- *N*-acetylglucosamine (GlcNAc) residue that are highly cross-linked by hydrogen bonds. It is found in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, lobsters and in the internal structures of other invertebrates (Bhattacharya, 2007). As for many other enzymes substrate, chitin is being used as a strong promoter to boost up extracellular chitinases formation. Chitinases are capable of degrading chitin directly to low molecular weight of chitooligomers, which have broad range of a agricultural, industrial and medical functions such as anti-tumor activity and elicitor action (Lee, 2007; Yuli, 2004).

Chitinases (EC 3.2.1.14) are glycosyl hydrolases group of enzymes that vary widely in size (20 kDa to about 90 kDa). Bacterial chitinases have a molecular weight range of ~20-60 kDa, which is similar to that of plant chitinases (~25-40 kDa) and are smaller than insect chitinases (~40-85 kDa) (Bhattacharya, *et al.* 2007). Chitinases can be produced by many bacteria, including *Aeromonas* (Sitrit, 1995), *Alteromonas* (Tsujiyo, *et al.* 1993), *Bacillus* (Watanabe, *et al.* 1990), *Serratia* (Jones, *et al.* 1986), *Streptomyces* (Blaak and Schrempf, 1995), *Enterobacter* (Chernin, *et al.* 1995), *Vibrio* (Bassler, *et al.* 1991.) and *Escherichia* (West and Colwell, 1984). Chitinase-producing bacteria were isolated from different environments including soil (Wang, 1997), garden and park waste compost (Poulsen, 2007), shellfish waste (Wang and Hwang, 2001) and from hot springs (Yuli, 2004).

This paper deals with the characterization of thermostable chitinase excreted from *Bacillus licheniformis* strains isolated from Red Palm Weavils' (RPW) gut (*Rhynchophorus ferrugineus*).

### MATERIALS AND METHODS

#### **Microorganisms:**

*Bacillus licheniformis* strains and others strains were isolated from Red Palm Weavils' gut. The isolates were identified by PCR amplification and sequencing of PCR product for analysis of 16S rDNA (Khiyami and Alyamani, 2008). Chitinolytic bacteria are typically detected and screened through the appearance of clearing zones when grown on chitin agar plates containing ( $g^{-1}$ )  $K_2HPO_4$  0.7,  $KH_2PO_4$  0.3,  $MgSO_4$  0.5,  $FeSO_4$  0.01,  $ZnSO_4$  0.01,  $MnCl_2$  0.01,  $(NH_4)_2SO_4$  0.25, yeast extract 0.2 and 1.0% colloidal chitin and maintained at pH 7. The plates were incubated at 30°C for 24 - 48 hr. The stock cultures were maintained at -80°C in 20% glycerol. The working cultures were maintained on agar slants of the same media at 4°C.

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**Enzyme production:**

The production of chitinases was carried out in a basal medium containing ( $\text{g}^{-1}$ )  $\text{K}_2\text{HPO}_4$  0.7,  $\text{KH}_2\text{PO}_4$  0.3,  $\text{MgSO}_4$  0.5,  $\text{FeSO}_4$  0.01,  $\text{ZnSO}_4$  0.01,  $\text{MnCl}_2 \cdot 0.01(\text{NH}_4)_2\text{SO}_4$  0.25 and yeast extract 0.2, with 0.5% colloidal chitin and maintained at pH 7. The inoculated flasks were incubated at 50°C on a rotary incubator shaker operated at 100 rpm. The maximum production of chitinase was monitored during a period of 6 days using the supernatant fluid as the source of chitinase and colloidal chitin as the substrate. The enzyme activity was measured by incubating one ml of supernatant with one ml of 0.5% colloidal chitin in 50 mM citrate phosphate buffer, pH 7.0, at 50°C for 15 min. The mixture was centrifuged immediately at 10,000 rpm for 5 min.

The amount of the reducing sugars released was determined by Imoto and Yagishita method (Imoto and Yagishita, 1971). The amount of monomer released was extrapolated from the standard graph of GlcNAc. A unit of enzyme activity was defined as the amount required for producing one micromole of GlcNAc per minute under the specified conditions. The activities in both assays were mean of three independent values and standard deviations were less than 5% of the mean.

**Extracellular protein:**

The samples were centrifuged at 3,000×g, 4°C for 25 min and neutralized by 0.01 M KOH. The concentrations of extracellular protein were determined using Bradford reagent. Protein concentration was determined spectrophotometrically at 595 nm by using a spectrophotometer (model PD-303, APEL Co. Ltd). Bovine serum albumin was used to establish a standard curve for extracellular protein concentration (50-400  $\mu\text{g}/\text{ml}$ ;  $r = 0.999$ ).

**Purification of chitinase:**

Chitinase Purification was carried out at 4°C. After the growth of *Bacillus licheniformis* strains for 6 days in the colloidal chitin medium, the culture medium was centrifuged at 10,000 X g for 20 min to sediment cells and any undegraded chitin. To obtain crude enzyme, the supernatant fluid was subjected to precipitation with ammonium sulphate to 80% saturation and stirred for 2 hr. The precipitate was allowed to stand overnight and then collected by centrifugation at 10000 g at 4°C for 30 min. The precipitated pellets were dissolved in 50 mM citrate phosphate buffer, pH 5.0 and dialyzed against the same buffer which was then partially purified by heating for 3 hr at 70°C.

The enzyme mixture was loaded onto the DEAE Sepharose CL-6B column equilibrated with the 50 mM citrate phosphate buffer. The enzyme was eluted with a linear gradient of 0–1M NaCl in 50 mM citrate phosphate buffer, pH 7.0. The active fractions were pooled, dialyzed against the same buffer and concentrated through freeze dry.

**Enzyme assay:**

The enzyme reaction mixture was consisted of 200  $\mu\text{l}$  of partially purified enzyme solution, 200  $\mu\text{l}$  of 50 mM citrate phosphate buffer, pH 5.0 and 300  $\mu\text{l}$  of 0.5 % colloidal chitin. The reaction mixture was then incubated for 15 min at 70°C. Finally, the mixture was centrifuged immediately at 10,000 X g for 5 min.

The amount of the reducing sugars released was determined by Imoto and Yagishita method (Imoto and Yagishita, 1971). The amount of monomer released was extrapolated from the standard graph of GlcNAc. A unit of enzyme activity was defined as the amount required for producing one micromole of GlcNAc per minute under the specified conditions. The activities in both assays were mean of three independent values and standard deviations were less than 5% of the mean.

**Characterization of chitinase enzyme:**

The heat stability of enzyme was analyzed by measuring the residual activity at different times of incubation at an optimum pH 5 and 70°C. The pH / buffer optimum of the enzyme was measured at 70°C with 50 mM citrate phosphate buffer, phosphate buffer and Tris-Cl buffer pH 3 to 9. The influence of metal ions on the activity of chitinase was investigated in 50 mM citrate phosphate buffer (pH 5.0). The purified chitinase was incubated with 1 or 5 mM of metal salt for 30 min at room temperature and the remaining chitinase activity was measured under the optimum conditions.

**RESULTS AND DISCUSSION**

**Results:**

In the previous study, the distribution of aerobic and facultative anaerobic bacteria in RPW larvae gut was investigated. Limited number of bacteria were isolated and identified from the gut of larvae grew on artificial diet. Analysis of 16S rDNA sequences revealed that bacterial isolates represented four genus including *Salmonella* sp, *Enterococcus* sp and *Xanthomonas* sp and *Bacillus* sp and six other species. The analysis also showed that two strains were belonging to *Bacillus licheniformis* (Khyami and Alyamani, 2008). The isolates were screened for chitinase activity detected via the appearance of clear zones. *B. licheniformis* A2 and A35 showed different chitinase activity. The enzymes were produced in the basal medium containing 0.5% colloidal chitin. At the beginning of incubation days, minimal chitinase activity was detected in the culture; however, after two days of incubation a significant chitinase activity was observed which continued increasing. The highest chitinase activity was observed on day four for strain A2 and on day six for strain A35. After reaching the maximum activity it decreased slightly (Fig. 1).

In our study, we focused on the thermostable chitinase. It was partially purified by heating for 3 hr at 70°C. In Table 1, we evaluated the effect of different treatment on enzyme activity has been evaluated. The enzymes were active between 50-70°C. After 3 hrs of heating, the activity of chitinase from strain A2 and A35 was still higher than the control (before heating). The activity of chitinase was 25.3 for strain A2 and 9.2 for A35 at 70°C. Thermostability of purified enzymes was also measured by incubation at 65°C for seven hours. The enzyme remained active for 5 hr for strain A2, compared to enzyme activity for strain A35 (Fig. 2).

**Table 1:** Purification of chitinase from *Bacillus licheniformis* (A2) and (A35).

Purification step	<i>B. licheniformis</i> (A2)			<i>B. licheniformis</i> (A35)		
	Protein (mg/ml)	Activity (x100) U/ml	U/mg protein	Protein (mg/ml)	Activity (x100) U/ml	U/mg protein
Ammonium sulfate	0.513	3.81	9.1	0.533	3.42	7.54
Heat treatment (70°C, 3h)	0.148	5.53	25.3	0.085	1.73	9.21
DEAE Sepharose	0.004	1.72	282	0.003	1.12	170

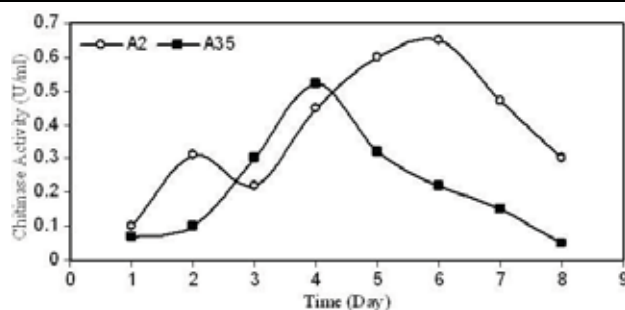


Fig. 1: Chitinase production by *Bacillus licheniformis* (A2) and (A35).

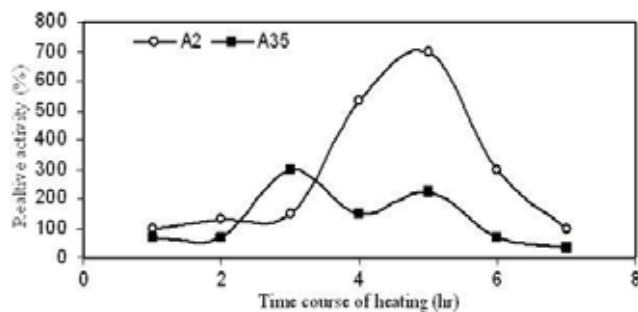


Fig. 2: Enzyme stability for the purified enzyme from *Bacillus licheniformis* (A2) and (A35).

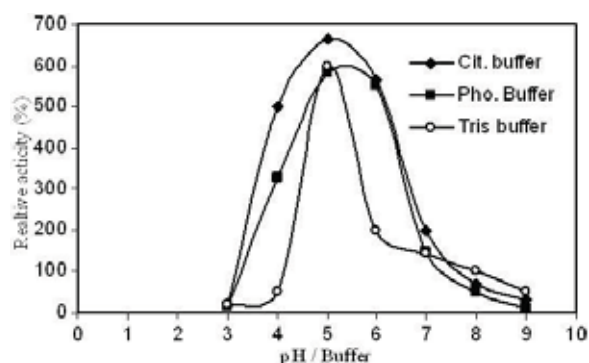


Fig. 3: Effect of pH upon enzyme activity, the purified enzyme of *Bacillus licheniformis* (A2) was incubated with the colloidal chitin substrate at the indicated pH using 50 mM citrate phosphate buffer, phosphate buffer and Tris-Cl buffer at pH 3 to 9

The extracellular chitinase from *B. licheniformis* (A2) was natural type enzyme, which was being active with all buffers at optimum pH range between 5 and 6 (Fig. 3). Certain metal ions affect the activity of chitinase of *B. licheniformis*. The enzymes from A2 and A35 were precipitated by adding  $Mn^{2+}$ ,  $Zn^{2+}$  and  $Co^{2+}$  at 1 mM concentration, but they could not be used in further assays. The  $Mg^{2+}$  activated 50% of chitinase enzyme activity of A2, but had no effect on enzyme of A35. The  $Ca^{2+}$  increased the enzyme activity in both A2 and A35 about 45 and 30% respectively.

## DISCUSSION

In all probability, this is the first study of extracellular chitinase produced by *Bacillus licheniformis* isolated from Red Palm Weevil larvae gut. Insects provide a fascinating system for microorganisms and the largest population of microbes is located in their intestinal tract. Intestinal microbiota produces useful compounds such as vitamins and enzymes.

In this study *B. licheniformis* was investigated for thermostable chitinase. Thermophilic microorganisms have been recognized for several decades and considered as natural sources for thermostable enzymes. Several bacteria can produce thermostable chitinase such as *Pseudomonas aeruginosa* K-187 and bacillus strain MH-1 (Sakai, *et al.* 1998). The highest chitinase activity for strain A2 was observed on day four of incubation, while for strain A35 was on day six (Fig. 1). It seems that the production of thermostable enzymes needs a period of time. *Pseudomonas aeruginosa* K-187 and *Bacillus* strain MH-1 required 3–4 days to produce its thermostable chitinase when incubated at 58°C (Sakai, *et al.* 1998; Wang and Chang, 1997). Thermostable chitinase from *Bacillus licheniformis* was reported of multiple kinds, I (98 kDa), II (76 kDa), III (66 kDa) and IV (59 kDa), of thermostable chitinase with optimum temperatures of 70–80°C (Takayangi, *et al.* 1991; Tantimavanich, *et al.* 1998). Therefore, the fascinating aspect of purification of the heat stable enzyme is that while other heated labile proteins denature at elevated temperature, the thermostable enzyme retains its active conformation during the enzyme purification (Yuli, *et al.* 2004). This strategy was applied in the purification of chitinase from *Bacillus licheniformis* A2 and A35 and heated the enzyme solution at 70°C for 3 hrs. Amazingly the specific activity of the enzyme increased (Table 1). The result of enzyme stability ensured that the remaining enzyme was active even after being heated at 70°C for 4-5hr. Also, the results confirmed that thermostable chitinase has several kinds and some molecule of enzyme was found as a major protein, even though other proteins are still present (Fig. 2).

The effect of pH on the chitinolytic activity was studied with 50 mM citrate phosphate buffer, phosphate buffer and Tris-Cl buffer at a pH range from 3 to 9. Under standard conditions the pH profile was normal (bell shaped) with a maximum value at pH 5.0. (Fig. 3). The chitinase was produced by different bacteria at a pH range between 5 to 10 (Ohtakara, *et al.* 1979; Wang, *et al.* 1997). For instance, *Microbiospora* sp. V2 produced chitinases at 60°C and

a wide range of pH from 3.0 to 11.0 (10); marine bacterial strain 98CJ11027 produced chitinase I at pH 6.0 in the presence of acidic amino acids (Inglis and Peberdy, 1997).

The chitinases in this study particularly due to its thermophilic and acidophilic characteristics suggest a potential future application in industrial and medical sectors, such as production of the chitooligosaccharide. The effect of metal ions upon chitinase is quite diverse between bacteria even between the same species. This study showed that the chitinase of *B. licheniformis* A2 and A35 were different. The chitinases form A2 and A35 were activated by  $\text{Ca}^{2+}$  similar to *Bacillus* MH-1 (Sakai, *et al.* 1998), yet only chitinase form *B. licheniformis* A2 was activated by  $\text{Mg}^{2+}$ . On the other hand, both chitinases from A2 and A35 were inhibited by  $\text{Mn}^{2+}$  and  $\text{Co}^{2+}$  similar to the thermostable chitinase isolated from Indonesian *Bacillus* (Yuli, *et al.* 2004).

## CONCLUSION

The current strategy to manage the weevil in the Gulf and Asia is largely based on insecticide applications despite the deep concerns about environmental pollution. Biopesticides are the new approach to controlling the damage caused by weevils and reducing the pollution that results from the use of pesticides. Nowadays, the use of fungi as biopesticides to control weevil is well established. However, no study used bacteria as biological control agents. This study will help in the development of new technique for controlling the red palm weevil- that is biopesticides.

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