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Contributed Paper

Purification and Characterization of an Extracellular Chitinase from *Bacillus thuringiensis* R 176 using Solid State Fermentation with Shrimp Shells Waste

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ABSTRACT

The chitinase-producing strain R 176 was isolated from paddy soil in Chiang Mai province, Thailand, and it was identified as *Bacillus thuringiensis*. The optimal condition of the strain suitable for production of extracellular chitinase was investigated to be a solid state fermentation using a medium (pH 7.0) containing 50 g of shrimp shell powder mixed with 10 mL basal medium (pH 7.0) containing 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) yeast extract, 0.028% (w/v) KH_2PO_4, 0.025% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.007% (w/v) $CaCl_2 \cdot 2H_2O$. The medium yielded the chitinase of 1.36 U/g IDS, which was 0.36-fold higher than the productivity in a liquid culture with colloidal chitin. The chitinase was purified from the culture broth of strain R 176 by ammonium sulphate precipitation, ion-exchange, and gel filtration. Molecular weight of the chitinase was 40 and 47 kDa compared with standard proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Optimal activity of the purified chitinase was pH 7.0 and 37ºC. More than 80% of R 176 was stable at pH 6.0 to 8.0 and more than 90% at 40ºC. Ca^{2+} ions, Cu^{2+} ions and Mg^{2+} ions inhibited the chitinase activity about 20% and EDTA and p-CMB by 21% and 40%, whereas Ag^{+} and Zn^{2+} inhibited the activity up to 65%. Substrate specificity tested indicated that, ball milled chitin (100% relative activity) was the best substrate followed by colloidal chitin (89% relative activity), chitosan, carboxymethyl cellulose, ethylene glycol chitin, glycol chitin and swollen chitin. These results suggested that the substrate specificity of this chitinase was due to the hydrolyzation of glycosidic bond linked between GlcNAc-GlcNAc. For the production of any industrial enzymes, the inexpensive substrates and cost-reducing process like solid state fermentation have been shown several advantages for the upscale production and giving value-added of solid waste.

Keywords: *Bacillus thuringiensis*, chitinase, chitin, shrimp shells wastes, solid state fermentation

1. Introduction

Chitin, *a*-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc) is the second most abundant polysaccharide in nature [1]. It is a major component to form the structure of shell and cuticle of arthropods, crustaceans and insects, and cell wall of fungi [2]. Shrimp shells contain chitin, protein and inorganic compounds, which are mainly composed of calcium carbonate [3]. The production of chitin and its hydrolysates from shellfish waste has been limited because of the high cost of chitinase and the chitin-rich waste treating process. Industrial preparation of chitin has still involved with demineralization and deproteinization of shellfish waste using strong acids or bases, therefore direct utilization of shrimp shell to produce chitinase may solve environmental problems and reduce the costs of chitinase production [4]. Nowadays, chitinase is very important in various fields including pharmaceutical and agriculture. In pharmaceutical, ketooligosaccharide, a result of chitin hydrolysis, showed antitumor activity [5]. Chitinase has an ability to degrade shrimp waste containing chitin [6]. In agriculture, chitinase is used as to control plant diseases [7]. Purified chitinase from *Cellulosimicrobium cellulans* strain 191 presents potential for application in fungal control and protoplast formation of *Rhizopus oligosporus*, *Mucor miechei*, *Penicillium* sp. and *Streptomyces phaerochromogenes* [8].

Biodegradation of chitin using microbial enzyme is the alternative way to solve this problem which is performed by chitinases and appears to occur in two steps. An endochitinase (EC 3.2.1.14) degrades the polymer to oligomers, which are subsequently degraded to monomers by exochitinase; *β-N*-acetylhexosaminidase (EC 3.2.1.52). These enzymes are found in a wide variety of organisms such as bacteria, fungi, insects, plants and animals [9]. Chitinases have been isolated from a variety of bacteria including *Bacillus* spp. and some of them are reported to produce multiple forms of chitinase with different molecular masses [10]. Chitinase production by different species of this genus was studied such as *B. amyloliquefaciens* [11], *B. thuringiensis* sub. sp. *kurstaki* [12], *B. licheniformis* [13], *B. subtilis* [14] and *B. cereus* [15].

Nowadays, solid state fermentation (SSF) has emerged as an appropriate technology for management of agro-industrial residues and for their value addition. SSF has recently gained for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (SmF) [16]. The capacity comparison of SSF and SmF suggested that SSF provided higher volumetric productivity, less problem on substrate inhibition and yielding enzymes with a higher temperature or pH stability [16]. However, use of SSF for chitinase production still lacks supporting knowledge, even though study and production of chitinase based on the other fermentation techniques is better understood.

This work reports the progress on production, purification and biochemical characterization of chitinase produced from thermotolerant *B. thuringiensis* R 176, which was isolated from a rice rhizospheric soil collected from paddy field of Chiang Mai province, Thailand. SSF using shrimp shell powders as a chitin source for the production of chitinase derived from *B. thuringiensis* R 176 was evaluated. Purification of produced chitinase was performed based on ion-exchange and gel filtration chromatography. The enzyme activity and its optimized conditions, i.e. pH-stability profile, temperature profile, effect of metal ions and chemical and substrate stability were investigated and revealed in this article.

2. Materials and Methods

2.1 Substrate for SSF

Sun-dried shrimp-shells waste were collected from a local market and transported to the laboratory under chill condition. These materials

were dried in an oven at 50°C to a constant moisture content (10%), milled to < 400 μM particle size and stored in a dry place at room temperature. It was used as solid substrate for SSF.

2.2 Isolation of Chitin-degrading Bacteria

One hundred rhizosphere soil samples were collected from paddy fields in Chiang Mai province, Thailand. Chitin-degrading bacteria were isolated by serial dilutions of soil samples and spread on 1% chitin agar plates (pH 7.0) containing 1% (w/v) shrimp shell powder, 0.3% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4$ $7H_2O$ and 2% (w/v) agar powder. After incubation for 72 h at 30°C, the isolates capable of degrading chitin with distinctive zone of clearance on agar medium were selected and sub-cultured onto nutrient agar (NA) slants (Difco, UK) as working stocks. The bacterial isolates were then checked for their thermotolerant activity by cultivating on the agar plates and incubated at 30°C to 60°C for 48 h. Those organisms capable of growing at all temperature settings were regarded as thermotolerant strains. These bacterial strains were also maintained on NA slant for further studies. The bacteria that showed large clear zone more than 1cm in diameter, were tested for chitinase production on SSF using shrimp shell powder as a sole source of carbon and energy.

2.3 Identification and Classification

Morphological, physiological and biochemical characteristics of the highest chitin-degradable strain were studied according to the 9^{ed} Bergey's Manual of Determinative Bacteriology and 16S rDNA gene sequencing with eubacteria specific primer set 16F27N (5'-CCAGA GTTTGATCMTGGCTCAG-3') and 16R1525XP(5'-TTCTGCAGTCTAGAAGG AGGTGWTCCAGGC-3') [17]. PCR amplification and sequencing of PCR product for analysis of 16S rRNA nucleotide sequence was carried out using BLAST search at NCBI [18].

2.4 Enzyme Production

2.4.1 SSF and preparation of crude chitinase

Experiments were carried out in 250-mL Erlenmeyer flasks which contained 50 g of shrimp shell powder mixed with 10 mL basal medium (pH 7.0) containing 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) yeast extract, 0.028% (w/v) KH_2PO_4, 0.025% (w/v) $MgSO_4 \cdot 7H_2O$, 0.007% (w/v) $CaCl_2 \cdot 2H_2O$. After sterilization, the moisture content of solid medium was 70% and the medium was inoculated with 100 μL of bacterial suspension (10^8 CFU/ mL) and incubated at 30°C in the rotary shaker at 150 rpm for 14 days. The culture was extracted in 100 mL of 0.1 M phosphate buffer (pH 7.0) by stirring for 1 h in ice bath then centrifuged at 8,000 *x* g (4°C) / min for 15 min. The supernatant was filtered by the filter paper (0.25 μM) to remove bacterial cells and used for enzyme purification. The supernatant was brought to 60% saturated with ammonium sulfate and left standing overnight at 4°C. The precipitant was collected by centrifugation at 8,000 *x* g (4°C) / min for 20 min and re-dissolved in a small amount of 50 mM phosphate buffer (pH 7.0). The solution was dialyzed for 12 h at 4°C to remove ammonium sulfate in the same buffer used. The resultant dialyzed was chitinase crude extract ready for further purification steps.

2.5 Purification and Purity of Chitinase

2.5.1 Ion-exchange chromatography

The crude enzyme solution obtained above was loaded onto a DEAE-Sephadex A (Pharmacia, UK) column (5×30 cm) equilibrated with the dialysis buffer. The enzyme was eluted with a linear gradient from 0.2 to 1.5 M of NaCl in 50 mM phosphate buffer (pH 5.8) at the flow rate of 20 mL/h, and the fractions were collected in 3 mL size. The active fractions were pooled and concentrated with ultra-filtration, followed

by dialysis against 50 mM phosphate buffer (pH 5.8) containing 0.1 M NaCl. All procedures were carried out at 4°C.

2.5.2 Gel filtration chromatography

The concentrated active fractions from the ion-exchange chromatography were loaded onto a Toyopearl HW 40C (Pharmacia, UK) column (1.5×60 cm) equilibrated with 50 mM phosphate buffer (pH 5.8) containing 0.1 M NaCl. The enzyme was eluted with the same buffer at the flow rate of 10 mL/h, and fractions were collected in 2 mL size. All procedures were carried out at 4°C.

2.5.3 Protein determinations

Protein content was determined by the method of Bradford [4] using bovine serum albumin (BSA) as standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

2.6 Characterization of Purified Chitinase

2.6.1 Molecular weight of chitinase

The molecular mass of the purified chitinase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [19]. The standard protein used for calibration was Prestained Protein Marker (Pacific Science Co., LTD, THAILAND). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7.0) containing *β*-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol: acetic acid: water (5:1:5 (v/v/v)), and decolorized in 7% (v/v) acetic acid. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility) on Minibis Bioimaging system (MiniBIS, UK). Following proteins were used as standards: BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and *a*-lactalbumin (14.4 kDa).

2.6.2 Chitinase activity

Chitinase activity was determined colorimetrically by detecting the amount of GlcNAc released from colloidal chitin substrate. The reaction mixture consisted of 0.3 mL of crude enzyme and 0.3 mL of 1.0% (w/v) colloidal chitin in 50 mM phosphate buffer (pH 7.0). The reaction was performed at 37ºC for 30 min. The mixture was boiled for 10 min, chilled and centrifuged to remove insoluble chitin. The supernatants were used for reduced sugar analysis using the dinitrosalicylic acid (DNS) method [20]. GlcNAc (Sigma, UK) was used as standard. One unit (U) of chitinase activity was defined as the amount of enzyme that released 100 μg equivalent of reducing sugar under the reaction conditions. Enzyme yield was expressed as U/g initial dry substrate (U/g IDS) [21].

2.6.3 Substrate specificity of chitinase

The purified enzyme was tested for hydrolysis of various substrates (1.0% v/v) including ball milled chitin, carboxymethyl cellulose (CMC), colloidal chitin, chitosan (95% degree of deacetylation), ethylene glycol chitin, glycol chitin and swollen chitin, which prepared in 50 mM phosphate buffer (pH 5.8). After incubation at 37ºC for 30 min, reducing sugars were measured by DNS method. One unit of chitinase activity was defined as the amount of enzyme that released 1 μmole of reducing sugar per minute [22].

2.6.4 Effect of temperature on chitinase activity

In order to determine the optimal temperature, chitinase activity in 50 mM phosphate buffer (pH 7.5) was examined at various temperatures (40, 50, 60 and 70ºC) for 1 hr. Thermal stability of the chitinase was also

measured in term of relative activity (%) after incubation of the purified chitinase at various temperatures ranging from 25ºC to 80ºC for 1 h. Residue enzyme activity was then measured under the standard assay condition using colloidal chitin as a substrate.

2.6.5 pH-activity and pH-stability of chitinase

The activity of the purified chitinase was measured at different pH values. The optimum pH for the enzyme reaction was investigated after incubation in various pH at 40ºC for 1 hr. The buffers used were: 1M sodium acetate-HCl buffer (pH 2.0-5.0), 1/20 M phosphate buffer (pH 5.0-8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (pH 8.0-9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (pH 9.0-12.0). pH stability of the enzyme was measured from the residual activity after preincubation in buffers with various pH for 12 hr at 4ºC.

2.6.6 Effect of several metal ions and chemicals

The effects of several metal ions and chemicals on the activity were measured. The enzyme solution at a final concentration of 1 mM was preincubated at 4ºC for 12 hr in the presence of metal ions and at 40ºC for 1 hr in the presence of chemicals. Then the residual activity was measured using the standard assay conditions.

2.7 Statistical Analysis

The enzyme production in different condition was compared by Analysis of Variance (ANOVA) using the statistical software. All experiments were conducted in triplicate and the mean values are reported.

3. Results and Discussion

3.1 Screening and Classification of Chitin-degrading Bacterium

A total of 200 chitinolytic bacteria were isolated from 120 soil samples collected from Chiang Mai province, Thailand. Of which, only 30 isolates produced zone of clearance over 1 cm, which accounted for 15% of total isolated bacteria. Thus, we evaluated all 30 chitinolytic bacteria in secondary screening and compared chitinase production on liquid culture and SSF for searching the best isolate which produced higher amount of chitinase activity on SSF. After fermentation, their culture filtrates were extracted and checked for chitinase activity. The result was shown on Table 1.

We selected an isolate R 176 because of its hydrolyzing capacity to use shrimp shell powder by means of SSF. This strain gave the highest chitinase productivity in the solid culture (1.36 U/g IDS) and higher than in liquid culture (1.00 U/g IDS) (Table 1). Bacterium strain R 176 was subjected to taxonomic analysis based on Bergey's Manual of Determinative Bacteriology and the 16S rDNA gene sequencing with eubacteria specific primer. The 16S rDNA sequence (1,488 bp) of strain R 176 showed high similarity with 98% identity to *Bacillus thuringiensis*. The bacterium was Gram-positive, rod shape and spore-forming. It was a facultative anaerobe, grew in the temperature range of 30-60ºC and hydrolyzed gelatin, mannitol, starch and xylose. This strain was also the most potent-chitinase producer by chitinase activity after incubation for 5 days in liquid medium containing 1.0% (w/v) shrimp shell powder.

The chitinolytic bacteria were found from rhizospheric soils more than in agricultural fields [23]. Interestingly, a bacterial isolate obtained from the rhizospheric soil of rice, designated as strain R 176 remarkably hydrolyzed the shrimp shell powders and produced a maximum clear zone (2.0 cm) in chitin agar plate using shrimp shell powders. The presence of chitinolytic bacteria in the crop rhizosphere soils is highly beneficial as they could suppress the plant pathogenic fungi near the root zone and provide sustainable plant protection against root diseases [24]. Further, we evaluated all the 30 chitinolytic bacteria in secondary screening

Table 1 Productivity of chitinase in liquid medium and solid state medium of some isolated bacteria.

Isolates	Chitinase activity (U/g IDS)	
	Liquid Medium	Solid State Medium
R 74	0.15 ± 0.15 f	ND
R 75	0.23 ± 0.25 f	ND
R 88	4.12 ± 0.10 a	ND
R 108	0.35 ± 0.05 e	ND
R 110	0.80 ± 0.15 d	1.25 ± 0.23 c
R 111	0.95 ± 0.23 c	1.18 ± 0.10 c
R 113	0.32 ± 0.05 e	0.65 ± 0.05 d
R 117	3.65 ± 0.35 b	0.60 ± 0.05 d
R 118	0.14 ± 0.05 f	ND
R 171	0.35 ± 0.05 e	ND
R 176	1.00 ± 0.15 c	1.36 ± 0.25 c
R 181	0.25 ± 0.05 f	ND
R 190	0.87 ± 0.15 d	0.35 ± 0.05 e

ND = not detected. The results are mean of three replicates. Different letters indicate the difference between chitinase activity results ($P \geq 0.01$)

using their culture filtrate extraction and selected an isolate R 176 because of its hydrolyzing capacity to use shrimp shell powder by means of SSF.

3.2 Purification of Chitinases

The chitinase crude extract was purified by a two-step procedure, using an ion exchange chromatography followed by gel filtration chromatography. After ammonium sulphate precipitation, the concentrated supernatant exhibited chitinase activity. The ion exchange chromatography eluted with 0.25-1.5 M NaCl resulted in three peaks and yielded 19.8 protein peaks (Figure 1). The second peak showed chitinase activity which was eluted by 1.5 M NaCl solution. One major peak containing the highest chitinase activity was collected and concentrated, then loaded onto a Toyopearl 40W column. After gel filtration chromatography, three peaks appeared and the second peak exhibited chitinase activity (Figure 2). The purification procedures were summarized in Table 2, where 6.6-fold of purification with overall yield of 3% derived from our chitinase was observed. The specific activity of this chitinase was 10 U / mg of protein. The final amounts of the chitinase obtained were 10 mg. This purified enzyme was confirmed to be homogeneous by SDS-PAGE. The molecular masses of R 176 chitinase were 40 and 47 kDa as determined by SDS-PAGE (Figure 3). Although the yield of enzyme was relatively low for the commercial production, the method gave sufficient pure enzyme for initial characterization studies.

Chitinase was separated by column chromatography, the ion exchange, chitinase

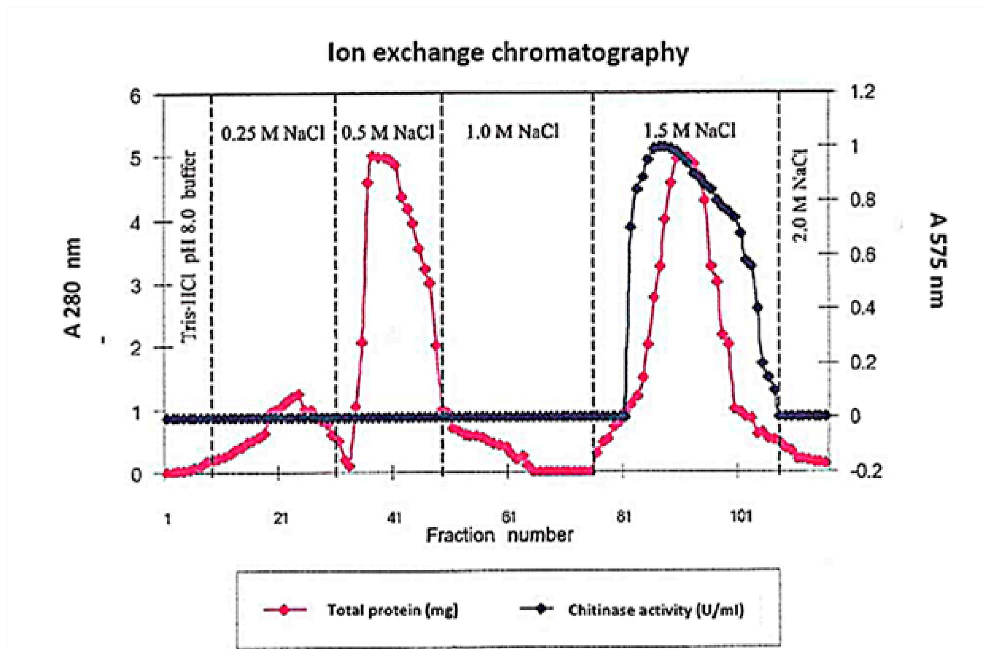

Figure 1. Ion exchange chromatograph of the chitinase from *Bacillus thuringiensis* R 176 using DEAE-Sepharose column with the flow rate at 0.5 mL/min.

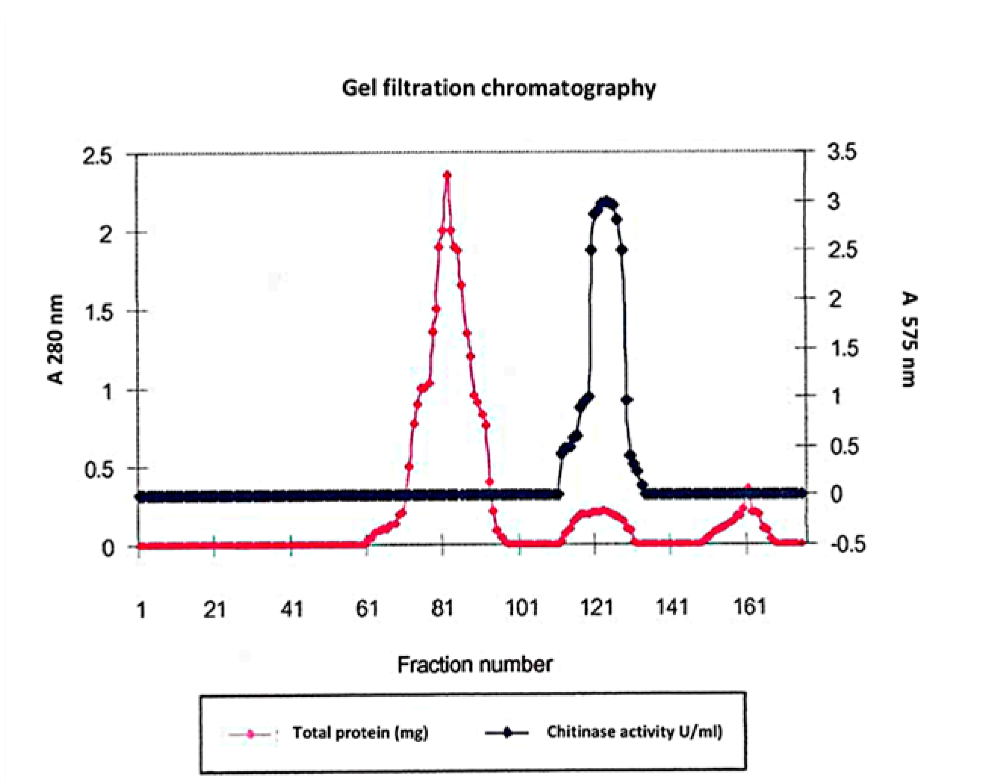

Figure 2. Chromatograph of chitinase by Toyopearl HW 40C column resolution at 0.2 mL/min.

Table 2. Purification capacity of a chitinase from *Bacillus thuringiensis* R 176.

Step	Total (mg)	Total (U)	Specific (U / mg)	Purification (fold)	Yield (%)
Culture supernatant	500	1,000	1.51	1	100
$(NH_4)_2SO_4$	100	215	2.15	1.4	21.5
DEAE-Sephadex	150	198	2.58	1.7	19.8
Toyopearl HW 40C	10	30	10	6.6	3

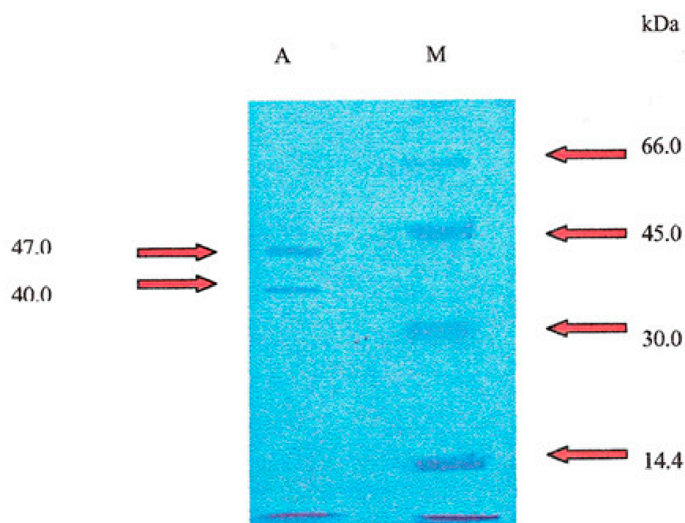

Figure 3. SDS-PAGE analysis of purification fraction of chitinase. Lane 1 (A): gel filtration chromatography fraction of *Bacillus thuringiensis* R 176 chitinase; Lane 2 (M): molecular weight marker proteins.

activity was detected in the high-salt fraction, suggesting that the enzyme surface is rather hydrophilic and this method produced 19.8% yield of chitinase. This fraction was then applied for further separation on gel-filtration chromatography. Several bacterial chitinases have been reported, these include multiform chitinases in the range of 30-81 kDa produced by bacteria [23]. In the case of *Bacillus* MH-1, 71 kDa was found [24]. The molecular weight of chitinase derived from strain R 176 was approximately 40 and 47 kDa by SDS-PAGE, which was similar to those of *Bacillus* chitinases such as 35, 47, 58 and 64 kDa from *Bacillus cereus* YQ 308 [25]. Chitin molecules can vary depending on the arrangement of GlcNAc strands, degree of deacetylation, the presence of cross-linked structural components: protein and glucans, which are different concerning the species of microorganisms [26]. In recent study, the semi purified chitinase from *B. thuringiensis* R 176 showed double bands with molecular masses of 40 and 47 kDa on SDS-PAGE. This was different from other chitinases from various other bacteria. The bacterial chitinase from different bacterial species presented different molecular masses and other characteristic as shown in Table 3.

Table 3. Comparison of some properties of chitinase obtained from *Bacillus thuringiensis* R 176 to some bacterial chitinases.

Source	Molecular mass (KDa)	Optimum pH	Optimum temperature (°C)	Thermal stability (°C, min)	Substrate	Reference
Bacillus thuringiensis R 176	40 (F1, SDS, GF) 47 (F2, SDS, GF)	7.0	37	60, 10	Ball-milled chitin	This article
B. licheniformis MB-2	67 (SDS, GF)	6.0	70	60, 30	Glycol chitin	Toharisman et al., 2005
Bacillus sp. MH-1	71 (L, SDS)	6.5	75	80, 10	PNP-(GlcNAc)2	Sakai et al., 1998
Bacillus sp. NTCU 2	36.5 (SDS)	7.0	50-60	60, 30	Colloidal chitin	Wen et al., 2002
Pseudomonas aeruginosa K187	30 (F1, SDS) 32 (F2, SDS)	8.0 (F1) 7.0 (F2)	50 (F1) 40 (F2)	50, 10	Colloidal chitin	Wang and Chang 1997

Note: SDS; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, GF; Gel filtration, L; Liquid culture F1; fraction 1 and F2; fraction 2.

3.3 Enzyme Characterizations

3.3.1 Effect of pH on activity and stability of R 176 chitinase

To investigate the optimum pH of R 176 chitinase, chitinase activity was measured at various pH ranging from 2.0 to 12.0. The optimum pH of R 176 chitinase was 7.5, and it was stable at pH 6.0-8.0, as shown in Figure 4. The optimal pH of R 176 chitinase was similar to that of *Enterobacter* sp. (pH 7.0) [27] and *Streptomyces* (pH 8.0) [28] but different from *Penicillium* (pH 5.0) [29] with acidic optimum pH.

To investigate the effect of pH on the stability of R 176 chitinase, the enzyme was preincubated at various pH for 12 hr at 4°C, and the remaining activity was determined. The chitinase was stable between pH 6.0-8.0, and more than 80% of the original activity remained (Figure 4). Results indicated that R 176 chitinase was stable in a relatively broad pH range. These results agreed with the chitinase from *Pseudomonas* (a broad optimum pH 5.0-9.0) [29], *Streptomyces* (pH 4.0-9.0) [28] and *Trichoderma* (pH 2.0-8.0), respectively.

3.3.2 Effect of pH on stability of R 176 chitinase

The effect of pH on the catalytic activity was studied by using colloidal chitin as a substrate under the standard conditions. The effects of pH on activity and stability of purified chitinase were examined at optimum temperature (37°C). The pure enzyme was active at pH 3.0 to 8.0 and the optimum activity was at pH 7.0 (Figure 5).

3.3.3 Effect of temperature activity and thermostability profile of R 176 chitinase

The enzyme activity was measured between 40°C and 70°C for 1 hr. The optimum temperature of R 176 chitinase was observed at 45°C (Figure 6). Most of the chitinases from other bacteria including *Bacillus* [30], *Pseudomonas* [31] and *Streptomyces* [28], showed optimum temperature in the range of 40°C to 50°C. To investigate the heat stability of R 176 chitinase, the enzyme was preincubated at various temperatures (40°C, 50°C, 60°C and 70°C) for 1 hr and the remaining activity was determined. About 80% of the initial activity remained after incubation for 1 hr at 40°C. R 176 chitinase lost its activity

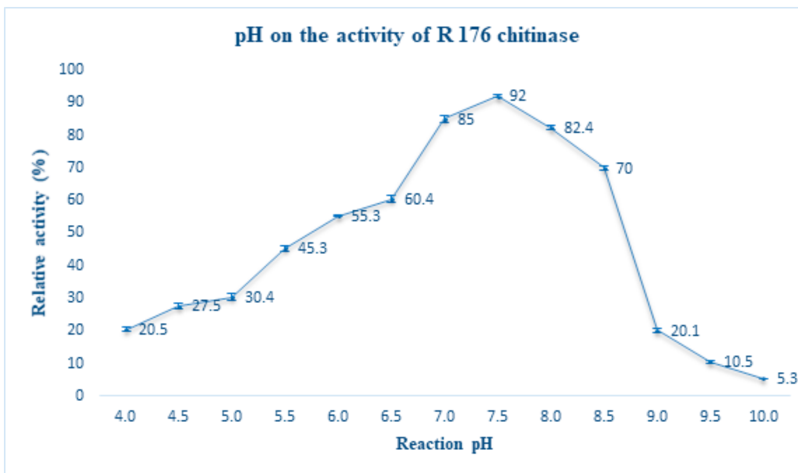

Figure 4. Effect of pH on the activity of R 176 chitinase.

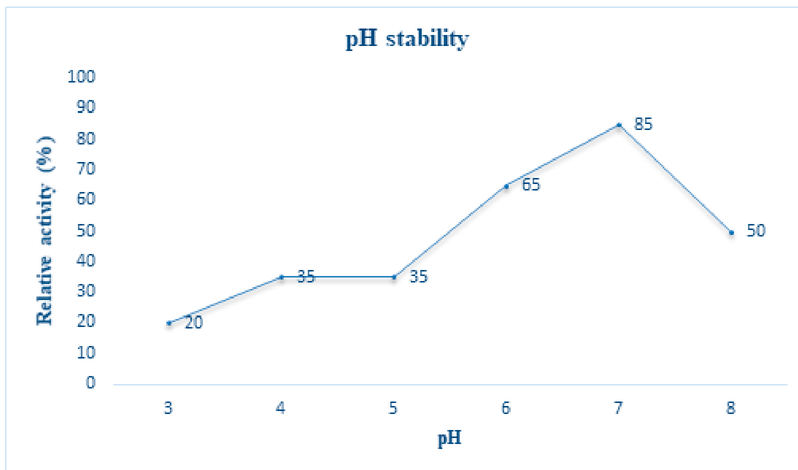

Figure 5. Effects of pH stability on the chitinase activity.

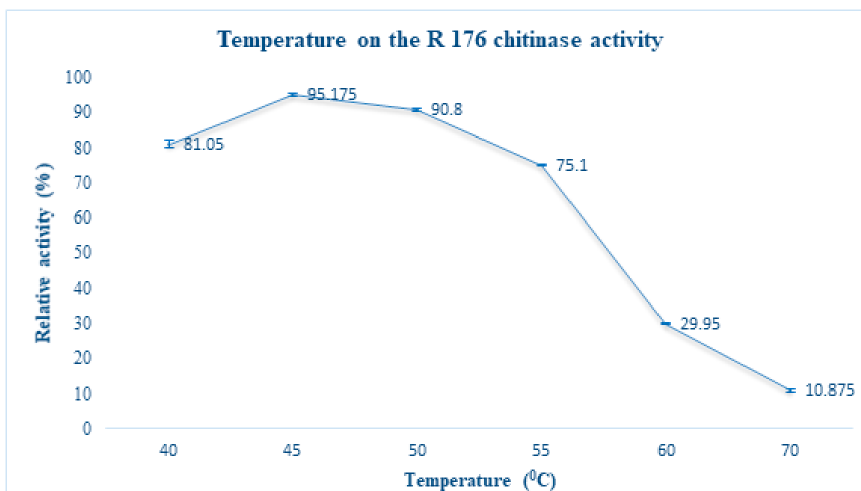

Figure 6. Effect of temperature on the activity of R 176 chitinase.

at above 50 °C and it was inactivated by preincubation at 70ºC (Figure 7). Similarly, the chitinase from *Ewigella* [32] was stable at 40ºC and became inactivated at 70ºC.

The chitinase maintained its stability in the range of 25ºC to 70ºC. The enzyme was completely inactivated at 80ºC (Figure 7).

The optimum pH for the chitinase produced by strain R 176 was neutral (approximately pH 7.0), whereas the chitinase of *Bt.* 15A3 is 5.0 [3], the Chi 36 of *Bt.* HD-1 is 6.5 [33] and pH 6.0 for the Chi A of *B. cereus* [34]. This character of *Bt.* R 176 chitinase was similar to that of various bacterial chitinases; *B. licheniformis* X-70 (pH 7.0-8.0) [35], *Pseudomonas aeruginosa* K-187 (pH 7.0-8.0) [36], *Enterobacter* sp. G-1 (pH 7.0) [37] and *Aeromonas hydrophila* subsp. *anaerogenes* A (pH 7.0). Several chitinases have broad pH optima, including the chitinase of *B. cereus* (pH 4.0-7.0) [22] and an exochitinase from *Bt.* subsp. *aizawai* (pH 5.0-8.0) [38]. Compared with the previously reported chitinases, however, the chitinase of *Bt.* R 176 showed a broader pH range with pH 3.0-8.0. The optimal pH for the chitinases of actinomycetes and fungi are acidic [23], unlike the chitinase derived from strain R 176. Therefore, the chitinase from thermotolerant *B. thuringiensis* R 176 would be very useful for industrial applications such as production of the chitin oligosaccharide for medical purpose [3] because it could be used in broad pH and temperature conditions.

The effect of temperature on the activity of chitinase was studied with colloidal chitin as a substrate. Examination of heat stability, the chitinase derived from strain R 176 held its activity from 25ºC to 70ºC and lost its activity at 80ºC. Similar thermostability profile was obtained at 50ºC for other bacterial chitinase such as *B. circulans* WL-12 (completely inactivated at 75ºC) [33] and *Aeromonas* sp. No.10S-24 (stable up to 50ºC) [34]. The high temperature optimum and the thermalstability profile of the chitinase from *B. thuringiensis* would particularly be advantageous for its applicability in recycling chitin wastes. The characterization of the chitinolytic system at molecular levels would extend the prospects for the enzyme, particularly due to its extreme pH and temperature optima and relatively good stability.

3.4 Substrate Specificity of the Chitinase

The ability to hydrolyze several carbohydrate substrates is an important criterion of chitinase potency. Table 4 compares the digestive capability of chitinase on six substrates. It is evident that

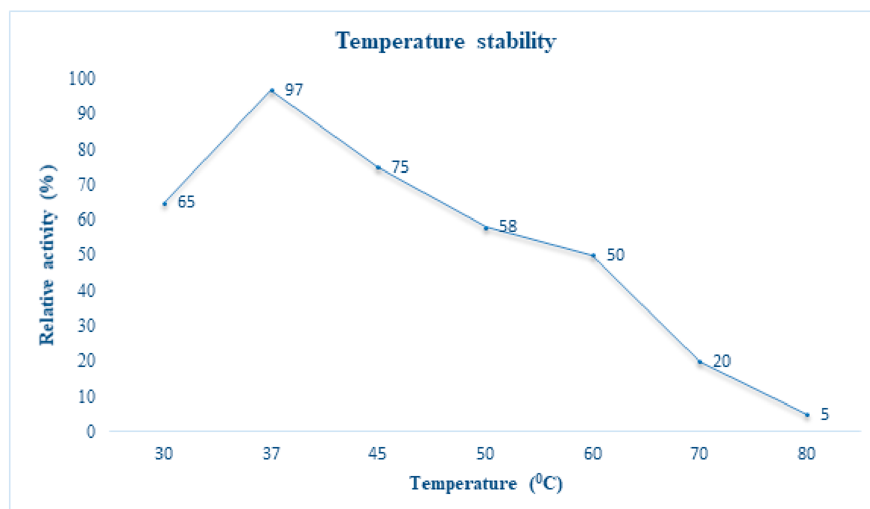

Figure 7. Effect of temperature on the stability of chitinase activities.

Table 4. Enzyme activities of *Bacillus thuringiensis* R 176 chitinase toward various polysaccharide substrates.

Substrate	Relative activity (%)
Ball-milled chitin	100 ± 0.74 a
Chitosan	54 ± 0.37 c
Colloidal chitin	89 ± 0.45 b
Carboxymethyl cellulose (CMC)	1 ± 0.27 f
N-acetyl glucosamine	33 ± 0.32 d
Swollen chitin	18 ± 0.27 e

The results are mean of three replicates. Different letters indicate the difference between chitinase activity results ($P \geq 0.01$).

the chitinase had better digestive ability on ball-milled chitin (100% relative activity) than other carbohydrates under the same assay condition. Relative activity of the chitinase only reached for swollen chitin, however, there was less hydrolysis activity when CMC was used as substrates. It is therefore reasonable to conclude that, the chitinase of this study has high specificity which hydrolyzes glucosidic bond between GlcNAc-GlcNAc.

In this study, ball-milled chitin and colloidal chitin were found to be more specific carbon sources and inducers for chitinase production than other forms of chitin. Our results agree with others as the reports of optimal carbon source for chitinase production by *B. licheniformis* X-7u was 0.3% of colloidal chitin [33] and *Enterobacter* sp. G-1 with 0.4% of the same chitin [33], which could decrease the productions when the concentration of chitin was increased. Chitinase derived from strain R 176 exhibited higher activity toward colloidal chitin and regenerated chitin than chitosan. The enzyme did not show activity toward ethylene glycol chitin and glycol chitin, suggesting that it might not possess GlcNAc activity (Table 4).

The product hydrolyzed by semi purified enzyme using colloidal chitins as a substrate was *N*-*N*-diacetylchitobiose as a major product and a minor of GlcNAc. Colloidal chitin and regenerated chitins are useful substrates for enzyme assays of endotype chitinase [38].

3.5 Effects of Metal ions on R 176 Chitinase

The enzyme solution at a final concentration of 1 mM was preincubated at 4ºC for 12 hr in the presence of metal ions, and at 40ºC for 1 hr in the presence of chemicals. Then the residual activity was measured. Ca^{2+} ions, Cu^{2+} ions and Mg^{2+} ions inhibited the enzyme activity by 20%. Fe^{2+} ions and Mn^{2+} ions inhibited the activity by 30% and Ag^{+} ions and Zn^{2+} ions inhibited up to 65% (Table 5).

The enzyme activity was assayed in a 1/20 M phosphate buffer (pH 5.0-8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (pH 8.0-9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (pH 9.0-10.0) at various pH under the standard conditions.

3.6 Effects of Chemicals on R 176 Chitinase

The enzyme solution at a final concentration of 1 mM was preincubated in 1/20 M phosphate buffer (pH 7.5) containing various chemicals at 4ºC for 1 hr, and the residual activity was measured using the standard assay conditions. EDTA and p-CMB inhibited the activity by

Table 5 Effect of various metal ions on the activity of R 176 chitinase.

Metal ion	Residual activity (%)
$BaCl_2$ $2H_2O$	63.4 ± 0.37 d
$FeSO_4$ $7H_2O$	75.4 ± 0.42 c
$CaCl_2$	85.2 ± 0.45 b
$CuSO_4$ $5H_2O$	82.7 ±0.37 b
$MgSO_4$ $7H_2O$	80.6 ± 0.31 b
$MnSO_4$	71.3 ± 0.47 c
$ZnSO_4$ $7H_2O$	30.5 ± 0.31 e
$AgNO_3$	30.0 ± 0.25 e
None	100.0 ± 0.54 a

The results are mean of three replicates. Different letters indicate the difference between chitinase activity results ($P \geq 0.01$).

21% and 40%, respectively. It had less inhibitory effects on SDS, L-Crysteine and sodium azide (Table 6).

The enzyme activity was assayed in a 1/20 M phosphate buffer (pH 5.0-8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (pH 8.0-9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (pH 9.0-10.0) at various pH under the standard conditions. Abbreviation: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; p-CMB, p-chloromercuribenzoic acid; AHA, acetohydroxamic acid.

Different from other reports the chitinase producing strain *B. thuringiensis* R 176 aimed for the microbial reclamation of shrimp processing wastes. Shrimp shells were used as the sole carbon source to screen the chitinase-producing bacteria. Consequently, strain R 176 belongs to *B. thuringiensis*, the same as the reported chitinase-producing strain of *B. thuringiensis* subsp. *colmeri* 15A3 [25] and strain *Bt*. HD-1 [26], however, the results regarding to the properties (such as molecular mass and pH stability) of the produced chitinase reported here were different.

Table 6 Effect of various chemicals inhibited by the activity of R 176 chitinase.

Chemicals	Residual activity (%)
EDTA	78.6 ± 0.32 c
SDS	98.2 ± 0.52 a
L-Cysteine	98.0 ± 0.47 a
Sodium azide	96.7 ± 0.41 ab
AHA	80.5 ± 0.37 b
p-CMB	60.2 ± 0.31 d
None	100.0 ± 0.57 a

The results are mean of three replicates. Different letters indicate the difference between chitinase activity results ($P \geq 0.01$).

Conclusion

In conclusion, with the research aimed for the microbial reclamation of shrimp shell wastes, we used these wastes as a sole source of carbon and energy to screen the chitinase-producing bacteria. *Bacillus thuringiensis* R 176 was a distinctive strain for chitinase production isolated from Thai paddy soil. The medium containing 1% (w/v) shrimp shell powder showed to be an optimal medium to maintain the chitinase-producing strain with low cost and no losing in the productivity of chitinase. The two-step purification procedure could be useful to purify apparently the produced chitinase with simple and convenient. The chitinase derived from strain R 176 chitinase was stable in the pH 6.0-7.0, and was active at 37ºC. The development of procedures for purification and characterization of physiochemical properties and structural elucidation will be studied further as a prospect to apply this enzyme in biotechnological and industrial fields.

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