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Production and Partial Characterization of Chitosanases from a Newly Isolated *Bacillus cereus*

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ABSTRACT

The production of chitosanases by a newly isolated *Bacillus cereus* TP12.24 was studied both in shake flask and fermenter cultures. The M9-chitosan medium was found most suitable with 0.5% chitosan as a sole carbon source optimized under aerobic growth conditions at pH 6.0 and 30°C. The specific rates of growth, substrate consumption, and enzyme production were improved using controlled completely aerobic conditions in 2-l fermenter. While the yield of biomass was considerably increased, the enzyme yield was on the contrary decreased. As a result, the volumetric chitosanases productivity was 43.55 U/l h, which was 1.2 times that obtained from shake flask culture due to higher specific rates of chitosan consumption and chitosanases production. In this work, the crude chitosanases from *Bacillus cereus* TP12.24 showed their optimal pH and temperature at 6.5 and 55°C, while the stabilities to pH and temperature were found at 3.0-8.0 and 30-50°C, respectively. The *Bacillus cereus* chitosanases could be used for preparing the chitosano-oligosaccharides under mild temperature.

Key words: chitosanases, chitosan, *Bacillus cereus*, optimization, batch culture

INTRODUCTION

Chitosan (poly-β-(1→4)-2-amino-2-deoxy-D-glucose) is a long chain polymer derived from chitin by deacetylation (Kumar *et al.*, 2000). Mostly, the sources of chitin in Thailand are solid wastes derived from the shrimp processing industries. Chitosan has been utilized as multi-purpose products in food, semi-food and non-food industries. Whereas the production of chitosan-derived oligosaccharides shows its potential as high value added food product, the enzymatic hydrolysis rather than chemical degradation that provides an attractive process is obviously limited. Chitosanase (EC 3.2.1.132) is exploited for the production of chitosano-oligosaccharides. Various sources of enzyme could be obtained from soil fungi and bacteria, such as *Bacillus circulans* MH-K1 (Yabuki *et al.*, 1988), *Bacillus* sp. No.7-M (Uchida and Ohtakara, 1988), *Bacillus licheniformis* UTK (Uchida *et al.*, 1992), *Bacillus cereus* S1 (Kurakake *et al.*, 2000), *Streptomyces* N-174 (*Boucher et al.*, 1992), *Streptomyces* sp. No.6 (Price and Storck, 1975), *Amycolatopsis* sp. CsO-2 (Okajima *et al.*, 1994), and *Burkholderia gladioli* strain CHB101 (Shimosaka *et al.*, 2000).

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The chitosano-oligosaccharides are water-soluble and possess versatile bioactivities such as immunopotentiating, bacteriostatic activities which have their advantages in food materials, agricultural and medical, and antitumor activity (Tominaga and Tsujisaka, 1975; Price and Storck, 1975; Suzuki *et al.*, 1984; Papineau *et al.*, 1991; Somashekar and Joseph, 1996; Jeon and Kim, 1998, 2000). The purpose of the present work was to optimize the production of chitosanases from the newly isolated *Bacillus cereus* TP12.24 (Wangtueai *et al*., 2006). The crude chitosanases were also partly characterized for their optimal and stability based on pH and temperature.

MATERIALS AND METHODS

Microorganism

Bacillus cereus TP12.24, a newly isolated soil bacterium (Wangtueai *et al*., 2006) was used throughout the experiments. The stock culture was maintained on the chitosanase-detection agar medium (CDA) (Cheng and Li, 2000) and freshly transferred every 2 weeks.

Factors affecting enzyme production in shake flask culture

All experiments were carried out in shake flask cultures using 500-ml Erlenmeyer flask containing 250-ml M9-chitosan medium at 250-rpm for 72 h. Samples were taken every 6 h for determining total viable cells, dry cell weight, residual chitosan and enzyme activity. The culture conditions and all analyses have been described previously (Wangtueai *et al*., 2006).

Effect of pH

The study on pH optimum for enzyme production was carried out by varying the pH values of M9-0.5% chitosan medium at 4.0 to 8.0 at 30°C.

Effect of chitosan

The M9-chitosan media containing 0.1, 0.5, 1.0 and 2.0 % chitosan were used for the production of chitosanases under optimized initial pH 6.0 at 30°C.

Effect of temperature

The temperatures of 30, 40 and 50°C were investigated for growth and enzyme production under optimized initial pH 6.0 and 0.5% chitosan concentration.

Enzyme production in 2-l fermenter

The 2-l fermenter (EYELA Mini jar fermenter, Model M-100, Tokyo Rikakikai Co., Ltd.) which contained 1.5-l M9-chitosan medium with 0.5% chitosan was used for the production of chitosanases from *Bacillus cereus* TP12.24. The fermentation conditions were controlled automatically at 30°C, pH 6.0, 1 vvm aeration rate and 400 rpm agitation rate for 58.5 h of cultivation time. The samples were taken every 6 h for determining the total viable cells, dry cell weight, residual chitosan and enzyme activity (Wangtueai *et al*., 2006). The fermentation kinetics of bacterial growth and chitosanases production were studied based on the experimental results.

Characterization of crude chitosanases

The crude chitosanases were prepared by growing cells in 2-l fermenter under the optimal conditions obtained in this work. The enzyme supernatant was collected from the culture broth after centrifugation at 8,000 rpm, 4°C for 20 min. This supernatant as crude chitosanases was used for the determination of enzyme optimal and stability on the basis of pH and temperature.

Optimal pH

The crude chitosanase activity was measured at various pH values, using 80% deacetylated chitosan as a substrate. The reaction mixtures consisting of 1.0 ml of 1% soluble

chitosan and 1.0 ml of the crude enzyme solution were incubated for 10 min at 30°C. The extended pH ranges of 3.0-7.5 and 8.0-9.0 were monitored by 0.05 M citrate phosphate buffer and 0.05 M carbonate bicarbonate buffer, respectively.

Optimal temperature

The temperatures were varied from 30-70°C for optimizing the enzyme activity for 10 min at the optimal pH 6.5 obtained in this work. The reaction mixture prepared was the same as mentioned above.

pH stability

The prepared crude enzyme was diluted 5 times with buffers at various pH's (crude enzyme:buffer, 1:4) using 0.05 M citrate phosphate buffer for pH 3.0-8.0 and 0.05 M carbonate-bicarbonate buffer for pH 9.0-11.0. The diluted enzyme solutions at these various pH's were incubated at 40°C for 60 min. Then the residual activities of chitosanases were determined under the specified conditions modified from Shimosaka *et al.* (1995) and Cheng and Li (2000).

Temperature stability

The diluted crude enzyme solutions were prepared with 0.05 M citrate phosphate buffer pH 6.5 and incubated at different temperatures varying from 30-80°C for 30 min. The residual enzyme activities of chitosanases were determined under the specified conditions modified from Shimosaka *et al.* (1995) and Cheng and Li (2000).

Analyses

Determination of growth

The total number of viable cells was determined by spread plate technique and the dry cell weight was calculated from the prepared standard curve of dry cell weight and total viable cells.

Determination of chitosan

The concentration of chitosan in culture broths was measured by the procedure described by Kobayashi *et al*. (1988).

Chitosanase assay

The 1% soluble chitosan was prepared by dissolving one gram of chitosan in 40 ml of deionized water and 9 ml of 1.0 M acetic acid. The solution was stirred for 2 h and the pH was adjusted to 6.0 with 1.0 M sodium acetate. This solution was finally made up to 100 ml by adding 0.05 M acetate buffer pH 6.0.

Chitosanase activity was analyzed by estimating the reducing ends of chito-oligosaccharides produced from the catalytic hydrolysis of chitosan. The assay was performed by mixing 1.0 ml of 1 % chitosan solution at pH 6.0 and 1.0 ml of suitably diluted enzyme. After 10 min incubation at 30°C, the reaction was stopped by boiling the mixture for 3 min. A 1.0 ml sample of the reaction mixture was taken for determining reducing sugar by the procedure described by Miller (1959). One unit chitosanase activity was defined as the amount of enzyme required to release 1 μmol of detectable reducing sugar at 30°C in 1 min.

RESULTS AND DISCUSSION

Optimizing chitosanases by shake flask culture
Effect of pH

Bacillus cereus TP12.24 grown in the M9-chitosan medium with varying initial pH 4.0, 5.0, 6.0, 7.0 and 8.0 at 30°C, gave the highest enzyme activities of 336.24 U/l in 24 h, 503.31 U/l in 30 h, 2,040.64 U/l in 54 h, 428.71 U/l in 30 h and 567.40 U/l in 48 h, respectively. While the maximal dry cell weights were 0.421 g/l at 24 h, 0.503 g/l at 30 h, 2.125 g/l at 54 h, 1.246 g/l at 30 h and 1.733 g/l at 30 h, respectively. Mostly, the production of chitosanases was associated with the bacterial growth, in which the concentrations of

enzyme and cells were maximized by using the initial pH of 6.0. The maximal specific growth rate obtained was 0.260 h^{-1} at the initial pH 6.0 (Table 1). Higher or lower initial pH's gave less favorable specific growth rates. At this optimal initial pH 6.0, the specific rates of chitosan consumption and chitosanases production were 0.091 g/g h and 31.99 U/g h, respectively. As a result, the yield and volumetric productivity of chitosanases were 247.59 U/g and 35.29 U/l h, respectively (Table 1). The optimal pH obtained in this work was quite similar to the results reported by Yoshihara *et al*. (1990) culturing *Pseudomonas* sp. at pH 6.3 and Tominaka and Tsujisaka (1975) producing *Bacillus* R-4 chitosanases at pH 6.0. Moreover, at higher pH 6.5 chitosan was difficult to dissolve and could not provide a useful carbon source for the bacterial growth. Especially, at initial pH 7.0 and 8.0, chitosan appeared in large particle sizes, which was barely consumed by the bacterial cells. The solubility of commercial chitosan being most excellent in diluted organic acids has been also reported (Kim *et al*., 2001). In particular, it is clear that the specific rate of chitosan consumption was enhanced 2.3-7.0 times higher at pH 4.0 than those at elevated pH's. Nevertheless, the specific growth rate maximized at pH 6.0 dictated the production yields of both cells and enzymes, so that the better substrate consumption could no longer monitor the production of enzymes.

Effect of chitosan

With 0.1 % chitosan, the highest concentrations of cells and enzymes were 0.474 g/l and 475.70 U/l at 66 and 54 h, respectively. The cell and enzyme concentrations were increased to 2.125 g/l and 2,040.64 U/l at 54 h, respectively, when using 0.5% chitosan as the main substrate. No bacterial growth was found at 1.0 and 2.0% chitosan because high viscosity of the culture medium limited oxygen availability for the bacterial growth. It was also reported that high chitosan concentration can inhibit the bacterial growth (No *et al*., 2001). In this study, the chitosan concentration of 1.0 and 2.0% could not be used as appropriate substrate concentration for the production of chitosanases. Therefore, 0.5% chitosan was finally selected for the optimal growth and chitosanases production from *Bacillus cereus* TP12.24. The specific growth rate and the

Table 1 Factors affecting growth and chitosanases production by *Bacillus cereus* TP12.24 using shake flask culture.

Factors	Variables	μ (h^{-1})	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (U/g)	q_S (g/g h)	q_P (U/g h)	Q_P (U/l h)
pH	4.0	0.043	0.112	122.19	0.218	36.13	9.81
	5.0	0.155	0.046	37.74	0.094	45.91	13.06
	6.0	0.260	0.352	247.59	0.091	31.99	35.29
	7.0	0.138	0.395	39.89	0.031	13.46	5.64
	8.0	0.126	0.739	107.27	0.068	25.28	8.91
Chitosan (%)	0.1	0.111	0.221	222.15	0.253	57.39	7.59
	0.5	0.260	0.352	247.59	0.091	31.99	35.29
Temperature	30	0.260	0.352	247.59	0.091	31.99	35.29
(°C)	40	0.175	0.032	168.62	1.388	285.06	23.01
	50	0.208	0.025	167.59	2.428	441.38	22.14

Note: Specific growth rate (μ) obtained from plotting the graph between log dry cell weight and culture time, the yields ($Y_{X/S}$, $Y_{P/S}$) obtained from plotting the graph of dry cell weight or enzyme activity with substrate, and the specific rates (q_S, q_P) calculated at the maximal enzyme production with culture time using average dry cell weight.

volumetric enzyme productivity were 2.3 and 4.6 times higher, respectively, as compared to 0.1% chitosan (Table 1). As discussed above, the more chitosan consumption, shown as the higher specific rate of chitosan consumption, did not favor the production of cells and enzymes even at optimal pH 6.0. Here, the limiting substrate at 0.5% chitosan which maximized the bacterial growth played an important role instead, for the production of chitosanases.

Effect of temperature

The maximal concentrations of dry cell weight and chitosanases were 2.125, 0.169 0.154 g/l and 2,040.64, 1,433.09 and 1,444.13 U/l at 30, 40 and 50°C in 54 h culture, respectively. The bacterial growth was clearly retarded at higher temperatures of 40 and 50°C, in which the cell concentrations decreased markedly after 18 and$12 h of culture times, respectively (data not shown). Both specific growth rate and the volumetric enzyme productivity decreased when increasing the growth temperatures beyond 30°C. Therefore, growth and enzyme production were found optimum at 30°C, as shown in Figure 1. Although the specific rates of substrate consumption were much higher at elevated temperatures (Table 1), these higher temperatures inhibited the bacterial growth and resulted in lower specific growth rate and the yields of cell and enzyme production. In conclusion, the factors that maximized the bacterial growth affected the production of both cells and enzymes. This revealed that chitosanases from the newly isolated *Bacillus cereus* TP12.24 was the growth associated enzymes.

The production of chitosanases in 2-l fermenter

Optimal conditions obtained from the shake flask culture were applied for kinetic study of the production of chitosanases in a laboratory fermenter, using the M9-chitosan medium containing 0.5% chitosan. The conditions were controlled at 30°C and pH 6.0 under completely aerobic cultivation (1 vvm aeration and 400 rpm agitation). *Bacillus cereus* TP12.24 produced highest dry cell weight at 0.904 g/l in 21 h, enzyme activity at 1,562.12 U/l in 28.5 h (Figure 2). However, the enzyme was harvested at 58.5 h at the end of cultivation for studying the properties

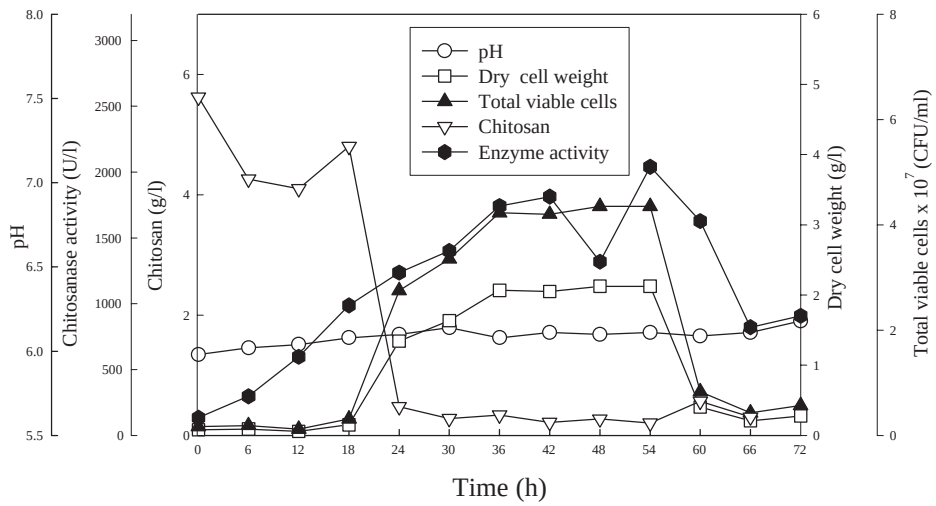

Figure 1 The production of chitosanases by *Bacillus cereus* TP12.24 in shake flask culture controlled at 30°C.

of crude chitosanases. Fortunately, the enzyme activity was found stable after its maximal at 28.5 h.

The kinetic parameters for growth and enzyme production were summarized in Table 2. The bacterial growth was promoted noticeably in fermenter cultivation, resulting in rapid production of chitosanases. As the specific growth rate increased, the high yield of cells provided higher cell concentration with higher specific rates of chitosan consumption and chitosanases production. As a result, the volumetric productivity of chitosanases was 1.2 times increased under aerobic conditions in fermenter. This indicated that oxygen plays a very important role in promoting the bacterial growth and the production of chitosanases. More or less, any suitable parameters for monitoring the supply of oxygen during cultivation, such as DO or K_La might be a key strategic optimization for scaling up the production of chitosanases in a large scale fermenter.

Moreover, when compared to the shake flask culture, the lag period of bacterial growth in fermenter culture was reduced to 6 h from 18 h (Figure 1 and 2). Substrate was also rapidly consumed under aerobic condition in fermenter. Chitosanases were produced in 18-36 and 6-20 h in shake flask and fermenter cultures, respectively.

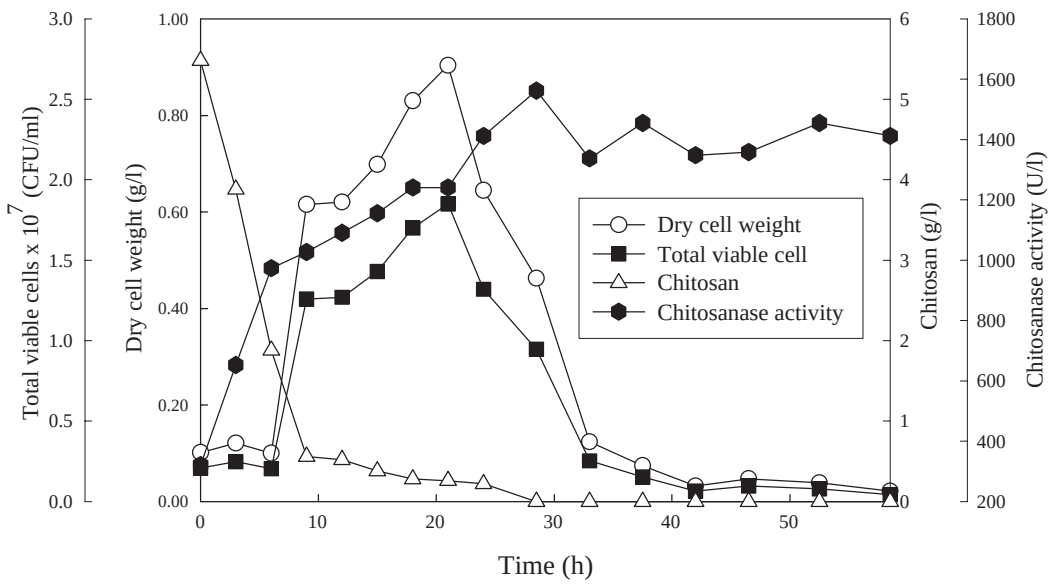

Figure 2 The production of chitosanases by *Bacillus cereus* TP12.24 in fermenter culture controlled at 1 vvm aeration, 400 rpm agitation, pH 6.0 and 30°C.

Table 2 Fermentation kinetics of *Bacillus cereus* TP12.24 from shake flask and fermenter cultures.

Culture conditions	μ (h^{-1})	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (U/g)	q_S (g/g h)	q_P (U/g h)	Q_P (U/l h)
Flask	0.260	0.352	247.59	0.091	31.99	35.29
Fermenter	0.304	0.447	181.01	0.682	154.37	43.55

Note:

(1) Flask culture referred to optimized conditions at initial pH 6.0, 0.5% chitosan and 30°C.

(2) The optimal conditions for fermenter culture were pH 6.0, 30°C, 400 rpm and 1 vvm.

(3) Calculations were done at maximal chitosanase activity obtained.

The enzymes were also increased at stationary growth phase to show the non-growth associated enzyme production. However, enzyme was quite stable in fermenter culture. Oxygen might confirm its important role during declining growth phase in promoting enzyme stability. Further investigation will be conducted on optimizing an effect of oxygen for the production of chitosanases.

The properties of crude chitosanases

The crude chitosanases after cell removal, prepared from the 2-l fermenter mentioned earlier were used without any further treatment for studying the pH and temperature optimum and stability of enzyme.

Effect of pH

The optimal pH of crude chitosanases was at pH 6.5 (Figure 3). At lower pH 3.0 and higher pH 9.0, the relative enzyme activities were 47.18 and 56.64%, respectively. This optimal pH was comparable to *Bacillus cereus* S1 chitosanases (pH 6.0) (Kurakake *et al*., 2000) and similar to chitosanases from *Bacillus circulans* MH-K1 (Yabuki *et al*., 1988) and *Bacillus* sp. No. 7-M (Uchida and Ohtakara, 1988). This, however, differed totally from those produced by *Bacillus subtilis* IMR-NK1 (Chiang *et al*., 2003) and *Bacillus megaterium* P1 (Pelletier and Sygusch, 1990) which were optimized at pH 4.0 and 4.5-6.5, respectively. As previously reported, the optimal pH's for various chitosanases were in a broad range of 4.0-8.0 (Somasheka and Joseph, 1996) depending on the bacterial strains.

Bacillus cereus TP12.24 chitosanases were found stable at a wide pH range of 3.0-8.0 retaining more than 70% activity after preincubation at 40°C for 60 min. However, at pH 9.0 and 11.0, the relative activities were decreased to 47.14 and 34.29%, respectively. Different chitosanases showed different pH stability, such as pH 6.0-11.0 for *Bacillus cereus* S1 chitosanases (Kurakake *et al*., 2000) and pH 5.0-9.0 for *Bacillus subtilis* IMR-NK1 chitosanases after preincubation at 25°C for 1 h (Chiang *et al*., 2003).

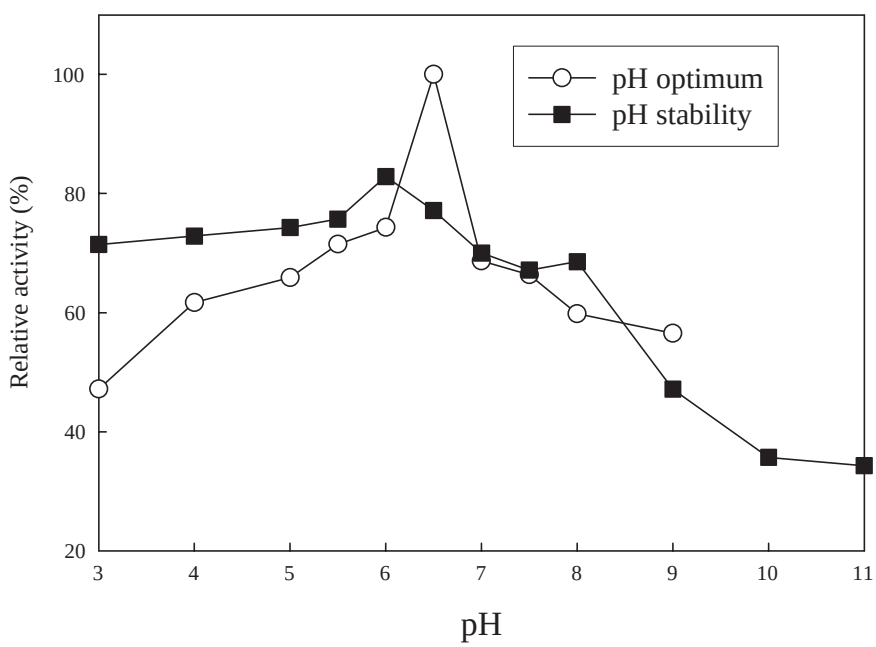

Figure 3 Optimal pH and pH stability of *Bacillus cereus* TP12.24 chitosanases.

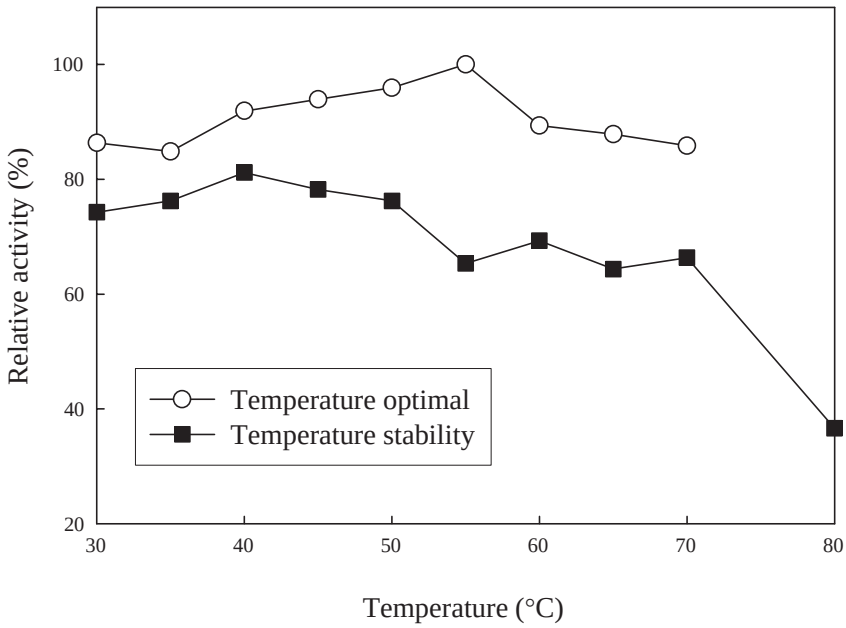

Figure 4 Optimal temperature and temperature stability of *Bacillus cereus* TP12.24 chitosanases.

Effect of temperatures

The activity of crude chitosanases from *Bacillus cereus* TP12.24 was found optimal at 55°C (Figure 4). At lower or higher temperatures, the relative activities were reduced to 86.36 and 85.86% at 30 and 70°C, respectively. This optimal temperature was slightly lower than that of *Bacillus cereus* S1 (60°C) (Kurakake *et al*., 2000), but higher than those of *Bacillus subtilis* IMR-NK1 (45°C) (Chiang *et al*., 2003) and *Bacillus megaterium* P1 (50°C) (Pelletier and Sygusch, 1990).

Bacillus cereus TP12.24 chitosanases were stable at temperature of 30-50°C showing 74.26-81.19% activity. However the enzyme activity was decreased at temperature higher than 50°C. Chitosanases from *Bacillus cereus* S1 were ever reported to be stable at temperature higher than 60°C at pH 5.0 for 30 min (Kurakake *et al*., 2000). Therefore, *Bacillus cereus* TP12.24 chitosanases were not the thermostable enzyme. The enzyme could be used at moderate temperature and neutral pH under the wide pH range of stability.

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