

## Characterization of Extracellular Chitinase from Bacterial Isolate 99 and *Enterobacter* sp. G-1 from Matsue City, Japan

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One hundred and twenty isolates of chitosanase producing bacteria were screened from water and soil from localities around Matsue city, Japan. In previous experiments, four isolates (isolates 96, 97, 99, and 100 strain) were analyzed for their chitosanase characteristics, and one of the isolates (99) was detected as being both a chitosanase and a chitinase producer. Characteristics of the chitinase enzyme were analyzed in this study. Chitinase from bacterial isolate 99 showed higher activities compared to that *Enterobacter* sp. G-1 (isolated from water in Matsue city, Japan), the activity was 0.039 U/ml and the specific activity was 0.56 U/mg protein, while those from *Enterobacter* sp. G-1 were 0.029 U/ml and 0.48 U/mg protein respectively. Chitinase from isolate 99 was stable in a pH range between 4-7, while that from *Enterobacter* sp. G-1 was stable in pH range 3-7. Optimum pH of the chitinase produced by isolate 99 was 5 whereas the chitinase from *Enterobacter* sp. G-1 it was pH 7. Chitinase from isolate 99 was stable at temperature 20-60°C, while that from *Enterobacter* sp. G-1 at 20-50°C. Chitinase secreted by isolate 99 showed optimum temperature of 50°C while chitinase from *Enterobacter* sp. G-1 was optimal at 40°C. Several ions (Fe<sup>2+</sup>, Ba<sup>2+</sup>, Co<sup>2+</sup>) increased the activity of the enzyme from isolate 99 whereas Ca<sup>2+</sup> and Co<sup>2+</sup> increased activity of the *Enterobacter* sp. G-1 chitinase.

Key words: chitinase activity, pH, temperature, metal ion

Chitinases (EC 3.2.1.14) are enzyme that catalyze the degradation of chitin into the monomer *N*-acetyl-D-glucosamine (Park *et al.* 1997; Yi-Wang *et al.* 2001). While chitosanases (EC 3.2.1.132) are glycosyl hydrolase that catalyze the degradation of chitosan into  $\alpha$ -D-glucosamine monomers. Chitin is a linear polymer of *N*-acetyl-D-glucosamine units linked through  $\alpha$  (1-4) glycosidic bonds and distributed widely in nature as the skeletal materials of crustaceans and insects (Minoru *et al.* 2002), and also as a cell wall component of bacteria and fungi. Chitosan is a partially or fully deacetylated chitin. The  $\alpha$  1.4 glycosidic bond at linear polymer of *N*-acetyl-D-glucosamine of chitin is very strong and the chitinase or a specific chitosanase can catalyze degradation of the bond into a simple monomer. Chitin combined with protein and (organic salt) CaCO<sub>3</sub> form the skeletal material of crustacean and insects and this structure is involved in self defence mechanism against pathogenic bacteria and evaporation (Yamasaki 1993).

The use of protein of skeletal crustacean as a protein source in poultry feed is inhibited by chitin compounds, because the poultry's digestive tract does not produce chitinase to hydrolyze chitin. Therefore, before adding to poultry feed, the crustacean skeleton should be hydrolyzed by chitinase into simple monomers, so that poultry can then digest.

Generally, bacteria use their chitinase for degrading chitin as their carbon source, but some of them use chitinases for their self defence mechanism against pathogenic microorganisms. The characterization of chitinase from some bacteria has been undertaken, for example from *Bacillus circulance* WL-12, *Enterobacter* sp. G1, *Stenotrophomonas maltophilia* C3, *Bacillus* sp. NCTU2, *Aeromonas* sp. 10 S-24, *Enterobacter* sp. NRG4, (Park *et al.* 1997; Watanabe

*et al.* 1999; Zhang *et al.* 2001; Min-Wen *et al.* 2002; Ueda *et al.* 2003; Dahiya *et al.* 2005). In nature, some bacterial species can produce chitosanases which hydrolyze chitin (Shimosaka *et al.* 1995) and cellululose (Reyes and Corona 1997) as their substrates. Mahata *et al.* (2005) reported that isolate 99 could produced both chitosanase and chitinase (at first, it was found that this bacterium could only produced chitosanase, whose activity was lower than that of isolate 97). Both chitosanase activities from isolate 99 and 97 were higher than the activity of *Matsuebacter chitosanotabidus* 3001 (a novel chitosanase bacterium isolated from water from Matsue city, Japan) used as control bacterium. The growth rates of isolate 99 in colloidal chitin solution and solid colloidal-chitin-agar medium were higher than that of other chitinase producing bacteria (119, 130, 136, and *Enterobacter* sp. G-1) in the same medium. Wide clear zones were produced by isolate 99 in solid colloidal chitin agar media. So far, we do not know how many types of chitinases and chitosanases are present in nature. The aims of this research was to characterize the chitinase from isolate 99 compared with the characteristics of chitinase from chitinolytic bacterium *Enterobacter* sp. G-1 as a control bacterium since it produces both chitinase and chitosanase.

### MATERIALS AND METHODS

**Materials.** The bacteria used in this experiment were isolate 99 and *Enterobacter* sp. G-1 from the laboratory collection of the Department of Biochemistry and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Japan.

**Bacteria Culture and Chitinase Production.** Single colonies of isolate 99 and *Enterobacter* sp. G-1 were cultured in 2 ml of Luria Bertani medium (1% polypepton, 0.5% yeast extract, and 1% NaCl) at pH 7.2 and 30°C for 24 h. One ml of each bacteria culture (isolate 99 and *Enterobacter* sp. G-1) were

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taken from Luria Bertani medium and cultured in 200 ml colloidal chitin solution. Every day, 2 ml of each bacteria culture were taken and centrifuged at 3 500 rpm for 5 min. After centrifugation, the supernatant was separated from the pellet (bacteria) and kept at 4°C for the analysis of enzyme activity, specific activity, optimum temperature, and stability, optimum pH and stability and also to analyze the effect of some metal ions on chitinase activity.

**Chitinase Activity and Specific Activity.** Chitinase activity was determined employing modified Schales method with colloidal chitin as substrate (Imoto and Yagashita 1971). A mixture of 0.5 ml colloidal chitin solution (pH 5.2), 1.48 ml McIlvaine buffer pH 7.0 and 20  $\mu$ l chitinase sample from isolate 99 and *Enterobacter* sp. G-1 were incubated at 30°C for 30 min in shaking incubator. The reaction was stopped by boiling the mixture (100°C) for 15 min, and then centrifuged at 3 500 rpm for 5 min. As much as 1.5 ml of the supernatant was placed in a test tube into which 2 ml Schales reagent was added. The reducing sugar (product of the reaction) in the supernatant was detected spectrophotometrically (spectronic 21) at A 420 nm. One Unit of chitinase activity equals the amount of chitinase needed to produce 1  $\mu$ mol reducing sugar which was equivalent to N-D, Acetyl glucosamine per min. Specific activity is measured by comparing chitinase activity (U) with protein content of the enzyme ( $\mu$ g protein), the protein content is measured by Lowry *et al.* (1951) method.

**Optimum Temperature and Stability.** Optimum temperature for chitinase activity from isolate 99 and *Enterobacter* sp. G-1 were determined at 20, 30, 40, 50, 60, and 70°C, and enzyme stability was determined at 20, 30, 40, 50, 60, 70°C for 60 min.

**Optimum pH and Stability.** Optimum pH of chitinase from isolate 99 strain and *Enterobacter* sp. G-1 were measured at pH 2 to 8 by using McIlvaine buffer. The chitinases sample from both bacteria were added to colloidal chitin substrate and incubated at 30°C for 30 min after which chitinase activity was analyzed. The pH of chitinase stability was measured at pH 2-8 and 30°C for 60 min and the colloidal chitin substrate was added before measuring chitinase activity.

**The Effect of Metal Ions on Chitinase Activity.** The effect of metal ions on chitinase activity produced by isolate 99 and *Enterobacter* sp. G-1 were determined employing Schales method (Imoto and Yagashita 1971). The chitinase sample from both bacteria were preincubated with certain ions ( $Mg^{2+}$ ,  $Na^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ , and  $Fe^{2+}$ ) in McIlvaine buffer pH 7.0 at 30°C for 30 min. The final concentration of metal ion in mixed solution was 1 mM. All ions were as chloride with the exception for  $Cu^{2+}$  which was a sulphate.

## RESULTS

**Chitinase Activity and Specific Activity.** The highest chitinase activity from isolate 99 was found on fourth day incubation while that from *Enterobacter* sp. G-1 on the third day (Fig 1). Chitinase activity from isolate 99 was 0.039 U/ml and its specific activity was 0.56 U/mg protein. Chitinase activity from isolate 99 and its specific activity were higher than those of *Enterobacter* sp. G-1 which

were 0.029 U/ml (Fig 2) and 0.48 U/mg protein (Fig 3).

**Optimum Temperature and Stability.** The optimum temperature of chitinase from isolate 99 was 50°C, and the temperature stability range was 20-60°C. The optimum temperature of chitinase from *Enterobacter* sp. G-1 was detected at 40°C and its temperature stability was at 20-50°C after 60 min incubation (Fig 4 and 5).

**Optimum pH and Stability.** The optimum pH of chitinase from isolate 99 was 5 and its pH stability range was 4 to 7, while the optimum pH of chitinase from *Enterobacter* sp. G-1 was 7 and its pH stability was 3 to 7 after 60 min incubation (Fig 6 and 7).

**The Effect of Metal Ions on Chitinase Activity.** Several ions ( $Fe^{2+}$ ,  $Ba^{2+}$ ,  $Co^{2+}$ ) increased the activity of enzyme from

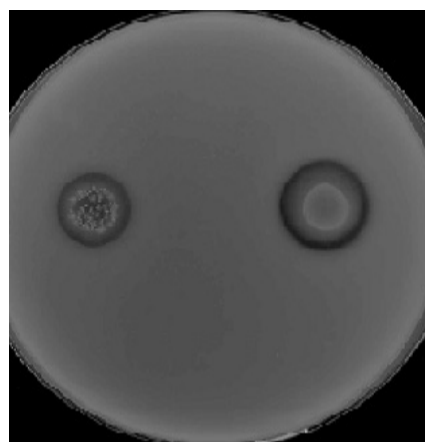


Fig 1 The clear zone of chitinase activity from isolate 99 on a solid chitin colloidal media on day 3 incubation. Right the clear zone of 10  $\mu$ l of isolate 99 culture with diameter 1.10 cm. Left the clear zone of 10  $\mu$ l chitinase from isolate 99 with diameter 0.90 cm.

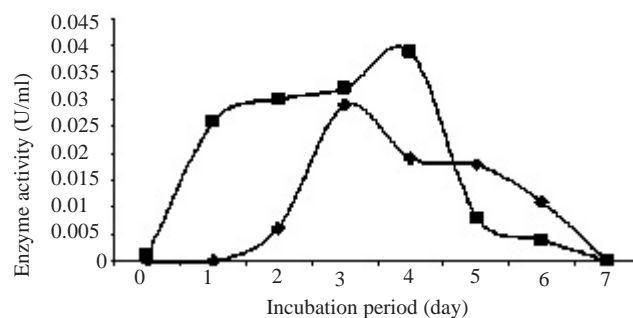


Fig 2 Chitinase activity from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

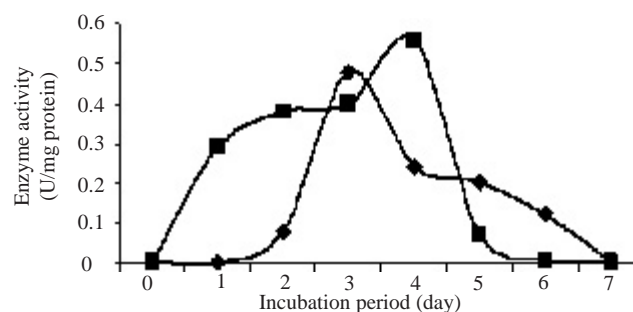


Fig 3 Chitinase specific activity from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

isolate 99 while Ca<sup>2+</sup> and Co<sup>2+</sup> increased activity of the *Enterobacter* sp. G-1 chitinase (Table 1).

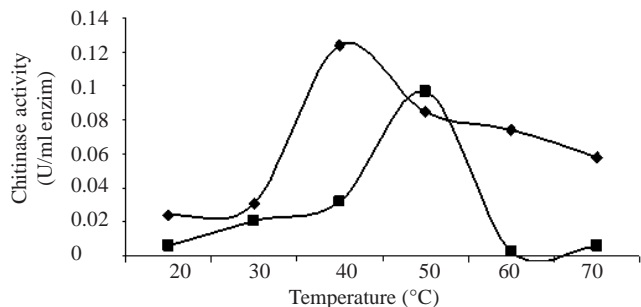


Fig 4 The effect of temperature on chitinase activity from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

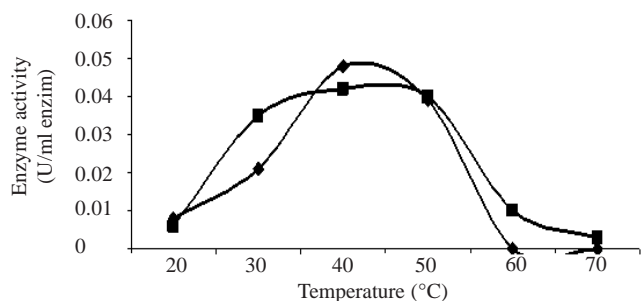


Fig 5 Chitinase temperature stability (°C) curve from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

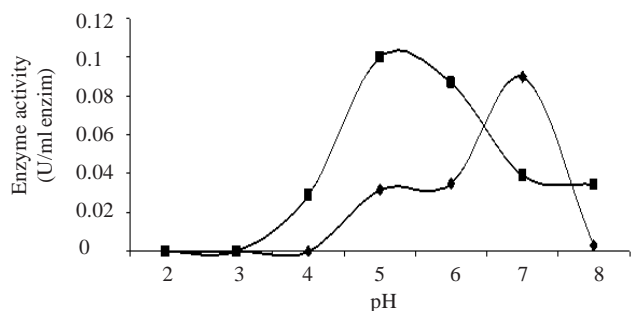


Fig 6 The effect of pH upon chitinase activity from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

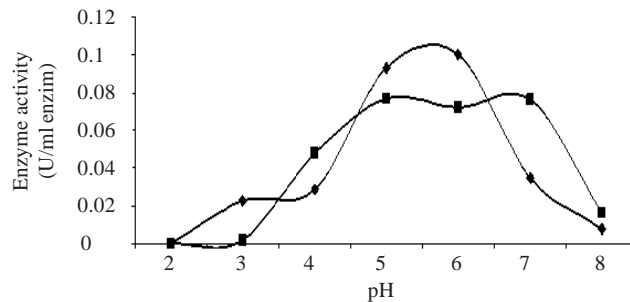


Fig 7 Chitinase pH stability from isolate 99 and *Enterobacter* sp. G-1: —◆— chitinase 99, —■— chitinase *Enterobacter* sp. G-1.

DISCUSSION

This study revealed that the activity of chitinase from isolate 99 (0.039 U/ml) was higher than that of *Enterobacter* sp. G-1 (0.029 U/ml), but its activity was lower than chitinase activity from *Paneibacillus illinoisensis* (3.4 U/mg) which was isolated from a garden soil containing crab shell on the west coast of Korea (Hwan *et al.* 2006). The chitinase from isolate 99 later will be used for hydrolyzing chitin in shrimp waste, and then the shrimp waste is used for poultry feed. Yamasaki *et al.* (1993) reported that *Enterobacter* sp. G-1 was both a chitosanase and a chitinase producing bacterium, but its chitinase activity was lower than its chitosanase activity. The highest chitinase specific activity from isolate 99 was 0.56 U/mg protein which was higher than the highest chitinase activity from *Enterobacter* sp. G-1 (0.48 U/mg protein). This fact indicated that chitinase protein content from both bacteria was equivalent with its activity. Compared with other bacteria, chitinase specific activity from isolate 99 in this experiment was higher than that of *Stenotrophomonas maltophilia* C3 (0.14 U/mg protein), but lower than purified chitinase from *Pseudomonas aeruginosa* Strain 385 (1.12 U/mg protein) (Thompson *et al.* 2001),

Table 2 Substrate specificity of chitosanase from isolate 97, 99, and *Matsuebacter chitosanotabidus* 3001

Substrate	Relative activity (%)		
	<i>M. Chitosanotabidus</i> 3001	99	97
80% deacetylated chitosan	100	100	100
70% deacetylated chitosan	50	118	0
90% deacetylated chitosan	31	30	1
100% deacetylated chitosan	8	13	20
0.5% Colloidal chitin	0	21	0

Table 1 The effect of metal ions on chitinase activity from isolate 99 and *Enterobacter* sp. G-1

Ion	Bacteria	Chitinase activity (U/ml)		Percentage increase or decrease* after incubation with ion
		before incubation	with ion after incubation	
Fe <sup>2+</sup>	Isolate 99	0.039	0.055	41.03
	<i>Enterobacter</i> sp3 G-1	0.029	0.040	37.93
Ba <sup>2+</sup>	Isolate 99	0.039	0.089	128.21
	<i>Enterobacter</i> sp3 G-1	0.029	0.023	20.69*
Ca <sup>2+</sup>	Isolate 99	0.039	0.040	2.561
	<i>Enterobacter</i> sp3 G-1	0.029	0.064	20.69
Cu <sup>2+</sup>	Isolate 99	0.039	0.040	2.56
	<i>Enterobacter</i> sp3 G-1	0.029	0.049	68.97
Co <sup>2+</sup>	Isolate 99	0.039	0.221	466.67
	<i>Enterobacter</i> s3p G-1	0.029	0.246	748.28

and purified chitinase from *Aspergillus fumigatus* YJ-407 (3.36 U/mg protein) (Xia *et al.* 2001). This experiment showed that isolate 99 was a potential chitinase producing bacterium. Its ability to produce chitinase was better than that *Enterobacter* sp. G-1, and it was also a potential chitosanase producing bacterium because its chitosanase activity was higher than that of *Matsuebacter chitosanotabidus* 3001 in previous experiments (Table 2) (Mahata *et al.* 2005). This experiment found that the isolate 99 is a bacterium that can produce both chitinase and chitosanase.

The optimum temperature of chitinase from isolate 99 was 50°C which was higher than the optimum temperature of chitinase from *Enterobacter* sp. G-1 (40°C). This data shows that chitinase from isolate 99 was more tolerant to high temperature than chitinase from *Enterobacter* sp. G-1, but that its activity in optimum temperature was lower than that of *Enterobacter* sp. G-1. Yi-Wang *et al.* (2001) stated that the optimum temperature of exochitinase from *Bacillus cereus* was 35°C who it was lower than chitinase from isolate 99, but relatively similar to chitinase from *Enterobacter* sp. G-1. Min-Wen *et al.* (2002) also found that the optimum temperature range of chitinase from *Bacillus* sp. NCTU2 was 50 to 60°C, and that the chitinase optimum temperature from isolate 99 was within in this range. This experiment also found that the temperature stability (incubated at 60 min) of chitinase from isolate 99 was 20 to 60°C, and for *Enterobacter* sp. G-1 it was between 20 to 50°C. This data showed that chitinase from isolate 99 was more tolerant and stable at high temperature compared with chitinase from *Enterobacter* sp. G-1, but that its activity was not as high as chitinase activity from *Enterobacter* sp. G-1. Yi-Wang *et al.* (2001) reported *Bacillus cereus* had a wide range of temperature stability, the range was from 4 to 70°C. The temperature stability of chitinase from isolate 99 and *Enterobacter* sp. G-1 in these studies were within this range. Apparently, the chitinase from isolate 99 can be characterized as a thermotolerant enzyme and its stability is adequate for industrial application.

In acid conditions, chitinase from isolate 99 degraded chitin more actively than chitinase from *Enterobacter* sp. G-1. In general, the optimum pH of chitinase from microorganisms (bacteria, yeast, fungi) is 3.5 to 8 (Koga *et al.* 1999), and the optimum pH of chitinase from isolate 99 and *Enterobacter* sp. G-1 in these experiments were within the range reported. Chitinase activity from isolate 99 was stable at pH 4 to 7, while *Enterobacter* sp. G-1 was stable at pH 3 to 7. Chitinase from both bacteria were stable in acid or alkaline conditions. The pH stability of chitinase from *Bacillus cereus* and *Bacillus* sp. NCTU2 were 2.5 to 8 (Yi-Wang *et al.* 2001 and Min-Wen *et al.* 2002). The pH stability of chitinase from isolate 99 and *Enterobacter* sp. G-1 in this experiment was within this reported range.

Chitinase activity from isolate 99 was increased to 41.03, 128.21, and 466.67% respectively after being incubated with  $\text{Fe}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{Co}^{2+}$ , while chitinase activity from *Enterobacter* sp. G-1 was increased up to 120% after being incubated with  $\text{Ca}^{2+}$  and 748.28% with  $\text{Co}^{2+}$ . Chitinase activity from *Enterobacter* sp. G-1 was decreased to 20.69% after being incubated with  $\text{Ba}^{2+}$ . In general chitinase activity was inhibited by  $\text{Hg}^{2+}$  and  $\text{Ag}^+$ , while  $\text{Cu}^{2+}$  could

increase or decrease chitinase activity. In some fish species and microorganism like *Pseudomonas aeruginosa*, their chitinase activities are increased by  $\text{Cu}^{2+}$  (Jolle's and Muzzarelli 1999). Some chitinase enzymes are very sensitive to metal ions. For example, chitinase activity from *Aspergillus fumigatus* was inhibited strongly by  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  (Xia *et al.* 2001). Chitinase activity from *Bacillus brevis* was inhibited by  $\text{Ag}^+$  after incubated in 1 mmol l<sup>-1</sup>  $\text{Ag}^+$  at pH 8 and 30°C for 30 min and only 60% of the enzyme activity remained (Sheng *et al.* 2004). Howard *et al.* (2004) reported that chitinase B of *Microbulbifer degradans* 2-40 has two catalytic domains (GH18N and GH18C), and the activity of each domain was not affected by a 10 mM concentration of various chloride salts:  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ , but the activity of GH18N was reduced 36% by  $\text{Ni}^{2+}$ , 8% by  $\text{Sr}^{2+}$ , and 41% by  $\text{Cu}^{2+}$ , while the activity of GH18C was reduced 14% by  $\text{Ni}^{2+}$ , 5% by  $\text{Sr}^{2+}$  and 53% by  $\text{Cu}^{2+}$ .  $\text{Hg}^{2+}$  completely inhibited the activities of both domains. The metal ion sensitivity of chitinase from isolate 99 strain and *Enterobacter* sp. G-1 in this experiment was not the same as reported previously, its activity was not inhibited by  $\text{Fe}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Co}^{2+}$ , except that for chitinase from *Enterobacter* sp. G-1 which was decreased by  $\text{Ba}^{2+}$  (Table 1). Its response to other ions is still unknown.

In conclusion, the chitinase characteristics (enzyme activity, temperature, pH, and the effect of metal ion on chitinase activity) from isolate 99 were better and it was more tolerant than those of *Enterobacter* sp. G-1. Several ions ( $\text{Fe}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Co}^{2+}$ ) increased the activity of the enzyme from isolate 99, while  $\text{Ca}^{2+}$  and  $\text{Co}^{2+}$  increased the activity of chitinase from *Enterobacter* sp. G-1 but  $\text{Ba}^{2+}$  decreased its activity.

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