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SCREENING AND CHARACTERIZATION OF BACTERIAL CHITOSANASE FROM MARINE ENVIRONMENT

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ABSTRACT

Screening of extracellular chitosanase from bacterial isolates associated with marine sponges have been done. Out of 100 bacterial isolates, forty isolates were capable of forming clearing zones on the chitin media and one isolate, 34-b, produced the highest chitinolytic index. The enzymes was produced on chitin liquid medium at 37°C in a shaking waterbath for a five-day cultivation. Crude enzymes were prepared by cell-free supernatant (CFS) and concentrated through 70% (saturated) ammonium sulphate percipitation followed by dialysis. The enzymes worked best at pH and temperature of 6-7 and 60°C, respectively. The half-life ($T_{1/2}$) for chitosanase activity was 500.2 min or 8.34 hours (at 37°C) and 55.12 min (at 50°C), indicating the enzyme are quite stable at that temperature. However, around 80% of the original activity was lost at 60°C after 15 min of incubation.

Key words: chitosanase, marine environment

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INTRODUCTION

Chitosanase (EC 3.2.1.132) has attracted much scientific and industrial attentions in recent years. This enzyme catalyzes the degradation of chitosan into *N*-chitooligosaccharides via the hydrolysis of 2-amino-2-deoxy-*D*-glucoside linkages (Tanabe, *et al.*, 1992; Hutadilok, *et al.*, 1995). Promising application of this enzyme in food and pharmaceutical industries has increased due to the popularity of chito-oligosaccharides and their bioactive properties possessed. Chito-oligosaccharide possesses higher advantages over its polymer chitosan, i.e. biologically more active and water soluble, therefore, chito-oligosacharide is widely used for functional food ingredient and other pharmaceutical products. Chito-oligosaccharides have been claimed to have ability to bind excess of fat, support immunity, lower blood sugar, controls blood pressure, prevent constipation, enhances calcium absorption, prevent heart disease and reduce blood levels of uric acid and posses anticancer and antibacterial activity

In the course of our screening for a novel type of chitinolytic enzymes, i.e. chitosanase and chitinase, we screened bacteria from marine environment. Marine environment is rich in chitinous material

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(Svitil, *et al*. 1996). Therefore, as nature work, chitinolytic enzyme should be abundant in marine environment. However, researches on chitinolytic enzyme from marine are scarce. Murao *et al*. (1992) isolated *Vibrio alginolyticus* TK-22 chitinase from marine environment, i.e. seawater of Ariakekai, Japan, while *Vibrio alginolyticus* H-8 chitinase have been isolated from seashore of Shizuoka Prefecture (Ohishi *et al*., 1996). Our previous work on screening of chitosanase from marine environment, i.e. Mentawai waters, revealed that of 86 bacteria isolated from 24 marine sponges, *Bacillus* sp KBJ 12 SB, produced the best performed chitosanase (Chasanah *et al*., 2007).

In this report, chitosanase is screened from the bacteria associated with marine sponges from Binuangen (West Java) and Nias (Sumatera) surrounding waters. The marine sponges have been well known as the richest sources of microbial communities such as bacteria either ingested as food or permanently inhabitants. It was reported that around 40% of sponge volume is microbes (Taylor *et al*., 2007).

MATERIALS AND METHODS

Isolation of potential isolates

Sponges were collected from Binuangen and Nias island waters by diving, and washed with sterile sea water before they were transported to laboratory in a cool box. Each sponge sample (ten grams) was cut aseptically into small pieces, mixed with 90 ml of 0.9% NaCl, and then homogenized in a sterile plastic sac. The homogenized sample (0.1 ml) was spread on the solid MSM media (0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO$, 0.05% yeast extract, 2% agar) and then incubated at 37^{o}C for 2 days.

To obtain pure cultures, the resulting mixed cultures were streaked on the MSM media, which was followed a 48-hour incubation at 37^{o}C. Single colonies appeared were individually transferred using sterile tooth sticks to new solid media. After incubating at 37^{o}C for 1 day, the pure cultures were kept on 40% glycerol solution as stock cultures and stored at -80^{0}C. The strains were then observed for the hydrolytic ability towards chitin by growing them on the same media containing colloidal chitin. The positive cultures were marked with a clearing zone surrounding their colonies. The chitinolytic index (CI) was then determined as a ratio of the diameter of clearing zone around the colony over the colony's diameter. The strains with relatively high hydrolytic index were used further for enzyme production.

Preparation of substrates for enzyme production and assays

The substrate for enzyme production, colloidal chitin, was obtained from commercially available chitin powder (Sigma) based on the method described by Arnold and Solomon (1986). Whereas, the substrate for chitosanase assay, 1% soluble chitosan, was prepared according to Uchida and Ohtakara (1998) using 85% deacetylated chitosan (Sigma).

Production and extraction of enzyme

Bacterial cells were cultivated in the Minimal Synthetic Medium (MSM) supplemented with 0.5% colloidal chitin as the sole carbon source. The medium consisted of 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO$, 0.05% yeast extract. One full loop of the fresh bacterial cells grown on an agar plate was picked up and inoculated into the 20-ml starting medium. After 15 hours of incubation in the shaking water bath (37^{o}C, 100 rpm), 10% (v/v) of the resulting culture was transferred into the 80-ml production medium, followed with one-day incubation at the same temperature and shaking as

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mentioned above. Crude chitosanase enzyme obtained by centrifuging the resulting culture at 9.000 *x g* (4°C, 25 min), followed by concentrating the protein enzyme using ammonium sulphate (70% (w/v) ammonium sulfate was added slowly, kept overnight at 4°C, and centrifuged at 12.000 *x g* (4°C, 6 min). The precipitate obtained was suspended with 15 ml phosphate buffer pH 7.0; and the suspension was dialyzed with the same buffer to remove the salt.

Assay of chitosanase activity

Assay of chitosanase activity was done based on the method described by Yoon, *et al.* (2000), in which it involved: (i) hydrolytic reaction of soluble chitosan, and (ii) staining and spectrophotometric measurement of reaction product. The substrate, 0.1% soluble chitosan (100 µl) was added with phosphate buffer (0.05M,) pH 6.0 in an Eppendorf tube. Then 100 µl of enzyme solution was added into the tube followed with a 30-min incubation at 37°C in order to let the enzymatic reaction take place. After a short mixing by vortex, the resulting mixture (200 µl) was put in a reaction tube filled with 800 µl water. After adding 1 ml of Schales reagent, it was boiled 15 min. and then centrifuged at 8,000 rpm for 10 min. The absorbance was measured at 420 nm by using spectrophotometer. One unit of chitosanase activity was defined as the amount of enzyme that produces reducing sugar corresponding to 1 µmol of D-glucosamine per minute.

Measurement of protein content.

The protein content of the crude enzyme was estimated according to the Lowry method with bovine serum albumin as the standard solution(Bollag and Edelstain, 1991).

Determination of optimum pH.

The optimal pH of chitosanase activities was determined at 37°C by using overlapping pH ranges. To generate such pH ranges, the following buffer systems were used: 0.2 M citrate buffer (pH 4.0-6.0), 0.2 M phosphate buffer (pH 6.0-8.0), 0.2 M borate buffer (pH 8.0-9.0), 0.2 M glycine buffer (pH 9.0-10), 0.2 M NaOH-borate buffer (pH 10). The measurement procedure and conditions followed the standard assay as described previously.

Determination of optimum temperature.

The optimal temperature of the enzyme was measured at different temperatures (30, 37, 50, 60, 70°C). The measurement was initiated by adding the sample with the substrate and phosphate buffer in an eppendorf tube. After mixing by inverting the tube several times, the mixture was incubated for 30 min at a desired temperature in a shaking waterbath. The product staining and spectrophotometric measurement steps were done as the standard assay described above.

Thermostability study.

Enzyme thermostability was determined as follows. An enzyme solution was firstly distributed into tightly closed eppendorf tube, in which each vial contained 50 µl enzyme. Then the tubes were incubated at a desired temperature in the water bath. Every 15 minutes, one of the tubes was withdrawn and immediately cooled in ice for a short time. The enzyme activity was assayed at the optimal temperature using the standard assay as described before.

Effect of metals.

The effects of divalent cations as chlorides metals on the oxidative and reductive activities were measured at the final concentration of 1 mM. The tested divalent

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cations included Mg^{2+}, Mn^{2+}, Li^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ba^{2+} and Ca^{2+}. As the control in this experiment, the enzyme solution without containing any metals was used. In addition, the enzyme preparations with monovalent cations (K^+, Na^+, $NH4^+$) as well as a trivalent cation (Fe^{3+}) were also used as the control in comparison with the divalents cations. The inhibitory influence of EDTA on the activity was also determined in order to check the metal dependency of the enzyme.

RESULTS AND DISCUSSION

Results

Screening of chitinolytic bacteria

Screening of chitinolytic enzyme-producing microorganisms from marine sponge samples resulted in 100 different bacterial isolates obtained from four different specimens of sponges. Of such number, forty are capable of secreting chitin-degrading enzymes into the culture medium. Then five of them with relatively higher clearing zones were selected further for the production of chitosanase. Among the five chosen strains, the strain designed as 34bs is considered to be the most suitable for further chitosanase production based on its promising properties, including: (i) the relatively higher of CI value, i.e. 11.0 (ii) the shorter cultivation time needed to achieve an optimal enzyme activity, and (iii) the relatively higher chitosanase activities. The 34bs chitosanase was optimally produced at 1st day of incubation with enzyme activity being produced of 4.0 U/mg. The information on its microscopic, growth and biochemical characteristics is summarized in Table 1, which has not given yet a convincing clue about its identity. Identification based on the 16S rRNA gene is in progress.

Table 1. Microscopic, morphological and biochemical characteristics of isolate 34bs

Characteristic	Result
Colony color	Pale yellow with white in the middle
Colony shape	Irregular (4 mm in diameter)
Gram	Negative
Cell shape	Small short rod
Chitinolytic index (CI)	11.0
Catalase	Positive
Oxidase	Negative
Glucose fermentation	Negative
Citrate	Positive
Peptone	Negative
Urease	Negative
Methyl Red	Negative
Voges Proskauer	Negative
Gelatinase	d (dubius)
Nitrate	d (dubius)

Enzyme preparation.

Preparation of crude chitinolytic enzymes was performed in two steps which was started with cell-free supernatant (CFS) preparation, followed with ammonium sulphate percipitation (70% saturation) and dialysis. The results (Table 2) shows that the total activity of chitosanase in the 800-ml CFS and the 15-ml dialysate was 4.8 U and 0.67 U, respectively. The dialysate (CFS) obtained (15 ml) indicated a relatively high specific activity (0.339 $U.mg^{-1}$) as shown in Table 2, suggesting a large amount of

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chitinolytic enzymes present in it. Particularly, the specific chitinase activity of the dialysate showed an increase up to 2 times compared with that of the CFS. The activity recovery of the dialysate was approximately 18.75% of the CFS activity.

Table 2. Summary of extraction of chitosanase produced by strain 34bs

Parameter observed	CFS	Dialysat
Total volume (ml)	800	15
Total Protein (mg)	28	1
Total activity (U)	4.8	0.67
Specific activity ($U.mg^{-1}$)	0.166	0.339
Yield (%)	100	18.75
Purity (fold)	1	2.042

Optimal pH

The optimal pH of chitosanase activity was observed in the neutral pHs of 6-7. The chitosanase activity reduced a lot at the pH of higher than 8, but was completely inactivated at the pH 5 or lower and the pH 9 or higher.

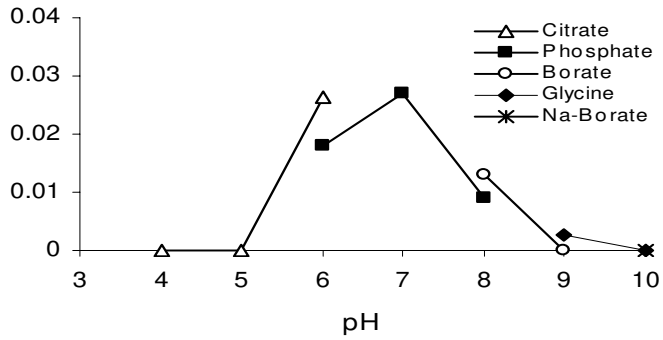

Fig 1. The 34bs enzyme activity at different pHs, showing optimal activity for chitosanase .

Temperature and thermostability.

It was found that the chitosanase activity of our enzyme sample revealed the optimal temperature of 60°C. The enzyme was not stable at the optimum temperature but quite stable at temperature below 60°C. had half-life ($T_{1/2}$) of 500.2 min or 8.34 hours at 37°C and 55.12 min at 50°C

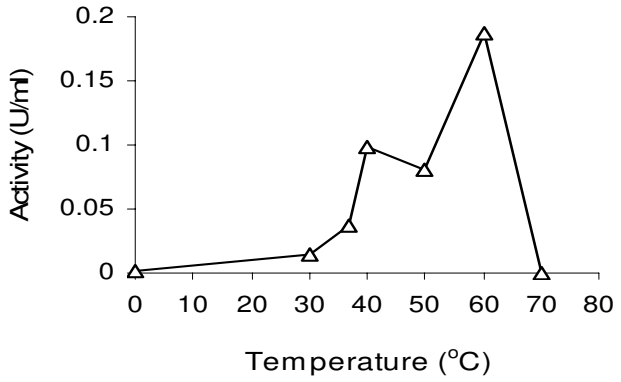

Fig 2. The activity of 34bs chitosanase at various incubation temperatures.

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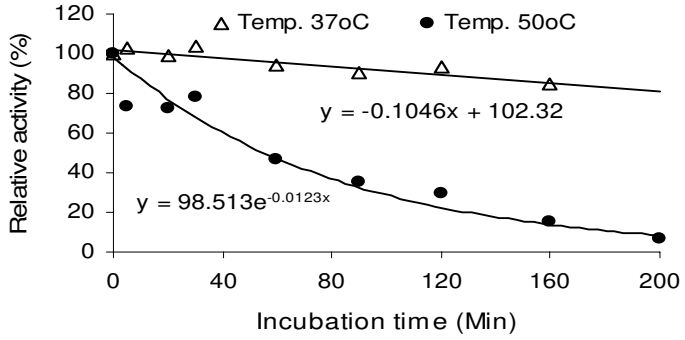

Fig 3. Thermal stability of 34bs chitosanase at 37 ^{o}C and 50 ^{0}C

Effects of metals ion

The effect of bivalent metal ions (Mg^{2+}, Mn^{2+}, Li^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}) in the salt form (Cl_2) on the chitosanase activity was measured at the final concentration of 1 mM. The results obtained showed that all metal ion added were able to increase the enzyme activity.

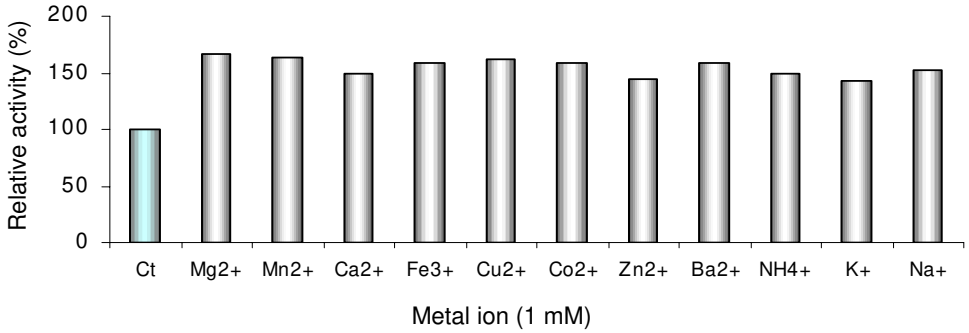

Fig. 4. Effect of additives on enzyme activity

Kinetic properties.

The effect of the substrate at different concentrations on the enzyme activity was investigated at 60^{o}C. The results indicated that chitosanase activities obeyed the Michaelis-Menten hyperbolic curve. If the curve is converted into the lineweaver-Burk plot, it was found that the apparent V_{max} and K_m values of the chitosanase for soluble chitosan were 1.251 U/mg and 0.33 mg/ml.

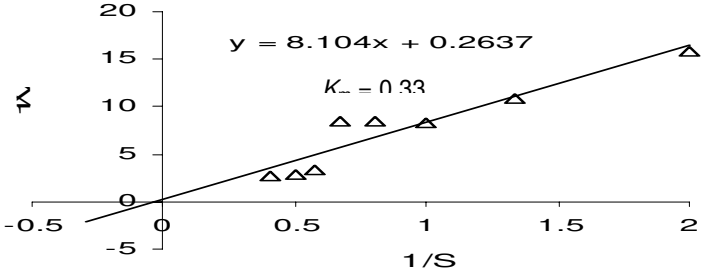

Fig. 5. The enzyme kinetics of crude 34bs chitosanase

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Discussion

Our attempt to screen chitosanase enzymes from marine environment was based on the supported data on answering the hypothesis that marine environment is rich in extracellular chitinolytic activity. Study by (Cottrell *et al*., 1999) revealed that marine bacterial communities capable of chitin degradation which is abundantly available in marine environment could be assessed by culture-dependent methods, and it did not greatly underestimate the portion of chitin degrader in the communities.

Isolate 34bs, isolated from sponge from Binuangen waters, was choosen as the best chitosanase producer isolate among forty isolates capable of secreting chitin-degrading enzymes into the solid culture medium. In previous report, isolate 34bs produce both chitosanse and chitanase. Among the 5 isolates producing relatively higher clearing zones, the isolate 34bs were selected further due to the highest activity of chitosanase. Chitosanase produced in liquid medium with addition of 2 % choloidal chitin which confirm that this enzyme like most of chitosanases is inducible enzyme. However, some of microorganisms have been reported to produce chitosanase without inducer such as *Bacillus* sp. KCTC 0377BP (Choi *et al*., 2004), *Bacillus cereus* S1 (Kurakake *et al*., 2000) *Bacillus* sp. HW-002 (Lee *et al.,* 1996) and *Acinetobacter* sp. strain CHB101 (Shimosaka *et al.* 1995).

The activity of chitosanase from 34bs, 0.67 in dialysates, is higher than some of chitosanases which have the activity usually less than 0.5 U/ml. (Zhu *et al.,* 2003). Higher activities have been found, 0.8 U/ml from *Entererobacter* sp. G-1 (Yamasaki *et al*., 1994), 1.1 U/ml from *Bacillus circulans* WL-12 (Mitsutomi *et al.,* 1998). and 2.8 U/ml from *Acinetobacter* sp. C-17 (Zhu *et al,*. 2003).

The chitosanase produced by 34bs worked well at pH 6-7. The activity was drop at pH value higher than 7. Similar results were also reported for chitosanase from *Bacillus* sp. No. 7-M and *Nocardia orientalis* isolated from Matsue, Japan (Somashekar and Joseph, 1995), chitosanase from *Streptomyces griceus* (Tanabe *et al.*, 2003) as well as from *Bacillus* sp.

Strain KCTC 0377BP (Choi *et al.,* 2004). Other chitosanases have different optimum pH such as produced by *Bacillus subtilis* IMR-NK1 at 4.0 (Chiang *et al.* 2003), *Bacillus* sp. P16 at 5.5 (Jo *et al.* 2003) and by *Bacillus* sp. KCTC 0377BP at pH 4-6 (Choi *et al.* 2004). The optimum temperature for chitosanase from 34bs is 60^0C. It is similar to chitosanase from some microorganisms, such as *B. circulans* and *Bacterium* sp. K-1 (Somashekar and Joseph, 1996).

Thermal stability at three different temperatures (37, 50, 60^{o}C) was estimated by incubating the enzyme for 200 minutes at those temperature. The results showed that the half-life ($T_{1/2}$) of this enzyme was 500.2 min or 8.34 hours at 37^{o}C and 55.12 min at 50^{o}C. The chitosanolytic activity of the enzyme lost significantly by around 85% compared with that of the non-heated enzyme after 15 min of incubation and completely zero after 60 min of heating at 60^{o}C. Several factors have been suggested to contribute to the thermostability of enzymes, in general including amino acid composition and intrinsic properties and/or structural organisation (Radianingtyas and Wright, 2003). The others chitosanases was stable at lower temperature such as chitosanase produced by *Streptomyces griceus* which was stable at 40^{o}C. (Tanabe *et al.* 2003) and by *Bacillus* sp.

Strain KCTC 0377BP at 55^{o}C (Choi *et al.* 2004). Chitosanases that were reported not inhibited by metal ion (Choi *et al.,* 2003). It was similar to chitosanase from 34bs where all of metal ion which are measaured able to increase the enzyme activity. Different result showed when chitosanase from *Bacillus* sp. Strain KCTC 0377BP added by NaCl and KCl which loss its activity around 50% (Choi *et al.,* 2004)

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while chitosanase from 34bs was not affected even able to increase the enzyme activity. Mn does not inhibit chitosanase generaly including from 34bs but exception from *Bacillus* sp. Strain KCTC 0377BP. Addition of Mn increasing the activity of 34bs chitosanase up to 50%.

CONCLUSION

Chitosanase produced by 34bs, one of 100 isolates which was capable of forming clearing zone on colloidal chitin media, worked best at pH and temperature of 6-7 and 60°C, respectively. The half-life ($T_{1/2}$) for chitosanase activity was 500.2 min or 8.34 hours (at 37°C) and 55.12 min (at 50°C), indicating the enzyme are quite stable at that temperature. However, around 80% of the original activity was lost at 60°C after 15 min of incubation.

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