Malays. Appl. Biol. (2020) 49(2): 37–42

ISOLATION AND CHARACTERIZATION OF A SURFACTANT-STABLE PROTEASE FROM HALOPHILIC BACTERIA *Chromohalobacter Japonicus* BK-AB18

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Accepted 23 April 2020, Published online 6 July 2020

ABSTRACT

The protease from *Chromohalobacter japonicus* BK-AB18 was produced by growing bacteria in a LB medium containing 5% casein and 5% NaCl. The crude protease was partially purified by three levels of ammonium sulfate concentration (ranges of 0–70%, 70–75% and 75–80%) and the highest specific activity was exhibited in the range of 75–80%. The enzyme has a relative molecular weight of 65 kDa. The protease in this fraction had the highest activity in the following optimum conditions: 7.5% NaCl, a pH of 9.0 and a temperature of 45°C. The activity of the enzyme at the optimum pH and temperature was enhanced by the addition of a Ca^{2+} ion, but its activity was significantly inhibited by EDTA, hence this enzyme is included as metalloenzyme. Interestingly, the protease activity increased when exposed to a concentration of 0.01% and 0.05% SDS, and was relatively stable in this solution up to a concentration of 10%. It is thus demonstrated that *C. japonicus* BK-AB18 is a potential source to produce extracellular protease that can be applied in the surfactant/detergent industry.

Key words: *Chromohalobacter japonicus*, protease, halophilic, surfactant-stable

INTRODUCTION

Proteases are hydrolysis enzymes that cleave proteins into shorter peptides (Motyan, *et al*., 2013). Proteases represent the most used industrial enzyme applied in various types of industries, such as the manufacture of surfactants and detergents. These industries require proteases that have a high activity and good performance in various conditions, from mild to extreme, such as in detergents and also in conditions of high salinity (Rao *et al*., 1998). In this study, we are interested in exploring the potential of protease produced by local halophilic bacteria in Indonesia. Halophilic bacteria have the ability to live in extreme conditions, and the biomolecules of these bacteria have good stability and unique character. *Bacillus* sp., *Halobacillus karajensis*, *Salicomarasensis* sp. IC10 and *Chromohalobacter* sp. TVSP101 are halophilic bacteria that produce extracellular proteases (Rao *et al*., 1998; Vidyasagar *et al*., 2009) showing unique biochemical and biophysical properties. The extracellular protease isolated from halophilic bacteria *Bacillus* sp. and *Bacillus mojavensis* A21 has been reported to be stable in both SDS solutions and detergent solutions, therefore having potential to be applied in the surfactant/detergent industry (Gupta *et al*., 2002; Haddar *et al*., 2009).

In the present study, we attempted to explore the potential of five halophilic bacteria isolated from the mud crater Bledug Kuwu in Purwodadi, Central Java, Indonesia (Asy'ari *et al*., 2014). We used four isolates of the *Halomonas* genus of bacteria: *Halomonas elongata* BK-AB8, *Halomonas meridian* BK-AB4, *Halomonas elongata* BK-AG18 and *Halomonas eurihalina* BK-AB15, as well as the isolate *Chromohalobacter japonicus:* BK-AB18 (Asy'ari *et al*., 2014). In this study, we reported that *C. japonicus* BK-AB18 is the most potent among halophilic bacteria producing protease that is stable in surfactant solutions.

MATERIALS AND METHODS

Chemicals

Ammonium sulfate, Luria-Bertani (LB) medium, NaCl, SDS, Tris-HCl buffer, KCl, $MgCl_2$, various

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metal ions (EDTA, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+} and Zn^{2+} ions).

Bacterial cultivation

The medium used for the cultivation of bacteria was Luria-Bertani (LB) liquid medium containing 1% tryptone, 0.5% yeast extract and 10% NaCl. Bacterial isolates were transferred into 5 ml LB medium and incubated at 150 rpm at 37°C in an incubator shaker for 24 hours.

Screening of proteolytic activity

The screening for proteolytic activity was assayed by a modification of agar diffusion method using a medium containing 2% bacto agar and 2% casein (Sneha *et al*., 2014). After incubation for 24 hours, the culture was centrifuged at 9.820×*g* for 10 minutes. Twenty microliters of free-cell supernatant from each culture was placed on paper discs on agar plates and then incubated at 37°C. The casein hydrolysis detected a clean zone in an area around the paper disc as an indication of proteolytic activity.

Production and purification of the protease

A protease production medium containing 3 gm/l yeast extract, 5 gm/l KCl, 5 gm/l casein, 10 gm/l $MgCl_2$, 10 gm/l peptone and 5% NaCl was used. A measure of 1% of the culture was transferred into a protease production medium and then incubated at 150 rpm at 37°C for 16 hours (incubation timing for optimum protease activity). The culture was centrifuged at 9.820×*g* at 4°C for 15 minutes. The free-cell supernatant was used and partially purified by ammonium sulfate precipitation as described by Scopes, (1994). The crude protease (the free-cell supernatant) were partially purified by three levels of ammonium sulfate concentration ranges, 0–70%, 70–75% and 75–80%. The supernatant was slowly mixed with ammonium sulfate in a cold condition and then centrifuged at 12.100×*g* at 4°C for 20 minutes. The protein precipitate was dissolved in 50 mM Tris-HCl buffer pH 7.5 and dialyzed against 20 mM Tris-HCl buffer with a pH of 7.5.

Protein concentration and protease activity assay

Protein concentration was assayed by the Bradford method (1976), while the protease activity was assayed as described by Anson, (1938). Protease activity assay was conducted using casein prepared in 20 mM carbonate-bicarbonate buffer, while trypsin assay was conducted at 37°C using casein; prepared in 100 mM Tris buffer. One unit of protease activity was defined as the amount of enzyme required to produce peptides equivalent to 1 µg of tyrosine in the filtrate per minute per ml. Similarly, one unit of trypsin activity was defined as the amount of enzyme required to produce peptides equivalent to 1 µg of tyrosine in the filtrate per minute per ml.

Determination of molecular weight protease

The protease molecular weight was determined using the SDS-PAGE technique as described by Laemmli (1970). Five microliters of protein marker (*Page Ruler Unstained Protein Ladder*) was used as an indicator to determine the molecular weight of the sample protein.

Optimization of conditions

The optimum condition was determined as described by Ghafoor and Hasnain, (2009). The protease activity was measured in different concentrations of NaCl (0–10%), with various metal ions (5 mM EDTA, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+} and Zn^{2+} ions), varying pH levels (4.0-12.0) and at different temperatures (25-75°C).

The effect of using SDS for protease activity

The effect of SDS was measured by determining protease activity after an enzyme and substrate were mixed and incubated at various SDS concentrations (0–10%) at optimum conditions for 30 minutes.

RESULTS AND DISCUSSION

Screening of proteolytic activity

The proteolytic potential of 5 halophilic isolates from the Bledug Kuwu mud crater was assayed by the agar diffusion (paper disc) method (Figure 1). The results showed that all five isolates have proteolitic ability on a solid medium and were observed as clear zones. Based on the results, it

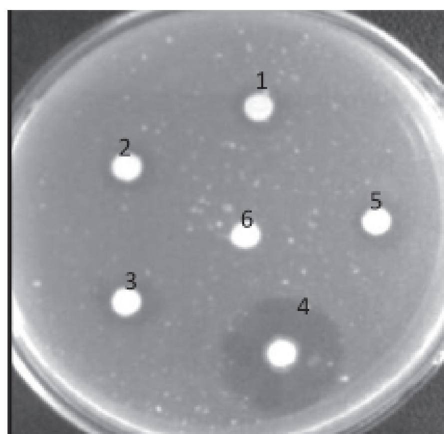

Fig. 1. Clear zone as proteolytic potential of five halophilic isolates from Bledug Kuwu mud crater, (1) *Halomonas elongata* BK-AB8, (2) *Halomonas meridiana* BK-AB4, (3) *Halomonas elongata* BK-AG18, (4) *Chromohalobacter japonicus* BK-AB18, (5) *Halomonas eurihalina* BK-AB15 and (6) Control.

appears that the clear zone around the disc of *Chromohalobacter japonicus* BK-AB18 supernatant was much larger than other isolates.

The growth and protease activity profile of *Chromohalobacter japonicus* BK-AB18

Based on the proteolytic screening, next, we used *C. japonicus* BK-AB18 as isolate to produce extracellular protease. The growth and protease activity profile of *C. japonicus* BK-AB18 created the best incubation time for protease production. The OD and protease activity of the bacterial culture in the medium were measured continuously (Figure 2). The result showed that the highest extracellular protease activity from *C. japonicus* BK-AB18 was produced at the log phase of 16 hours of bacterial growth which was the highest peak of activity, as shown in the graphic. Based on this, the extracellular protease from *C. japonicus* BK-AB18 was harvested at 16 hours of inoculation time.

Production and purification of protease

The free-cell supernatant of bacterial culture was harvested and then partially purified by three levels of ammonium sulfate concentration. The partially purified protease results are shown in Table 1. All of the ammonium sulfate fractions had protease activity, but the highest specific activity was exhibited by the fraction of 75–80%, which was 2129.98 Units/mg. A unit of protease activity is the amount of protease enzyme used to produce 1 mmol of tyrosine (a product of casein hydrolysis) for every minute.

Based on the protease activity results shown in Table 1, the fraction of 75–80% was used for protease characterization. The molecular weight (MW) protease was measured by SDS-PAGE and the protease in this fraction had a relative MW of 65 kDa (Figure 3). The extracellular protease was also isolated from the halophilic bacteria, *Chromohalobacter* sp. TVSP101 and had a relative MW of 66 kDa (Vidyasagar *et al*., 2009).

The optimum condition of protease activity

The ammonium sulfate fraction of 75–80% from *C. japonicus* BK-AB18 was then characterized using biochemical parameters. The optimization of NaCl is very important because the bacteria used in

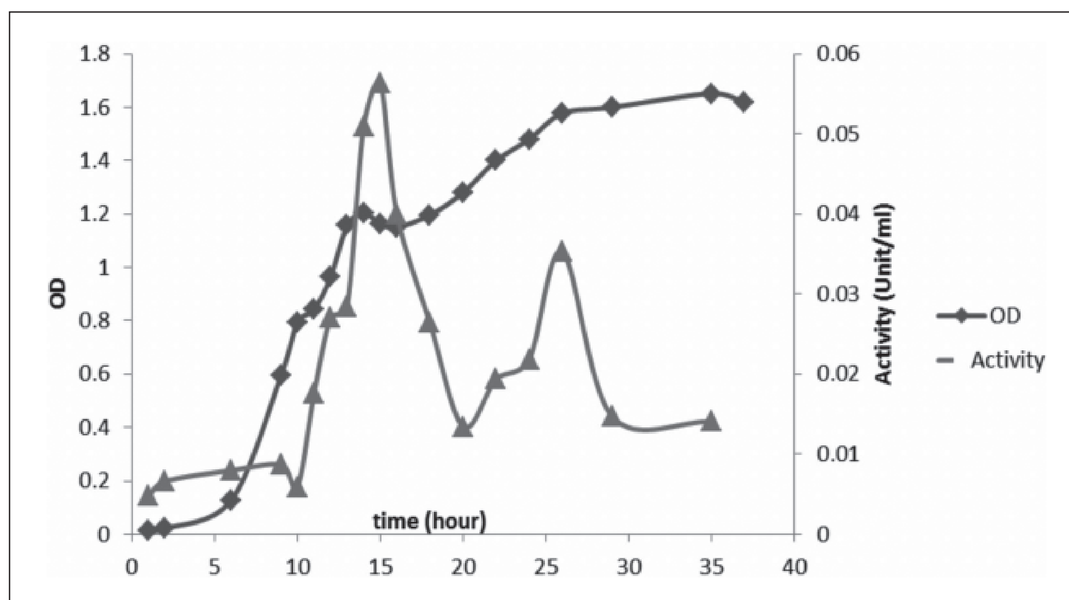

Fig. 2. Clear zone as proteolytic potential of five halophilic isolates from Bledug Kuwu mud crater, (1) *Halomonas elongata* BK-AB8, (2) *Halomonas meridiana* BK-AB4, (3) *Halomonas elongata* BK-AG18, (4) *Chromohalobacter japonicus* BK-AB18, (5) *Halomonas eurihalina* BK-AB15 and (6) Control.

Table 1. The result of ammonium sulfate precipitation of crude enzyme from *C. japonicus* BK-AB18

Ammonium Sulfate Precipitation	Total Protein (mg)	Unit Activity (Unit)	Specific Activity (Unit/mg)	Purification Factor
Crude Enzyme	12.29	1956.55	159.20	1
0–70%	0.18	81.82	454.54	3
70–75%	0.10	65.45	654.54	4
75–80%	0.04	85.20	2129.98	13

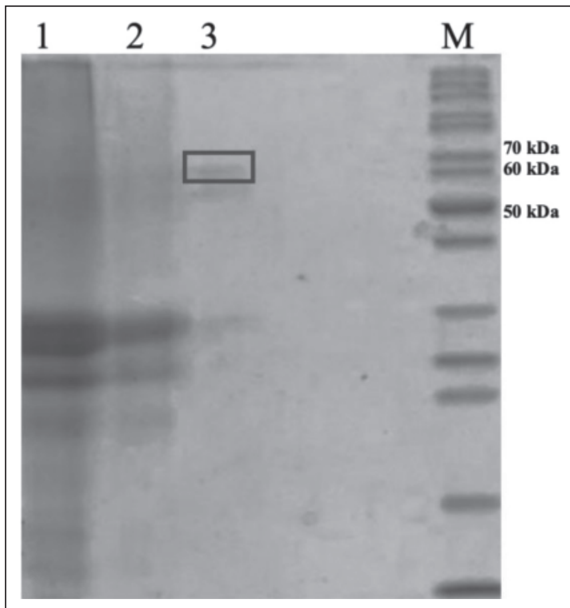

Fig. 3. The result of SDS-PAGE, the fraction of (1) 0–70%, (2) 70–75%, (3) 75–80%, and (M) Protein Marker.

this study are halophilic bacteria, and the enzyme stability and activity are strongly influenced by the NaCl concentration in their environment.

The results obtained showed that this protease has good stability at a low NaCl concentration (up to a 30% NaCl concentration) but the highest activity was shown at a concentration of 7.5% NaCl (Figure 4). *C. japonicus* BK-AB18 itself is a halophilic bacterium that can grow well in a concentration of 5.0–22.5% NaCl (Asy'ari *et al.*, 2014). The optimum condition of NaCl was used to determine the effect of EDTA and metal ions (Figure 5). EDTA is an inhibitor of metalloprotease that creates an interaction (chelate) with a metal ion. Based on this assay, the protease activity from *C. japonicus* BK-AB18 was inhibited by EDTA. This shows that this protease is metalloprotease. The enzyme activity is highly affected by metal ions as a cofactor. Based on the assay of the effect of metal ions, we determined that Fe^{2+}, Zn^{2+} and Ni^{2+} ions do not significantly affect protease activity. The protease activity can be increased by the addition of Ba^{2+}, Mg^{2+} and Ca^{2+} ions. The activity is more significantly increased by the addition of Ca^{2+} ions than the addition of Ba^{2+} and Mg^{2+} ions.

The effects of pH and temperature were measured in two different conditions: a buffer solution with the addition of the Ca^{2+} ion and a buffer solution without the addition of the Ca^{2+} ion

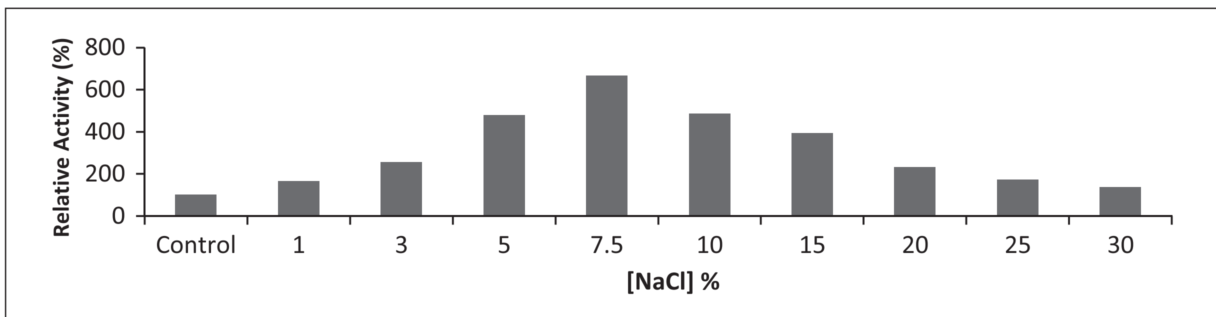

Fig. 4. The effect of various levels of NaCl concentration on protease activity.

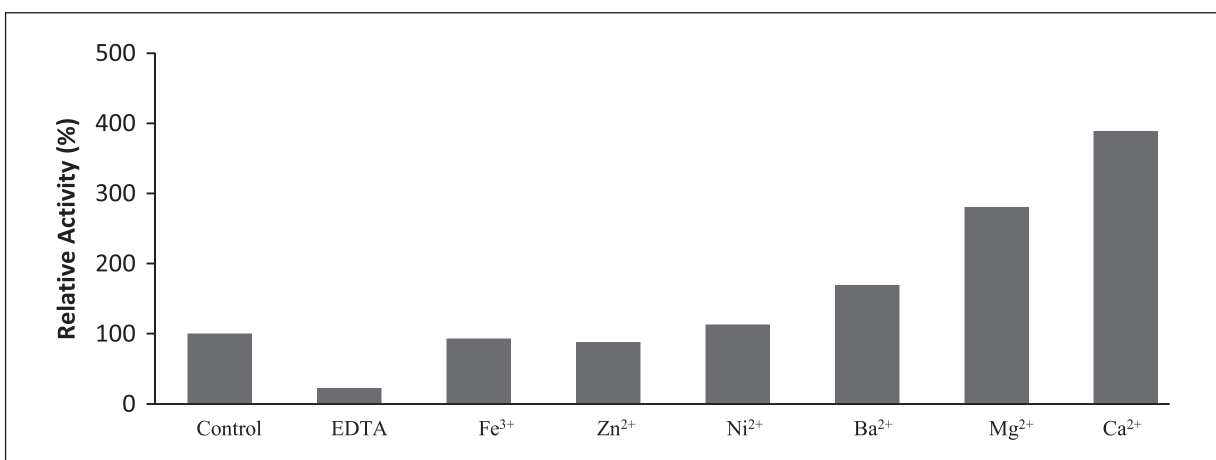

Fig. 5. The effect of EDTA and metal ions on protease activity.

(Figure 6). The optimum pH at both conditions is pH 9.0, and therefore, this protease was classified as an alkaline protease. An alkaline protease has the potential to be applied in the detergent industry (Khan, 2013). The protease activity was significantly decreased at extreme pH levels, such as between pH 4.0 and 12.0, because the sensitive site of protein molecules is generally uncharged residues and at an extreme pH will change conformation due to changes in the cargoof residues (Scope, 1994). The conformation improperly influences the binding of the substrate at the catalytic site and decreases protease activity. Based on the assay of the effect of pH, the results show that the addition of a Ca^{2+} ion is able to increase the protease activity at all pH range variations (4.0–12.0). This indicates that the Ca^{2+} ion is able to increase the stability of the protease structure. The result showed that the optimum temperature of this protease measured at the two different conditions is 45°C (Figure 7). The addition of Ca^{2+} ions can significantly improve protease activity at all temperature variations. The existence of a Ca^{2+} ion stabilizes the structure of the protease, allowing for good protease activity in a wide temperature range.

The effect of SDS concentration on protease activity

The effect of the SDS concentration on protease activity was assayed at the optimum conditions, as follows: 7.5% NaCl, the addition of 5 mM of a Ca^{2+} ion, a pH level of 9.0 and a temperature of 45°C (Figure 8). At these conditions protease activity is optimum, allowing for the protease to be resistant in extreme environments, such as in a detergent or SDS solution. SDS is an ionic surfactant, which has both ionic hydrophobic and hydrophilic sides, so it can inhibit protease activity. The results of assaying the effect of various SDS concentrations showed that the protease from *C. japonicus*

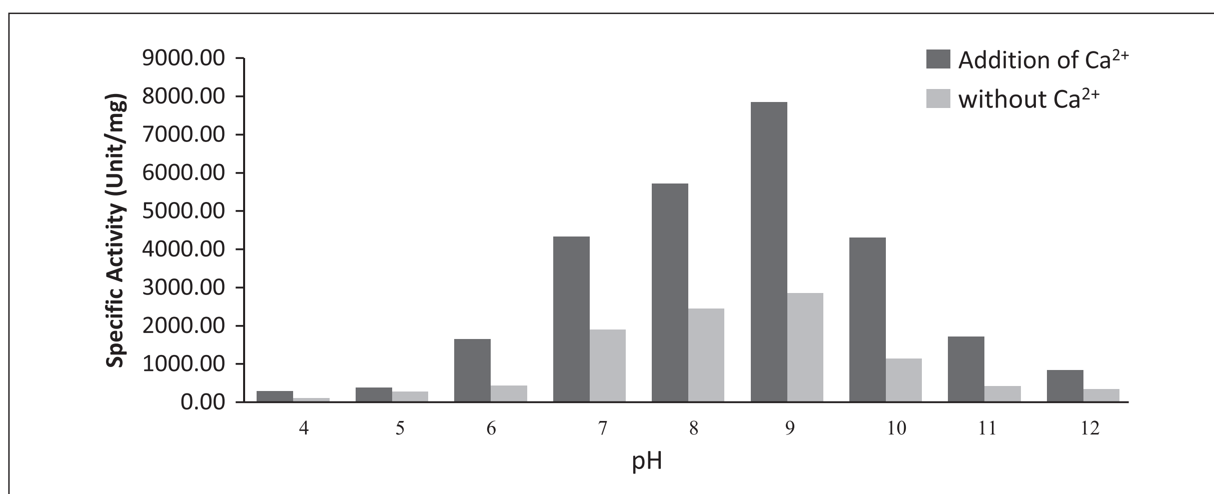

Fig. 6. The effect of pH to protease activity with added Ca^{2+} ions and without Ca^{2+} ion.

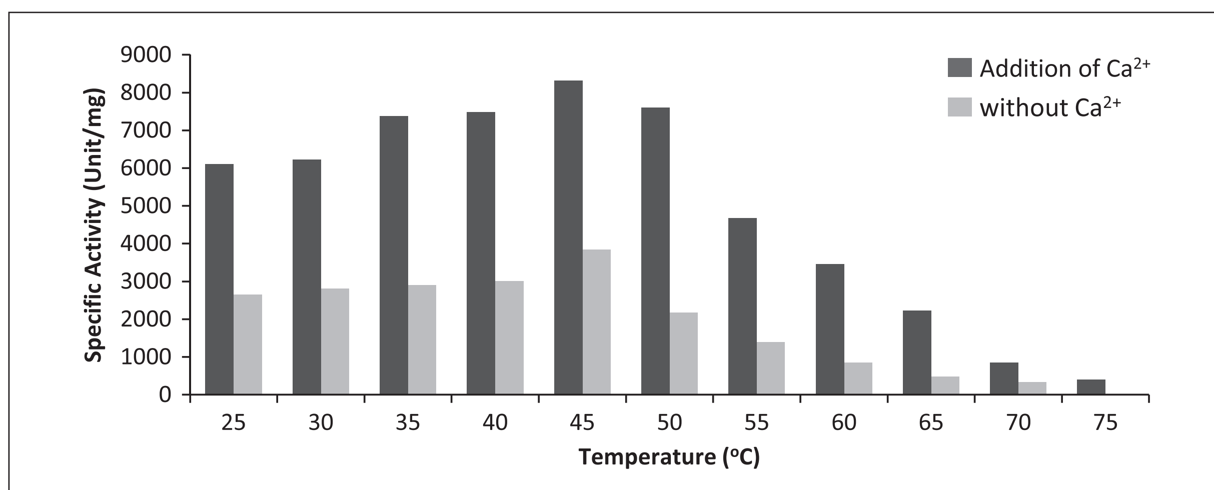

Fig. 7. The effect of various temperatures to protease activity at optimum pH.

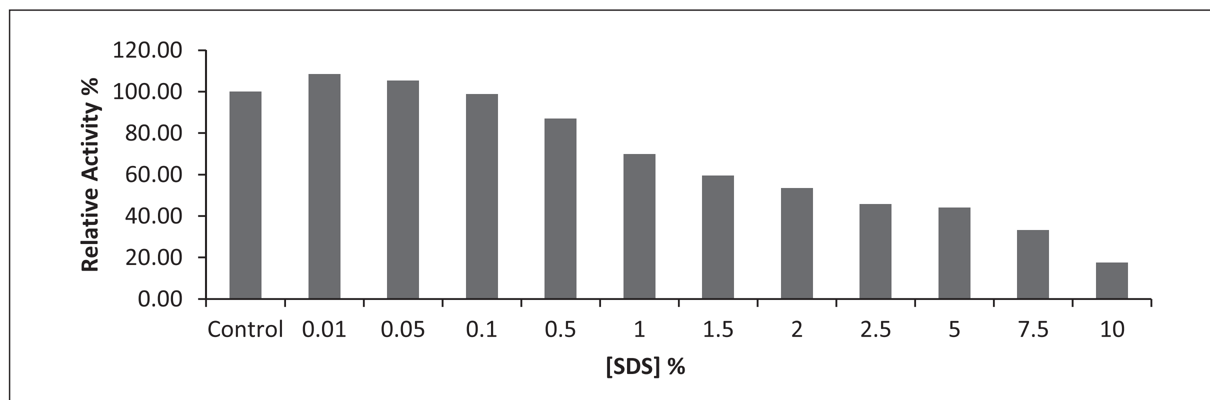

Fig. 8. The effect of various SDS concentrations at optimum condition to protease activity.

BK-AB18 has good stability in an SDS solution. The protease activity increased in low SDS concentrations (0.01 and 0.05% solutions). This indicated that protease flexibility increased as an effect of hydrophobic interaction between the enzyme and the SDS molecules, and that interaction of enzyme intramolecular activity was decreased (Oberoi *et al*., 2001). The activity of protease was relatively stable in an SDS solution up to a concentration of 10%. This showed that the protease in this fraction had high stability in the SDS solution.

ACKNOWLEDGEMENT

This work is funded by Priority Research Grant from Indonesian Ministry of Research and Technology with the contract number of 585u/l1.C01/PL/2016.

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