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A novel alkaline serine protease from *Bacillus amyloliquefaciens* strain S1-13

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Abstract

Bacillus sp. strain S1-13 was identified as *Bacillus amyloliquefaciens* with 16s rRNA gene (Accession number: JX441363). The strain S1-13 was expressed and secreted alkaline serine protease (called ASP1-13) when growth in nutrient broth containing with 1% skim milk. ASP1-13 was partial purified with a specific activity of 1,324 U/mg and 2% yield. The molecular weight and isoelectric point of ASP1-13 was determined about 40 kDa and 8, respectively. It was indicated as alkaline serine protease with a broad range of activity at alkaline condition (pH 7-12) and completely inhibited with serine protease inhibitor. ASP1-13 was also active in high temperature (50-60°C) and stabilizes with broad range of pH (5-12), surfactant, oxidant, reducing agent and organic solvent. Finally, the partial amino acid sequence from LC/MS-MS was confirmed similarity with sequence of neutral protease precursor from *Bacillus subtilis*.

Keywords : *Bacillus amyloliquefaciens, 16 rRNA gene, alkaline serine protease, metal-dependent enzyme, and LC/MS-MS.*

1. Introduction

Proteases are enzymes which naturally produced by animals, fungal, plants, and microbes (1)Its catalyze the digestion of long polypeptide chain into short peptide fragments, splitting the peptide bond that link amino acid residues (1-4). The extracellular proteases are enable the cell to absorb and utilize hydrolytic products. At the same time, they have also been commercially exploited to assist protein degradation in various industrial processes. Today, proteases account for 40% of the total enzyme sales in various industrial market such as detergent, food, leather, waste management, and pharmaceutical (3, 4). Most of total protease market are microbe alkaline serine protease. The alkaline serine proteases, presented serine group in active site, are defined as those proteases which are active and stable in high pH value (pH 10) (1, 3, 4). Their major application is in detergent industry because

the pH of laundry detergents is generally in the range of 9.0–12.0. Alkaline proteases used as cleaning additives in detergents to facilitate the release of proteins. The most of the commercially alkaline serine proteases are produced by *Bacillus* sp. (3). Although alkaline serine proteases have already been found in some strains of *B. mojavensis, B. subtilis, B. licheniformis,* and *B. amyloliquefaciens* (1, 4-6). The present study was described the purification and characterization of novel alkaline serine protease from *B. amyloliquefaciens* strain S1-13 which could applied for industrial processing.

2. Materials and Methods

B. amyloliquefaciens strain S1-13 was isolated from Indonesia terasi shrimp paste and obtained from Mae Fah Luang culture bank.

2.1 16S RNA gene amplify and sequence analysis

Genomic DNA of *Bacillus* sp. strain S1-13 was extracted as described by Malik method (7). The part of the 16s rRNA gene was amplified and sequenced. The primers used were: 117F (GGC GGA CGG GTG AGT AA) and 1,472R (GTG TGA CGG GCG GTG TG). Then, the 16s rRNA sequence were compared using BlastN program to GenBank database. Finally, the phylogenic tree was constructed by MEGA (version 5.0) software with all reference database dedicated to 16s rRNA of *Bacillus* sp., neighbor-joining method and bootstrap analysis for 1,000 replications (8, 9)

2.2 Optimization of media composition for protease production

Nutrient broth (NB), NB+1% casein, and NB+1% skim milk were used in this study (10, 11). The 2.5 ml of overnight cultured strain S1-13 was inoculated into 250 ml those media and incubated at 37°C, 180 rpm. After incubation 6, 12, 24, 30 and 36 hr, crude enzyme was separated by centrifugation at 5,000 g for 10 min and stored at -20°C until used.

2.3 Protease activity assay

Protease activity was determined using azo-casein as substrate. Sample (20 µl) was mixed with 380 µl of 2% azo-casein solution in 0.1 M phosphate buffer pH 6.5, and incubated at 37°C for 30 min. After incubated, reaction was terminated with 1 ml of 0.44 M tri-chloroacetic acid (TCA) and centrifuged at 12,000 g for 10 min. Then, 0.5 ml of supernatant was mixed with 0.5 ml of 1 M NaOH and incubated at 37°C for 30 min. Finally, the optical density at 440 nm of the reaction was determined (6, 10, 12, 13). One unit of protease activity was defined as amount of enzyme that hydrolyzed azo-casein to produce a change 0.001 optical density at 440 nm (A_{440}) per minute under assay condition (6, 10, 12).

2.4 Protein determination

Total protein concentration was determined according to the method of lowry (14), with bovine serum albumin (BSA) as standard.

2.5 Partial purification steps

The crude enzyme was brought to fresh -20°C acetone solution and kept at-20°C for 2 hr. After centrifugation at 10,000 g, 4°C for 10 min, the precipitate was dissolved and dialyzed in 10 mM phosphate buffer pH 6.0. This preparation was applied to SP-Sepharose strong cation exchanger, (GE Healthcare, USA), that equilibrates with 10 mM phosphate buffer pH 6.0. Then, ASP1-13 was eluted with step

gradient NaCl (0.1 to 1.0 M) in the same buffer. Finally, the active fraction were collected, concentrated by ultra-filtration (cut off 10 kDa), and stored at 4 °C for further use.

2.6 Gel electrophoresis

SDS-PAGE was carried out at 12.5% gel according to the method of Laemmli (15). The protein molecular weight was determined using LMW-SDS Marker Kit (GE Healthcare, USA) with coomassie staining method (16).

2.7 Zymogram

Protease activity was visualized on SDS-PAGE as described by Garcia method (17, 18). In brief, after standard electrophoresis, gels were immersed with 2% casein solution in 50 mM Tris-HCl buffer pH 7.5 at 4°C overnight. Then, it was incubated at 37°C for 90 min. The protease activity was visualized as clear bands against a blue background when stained with 0.1% coomassie brilliant blue R250 and destained with 40% methanol and 10% acetic solution.

2.8 Isoelectric focusing

The partial purified ASP1-13 was mixed with 2% immobilized pH gradient buffer (IPG buffer) before loaded into isoelectric focusing gel (IEF) with a linear pH gradient (3-10) (GE Healthcare, USA). Then, the IEF gel was performed according to the instructions of the manufacturer. Finally, the IEF gel was applied to zymogram for determining the isoelectric point.

2.9 Effect of temperature and pH on protease activity and stability

The optimum temperature was examined in the varied temperature (30°C to 90°C) (19). The thermal stability was carried out by incubating ASP1-13 at various temperatures (30°C to 80°C) for 2 hr before standard protease assay (19, 20). To study of optimum pH, ASP1-13 was measured at different pH value (4.0 to 12.0). For pH stability, ASP1-13 was incubated at various pH buffers (4.0 to 12.0) at 4°C for 12 hr. Finally, the residual enzyme activity was determined under protease assay condition (19-21).

2.10 Effect of protease inhibitors and metal ions

The effect of different additives on protease activity was investigated by incubating the enzyme sample in the presence of different protease inhibitor and metal ion at 37°C for 30 min. The 10 mM of aspartic protease inhibitor (pepstatin), serine protease inhibitor (PMSF), metallo protease inhibitor (EDTA), cysteine protease inhibitor (IAA), and metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Na^{+} and Zn^{2+}) were used in this study. After incubated, the remaining activity was measured under protease activity assay conditions (2, 20, 22).

2.11 Effect of various chemical agents on protease stability

The stability of surfactant, oxidant, reducing agent, ASP1-13 was pretreatment with 5% SDS, 1% H_2O_2, 5% triton x100, 5% tween 80, 5% CTAB, and 10 mM DTT at 4°C for 1 hr before measured the protease activity (6, 20, 22, 23). In order to determine the effect of various organic solvent, the ASP1-13 activity was measured at different concentration (0-80%) of organic solvents (ethanol, acetone, acetonitrile, isopropanol and methanol) under protease activity assay (24-26).

2.12. Protein identification by LC/MS-MS

The protein band on SDS-PAGE was cut, excited, and cleaved into peptide with modify in-gel digestion method

described by Shevchenko (27). Then, the digested protein sample was applied to liquid chromatography mass spectrometry analysis (LC/MS-MS). Finally, the MS-MS data was acquired and analyzed using MASCOT software (Matrix Science, London, UK) and the NCBI database.

3. Results and discussions

3.1 Identification of bacterial strain

The 16s rRNA sequence of strain S1-13, which is 1,256 bp, was initially identified species using BlastN program against Genbank database. Results showed high similarities (Max identity > 99%) to *B. amyloliquefaciens*, *B. atrophaeus*, *B. subtilis* and *B. vallismortis*. Those species was subtilis-like bacteria. They are phenotypically very similar and share almost identical 16S rRNA sequences. It was also classified in group VI of genus *Bacillus* sp. (8, 9)

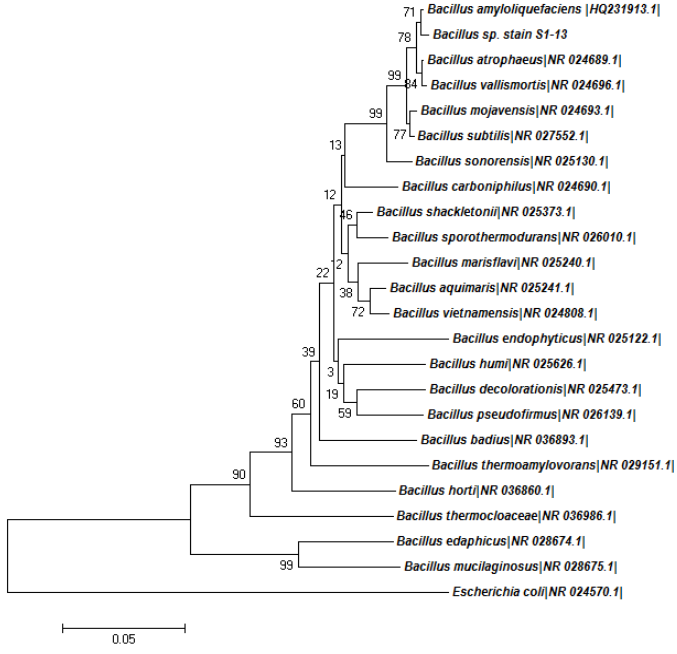

Figure 1. Phylogenetic tree based on 16s rRNA gene of reference sequences of Bacillus sp. and strain S1-13**.**

Phylogenic tree was constructs based on comparison of all reference 16s rRNA gene of *Bacillus* sp. database, neighbor-joining method, and bootstrap values from 1,000 replicates using MEGA (version 5.0) software. The strain S1-13 was most closely associated with *B. amyloliquefaciens* (Figure 1). Finally, the 16s rRNA sequence of strain S1-13 was submitted into Genbank database (Accession number: JX441363).

3.2 Optimization of media composition

Protease production was estimated by using nutrient broth (NB) as culture media and casein, skim milk as media

composition. 1% of overnight cultured strain S1-13 was inoculated in 3 different media (NB, NB+1% casein and NB+1% skim milk) and incubated at 37°C, 180 rpm. From previous researchers were showed that protease production was depended on growth condition, media and media composition (10, 11). Casein and skim milk are an organic nitrogen source that enhanced microbe growth and used as inducing protease production (11, 28).

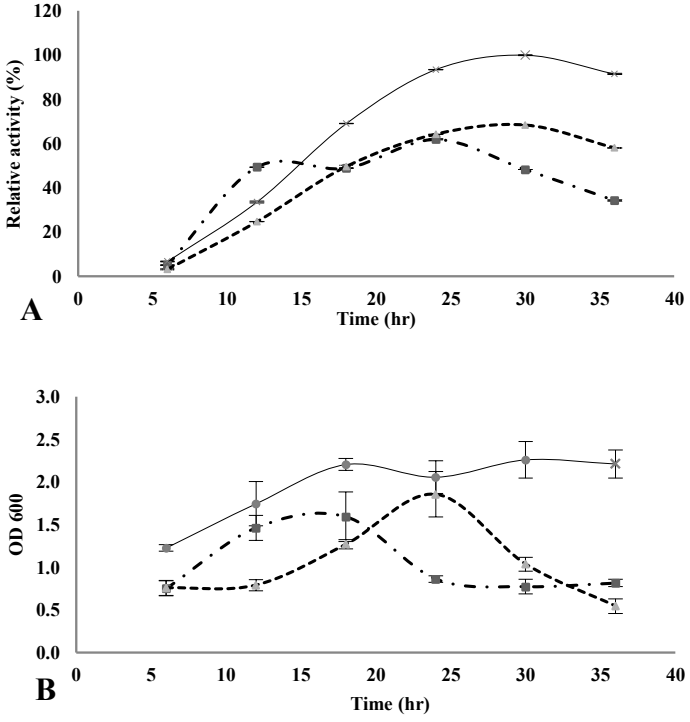

Figure 2. Effect of media composition on strain S1-13 protease production (A) and growth rate (B); (■-•-■): NB, (▲---▲): NB+1% casein, (X-X): NB+ 1% sk=im milk (Mean ± SD, n=3).

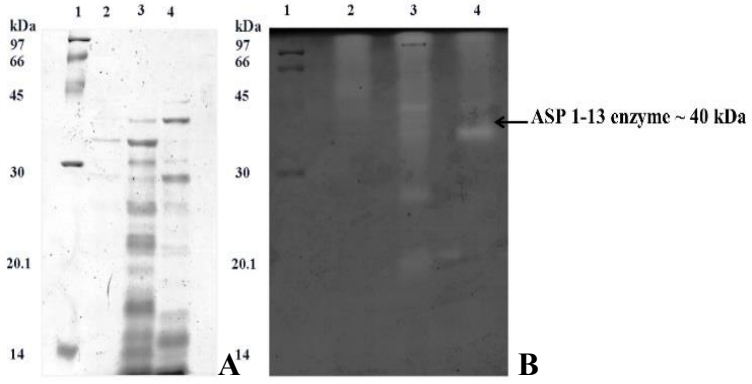

Figure 3. SDS-PAGE (A) and zymogram (B) of the crude enzyme and partial purified ASP1-13 from strain S1-13; lane 1: protein molecular weight markers, lane 2: crude ASP1-13, lane 3: acetone precipitation sample, lane 4: strong cation exchanger sample.

In this study, the maximum protease activity was observed in the present of 1% skim milk after 30 hr at the exponential phase (Figure 2). Similar pattern of protease production was also observed by Shahee M. et al. (2008) in *B. subtilis* strain BS1 [28]. The relationship between protease production and growth was comparable to other published reports on microbe protease. Mostly microbe protease are partially produced during stationary phase. In contrast, stationary phase, protease production was decreased because of depletion of nitrogen source and inactivation of protease by acidic media (10, 11, 28).

3.3 Partial purification of ASP1-13

The purification table and relevant data was summarized in Table 1. It was showed that purified 15 fold with specific activity 1,324 U/mg and 2% yield after passed through strong cation exchanger (SP-Separose). Analysis of the ASP1-13 on both SDS-PAGE and zymogram was appeared ~ 40 kDa (Figure 3A-B). This enzyme is slightly larger than normal molecular weight of *Bacillus* alkaline serine protease (18-35 kDa). The isoelectric point of ASP1-13 was about 8, which was closed to those reported for, isoelectric point around 9, *Bacillus* alkaline serine protease (1, 3, 6, 12).

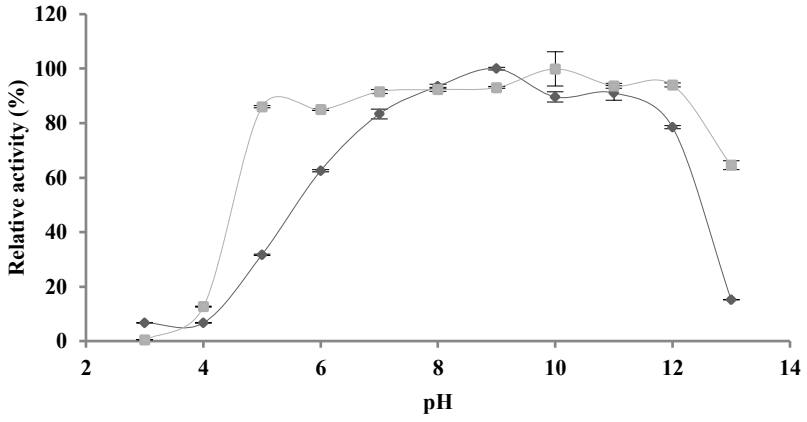

Figure 4. Effect of pH on the activity and stability on ASP1-13; (♦-♦): effect of pH on ASP1-13 activity, (■-■): pH stability (Mean ± SD, n=3).

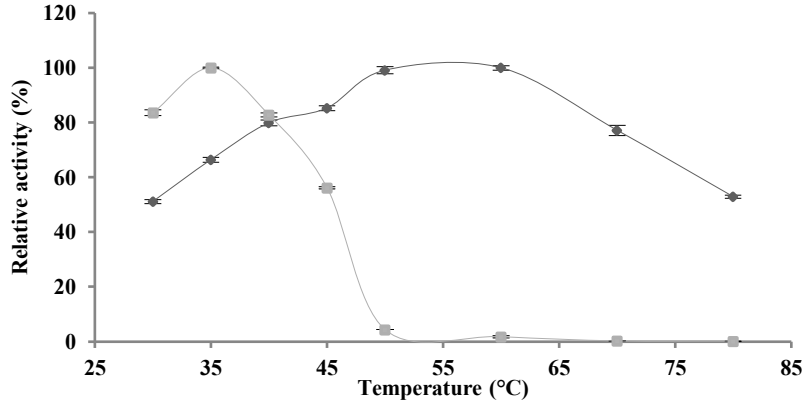

Figure 5. Effect of temperature on the activity and stability on ASP1-13; (♦-♦): effect of temperature on ASP1-13 activity, (■-■): temperature stability (Mean ± SD, n=3).

Table 1. Purification step of protease ASP1-13

Purification step	Total volume (ml)	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Crude ASP1-13	1,000	6641	591,667	89	1	100
Acetone precipitation	50	92	56,750	620	7	10
Strong cation exchanger	20	7	9,400	1,324	15	2

3.4 Effect of temperature and pH on protease activity and stability

The optimum pH of ASP1-13 was determined with various pH buffers (3 to 13). ASP1-13 activity was not significant decrease between pH 7 to 12. Maximum protease activity was found at pH 9 (Figure 4). ASP1-13 was stabled between pH 5 to 12 after incubated at 4°C for 12 hr. The actives in an alkaline condition could be classified as alkaline protease when compared with previous researcher (11, 20, 21). Furthermore, ASP1-13 activity was increased at temperature between 50°C to 60 °C and abruptly lost at 65°C for 30 min (Figure 5).

The maximum stability of this enzyme was showed at 30°C to 40°C for 2 hr. Similar to number of alkaline protease from *Bacillus* sp. that have high optimum temperature (4, 6, 20)

3.5 Effect of protease inhibitors and metal ion

The effect of various protease inhibitors on ASP1-13 are summarized in Figure 6. In the presence of EDTA, the activity of ASP1-13 was decresed about 60% of the protease activity. These results suggest that ASP1-13 may not be a metalloprotease. The completely inhibition by PMSF suggests that ASP1-13 could be a serine (2, 20). The inhibitory effect of EDTA was indicated the requirement of metal ions for the enzyme activity. Similar effect of EDTA on serine protease activity produced by *Bacillus* sp. strain B001 and *B. aquimaris* strain VITP4 (20, 29).

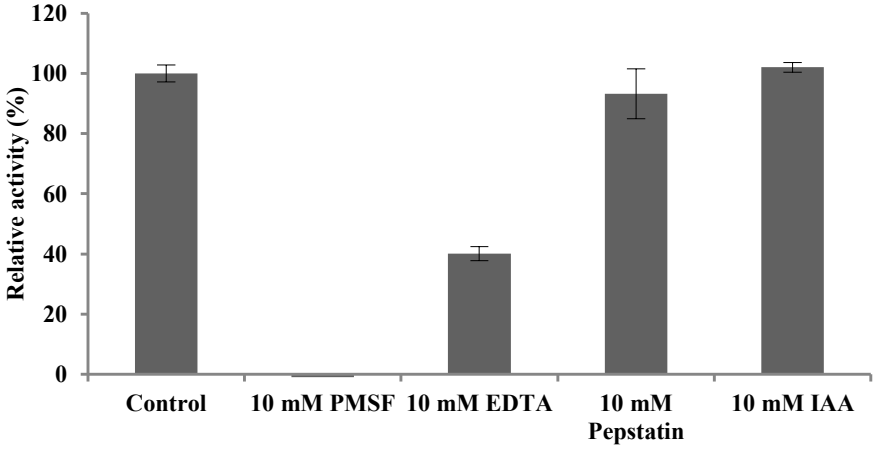

Figure 6. Effect of protease inhibitor on ASP1-13 activity (Mean ± SD, n=3).

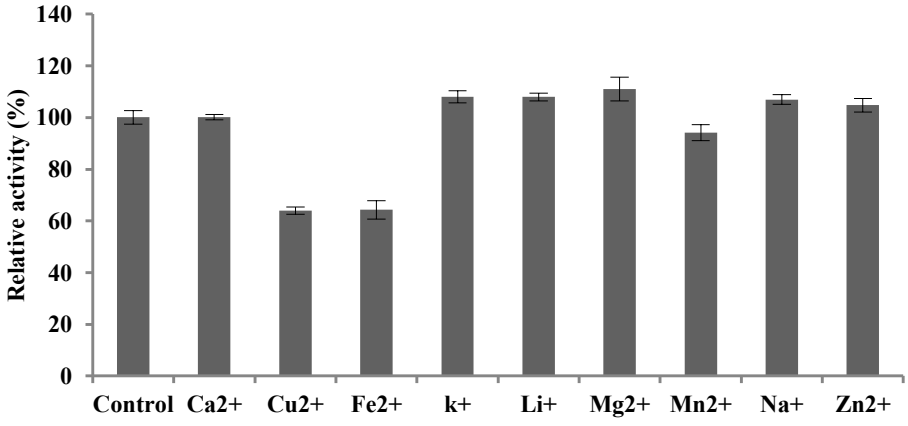

Figure 7. Effect of metal ion on ASP1-13 (Mean ± SD, n=3).

Metal ion is an external factor that affect with the protease activity or the stability of the protease (20, 22, 29). K^+, Li^+, Mg^{2+}, Na^+, and Zn^{2+} were slightly enhanced APS1-13 activity (Figure 7). The ASP1-13 activity was not effect with Ca^{2+}. But, Cu^{2+}, Mn^{2+}, and Fe^{2+}. The same effects of Fe^{2+}, and Cu^{2+} on an alkaline protease are also found in Bacillus sp. strain B001 (20).

3.6 Effect of various chemical agents on protease stability

Organic solvent was enhanced performance of ASP1-13. The protease activity was slightly enhanced at 5% concentration of isopropanol acetone, acetonitrile, ethanol, and methanol (Figure 8). The maximum activity (162%) was presented in 10% acetonitrile. Above 20%, increasing concentration of organic solvent caused a continuous loss of protease activity. Different to the mentions above, methanol was showed a maximum activity (135%) at 40% concentration. Similarly to the effects of various organic solvents have previously been reported (25).

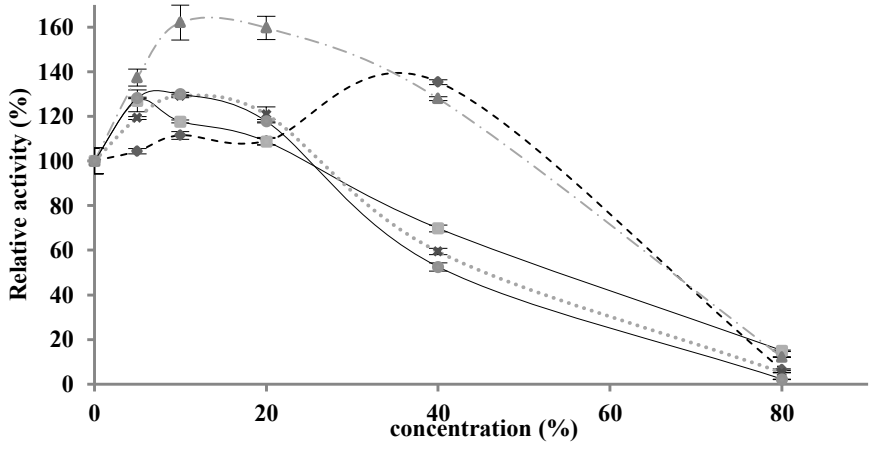

Figure 8. Effect of organic solvent on ASP1-13 activity; (●–●): acetone, (▲-•-▲): acetonitrile, (■–■): ethanol, (×•••×): isopropanol, (♦--♦): methanol (Mean ± SD, n=3).

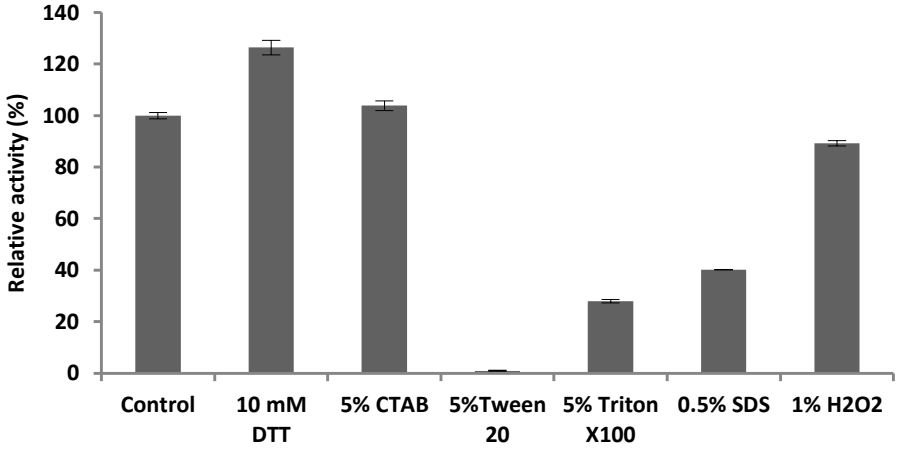

Figure 9. Effect of surfactant, reducing and oxidant agent on ASP1-13 activity (Mean ± SD, n=3).

Organic solvent could be enhance protease activity by increase the solubility of non-polar substrates, increase the thermal stability, and decrease water dependent side reaction (24, 25). In addition of other chemical, high stability was observed for ASP1-13 toward various reducing agent, surfactant, and oxidant (Figure 9). Upon incubation with 10 mM DTT and 5% CTAB, ASP1-13 was exhibited enhanced residual activities to 103% and 126%, respectively. These results were consistent with reported for alkaline proteases from Bacillus sp. (3, 20). The present of 5% tween 20, 5% triton X100 and 0.5% SDS caused a loss of protease activity that similarly to the other reported. The surfactant can be inhibited protease activity by reduction of the hydrophobic interactions and the direction interaction with protein molecule (20, 23). Although, oxidizing agent had been reported to destabilize alkaline serine protease (20, 22), ASP1-13 was retained 89% activity after treated with 1% H_2O_2. All data shown that, ASP1-13 could use in various industrial applications

3.7 Protein identification

Protein identification of target enzyme band on SDS-PAGE was analyzed by using LC/MS-MS. MS-MS data was applied to MASCOT MS/MS ion search software against NCBI data base. The results were showed this ASP1-13 has high similarities to neutral protease precursor from Bacillus subtilis (accession number: gi|83972424).

4. Conclusion

In this work, the strain S1-13 was identified as Bacillus amyloliquefaciens based on 16s rRNA gene. This strain was produced a newly protease (ASP1-13). The ASP1-13 was indicated as alkaline serine protease with the active condition (pH 7 to 12) and protease inhibitor study. This enzyme was also active in high temperature (50°C to 60 °C) and stabilized at pH 5 to 12, 30°C to 40°C for 2 hr. It also stabilized with surfactant, oxidant, reducing agent and organic solvent. These properties of ASP1-13 are potentially useful for various industrial applications.

5. Acknowledgement

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