International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **2(2),** 29-33, February **(2013)** *Int. Res. J. Biological Sci.*

Thermostable Alkaline Serine Protease from Thermophilic *Bacillus* Species

Ibrahim Noor Azlina* and Yusoff Norazila
Faculty of Agro Based Industry, Universiti Malaysia Kelantan (Jeli Campus), Locked Bag No. 100, 17600 Jeli, Kelantan, MALAYSIA

Available online at: www.isca.in
Received 14th November 2012, revised 20th November 2012, accepted 15th December 2012

Abstract

Screening and isolation of proteolytic bacteria were carried out from water samples of Lojing hot spring (Kelantan, Malaysia). Based on the qualitative screening on Skim Milk Agar (SMA), two isolates showed positive results by forming clearing zones around the colonies on SMA. The isolates were identified as Bacillus subtilis 50a and B. licheniformis 50b on the basis of the 16s rRNA gene sequencing. The effect of temperature, pH and inhibitors on enzymes activity and stability were investigated. The crude proteases for both isolates displayed maximal activity at 70˚C and showed characteristic pH optima at pH 9.0. Enzymes activities were totally inhibited by phenylmethyl sulphonyl fluoride (PMSF), suggested that the proteases from B. subtilis 50a and B. licheniformis 50b belong to the family of serine protease. The thermostability profile exhibited the protease from B. subtilis 50a was very stable at 50˚C (maintain 100% relative activity) and the protease activity retained 89% of its original activity after heat treatment at 60˚C for 30 min. Meanwhile, protease activity for B. licheniformis 50b retained 96 and 72% of the original activity after heat treatment at 50 and 60˚C, respectively. Considering their promising properties, B. subtilis 50a and B. licheniformis 50b could be a potential sources of enzymes for industrial applications.

Keywords: Alkaline protease, thermophilic, *Bacillus subtilis*, *Bacillus licheniformis,* thermostable.

Introduction

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of total worldwide enzyme sales[1]. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. And among bacteria, *Bacillus* strains are one of the most important producers of commercially applicable proteases among various microorganisms[2]. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries. It was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources[3]. Thermophiles such as *Bacillus stearothermophilus*[4] have been studied for their capability in producing thermostable protease. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, with the consequences of faster reaction rates, increase in the solubility of nongaseous reactants and products, and reduced incidence of microbial contamination from mesophilic organisms[4]. Thus it is desirable to search for new proteases with novel properties from as many different sources as possible.

Material and Methods

Bacterial strain: The microorganisms used in this study were isolated from water samples collected from hot spring in Lojing Highlands (Kelantan, Malaysia) after extensive screening at various temperatures (50, 55 and 60˚C) on Skim Milk Agar (SMA) containing (g/l): nutrient agar, 13.8 and skim milk powder, 12.0. Isolates that showed positive results on the SMA plate were then tested for their enzyme production in liquid medium. The organisms were maintained on nutrient agar plates and stored at 4˚C, while the stock was maintained in 15% glycerol at -80˚C.

Bacterial identification: Two out of seven different types of bacteria isolates showed positive results by forming clearing zones around the colonies on SMA. The isolates were then identified by 16S rRNA gene identification. The 16S rRNA genes were amplify by polymerase chain reaction (PCR) with two universal primers, forward: 5'-GAG TTT GAT CCT GGC TCA-3' and reverse: 5'-CGG CTA CCT TGT TAC GAC TT-3'. Genomic DNA of isolates were then subjected to PCR reactions containing the above forward and reverse primers. The reaction mixture were initially heated for 4 min at 94°C, followed by 30 cycles of PCR including 1 min at 94°C (denaturation), 1 min at 58°C (annealing) and 1 min at 72°C (extension). This was followed by one cycle of 7 min at 72°C and held at 4°C. The PCR products was examined by electrophoresis and detected using ethidium bromide fluorescence. QIAquick PCR Purification Kit (Qiagen, Germany) methods were used for the purification of PCR product and the purified PCR product was sent for sequencing. The sequence for DNA homology was matched with the Genebank database that was available at http://www.ncbi.nlm.nih.gov/BLAST/.

Protease production: The inoculums were prepared by inoculating a single colony from the culture of nutrient agar into 10 ml nutrient broth (g/l: bacteriological peptone,5; meat extract,1; yeast extract,2; 5 g of NaCl,5) in universal bottles and

incubated at 150 rpm in a shaker for 24 hours at 50˚C. The cells were harvested by centrifugation at 10000 rpm, 4˚C for 10 min. The bacteria pellet was dissolved in saline (0.85% NaCl) to give an absorbance reading of 0.5 at 540 nm[5]. Inoculum (4%) was then inoculated into 100 ml growth medium and incubated at 50˚C for 24 h. 10 ml of 24h culture was harvested by centrifuged at 10000 rpm for 10 min at 4˚C. The cell free supernatant was used for the assay of protease activity.

Assay of protease activity: Protease activity was determined by a modification of the method described by Rahman *et al*.[6]. Azocasein (0.5%, 1 ml) was dissolved in 0.1 M Tris-HCl, pH 9 and preincubated at 50˚C. The reaction was initiated by addition of 100 µl of enzyme solution, and assayed at 50˚C for 30 min. An equal volume of 10% trichloroacetic acid (TCA) was added to terminate the reaction and the mixture allowed to stand at room temperature for 30 min and then centrifuged at 13000 rpm for 10 min. The absorbance of the supernatant was determined at 450 nm. All enzyme assays were carried out in triplicates.

One unit (U) of azocaseinase activity was defined as the amount of enzyme activity that produces a change of absorbance (0.001 per min) at 450 nm at 50˚C under the standard assay conditions.

Characterization of crude protease: Effect of pH on protease activity: The effects of pH on the hydrolysis of azocasein by crude proteases were studied by varying the pH from 5 to 12. Phosphate buffer (pH 5–6), Tris–HCl buffer (pH 7–9) and glycine-NaOH buffer (pH 10-12) were employed to regulate the pH. Reaction was initiated by the addition of 100 µl enzyme to 1 ml of pre-incubated substrate dissolved in the above buffer at 50°C for 30 min, and the activity was assayed.

Effect of temperature on protease activity: Effect of temperature on crude proteases activity was determined by using 0.5% (w/v) of azocasein dissolved in 0.1M Tris-HCl buffer (pH 9.0) at various temperatures ranging from 40°C to 80°C. The incubation was carried out for 30 min.

Effect of inhibitors on protease activity: Patterns of inhibition were determined using inhibitors such as, 10 mM phenylmethylsulphonyl fluoride (PMSF), 10mM ethylenediaminetetracetic acid (EDTA), 10mM iodoacetic acid (IAA), 2mM pepstatin, 2mM elastatin, 1.5 mM bestatin and 1 mM antipain. The protease preparation was incubated with the inhibitors in the ratio 1:1 for 30 min at room temperature. The activity was determined as above.

Thermostability: For thermostability studies, the crude proteases were pre-incubated at different temperatures such as 50˚C, 60˚C, 70˚C and 80˚C. After 30 min, the sample were cooled rapidly in ice and assayed.

Results and Discussion

Identification of bacterial strain: The partial 16S rRNA nucleotide sequence (1192 bp and 1180 bp) from isolates 50a and 50b has been analyzed using Blast from National Center of Biotechnology (http:// www.ncbi.nih.gov). From the analysis, isolate 50a was closely related to *Bacillus subtilis* and isolate 50b was closely related to *B. licheniformis.* The homology among these bacteria showed a significant value of 99% homology.

Effect of pH on protease activity: The effects of pH on enzyme activity was examined in the pH range 5-12 (figure-1). The highest activity of *B. subtilis* 50a and *B. licheniformis* 50b crude proteases were found to be at pH 9 using Tris-HCl buffer, indicating that the enzymes belonged to alkaline protease group. These findings are in accordance with several earlier reports showing pH optima of 9 for protease from *Bacillus licheniformis* LHSB-05[7] and *B. laterosporus* AK-1[8]. Meanwhile, several reports are showing the pH optima of 8-10 for protease obtained from *B. subtilis* P-2[9], *Bacillus subtilis* DM-04[10] and *B. licheniformis* Lbb1-11[11]. Considering maximum enzyme activity was observed at alkaline pH, the 50a and 50b proteases may find potential application in detergent industry because the pH of laundry detergent is generally in the range of 9.0 – 12.0[12]. Alkaline proteases are used as cleaning additives in detergents to facilitate the release of proteins.

Effect of temperature on protease activity: The studies of effect of temperature on the protease activity were carried out at temperature ranging from 40 to 80°C. As shown in figure-2, the enzymes produced by *B. subtilis* 50 and *B. licheniformis* 50b manifested their maximal activity at 70 °C. The protease activity gradually declined at temperature beyond 70°C. The performance of alkaline protease in detergent is influenced by several factors such as pH and temperature of washing solution as well as detergent composition. According to Beg and Gupta[13], all currently used detergent compatible enzymes are alkaline and thermostable in nature with a high pH optimum and varying thermostabilities at laundry temperatures (50-70°C). Thus, the thermostable alkaline proteases of *B. subtilis* 50a and *B. licheniformis* 50b can be considered as a suitable candidates as an additive for commercial detergents. Similar results were obtained for some proteases from *Bacillus* species showing temperature optima of 70 °C[14,15,16].

Effect of inhibitors on protease activity: Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center. The effect of different inhibitors on the enzyme activity of both crude proteases were studied. Inhibitors of EDTA, IAA, antipain, pepstatin, bestatin and elastatin did not inhibit the protease activity (figure-3). However, the protease activity of both proteases were inhibited by the serine protease inhibitor, PMSF, which resulted 100% inhibition at concentration of 10 mM. In this regard, PMSF sulphonates the essential serine

residue in the active site of the protease and has been reported to result in the complete loss of enzyme activity[17]. The results suggested that these enzymes belong to the serine protease.

Effect of temperature on protease stability: The thermal stability of proteases in the range of 50 to 80°C was studied and results are shown in figure-4. The results indicated that within 30 min, protease from *B. subtilis* 50a was stable at 50˚C (maintain 100% relative activity) while at 60°C, the enzyme retained 89% of the original activity. Protease activity for *B. licheniformis* 50b protease retained 96 and 72% of the original activity after heat treatment at 50 and 60°C, respectively. In contrast, protease from *B. subtilis* DM-04[10] retained 67% of original activity after 30 min heat treatment at 60°C. Whereas, protease activity of *B. cereus* BG1[18] lost 100% of its initial activity after 15 min incubation at 55°C.

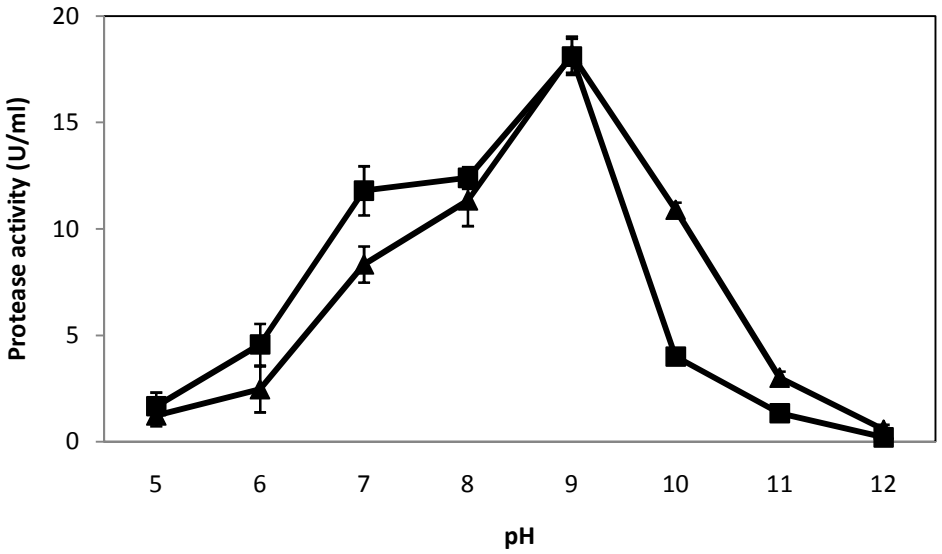

Figure-1
Effect of pH on activity of proteases from *B. subtilis* 50a (▲) and *B. licheniformis* 50b(■). Protease activity was determined by assayed at various pHs from pH 5-12 at 50˚C

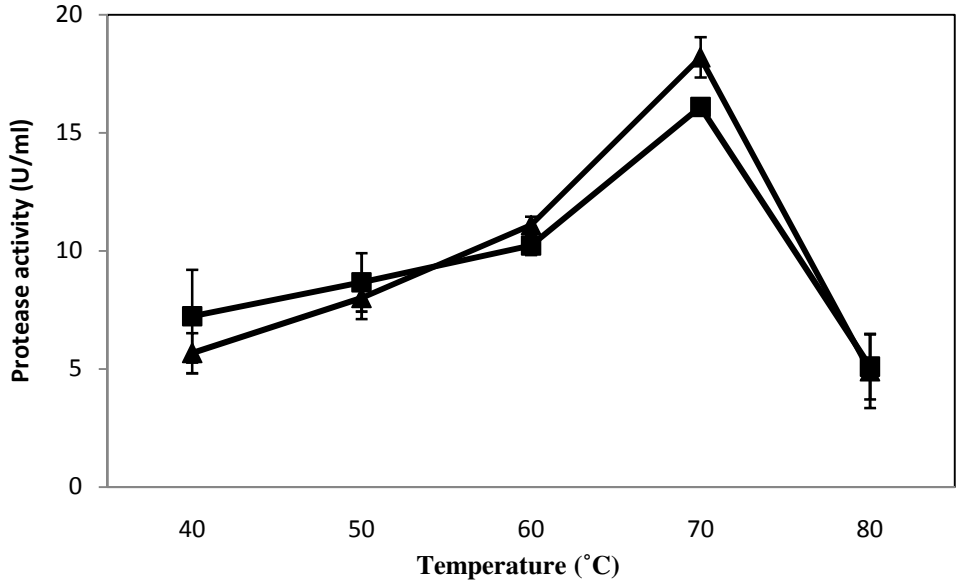

Figure-2
Effect of temperature on activity of proteases from *B. subtilis* 50a (▲) and *B. licheniformis* 50b (■). Protease activity was determined by assayed at pH 9

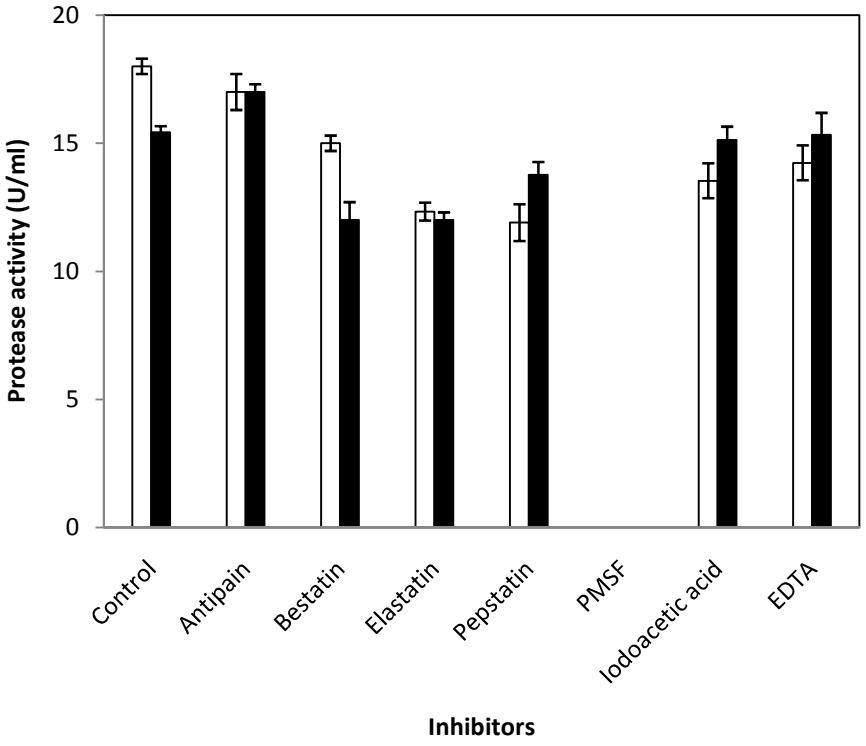

Figure-3
Effect of inhibitors on activity of proteases from *B. subtilis* 50a (□) and *B. licheniformis* 50b (■)

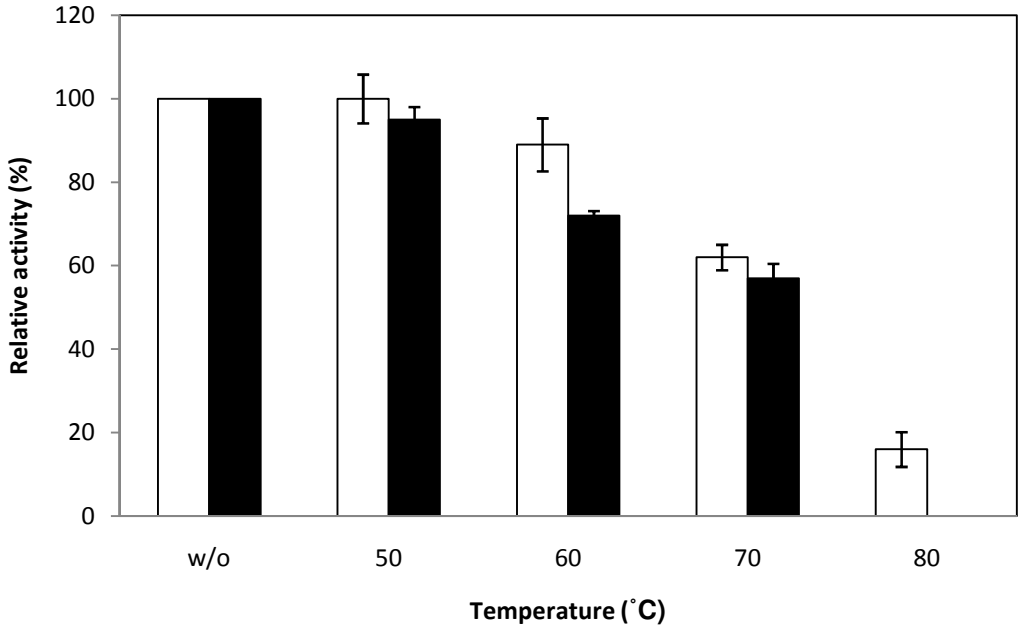

Figure-4
Thermostability of *B. subtilis* 50a (□) and *B. licheniformis* 50b(■) proteases after heat treatment at various temperature for 30 min.

Conclusion

Thermophilic proteolytic microorganisms identified as *B. subtilis* 50a and *B. licheniformis* 50b producing thermostable alkaline serine protease were screened and isolated. The characteristics of both proteases showed *B. subtilis* 50a and *B. licheniformis* 50b are good producers of extracellular protease at high temperature. Thus promising thermostable alkaline serine protease producers for industrial use especially in detergent industry.

Acknowledgement

This research was supported by Fundamental Research Grant Scheme (FRGS) funded by Ministry of Higher Education (MOHE) of Malaysia Government.

References

1. Nascimento W.C. and Martins M.L., Production and Properties of an Extracellular Protease from Thermophilic *Bacillus* sp. SMIA2, *Braz. J. Microbiol*., **35**, 91-96 **(2004)**

2. Kazan D., Denizci A.A., Oner M. and Erarslan A., Purification and Characterization of a Serine Alkaline Protease from *Bacillus clausii* GMBAE 42, *J. Ind. Microbiol Biot*., **32**, 335–344 **(2005)**

3. Huang G., Ying T, Huo P. and Jiang Y.Z., Purification and Characterization of a Protease from Thermophilic *Bacillus* strain HS08, *Afr. J. Biotechnol*., **5**, 2433-2438 **(2006)**

4. Boonyanas S., Supachok S., Suree P. and Shuitein C., Purification and Characterization of the Highly Thermostable Proteases from *Bacillus stearothermophilus* TLS33, *Protein Expres. Purif*., **20**, 142–151**(2000)**

5. Razak C., Rahman R., Salleh A.B., Yunus W., Ampon K. and Basri M., Production of a Thermostable Protease from a New High pH Isolate of *B. stearothermophilus, J. Biosciences*, **6**, 94-100 **(1995)**

6. Rahman R.N.Z.A, Razak C.N, Ampom K., Basri M., Yunus W.M.Z. and Salleh A.B., Purification and Characterization of a Heatstable Alkaline Protease from *Bacillus stearothermophilus* F1, *Appl. Microbiol. Biotechnol*., **40**, 822-827 **(1994)**

7. Olayujigbe F.M. and Kolawole A.O., Purification and Partial Characterization of a Thermostable Alkaline Protease from *Bacillus licheniformis* LHSB-05 Isolated from Hot spring, *Afr. J. Biotechnol.,* **10(55)**, 11703-11710 **(2011)**

8. Arulmani M., Aparanjini K., Vasanthi K., Arumugam P., Arivuchelvi M. and Kalaichelvan P.T., Purification and Partial Characterization of Serine Protease from Thermostable Alkalophilic *Bacillus laterosporus*-AK1, *World J. Microb. Biot*., **23**, 475-481 **(2007)**

9. Kaur S., Vohra R.M., Kapoor M., Beg Q.K. and Hoondal G.S., Enhanced Production and Characterization of a Highly Thermostable Alkaline Protease from *Bacillus* sp. P-2, *World J. Microb. Biot*., **17**, 125-129 **(2001)**

10. Mukherjee A.K., Adhikari H. and Rai S.K., Production of Alkaline Protease by a Thermophilic *Bacillus subtilis* under Solid-state fermentation (SSF) condition using *Imperata cylindrical* grass and Potato peel as Low-cost medium: Characterization and Application of Enzyme in Detergent formulation, *Biochem. Eng. J*., **39**, 353-361 **(2008)**

11. Olajuyigbe F.M. and Ajele J.O., Some Properties of Extracellular Protease from *Bacillus licheniformis* Lbbl-11 Isolated from "iru", a Traditionally Fermented African Locust Bean Condiment, *Global J. Biotech. Biochem*., **3(1)**, 42-46 **(2008)**

12. Hajji M., Hmidet N., Jellouli K., Vallaeys T., Nasri M. and Kamoun A.S., Gene Cloning and Expression of a Detergent Stable Alkaline Protease from *Aspergillus clavatus* ES1, *Process Biochem*., **45**, 1746-1752 **(2010)**

13. Beg Q.K. and Gupta R., Purification and Characterization of an Oxidation-stable, Thiol-dependent Serine Alkaline Protease from *Bacillus mojavensis*, *Enzyme Microb. Technol*., **32**, 294-304 **(2003)**

14. Jellouli K., Ghorbel-Bellaaj O., Ayed H.B., Manni L., Agrebi R. and Nasri M., Alkaline-protease from *Bacillus licheniformis* MP1: Purification, Characterization and Potential Application as a Detergent additive and for Shrimp waste Deproteinization, *Process Biochem*., **46**, 1248-1256 **(2011)**

15. Rao C.S., Sathish T., Ravichandra P. and Prakasham R.S., Characterization of Thermo and Detergent Stable Serine Protease from isolated *Bacillus circulans* and Evaluation of Eco-friendly Applications, *Process Biochem*., **44**, 262-268 **(2009)**

16. Johnvesly B. and Naik G.R., Studies on Production of Thermostable Alkaline Protease from Thermophilic and Alkaliphilic *Bacillus* sp. JB-99 in a Chemically defined medium, *Process Biochem.*, **37**, 139-144 **(2001)**

17. Adinarayana K., Ellaiah P and Prasad D.S., Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis* PE-11, *AAPS PharmSciTech*., **4(4)**, article 56 **(2003)**

18. Ghorbel B., Kamoun A.S. and Nasri M., Stability Studies of Protease from *Bacillus cereus* BG1, *Enzyme Microb. Technol*., **32**, 513–518 **(2003)**