IJABBR- 2014- eISSN: 2322-4827

International Journal of Advanced Biological and Biomedical Research

Journal homepage: www.ijabbr.com

Original Article

Study on Activity and Stability of Proteases from *Bacillus* Sp. Produced by Submerged Fermentation

Behzad Otroshi[1], Masumeh Anvari*[2], Mehdi Shariarinour[1]

[1] Department of Microbiology, Sciences and research branch, Islamic Azad University, Rasht Iran
[2] Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, Rasht, Iran

ARTICLE INFO

Article history:
Received: 3 April, 2014
Revised: 23 April, 2014
Accepted: 28 June, 2014
ePublished: 30 July, 2014

Key words:
Protease
Submerged fermentation
Enzyme activity
Stabil

ABSTRACT

Objective: Investigations were carried out to isolate bacteria from saline-alkali soils and determined optimized alkaline protease activity and stability produced by a wild strain of *bacillus* sp. in submerged fermentation (SMF). **Methods:** Optimum temperature for enzyme activity in the crude extract was 40 ◦C at a pH between 8.0 and 9.0. The studies on pH stability showed that the enzyme was stable in a range of pH 7.0–10.0 and the effect of the inhibitors showed it to be possibly an alkaline protease. Stability studies revealed temperatures around 40–60 ◦C. **Results:** The activity was reduced in the presence of Co^{2+}, Fe^{2+} and Zn^{2+} ions, while the presence of K^{+} resulted in a discreet increase in proteolytic activity. The enzyme presented good stability towards organic solvents. Organic solvents such as 2-ethyl-1-hexanol and oleyl alcohol enhanced the activity of enzyme. The enzyme presented good stability towards oxidizing agent. The crude enzyme preparation was compatible with commercial detergents, retaining their 50–60% activities. **Conclusions:** The results demonstrated the importance of submerged fermentation for the production of protease.

INTRODUCTION

Proteases are the most important industrial enzymes, accounting for about 60% of the total enzyme market (Ng and Kenealy, 1986). Microbial proteases represent one of the three largest groups of industrial enzymes and they have extensive applications in a range of industrial and household products including detergents, food, leather, silk and in pharmaceuticals industries (Mukherjee, et al. 2008; Rai and Mukherjee, 2009; Rai and Mukherjee, 2010). Of all proteases, alkaline proteases produced by *Bacillus species* are of great importance in detergent industry due to their high thermo-stability and pH stability, for production of enzymes in industrial use (Asokan and Jayanthi, 2010). Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. Hence, rapid and sensitive techniques for the detection and characterization of microbial proteases are highly desirable (Germano et al. 2003). The present study is undertaken to investigate the alkaline protease production from soil sample of screened bacterial isolates and to investigate the characteristics of the protease produced by *bacillus* sp. in submerged fermentation.

***Corresponding Author:** Masumeh Anvari, *Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Rasht, Iran P.O. Box 41335-3516* (anvari@iaurasht.ac.ir)

2. MATERIALS AND METHODS

2.1. Microorganism

Fifty-two alkalotrophic bacterial strains were isolated, employing an enrichment culture technique (Boyer et al., 1973) from saline-alkali soils of Manjil, Guilan province Iran. The research was conducted in Department of Microbiology, Sciences and research branch, Islamic Azad University, Rasht Iran.

2.2. Screening method

All the strains were screened for their alkaline protease production on milk agar medium containing (g/l): skimmed milk, 100; yeast extract, 10; agar, 20; pH 8.0 (adjusted after autoclaving). Fifteen isolates found positive for alkaline protease were again tested for their enzyme production in the growth medium containing (g/l): glucose, 10; peptone, 5; yeast extract, 5; KH2PO4, 1; MgSO4.7H20, 0.2. The pH of the broth was adjusted to 10.0 after autoclaving by adding sterilized NaeCO3 solution (20% w/v) at 10% (v/v). The above medium (50ml) was inoculated at 1.0% (v/v) with a 24 h old (O.D. 0.6) culture and incubated at 37°C in a shaker (120 rpm) for 18 h. The grown cultures were then centrifuged at 10000g for 25 min and the supernatants used for alkaline protease assay. The promising strain, identified as *Bacillus* sp. (Irost Iran), was selected for protease production. The culture was maintained on slants containing the yeast extract peptone growth medium with agar and stored at 4°C.

2.3 Submerged fermentation

The medium for pre-inoculum, inoculum as well as enzyme production consisted of glucose (10.0 g l−1), biopeptone (5.0 g l−1), yeast extract (5.0 g l−1), KH2PO4 (1.0 g l−1), MgSO4·7H2O (0.2 g l−1) and Na2CO3 (10.0 g l−1). A solution of Na2CO3 was sterilized separately and then added to the medium. Pre-inocula were developed by transferring freshly grown cells from a skim milk agar plate into 250 ml flasks, each containing 50 ml of medium and incubating the flasks at 30 ◦C and 200 rpm on a rotary shaker. 10 ml of this pre-inoculum was used to inoculate each of 500 ml flasks containing 100 ml medium and the flasks were incubated an a rotary shaker at 200 rpm and 30 ◦C for 12 h to serve as inoculum for the fermenter with 5 l working volume was used in this investigation. The initial pH of the medium was adjusted to 10.0 with sterile Na2CO3 solution and was not controlled during the course of fermentation. Temperature was controlled at 30 ◦C and air flow rate (5 l min−1) and agitation speed (300 rpm) were kept constant during batch stage. After fermentation, the supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4°C) and was used as crude enzyme extract.

2.4. Alkaline protease assay

Protease activity was determined using sulphanilamide azocasein substrate according to the method of Leighton et al. (1973). The reaction mixture containing 250 µl 1% (w/v) substrate in 0.1M carbonate/bicarbonate buffer (pH 10.0) and 150_l of enzyme solution was incubated for 30 min at 37 ◦C. After incubation, the enzyme was inactivated by addition of 1.2 ml trichloroacetic acid solution (10%, v/v) and then the solution was neutralized using 800 µl of 1.8N NaOH solution. The absorbance was read at 420 nm. One unit of proteolytic enzyme activity was defined as the amount of azocasein that hydrolyzed during 1 h incubation at 37 °C for milliliter of solution of extract.

2.5. Biochemical characterization of enzyme

2.5.1 Effect of pH and temperature on protease activity and stability

In order to find the optimum pH, protease activity was tested at different pH from 6 to 10 in a suitable buffer by incubating enzyme-substrate under standard assay condition. The optimal temperature enzyme activity was determined by incubating the reaction mixture at 30, 40, 50 and 60 °C.

To investigate the effect of pH stability, enzyme solutions were incubated at various PHS (4–10.6) at 30 °C for 60 min and the residual activity was measured. Thermo-stability (at pH=8.0) of enzyme was measured by incubating the protease alone at different temperatures (40–80 °C) for 60 min followed by standard assays.

2.5.2 Effect of metal ions and organic solvents on enzyme activity

The extracted enzymes were incubated with various metal ions (10 mM) and organic solvents (50% v/v) at 30°C with shaking at 150 rpm for 60 min. At the end of the incubation period, residual protease activity was measured under standard assay conditions (see above). Control experiments were performed without any compound.

2.6. Statistical analysis

The Data was statistically analyzed using one-way ANOVA. Post hoc tests like Duncan multiple range test (DMRT) and least significant differences (LSD) were conducted to find out whether variations existed between the treatments. A probability level of $p < 0.05$ was considered statistically significant. All statistical

analyses were conducted using SPSS for windows and Microsoft Excel.

3. Results and Discussion

3.1. Biochemical characterization on hydrolytic activity

3.1.1. Effect of pH and temperature on protease hydrolytic activity and stability:

The optimum activity of protease was observed between pH 8.0 and 9.0. In compare with pH=7, there was a 25% increasing rate on the protease activity. But the hydrolytic activity decreased sharply when the enzyme-substrate solution incubated at acidic PHS (below 7.0), since over 75% of the original activity was lost (Figure 1).

In the pH range from 7.0 to 10.0, the enzyme was more stable when were incubated for 1 h (Figure 1). This enzyme is active and stable in alkaline media and it probably has a potential for different industrial applications such as leather manufacture, detergent formulation, pulp and paper manufacture, and dairy industry. The optimum temperature for the protease activity was observed at 40° C with substantial activity between 30 and 50° C (Figure 2). At 50 and 60° C the activity decreased to 97% and 85%, respectively.

Thermo stability was examined by measuring the residual activity after 1h of incubation at 40–80° C at pH=8. It was stable at 40-60 ºC, since it retained greater than 75% of the initial activity after 1 h incubation (Figure 2). At 70 and 80 °C the stability decreased to 65% and 53%, respectively. Our results showed that enzyme should be thermos table. Some works presented in the literature report that the optimum temperature and pH for protease activity is approximately 40 °C and 8, respectively (Sun and Xu, 2009; Sun et al. 2009).

3.1.2. Effect of organic solvent on the protease stability

In addition to activity, the stability of protease in organic solvents is an important parameter for industrial applications. Log $P_{o/w}$ value is generally used to correlate solvent polarity with enzyme activity and stability in non-aqueous phases (Hernandez-Rodriguez et al. 2009). Generally, the less hydrophobic the solvent (related to lower log $P_{o/w}$ values) the higher its affinity to water and the more likely to strip the essential water from the enzyme molecules (Sun and Xu, 2009).

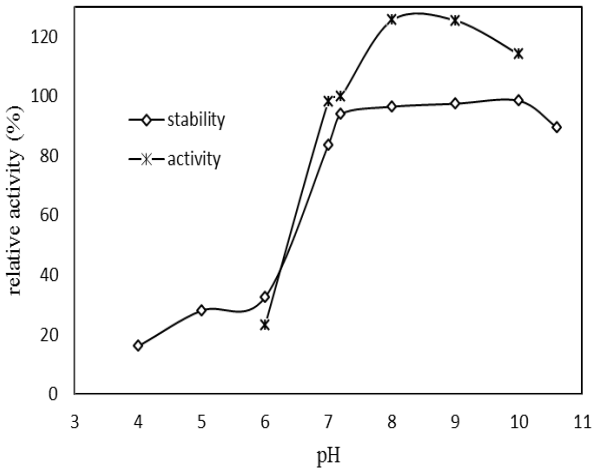

Figure1:
Effect of pH on the stability and activity of the protease produced by *Bacillus*

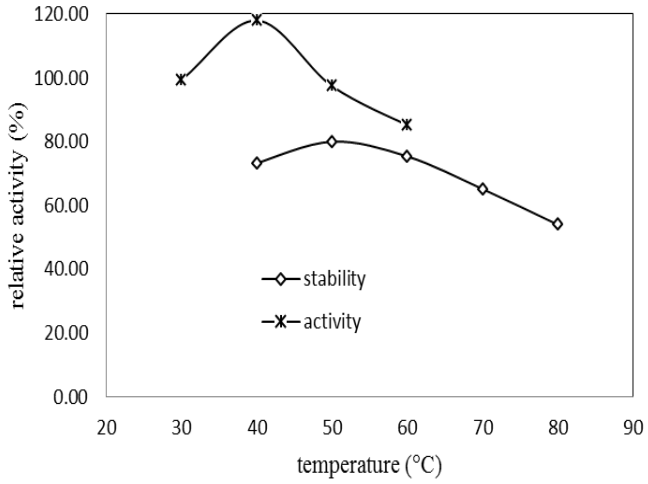

Figure2:
Effect of temperature on the stability and activity of the protease produced by *Bacillus*

Table 1:
Effect of organic solvents on activity of protease

Organic solvents	log $P_{o/w}$	Residual activity (%)
2,3-butanediol	- 0.36	43.60
1,3-butanediol	0.74	36.65
1-propanol	0.33	43.69
2-methyl-1-propanol	0.76	78.82
1-Hexanol	1.85	98.64
2-ethyl-1-hexanol	2.71	123.88
Oleyl alcohol	7.50	108.01

In order to determine protease stability, each organic solvent was incubated for 1 h in different organic solvents at 30 °C; then the residual hydrolytic activity was measured. The effects of various organic solvents on the stability of the protease were shown in Table 1. Organic solvents such as 2-ethyl-1-hexanol and oleyl alcohol enhanced the activity of enzyme by 23.88%, and 8.01%, respectively, when compared to the control. 1, 3-butanediol, 2,3-butanediol and 1-propanol were destabilizing enzyme activity, resulting in separately 63.35%, 56.40% and 56.31% of activity loss. Lower relative stability was also observed in 2-methyl-1-propanol. In summary, the stability of our protease in organic solvents follow the log $P_{o/w}$ trends and there was clear correlation between the stability of enzyme and the log $P_{o/w}$ values (exception for oleyl alcohol) (Table 1). The obtained results suggest that the activity values are influenced by several phenomena, including not only inhibition and activation, but also denaturation.

3.1.3 Effects of metal ions on protease hydrolytic activity

The effect of various ions at a concentration of 10 mM on protease hydrolytic activity was assessed (Figure 3). Inhibitory effects were observed in the presence of, Zn^{2+}, Co^{2+} and Fe^{3+} after 1 h-incubation with 35– 50% for enzyme. On the other hand, the enzyme was positively activated by 39% in the presence of K^{+} in comparison to the activity in the absence of this ion.

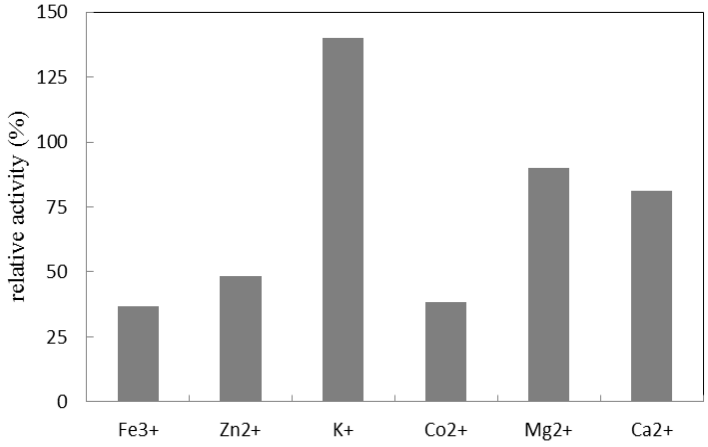

Figure 3:
Effect of various ions on the hydrolytic activity of the protease produced by *Bacillus*

Many enzymes have been found to require certain metals to sustain or enhance activity and/or stability (Salameh and Wiegel, 2007). Metal enzymes and metal-activated enzymes both belong to this group. Metal enzymes contain tightly bound metal ion cofactors, mostly commonly transition ions such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+} and Zn^{2+}. In contrast, metal-activated enzymes loosely bind metal ion from solution, usually the alkali metal ions Na^{+} and K^{+} (Rahman et al., 2005). The protease produced by *Bacillus* , in this study, was strongly inhibited by Fe^{3+}, $Zn^{2+}$and Co^{2+} but stimulated by alkaline metal ions, K^{+} ,after 60 min of incubation, thus, this enzyme is suggested to be a metal-activated enzyme.

Conclusion

Partial characterization of the crude extract, without any purification, suggested the presence of a alkaline proteases produced by *bacillus* sp. The crude extract showed that the optimum pH was 8.0-9.0 in a 0.2M buffer; the optimum temperature was 40 ◦C. It was stable in the pH range from 7.0 to 10.0. With respect to temperature, the studies revealed that the enzyme was quite stable in the temperatures of 40-60◦C. The activity increased in the presence of metallic ions such as Co^{2+}, Fe^{2+} and Zn^{2+}. Enzyme produced was compatible with commercial detergents and oxidants agents. The results presented in this study suggested the possibility to produce this enzyme by submerged fermentation and it's industrially usage with commercial detergents.

References

Asokan, S., Jayanthi, C., 2010. Alkaline protease production by *bacillus licheniformis* and *bacillus coagulans*. *J. Cell Tissue Res*. 10, 2119-2123.

Boyer, E.W., Ingle, M.B., Mercer, G.D., 1973. *Bacillus alcalophilus* sub sp. *halodurans* sub sp. nov: an alkaline amylase-producing, alkalophilic organism. *Int. J. Syst. Bacteriol*. 23, 238-242.

Germano, S., Pandey, A., Osaku, C.A., Rocha, S.N., Soccol, C.R., 2003. Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation. *Enzyme Microbial Technol*. 32, 246–251.

Hernandez-Rodriguez, B., Cordova, J., Barzanac, E., Favela-Torres E., 2009. Effects of organic solvents on activity and stability of lipases produced by thermotolerant fungi in solid-state fermentation. *J. Mol. Catal. B: Enzym*. 61, 136–142.

Leighton, T.J, Doi, R.H., Warren, R.A.J., Kelln, R.A., 1973. The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*. *J. Mol. Biol*. 76, 103–122.

Mukherjee, A.K., Adhikari, H., Rai SK., 2008. Production of protease by a thermophilic Bacillus subtitles under solid-state fermentation (SSF) condition using Imperata cylindrical grass and potato peel as low-cos t medium: characterization and application of enzyme in detergent formulation. *Biochem. Eng. J*. 39, 353–361.

Ng TK, Kenealy, W.R., 1986. Industrial applications of thermostable enzymes. In: Brock, T.D. (Ed.), Thermophiles General, Molecular and Applied Microbiology, John Wiley, New York, 197-205.

Rahman, R., Baharum S.N., Basri M., Salleh A.B., 2005. High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. Strain S5. *Anal. Biochem*. 341, 267–274.

Rai, S.K., Mukherjee, A.K., 2009. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from Bacillus subtilis strain DM-04. *Bioresour. Technol*. 100, 2642–2645.

Rai, S.K., Mukherjee A.K., 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin like serine protease (Alzwiprase) from Bacillus subtilis DM-04. *Biochem. Eng. J*. 48, 172–180.

Salameh, M.A., Wiegel, J., 2007. Purification and characterization of two highly thermophilic alkaline lipases from *Thermosyntropha lipolytica*. *Appl. Environ. Microbiol*. 73, 7725–7731.

Sun, S.Y., Xu, Y., 2009. Membrane-bound 'synthetic lipase' specifically cultured under solid-state fermentation and submerged fermentation by *Rhizopus chinensis*: A comparative investigation. *Bioresour. Technol*. 100, 1336–42.

Sun, S.Y., Xu, Y., Wang, D., 2009. Novel minor lipase from *Rhizopus chine sis* during solid-state fermentation: Biochemical characterization and its esterification potential for ester synthesis. *Bioresour. Technol*. 100, 2607–2612.