Journal of Bio-Molecular Sciences (JBMS) (2014) 2(1): 1-5.

Journal of Bio-Molecular Sciences
(JBMS)
ISSN: 2311-4630
www.directsciences.com/jbms

# Isolation and Characterization of Protease Producing Bacteria from Soil Samples of District Kohat, Pakistan

Imran Shah[1], Nasir Azam[1], Ghias ud Din[1], Nourin Ali[1], Waheed ullah[1], Muhammad Qasim[2], Aamir Shehzad[1] and Noor Muhammad[1]

[1]Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan
[2]Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan

Received 05 December 2013; Accepted 30 January 2014; available online 03 March 2014

**Abstract:** In this study protease producing bacteria from different regions of Kohat, Khyber Pakhtunkhwa, Pakistan were isolated and screened for protease production on skimmed milk agar and casein agar plates. Out of 100 isolates, 12 were found to be protease producing on skimmed milk/casein agar plates. These isolates were subjected to several biochemical tests and gram staining and were identified to be mainly *Bacillus* species. Optimum enzymatic activity was found to be at 55˚C and pH 8-9.SDS-PAGE and Zymographic analysis showed that there is mainly one protease with molecular weight of around 50 kDa.
**Keywords:** Protease; *Bacillus* species; Casein and skim milk agar

## Introduction

Proteases are the oldest and the most common family of enzymes that are involved in almost every process of organism's physiology. Due to their wide range substrate specificity, proteases are used in many industrial applications such as leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, and recovery of silver from waste photographic film. Proteases have also been used for analytical purposes in basic research. Isolation of proteases from new and novel extremophiles organisms that are active and stable under harsh industrial conditions has resulted in renewed interest in microbial proteases. Most of the commercial proteases are of bacterial origin (Prakashamet al., 2006). Proteases can be effectively used for degradation of protein containing wastes, and to remove clogs in blocked drainage pipes (Kumar and Takagi, 1999; Gupta et al., 2002). Proteases from the genus *Bacillus* are industrially important as they have

*Correspondence to: Noor Muhammad, Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan

usually have substrate specificity and can be used in leather industries, detergent formulation, baking and peptide synthesis etc. (Beg et al., 2003). These strains can produce proteases that are stable in harsh conditions of temperature and a wide range of pH.

In this study we have isolated and screened protease producing soil bacteria from Kohat, Pakistan. These proteases may be useful for working under harsh condition like, various pH and higher temperature and varied ionic concentrations.

## Material and Methods

### Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade and were purchased from Sigma and Oxoid, UK.

### Soil Sample Collection and Culturing

Soil samples from different areas of Kohat district (butcher shops of various markets) were collected in sterile polyethylene plastic bags and immediately taken into the laboratory. Soil samples were serially diluted with distilled water, cultured on nutrient agar plates at 37˚C and then sub-cultured for getting single colonies at the same temperature for 24 hours.

### Screening for Protease Producing Bacteria

Initial screening was done on 1 % skimmed milk agar plate. One gram of skimmed milk was added into 100 ml LB medium containing agar as solidifying agent. Total of 100 single colonies were picked through sterilized tips and inoculated on the skimmed milk agar plate and then incubated for 48 hours at 37˚C. Clear zones were taken as an indication of casein hydrolysis (Olajuyigbe and Ajele, 2005).

### Protease Production

Colonies showing clear zones on the skimmed milk agar were used for the production of proteases in LB medium containing casein or skimmed milk in 100 ml flask. After 48 hours shaking at 200 rpm and 37˚C, cultures were centrifuged at 4000 rpm and 4˚C. Only the supernatant was used for proteolytic activity.

### Protease Production at Different pH Values

The pH was adjusted using sodium acetate (pH 5-6), sodium phosphate (pH 6-7), simple phosphate buffer (pH 7) and sodium carbonate (pH 8-9). After incubation, the cell culture was treated as described above and cells-free supernatants were used for enzymatic activity (Sevinc and Demirkan, 2011).

### Protease Production at Different Temperatures

The optimum temperature for protease production was determined by culturing of the protease producing strains at different temperatures such as 37, 40, 45, 55 and 65˚C.

### Identification of Proteolytic Strains

Colony morphology, standard Gram's staining protocol and biochemical tests such as catalase, DNase and oxidase tests were performed for the identification of bacterial strains.

### Characterization of Protease

After partial purification with 75% ammonium sulfate and centrifugation at 13000 rpm at 4˚C, the precipitate was collected and dissolved in 0.01 molar phosphate buffers. The concentration of the protein was measured by Bradford method and the enzyme was characterized. SDS gel electrophoresis was performed for identification of the molecular weight.

Composition of 12% SDS running gel is given in (Table 1).

**Table 1.** Composition of 12 % SDS-acrylamide gel

| **Chemical** | **Amounts (µl)** |
|---|---|
| $dH_2O$ | 3075 |
| 0.5 M Tris-HCl [pH: 6.8] | 1250 |
| 20 % SDS | 25 |
| 30% Acrylamide/Bisacrylamide (29.2 % / 0.8 % ) | 670 |
| 10 % Ammouniumpersulfate | 25 |
| TEMED | 5 |

**ZymographicAnalysis**

For zymographic analysis, 0.30 % skim milk was copolymerizied in SDS gel. Samples used this time were not pre-heated. Anionic SDS was removed after completion of the electrophoresis by using TritonX-100. For this purpose gel was kept in 50 mM (Tris-Cl, pH 7.5) with 2.5 % TritonX-100 for 24 hours at 4˚C. The gel was then incubated in a zymogram reaction buffer (30 mMTris-HCl, pH 7.4, 200 mM NaCl and 10 mM $CaCl_2$) left at 37°C for 12 hours on a shaker.

**Results and Discussion:**

**Screening of Proteolytic Bacteria**

Screening of proteolytic bacteria was performed on 1% skim milk agar plates. Out of 100 isolates from the initial soil samples 15 isolates showed proteolytic activity (Fig. 1). These isolates were then subjected to further analyses.

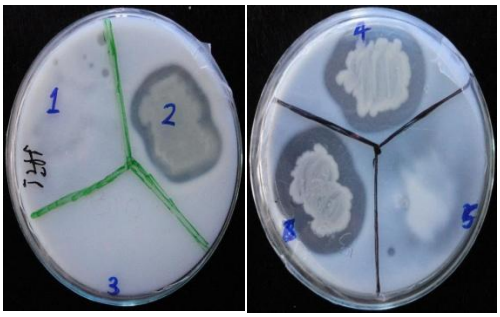


**Fig. 1.** Clear zones produced upon proteolysis by isolates (number 2, 4 and 8) on 1% skim milk agar plates.

**Identification of Bacterial Strain:** Colony morphology, Gram's staining and biochemical tests such as catalase, oxidase and DNase revealed that the isolates were different *Bacillus* species (Fig. 2).

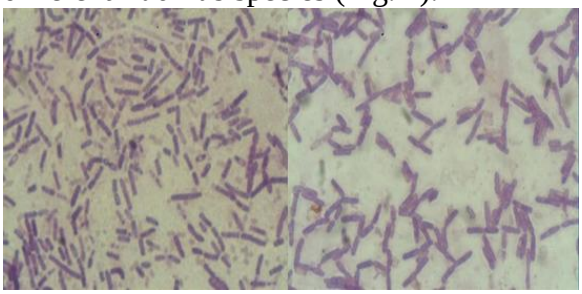

**Fig. 2.** Gram's staining of proteolytic bacteria.

**Effect of pH on Proteolysis of Proteases Production**

Proteolytic activity of 1 ml of cells-free supernatant was measured at pH values 5, 6, 7, 8 and 9. Maximum activity was found to be at pH 9 (Fig. 3). This observation is in agreement with other studies which show that the optimum pH for the activity of proteases from *Bacillus* sp. ranges between 9 – 11 (Durham et al., 1987; Bundela and Mandal, 2013).

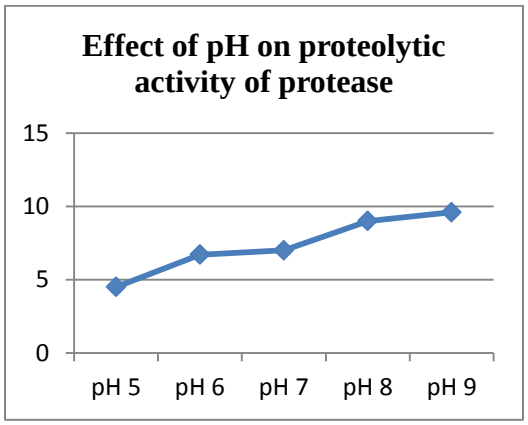


**Fig. 3.**Protease production at different pH values.

**Effect of Temperature on the Production/Proteolytic Activity of Proteases**

The proteolytic activity of 1 ml cells-free supernatant cultured at different temperatures was measured. Maximum production of the proteases was observed at 55˚C (Fig. 4). The optimum temperature for the production of proteases from majority of *Bacillus* sp. ranges from 30 – 60˚C (Kumar and Takagi, 1999). The optimum temperature (55˚C) is equal to that of *B. subtilis* for the production of alkaline proteases (Bundela and Mandal, 2013).

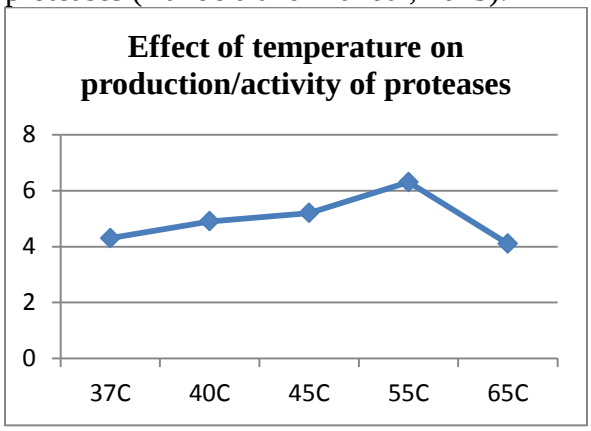


**Fig. 4.** Proteolytic activity measured from 1 ml cell-free supernatant at different temperatures.

**SDS PAGE and Zymographic Analysis**

SDS PAGE was performed using 12 % gel after purification, and the size for the protease was determined to be ~ 50 kDa (Fig. 5). Zymographic analysis showed the activity of the protease on skim milk for the same molecular weight of the protease (results not shown). The molecular weight of majority of proteases from *Bacillus* sp. ranges between 15 and 45 kDa (Kumar and Takagi, 1999; Jisha et al., 2013; Olajuyigbeet al., 2005).

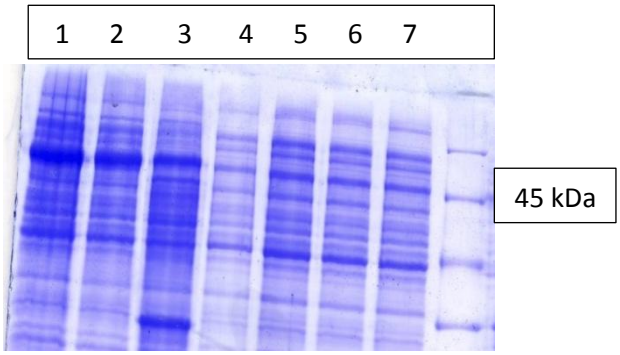


**Fig. 5.** SDS gel electrophoresis: Higher expression of proteases of around 50 kDA can be seen in Lane 1, 2 and 3 at above 50˚C. Lane 4, 5, 6 and 7 show less expression of the protease at 30, 37, 40 and 45˚C respectively.

**References**

Beg, Q. K., Sahai, V. and Gupta, R. 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem.38:1 – 7.

Bundela, V. and Mandal, S. K. 2013. Purification and characterization of an extracellular alkaline protease produced from an isolated *Bacillus subtilis*. Int. J. Appl. Biol. Pham. Tech. 4:112-119.

Durham, D. R., Stewart, D. B. and Stelwag, E.G. 1987. Novel alkaline and heat stable protease from alkalophilic *Bacillus* species strain GX6638. J. Bacteriol. 169:2262-2268.

Gupta, R., Beg, Q. K. and Lorenz, P. 2002. Bacterial alkaline proteases: molecular

approaches and industrial applications. Appl. Microbial Biotechnol. 59:15-32.

Gupta R., Beg Q. K., Khan S. and Chauhan B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl. Microbial Biotechnol. 60:381-395.

Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., Priji, P., Josh, M. S. and Benjamin, S. 2013. Versatility of microbial proteases. Adv. Enz. Res. 1:39-51.

Kumar, C. G. and Takagi, H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv. 17:561–594.

Olajuyigbe, F. M. and Ajele, J. O. 2005. Production dynamics of extracellular protease from *Bacillus* species. Afr. J. Biotechnol. 4:776-779.

Prakasham, R. S., Rao, C. S. and Sharma, P. N. 2006. Green gram husk – an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid state fermentation. Biores. Technol. 97:1449-1454.

Sevinc, N. and Demirkan, E. 2011. Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J. Biol. Environ. Sci. 5:95-103.