African Journal of Biotechnology Vol. 4 (8), pp. 776-779, August, 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Production dynamics of extracellular protease from *Bacillus* species

Folasade M. OLAJUYIGBE* and Joshua O. AJELE

Department of Biochemistry, School of Sciences, Federal University of Technology, Akure, Nigeria.

Accepted 27 June, 2005

Screening and isolation of proteolytic bacteria were carried out from soil samples of Ikogosi warm spring (SW, Nigeria). Eighteen isolates were positive on skim milk agar (10%) of which fifteen produced protease in culture broth. Three isolates, identified as *Bacillus macerans* IKBM-11, *B. licheniformis* IKBL-17 and *B. subtilis* IKBS-10, were selected for further study. These *Bacillus* species could grow up to 65°C within a broad pH range of 5 to 10 with an optimal growth temperature and pH at 60°C and 8.0, respectively. For the three *Bacillus* species, protease production occurred between 37°C and 65°C and pH 5 to 10. Maximum growth and maximum enzyme production was observed at 48 h when grown in 50 ml medium (pH 8.0) under shaking condition at 60°C. The results showed that *Bacillus* species under study are good producers of extracellular protease at high temperature. This might be an indication that proteases produced would be thermostable.

Key words: Protease, proteolytic bacteria, *Bacillus macerans*, *Bacillus licheniformis*, *Bacillus subtilis*.

INTRODUCTION

Proteases (serine protease (EC. 3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic proteases (EC. 3.4.23) and metallo-protease (EC. 3.4.24) constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Nascimento and Martins, 2004; Beg and Gupta, 2003; Ellaiah et al., 2003). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. And among bacteria, *Bacillus* species are specific producers of extracellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Pastor et al., 2001; Ward, 1985).

Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two thirds (Beg et al., 2003). Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in solubility of nongaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms. Thermophilic bacteria from hot springs produced unique thermostable enzymes (Rao et al., 1998).

In this study, bacteria isolates from Ikogosi warm spring, S.W. Nigeria were screened for proteolytic activity. Dynamics of protease production from *B. macerans* IKBM-11, *B. licheniformis* IKBM-17 and *B. subtilis* IKBM-10 with growth temperature range of 35 to 65°C was examined at different pH and temperature.

MATERIALS AND METHODS

Isolation and identification

Soil samples were taken from 0-5 cm layers at the point of outflow of the Ikogosi warm spring, SW Nigeria for preparation of initial cultures. These were subcultured to obtain pure isolates of bacteria species using Aslim et al. (2002) methods. The isolated bacteria were identified based on cellular morphology, growth conditions,

*Corresponding Author E-Mail: sadeolaj@yahoo.com.

Table 1. Identification of bacteria species.

Isolate code	Gram Reaction	Cellular Morphology	Catalase	Oxidase Test	Indole Prod.	Motility Test	Methyl Red Test	Voge's Proskaver Test	Citrate Utilization	Urease Activity	Starch Hydrolysis	Gelatin Hydrolysis	NO_3^- Reduction	Spore test	Growth on MacConkey	Glucose	Arabinaose	Xylose	lactose	Sucrose	Raffinose	Galactose	Salicin	Maltose	Mannitol	Probable Identity
1	+	Cocci	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	+	-	-	-	-	*Micrococcus leteus*
2	-	Rods	+	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-	-	+	-	-	-	-	+	*Flavobacterium rigense*
3	+	Rods	+	+	-	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-	-	-	*Bacillus brevis*
4	-	Rods	+	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	*Enterobacter cloacae*
5	+	Rods	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	*Bacillus licheniformis*
6	-	Rods	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	*Alcaligenes eutrophs*
7	-	Rods	+	-	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	*Klebsiella aerogenes*
8	-	Rods	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	*Alcaligenes eutrophs*
9	-	Rods	+	-	-	+	+	+	+	-	+	+	-	-	-	+	-	+	+	+	-	-	+	+	+	*Serratia liquefasciens*
10	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+	*Bacillus subtilis*
11	+	Rods	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	-	+	+	-	-	+	-	+	*Bacillus macerans*
12	-	Rods	+	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	*Acinetobacter moffi*
13	+	Rods	+	+	-	-	-	+	-	-	+	-	+	+	-	+	-	-	+	+	-	-	-	-	+	*Bacillus mycoides*
14	-	Rods	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	*Acinetobacter mallei*
15	+	Rods	+	+	-	+	-	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-	*Bacillus coagulans*
16	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Bacillus polymyxa*
17	+	Rods	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	+	*Bacillus licheniformis*
18	+	Rods	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	*Bacillus cereus*
19	-	Rods	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	-	*Chromobacterium violaceum*
20	+	Cocci	+	+	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	+	+	*Micrococcus roseus*
21	-	Rods	+	+	-	+	-	-	+	-	+	+	+	-	+	+	+	-	+	+	+	-	-	+	-	*Pseudomonas aeruginosa*
22	+	Rods	+	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	*Corynebacterium pilosum*
23	+	Cocci	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	+	-	-	-	+	-	*Micrococus varians*
24	+	Rods	+	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	-	+	*Bacillus megaterium*
25	-	Rods	+	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	*Alcaligenes faecalis*

gram stain, motility and biochemical tests (Sneath and Halt, 1986; Watanabe and Hayano, 1993). Growth in 5% nutrient broth at pH 5 - 8 and temperature of 37 - 70°C were examined.

Screening for proteolytic activity

The identified bacterial isolates were plated onto skim milk agar plates and were incubated at 37°C for 24 h. A clear zone of skim hydrolysis gave an indication of protease producing organisms. Depending on the zone of clearance and growth temperature of organism, three *Bacillus* species, *B. macerans* IKBM-11, *B. licheniformis* IKBL-17 and *B. subtilis* IKBS-10 were selected for further experimental studies.

Protease production

The culture medium used in this work for protease production contained 0.5% glucose (w/v), 0.75% peptone (w/v), 0.5% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) KH_2PO_4, and 0.01% (w/v) $FeSO_4.7H_2O$ maintained at 37°C for 24 to 72 h in a shaking incubator (140 rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C for 15 min and the clear supernatant was used as crude enzyme preparation.

Determination of protease activity

Protease activity was determined in triplicate by incubating 500 µL of 0.5% azocasein in Tris-HCl buffer with 100 µL enzyme solution for 60 minutes at 37°C. Reaction was stopped by adding 500 µL of 15% Trichloroacetic acid (TCA) with shaking. This was left for 15 min and centrifuged at 4°C for 15 min at 3000 rpm. 1 ml of supernatant was added to 1 ml of 1 M NaOH and absorbance was read at 440 nm. One unit (U) of protease activity was defined as micromole of substrate converted per minute under standard assay conditions.

Effect of pH on protease production

The effect of pH on protease production from *Bacillus* species under study was determined by growing each specie in fermentation media of different pH using appropriate buffers, phosphate buffer (pH 5 – 6.0), Tris-HCl buffer (pH 7.0 - 8.0) and glycine-NaOH buffer (pH 9-10). Protease production was measured and monitored at 6 hours intervals over a 72 h fermentation period through assay of protease activity.

Effect of temperature on protease production

The effect of temperature on protease production was studied by growing each *Bacillus* specie in fermentation media set at different temperatures (37, 45, 50, 55, 60 and 65°C). Protease production was monitored at 6 h intervals over a 72 h fermentation period through assay of protease activity.

RESULTS AND DISCUSSION

Twenty five (25) bacterial isolates were obtained from soil samples of which nine isolates were identified as *Bacillus* species. They were *Bacillus brevis, B. licheniformis* (2 strains)*, B. subtilis, B. macerans, B. mycoides, B. coagulans, B. polymyxa, B. cereus* and *B. megaterium* (Table 1). Watanabe and Hayano (1993) identified *B. subtilis*, *B. licheniformis*, *B. cereus* and *B. megaterium* in soil isolations. In another study, Waksman, (1961) identified 29 isolates as *B. megaterium* and 24 isolates as *B. subtilis* out of 306 soil samples. These agree with the results of this study that *Bacillus* genera are widespread among bacteria is soil.

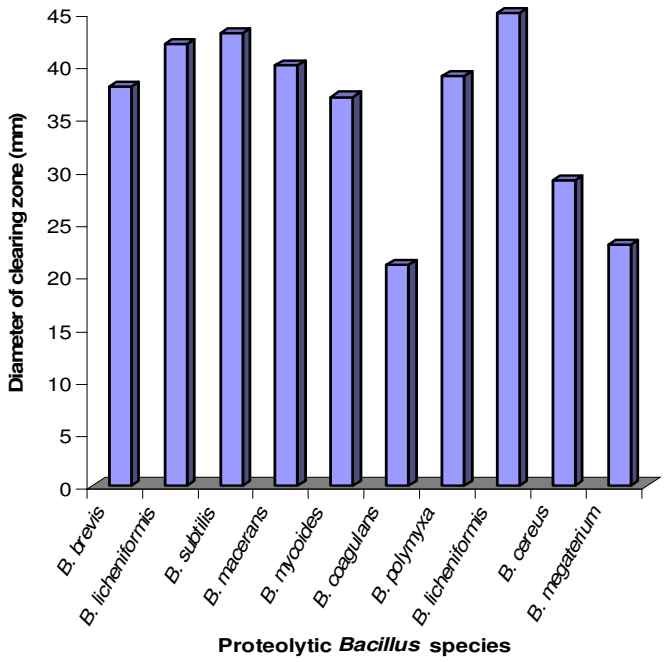

Figure 1. Clear zone of proteolytic *Bacillus* species on skim milk agar.

The proteolytic activity was assayed using skim milk agar and expressed as diameter of clear zone in mm. *B. licheniformis* IKBL-17 exhibited the highest proteolytic activity with a clear zone diameter of 45.00 mm followed by *B. subtilis* IKBS-10 with clear zone of 43.00 mm and *B. macerans* IKBM-11 zone diameter of 39.00 mm (Figure 1). These *Bacillus* species were selected for further studies.

The three *Bacillus* species produced protease over the entire range of pH investigated (pH 5 – 10). However, maximum protease production was observed at pH 8.0 while at pH 10, the protease production was about 60% (Figure 2) Most commercial proteases mainly neutral and alkaline have been reportedly produced from the genus *Bacillus* (Rao et al., 1998).

Based on exhibited proteolytic activity and growth temperature range of 37 – 65°C, the effect of temperature on protease production was determined. The three *Bacillus* species had maximum protease production at 60°C. Protease production was about 70% even at 65°C (Figure 3). Results showed that the *Bacillus* species under study from Ikogosi warm spring are thermophiles. These might be sources of thermostable proteases.

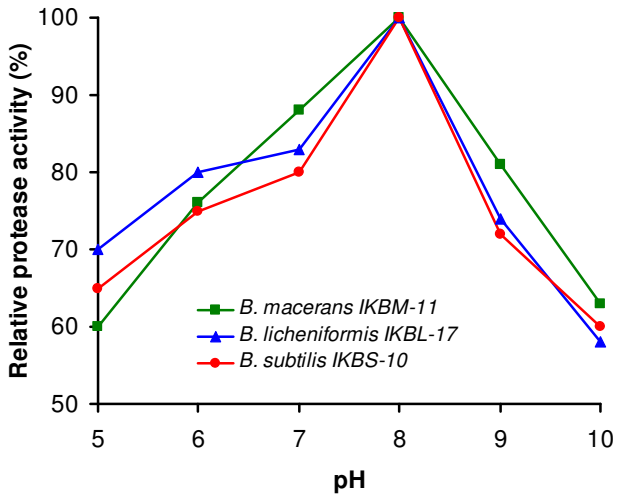

Figure 2. Protease production from *B. macerans* IKBM-11, *B. licheniformis* IKBL-17 and *B. subtilis* IKBS-10 at different pH.

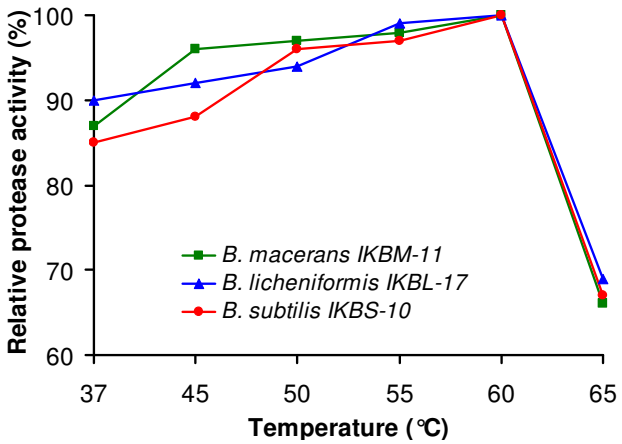

Figure 3. Protease production from *B. macerans* IKBM-11, *B. licheniformis* IKBL-17 and *B. subtilis* IKBS-10 at different temperatures.

Other workers have also isolated thermophilic bacteria in the Thai hot spring (Thailand), which were reported to produce thermostable proteases (Sookkheo et al., 2000).

The results obtained in this study show that *B. macerans* IKBM-11, *B. licheniformis* IKBL-17 and *B. subtilis* IKBS-10 are good producers of extracellular protease at high temperature over a broad pH range. This might be an indication that the *Bacillus* species would produce thermostable neutral and alkaline proteases which could find applications in industry and biotechnology. Proteases from the *Bacillus* species are presently being characterized.

ACKNOWLEDGEMENT

This research was supported by the International Foundation for Science, Stockholm, Sweden, through a research grant to Folasade Mayowa Olajuyigbe.

REFERENCES

Aslim B, Yuksekdag ZN , Beyatli Y (2002). Determination of PHB growth quantities of certain *Bacillus* species isolated from soil. Turkish Electronic J. Biotechnol. *s*pecial issue, 24 – 32.

Beg KB, Gupta R (2003) . Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enz. and Microbial Technol., 32: 294 – 304.

Beg KB, Sahai V, Gupta R (2003) . Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem. 39: 2003 – 2009.

Ellaiah P, Adinarayana K, Rajyalaxmi P, Srinivasulu B (2003) . Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic Bacillus sp. Asian J. Microbial Biotechnol. Environ. Sc. 5: 49-54.

Nascimento WCA, Martins MLL (2004) . Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz.* J. Microbiol. 35: 1 – 2**.**

Pastor MD, Lorda GS, Balatti A (2001) . Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration rates. Braz. J. Microbiol., pp. 32: 1-8.

Rao BM, Tanksale MA, Ghatge SM, Deshpande VV (1998). Molecular and Biotechnological Aspects of Microbial Proteases: Microbiol. and Mol. Biol. Rev. 62 (3): 597-635.

Sarath G,De la Monte SR, Wagner WF (1994) . Protease assay methods in R.J. Beynon and J.S. Bond (Ed.), IRL, Oxford, U.K.

Sneath HAP, Halt GJ (1986). Bergey's manual of systematic bacteriology Vol. 2 Baltimore, M.D.: Williams and Wilkins.

Sookkheo B, Sinchaikul S, Phutrakul S, Chen ST (2000). Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS 33, Protein expression and purification, 20: 142 – 151.

Ward OP (1985). Proteolytic enzymes. In: Blanch, H.W., Drew, S., Wang, D.I., eds. Comprehensive Biotechnology. Vol. 3. Oxford U.K. Pergamon Press; 789 –818.

Waksman SA (1961). *Microbiol* Rutgers Univ. Copyright, 3rd print.

Watanabe K, Hayano K (1993) Distribution and identification of proteolytic *Bacillus* species in paddy field soil under rice cultivation. Can. J. Microbiol. Vol. 41 pp. 674 – 680.