INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-2, April-June-2012 Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Received: 19th April-2012 *Revised: 23th April-2012* *Accepted: 28th April-2012*

Research article

BIOSYNTHESIS AND CHARACTERIZATION OF KERATINOLYTIC PROTEASE FROM *ACTINOBACTERIUM* SP. IN SOLID STATE CULTURE

Ponnuswamy Vijayaraghavan[1*], J. Lavanya[2], and Samuel Gnana Prakash Vincent[3]

[1]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam - 629 502, Kanyakumari District, Tamil Nadu, India
[2]Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu, India
[3]International Centre for Nanobiotechnology, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam – 629 502, Kanyakumari District, Tamil Nadu, India

E-mail: venzymes@gmail.com

ABSTRACT: The aim of this study was to use agro-industrial residues for the production of a halotolerant keratinolytic- protease by *Actinobacterium* sp. in solid-state fermentation. Among various agro-industrial residues that were evaluated, apple pomace supported maximum protease production (8400 U/g material). The optimum conditions required for enzyme production were a fermentation period of 72 h, 10% (w/v) NaCl, pH 7.0, 120% (v/w) moisture and 10% (v/w) inoculum. The enzyme exhibited activity to a range of pH (7.0-9.0) and temperature (30-45°C), with optima at 8.0 and 40 °C, respectively. Most of the divalent ions tested stimulated the protease activity and Ca^{2+} ion was required for its activity and stability. The enzyme was widely active at the range of NaCl concentration (5%-15%, w/v) and effectively degraded chicken feather. This protease could be useful in fish sauce fermentation and also in feed industry.

Keywords: *Actinobacterium* sp., agro-residues, solid-state fermentation, halotolerant protease

INTRODUCTION

Proteases constitute one of the most important groups of enzymes and their annual sales account for 60% of the world enzyme market (Turk, 2006). The physical, biochemical, molecular and catalytic properties of proteases vary from organism to organism (Geok et al., 2003; Ghorbel et al., 2003; Gupta et al., 2002). Generally, most industrial proteases have some limitations (Joo et al., 2003) and their use highly depends on their stability during isolation, purification and storage, in addition to their robustness against solvents, surfactants and oxidants (Gupta et al., 2005; Joo et al., 2003; Sivasubramaniam et al., 2008). Hence, an in-depth knowledge of kinetics and catalytic behavior during protease production from any new strain is a pre-requisite for evaluating its biotechnological potential (Prakasham et al., 2006; Subba Rao et al., 2008).

Extremophiles, the microbes dwelling in unusual habitats, can potentially serve in a variety of industrial applications. Halotolerant proteases have many applications including saline fermentation process and are thus involved in the production of various protein-rich foods. These proteases are essential not only in preventing putrefaction and the development of food poisoning, but also to produce desired flavors (Margesin and Schinner, 2001).

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

The main step in this fermentation process concerns the hydrolysis of protein. Thus, proteases that are able to maintain high activities under moderate saline conditions are essential. Halophilic proteases are less suited for this process, because they need at high concentration of NaCl for expressing high activity (Ventosa et al., 1998). Therefore, halotolerant proteases, which are active at both low and high concentrations of NaCl, are needed (Setyorine et al., 2006). So the aim of the present study was to isolate a potent isolate from the saline environment for the production of halotolerant protease.

The cost of the production medium which is used for submerged fermentation is considerably high. Solid-state fermentation has gained renewed interest over submerged fermentation and fresh attention is being paid to it from researchers, owing to its importance in recent developments related to biomass energy conservation, in solid waste treatment and in the production of secondary metabolites (Prakasham et al., 2006). An important factor to be monitored while developing a production medium is the cost effectiveness of the medium and this can be achieved by using cheaply available agro-industrial residues. There are several reports that describe the use of agro-industrial residues for the production of alkaline protease, e. g., pigeon pea and *Bacillus* sp. JB-99 (Johnvesly et al., 2002), green gram husk and *Bacillus* sp. (Prakasham et al., 2006). In the Actinobacteria family, very few organisms were subjected to solid state fermentation for the biosynthesis of protease e.g. *Streptomyces albidoflavus* (Bressollier et al., 1999) *Streptomyces* sp. 594 (De Azeredo et al., 2006) and *Streptomyces* sp. CN902 (Lazim et al., 2009). Therefore, considering the applications of halotolerant proteases in industry and the solid wastes generated in agro-industries, the present study was carried out using a newly isolated actinobacterium, *Actinobacterium* PVJL.

MATERIALS AND METHODS

Screening of halotolerant protease secreting organism

The soil sediment was collected at a depth of 2-3 cm from a solar salt pan in Tamilnadu (India). One gram of the soil sample was serially diluted in sterile double distilled water from 10^{-1} to 10^{-7} preparations. The organisms were cultivated at 37 °C in nutrient agar, which contained 5 g of peptone, 5 g of yeast extract, 10 g of KH_2PO_4, 0.2 g of $MgSO_4$, 10 g of skimmed milk, 1-25% (w/v) sodium chloride, and 15 g of agar per liter. The pH of the culture medium was adjusted (7.0 to 11.0) by 1 N NaOH. The plates were incubated at 37 °C for 3 days and examined for clear zone formation on the skimmed milk agar plates.

Identification of organism

The organism was tentatively identified as *Actinobacterium* sp. based on morphology and biochemical characteristics (Holt et al., 1994). Further, this organism was identified by molecular approach. The 16S rRNA gene was sequenced after the genomic DNA extraction and the PCR amplification as described elsewhere (Riffel et al., 2003). The 1395 bp sequence was submitted to Genbank with the accession number JF 499686. The BLAST algorithm was used to search for any homologous sequences in Genbank. This organism was found to be homologous with the sequence of *Actinobacterium* and thus named as *Actinobacterium* PVJL.

Substrates

The agro-industrial residues such as apple pomace, banana peels, cabbage leaves, maize husks, pineapple peels, sugarcane waste, wheat bran and tapioca peels were collected from the local market. The coir pith and paddy straw were collected locally. They were dried for several days, and powdered in a mixer grinder. These were evaluated for their potential as substrates in the solid state fermentation for alkaline protease production.

Solid-state fermentation (SSF)

SSF was carried out separately in a 250 ml Erlenmeyer flask containing five grams of the above solid substrate moistened (100%, v/w) with a 50 mM tris-HCl buffer (pH 8.0). The medium was sterilized at 121°C for 30 min, the flasks were inoculated with one milliliter of an 18 h grown (0.763 OD at 600 nm) culture broth and incubated at 37±1°C for 72 h. Among the all substrates, the best substrate was used for further optimization studies.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

For investigating the effect of the fermentation period on enzyme production, the entire contents of a flask were used for the extraction and for the estimation of protease activity every 12 h. To evaluate the effect of sodium chloride on enzyme production, NaCl was added as 5-25% (v/w). To evaluate the effect of pH on enzyme production, the fermentation pH adjustment was achieved by moisturizing the medium with a definite buffer on the solid material. In order to study the moisture content, the quantity of water was calculated with respect to solid material being used. To evaluate the effect of inoculum on enzyme production, the 18 h grown culture broth was introduced onto the solid medium accordingly. The results reported in this study are the mean of three different repeats.

Enzyme extraction

The fermented substrates were mixed thoroughly with a 50 ml tris-HCl buffer (50 mM, pH 8.0). The mixture was shaken for 30 min at room temperature in an orbital shaker (155 strokes/min) and the process was repeated three times. The filtrate thus obtained was used as a crude enzyme for protease activity.

Enzyme assay

Keratinolytic and caseinolytic activities were determined by using keratin and casein as substrates. The protease activity was assayed according to the method of Kim et al. (2004). One unit of caseinolytic and keratinolytic activity was defined as the amount of enzyme required to release 1 µg of tyrosine min^{-1} under experimental condition. The protein content was determined by the method of Lowry et al. (1951).

Partial purification of protease from *Actinobacterium* PVJL

The crude enzyme obtained previously was precipitated by ammonium sulphate salting-out procedure (30-70% saturation) and the enzyme precipitate obtained was centrifuged at 10000× g for 15 min at 4 °C. The resulting pellet was dissolved in a small amount of 50 mM tris-HCl buffer (pH 8.0), and this enzyme solution was loaded for gel-filtration chromatography using a sephadex G-75 column (0.9×40 cm) (Amersham Biosciences, Sweden) equilibrated with the same buffer. The flow rate was adjusted to 1.0 ml min^{-1}. The fractions containing protease activity were pooled and used for characterization studies.

Determination of the pH optimum and stability

The effects of pH on the enzyme activity of protease were determined at various pH ranging from 4.0–10.0 using a 50 mM citrate buffer (pH 4.0), a succinate buffer (pH 5.0), a sodium phosphate buffer (pH 6.0–7.0), a tris-HCl buffer (pH 8.0) and a glycine–NaOH buffer (pH 9.0-10.0). The pH stability studies were performed by pre-incubating the partially purified enzyme preparation in the above buffers at 37 °C for 1 h and subsequent analysis of residual activity under standard assay was carried out.

Determination of optimum temperature and thermal stability

The optimum temperature of the enzyme was measured by incubating the reaction mixture of the enzyme at different temperatures (30-80°C) at pH 8.0. The thermal stability of the protease was evaluated by incubating the enzyme without any substrate at different temperatures ranging from 30-80 °C. The remaining enzyme activity was determined under standard assay conditions.

Effect of divalent ions on protease activity

The protease activity in response to divalent ions (5 mM) was evaluated with Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+} and Zn^{2+}. The catalytic behavior of enzyme was studied by pre-incubating (1 h, 37 °C) these divalent ions with the partially purified enzyme sample and the residual activity of the enzyme was measured.

Effect of surfactants on enzyme stability

The effect of surfactants (1%) (sodium dodecyl sulphate, Tween-20, Tween-80, Triton X-100 and Brij-35) on the proteolytic activity was evaluated by pre-incubating it for 1 h in the above surfactant at 37 °C before assaying the residual enzyme activity. A control was kept with the enzyme and the substrate (without detergents) and the value of the control activity was considered as 100%.

Effect of solvent on enzyme stability

To evaluate the effect of solvent on enzyme stability, the enzyme preparation was incubated with solvent (1%, v/v) such as benzene, toluene, diethylether, and acetone at 37 °C for 1 h. The enzyme activity without any solvent was taken as 100%.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Effect of alkaline protease for its activity on chicken feather

To evaluate the keratinase property of this enzyme, broiler chicken feathers were selected. Five feathers were taken and supplemented with 10 mg of the partially purified protease enzyme in the tris-HCl buffer (50 mM, pH 8.0) and incubated at 32±2 °C for 48 h in order to analyse keratinase activity.

RESULTS AND DISCUSSION

Selection of the alkaline protease-producing organism

From the isolates that were tested, five had the ability to produce alkaline proteases. The best alkaline protease producer was selected and identified as *Actinobacterium* based on morphology and biochemical characteristics. The identified organism was Gram-positive, hydrolysed casein and gelatin, catalase-, and oxidase positive. Acid is produced from D-fructose, D-galactose, D-glucose, D-maltose, D-Mannitol, negative for H_2S-, indole production and urease activity. This organism was selected for the biosynthesis and characterization of the alkaline protease. It was identified as *Actinobacterium* PVJL based on 16 S rRNA sequence analysis. There are reports describing the use of *Actinobacterium* sp. for the production of proteases that correlate with and support the present study. Saha and Dhanasekaran (2011) recently pointed out that the potential application of Actinobacterial isolates involves various biotechnological applications including keratin hydrolysis.

Solid-state fermentation

The selection of an ideal agro-industrial residue for enzyme production in an SSF process depends on several factors, mainly related to the cost and availability of the substrate material. Thus, it may involve screening of several agro-industrial residues (Kim et al., 2004). The present results revealed that the maximum production of enzymes was observed with apple pomace (8400 U/g material), while the minimum protease production (10 U/g material) was noticed with coir pith as the substrate. Wheat bran showed only 6625 U/g material protease production (Fig. 1). Apple pomace is a left over residue represents about 25-35% of the weight of the fresh apple processed (Wang and Thomas, 1989). It is a rich source of nutrients including vitamins and trace elements. These results are in accordance with the observations made by Prakasham et al. (2006), found that the alkaline protease production varies with the type of agro-waste used. Many previous studies have suggested that using various agro-wastes for protease production in solid-state culture other than wheat bran: pigeon pea in *Bacillus* sp. JB-99 (Johnvesly et al., 2002), green gram husk in *Bacillus* sp. (Prakasham et al., 2006); feather meal in *Streptomyces* sp. 594 (De Azeredo et al., 2006); wheat bran and chopped dates stones in *Streptomyces* sp. CN902 (Lazim et al., 2009) were found to be effective for protease biosynthesis. Based on the observation, apple pomace was selected and used as a substrate for optimization studies.

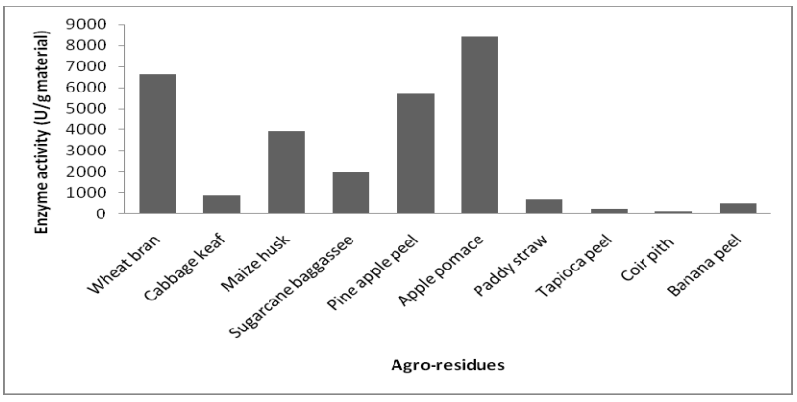

Figure. 1. Evaluation of agro-industrial residues for the production of alkaline protease.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Fermentation period

The results of protease production had a positive impact on the fermentation period. Enzyme production was high after 72 h of incubation at 37 °C (Fig. 2). The maximum rate of enzyme production occurred during the early stationary phase of growth resembling *Micrococcus* sp. INIA (Mohedano et al., 1997) and *Bacillus* sp. JB-99 (Johnvesly et al., 2002).

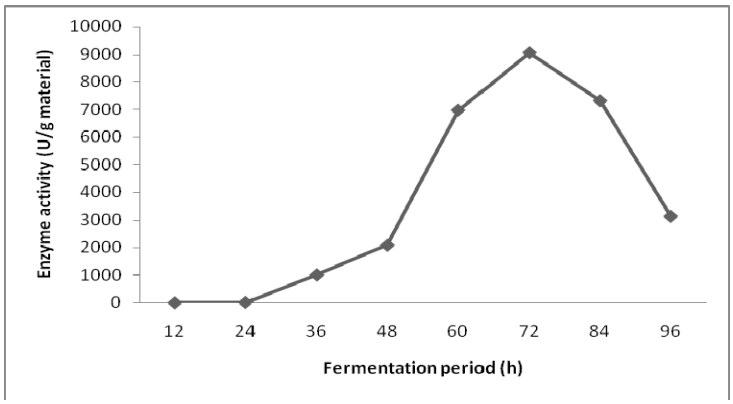

Figure. 2. Effect of incubation time on alkaline protease production under solid-state fermentation with apple pomace.

Effect of NaCl on protease production

The present study strongly suggested that, for the production of protease, NaCl was required. The enzyme production increased in the medium containing 10% (w/v) NaCl (4590 U/g material) resembling *Halomonas campaniensis* sp. (Romano et al., 2005) and decreased (3545 U/g material) at 15% NaCl (Fig. 3). Moreover, the bacterial growth was also observed at higher concentrations of NaCl (15–25%), but enzyme biosynthesis declined considerably. This may be attributed to growth reduction and enzyme inactivation at higher concentration of NaCl.

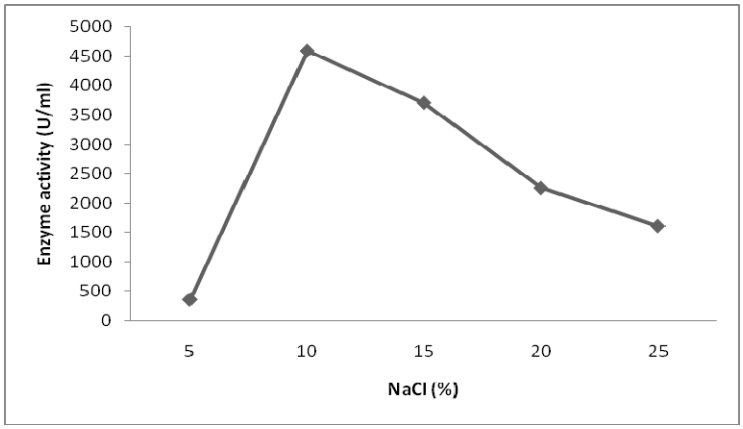

Figure. 3. Influence of NaCl on alkaline protease production in solid-state fermentation.

Effect of pH on enzyme production

Alkaline protease production by microbial strains strongly depends on the extra-cellular pH, because culture pH strongly influences many enzymatic processes and the transport of various components across cell membranes, which in turn supports cell growth and enzyme production (Ellaiah et al., 2002).

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

In this study, optimum biosynthesis of protease was observed at a pH of 7.0 and decreased thereafter; this result was in accordance with the results obtained with *Micrococcus* sp. INIA 528 (Mohedano et al., 1997). The enzyme production was remarkably low at acidic pH (4.0-6.0). And, unlike the acidic range, enzyme biosynthesis was not affected strongly in the alkaline range (7.0-9.0). At lower pH, the metabolic action of a bacterium may be suppressed and thus enzyme production inhibited. Hence this protease can be considered as an alkaline protease.

Effect of moisture content

The moisture content is one of the critical factors in SSF (Kim et al., 2004; Nigam and Singh, 1994). In the present study, the maximum biosynthesis of the enzyme was observed with 120% moisture (1820 U/g material) and there after it dramatically decreased (700 U/g material at 180%). Moisture is an important factor affects enzyme production and it varied depending on the substrates used. Similar trends have also been observed in SSF of protease by some bacterial species. Lazim et al. (2009) stated that SSF of protease by *Streptomyces* sp. CN902 increased at 60% moisture with wheat bran, and 140% was found to be optimum for the production of alkaline protease by *Bacillus* sp. on green gram husk (Prakasham et al., 2006).

Effect of inoculum on enzyme production

Physio-chemical factors such as inoculum levels strongly affect the fermentation process (Dodia et al., 2006). In this study, the inoculum level showed diverse effects on enzyme biosynthesis. When a wide inoculum (5-25%) was applied, a positive response was observed upto 10% inoculum and it decreased thereafter. At 5%, the enzyme production was 4872 U/g material and it increased to 5398 U/g material at 10%. The enzyme production was strongly affected at 25% inoculum (1420 U/g material). This may be attributed to growth reduction and enzyme inactivation at higher inoculum level (>10%).

Effect of pH on enzyme activity and stability

The partial purification step yielded 29.1% and purification fold of 17. The partially purified protease enzyme was used to study its properties. The enzyme produced by *Actinobacterium* sp. demonstrated optimum activity at a pH of 8.0 (Fig. 4). The enzyme activity was high for alkaline pH; similar finding was reported in *Streptomyces* sp. (De Azeredo et al., 2006). Incubating the protease for 1 h with the pHs ranging between 7.0 and 8.0 pH solutions did not show much reduction in the activity profile, whereas, around 61% and 55% reductions in activity were noticed when incubated at pHs of 6.0 and 9.0 respectively. There are reports describing the effect of pH on enzyme activity and stability of protease that correlates with and support the present study. Dodia et al. (2008) reported that the protease was stable at this range of pH with the haloalkaliphilic bacterium sp. AH-6.

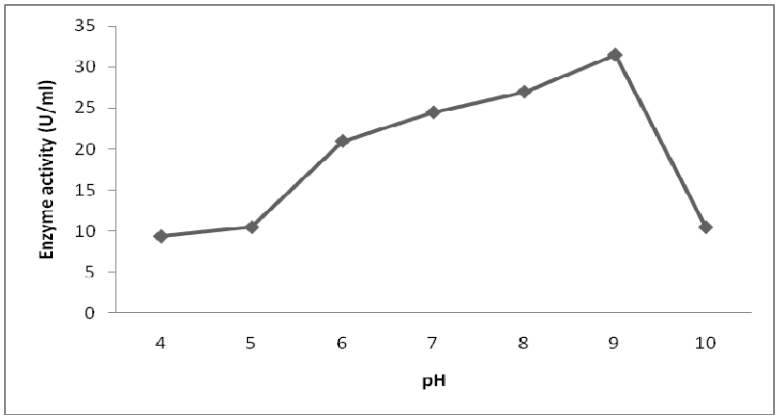

Figure. 4. Effect of pH on activity of the enzyme

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Effect of temperature on enzyme activity and stability

The analysis of temperature dependent *Actinobacterium* PVJL protease activity revealed that the catalytic behavior of the enzyme increased with increasing temperature, up to 40°C (Fig. 5) and incubation temperatures beyond 40°C drastically reduced enzyme activity. Similar kind of protease properties were studied on *Bacillus subtilis* (Setyorine et al., 2006). This enzyme was stable upto 50°C, and enzyme activities were 100%, 98.5% and 67% at temperatures of 30, 40, and 50°C respectively. This result was in accordance with the *Streptomyces* sp. strain AB1 (Jaouadi et al., 2010).

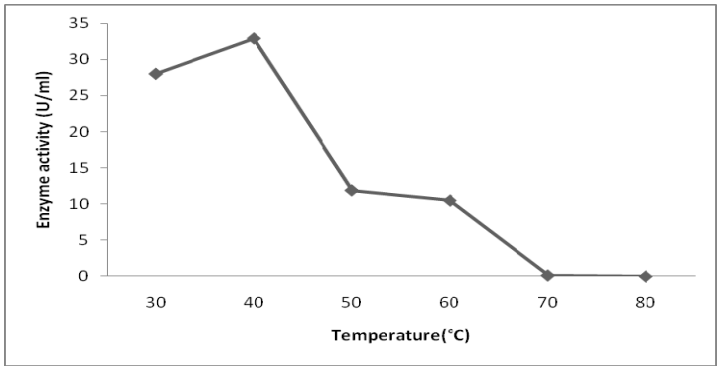

Figure. 5. Effect of temperature on protease activity from 30 to 80°C

Effect of divalent ions, surfactants and solvent on enzyme stability

The results of enzyme activity of protease towards ions (5 mM) such as Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} were evaluated and stimulated effects were observed with Ca^{2+}, Mn^{2+}, and Mg^{2+} (135%, 103%, and 116.8%) ions. An inhibitory effect was reported with ions such as Cu^{2+} (80%), Hg^{2+} (41%) and Zn^{2+} (73%). Experiments revealed that Ca^{2+} was required for enzyme activity and for the maintenance of an active conformation of this enzyme. This result was in accordance with haloalkaliphiic *Bacillus* sp. (Gupta et al., 2005).

The experimental data suggested that this protease had activity on surfactants such as SDS (62%), Tween 20 (4.6%), Triton X-100 (32%) and Brij35 (60%) (Table. 1). There are reports describing the effect of surfactant on protease activity that correlate with and support the present study. Dodia et al. (2008) reported that protease activity of haloalkaliphic bacterium sp. AH-6 was marginally decreased by SDS, which was also supported by Nascimento and Martins (2006).

Table 1. Relative activity of the alkaline protease on surfactants and solvent

Surfactants (1%)	Relative activity (%)
SDS	62
Tween-20	66
Tween-80	4.2
Triton X-100	32
Brij-35	60
Control	100
Solvent (1%)	Relative activity (%)
Benzene	97
Toluene	72
Diethyl ether	46
Acetone	72.4
Control	100

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

The *Actinobacterium* PVJL protease was moderately stable against various organic solvent. The enzyme activity was 97% in benzene while it was 46% for diethyl ether (Table 1). This result was in contrast to some of the earlier reported results. Actinobacteria such as *Streptomyces* sp. strain AB1 showed high resistance against organic solvents (Jaouadi et al., 2010). In *Halobacterium* sp. SP1(1), the extracellular protease was highly tolerant to organic solvents, the majority of which are frequently used in paints (Akolkar et al., 2008).

Effect of NaCl on enzyme activity

This enzyme was broadly active in a range of 5-15% (w/v) NaCl and decreased thereafter. 12.5% NaCl was found to be optimum for protease activity. The results suggest that NaCl is important for its better activity, which may have wide spread applications in the preparation of fish sauce. This result was in accordance the observation made with halophilic bacterium (Dodia et al., 2006). It was previously reported that 150 mM was found to be optimum for Haloalkaliphilic bacterium sp. AH-6 (Dodia et al., 2008) indicates that varying concentrations of NaCl among halotolerant isolates were required.

Keratinolytic activity of Actinobacterium sp.

Results regarding keratinolytic activity revealed that the partially purified protease was capable of degrading broiler feathers after 48 h of incubation in room temperature (30±2 °C) (Fig. 6). This degradation suggested that this enzyme possessed both keratin disulfite reductase and keratinase activity. It was evident that the Actinobacteria such as the *Streptomyces* sp. strain AB1 protease were able to degrade keratin (Jaouadi et al., 2010). The keratinases were optimally active in the neutral to alkaline pH range and temperatures ranging from 40-60 °C and the present study correlated with the earlier finding.

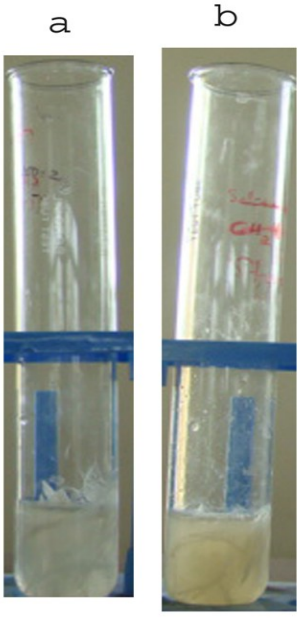

Figure. 6. Feather degradation of *Actinobacterium* protease at pH 9.0 at room temperature (30±2 °C) after 24 h (a-Control; b-Experimental).

Solid-state fermentation of agro-residues has a large economic potential for conversion into proteases. The use of low cost agro industrial wastes such as apple peel leads to reduction of total production cost (25-50%). The protease produced by *Actinobacterium* PVJL was active at wide range of NaCl concentrations (5-15%) may have application in fish-sauce preparation. This protease effectively degraded feather and could be useful in feed industry.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

REFERENCES

Akolkar. A.V., G.M. Deshpande, K.N. Raval, D. Durai, A.S. Nerurkar and A.J. Desai (2008). Organic solvent tolerance of Halobacterium sp. SP1(1) and its extracellular protease. Journal of Basic Microbiology 48(5):421-425.

Bressollier. P., F. Letourneau, M. Urdaci, and B. Verneuil (1999). Purification and characterization of a keratinolytic serine proteinase from Streptomyces albidoflavus. Applied Environmental Microbiology 65(6):2570-2576.

De Azeredo, L.A. M.B. De Lima, R.R. Coelho and D.M. Freire (2006). Thermophilic protease production by Streptomyces sp. 594 in submerged and solid-state fermentations using feather meal. Journal of Applied Microbiology 100(4):641-647.

Dodia M.S., C.M. Rawal, H.G. Bhimani, R.H. Joshi, S.K. Khare and S.P. Singh (2008). Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp. AH-6: Journal of Industrial Microbiology 35(2):121-131.

Dodia M. S, R.H Joshi, R.K Patel and S. P, Singh (2006). Characterization and stability of extracellular alkaline protease from halophilic and alkaliphilic bacteria isolated from saline habitat of coastal Gujarat, India. Brazilian Journal of Microbiology 3 (37):276-282.

Ellaiah, P. B. Srinivasulu and K. Adinarayana (2002). A review on microbial alkaline proteases: Journal of Scientific and Industrial Research 61:690-704.

Geok, L.P. C.N.A. Razak, R.N.Z.A. Rahman, M. Basri and A.B. Salleh (2003). Isolation and screenings of an extracellular organic solvent-tolerant protease producer. Biochemical Engineering Journal 13:73-77.

Ghorbel, B. A. Sellami-Kamoun and M. Nasri (2003). Stability studies of protease from Bacillus cereus BG1. Enzyme and Microbial Technology 32:513-518.

Gupta. R., Q.K. Beg and P. Lorenz (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbiology and Biotechnology 59:15-32.

Gupta.A., I. Roy, S.K. Khare and M.N. Gupta (2005). Purification and characterization of a solvent stable protease form Pseudomonas aeruginosa Pse A. Journal of Chromatography 1069:155-161.

Holt, J.G. N.R. Krieg, P.H. Sneath, J.J. Stanley and S.T. Williams. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimere, USA, (1994).

Jaouadi, B.B. Abdelmalek, D. Fodil, F.Z. Ferradji, H. Rekik, N. Zaraî and S. Bejar (2010). Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from Streptomyces sp. strain AB1 with high stability in organic solvents. Bioresource Technology 101(21):8361-8369.

Johnvesly, B. B.R. Manjunath and G.R. Naik (2002). Pigeon pea waste as a novel, inexpensive, substrate for production of a thermostable alkaline protease from thermoalkalophilic Bacillus sp. JB-99. Bioresource Technology 82:61-64.

Joo H-S., C.G. Kumar, G-C. Park, S.R. Paik and C-S. Chang (2003). Oxidant and SDS-stable alkaline protease from Bacillus Clausii I-52: production and some properties. Journal of Applied Microbiology 95:267-272.

Kim D.C., N.S. Oh and M.J. In (2004). Effect of carbon and nitrogen sources on cell growth and halotolerant alkaline protease production in Halomonas marisflava isolated from salt-fermented food. Food Science and Biotechnology 13:837-840.

Krishna.C. (2005). Solid-state fermentation systems-an overview. Critical Review in Biotechnology 25(1-2):1-30.

Lazim H., H. Mankai,N. Slama, I. Barkallah and F. Limam (2009). Production and optimization of thermophilic alkaline protease in solid-state fermentation by Streptomyces sp. CN902. Journal of Industrial Microbiology and Biotechnology 36(4):531-537.

Lowry O.H., N.J. Rasebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with Folin Phenol reagent. Journal of Biological Chemistry 193:256-275.

Margesin.R.and F. Schinner (2001). Potential of halotolerant and halophilic microorganisms for biotechnololgy. Extremophiles 5:73-83.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Mohedano A.F., J. Fernández, P. Gaya, M. Medina and M. Nuñez (1997). Effect of pH, temperature and culture medium composition on the production of an extracellular cysteine proteinase by Micrococcus sp. INIA 528. Journal of Applied Microbiology 82(1):81-86.

Nascimento and M.L.L. Martins. W.C.A. (2006). Studies on the stability of protease from Bacillus sp. and its compatibility with commercial detergent. Brazilian Journal of Microbiology 37:307-311.

Nigam.P. and D. Singh (1994). Solid state (substrate) fermentation system and their applications in biotechnology. Journal of Basic Microbiology 34:405-423.

Pandey, A. C.R. Soccol, P. Nigam, D. Brand R. Mohan and S. Roussos (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochemical Enginnering Journal 6:153-162.

Prakasham, R.S. Ch. Subba Rao and P.N. Sarma (2006). Green gram husk: an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation.Bioresource Technology 97:1449-1454.

Rajkumar, Rengananthan, J.K. Ranishee and R. Ramasamy (2011). Production and characterizaion of a novel proteases from Bacillus sp. RRM1 Under Solid State Fermentation. Journal of Microbiol Biotechnology 21(6):627-636.

Riffel, A. F.S. Lucas, P. Heeb and A. Brandelli (2003). Characterization of a new keratinolytic bacterium that completely degrade native feathers. Archives in Microbiology 179:258-265.

Romano, I. A. Giordano, L. Lama, B. Nicolaus and A. Gambacorta (2005). Halomonas campaniensis sp. nov., a haloalkaliphilic bacterium isolated from a mineral pool of Campania Region, Italy. Syst Applied Microbiology 28:610-618.

Saha S. and D. Dhanasekaran (2011). Isolation and Screening of Keratinolytic Actinobacteria form Keratin Waste Dumped Soil in Tiruchirappalli and Nammakkal, Tamil Nadu, India. Current Research Journal of Biological Sciences 2(2):124-131.

Setyorine, E. S. Takenaka, S. Murakami and K. Aoki (2006). Purification and Characterization of Two Novel Halotolerant Extracellular Protease from Bacillus subtilis Strain FP-133. Bioscience Biotechnology and Biochemistry 70(2):433-440.

Sivasubramanian, S. B. Murali Manohar, A. Rajaram and R. Puvanakrishna (2008). Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. Chemosphere 70:1015-1024.

Soares, V.F.L.R. Castilho, E.P. Bon and D.M. Freire (2005). High-yield Bacillus subtilis protease production by solid-state fermentation. Applied Biochemistry and Biotechnology 121-124:311-319.

Subba Rao, Ch. S.S. Madhavendra, R. Sreenivas Rao, P.J. Hobbs and R.S. Prakasham (2008). Studies on improving the immobilized bead reusability and alkaline protease production by isolated immobilized Bacillus circulans (MTCC 6811) using Overall Evaluation Criteria. Applied Biochemistry and Biotechnology 15:65-83.

Turk. B. (2006). Targeting proteases: successes, failures and future prospects. Nature Reviews Drug Discovery 5:785-798.

Ventosa, A. J.J. Nieto and A. Oren (1998). Biology of moderately halophilic aerobic bacteria. Microbiology and Molecular Biology Reviews 62:504-544.

Wang H.J. and R.I. Thomas (1989). Direct use of apple pomace in bakery products. Journal of Food Science 54(3): 618-620.