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A COMPARATIVE ANALYSIS OF PROTEASE PRODUCING MICROBES ISOLATED FROM TANNERY EFFLUENT

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ABSTRACT
Proteases are well known enzymes for their wide range application in food industry, detergent industry and pharmaceuticals industry for the preparation of ointments and medicine. They are also widely used in leather industry for dehairing and bating of hides as an alternative for toxic chemicals which in turn hamper the environment. As enzymes have proved to solve unresolved technical issues relating to pollution control the present study was undertaken to screen for organisms which could produce extracellular proteases. Organisms were isolated from tannery effluent, in which 13 isolates were obtained and among which 5 isolates were found to produce extracellular proteases. Biochemical test revealed the organisms to be *Aeronomas sp., Alcaligenes sp., Bacillus sp., Staphylococcus sp. and Pseudomonas sp.* The stability of the protease was also studied under temperature and pH. The organism was found to grow over a wide range of pH and temperatures. Among the 5 isolates *Aeronomas sp., Bacillus sp., Staphylococcus sp. and Pseudomonas sp.* showed enzyme production at pH 8.0 and *Alcaligenes sp* at pH 9.0. The enzyme production for all the isolates was found to be between 45°C to 65°C with ane optimum temperature of 55°C. Zymography analysis in this present study showed extracellular caseinase activity by substrate hydrolysis.

KEYWORDS: extracellular proteases, proteolytic activity, tannery effluent, zymography

INTRODUCTION

The amount of pollutants and waste generated by industries has become an increasingly costly problem for manufacturers and adding significant stress on the environment. Industries, therefore, are looking for ways to reduce pollution at the source as a way of avoiding costly treatment and reducing environmental liability and compliance costs. Understanding the microorganisms and the ability to genetically manipulate the microorganisms by infusing engineering principles into biology has led to novel strategies for combating environmental problems with enzymes. The scope of Industrial enzymes is growing because they offer less polluting processes than chemical catalyst and perform reactions with higher specificity than chemical catalyst. Among these enzymes, protease is the most important enzyme and accounts for about 60% of the total worldwide sale (Gupta *et al.,* 2002). Proteases find application in the food, pharmaceutical and detergent industries and are important tools in studying the structure of proteins and polypeptides (Bhosale *et al.,* 1995). Malathi and Dhar (1987) reported that in leather processing enzymatic process is favorable when compared to that of lime-sulphide method. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations. (http://www.ias.ac.in/currsci/jul10/articles16.htm).

Therefore, enzymatic dehairing is an efficient method to produce quality leather without causing pollution to the environment. 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 (Ciborowsky *et al.,* 1994). Proteases are extracted from many organisms like *Bacillus, Aspergillus, Pseudomonas* etc. In bacteria, serine- and metalloproteases are the principal classes of proteases found in several species such as *Bacillus subtilis, B. amyloliquefaciens, Pseudomonas sp., Lysobacter enzymogenes* and *Escherichia coli* (Fujishige *et al*., 1992).

MATERIALS AND METHODS

Isolation of protease secreting organisms

Samples were collected from leather industrial waste water at Common Effluent Treatment Plant (CLRI), Chennai and transported to laboratory in sterile conditions. The organism thus isolated from tannery effluent was cultured in skim milk agar plates, and incubated at 30ºC for 48 h. Individual colonies producing clear zones were purified by subculturing and those isolates producing the largest clear zones were selected. Among the 13 isolates obtained from the tannery effluent treated sample and only 5 isolates were found to produce protease. Morphological characters were analysed with gram staining and biochemical characters such as catalase test, oxidase test, coagulase test, urease test, phenylaline deamination, indole test, methyl red test, voges prosakeur, citrate utilization, lysine utilization, ornithine utilization, glucose utilization, adonitol utilization, lactose utilization, arabinose utilization, sorbitol utilization, H_2 production, nitrate test and triple sugar iron agar test were performed.

Assay for protease activity

Enzyme activity was assayed using casein as the substrate with slight modification to the method of Sarath *et al.*(1989). The reaction mixture consisted of 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2.0% (w/v) of casein and 0.15 ml of enzyme solution. After incubating at 25ºC for 15 min, the reaction was stopped by adding 1.2 ml of 10.0% (w/v) TCA then it was incubated

at room temperature for an additional 15 min, and the precipitate was removed by centrifugation at 8,000 ´ g for 5 min. 1.4 ml of 1.0 M NaOH was added to 1.2 ml of the supernatant, and its absorbance was measured at 440 nm. The activity was determined by detecting the release of amino acids (tyrosine) from casein and the amount of tyrosine released was calculated from the standard curve constructed with tyrosine. One protease unit is defined as the amount of enzyme that releases 1µg of tyrosine per ml per minute under the above assay condition. Lowry *et al.*(1951) method was followed for the estimation of protein using bovine serum albumin as a standard.

CHARACTERIZATION OF ENZYME ACTIVITY

Effect of temperature on the activity and the stability of protease

The effect of temperature on pure enzyme was studied by assaying the enzyme at different temperatures in the range of 45-65°C with a of pH 8 using azocasein as substrate. The thermo-stability of enzyme was studied by incubating the enzyme preparation at varying temperatures ranging from 25-75°C for 1 h. Subsequently, the enzyme activity was assessed as above (Ganesh Kumar *et al.,* 2007).

Effect of pH on the activity and the stability of protease

The effect of pH on the protease activity was determined by incubating the reaction mixture at pH values ranging from 7.0 to 10.0. To check the effect of pH on the stability, the crude enzyme solution (50 µl) was mixed with 150 µ different buffer solution and was incubated at room temperature for 1h after which the proteolytic activity was measured under standard assay conditions (Shahanara Begum *et al.,* 2007).

Zymographic analysis

Casein (0.05%) was co-polymerized with polyacrylamide gel and SDS-PAGE was performed according to the method described by Heussen *et al.,* (1980). Following electrophoresis, the gels were washed successively with 2.5% (v/v) Triton X-100 and then with Triton X-100 containing 20mM sodium phosphate buffer (pH 7.0) for 10 min each. Finally, the gels were equilibrated for 10 min with the same buffer, and incubated at 37ºC for 5 min. The gels were stained with Coomassie brilliant blue, and destined to reveal zones of substrate hydrolysis.

RESULTS

Characterization of the isolates

In the present study 13 isolates were isolated from tannery effluent and was screened for protease production by inoculating them in casein agar and skim milk agar. Among which 5 organisms showed zones of substrate hydrolysis which were named P1, P2, P3, P4 and P5. The zone of hydrolysis was found to be 0.9 mm by P2 followed by 0.8 mm by P1, 0.6 by P3, P4 and P5 showed 0.4mm. Physical and biochemical test showed that the organisms were *Aeronomas sp., Alcaligenes sp., Bacillus sp., Staphylococcus sp. and Pseudomonas sp.* respectively.

Effect of pH on protease production

The protease production by bacterial strains is strongly controlled by the extracellular pH, as the culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes which in turn sustain the cell growth and product formation (Ellaiah *et al.,* 2002). In order to determine the influence of pH and temperature the production medium was adjusted to various levels of pH. The organism was found to grow over a wide range of pH and temperatures. Among the 5 isolates P1, P3, P4 and P5 showed enzyme production at pH 8.0 and P2 at pH 9.0. Similar findings were reported by Shimogogi *et al.*(1991) and Manjeet Kaur *et al.,* (1998) from some thermophilic *Bacillus sps.* The maximum of enzyme production was observed at pH 8.0. Majority of *Bacillus* sps. showed growth and enzyme production under alkaline conditions, Manjeet Kaur *et al.*(1998) have reported pH 9.0 as optimum condition for production of alkaline proteases. The results showed that at 50°C with pH 8.0 the protease production was at the highest (211.1 U /mL). Our findings were found to coincide with the earlier reports of Meenumadan *et al.*(2000) and Ellaiah *et al.*(2002) (Fig.1).

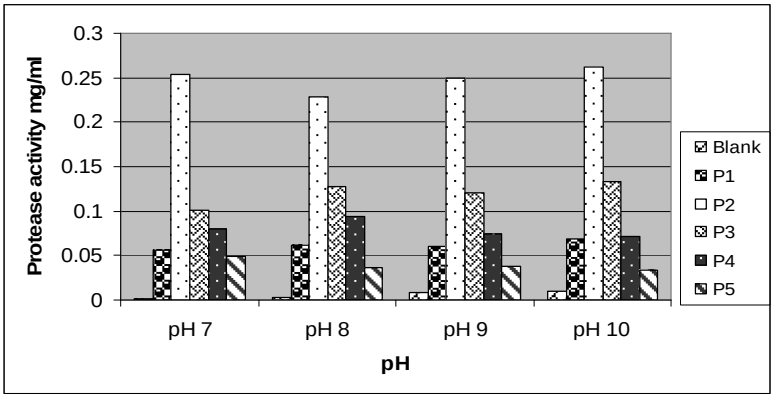

FIGURE 1. Effect of pH on enzyme protease isolated from organisms from tannery effluent
(P1: *Aeromonas sp.*, P2: *Alcaligenes sp.*, P3: *Bacillus sp.*, P4: *Staphylococcus sp.*, P5: *Pseudomonas sp.*)

Effect of temperature on protease production

The enzyme production for all the isolates was found to be between 45°C to 65°C and the optimum temperature was 55°C. Above 65°C there was a sharp decline in the enzyme production (Fig.2)

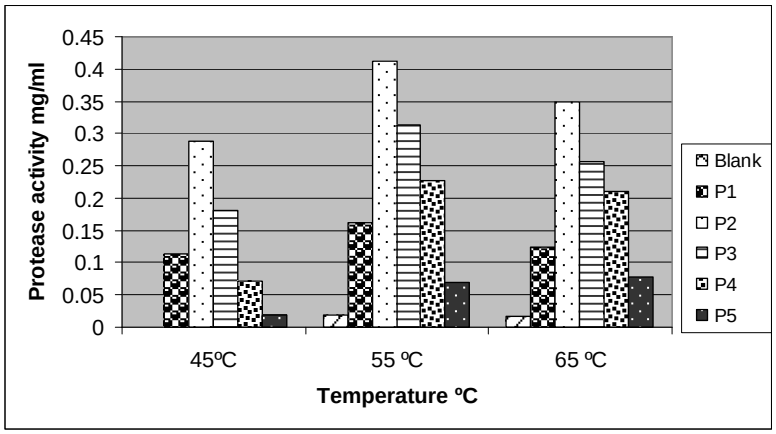

FIGURE 2. Effect of temperature on enzyme protease isolated from organisms from tannery effluent (P1: *Aeromonas sp*., P2: *Alcaligenes sp*., P3: *Bacillus sp*., P4: *Staphylococcus sp.,* P5: *Pseudomonas sp.*)

Our findings coincide with earlier works were the enzyme production was maximum at temperature of 50°C for 72 h, it was found to greatly differ from the production of alkaline proteases from *Bacillus polymyxa* reported by Meenumadan *et al*.(2000) where maximum temperature for enzyme production was found to be 70°C.

Zymography analysis

The extracellular enzyme solutions prepared from the five isolates had different caseinolytic activities, as determined by zymography (Fig. 3). On the zymogram obtained for P1 showed blurred band of approximate molecular weight of 90KDa indicating protease being synthesized by *Aeromonas* sp. In the lane of P2 the recovery of enzymes was roughly at 116 KDa, 60 KDa and 35 KDa indicating the presence of numerous proteases. P3 showed elution of enzymes at 50 KDa and 30 KDa. P4 had visualization of bands of distinct proteins with protease activities to have molecular masses of approximately 116kDa and at 29KDa. Similar work was done by Prakash *et al.,* 2005 with *Bacillus cereus* were the organism produced protease enzyme in large amounts by the method of extractive fermentation.

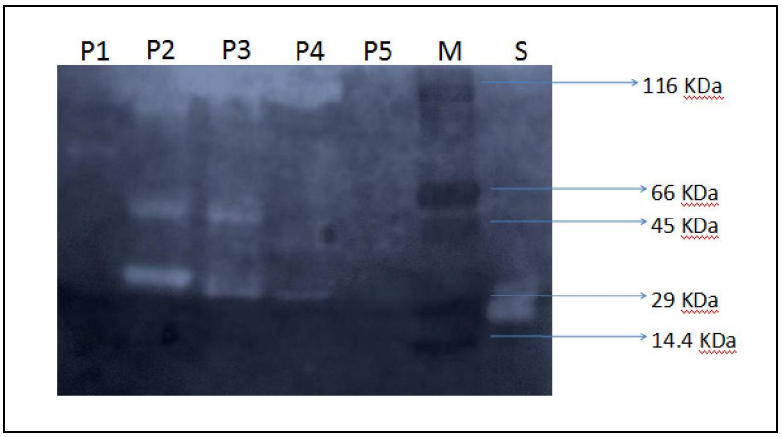

FIGURE 3. Zymogram of purified protease in SDS–PAGE (P1: *Aeromonas sp*., P2: *Alcaligenes sp*., P3: *Bacillus sp*., P4: *Staphylococcus sp*., P5: *Pseudomonas sp.*, M: Marker, S: Standard)

They also found protease purified and characterized in their study was superior to endogenous protease already present in commercial laundry detergents which adds the scopes of extracellular proteases. In the above mentioned study the purified enzyme had a specific activity of 3256.05µg/ml. It is desirable that the enzymes show good activity in the temperature range and pH. The chosen strategy for the study was the optimization of temperature and pH according to the maximum value of the production obtained. It was also concluded that the protease production is much more effective when growth at pH 8 and temperature of 55°C.

CONCLUSION

It is thus, imperative to find new methods in order to decrease and to re-use these wastes. The use of proteases would also make it possible to reduce the use of harmful chemicals and wastes in the environment because these chemicals can be replaced by proteases which are more efficient. It is evident from this examination of the possible production and purification of enzyme and to apply enzymes from different microbes.

REFERENCES

Bhosale, S.H., Rao, M.B., Deshpande, V.V., Srinivasan, M.C. (1995) Thermostability of high activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20), *Enzyme Microbiology and Technology,* **17**, 136-139.

Ciborowsky, P., Nishikata, M., Allen, R.D., Lantz, M.S. (1994) Purification and characterization of two forms of a high molecular-weight cysteine protease (porphypain) from Porphyromonas gingivalis, *Journal of Bacteriol*ogy, **170**, 4549-4557.

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

Fujishige, A., Smith, K.R, Silen, J.L., Agard, D.A. (1992) Correct folding of a-lytic protease is required for its extracellular secretion from *Escherichia coli*, *Journal of Cell Biology*, **118**, 33-42.

Ganesh Kumar, A., Nagesh, N., Prabhakar, T.G., Sekaran, G. (2008) Purification of extracellular acid protease and analysis of fermentation metabolites by Synergistes sp. utilizing proteinaceous solid waste from tanneries, *Bioresource Technology*, **99**, 2364–2372.

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

Heussen, C., Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substratesm, *Analitical Biochemistry,* **102**, 196-202.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. (1951) Protein measured with the folin phenol reagent, *Journal of Biological Chemistry,* **193**, 265-273.

Malathi, S., Chakraborty, R. **(**1991) Production of alkaline protease by a new *aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent, *Applied and Environmental Microbiology*, **57,** 712-716.

Malathi, S., Dhar, S.C. (1987) Production of extracellular protease by an *Aspergillus flavus* isolate and its application in the depilation of skins, *Leather Science,* **34**, 67-76.

Manjeet, K., Dhillon, S., Chandhary, K., Randhir, S. (1998) Production, purification and characterization of alkaline protease from *Bacillus polymyxa*, *Indian Journal of Microbiology,* **38**, 63-67.

Meenumadhan, K., Dhillon, S., Randhir, S. (2000) Production of alkaline protease by a UV-mutant of *Bacillus polymyxa*, *Indian Journal of Microbiology,* **40**, 25-28.

Prakash, M., BanikRathindra, M., Koch Bradt, C. (2005) Purification and characterization of *Bacillus cereus* protease suitable for detergent, *Applied Biochemistry and Biotechnology,* **127,** 143-156.

Sarath, G., Dela Motte, R.S., Wagner, F.W. (1989) *Proteolytic Enzymes: A Practical Approach*, IRL Press, Oxford, England.

Shahanara, B., Iftikhar, A., Faisal, A., Samsuzzaman Parvez, H., Nurul, A., Jalaluddin Ashraful, H. (2007) Characterization of an intracellular protease from pseudomonas aeruginosa, *Pakistan Journal of Medical Sciences,* **23,** 227-232.

Shimogagi, H., Takeuchi, K., Nishino, T., Ohdera, M., Kudo, T., Ohba, K., Iwama, M., Irie, M. (1991) Purification and properties of a novel surface active agent and alkaline resistant protease from Bacillus sp., *Agricultural and Biological Chemistry,* **55**, 2251-2258.