

Original Research Article

Identification and Characterization of Thermostable Protease Producing Organism and Partial Purification of Protease Enzyme

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ABSTRACT

Thermophilic bacteria were isolated from soil sample by serially diluting the sample followed by spread plate technique. Isolated stains were screened for protease production and maximum enzyme producing strain was selected for further studies. The strain was identified as *Saccharomonspora sp.* by using Bergey's manual of determinative bacteriology. Different fermentation parameters such as media, pH, temperature, metal ions, carbon source and nitrogen source were studied for maximum production of protease enzyme. Maximum enzyme production was observed in nutrient broth containing glucose as carbon source, NaNO_3 as nitrogen source, CaCl_2 , pH 7 at 60°C after 96 hrs of incubation. The enzyme was partially purified using ammonium sulphate and acetone precipitation. 70% ammonium sulphate precipitation fraction showed maximum enzyme activity (3.45 mM/ml/min) and 1:2 acetone fraction showed maximum enzyme activity (2.8 mM/ml/min).

Keywords

Actinomycetes,
Thermostable
protease

Introduction

Protease is an enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Sources of proteases include plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases have pH optima in the range of 2.0–5.0 and are mostly produced by fungi. Neutral proteases have pH optima in the range of 7.0. Alkaline

proteases have pH optima in the range of 8–12 (Hanan, 2012).

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes (Abirami *et al.*, 2011) Microorganisms are capable of producing these enzymes intracellularly and extracellularly. The isolation of proteases especially the extracellular proteases of microbial origin is easy and economical. Seasonal fluctuations in the availability of raw material usually do not affect the

enzyme production by microbes (Roja Rani *et al.*, 2012). Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes in the world (Radha *et al.*, 2011) Proteases represent an important group of enzymes produced industrially and account for 60% of the worldwide sales value of the total industrial enzymes (Abirami *et al.*, 2011).

Proteases are thus the most important group of the enzymes produced commercially and for industrial purpose. They have extensive applications in a range of industrial products and processes including detergents, food, pharmaceuticals, leather and bioremediation process. They are classified into various groups such as alkaline protease, serine protease, cystein protease, aspartic protease and metallo proteases (Balachandran *et al.*, 2012; Sathiya *et al.*, 2013)

Actinomycetes are Gram-positive, mycelium-forming soil bacteria that include many species considered to be among the most important producers of antibiotics. However, the present knowledge concerning proteases of actinomycetes is much less than that of fungi and other bacteria. Proteases produced by actinomycetes are the most important group of secondary metabolites that are widely exploited (Balachandran *et al.*, 2012)

Materials and Methods

Sample collection

The compost sample was collected from Mahatma Phule Agriculture College, Shivajinagar, Pune, Maharashtra, India.

Isolation of organism

The compost sample was pretreated at 60°C for 1 hour and was crushed using mortar

pestle. The sample was homogenised using distilled water and was further used for isolation of bacteria. One ml of homogenised sample was serially diluted (10^{-1} to 10^{-10}) and aliquots of 0.1ml were spread plated on the skimmed milk agar plates at pH (Ferrero *et al.*, 1996).

The plates were incubated at 60°C for 2 days. The isolates which exhibited protease activity on skimmed milk agar plates were subcultured on nutrient agar medium and stored at 4°C for further studies (Balachandran *et al.*, 2012)

Morphological and biochemical characterisation of isolates

Isolates were characterized up to genus level based on morphological and biochemical characters, following the Bergey's manual of systematic bacteriology.

Morphological characterisation was done by slide culture and Gram's staining technique using light microscope. Morphological characters of isolates such as morphology of spore bearing hyphae with entire spore chain and structure of spore were observed.

Biochemical characters of isolates such as various sugar utilization tests viz. glucose, galactose, xylose, mannitol, sucrose, mannose and arabinose, rannose were performed.

Detection of protease activity of the isolates

Detection of protease activity by strain fresh culture of the isolates was done. Nutrient gelatin agar plates were spot inoculated with the culture and incubated at 60°C for 24hrs (Bholay *et al.*, 2012; Shilpa Jani *et al.*, 2012). After incubation plates was observed for zone of clearance around the colony.

Production medium and culture conditions

The isolate was inoculated in 250ml Erlenmeyer flasks containing 100ml of production medium, pH-7. The flask was incubated in shaker incubator at 55°C, 100 rpm for 144 hrs. The aliquots were withdrawn at interval of 24 hrs and centrifuged at 5000 rpm for 30 min. The pellet and supernatant obtained were subjected for detection of enzyme activity using enzyme assay (Shilpa Jani *et al.*, 2012).

Protease assay

Proteolytic activity in the pellet and supernatant was determined by using spectrophotometer method. 0.5 ml of crude enzyme solution was allowed to react with 2.0 ml of 0.6% casein in phosphate buffer (50 mM, pH 9) at 45°C for 20 min. The reaction was terminated by the addition of 4 ml 10% trichloroacetic acid. The reaction mixture was allowed to stand for 15 minute before centrifugation. The mixture was then centrifuged at 5000 rpm for 15 minutes at 4°C and 1 ml of supernatant was taken as the enzyme source. The supernatant was mixed with 2.5 ml of 0.4M Na₂CO₃, 0.5 ml of 1:1 FollinCiocalteus phenol reagent and distilled water. The reaction mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 660nm wavelength. The amount of amino acid released was quantified from standard graph of tyrosine (Mohsen *et al.*, 2013; Sathiya, 2013)

Optimization of fermentation parameters for maximum enzyme production

Optimization of fermentation media

The effect of different media such as nutrient broth, skimmed milk broth, Luria-

Bertani broth and nutrient gelatin broth on the enzyme activity was determined. The media were incubated at 55°C for 96 hrs (Hanaa *et al.*, 2010; Alya Sellami-Kamoun *et al.*, 2008). The enzyme activity was determined by performing the standard assay procedure.

Optimization of pH

The effect of different pH was determined by preparing nutrient broth in different buffers such as sodium phosphate (pH 7.0), Tris-HCl (pH 8–10).

The media were incubated at 55°C for 96 hrs (Titilayo Olufunke Femi-Ola and Desmond Olayinka Oladokun, 2012). The enzyme activity was determined by performing the standard assay procedure.

Optimization of temperature

The effect of different temperature conditions on the enzyme activity was determined. Nutrient broth at pH7 was incubated at different temperatures viz. 40 to 80°C was determined by incubation of crude enzyme at temperatures for 96 hrs (Camila Rocha *et al.*, 2007). The enzyme activity was determined by performing the standard assay procedure.

Optimization of metal ions

The effect of different metal ions on enzyme activity was determined by the addition of CaCl₂, MgSO₄, FeSO₄, HgCl₂, EDTA at a final concentration of 1.0 mM to the nutrient broth at pH 7.

The media were incubated at 55°C for 96 hrs (Parawira Wilson and Zvauya Remigio, 2012). The enzyme activity was determined by performing the standard assay procedure.

Optimization of Nitrogen source

The effect of different nitrogen sources on enzyme activity was determined. Nutrient broth of pH 7, containing different nitrogen sources such as NaNO₃, casein, asparagine, ammonium sulphate, urea and yeast extract were incubated at 55°C for 96 hrs (Shilpa Jani *et al.*, 2012). The enzyme activity was determined by performing the standard assay procedure.

Optimization of carbon source

The effect of different carbon sources on the enzyme activity was determined. Nutrient broth of pH 7, containing different carbon sources such as glucose, starch, sodium citrate, lactose and sucrose, were incubated at 55°C for 96 hrs (Shilpa Jani *et al.*, 2012). The enzyme activity was determined by performing the standard assay procedure.

Partial purification of protease enzyme

Nutrient broth, pH 7 containing CaCl₂, NaNO₃ was inoculated with 24hrs old preculture. The fermented broth was harvested after 96 hrs of incubation at 60°C by centrifugation at 5000 rpm 30 min, 4°C.

Precipitation by ammonium sulfate

Cell-free supernatant was collected after centrifugation. Different concentrations of ammonium sulphate salt from 30% to 90% of saturation were slowly added to the aliquots of supernatant. The mixture was incubated overnight at 4°C. The precipitated proteins were obtained by centrifugation at 5000 rpm for 20 min, 4°C. The resulted pellet was re-suspended in ice-cold 0.05 M phosphate buffer pH 9. The protease activity and protein concentration were determined for pellet and supernatant of each fraction. (Nihan *et al.*, 2011)

Precipitation by acetone

Cell-free supernatant was collected after centrifugation. Acetone in proportion of 1:1 and 1:2, were added to the aliquots of supernatant. The mixture was incubated overnight at 4 °C. The precipitated proteins were obtained by centrifugation at 5000 rpm for 20 min, 4°C. The obtained pellet was re-suspended in ice-cold 0.05 M phosphate buffer, pH9. The protease activity and protein concentration were determined for pellet and supernatant of each fraction. (Ekundayo *et al.*, 2012).

Results and Discussion

Isolation of organisms

The extracellular protease enzyme was synthesized by the isolates, as indicated from the zone of clearance exhibited on skimmed milk agar medium due to casein hydrolysis. Three isolates exhibiting protease activity were selected for further studies (Fig.1).

Morphological and biochemical characterization of isolates

Morphological characterization of colonies showed filamentous colony margin, mycelia growth, and aerial hyphae bearing spores. Morphological characterization showed Gram positive rods, non-motile, gray color spore forming organisms. Based on morphological and biochemical characterization (Table: 1) the isolates were identified as actinomycetes belonging to genus *Saccharomonospora* sp. by using Bergey's manual of determinative bacteriology.

Detection of protease activity of the isolates

Saccharomonospora sp. showed the ability to hydrolyse gelatin and protease enzyme production, as indicated by zone of clearance exhibited by the organism around the colony on the nutrient gelatin agar plates (Fig.2).

Production medium and culture conditions

In quantitative analysis of enzyme, by protease assay, *Saccharomonospora sp.* showed maximum enzyme activity after 96 hrs of incubation (Fig. 3).

Optimization of fermentation parameters for maximum enzyme production

Optimization of fermentation media

Different fermentation parameters were used to obtain the maximum production of protease enzyme by *Saccharomonospora sp.* The effect of different media on protease production was determined. Maximum enzyme production was observed in nutrient broth (4.5mM/ml/min) and minimum production was observed in skimmed milk broth (2.3 mM/ml/min) (Fig.4).

Table.1 Biochemical characterisation of the isolates

Biochemical Test	Observation
Sugar Utilisation Test	
D- arabinose	+
Glucose	+
Ramnose	-
Mannose	-
Mannintol	+
Sucrose	-
Galactose	+
Xylose	+

Note: + Psotive, - Negative

Fig.1 The clear zone showing the ability of the colonies to hydrolyze casein due to protease enzyme production



Fig.2 The clear zone showing the ability of the isolates to hydrolyze gelatin due to protease enzyme production

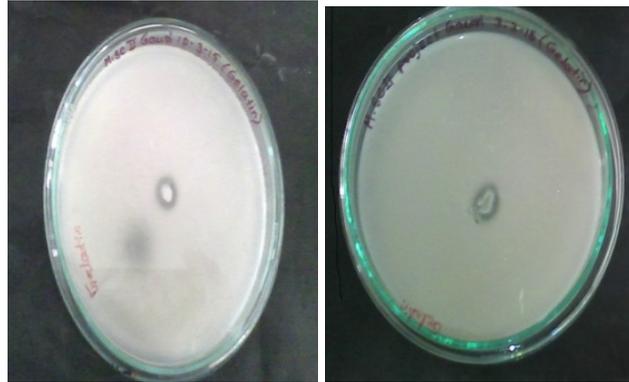


Fig.3 Protease assay of *Saccharomonospora* sp.

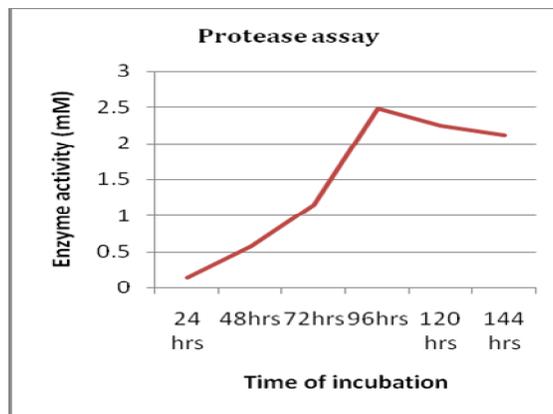


Fig.4 Protease activities in different media

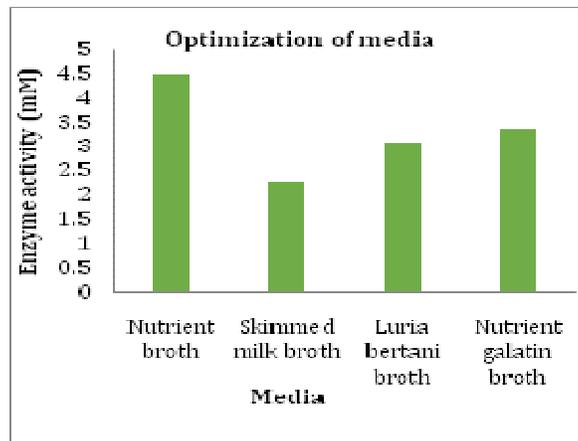


Fig.5 Protease activities in presence of different pH

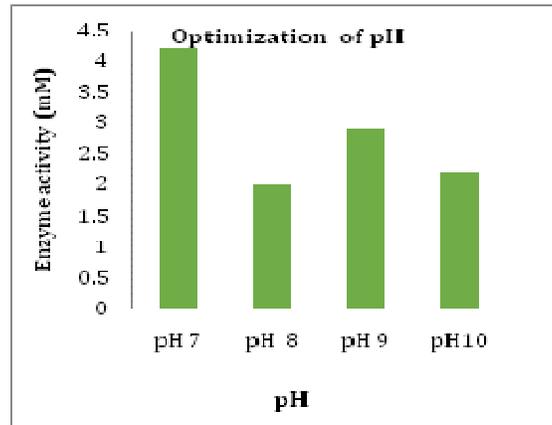


Fig.6 Protease activities at different temperatures

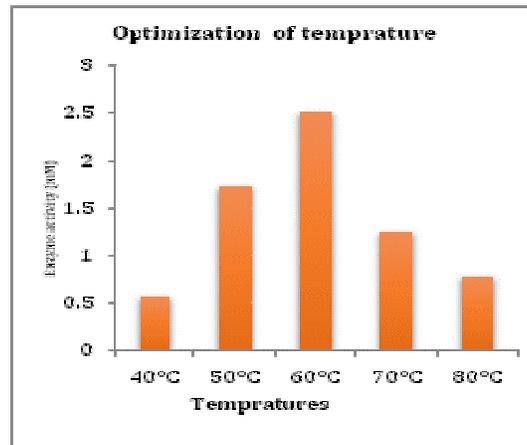


Fig.7 Protease activities in presence of different metal ions

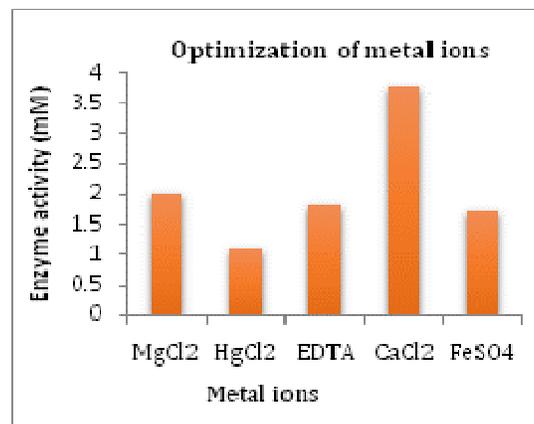


Fig.8 Protease activities in presence of different nitrogen sources

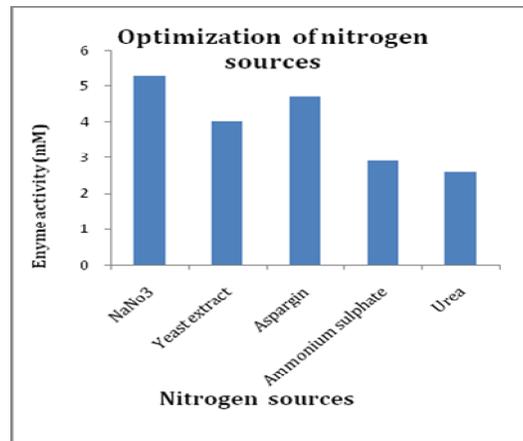
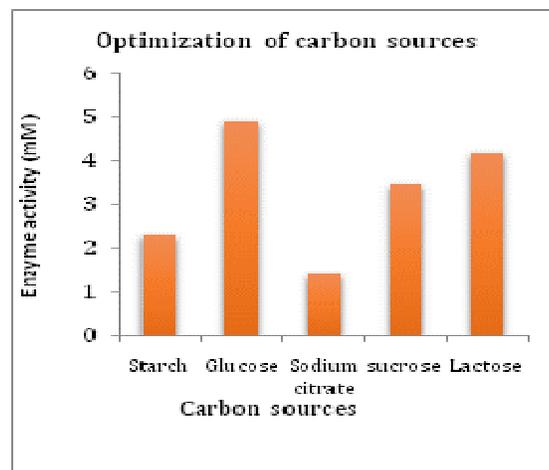


Fig.9 Protease activities in presence of different carbon sources



Optimization of pH

The effect of pH on protease production was determined. Maximum enzyme production was observed at pH 7 (4.2mM/ml/min) and minimum production was observed at pH 8 (2 mM/ml/min) (Fig. 5).

Optimization of temperature

The effect of temperature on protease production was determined. Maximum enzyme production was observed at 60°C (2.5mM/ml/min) and minimum production

was observed at 40°C(0.57mM/ml/min) (Fig. 6).

Optimization of metal ions

The effect of metal ions on protease production was determined. Maximum enzyme production was observed in nutrient broth containing CaCl₂ (3.8mM/ml/min) and minimum production was observed in nutrient broth containing HgCl₂ (1.1mM/ml/min) (Fig. 7).

Optimization of Nitrogen source

The effect of nitrogen sources on protease production was determined. Maximum enzyme production was observed in nutrient broth containing NaNO_3 (5.3mM/ml/min) and minimum production was observed in nutrient broth containing urea (2.6mM/ml/min) (Fig. 8).

Optimization of carbon source

The effect of carbon sources on protease production was determined. Maximum enzyme production was observed in nutrient broth containing glucose (4.9mM/ml/min) and minimum production was observed with in nutrient broth containing sodium citrate (1.4mM/ml/min) (Fig. 9).

Partial purification of protease enzyme

Precipitation by ammonium sulfate

The culture supernatant of *Saccharomonospora sp.* was concentrated by ammonium sulphate precipitation. Ammonium sulphate concentration of 70% totally precipitated protease enzyme protein (3.45 mM/ml/min).

Precipitation by acetone

The culture supernatant of *Saccharomonospora sp.* was concentrated by acetone precipitation. Acetone concentration of 1:2 totally precipitated protease enzyme protein (2.8mM/ml/min).

Thermophilic microorganisms have the adaptability to survive in high temperature environmental conditions.

Many researchers believed that such capability maybe due to their molecular modifications at cellular and sub cellular level. The present study, thermophilic

actinomycete producing protease enzyme was isolated. Identical observations were recorded by in *Streptomyces thermoviolaceus* and *Actinobacillus* (Aliya *et al.*, 2001; Megha *et al.*, 2014)

Time course for the production of protease by *Saccharomonospora sp.* was studied upto 6 days. Maximum protease enzyme production was observed on 4th day of incubation. These results are supported by (Shilpa Jani *et al.*, 2012) who reported the maximum protease enzyme production on 4th day of incubation by using *Saccharomono sporaviridis SJ-21*. The incubation period is directly related to production of enzymes.

Protease production by microbial strains strongly depends on the extra-cellular pH because, culture pH, strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production. (Chinnasamy *et al.* 2011). *Saccharomonospora sp.* showed maximal protease production at pH - 7 (Fig. 5).

Higher enzyme production was found to be at temperature 60°C. Earlier studies have reported that species of *Saccharomonospora viridis SJ-21* produced highest yield of protease when incubated at 55°C and the enzyme production was reduced when the incubation temperature was increased above 55°C (Shilpa Jani *et al.*, 2012).

There are reports showing that different carbon sources, nitrogen sources and metal ions have different influences on extracellular enzyme productin by different organisms. In the present study glucose, NaNO_3 and CaCl_2 showed the maximum protease production (Parawira Wilson and

Zvauya Remigio, 2012; Shilpa Jani *et al.*, 2012).

Studies on production of proteases reveal that media optimization influences the production of the protease enzyme. In this study the organism showed maximum enzyme production in nutrient broth. (Amro A *et al.*, 2009; Wellington *et al.*, 2004; Shilpa Jani *et al.*, 2012).

The purification of proteases is important from the perspective of developing a better understanding of the functioning of the enzyme (Tsai *et al.*, 1988). Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures. It also performs both purification and concentration steps. (Bell *et al.*, 1983). Our data showed that the 70% ammonium sulfate saturation fraction correlated with highest proteolytic activity compared with the crude protease and other fractions. (Nihan Sevinc and Elif Demirkan, 2011). The similar observation was reported for *Aspergillus flavus* (Chinnasamy *et al.*, 2011).

In the present study thermophilic actinomycete *Saccharomonospora sp.* producing extracellular protease enzyme was isolated from compost sample. Optimum fermentation parameters for maximum enzyme production by *Saccharomonospora sp.* were nutrient broth containing glucose as carbon source, NaNO₃ as nitrogen source and metal ion CaCl₂, pH 7 at 60°C. 70% concentration of ammonium sulphate precipitation showed better activity of the enzyme as compared to acetone precipitation.

Acknowledgements

The authors are thankful to P.E.S. Modern College of Arts, Science and Commerce,

Shivajinagar, Pune, affiliated to Savitribai Phule University, Pune, Maharashtra, India for providing the infrastructure facilities for the present study.

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