

Protease Characteristics of a Bacteriocin Producing *Lysinibacilli* (HM359123) Isolated from Nagavali River Basin India

Ashok Kumar Panigrahi¹, Amrita Kumari Panda² and S.P.S. Bisht^{2*}

¹Department of Botany, Berhampur University, Berhampur-760007, Odisha, India.

²Department of Zoology, Kumaun University, Nainital-263002, Uttarakhand, India.

(Received: 11 June 2014; accepted: 30 July 2014)

Ten strains were isolated from first rain soil deposits in and around Nagavali River basin and screened for protease production. One strain producing maximum protease was selected and identified as *Lysinibacillus* sp. by 16S rRNA gene sequencing. In order to enhance the production of protease, the effects of major medium ingredients carbon and nitrogen sources on the production of the enzyme were investigated. Amongst all carbon sources used, fructose showed the highest potential for the production. The best organic nitrogen source observed was skim milk. Inorganic nitrogen sources were not as effective as organic sources. Increased production (51%) of the enzyme was obtained by manipulating the medium composition. The optimum pH and temperature for the purified enzyme activity was 7.0 and 55°C respectively. The study of its stability showed that the enzyme is stable in the alkaline pH range i.e. 6.0-9.0. A single band with a molecular weight of 25 kDa was resolved employing SDS-PAGE analysis.

Key words: Protease, 16S rRNA, river basin, *Lysinibacillus*.

Proteases are the most important class of industrial enzymes occupying a major share (60%) of the total enzyme market (Gupta *et al.* 2005) having wide applicability in detergent, food, pharmaceutical, leather, laundry and food processing industries. These enzymes have been reported for various biological processes i.e. in the dairy industry as milk clotting agent and meat tenderizing agent in food industry (Kumara Swamy 2014) etc. Microorganisms are the preferred source for obtaining proteases because of their fast growth rate, easy manipulation, shorter time for production and purification processes (Rao *et al.* 1998). Although a wide range of microorganisms

such as bacteria, fungi, actinomycetes and yeasts are known for the production of proteases, a large potential of commercially available alkaline protease are derived from gram positive *Bacillus* strains due to their ability to secrete large amounts of alkaline protease with significant proteolytic activity and stability at a high pH and temperature range (Yang *et al.* 2000; Kumar 2002).

Bacillus is highly favorable bacterium for protease production being non-pathogenic and well explored for producing various types of proteases. Among *Bacillus* strains, *B. licheniformis*, *B. subtilis*, *B. acidophilous* and *B. lentus* are important strains exploited at industrial scale (Bhunja *et al.* 2010). *Bacillus* species produce two types of proteases, alkaline and neutral. Many other applications of proteases of another bacterium *Brevibacillus* has been described in detail (Panda *et al.* 2014). The neutral bacterial proteases are active in narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. Due to increased industrial demand for proteases it is

* To whom all correspondence should be addressed.
E-mail: sps.bisht@gmail.com

required to screen and investigate some hyperactive strains those can be commercially exploited as biocatalyst producer in the biotechnology based industries (Ellaiah *et al.* 2002; Prakasham *et al.* 2005. Present investigation reports on the isolation and proteolytic characterization of a hyperactive bacterium from a river basin.

MATERIALS AND METHODS

Site description

Water samples were collected from Nagavali river basin (18° 10' to 19° 44' N, 82° 53' to 84° 05' E), Srikakulam district of Andhra Pradesh in sterile capped collection bottles and transported to laboratory for further analysis.

Isolation of Proteolytic bacteria

Soil samples from 3 different sites of Nagavali River basin were collected, serially diluted and plated on nutrient agar plates. A total of 10 isolates were screened for protease production using casein agar (0.5% casein in nutrient agar) and skim milk agar (1% skim milk in nutrient agar). Proteolytic activities of isolates were detected on the basis of appearance of clear zones around the bacterial colonies. The protease positive colonies were pure cultured and the hyperactive strain was selected for quantitative tests of protease.

Biochemical and Molecular Characterization

The hyperactive isolate was characterized using 16S rRNA sequencing (Bisht *et al.*, 2012), revival of the strain was performed and assayed for the enzyme activity.

Assay of proteolytic activity

Total protease activity was measured using a casein substrate Anson Method (Key and Wildi 1970) with few minor modifications. One ml of the culture supernatant was mixed with one ml 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 ml 0.4 M TCA. After 30 min incubation at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0–60 µg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/mL tyrosine under the experimental conditions.

Determination of pH and temperature optimum

The optimum temperature and pH of protease was determined at 35 to 75°C and pH 3 to 10 respectively. To determine the effect of temperature on protease activity, enzyme and substrate were incubated at various reaction temperatures before the start up of the experiment and the enzyme assay was performed to determine the optimal temperature by spectrophotometry. The optimal pH was determined by incubating the enzyme-substrate at pH 3-10 and assayed for protease activity.

Effect of nutrients on enzyme production

The effect of some nutrients such as carbon and nitrogen sources on enzyme production was investigated. Glucose (0.1% w/v) was replaced in the production medium with maltose, sucrose, fructose and starch. Different organic and inorganic nitrogen sources including corn steep liquor, skim milk, tryptone, soyabean, casein, (NH₄)₂SO₄ and KNO₃ were tested. These nitrogen sources were used to replace peptone and yeast extract those were the original nitrogen source in growth medium. Incubation periods set as 0,4,8,12,16, 20, 24, 28, 32, 36, 40, 44, 48h. A new medium including the best source of carbon and nitrogen for protease production was achieved and bacterium was grown in this modified medium.

Partial purification of crude protease

The crude protease extract was partially purified by ammonium sulphate (80%) precipitation followed by dialysis. The precipitate was collected by centrifugation at 12,500 rpm for 20 min at 4 °C and dissolved in 0.01 M phosphat buffer (pH 7.0). The solution was dialyzed against the same buffer at 4 °C for 8 h with 3 changes of the dialysis buffer and protein concentration was measured by Lowry *et al.* (1951) using BSA as standard protein. The approximate molecular weight of partially purified enzyme was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970).

Effect of organic solvent

Protease enzyme was separately mixed with 50% v/v each of methanol, ethanol, formaldehyde, chloroform, isopropyl alcohol, n-butanol, diethylether and acetone for 2h at 4°C before adding the required substrate for incubation. The enzyme activity was determined in each case.

Effect of detergent

The enzyme assays were performed in the presence of various domestic detergents (1% w/v) like Ariel, Fena, Surf Excel, Rin Shakti, and Tide. The activity of the enzymes in the presence of these detergents was checked.

Effect of EDTA, PMSF and SDS

The enzyme substrate reactions were separately performed in the presence of EDTA (5mM), PMSF (5 mM) and SDS (1 %) in the reaction mixture. The activity of the enzymes was checked in each case.

RESULTS AND DISCUSSION

Ten isolates grown in the skim milk agar medium were found to produce protease and their enzyme producing efficiency was evaluated based on the clear zone around the colony. The result of isolate 2 – whose protease production was outstanding identified as a member of

Table 1. Effect of various organic solvent on protease activity

Solvent	Residual protease activity (Mean %)
Control	100
Methanol	50.66±1.12
Ethanol	70.41±1.72
Chloroform	66.42±2.30
n-Butanol	48.44±0.58
Acetone	62.30 ± 1.46
Diethyl ether	44.34 ± 1.72

Table 2. Effect of various detergents on protease activity

Detergents	Residual protease activity (Mean %)
Control	100
Surf excel	85.36±1.15
Rin shakti	77.23±0.68
Ariel	71.45±1.30

Table 3. Effect of various inhibitors on protease activity

Inhibitors	Residual protease activity (Mean %)
Control	100
EDTA	53.72±3.46
SDS	69.22±1.10
PMSF	22.10±0.80

Lysinibacillus genus. This strain was also reported to have significant inhibitory effect on various pathogenic test strains i.e. *E.coli* (MTCC 40), *Staphylococcus aureus* (MTCC87), *Proteus vulgaris* (MTCC426) and *Pseudomonas aeruginosa* (MTCC 424) (Bisht *et al.* 2012). The correlation between cell growth and protease production was investigated; it was observed that the enzyme production was maximum during 24-36 hr of incubation (Fig. 1).

Initial pH of the culture broth is one of the most critical environmental parameters affecting both growth and protease production. The results show that *Lysinibacillus* have shown maximum protease activity of 27 U/ml at temperature 60°C (Fig. 2) and pH 7 (Fig. 3). A decrease in the proteolytic activity was observed at above 65°C.

In order to investigate the effect of organic solvents on protease activity, methanol, ethanol, chloroform, n-butanol, acetone and diethyl ether were used at a concentration of 30% (v/v). The enzyme retained about 66% and 70% of its activity in the presence of chloroform and ethanol respectively (Table 1). The enzyme retained 85% activity in the presence of commercially available detergent Surf excel and around 70% activity in the presence of Rin Shakti (Table 2) and inhibitor SDS (Table 3).

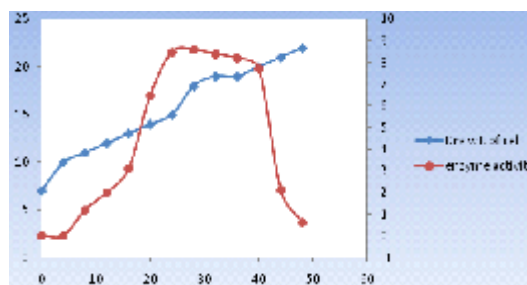


Fig. 1 Growth curve and protease production by *Lysinibacillus* sp.

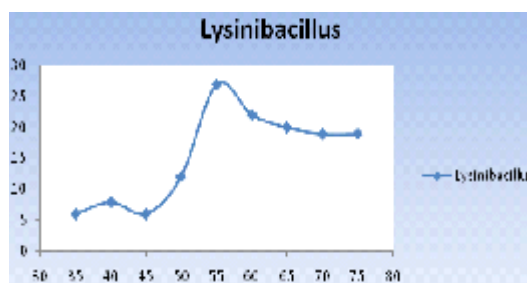


Fig. 2 Effect of temperature on protease activity

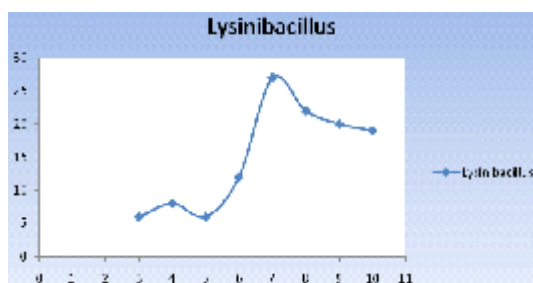


Fig. 3 Effect of pH on protease activity

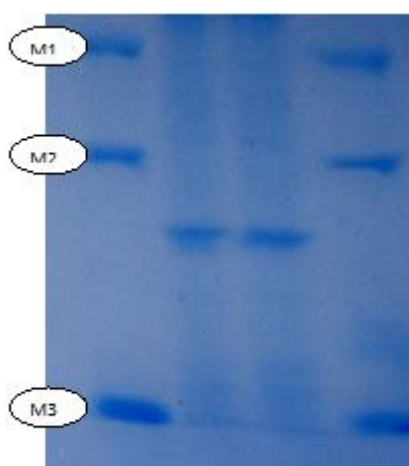


Fig. 4 SDS-PAGE electrophoregram showing the partially purified protease (Lane 1 & 4 Molecular Marker M1- Ovalbumin (43 kD), M2- carbonic anhydrase (29 kD), M3-Soyabean trypsin inhibitor (20.1 kD), Lane 2 & 3 Partially purified protease (25 kD)

Partial purification and SDS-PAGE analysis

In the present study, partial purification of protease enzyme was performed by ammonium sulphate precipitation followed by dialysis. Partially purified protease with a molecular weight of ~25 kDa was resolved in SDS-PAGE (Fig. 4). The present findings substantiated by the investigations made by Abou-Elela *et al.* (2011) in *Bacillus cereus* where they reported a protease of nearly 31.0 kDa and Usharani and Muthuraj (2010) with a protease of 15 kDa in *Bacillus laterosporus*.

Effect of nutrients

Various carbon, nitrogen sources and metal ions were used for the production of protease by *Lysinibacillus* sp. When glucose in basal medium was replaced by four various sugars (maltose, sucrose, fructose, starch), fructose was observed as the best source for protease production (Fig. 5). Fructose (130 U/ml) increased the production of protease by 30% when compared to control (glucose, 100 U/ml). The protease production was affected by carbon sources in the following order: fructose > sucrose > maltose > control > starch.

Starch, sucrose, and lactose proved appreciably good for the protease production by *Bacillus* sp. K-30 (Naidu and Devi 2005). Alkaline protease production was best in wheat bran, starch, glucose and dextrin by *Bacillus licheniformis* and *Bacillus coagulans* (Asokan and Jayanthi 2010). Madzak *et al.* (2000) recorded that the sucrose is good substrate for production extracellular proteases. Glucose was found to be the optimum

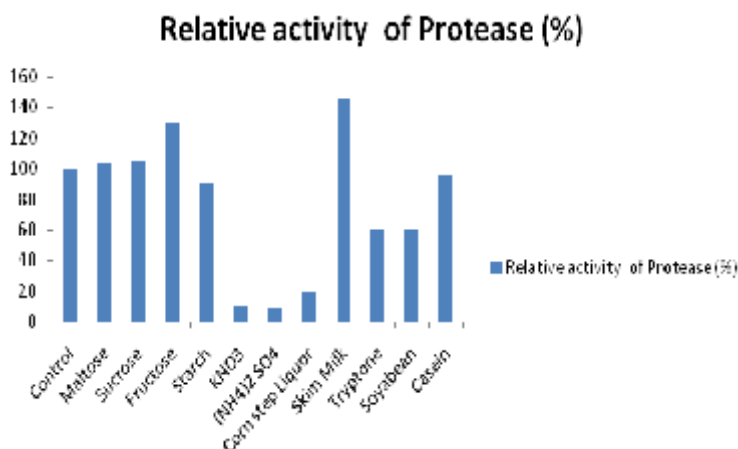


Fig. 5 Protease production in various carbon and nitrogen sources

carbon source for protease activity by all the four *Bacillus* isolates followed by sucrose, fructose, maltose, starch and cellulose (Boominadhan *et al.* 2009). However, other works reported that glucose drastically inhibited protease production (Puri *et al.* 2002). Nitrogen sources also affected enzyme production. Effects of various organic and inorganic nitrogen sources on production of protease were investigated. During the present investigation it was observed that the best nitrogen source for protease production is skim milk (145 U/ml) at 28 h and enzyme yield was 45% compare to control (100 U/ml). Inorganic sources were found to have no significant effect on protease production.

It has been reported that peptone, casein, skim milk, yeast extract, favored maximum protease production by *Bacillus* sp (Puri *et al.* 2002). Among the organic nitrogen sources, the effects of soybean flour, fish peptone, beef peptone and polypeptone were almost the same for *B. pumilus* c172-14 (Feng *et al.* 2001). Among the various organic and inorganic nitrogen sources, the maximum enzyme activity was obtained with ammonium nitrate followed by ammonium chloride, ammonium citrate and potassium nitrate were used as nitrogen sources (Nascimento and Martins 2004).

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