

Original Research Article

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Isolation and Characterization of Protease Producing *Bacillus* Species from Soil of Dairy Industry

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ABSTRACT

Proteases have a long history of application in food and detergent industries. The attempt of this study was to isolate the protease enzyme producing *Bacillus* species from various dairy industries. In the present study 25 soil samples were collected from various dairy industry and proceed for isolation and identification of protease producing bacteria specially *Bacillus* spp. In the present study a total of 28 *Bacillus* species were isolated which includes *Bacillus subtilis* was predominantly found which were 8 (28%) followed by *Bacillus megaterium* 7 (25%), *Bacillus cereus* 5 (18%), *Bacillus licheniformis* 5 (18%) and *Bacillus brevis* 3 (11%). Among the 28 *Bacillus* species 3 isolates such as SP3 (*Bacillus cereus*), SP10 (*Bacillus licheniformis*) and SP21 (*Bacillus megaterium*) showed significant protease enzyme production after 24h. SP3 showed excellent protease production after 72h followed by SP10 and SP21 which was 35mm, 26mm and 18mm respectively. The protease enzyme from *Bacillus* SP3 was partially purified by ammonium sulfate fractionation. The specific activity of crude protease enzyme was 7.71U/mg of protein. The specific activity of ammonium sulfate fractionation is found to be 10.32 and the fractionation is 1.32 fold purified from the crude enzyme preparation yielding 75.75% from the crude protein. The optimum pH of protease produced from *Bacillus cereus* (SP3) shown at the pH 7.0. The purified enzyme was loaded onto holes punched on skim milk agar. After 24-48 hours of incubation it was observed that clear zones were formed successfully. It can be produced in large scale for the potential industrial applications.

Keywords

Protease enzyme, *Bacillus* species, Dairy industry, Soil.

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Introduction

Microbial enzymes are often more useful than enzymes derived from plants or animals because of are more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1987). Only about 2% of the world's microorganisms have been tested as enzyme sources. Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins (Raju *et al.*, 1994; Haq *et al.*, 2006).

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta *et al.*, 2004). Of these, strains of *Bacillus* sp. dominate the industrial

sector (Gupta *et al.*, 2002). Several species of strains bacteria (*Bacillus licheniformis*, *B. firmus*, *B. alcalophilus*, *B. amyloliquefaciens*, *B. proteolyticus*, *B. subtilis*, *B. thuringiensis*, *B. cereus*, *B. stercorophilus*, *B. mojavensis* and *B. megaterium*) are reported to produce proteases (Beg *et al.*, 2003, Banik *et al.*, 2004, Gerze *et al.*, 2005, Soares *et al.*, 2005). Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale (Brown and Yada, 1991 and Escobar and Barnett, 1993).

The major uses of free proteases occur in the food, dairy, pharmaceutical, textile industries dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds and are produced in large amounts by microbial synthesis (Nout and Rombouts, 1990; Aleksieva and Peeva, 2000). Protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 and 10 (Ishikawa *et al.*, 1993). Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic deriders (Anwar *et al.*, 2000). The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material (Sjodahl *et al.*, 2002). Protease is also used for the deproteinization of marine crustacean wastes. Approximately 60% of the enzymes used commercially are proteases (Badgajar and Mahajan, 2010).

Proteases are broadly classified as endo or exoenzymes on the basis of their site of action on protein substrates. They are further

categorized as serine protease, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism (Geethanjali and Subash, 2011). Alkaline serine proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Salem *et al.*, 2009).

The demand for industrial enzymes, particularly microbial origin, is ever increasing owing to their applications in a wide variety of processes (Padmapriya *et al.*, 2012).

Materials and Methods

Collection of samples

For the isolation of the protease producing bacteria a total of twenty five soil samples were collected from different dairy industries of Aurangabad city (MS) India. The soil samples were collected in sterile plastic bags and immediately carried to the research centre, Aurangabad, (MS) India for further studies.

Isolation of the microorganisms

The primary screening was done by the skimmed agar plate method. The soil sample (1gm) was diluted in 100 ml of sterilized distilled water and heated at 80°C for 15 minutes. The skimmed milk agar plate was inoculated by the spread plate method with 0.1 ml of heated diluted soil sample and incubated aerobically at 37°C. Heat treatment to the dilution was killed all the vegetative cells and remain only spores of *Bacillus* species. Strains that were capable of producing protease enzymes were screened by

allowing them to grow for 24 hr on skimmed milk agar plate at 37°C. The plates were observed for zone of hydrolysis for protease production and were chosen for further investigation.

Identification of enzyme producers

Protease producer was identified on the skimmed milk agar by observing the zone of hydrolysis around the colony or growth. After identification of enzyme producer each colony was then isolated by streaking on the slants of nutrient agar. Then these slants were incubated at 37°C for 24–48 hrs and further proceed for identification of strains. Identification of bacterial strains was done on the basis of standard morphological, biochemical, and sugar fermentation test.

Production and extraction of protease enzyme

A 500 ml of Skimmed milk broth was prepared according to the composition in the 1000 ml of conical flask. All components were dissolved in the distilled water. The conical flasks containing medium were autoclaved at 121°C for 15 min at 15 LBS pressure. After the isolation and screening of protease producing microorganism, efficient protease producer microorganism was inoculated in 500 ml of three broths and it was then incubated in shaking water bath at 37° C for 72 hrs. It was observed daily for the growth in the flasks up to complete incubation period. After 3 days of incubation, content of flasks was centrifuged at 10000 rpm for 10 mins at 4°C. A clear supernatant was recovered after centrifugation. The crude enzyme supernatant was tested for the protein determination by Biuret test, protease assay (i.e. Folin-Lowry method). This isolated crude enzyme was processed for its purification by salting-out method using $(\text{NH}_4)_2\text{SO}_4$, an ammonium sulfate a neutral salt.

Partial purification by salting-out method

First of all, the volume of 3 crude enzymes were measured and then it was treated with 50% $(\text{NH}_4)_2\text{SO}_4$. The salt was slowly added to the volume of crude enzymes with continuous stirring, after completing the addition, the enzyme was kept at 4°C overnight precipitation. Next day, the enzyme was brought to room temperature and then proceeds for the centrifugation to separate the protein precipitated from the rest of the liquid. The precipitate was containing fractionated enzyme (protease) and supernatant contained other soluble proteins. Then both precipitate and supernatant were assayed for the enzyme activity by using protease assay. The supernatant was showing enzyme activity, thus it proceeded for further precipitation with the same salt with high concentration as 60%.

The process was continued till supernatant would not show any enzyme activity. Thus it was precipitated till 90% of $(\text{NH}_4)_2\text{SO}_4$ salt concentration. The protein/enzyme was resuspended in 0.1M Phosphate buffer, pH 7.0, and dialyzed against the same buffer.

Biuret test

Standard protein solution (BSA) was pipetted out into a series of tubes – 0.1, 0.2,..., 1 mL from stock solution of 100 mg/ml. 3 ml of biuret reagent was added in all different concentration tubes. These tubes were incubated at 37°C for 10 minutes.

Then 2 ml of distilled water was added to each tube except in blank to make up the volume 6 ml. The optical density for each tube was taken at 530 nm on UV-VIS Spectrophotometer. Then graph was drawn of Concentration of Standard protein against the respective O.D. at 530 nm. Same procedure was followed for the protein estimation of unknown sample.

Protease assay

One ml of enzyme was added in a tube containing 1 ml of buffer and 1 ml of substrate. A 5 reaction mixture were prepared with different pH buffer (7, 8, 9, 10, 11). Above reaction mixtures was incubated at 37°C for 20 minutes. After 20 min of incubation reaction was terminated by adding 1 ml of TCA in it. Blank was prepared by using distilled water in place of the casein substrate. Above terminated reaction mixture was filtered using filter paper. Filtrates prepared at different pH were tested for the Folin Lowry method of protein estimation. Highest enzyme activity was observed at optimized pH and noted down.

Folin Lowry method

Standard protein solution (BSA) was pipetted out into a series of tubes – 0.1, 0.2,..., 1 mL from stock solution of 200 mg/ml. A 5 ml of alkaline solution was added in each tube and allowed to stand for 10 minutes at room temperature. 0.5 ml of diluted F-C reagent was added with immediate mixing. Above reaction mixture was kept at room temperature for 30 mins. After 30 minutes, O.D. was taken at 750 nm on UV-VIS Spectrophotometer. Then graph was drawn of Concentration of Standard protein against the respective O.D. at 750 nm. Same procedure was followed for the protein estimation of unknown sample.

Isolated efficient protease producer identified by sequenced

The morphological characteristics of the isolates were identified by Gram staining and biochemical reactions (Balow *et al.*, 1992). The biochemical reactions included glucose fermentation, catalase and oxidase production. Confirmation was done by 16S rRNA sequencing from NCCS, University of

Pune. These isolates were initially identified by Ribosomal Database Project (RDP). Sequences obtained through RDP were subjected to Basic Local Alignment Search Tool (BLAST) to obtain significant relationships from chance similarities.

Results and Discussion

In the present study, a total of 25 soil samples were collected from dairy industry in sterilized polythene bag. From the 25 soil samples, 28 *Bacillus* species were isolated and identified on the basis of morphological and biochemical characteristics as well as screened for protease enzyme production activity. All the isolates were found gram positive rods, motile and spore forming. Isolates were produced hydrolytic enzymes such as gelatinase, catalase and amylase. They fermented sugars without production of gases and reduced nitrates to nitrites. Isolates SP1, SP5, SP9 SP11, SP16, SP20, SP25 and SP28 were identified as *Bacillus subtilis* whereas isolates SP3, SP6, SP8, SP18 and SP22 were identified as *Bacillus cereus*. Isolates SP2, SP12, SP14, SP17, SP21, SP23 and SP27 were identified as *Bacillus megaterium* whereas SP4, SP24 and SP26 were identified as *Bacillus brevis* and isolates SP7, SP10, SP13, SP15 and SP19 were identified as *Bacillus licheniformis*. Few moderate alkaliphilic strains especially representatives of the genus *Bacillus*, are able to produce extracellular amylase, lipase and proteases that are even active at alkaline pH (Martins *et al.*, 2001; Vargas *et al.*, 2004; Tambekar and Tambekar, 2012). A large number of alkaliphilic *Bacillus* strains have been isolated for industrial applications (Horikoshi, 1971).

From the (Fig. 1) it was observed that among the 28 *Bacillus* species, *Bacillus subtilis* was predominantly found which were 8 (28%) followed by *Bacillus megaterium* 7 (25%),

Bacillus cereus 5 (18%), *Bacillus licheniformis* 5 (18%) and *Bacillus brevis* 3 (11%). After the isolation and identification, isolated *Bacillus* species were used for screening of protease enzyme. Among the 28 *Bacillus* species 3 isolates such as SP3 (*Bacillus cereus*), SP10 (*Bacillus licheniformis*) and SP21 (*Bacillus megaterium*) showed significant protease enzyme production after 24h, hence these

isolates were incubated different time intervals (24h, 48h and 72h) to observe the maximum protease production. From the (Fig. 2), SP3 showed 15mm, SP10 showed 18mm and SP21 showed 9mm of casein hydrolysis after 24h while after 48h they showed 30mm, 22mm and 14mm respectively. SP3 showed significant protease production after 72h followed by SP10 and SP21 which was 35mm, 26mm and 18mm respectively.




Protease production activity of isolate <i>Bacillus cereus</i> SP3 after 24, 48 and 72 h. respectively	
	
Casein hydrolysis after 24 hour incubation	Casein hydrolysis after 48 hour incubation
	
Casein hydrolysis after 72 hour incubation	

Fig.1 Isolated *Bacillus* species from soil of dairy industry

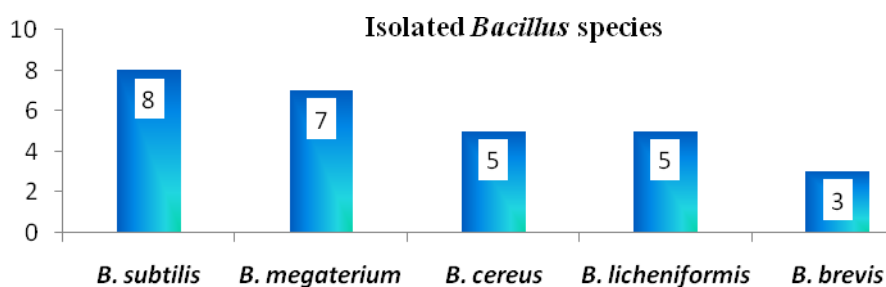
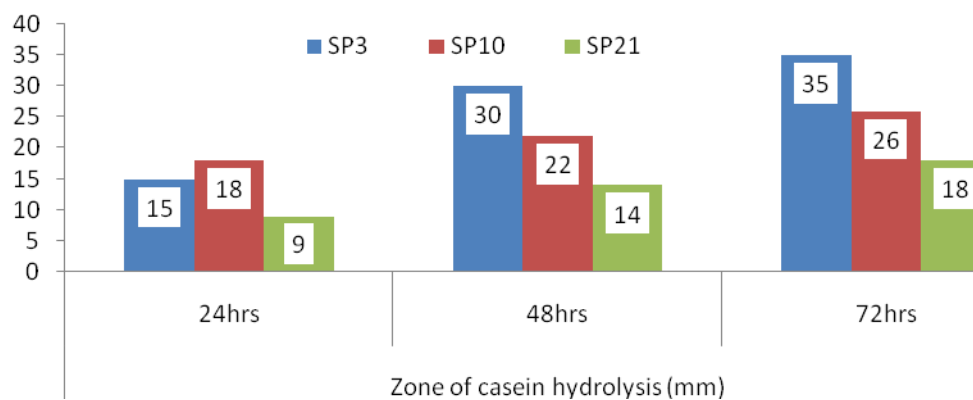


Fig.2 Zone of casein hydrolysis (mm) of isolated *Bacillus* species from soil of dairy industry after 24h, 48h and 72h



Purification Step	Total Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	% Recovery
Crude enzyme	30210	3920	7.71	1.0	100
(NH ₄) ₂ SO ₄ precipitation, dialyzed	15482	1500	10.32	1.32	75.75

After the isolation, identification of isolates and screening of protease production, the

significant protease producer isolates were used for sequencing. The isolates which have

protease enzyme producing activity were selected for confirm identification. These isolates were initially identified by Ribosomal Database Project (RDP). By sequencing these isolates were confirmed as *Bacillus* species and excellent protease producer strain *Bacillus cereus* (SP3) was used for extraction and partial purification purpose.

The isolated source of *Bacillus cereus* (SP3) has maximum protease enzyme production was observed at 72h. The protease enzyme from *Bacillus* SP3 was partially purified by ammonium sulphate fractionation. One liter of the bacterial broth was centrifuged at 10000 rpm for 10 min at 4°C. The specific activity of crude protease enzyme was 7.71U/mg of protein. The specific activity of ammonium sulphate fractionation is found to be 10.32 and the fractionation is 1.32 fold purified from the crude enzyme preparation yielding 75.75% from the crude protein.

The optimum pH of protease produced from *Bacillus cereus* (SP3) shown at the pH 7.0. The purified enzyme was loaded onto holes punched on skim milk agar. After 24-48 hours of incubation it was observed that clear zones were formed successfully. Other investigators, reported that both *Bacillus anthracis*, S-44 and *Bacillus cereus* var. *mycoides*, S-98 exhibited their maximum ability to biosynthesize proteases within 24 h incubation period since the productivity reached up to 126.09 units/ml for *Bacillus anthracis*, S-44 corresponding to 240.45 units/ml for *Bacillus cereus* var. *mycoides*, S-98 respectively. Moreover, Johnvesly *et al.*, (2002) found that a high level of extracellular thermostable protease activity was observed after 24 h incubation and hence our results are in complete accordance with earlier reports. Other investigators recorded optimum proteolytic activity at different pH values such as pH 7.8 (Tsujiibo *et al.*, 1990), 8.0 (Anwar and Saleemuddin, 1997), 9.8-10.2

(Kumar *et al.*, 1999) and 12-13 (Takami *et al.*, 1989).

In conclusion, the isolated new source of protease producing bacteria from the soil sample of dairy industry and partially purified protease may be alternative source and also used for the potential industrial applications. Although many potent strains are on market for enzyme production, scientists prefer studying on new isolates because they could be alternative for commercial use. This is very cheap source to enhance the Protease production and recommended that isolated potential protease producer can be used in various industries.

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