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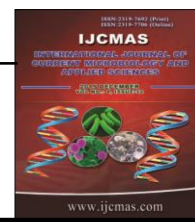

Original Research Article

Isolation and screening of *Bacillus subtilis* isolated from the dairy effluent for the production of protease

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Keywords

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A B S T R A C T

Proteases are a group of enzymes, found in all living organisms and are essential for the cell growth and differentiation. The proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries. Their catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. The present study was aimed at isolating *Bacillus subtilis* from a dairy effluent obtained from a local retailer. *Bacillus subtilis* was isolated using the serial dilution method and the identification of bacterial population was done using morphological examination and biochemical characterization. The culture was maintained in two different media that were prepared from rice bran and groundnut cake. The study of the factors namely pH and temperature, that influence protease activity was also done. Results show that maximum activity could be obtained in rice bran medium and shows that the rice bran medium to be the optimal medium for the growth of *Bacillus subtilis* isolated from the dairy effluent for the production of protease. The optimal conditions for maximal activity was found to be at a pH of 7 and at a temperature of 35°C.From the current study it is evident that the protease produced byisolated *Bacillus* species was a neutral protease that are of great importance in detergent industry due to their high thermostability and pH stability

Introduction

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59%of the global market of industrial enzymes, (Deng *et al.,* 2010).They has got a wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.,*2007).

Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base

behavior, proteases are classified into three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0 to 5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases.

Proteases occur naturally in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade). Proteases can either break specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). Bacterial and fungal proteases are particularly important to the global carbon and nitrogen cycles in the recycling of proteins, and such activity tends to be regulated in by nutritional signals in these organisms.

Proteases produced from microorganisms play an important role in several industries for example in detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Gupta *etal.,* 2002). Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kocher and Mishra, 2009)

The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Nout and Rombouts, 1990).Proteases are part of many laundry detergent. The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase by the year 2005 (Gupta *et al*., 2002).

The present investigation is aimed to isolate newer sources of extracellular protease from the dairy effluent collected from a Dairy Industry located at Madavaram, Chennai, Tamilnadu, India.

Materials and methods

Samples of dairy effluent were collected in sterile containers for the isolation of Bacillus sp, from a Dairy Industry located at Madavaram, Chennai, Tamilnadu, India.

Isolation of Bacteria: Bacteria were isolated for protease enzyme using a serial dilution method described by Sjodahl *et al.,* (2002). Samples were inoculated on skim milk agar plates containing peptone (0.1%), sodium chloride (0.5%) and skim milk (10%) medium prepared using saline, then incubated at 28 ± 2°C for three days (Uyar *et al.,*2011).Bacterial isolates were primarily purified on nutrient agar medium and routinely maintained at 4^0C on culture. Purity was determined from colony morphology.

Screening for the strain producing protease:

The isolates were screened for a good strain producing protease by plate assay using protease specific medium containing (g/L) of dipotassium hydrogen phosphate2.0, glucose 1.0, peptone 5.0, gelatin 15.0 and agars 15.The clear zone diameters were

measured after 24hours of incubation at28°C by flooding the plates with mercuric chloride solution, this method is referred to as gelatin clear zone method (Abdel Galil, 1992).

Identification of isolated bacteria

The isolated bacteria with strong productivity for protease was purified according to the procedure described by Peciulytė (2007).Finally, the isolated bacterial strain produced was identified by means of morphological examination, cultural studies and biochemical characterization according to the method of Buchanon and Gibbons (1974).

Assay of activity of protease in the isolated *Bacillus* sp

The isolated strains were inoculated in 50 ml of protease specific medium broth containing (g/L) glucose, 5.0; peptone, 7.5; (magnesium sulphate.7H_2O, 5.0; potassium dihydrogen phosphate, 5.0; and ferrous sulphate.7H_2O, 0.1, pH-7.0 and were culture in a rotary shaker (180 rpm) at 28°C for 3 days. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies (Josephine *et al.,*2012).Protease activity in the culture supernatant was determined according to the method of Tsuchida *et al.,* (1986) by using casein as a substrate. A mixture of 500µl of 1% (w/v) of casein in 50mM phosphate buffer, and 200µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 200 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the unreacted casein at 10,000rpm for 5 minutes. The supernatant was mixed with 2g sodium carbonate. 8.5 ml of diluted Folin Ciocalteu's phenol reagent, was added. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue colour developed was measured at 660 nm against a reagent blank using a tyrosine standard (Lowry *et al.,*1951). A standard graph was plotted using the concentration of tyrosine on x-axis and absorbance values on y-axis. Proteolytic activity was determined and is represented in terms of Units/ml of enzyme. One unit of protease is defined as the amount of enzyme that releases 1µg of tyrosine per ml per minute under the standard conditions of supernatant solution.

Proteolytic activity is represented in terms of U/ml enzyme and is derived by,

$$\frac{\mu\text{mole tyrosine equivalents released} \times \text{total volume(ml) of assay}}{\text{Volume of enzyme (ml)} \times \text{time of assay (min)} \times \text{volume(ml) used in colorimeter(ml)}}$$

One unit (Anson *et al*., 1938) of enzyme will hydrolyze casein to produce colour equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 8.0 at 37˚C with Folin and Ciocalteau's reagent. The µmoles of tyrosine equivalents liberated were calibrated using the standard curve. After the evaluation, the protease activity was determined using the above formula.

Protein Assay

Protein was measured by the method of Lowry *et al.,*1951 with bovine serum albumin as the standard. The concentration of protein studies was calculated from the absorbance at 660nm.

Study of optimisation of conditions for protease activity: The factors namely pH,

temperature were studied to obtain the optimal condition for the activity of protease. The specific activity of the protease enzyme protein was expressed in terms of units/mg protein/ml according to the following equation:-

Specific activity = protease activity / total protein content (mg/ml)

pH: Erlenmeyer flasks containing 50 ml quantities of modified standard broth medium containing the optimum casein concentration of pH 6.0, 6.5, 7.5, 8.0 respectively in different flasks were sterilized. The bacterial culture was inoculated and incubated at 30ºC for one day. At the end of incubation period the cell free culture filtrate was obtained and used as the enzyme source for studying the optimum pH at which maximum activity is shown by the protease isolated from the dairy effluent used in the present investigation.

Temperature: Fifty millilitre quantities of modified standard broth medium were dispensed in 100ml Erlenmeyer flasks. The flasks were sterilized, inoculated and incubated at different temperature namely, 25ºC, 35ºC, 45ºCfor one day. At the end of the inoculation period, the cell free culture filtrate was obtained and used for finding out the maximum activity of protease enzyme isolated from the dairy effluent.

Results and Discussion

Screening and isolation of proteolytic bacteria

Bacterial strain producing protease enzymes were isolated from a local dairy effluent isolated from Madhavaram, Chennai. Among them, culture grown in rice bran medium showed the highest proteolytic enzymes productivity. The potent *bacteria was* identified based on morphological characterizations using Bergeys manual, (Buchanan RE *et al*., 1974). The results showed that the strain is a Gram-positive, rod shaped, spore-forming bacterium and identified to be a strain of *Bacillus* (Kim *et al.,* 1998).

Fig.1 Media used for the growth of *B.subtilis*

Figure 1 shows the freshly prepared different kinds of media namely nutrient broth, rice bran and groundnut cake that were used for studying the medium suitablity for the optimal growth of the isolated *Bacillus subtilis* to be used for the study of protease in the present study.

Results as shown in figure 2 shows that the activity of protease was maximum in that grown in the rice bran medium.

Fig.2 Media used for the growth of *B.subtilis* media shown after inoculation

The isolates were also screened for an optimum medium for producing protease by plate assay using protease specific medium containing the bacterial inoculums that was

isolated in the present study. The proteolytic activities of *B.subtilis* was found using gelatin agar and exhibited as a specific diameter of clear zone.

Study of the biochemical characteristics of the isolated *B.subtilis* strain

Results of Biochemical tests for the identification of the Bacillus species isolated from the dairy effluent collected from the study area are shown in table 2.

Table.3 shows that the results obtained for the activity of protease grown in various pH values in the rice bran medium and that isolated from the dairy effluent collected from the study area.

Table.1 Study of the activity of protease grown in three different media

S.No	Sample	Zone(mm)
1.	Nutrient broth	29
2.	Rice Bran	30
3.	Groundnut cake	27

Fig.3 Zone of inhibition by the *B.subtilis* isolate from the dairy effluent grown three different media rice bran, groundnut cake and nutrient broth

Table.2 Biochemical tests for *Bacillus subtilis.*

S.No	Biochemical tests	Result
1.	Catalase test	positive
2.	Oxidase test	positive
3.	Urease test	negative
4.	Indole test	positive
5.	Methyl red test	negative
6.	VogesProskauer test	negative
8.	Nitrate Reduction Test	negative

Table.3 Study of the activity of protease grown in various pH values in Rice Bran medium

S.No	pH	Specific Activity (U/ ml/ min)
1.	6.0	0
2.	6.5	0.1091
3.	7.0	0.4125
4.	7.5	0.3677
5.	8.0	0

Fig.4 Study of the activity of protease grown in various pH values in Rice Bran medium

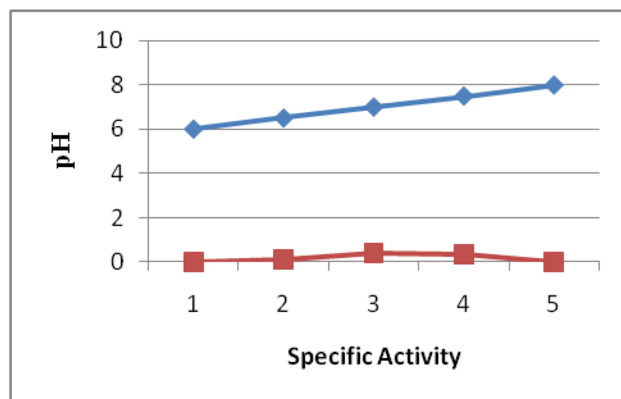
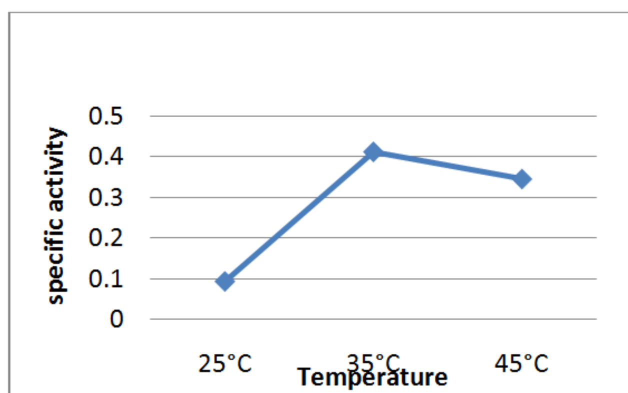


Table.4 Study of the activity of protease grown in different temperature in Rice Bran medium

S.No	Temperature	Specific Activity (U/ ml/ min)
1.	25^0C	0.0948
2.	35^0C	0.4128
3.	45^0C	0.3460

Fig.5 Study of the activity of protease grown in different Temperature in Rice Bran medium



Results of the study shows that maximum activity of protease was obtained at pH 7.0. The protease synthesized by the isolated Bacillus sp is found to be a neutral class of protease.

Figure 4 shows maximal activity for protease at p H 7, that shows that the secreted enzyme is a neutral protease. Results of the study shows that maximum activity of protease was obtained at a temperature of 35°C. Figure 5 shows the results of the temperature study for protease.

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. Extracellular enzymes such as proteases are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch, and the digested products are transported into the cell where they are used as nutrients for growth (Gibb *et al*, 1987).

In the present study the morphological examination and the biochemical tests for the bacterial strain had shown the presence of *Bacillus subtilis* in the effluent used for the study. The study on the optimization of the medium for the growth of *Bacillus subtilis* showed that rice bran, a rich source of carbon and nitrogen is the cheapest and most suited medium among the tested three different media used in the present study.

Results of the study of optimisation of the conditions for protease activity shows that maximum activity of protease was obtained at pH 7.0 and at the temperature 35°C.The protease synthesized by the isolated Bacillus sp is found to be a neutral class of protease.

Of all proteases, alkaline proteases produced by *Bacillus* species are of great importance in the detergent industry due to their high thermostability and pH stability.

In conclusion, the present investigation shows that *Bacillus subtilis* may be isolated from the dairy effluent collected from Madhavaram area, Chennai. The isolated Bacillus species can be grown in rice bran medium, that is a by product of the milling of rice. It is a cheap source of carbon, nitrogen and protein and is suitable for the growth of *Bacillus subtilis* isolated in the current study. Results also show that the optimum pH of 7 and an optimum temperature of 35°C are the conditions that may be used for obtaining maximal activity for protease enzyme, that is synthesised in the isolated Bacillus sp, isolated from the study area. The acid base behaviour of the protease is found to be a neutral protease and hence may be employed in detergent industries.

References

Abdel Galil O A. 1992.Fermation of proteases by *Aspergillus fumigates* and *pencillium* sp. J.king. Saud. Univ.; 4 (2):127-136

Bhaskar N, Sudeepa ES, Rashmi HN and Selvi AT. 2007. Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour. Technol. 98: 2758-2764. 25: (3) 469-477.

Buchanan RE and Gibbons NE.1974. Bergey's Manual of Determinative Bacteriology, 8th edition. Baltimore, The Williams and Wilkins Co. 15-32

Deng A, WU J, Zhang Y, Zhang G and Wen T. 2010. Purification and characterization of a surfactant-stable

high-alkaline protease from Bacillus sp. B001.Bioresour.Technol. 101: 7100-7116.

Gibb, G. D. and Strohl, W. R.1987. Physiological regulation of protease activity in *Streptomyces peucetius*. Can. J. Microbiol. 34: 187-190.

Gupta R, Beg QK, Lorenz P2002. Bacterial Alkaline Protease: Molecular approaches and industrial application. Appl. Microbial. Biotechnol. 59: 15-32.

Josephine S F, *et al*.2012. Isolation, production and characterization of protease from *Bacillus* Sp isolated from soil sample Microbiol. Biotech. Res.; 2 (1):163-168.

Kocher GS and Mishra S.2009: Immobilization of *Bacillus circulans* MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions. Internet J. Microbiol. 2009; 7: p. 2.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ.1951: Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Nout, M. J. R. and Rombouts, F. M. 1990. Recent developments in tempe of extracellular enzymes. In: *Bacillus subtilis* and other Gram positive proteases. Appl. Microbiol. Biotechnol. 2002. 60: 381-395.

Peciulytė D.2007**:** Isolation of cellulolytic fungi from waste paper gradual recycling materials. Ekologija.; 53: 11-18

Sjodahl J, Emmer A, Vincent J and Roeraade J.2010. Characterization of proteinases from *Antarctic krill* (*Euphausia superba*). Protein Expression and Purification.; 26:153-161.sp. B001. Bioresour. Technol.; 101: 7100-7116.

Tsuchida O, Yamagota Y, Ishizuka J, Arai J, Yamada J, Takeuchi M, and Ichishima E.1986: An alkaline protease of an alkalophilic *Bacillus* sp. Curr. Microbiol.; 14:7-12.

Uyar F, Porsuk I, Kizil G and Yilma, E. 2011: Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain C15. EurAsia. J. BioSci.; 5: 1-9.