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Optimization of Protease Production by *Bacillus subtilis* Isolated from Mid Gut of Fresh Water Fish *Labeo rohita*

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Abstract: Protease enzyme catalyzes the hydrolysis of protein. Among the various proteases, bacterial proteases are the most significant when compared with animal and fungal proteases. The aim of the present study was to isolate the protease producing *Bacillus* strain and to optimize the media composition that supports the protease production. The enzyme from fish intestinal bacteria is an untouched bio-source for enzyme production. Hence, the isolation and enumeration of heterotrophic bacteria from gastrointestinal tracts of rohu, *Labeo rohita* was carried out. The isolates were selected based on the cellular morphology, growth conditions and biochemical tests. The best protease producing organism was screened and found as *Bacillus subtilis* by 16S rRNA sequencing. Then, production medium for *Bacillus subtilis* were optimized by using different pH, temperature, carbon and nitrogen sources for 48 hours fermentation period. The results obtained in the present study revealed that protease production can be optimized at pH - 9.0, temperature 40°C by utilizing carbon source as glucose and nitrogen source as peptone.

Key words: Protease • Labeo rohita • *Bacillus subtilis* • Media optimization

INTRODUCTION

Microorganisms excrete a wide variety of proteolytic enzymes, which are also found in mammalian systems. They are molecules of small size, compact, spherical structures that catalyzes the peptide bond cleavage in proteins [1]. Commercially they are very important and isolated from various living sources such as plants, animals, bacteria and fungi. [2]

Proteases from microbial sources are preferred over the enzymes from plant or animal sources since they possess all most all the characteristics desired for their biotechnological applications [3]. Among bacteria, *Bacillus* species are specific producers of extracellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries [4]. In the present study, an alkaline protease producing bacterium was isolated from intestinal fluid of *Labeo rohita* and cultural conditions were optimized for the enzyme production. Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms. The importance of intestinal bacteria in the nutrition and well-being of their hosts has been established for homeothermic species, such as birds and mammals [5]. Endogenous digestive enzymes in fish have been studied by several workers [6, 7]. However, information regarding the enzyme producing intestinal bacteria, their source and significance in fish is scarce [8]. Hence an attempt has been made to investigate the protease producing organisms present in the gastro intestinal tract of *Labeo rohita* (Rohu).

MATERIALS AND METHODS

Isolation and Identification: *Labeo rohita* were purchased from the local market in Mettur Dam, Tamilnadu, India. The fish were kept in ice and transported to the research laboratory with in 1 hour. After washing the fish with

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distilled water, visceral organs were separated and GI tracts were removed.

A homogenate solution was made by adding GI tracts with 0.9% sodium chloride solution. (10:1; volume: weight) Serial dilutions were made by mixing this homogenate solution with sterilized distilled water. Diluted samples (0.3 ml) were aseptically poured in peptone gelatin agar and cultured plates were incubated at 37°C over night and examined for development of bacterial colonies after incubation period [8].Nine bacterial colonies were selected and tested for protease production on gelatin clearing zone technique [9]. Colonies with clear zones formed by the hydrolysis of gelatin were evaluated as protease producers. Depending on the zone diameter and clearance, isolate SG-1 was selected as good protease producer and used in all further investigations.

The isolated SG-1 was identified based on cellular morphology, growth condition, gram staining, endospore staining, capsule staining and biochemical tests [10].

Sequence Determination of 16S rRNA: Sequence analysis was performed on a 1492-bp PCR product using 2 primers for sequencing reaction. Primers were obtained from Sigma Genosys, Bangalore. Amplification was carried out Eppendorf Master cycler Personal PCR thermo cycler as follows: one cycle comprises of denaturation for 5 min at 95°C; cyclic denaturation of 45 sec at 95°C; annealing of 45 sec at 56°C; extention of 2 min at 72°C and a final elongation step of 10 min at 72°C. The amplified PCR product was purified by Perfect prep® Gel cleanup kit (Eppendorf AG, Germany). The purified PCR product were sequenced and analyzed. Analysis of the sequence was performed by comparing with the existing sequences in the Genbank database.

Protease Production [11]: The culture medium used in this work for protease production contained 0.5% glucose (w/v), 0.75% peptone (w/v), 0.5% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) KH_2PO_4 and 0.01% (w/v) $FeSO_4.7H_2O$ maintained at 37°C for 24 to 48 h in a shaking incubator (140 rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C for 15 min and the clear supernatant was used as crude enzyme preparation.

Protease Assay: Activity of protease was determined by Anson method [12] with some modifications. Enzyme solution (0.5 ml) was mixed with 5.0 ml substrate (0.65% casein in 25 mM Tris-HCl buffer) at 37°C for 30 minutes and after incubation TCA (110 mM) was added to attenuate the reaction. This mixture was centrifuged at 10000 rpm for 5 minutes and the released amino acids were measured as tyrosine by the method of Folin and Ciocalteu [13]

Protein Determination: Protein concentration was estimated by Lowry's method [14] using Bovine Serum Albumin as standard.

Effect of pH on Protease Production [11]: The effect of pH on protease production by SG-1 was determined by growth of organisms in fermentation media at different pH using appropriate buffers, phosphate buffer (pH 5.0 - 6.0), Tris-HCl buffer (pH 7.0 - 8.0) and glycine-NaOH buffer (pH 9.0-10.0). Protease production was measured and monitored at 6 hours intervals over 48 h fermentation periods through assay of protease.

Effect of Temperature on Protease Production: The effect of temperature on protease production was studied by growing SG-1 in fermentation media set at different temperatures (20, 30, 40, 50 and 60°C).The reaction was carried out at the optimum pH 9.0. The resulting culture was subjected to centrifugation at 10,000 rpm, 4°C for 15 minutes. Finally, the protease activity was assayed by above said procedures.

Effect of Carbon Source: The sterilized selective production broth was prepared with glycine-NaoH buffer (pH 9.0) with the various carbon sources like, fructose, sucrose, lactose and mannitol. These carbon sources were used to replace the carbon source available in the media. The isolated SG-1 was inoculated on different carbon source flasks and the flasks were incubated at 40°C for 48 hrs on a rotary shaker at 140 rpm. The resulting culture was subjected to centrifugation at 10,000 rpm, 4°C for 15 minutes. Finally, the protein and protease activity were assayed by above said method.

Effect of Nitrogen Source: The sterilized selective production broth was prepared with glycine-NaoH buffer (pH 9.0).The different nitrogen sources used in the experiment was gelatin, urea, sodium nitrate, ammonium sulphate. These nitrogen sources were used to replace the nitrogen source available in the media. The isolated SG-1 was inoculated on different nitrogen source flasks and was incubated at 40°C for 48 hrs on a rotary shaker at 140 rpm. The resulting culture was subjected to centrifugation at 10,000 rpm, 4°C for 15 minutes. Finally, the protein and protease activity were assayed.

Detection of Protease Activity by Zymography [15]: Zyomgraphy is a sensitive and rapid assay method for analyzing protease activity [16]. Proteases obtained from mid gut of *Labeo rohita* were resolved in 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels containing the following substrates: 0.1% gelatin, 0.1% casein. Samples (10 µl) were mixed with sample buffer. (Samples were not boiled). The protein sample was electrophoresed under standard conditions. After electrophoresis, SDS was removed from the gel by immersing in a solution containing 2.5% Triton X 100 and then the gels were incubated overnight at room temperature in 50 mM Tris Hcl (pH-8.0) buffer supplemented with $Cacl_2$(1 mM), $Zncl_2$ (0.001 mM) and Nacl (150 mM). The protease activity was visualized by staining the gels by coomassie brilliant blue.

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics: Morphological and physiological characteristics of bacterial isolate SG-1 were investigated according to the methods described in Bergey's Manual of Determinative Bacteriology [10]. It was identified as member of *Bacillus* species. The colonial appearance of the strain SG-1 was creamy yellow with 7 mm diameters in peptone gelatin agar (Plate 1). The phenotypic characteristics, Biochemical tests and carbohydrate utilization pattern of the isolate SG-1 are summarized in the tables 1 and plate 2. Isolate SG -1 was found as Gram positive, aerobic, catalase and oxidase-positive, motile, spore-forming and rod-shaped bacterium.

16S rDNA (rRNA) Sequence Analysis: We constructed a phylogenetic tree based on comparison of 16S rDNA sequences of reference *Bacillus* strains in order to understand the phylogenetic position of our strain. The sequence analysis for the 1500 bases of 16S rDNA gene of the isolate SG-1 was determined and compared with those of reference *Bacillus* spp. The sequence of isolate SG-1 showed 99% homology with the *B.subtilis* strain.

Optimization of Culture Conditions: The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the production of proteases [17].Hence proper combination of various cultural conditions can be established in order to suit the *Bacillus subtilis* for high secretion of alkaline protease.

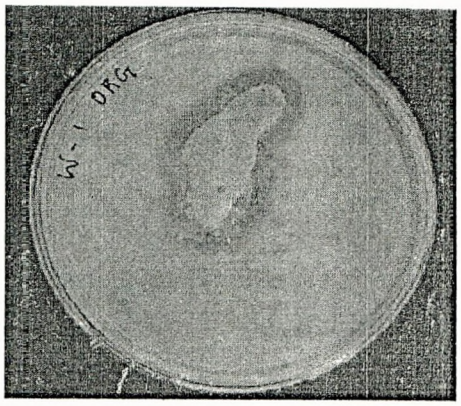


Plate 1: Qualitative assay protease by gelatin zone clearing technique

Plare 2: Gram-Positive Rod Shaped *Bacillus* Strain

Table1: Biochemical Charecterization

Cellular Morphology	Rods
Gram Staining	Positive
Spore Staining	Positive
Catalase Test	Positive
Oxidase Test	Positive
Indole Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Positive
Citrate Utilization Test	Positive
Starch Hydrolysis Test	Positive
Gelatin Hydrolysis Test	Positive
Litmus Milk Reduction	Positive
Carbohydrate Fermentation	
Glucose	
Positive	
Fructose	Positive
Xylose	Negative
Maltose	Positive
Mannito	Positive
Sucrose	Positive
Lactose	Negative

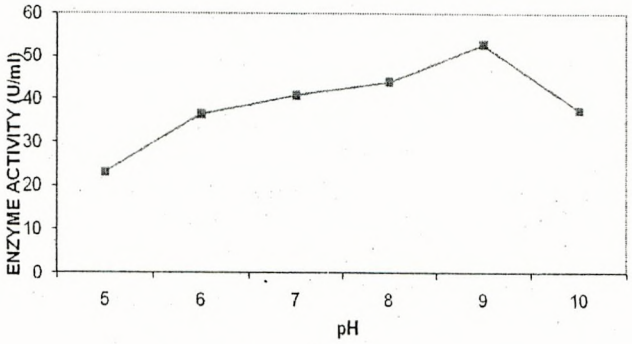


Fig. 1: Optimum pH

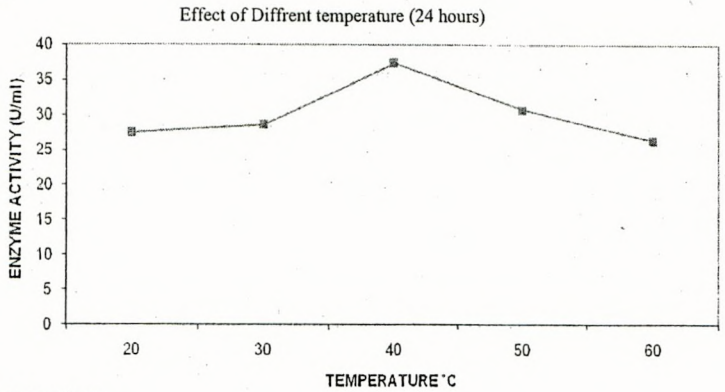


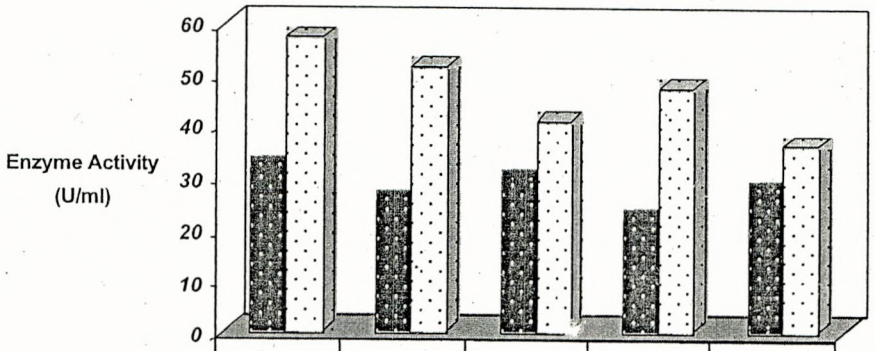
Fig. 2: Optimum temperature

Effect of pH: Bacillus subtilis was allowed to grow in media of different pH ranging from 5.0-10.0. Maximum enzyme activity was observed in medium of pH 9.0 (glycine-NaoH buffer) which was the optimum pH for *Bacillus subtilis* (Figure 1). Similar results were obtained for Bacillus proteolyticus CFR 3001 isolated from fish processing waste [18]. However majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline condition [19, 20]

Effect of Incubation Temperature: Enzyme activity recorded at different temperatures revealed that the *Bacillus subtilis* yielded maximum protease production at 40°C (Fig. 2).The temperature was found to influence extracellular enzyme secretion; possibly by changing the physical properties of the cell membrane [21]. El-Safey *et al.* [22] reported the same findings in production, purification and characterization of protease enzyme from *Bacillus subtilis*. Related studies also reported that protease production by *Bacillus laterosporus* was best at 40°C [23].

Effect of Carbon Source: Various sources of carbon such as fructose, lactose, manitol and sucrose were used to replace glucose which was the original carbon source in growth media. Results obtained were showed that, glucose brought the highest protease production compared to other carbon sources at 24 and 48 h of incubation. Figures 3&4 represent the protein and protease activity. Sucrose and Lactose also showed high protease production at 24 h but drastically reduced by 48 h of incubation. Hence, glucose was found to be the best source for protease production. For commercial production, sugars like fructose, lactose, manitol, sucrose will be prohibitive due to their cost [24]. Similar findings were observed by Jedeja and Bhatiya [25] in optimization of environmental and nutritional factors for alkaline protease production.

Effect of Nitrogen Source: Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources [26, 27]. The effect of nitrogen source was studied in the growth medium, where peptone was replaced with gelatin, sodium nitrate, ammonium sulphate and urea. Among the various nitrogen sources tested, peptone was found to be the best nitrogen source for alkaline protease production (Figs. 5&6).This findings were agreement with findings of Wang and Hsu [28] who found out that Casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicolo* 23. Gitishree and Prasad [29] optimized protease production with peptone



	Glucose	Fructose	Sucrose	Mannitol	Lactose
24 HOURS	31.9	25.3	29.7	22	27.5
48 HOURS	57.3	51.8	40.8	47.4	36.4

Fig. 3: Effect of carbon sources on protease production

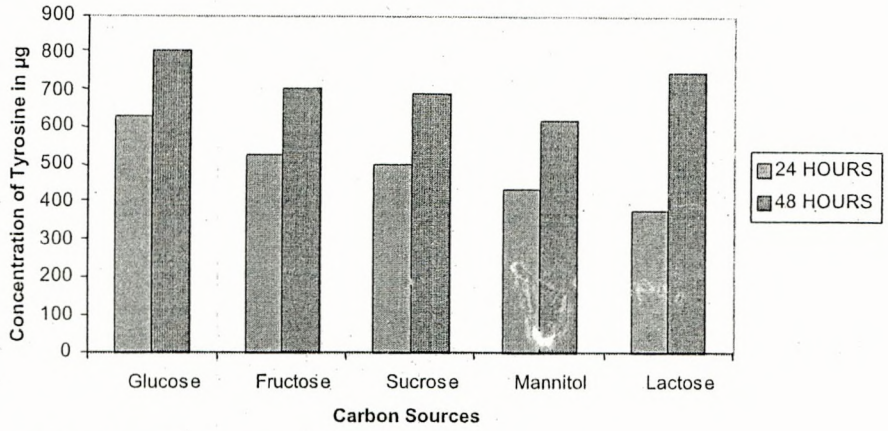
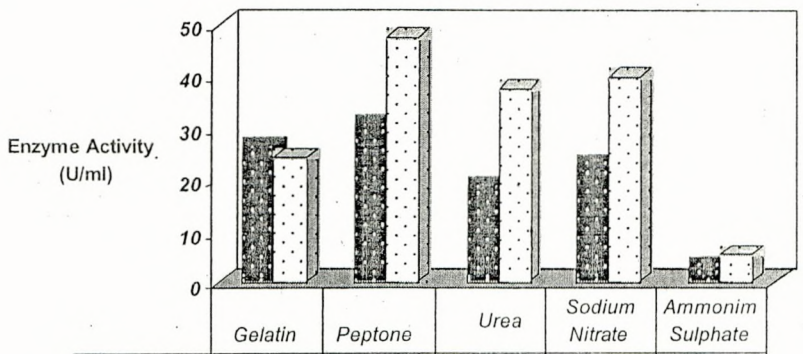


Fig. 4: Protein estimation for carbon sources



	Gelatin	Peptone	Urea	Sodium Nitrate	Ammonim Sulphate
24 HOURS	26.4	30.9	18.7	23.1	3.3
48 HOURS	24.2	47.4	37.6	39.7	5.5

Fig. 5: Effect of nitrogen sources on protease production

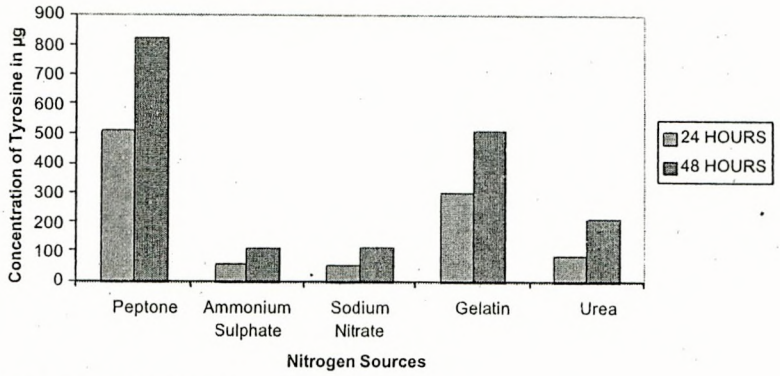


Fig. 6: Protein estimation for nitrogen sources

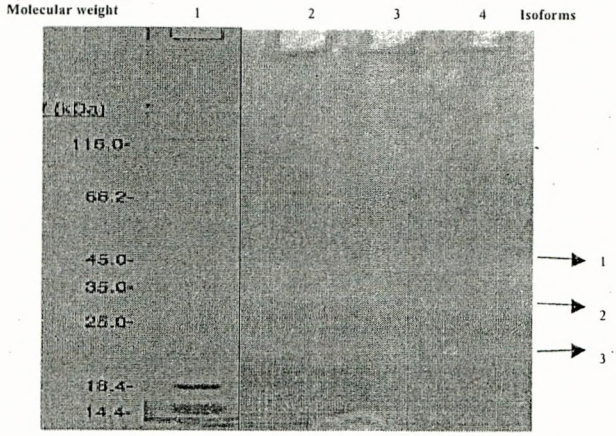


Fig. 7: Zymogram pattern of *Bacillus subtilis* isolated from mid gut of *Labeo rohita*
7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels
Lane 1- Markers molecular weight ranging from - 14.4 to 1[illegible]0 kDa
Lane 2,3 &4-Three isoforms of *Bacillus subtilis* proteases.

as nitrogen source; Kalaiarasi and Sunitha [30] reported that peptone was good nitrogen source for alkaline protease production from *Pseudomonas fluorescens*.

Zymogram Analysis: Zymography (in-gel activity staining), a two-stage technique involving protein separation by electrophoresis followed by in situ (in-gel) assay of enzymatic activities, has proved to be extremely useful for the detection of a wide range of microbial, animal and plant enzymes [31-35]. Certain advantages of zymography over conventional assays, such as the ability to assess the repertoire of enzymes that have a particular activity in nonfractionated cell extracts and to estimate the molecular weight and isoelectric point of the corresponding polypeptides and their isoforms, can be invaluable in identifying and monitoring specific and non-specific activities in the complex biological and clinical samples and developing purification schemes. Figure 7 shows that the *Bacillus subtilis* produced 3 different proteases (approximate molecular weight of 20, 35 and 45 KDa) under optimized condition which confirms the protease activity.

On conclusion, the aim of this research work was to isolate and identify high protease producer from local habitat. *Bacillus subtilis* was produced maximum yield of alkaline proteases and it was selected as a potent strain for further studies. The optimum temperature and pH were determined as 40°C and 9.0 and best carbon and nitrogen sources were Glucose and peptone. This information has enabled the ideal formulation of media composition for maximum protease production by this organism. After optimization, the mass production was carried in 1 liter of optimized media at 40°C for 48 hours at a pH of 9.0 on a rotary shaker at 140 rpm. Further studies were in progress in the purification and application of protease in different commercial fields.

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