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Alkalophilic Protease Enzyme Production from Estuarine *Bacillus aquimaris*

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Abstract: In the present investigation protease producing potent bacterial strain was isolated from the gut of estuarine fish *Etroplus suratesis*. The optimization of protease production in submerged fermentation at varying environmental parameters like pH, temperature, nitrogen, carbon, metal ions and NaCl concentration were tested. The isolated *Bacillus aquimaris* utilized sucrose and casein for maximizing the protease production 377.91 ± 4.14 and 165.36 ± 3.09 U/ml respectively. Among the metal ions used maximum protease production was observed in $CaCl_2$ (170.34 ± 6 U/ml). The effect of NaCl on protease production revealed that the *Bacillus aquimaris* is slightly halophilic and required 2.0% NaCl for the maximum production of protease 307.33 ± 4.94 U/ml. The optimum pH and temperature for maximum protease activity determined as 10 and 40°C respectively and the production realized were 234.54 ± 4.49 and 197.44 ± 3.46 U/ml. The isolated crude protease enzyme was partially purified by using DEAE Sephadex –A50 column chromatography. The partially purified products obtained the molecular weight around 37 KDa by using SDS-PAGE. It was concluded that the primary purpose of this report is to point out the original sources from which the isolates were made and to correlate the nature of the substrates with the extent of protease production by the *Bacillus aquimaris*.

Key words: Proteases · *Bacillus aquimaris* · *Etroplus suratesis* · DEAE Sephadex · SDS-PAGE

INTRODUCTION

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and are essential for cell growth and differentiation. Microbial proteases are among the most important hydrolytic enzymes and have been extensively studied since the advent of enzymology. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. Proteases encompass a group of enzymes, which hydrolyze peptide bonds in aqueous environment and synthesize peptide bonds in non-aqueous environment. These enzymes account for about 60% of total worldwide sales of enzymes [1] and have application in food, pharmaceuticals, detergent, leather industry, basic research and extraction of silver from used X-ray films [2]. Alkaline protease comprises 30% of the total worldwide enzyme production. It's particularly suitable for the detergent industry because of its stability at high pH [3]. Proteases are currently received more attention in the view of their stability, in high pH, temperature and in the presence of surfactants, organic solvents and denaturing agents. Proteases especially the alkaline proteases are physiologically and commercially important group of enzymes used primarily as detergent additives [4]. Bacterial proteases are easily produced in large amounts, thermostable and active at wider pH range. These properties make the bacterial proteases most suitable for wide industrial applications. The economic importance of proteases came to light when bacterial alkaline proteases from *Bacillus* species were introduced in 1960's to the detergent industry [5]. The advantage of using proteases for dehairing of skins are the reduction of the sulphide contents in the effluent recovery of the hair or wool which is of good quality, an increased yield of leather area, easy handling of the pelts by workman, simplification of the bate in the deliming stage and finally the production of a good quality pelts and leather [6]. Serine protease, cysteine protease, aspartic protease and metallo protease

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constitute one of the most important groups of industrial enzymes [1]. Protease may play a vital role in these treatments by replacing these hazardous chemicals especially involved in soaking, dehairing and bating. In generally proteases play a vital role in leather processing starting from soaking of hides to finished products. The proteases enzymes catalyze the breakdown of the protein keratin in the hair and allow the hair to be easily removed. The enzymes such as pepsin and papain can be used as neutral and alkaline enzymes from *Bacillus subtilis*. To isolate the potential protease producing bacterium from the gut of estuarine fish *Etroplus suratensis*. To perform the biochemical test for identification of protease positive bacteria using the Bergey's manual of determinative Bacteriology and based on 16S rRNA sequencing method. To optimize the identified bacterium with varying parameters such as carbon sources, nitrogen sources, sodium chloride, metal ions, pH and temperature through the submerged fermentation (SF) process. To purify the protease produced by the optimized bacterial strains through the DEAE Sephadex A50.

The present study dealt with the isolation of a *Bacillus aquimaris* and described the effect of cultural conditions on the activity of alkalophilic protease.

MATERIALS AND METHODS

Isolation of Potential Protease Producing *Bacillus aquimaris*: The organism used in the study was isolated as potent proteolytic bacterium from the gut of *Etroplus suratensis* collected from the Rajakkamangalam estuary of Kanyakumari district, Tamilnadu. The bacterium produced a clear zone size of more than 14mm, when streaked on skim milk agar after 24 hrs of incubation. The bacterium was identified according to the standard key of Bergey's manual of Determinative Bacteriology.

Genomic DNA Extraction, Cloning and Sequencing of 16S rRNA Gene: The isolated bacterial strain was grown in 2ml Zobell Marine Broth overnight at 27°C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 37°C. To this tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water. The amplified product (1,500-bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea). The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST [7].

Mass Culture of Protease Producing Organism: For mass culture of protease positive organism was enriched first using enrichment medium containing beef extract (0.5%) at pH 7 and incubated at room temperature for 24 hrs. Then 5ml of enriched seed culture was inoculated into 250ml flask containing 50 ml of basal medium. The culture was then incubated for 48hrs by reciprocal shaking at 40°C. The cells were then harvested by centrifugation at 10x rpm for 15minutes and the supernatant was used for further assay.

Protease Assay: The assay system consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein solution and 0.25 ml culture supernatant. The mixture was incubated for 30 min at 30°C. Then 3 ml of 5% TCA was added to this mixture and placed at 4°C for 10 min to form precipitate. Then it was centrifuged at 5x rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of Folin phenol reagent and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions.

Effect of pH on Protease Production: The effect of optimum pH for protease production by the experimental bacterium was determined by culturing the bacterium in the protease production media with different pH. The experiment was carried out individually at various pH such as 5, 6, 7, 8, 9, 10, 11 and 12. The enzyme assay was carried out individually after 48 hrs of incubation at 35°C.

Effect of Temperature on Protease Production: Temperature plays an important role for the production of extracellular protease by the test organism. The effect of temperature on protease production was studied by incubating the culture media at various temperatures such as 20°C, 30°C, 40°C, 50°C and 60°C.

Effect of Carbon Source on Protease Production: To identify suitable carbon source for the protease production by the test organisms, the following three different carbon sources were tested such as glucose, sucrose and maltose. The above mentioned carbon sources were tested individually at the concentration of 0.5% in the optimized basal medium. Simultaneously a control medium without addition of any of these carbon sources was also maintained. After screening, the maximum protease producing carbon source was taken for optimization. It was done by varying concentrations such as 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6% in the protease production medium.

Effect of Nitrogen Sources on Protease Production: The protease production by the selected bacterium was optimized by supplementing different organic nitrogen sources. For this, the following four organic nitrogen sources were tested individually by supplementing at the rate of 0.5% each in the basal medium such as ammonium sulphate, sodium nitrate, beef extract and casein. Simultaneously a control medium was also maintained without the addition of any organic nitrogen sources. After screening the maximum protease producing nitrogen source was taken for optimization.

Effect of Sodium Chloride on Protease Production: The effect of NaCl on protease production was studied by supplying various concentrations of NaCl to the production medium. The experiment was carried out individually at various concentrations of NaCl such as 1, 1.5, 2.0, 2.5, 3.0 and 3.5%.

Effect of Metal Ions on Protease Production: For the selection of suitable metal ion for protease production by the tested organisms four different metal ions were screened and are given below ferrous sulphate, calcium chloride, mercuric sulphate and KH_2PO_4. They were tested individually at the concentration of 5 mM in the lactose and yeast extract supplemented medium. Simultaneously a control was maintained without addition of any of the metal ions.

Partially Purification of Protease Enzyme: The supernatant were precipitated by adding two volumes of acetone and kept for 1 hr at 4°C to allow complete precipitation. The resulting precipitate was collected by centrifugation (10x rpm, 30 min) and the pellet was air dried and resuspended in a minimal volume of 20 mM Tris Hcl buffer (pH 7.2). Finally, the insoluble materials was removed by centrifugation at 10x rpm for 30 min, the supernatant was applied to ion exchange column chromatography, of DEAE Sephadex -A50 (2.5 -30 cm), which had been equilibrated with 20 mM Tris Hcl (pH 7.2). After loading the sample, the column was washed with the same buffer until the optical density of the elution was zero at 280nm. The bound proteins were then eluted with a linear gradient of sodium chloride in the range of 0.1-1M in the equilibrating buffer. 4 ml of each fraction were collected at a flow rate of 40 ml/h. The enzyme activity and protein content of the each fraction were determined.

Molecular Weight Determination by SDS-PAGE: SDS-PAGE (12%) was performed according to the method of Laemmli, [8] under reducing conditions. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular weight versus the Rf value (relative mobility) using a standard molecular weight marker range between 14 to 98 kDa.

RESULTS

Identification of Protease Positive Colony: Based on the morphological, physiological and biochemical characteristics, the protease positive colony was identified as *Bacillus* sp. by following the standard keys of Bergey's Manual of Determinative Bacteriology (Table 1).

Screening of Protease Production by Identified Organism: The isolated bacterium strain was screened for protease producing ability on skim milk agar which forms a zone due to hydrolysis of casein (Fig.1). Hence this strain was identified as a protease producer and it was used for further experimental studies. Phylogenetic studies revealed that the 16S rRNA gene sequence of the strain *Bacillus aquimaris* has 97% similarity with the nearest match in the Genbank.

Table 1: Biochemical characteristics of the *Bacillus aquimaris*

Biochemical tests	Results
Gram's staining	Gram Positive *Bacillus sp.*
Endospore staining	Central spores
Motility	+
Carbohydrate fermentation test	
a. D-glucose	+
b. Mannitol	+
c. Lactose	-
d. Sucrose	+
Indole Production	-
Methyl red test	-
Voges – Proskauer test	+
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	-
Catalase test	+
Oxidase	-
Nitrate utilization test	+

+ Positive Results

- Negative Results

Protease Production by *Bacillus aquimaris* in Different Culture Conditions

Effect on pH on Protease Production: Fig. 2 shows the effect of various pH on protease production. The maximum protease production was obtained at pH 10 (234.54 ± 4.49 U/ml). The minimum protease production was recorded at pH 5 (144.78 ± 3.16 U/ml).

Effect of Temperature on Protease Production: Temperature is one of the most critical parameters that have to be controlled in bioprocess. Fig. 3 shows the effect of various temperatures on protease production. Among the various temperature tested, the maximum (197.44 ± 3.46 U/ml) protease production was obtained at 40°C and minimum (2 U/ml) protease production recorded at 60°C temperature.

Effect of Carbon Sources on Protease Production: The effect of carbon sources on protease production by the candidate species after 48 hrs of incubation period at 40°C is given in fig. 4. Here the maximum (377.91 ± 4.14U/ml) protease production was recorded in sucrose supplemented medium. Minimum protease production was recorded in glucose (218.79 ± 4.07 U/ml) added medium.

Fig. 1: Protease activity of *Bacillus aquimaris* on skim milk agar plate

Effect of Nitrogen Sources on Protease Production: Fig. 5 shows the effect of different kinds of organic nitrogen sources on protease production after 48 hrs of incubation period at 40°C. The maximum (165.36 ± 3.09U/ml) amount of enzyme production was obtained in casein supplemented medium. While the minimum amount of protease production was observed in ammonium nitrate (60.93 ± 2.67 U/ml) supplemented medium.

Effect of Metal Ions on Protease Production: Fig. 6 shows the effect of metal ions on protease production after 48 hrs of incubation period 40°C. Among the tested metal ions, the maximum (170.34 ± 6 U/ml) amount of enzyme production was recorded in $CaCl_2$. Whereas the minimum (93.22 ± 3.17 U/ml) protease production was recorded in KH_2PO_4 supplemented medium.

Effect of Various Concentration of NaCl on Protease Production: Fig. 7 shows the effect of various concentrations of NaCl on protease production, after 48 hrs of incubation period at 40°C. This result indicated that the halotolerent nature was observed in the candidate bacterium. Among the tested concentrations of NaCl, the maximum (307.33 ± 4.94 U/ml) amount of enzyme production was observed at 2% NaCl supplemented medium. But the lowest (130.45 ± 3.09 U/ml) amount of enzyme production was recorded at 1% NaCl supplemented medium.

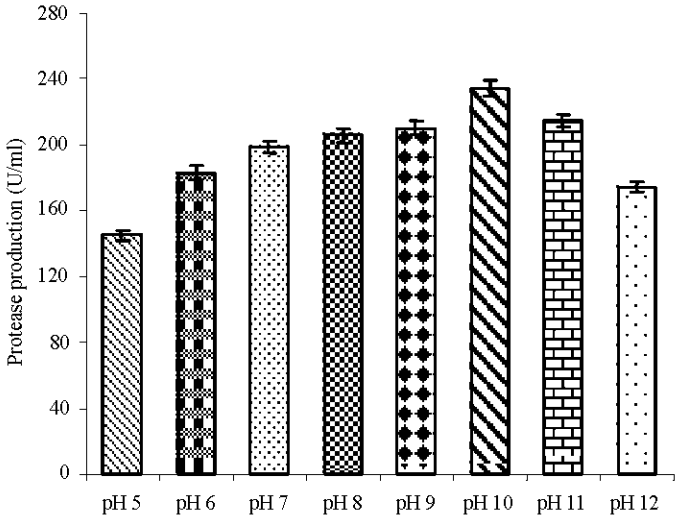

Fig. 2: Protease production on various pH

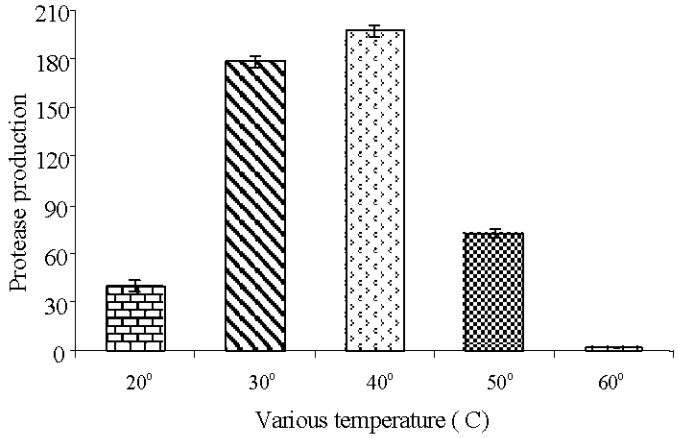

Fig. 3: Protease production on various temperatures

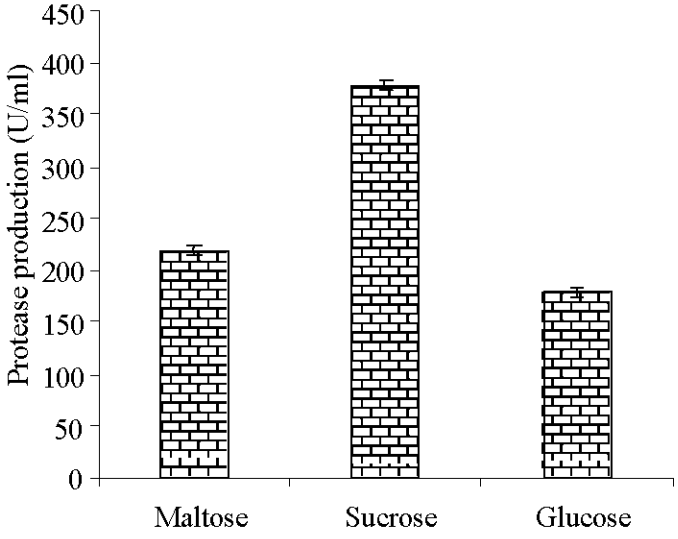

Fig. 4: Protease production on various carbon sources

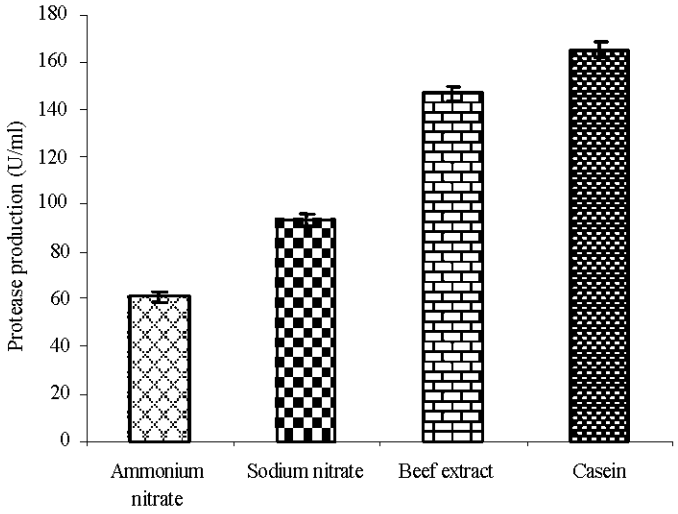

Fig. 5: Protease production on various nitrogen sources

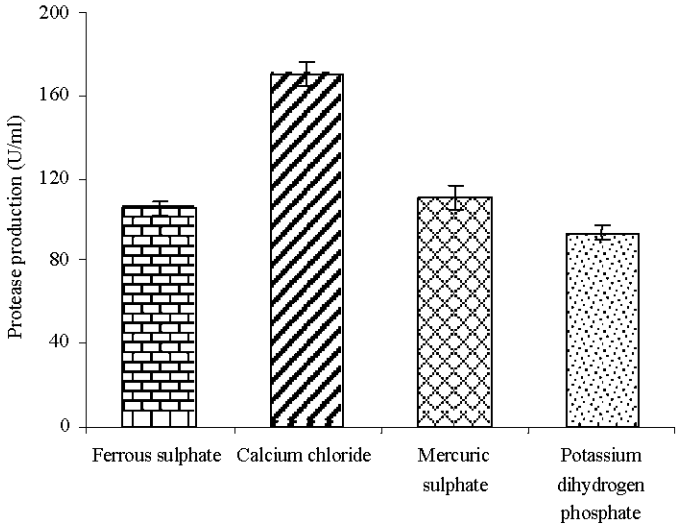

Fig. 6: Protease production on various metal ions sources

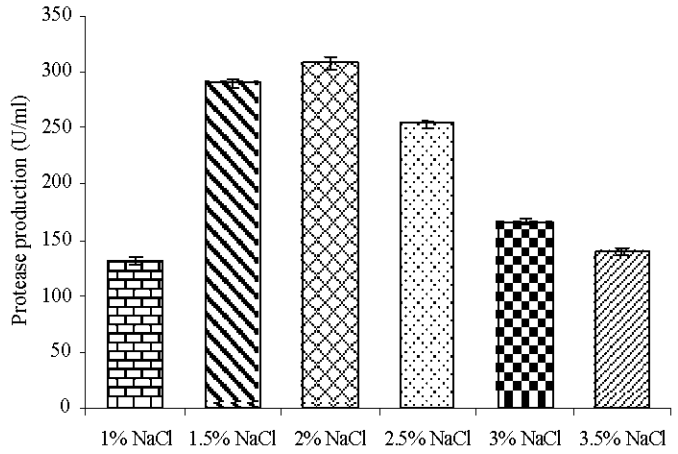

Fig. 7: Protease production on various NaCl concentrations

Table 2: Protease activity of *Bacillus aquimaris*

Purification steps	Volume (ml)	Total Protein (mg)	Total Activity (U/ml)	Specific Activity (U/mg protein)	Purification Fold	Yield (%)
Culture Supernatant	100	6.4	520	81.25	1	100
DEAE Sephadex A 50	50	2.24	225	937.5	1.6	43.26

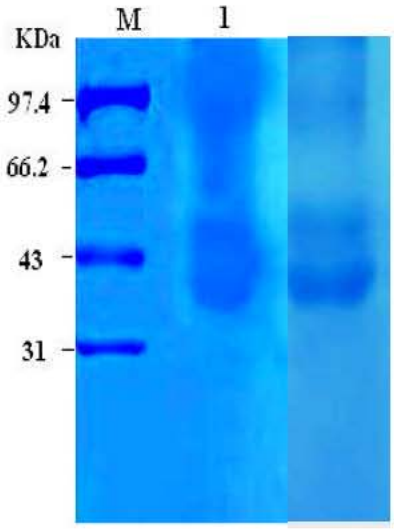

Fig. 8: SDS-PAGE Profile of Protease (37 KDa) enzyme Protein

Lane M : Standard Protein Molecular Weight Marker

Lane 1 : Showing Ammonium sulphate precipitated cell free extract after dialysis

Lane 2 : DEAE Sephadex A50 purified fractions of protease enzyme

Enzyme Activity of Partially Purified Compounds: The protease activity of crude enzyme extract was concentrated by acetone precipitation. Ninety percent protease recovery was achieved with 1.6 fold purification (Table 2). Following the acetone precipitation the resuspended solution was applied to a column of DEAE-Sephadex-A50. The anion gel filtration profile and most of the protease activity was observed in a single peak of the eluted fraction with 1.6 fold purification with the specific activity of 937.5 U/mg. Purified protease from *Bacillus aquimaris* migrated as a single band of 37 kDa in SDS-PAGE under reducing conditions, suggesting that the purified protein was homogeneous (Fig. 8).

DISCUSSION

In the present study, the effect of various carbon sources on protease production showed that sucrose yields maximize protease production than other supplied carbon sources. There are several reports showing that different carbon sources have different influence on enzyme production by different bacteria and fungi [9]. The maximum alkaline protease production was reported by several workers who used different sugars such as lactose, maltose, sucrose and fructose [10]. Considering the importance of sodium chloride, the present study was conducted and the result showed that the test organism can utilize the salt context within the range between 1-3% for maximizing the protease production and it was remarkably high in 2% sodium chloride supplied medium. At high concentration, the protease production decreased due to osmoregulation problems. These findings evidenced that the candidate bacterium is moderately halophilic because it is an estuarine isolate. Mussarat *et al.* [11] have been reported the protease production by marine bacterium *Roseobacter* sp. absolutely require 3.0% of NaCl concentration. Metal ions and trace elements are required for bacterial growth and physiological activities.

The present study on the effect on metal ions on protease production revealed that the calcium, mercury, ammonium ions had profound effect on the protease production. Folasade and Ajele [12] reported that the supplementation of the culture medium with a solution of metal ions improved substantially the growth of *Bacillus* sp. and also the enzyme production. The presence of Ca^{2+}, $Mg^{2+}$and Mn^{2+} not only protected the enzyme from undergoing denaturation but also enhanced the activity marginally. In the present study, the protease activity was higher at pH-10 (234.54 ± 4.49 U/ml) and its activity was lowered at pH-5 (144.78 ± 3.16 U/ml). Aunstrup *et al.*, [13] were granted by an Indian patent for the production of alkaline protease from alkalophilic *Bacillus* isolated on media adjusted to pH 9 to 11 with sodium carbonate.

In the present study, the protease activity was higher at 40°C (197.44 ± 3.46 U/ml) and its activity was lowered at 60°C (2 U/ml). Bhaskar *et al.*, [14] also reported the optimum temperature of 50°C for *Bacillus megaterium* which retained the activity at 30-45°C. The protease was purified by the acetone precipitation, gel filtration column chromatography and its homogeneity was tested by SDS-PAGE. Most of the purified protease from *B. cereus* was found to have a molecular weight between 34 to 45 KDa [15]. The molecular weight of alkaline protease from *Bacillus aquimaris* was estimated to be 37 KDa by SDS-PAGE.

It was concluded that from economic point of view *Bacillus aquimaris* was optimized in various production parameters like pH, temperature, carbon sources, nitrogen sources, NaCl concentration and metal ions. So it can be used for alkalophilic protease production on cheaper and more easily available recourses than on expensive and refined substrates.

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