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Production and optimization of extracellular Alkaline Protease from *Bacillus subtilis* isolated from dairy effluent

K. Krishnaveni¹, D. J. Mukesh kumar^{2*}, M. D. Balakumaran², S. Ramesh¹
and P. T. Kalaichelvan²

¹Department of Microbiology, PRIST University, Tanjavur, TN, India

²CAS in Botany, University of Madras, Guindy campus, Chennai, TN, India

ABSTRACT

*Proteolytic bacteria are most important for industries such as food and fermentation. Thus isolating and manipulating pure culture from various sources has importance for various biotechnology industries. In the present investigation a total of 26 bacteria were isolated from the dairy plant effluent. All isolates were screened for proteolytic activity using alkaline skim milk agar plate, among 26 bacterial isolates only 7 isolates showed alkaline proteolytic activity, one isolate was selected for further study. Characteristic feature and 16S rDNA analysis of the strain indicates that the organism was *Bacillus subtilis*. The culture and medium components was optimized further. The best enzyme activity was observed at pH 9 and temperature 45 °C, 5% MgSO₄ concentration, glucose as carbon source and beef extract as nitrogen source. Also maximum enzyme activity was observed when using wheat bran as the substrate. It is well proved that the dairy effluent can be used as the isolation source for alkaline protease production which in further having many industrial applications.*

Keywords: Dairy plant effluent, Skim milk agar medium, 16S rDNA analysis, Horikoshi I medium.

INTRODUCTION

Proteases are the class of enzymes which holds its application in detergent, pharmaceutical, photography, leather, food and agricultural industries. It plays a major role in Bioremediation [1]. Proteases represent one of the three largest groups of industrial enzymes. Proteases accounts for about 40 % of the total enzyme sales in different market sectors. The application of bacterial

proteases is more significant when compared to the proteases from other sources like fungal and human [2].

Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry [3]. The enzyme is widely distributed in nature, being found in various types of microorganisms, plant and animal tissues [4].

Microorganisms are considered potentially to be the most suitable sources of alkaline Protease for industrial application. Among the various bacteria *Bacillus* sp. was found to be the major group producing protease [5]. Hence the present study also deals with the isolation of Alkaline Protease producing *Bacillus* sp. from the dairy industry plant effluent. Previously, the identification of the organism was done by following Bergey's manual of classification. But nowadays a novel 16S rDNA based approach was done for the accurate identification of organism [6].

The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors the cultural parameters like temperature, pH, incubation time were also plays a major role in enzyme production [7] and as so the optimization of media components and cultural parameters is the primary task in a biological process. So, the media components and cultural conditions were optimized. The alkaline proteases were produced, extracted and purified and characterized.

MATERIALS AND METHODS

Isolation and Culture of Bacteria

The Dairy Plant Sludge sample was collected from the Aavin dairy plant, Sholinganallur, Chennai, in a sterilized container. Precautions were taken to minimize the contamination. The microorganisms were isolated from dairy plant sludge using spread plate technique. The mixed cultures obtained were differentiated based on colony morphology and further purified by quadrant streaking on nutrient agar plates. The purity of the cultures was checked using Gram's staining [3].

Screening for Alkaline protease activity

The purified cultures were screened for the production of Alkaline Protease by streaking them on Alkaline Skim milk agar plate (Skim milk 1.0%, Peptone 0.1%, NaCl 0.5%, Agar 2.0% and pH 10.0) [4]. The Alkaline Protease production was confirmed by the formation of clear zones around the colonies. The organism with maximum zone formation was further analysed.

Molecular Characterization of Proteolytic Bacteria

Pure culture of the bacteria was grown overnight on nutrient broth. The DNA was isolated from the bacteria using Cell Lysis method and 16S rDNA was amplified by Thermocycler (PTC – 100 TM Programmable Thermal Controller) using the primers, Forward: 5'-AGAGTTTGATCCTGGCTCAG-3' Reverse: 5'-TACCTTGTTACGACTT 3'. The amplified 16S rDNA PCR product was sequenced using automated sequencer (Chromus Biotech,

Chennai). The Sequence similarity search was done for the 16S rDNA sequence using online search tool called BLAST (<http://www.ncbi.nlm.nih.gov/blast/>). The unknown organism was identified using the maximum aligned sequence through BLAST search [6].

Production of Protease

The culture positive for Alkaline Protease was grown under optimal condition for the enzyme production in 100 ml of Horikoshi I medium [7] containing (g/l) Glucose–5g, Peptone– 7.5 g, NaCl–5g, MgSO₄·7H₂O–5g, FeSO₄·7H₂O–0.1g with the pH of 10.0. The medium was incubated for 48 h in shaker incubator (150 rpm) at 37 °C. The fermented broth was filtered and the filtrate was centrifuged at 5000 rpm for 5 minutes to extract the crude extracellular Protease. The culture filtrate was used for further assay procedures.

Assay for Proteolytic Activity

The assay of alkaline protease was carried out by the modified folins method [8]. 0.5% Casein was dissolved in 50 μM Glycine NaOH buffer pH 10.0 and the reaction mixture was kept for incubation at 80 °C for 30 minutes. The reaction was then stopped by the addition of 10% TCA. The tyrosine liberated was estimated using Folins reagent and OD was recorded at 670 nm. One unit of protease was defined as amount of protease required to release 1 μg of tyrosine under the assay condition in 1min.

Assay of Total Protein Content

The Total protein content from the sample was determined using Bradford method [9]. One ml of culture filtrate was taken and 5 ml of Bradford reagent was added. The tube was gently tilted once for mixing and the absorbency was taken at 595nm in UV–vis spectrometer. The protein concentration was determined by comparing the value with standard graph prepared using Bovine serum albumin.

Time course studies

Different incubation times (12, 24, 36, 48, 60, 72, 84, 96) were employed to study their effect on the Protease production. The culture filtrates were collected at respective time interval and assayed.

Determination of Optimum Temperature

The effect of Temperature on Protease production was studied by incubating the Culture media at different temperatures like 35, 40, 45, 50, 55, 60 °C and assayed.

Determination of Optimum pH

The optimum pH for the production of Proteases was determined by growing the Organism with different pH (7, 8, 9, 10, and 11). The enzyme activity was assayed further.

Analysis of Different Carbon Sources for Protease Production

To identifying the Carbon sources facilitating Protease Production, five different Carbon sources were used for the experimentation. They were Sucrose, Mannose, Glucose, Lactose, Starch and Fructose. The suitable Carbon source was identified by assaying the culture filtrate after incubation.

Analysis of Different Nitrogen Sources on Protease Production

The influence of Nitrogen sources on the catalytic activity of Proteases are determined by measuring the enzyme activity when the medium with different Nitrogen sources like Peptone, Yeast extract, Beef extract, Casein, Ammonium Chloride, Ammonium Carbonate, Sodium nitrate, Urea.

Role of Agrowastes as substrates for alkaline protease production

The renewable agro wastes like Groundnut cake, Coconut cake, Soy cake, Wheat bran, Rice bran were tested for their ability as substrates for alkaline protease production by replacing the peptone in the production medium and the enzyme activity with Total protein content were assayed.

Effect of different metal ions on Alkaline Protease production

The role of metal ions on alkaline protease production was analysed by the addition of metal ions like CaCl₂, MnSO₄, MgSO₄, COCl₂, MnCl₂ of 0.5% concentration and was assayed.

Extraction and Purification of Protease

The culture fluid from the production media was collected and centrifuged. The culture supernatant was collected as crude enzyme extract for purification. To the culture supernatant, three volumes of 95% cold ethanol was added and the mixture was maintained in ice for 1 h with agitation. The precipitated crude extract was harvested by centrifugation and dissolved in 0.1 M Tris-HCl buffer (pH 7.0). The precipitated enzyme was then subjected to ion exchange chromatography with DEAE Sephadex A-50 column, 50 mM phosphate buffer (pH 7.0) as Running buffer and 1M NaCl in 50 mM phosphate buffer as elution buffer (pH 7.0) for further purification [10, 11].

Zymogram Analysis

PAGE was executed according to the method of Mary *et al* [12] which was not boiled and it was electrophoresed at 4 °C and 20 mA through a 10% SDS-polyacrylamide gel containing 0.1% gelatin. The gel was removed, washed and incubated in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM CaCl₂, and 1 μM ZnCl₂ at 37 °C for 48 h. The gel was stained for 20 minutes in 0.5% Coomassie brilliant blue R-250 in glacial acetic acid:isopropanol:DH₂O (1:3:6). Washing with DH₂O revealed clear bands, where proteolysis of gelatin occurred, on a blue background.

Enzymatic Dehairing

Cow's skin was cut to (5 cm × 5 cm) 2 pieces and incubated with the 5ml of partially purified protease in 10ml of 50 mM Tris-HCl (pH 9) at 50 °C. The skin was checked for removal of hair at different incubation times. After incubation using blunt knife the hair removed from the skin [13].

RESULTS AND DISCUSSION

Proteases are the important biological enzymes involved in cell division, regulating protein turnover, activation of zymogenic preforms, blood clotting, lysis of blood clot, processing and transport of secretory proteins across membrane, nutrition, regulation of gene expression and in pathogenic factors. Extreme environments are important sources for isolation of micro organisms

for novel industrial enzymes production [14]. Hence, in this present study also the protease producing bacteria were isolated from Dairy Industry Sludge.

Out of 26 bacteria isolated, 7 bacteria were found to be positive for protease production on Skim milk agar medium (Fig. 1). The better zone producing strain was assigned name as RMK. Tsai et al. [15] isolated similar Protease producing *Bacillus coagulans* and *Bacillus megaterium* from fish sauce in Taiwan. Identification of Bacteria on the basis of morphological characteristics is not reliable for all groups of organisms, including bacteria which possess limited morphological differentiation [16] and can be rely on the basis of biochemical tests and assimilation assays [17].

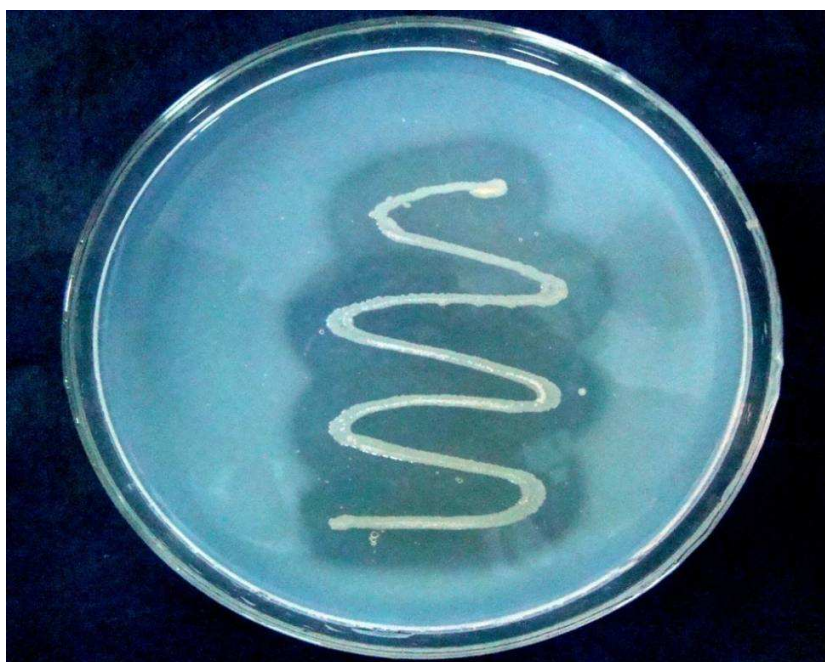


Fig. 1: *Bacillus subtilis* RMK strain in alkaline skim milk agar plate

When compared to morphological and biochemical characterization methods, 16S rDNA analysis is found to be the novel and accurate method for identifying unknown species. The DNA from the strain RMK was isolated and the 16S rDNA was amplified and sequenced. The BLAST analysis of RMK using its 16S rDNA sequence data showed that strain had highest homology (100 %) with *Bacillus subtilis*. The sequence has been submitted to the Genbank (GU047349).

Most of the Protease producing bacteria were reported as *Bacillus* and *Nesterkonia sp.* [18, 19]. 16S rRNA sequencing appears to have the potential ability to differentiate strains at the subspecies level. Several studies suggested that 16S rDNA sequences and phylogenetic relationships could be used to identify halotolerant/halophilic bacteria [20, 21, 22, 23].

Selection of suitable fermentation technique and optimization of culture conditions contribute much in achieving highest enzyme productivity [7]. At Present, enzyme production by microorganisms can be achieved using submerged fermentation. More than 90% of the commercial enzymes are produced through Submerged Fermentation [24]. As the environmental

parameters are essential for the production of Protease, they were optimized by shaken flask fermentation method. There is gradual increasing of production has occurred from beginning to 48 h (Fig. 2) and higher production has occurred at 48 h with the enzyme activity of 79.37 U/ml (Table 1). Different fermentation time has been also reported by other workers: 60 h in *Bacillus* sp. to produce subtilisin [25], and 36 h in *Streptomyces* sp. CN902 to produce alkaline protease [26] for maximum alkaline protease production under Submerged Fermentation.

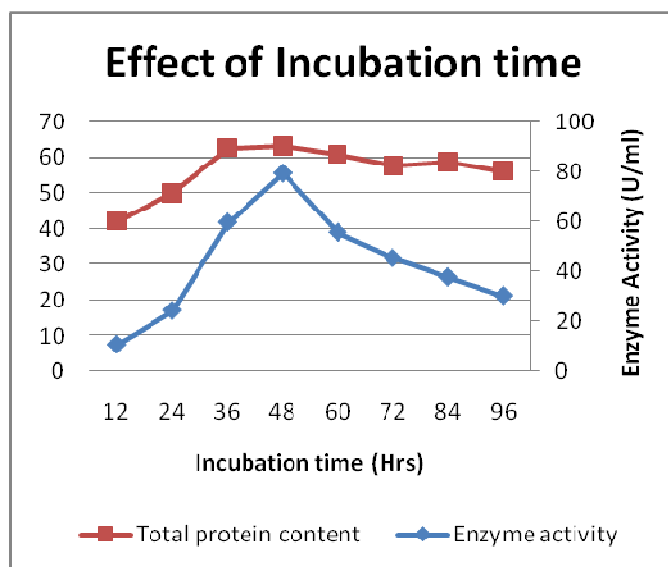


Fig. 2 Enzyme production and total protein content of bacterial isolate at different incubation time

To optimize the temperature for the better Protease production, medium were kept in various temperatures (Table 2). The higher protease activity was found as 80.56 U/ml at 45 °C for the Protease production (Fig. 3). The temperature requirement of the organism is based on the nature of organisms. Many reports showed bacterial and fungal alkaline protease production at lower and moderate temperatures (25–50 °C), like *Aspergillus* and *Bacillus* strains preferred such temperatures [27, 14, 28, 29, 30].

Medium composition, pH, and aeration are the important variables that affect the production of enzyme in Submerged Fermentation [31]. As the pH is found to be also important environmental parameter, varying pH were analyzed on Protease production (Table 3). Maximum production of the enzyme (84.09 U/ml) was obtained at the pH 9.0 (Fig. 4). Initial pH of the production medium is the most important factor that significantly influences the production of proteases [32]. Proteases that having optimum pH between 8 and 12 are having potential applications in the fields of detergent application, dehairing of hides, and silver recovery from waste X-ray and photographic films [33, 1, 31].

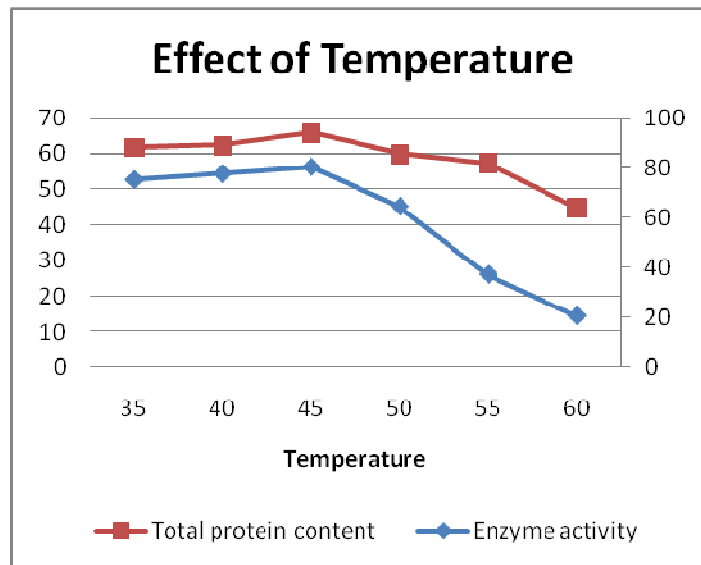


Fig. 3 Enzyme production and total protein content of bacterial isolate at different temperature

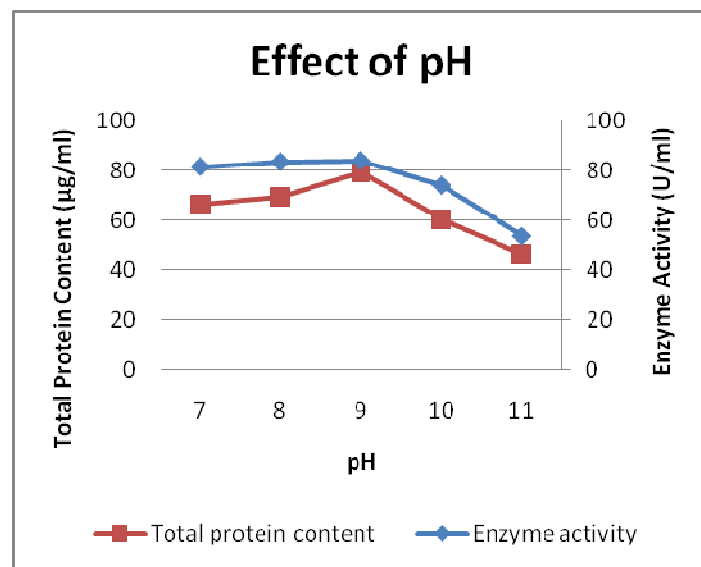


Fig. 4 Enzyme production and total protein content of bacterial isolate at different pH

Nutrient sources were found to be the next important factor for the Protease production. Since carbon is considered as the primary nutrient for the bacteria, different carbon sources like Sucrose, Mannose, Glucose, Lactose, Starch and Fructose were analyzed for the protease production (Table 4). Maximum production of Protease of 84.53 U/ml was observed when Glucose was served as the carbon source (Fig. 5). Hence, glucose was served as the better carbon source for the Protease production. Alkaline protease production was found to be best in wheat bran, glucose and dextrin by *Bacillus licheniformis* and *Bacillus coagulans* [34].

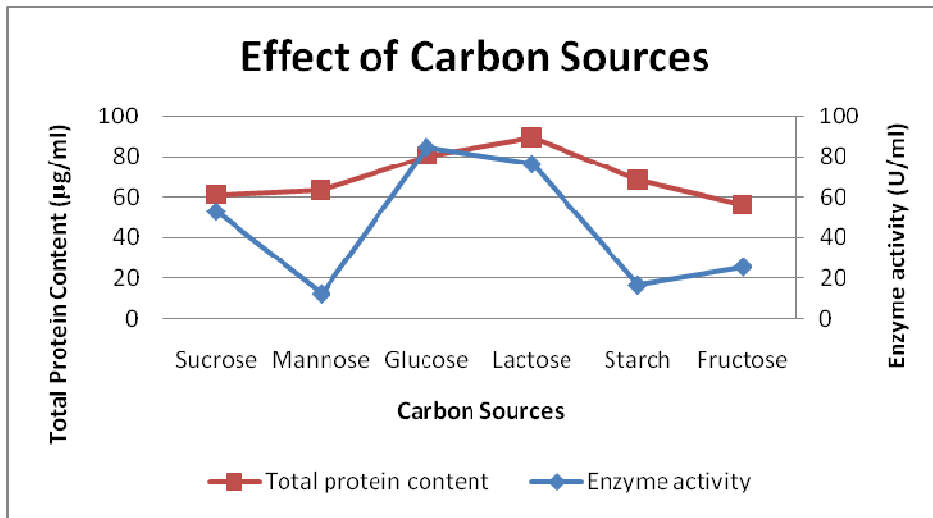


Fig. 5 Role of different Carbon sources on Enzyme production and total protein content

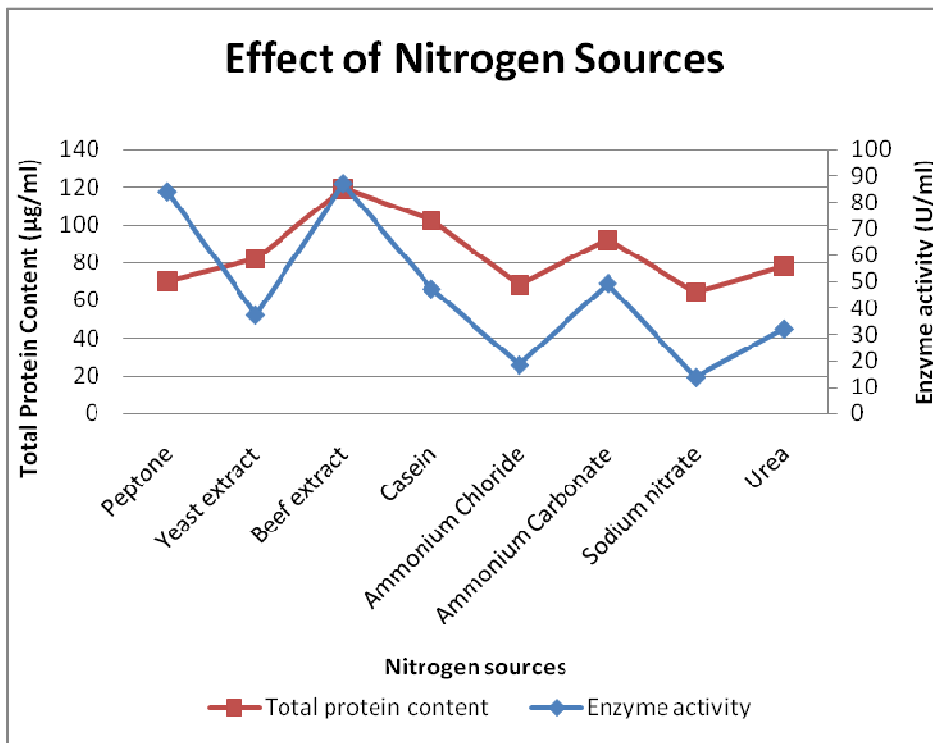


Fig. 6 Role of different nitrogen sources on enzyme production and total protein content

Next to the carbon, Nitrogen was served as important nutrient source for the Protease production (Table 6). Hence, different nitrogen sources like Peptone, Yeast extract, Beef extract, Casein, Ammonium Chloride, Ammonium Carbonate, Sodium nitrate, Urea were applied as nitrogen sources for the Protease production. Beef extract (Fig. 6) is found to be the better nitrogen source as it increases the production of Protease up to 86.87 U/ml. it has been reported that organic nitrogen sources like peptone, casein, skim milk, yeast extract, favored maximum protease

production by *Bacillus* sp. [35, 36]. Next to that, inorganic Nitrogen sources like ammonium carbonate followed by ammonium chloride, ammonium citrate and potassium nitrate were used as nitrogen sources [37].

Upto 40% of the total production cost of enzymes is due to the production the growth substrate. The real and beneficial production of enzyme is produced from the natural sources and industrial wastes. The production Medium costs of about 30–40% of the total production cost of the enzyme at industrial level [38]. The production of Protease was done by replacing the Nitrogen sources and common substrates with Groundnut cake, Coconut cake, Soy cake, Wheat bran, Rice bran. The maximum production was occurred when wheat bran used as natural substrate (75.96 U/ml) (Fig. 7). The result indicates that the wheat bran can also be used as the cheap substrate for Protease production.

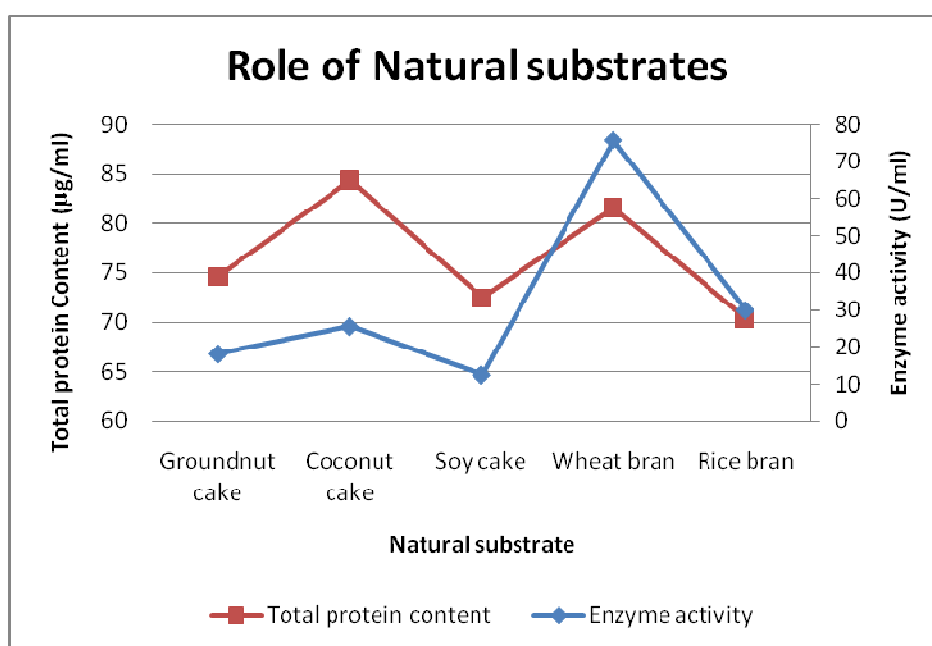


Fig. 7 Role of different natural substrates on enzyme production and total protein content

The metal ions were considered to be important cofactors for an enzyme to function and hence so analysed. From the result, it is clear that $MgSO_4$ (84.36) is found to be plays better role in the alkaline protease activity (Fig. 8). The result supported the statement that supplementation of Mg^{2+} , Ca^{2+} and K^+ salts to the culture medium exhibited slightly better production of protease [39].

The Protease enzyme produced was extracted and purified using ethanol precipitation and chromatography [11]. Molecular weight of Proteases isolated from *Bacillus* sp. was identified using SDS-PAGE analysis. Electrophoretic analysis of extracellular Protease from the isolate has been carried out. SDS-PAGE results showed the presence of multiple bands since along with protease some other proteins can be produced by the organisms. But the Presence of protein band nearing the molecular weight 27 kDa confirms the presence of enzyme. It has been previously

reported that the molecular weight of most of the alkaline proteases from *Bacillus sp.* Lies between 16 and 32kDa [40].

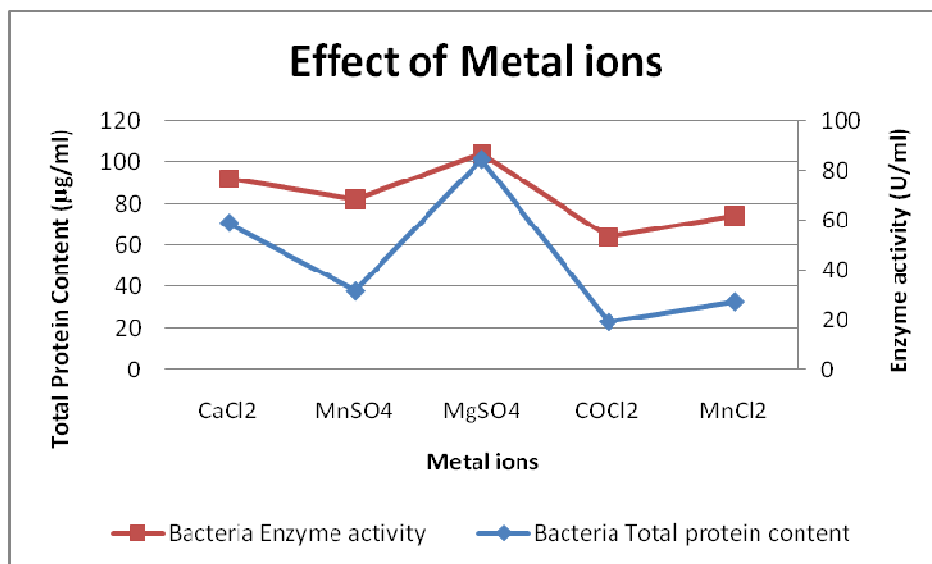


Fig. 8 Role of different metal ions on Enzyme production and total protein content

The zymogram of the present study shows the presence of Protease as green band due to staining with malachite green solution. In order to detect these *Bacillus* proteases, zymographic techniques are widely used [41, 42, 43].

In Tanneries, Dehairing using lime and sodium sulfide [44] brings about complete removal of hair, but the hair root remained within skin [45]. The Dehairing using Proteases have been proven to be superior for the complete removal of hair and also help in the closure of the pores.

When compared to the other proteases, the alkaline proteases were found to be having more applications in various industries. From the research, it has been well proven that alkaline protease producing bacteria can be isolated from dairy effluent. The dehairing application of alkaline protease was also evidenced.

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