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OPTIMIZATION OF PROTEASE ENZYME PRODUCTION BY MARINE ACTINOMYCETES

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ABSTRACT

A light yellow, whitish and greyish pigment, proteolytic *actinomycetes* strain was isolated from marine soil samples collected form the coastal area of Chennai, India. In this study 114 isolates of *actinomycetes* were isolated and identified by morphological studies. The isolate grows to produce circular colonies that had yellow, white and greyish substrate mycelium on starch casein agar. Its proteolytic activity was indicated by growth and clear zone appearance on casein skim milk agar plate method. From the total 114 isolates 8 showed proteolytic activity; among them one strain MB22 which showed higher protease activity with lower biomass and it was taken for further investigation. The growth conditions of protease production by identified *actinomycetes* were optimized with different fermentation period, pH, Temperature, carbon source, nitrogen source. Among the different carbon source, sucrose was found to be best source. Beef extract was found to be the optimum nitrogen source for protease enzyme production by the *actinomycetes*. The best source was found to be pH-4, for temperature 46˚C. The amount of protein was estimated by Bradford method and the molecular weight was determined by Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

KEY WORDS: ***Marine Actinomycetes, Protease activity, Optimization, SDS-PAGE.***

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INTRODUCTION

Marine Microorganisms have recently emerged as rich source for the isolation of industrial enzymes[1]. Proteases are also isolated from marine algae[2]. *Actinomycetes* are of universal occurrence in nature and are widely distributed in natural and man-made environments. They are found in large numbers in soils, fresh waters, lake, river bottoms, manures, composts and dust as well as on plant residues and food products. However, the diversity and distribution of *actinomycetes* that produce secondary metabolites can be determined by different physical, chemical and geographical factors[3,4]. Proteases have been partially purified from marine waste like crustaceans shells and fish scales[5]. Proteases are the single class to enzymes which occupy a pivotal position with respect to their applications in detergents, pharmaceuticals, brewing, leather, food industry and waste treatments[6]. *Actinomycetes* are excellent sources of biotechnologically important compounds and that still makes them one of the most sought after microbes to research on, despite decades of research dedicated to unravel their bioprospects. Proteases are classified based on chemical nature of the active site, the reaction they catalyze, their structure and composition[7]. The major classes are again classified into subclasses based on pH, catalytic site on polypeptide, occurrence and so on. Protease deficiency leads to arthritis, osteoporosis and other calcium deficient diseases. This leads to hypoglycemia, resulting in moodiness, mood swings and irritability. Proteases from marine microorganisms due to their inherent stability at different values of pH, temperature and salinity[9,10,11]. The production of extracellular thermostable alkaline protease by a thermoalkalophlic *Bacillus sp. JB-99* under solid state fermentation was investigated using various agro wastes as inexpensive solid substrate raw materials[12]. The present study was aimed to isolate the protease producing strain and to optimize its nutritional factors like optimum carbon and nitrogen sources and different cultural conditions such as temperature and pH. The resulting sample was characterized by Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis followed by Silver nitrate staining.

MATERIALS AND METHOD

All the chemical and culture media used in the present study were of the AR grade and protease, Starch, Casein, agar and agarose were purchase from Hi-Media Ltd, Mumbai, India. Buffer salts like Tris, ammonium sulfate, calcium carbonate, ferrous sulfate, potassium nitrate, potassium hydrogen phosphate, magnesium sulfate and sodium chloride were purchased from Merck India limited, Mumbai, India. Corning® 96 Well Clear Polystyrene Microplate was purchase from Corning Incorporated life Sciences, Acton, MA, USA. Acrylamide, bis acrylamide, TEMED, ammonium persulphate, ethanol, glacial acetic acid, glycine, Tris HCL, glycerol, bromophenol blue, 2-mercaptoethanol, comassie brilliant blue, Staining box, Silver nitrate and Bovine serum albumin were purchased from S.D. Fine Chemicals limited, Mumbai, India.

Sample Collection

The Marine soils were collected from Chennai coastal region namely from Marina Beach, Besant nagar beach and Neelankarai beach. The samples were collected using alcohol rinsed person grab and were transferred to New Zip lock bags using sterile spatula and were subjected to drying process[13,14].

Isolation of Actinomycetes

The collected soil was treated with calcium carbonate. Starch casein agar (SCA) medium and used for the isolation of actinomycetes[14,15]. The plates were incubated at 28˚C for 6 to 7 days[16]. The isolated agar plates were observed for the presence of actinomycetes colonies 3rd day onwards. Single separated colonies were selected and the subculture was maintained in SCA slants at 4˚C until further use.

Morphological Studies by Cover Slip Method

The isolated strains were confirmed as *actinomycetes* by studying their morphology under microscope.

Microscopic Examination

The following microscopic observations were recorded using cover slip culture.

1. Presence or absence of substrate mycelium.
2. Fragmentationof substrate mycelium
3. Sclerotia or sporangia
4. Spore chain morphology.

The generic level identification was carried out by using Bergey's manual of determinative bacteriology 8th edition (half 1994)[17].

Screening of Protease Activity

The Isolated *actinomycetes* colonies were plated onto skim-milk agar plate method. The plates were incubated for 5 days at 28˚C. A clear zone on skim-milk hydrolysis gave an indication of protease producing strains. Different colonies from the plates were purified through repeated streaking on fresh agar plates [18.] The enzyme activity was visualized as clear zone around the wells and the diameter of the proteolytic zone was measured. The strains with maximum protease activity were selected for optimization process.

Downstream processing - Protease production

The Starch casein broth was prepared [14,15]. and inoculated with actinomycetes inoculum and maintained at 37˚C for 24 to 72 hrs 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 20 minutes and the clear crude supernatant was stored at 40˚C for further studies [19]

Optimization parameters

Effect of Carbon source on protease production

The sterile production medium was prepared in different Erlenmeyer flasks. Each flask was amended with different carbon sources. The medium was supplemented independently with 1% of glucose, maltose, sucrose, lactose, dextrose. The individual medium was sterilized, test samples were inoculated and incubated at 37˚C and kept on the shaker for 48 hrs at 200 rpm. The culture was filtered by centrifugation process, the filtrate was collected and the enzyme activity was determined.

Effect of Nitrogen source on protease production

The sterile production medium was prepared in different Erlenmeyer flasks. Each flash was amended with 1% of different nitrogen source like ammonium chloride yeast extract, ammonium sulphate, potassium nitrate, peptone, beef extract. The individual medium was sterilized, test samples were inoculated and incubated at 37˚C and kept on the shaker for 48 hrs at 200 rpm. The culture was filtered by centrifugation process, the filtrate was collected and the enzyme activity was determined.

Effect of Temperature on protease production

The sterile production medium was prepared in different Erlenmeyer flasks and inoculated with 1% inoculated test sample, each flask was incubated at different temperature such as 44˚C, 46˚C, 48˚C, 52˚C, 54˚C, 56˚C and 58˚C for 48 hrs at 200 rpm. The enzyme activity was estimated.

Effect of pH on protease production

The sterile production medium was prepared in different Erlenmeyer flasks and was adjusted to different pH such as 4.0, 4.2, 4.4, 4.6, 4.8, 5.2, 5.4, 5.6 5.8, and 6.0. After sterilization flasks were inoculated with 1% inoculated test sample. The flaks were incubated at 37˚C shaker at 700 rpm for 48 hrs. The Enzyme activity was estimated.

Protein Determination

Protein content of the culture filtrate was estimated by Bradford method and the amount of protein was calculated using Bovine Serum Albumin Fraction as standard[20].

SDS-PAGE analysis

SDS-PAGE was performed using acrylamide gel (12.5%) by the method of Laemmli[21]. The protein markers were run along side of the sample (10 µg protein sample was used). Protein bands were stained with Silver nitrate.

RESULTS AND DISCUSSION

Screening and Identification of protease producing Actinomycetes

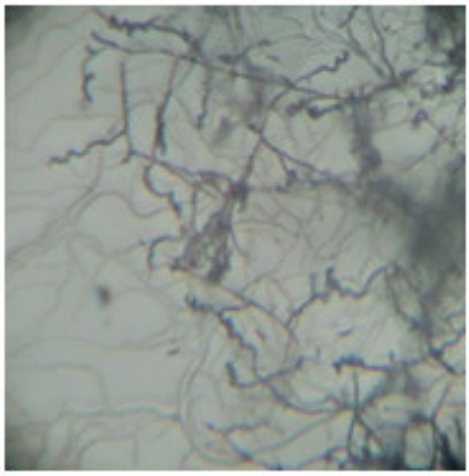
A total of 114 *actinomycetes* samples were isolated from marine soil by SCA plate method (Table 1). The *actinomycetes* strains were isolated by colony color and categorized into light whitish, greyish, light and dark brownish grey (Figure 1). The isolates were identified according to the morphological tests (Figure 2). Then it was tested for the ability to the produce protease enzyme by using skim milk agar plate method. The protease activity of *actinomycetes* was high in casein and the clear zone was observed. The appearance of clear zone around the colonies, which is surrounded by white color background with skim milk agar plate, indicated the presence of protease activity (Figure 3). The maximum protease producing strains were selected, depending upon the highest clear zone. In that 8 of them showed the highest protease activity of clear zone in mm. It was found that the *actinomycetes* strain MB22 showed that largest clear zone and it was selected for optimization process and production of protease enzyme.

Table 1
Collection of Soil Samples for isolation of Actinomycetes

S. No	No of Isolates	Symbol of Strain	Collection area
1	57	MB1-MB57	Marina Beach
2	40	BN1-BN40	Besant nagar
3	17	NK1-NK17	Neelankarai

A) Strain MB22-light whitish, B) Strain NK17- Greyish, C) Strain MB41-Brownish

Figure-1
Screening and Identification of protease producing Actinomycetes



Strain MB22

Figure 2
Photograph showing spore chain morphology of actinomyctes isolate No: MB22

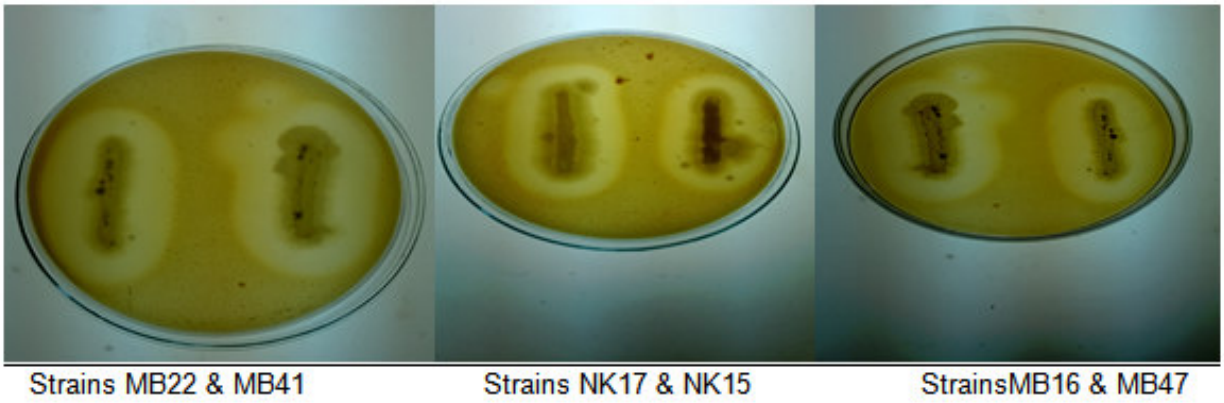


Figure-3
Screening of Protease Activity

Effect of Carbon source on protease activity

Carbon source of different origins were used in the production medium for determining the highest yield of enzyme production. Results indicated the role of carbon compounds in maximizing the production of protease enzyme, sucrose was found the optimum source (Figure 4).

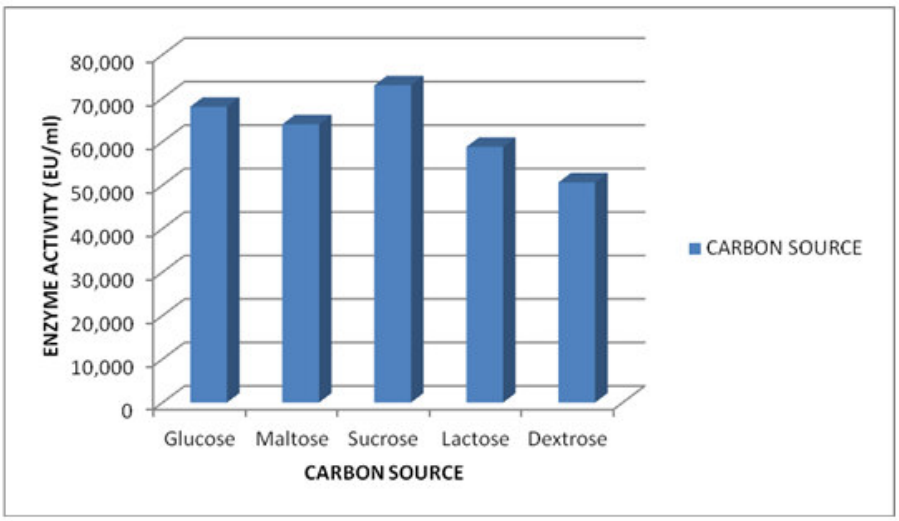


Figure 4
Effect of Carbon source on Protease activity

Effect of Nitrogen source on protease activity

The nitrogen source influence the production of protease. The varying natures of the nitrogen source were tested for the entire nitrogen source. The strain showed that the maximum activity of the protease production in the presence of beef extract (Figure 5).

Effect of Temperature source on protease activity

The optimal temperature for the production of protease is in the range 44-58˚C. This enzyme has significant activity over the wide range of temperatures. The maximum amount of protease production was found at 46˚C, and the minimum amount of protease production was found at 58˚C (Figure 6).

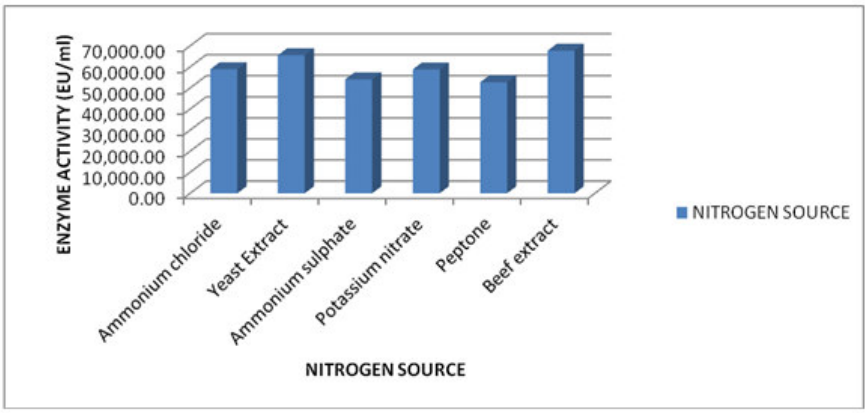


Figure-5
Effect of Nitrogen source on protease activity

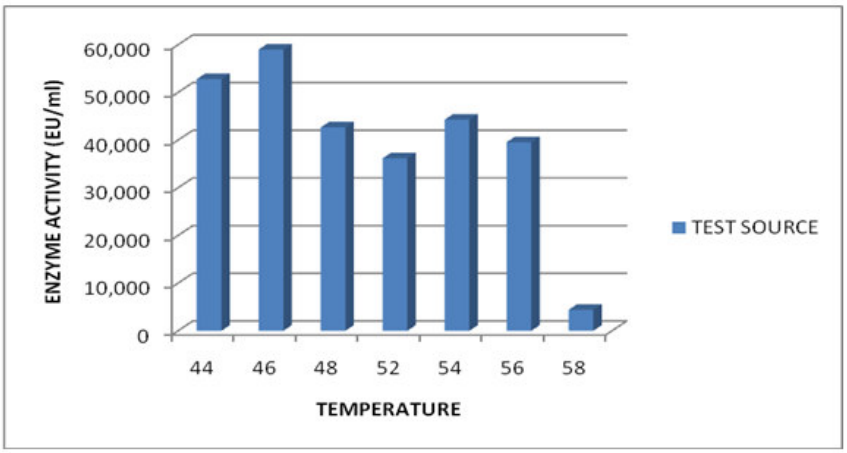


Figure-6
Effect of Temperature source on protease activity

Effect of pH source on protease activity

The pH raging from 4.0-6.0 was tested for the detection of optimum pH for high protease production and the result showed that the production of enzyme was maximum at pH 4.0 and minimum at pH 4.4 (Figure 7).

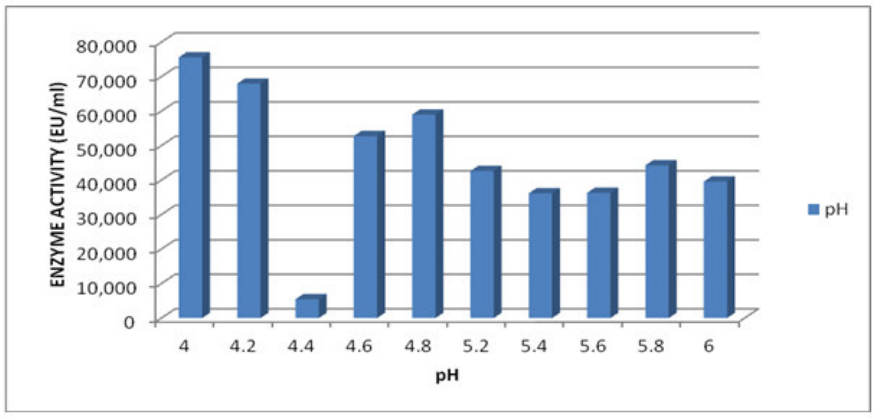


Figure-7
Effect of pH source on protease activity

Protein Estimation

Protein content of the crude enzyme was quantified by means of Bradford method 1976[20]. and it was found to the118.86 mg/ml.

Determination of Molecular weight by SDS-PAGE

The molecular weight (58 kDa) of the purified protease enzyme was analyzed using SDS-PAGE analysis (Figure 8).

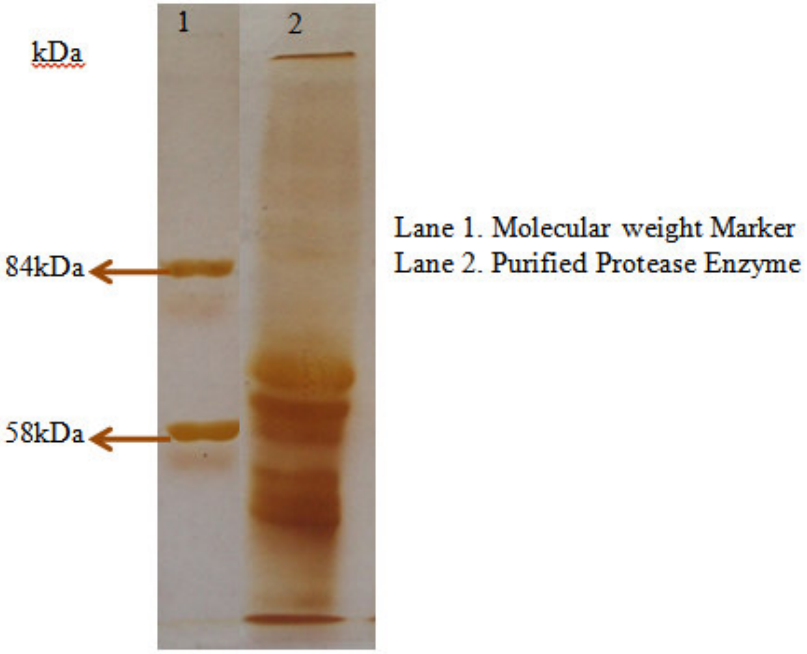


Figure 8
Determination of Molecular weight by SDS-PAGE

Protease is considered as an industrially important enzyme that showed a wider range of application in pharmaceutical, leather, laundry, food, waste processing as well as in textile industry. Preferable type of proteases used as detergent additive. In our search for protease, out of 114 actinomycetes strains isolated, only 8 strains showed protease activity as they qualitatively screened by using skim milk agar plate method. The 8 *actinomycetes* strain isolates MB41, MB16, MB47, BN3, BN16, NK17, NK15 and MB22 exhibiting higher protease activity with maximum yield for MB22 isolate. Several investigators used different screening plate media in their search for proteases[22, 23, 24]. By studying the effect of different carbon and nitrogen source, it was found that the optimum enzyme yield had been established in case of sucrose as the carbon source and beef extract as the nitrogen source. The optimum temperature and pH for protease production were determined, the actinomycetes showed optimum activity at 46˚C and pH 4.0. This study has given a hint that microbial wealth of protease producing from actinomycetes isolated from marine soils of Marina beach, Besant nagar, and Neelankarai.

CONCLUSION

The result obtained from the present investigation of the isolated *actinomycetes* strain MB22 was found to be having ability to produce economically important protease enzyme. The MB22 strain gave highest production of protease enzyme with high activity after optimization process. Further more the activity and stability profile of the protease enzyme from actinomycetes strain MB22 suggesting its potential for the further industrial application.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Adinarayana K, Ellaiah P. Response surface optimization of the critical medium components for the production ofalkaline protease by a newly isolated Bacillius sp. Indian J. Pharm.Sci. 2002 5(3): 272-278.

2. Swapnil Suresh Patil and Jeyanthi Rebecca L. Isolation and Characterization of Protease from Marine Algae. Int. J. Pharm, Sci. 2014.27(1): 188-190.

3. Gurung TD, Sherpa C, Agrawal VP, Lekhak B. Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar, Mount Everest Region. Nepal J Sci Technol. 2009; 10:173–182.
4. Ogunmwonyi IH, Mazomba N, Mabinya L, Ngwenya E, Green E, Akinpelu DA, et al. Studies on the culturable marine actinomycetes isolated from the Nahoon beach in the Eastern Cape Province of South Africa. Afr J Microbiol Res. 2010;4(21):2223–2230.
5. L Jeyanthi Rebecca L, Sharmila S, Merina Paul Das and Abraham Samuel F. Production and analysis of Protease from Aspergillus niger using fish scales as substrate J. Chem and Pharm Res. 2012; 4(10): 4597-4600.
6. Gupta A, Roy I, Patel RK, Singh SP, Khare SK, Gupta MN. One-step purification and characterization of an alkaline protease from haloalkaliphilic Bacillus sp.J Chromatogr. 2005; 1075 (1.2): 103- 108.
7. Rao, MB., Tanksale, AM., Ghatge, MS and Deshpande VV. Molecular and Biotechnological aspects of microbial proteases. Microbiol, Mol. Bol 1998; 62:597-635.
8. Bitange NT, Zhang Wang shi-Ying Xu and Wenbin Zhang. Therapeutic Application of Pineapple Protease. Pakistan Journal of Nutrition. 2008; 7(4): 513-520.
9. Naidu KSB, Devi KL. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. Afr. J. Biotechnol. 2005; 4: 724-726.
10. Sen S, Satyanarayana T. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. Indian J Microbiol 1993; 33:43–47.
11. Jayaraman G, Shivananda P. Isolation and characterization of a metal ion-dependent alkaline protease from a halotolerant Bacillus aquimaris VITP4. Ind J Biochem Biopsy. 2011:48(2) 95-100.
12. Patil J U., Bajekal S. Halostable alkaline amylase and protease from an extremely haloalkaliphilic archaeon, natrinema sp. Ssbjup-1 isolated from lonar lake. Int J Pharm Bio Sci. 2015; 6(1): (B) 777 – 786.
13. Dhevendaran K, Anithakumari K. L-asparaginase activity in growing conditions of streptomyces sp., associated with Therapon Jarb uo and Villiorita Cuprinoids of Veli lake, south India. Fish Technology. 2002; 39: 155-159.
14. Viswanathan K, Jeyanthi Rebecca L, Arumugam P, Anbarasu K, Isolation and screening of protease producing marine Actinomycetes from Chennai coastal region. Int.J.Adv Res: Biol Sci. 2015: 2(8) 153-157.
15. Radhakrishnan M, Balaji S, Balagurunathan R. Thermotolerant Actinomycetes From The Himalayan Mountain – Antagonistic Potential, Characterization And Identification Of Selected Strains. Malays.Appl. Biol. 2007; 36 (1), 59-65.
16. Savitri AN, Azmi W. Microbial L-asparaginase; a potent antitumour enzyme. Ind. J. Biotech. 2003; 2: 184-194.
17. Holt JG. Bergey's manual of determinative bacteriology 9th edition (Willian and Wilkin Baltimore), pp 1994; 667-669.
18. Panuwan Chantawannakula, Anchalec Oncharoena, Khanungkan Klanbuta, Ekachai Chukeatiroteb and Saisamorn Lumyonga. 2002.
19. Characterization of protease of Bacillus subtilis Strain 38 isolated from traditionally fermented soybean in Northern Thailand, Science Asia 2002; 28: 241-245.
20. Kumara swamy M, Kashyap SSN, vijay R, Rahul tiwari, Anuradha M. Production and optimization of extra cellular protease from Bacillus sp. Isolated from soil. Intl. J. Of adv. Biotec. And res, 2012; 3(2) 564–569.
21. Bradford M M, (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* 72: 248–254.
22. Laemmli U K, (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
23. Aftab S, Ahmed S, Saeed S and Razoo S A. Screening, isolation and characterization of alkaline protease producing bacteria. Pak. J. Biol. Sci. 2006; 9:2122-2126.
24. Rao K, Narasu M L. Alkaline Protease from Bacillus firmus 7728. Afr. J. Biotechnol. 2007; 6: 2493-2496.
25. Kasana RC, adav S K. Isolation of a psychrotrophic Exiguobacterium sp. SKPB5 (MTCC 7803) and characterization of its alkaline protease. Curr.Microbiol. 2007; 54: 224–229.

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