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RESEARCH ARTICLE

Optimization, Production and Partial Purification of an Extracellular Alkaline Protease from Halophilic Bacterium *Bacillus licheniformis* EBPL0007

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ABSTRACT:

The Halophilic bacterium EBPL0007 was obtained from the Eukpro Biotech Private Limited, R&D Lab, Chrompet, Chennai. The Halophilic bacterium screened for four industrially important enzymes, out of which protease showed the maximum zone of clearance. Halophilic bacterium EBPL0007 was identified as *Bacillus Licheniformis* by polyphasic taxonomical approach and submitted in GenBank with the accession number MW387224. The medium for protease production was optimized as Glucose as a carbon source, peptone as a nitrogen source, 0.05% of gelatin as a substrate, 3% NaCl, pH-9 and 36°C, *Bacillus Licheniformis* was mass cultured in optimized production medium, harvested after 9 days of incubation and the enzyme protease was partially purified by Acetone precipitation method. The partially purified enzyme was lyophilized and the effect of temperature, pH, NaCl and metal ions was analyzed. The enzyme activity was found to be 3150 units/ml in partially purified. The crude enzyme was used for the washing test to compare the results with the available detergents in the market as its application.

KEYWORDS: Halophilic bacterium, Alkaline Protease, *Bacillus licheniformis,* Polyphasic taxonomy.

INTRODUCTION:

Halophilic microorganisms thrive in salty and highly saline environments. Most belong to the Archaea domain, with some bacterial and eukaryotic halophiles. These organisms endure extreme conditions like high pH, salinity, and temperature, classifying them as poly-extremophiles[1]. Halophiles are grouped based on their salt requirements: (1) Extreme halophiles (15–30% NaCl), (2) Moderate halophiles (3–15% NaCl), and (3) Slight halophiles (1–3% NaCl). High salt levels cause osmotic imbalances in cells, but halophiles adapt by producing neutral organic molecules called compatible solutes, which protect cellular components[2,3,4].

Microbial proteases are vital hydrolytic enzymes, contributing ~59% of total enzyme applications[5] These

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enzymes are used in food, detergent, and medical industries. Halophilic enzymes, known for stability under extreme conditions, are ideal for laundry and food industries due to their thermostability and activity at high pH[6]. Proteases are classified by activity under acidic, alkaline, or neutral conditions and are used in brewing, meat tenderization, and leather processing[7,8]. Alkaline proteases are valued in industries for their stability across pH variations.

The demand for alkaline proteases has increased to address salinity and groundwater pH issues. Bacillus species, especially *Bacillus licheniformis*, are prominent producers of alkaline proteases, secreting large amounts of extracellular proteins[9]. Genetic engineering has improved strains for higher yields[10]. This study focuses on extracellular alkaline protease production from halophilic *Bacillus licheniformis* for industrial applications.

MATERIALS AND METHODS:

Halophilic Bacteria Isolate:

In this study Gram-positive, filamentous halophilic bacteria were procured from Eukpro Biotech Private Limited, chrompet and named as EBPL0007 as the source of protease production.

Maintenance of the Culture:

Screening of EBPL0007 for industrially important enzymes:

EBPL0007 was screened for industrial enzymes (amylase, cellulase, protease, and lipase) by streaking on nutrient agar with respective substrates. After 3 days of incubation at 37°C, indicators were added to detect clear zones around the colonies.

Primary screening for protease activity by plate assay:

The isolate was screened for protease production using a protease production medium and it was incubated at 37°C for 4 days. Then, clear zone was observed in addition of saturated ammonium sulphate in 1N HCL.

Biochemical characterization:

Microscopic and several biochemical reactions were carried out which includes cell shape, motility, Gram's staining, catalase, oxidase, Indole, MRVP, Citrate utilization, Triple sugar, Urease and Nitrate reduction tests were performed following standard procedures.

Molecular identification of halophilic bacteria:

DNA isolation from Halophilic Bacteria EBPL0007:

The DNA was extracted from the strain EBPL0007and then allowed to run in an agarose gel, a single DNA band was observed. The molecular weight of the DNA was identified as 1.3 kbps by comparing it with the marker DNA

Amplification of 16s rRNA gene:

The amplified PCR product was allowed to run in an agarose gel and the gel was observed under a UV trans-illuminator. A single band was observed its molecular weight was identified as 1200 bp by comparing it with the marker. The amplified product were sequenced and submitted in NCBI Gen Bank.

Optimization of production medium for increased protease production:

The culture was grown in different concentrations of gelatin (0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5, and 3%), different time interval (2, 4, 6, and 8 days), different temperatures like (16, 28, 36, 45 and 58°C) different concentrations of NaCl 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, and 20%. different pH ranges (5, 7, 9, 11, and 13). carbon Sources like Cellulose, Starch, Sorbitol, Mannitol and Glucose, nitrogen Sources like Yeast extract, Peptone, Ammonium sulphate and Beef extract were amended separately in protease production medium. The culture was inoculated and incubated. After incubation period, 0.1N HCL saturated with ammonium sulphate was added to observe the clear zone

Mass production of enzymes using optimization of media:

After the optimization formulate our own optimized media was used for the mass production of the enzyme. The produced enzyme was precipitated with acetone and lyophilized for further studies.

Production of protease enzymes:

The halophilic bacteria were grown in 0.05% of gelatin medium for protease production. After the incubation, the culture broth was centrifuged and the supernatant was estimated for protein content.

Partial purification of protease enzyme:

Acetone precipitation:

Proteins are insoluble in acetone at low temperatures. Acetone was pre-cooled to -15°C and it was added to the crude enzyme in 1:2 ratios. Then it was stirred and centrifuged at 4°C. Further the protein was precipitated and dissolved in 50mL of Tris HCL Buffer.

Qualitative plate assay:

A qualitative plate assay was carried out by adding different concentrations of culture filtrate into the wells in petriplate containing 0.05% gelatin. After incubation period, the enzyme activity was measured by adding the saturated ammonium sulphate in 0.1N HCL to observe the clear zone.

Estimation of total protein content:

100µL of the sample was mixed with 1mL of the tris HCL buffer and then 5mL of CBB 250 was added and

mixed well to develop colour. The absorbance was read at 595nm and the estimated protein was then compared with the standard graph. The enzyme activity in the culture filtrate was measured by the dye-binding method of Bradford[11].

Characterization of partially purified protease:
Effect of Different Temperature:
The effect of partially purified enzyme on different temperature from 4˚C, 24˚C, 37˚C, 54˚C, 80˚C and 100˚C for 15, 30, 60 min and the enzyme activity was determined.

Effect of different pH:
The Effect of different pH (3–13) by buffers: citric acid–sodium phosphate (pH 3), Sodium phosphate (pH 7), Glycine buffer (pH 9) and Borax buffer (pH 11). To measure the enzyme activity the partially purified enzyme was incubated with the respective buffer for 15, 30, 60 min.

Effect of metal ions and inhibitors:
For the effect of metal ion such as $CaCl_2$, $MgCl_2$, $CuSo_4$, $MgSo_4$ ZnCl2 and $FeCl_2$ were used for the study. The partially purified enzyme was incubated for 15, 30, 60 mins in 5mM concentration at room temperature, the enzyme activity was measured.

Application of protease enzyme:
Washing test with protease:
To analyze the effect of partially purified enzyme, it was used for washing test as a additive in detergent. The enzyme (100mg/ml) along with the detergent drastically improves the cleaning within 10 mins. The experiment was achieved by washing a cotton cloth pieces (10 x 10) stained with human blood, chocolate and Egg yolk.

RESULTS AND DISCUSSIONS:

Isolation and identification of halophilic bacteria:
EBPL0007 was identified as the best strain for protease production, yielding maximum activity in qualitative screening using gelatin agar plates (Fig. 1), consistent with previous studies using protease plate assays[12].

Fig. 1: Screeing for Protease Production

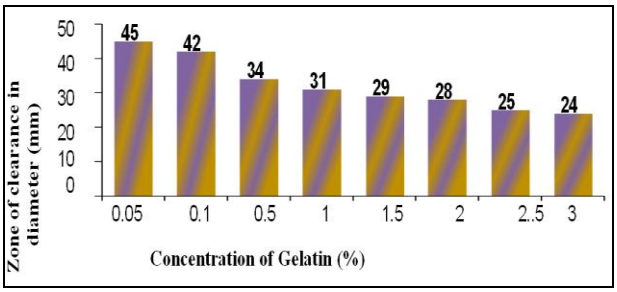

Fig. 2: Optimization of Protease Production in Different Concentrations of Gelatin

Biochemical tests were conducted for EBPL0007. DNA was extracted, and a single DNA band of 1.3 kbps was observed in agarose gel, confirmed using marker DNA. The PCR-amplified product was also run on agarose gel, revealing a single band with a molecular weight of 1200 bp. The amplified 16S rRNA gene was sequenced and identified as Bacillus licheniformis, with the sequence submitted to GenBank under Accession Number MW387224. This aligns with studies identifying high protease-producing bacteria through 16S rRNA gene sequence analysis[13].

Optimization of protease production in different concentrations of gelatin:
The culture was grown in different concentrations (0.05%, 0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, and 3%) of gelatin amended in a minimal medium to find the appropriate concentration for utmost

protease production. 45mm zone of clearance was observed in 0.05% gelatin. (Fig. 2)[14] state that *B. subtilis* possess 0.03% of gelatin concentration for higher proteolytic Activity.

Optimization of Protease Production In The Different Time Intervals:
The culture was streaked onto plates containing 0.05% gelatin and incubated at different time intervals. The maximum zone of clearance of 73mm was observed on Day 8. (Fig. 3). The metabolic and extrinsic parameters, such as cultivation duration, medium, and temperature, have an impact on microbial growth[15].

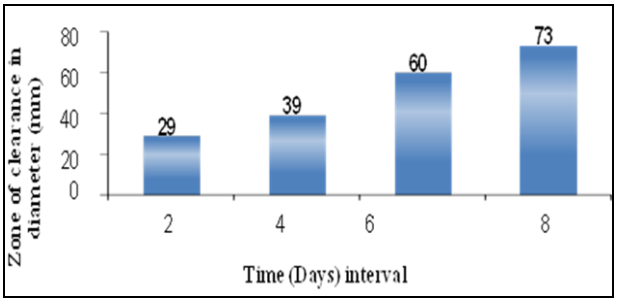

Fig. 3: Optimization of Protease Production in Different Time Intervals

Optimization of protease production in the different temperatures:

The culture was streaked onto plates with 0.05% gelatin and incubated at varying temperatures. The largest clearance zone (48mm) was observed at 36°C (Fig. 4). Temperature is a key factor in fermentation, influencing enzyme reaction rates, metabolic energy, and biomass synthesis for optimal protease production[16,17,18].

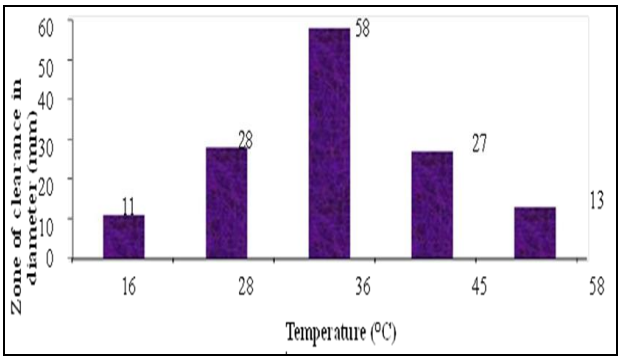

Fig. 4: Optimization of Protease Production in Different Temperature

Optimization of protease production in the different NaCl concentrations:

The culture was inoculated onto plates containing 0.05% gelatin and different concentration of NaCl. Among these concentrations, the maximum zone of clearance of 84mm was observed in 3% NaCl followed by 81mm observed in 2% NaCl. (Fig. 5). This similar salinity study was carried out in *Bacillus sonorensis* BH3 by[19].

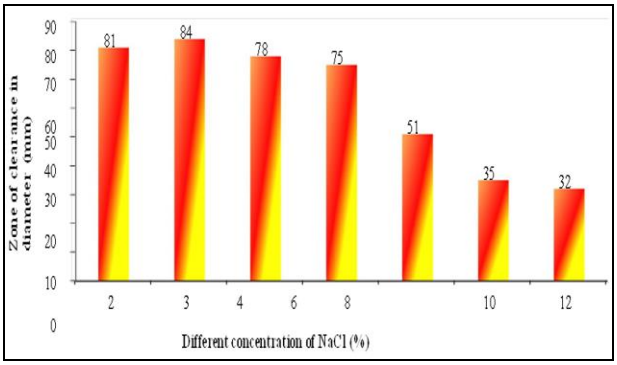

Fig: 5 Optimization of Protease Production in Different NaCl concentration

Optimization of protease production in the different pH:

The culture was inoculated into plates containing 0.05% gelatin, and 3% NaCl and the pH was adjusted in different plates to pH 5, 7, 9, 11 and 13. The maximum zone of clearance of 86mm and 85mm was observed in pH 9 and 11 respectively. (Fig. 6). [13],2022 showed that protease produced by *Bacillus licheniformis* using bran waste showed the maximum enzyme activity at pH 8.

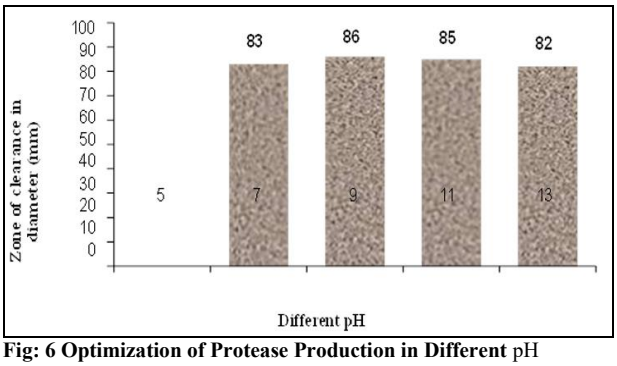

Fig: 6 Optimization of Protease Production in Different pH

Optimization of protease production using different carbon source:

The culture was inoculated into plates containing 0.05% gelatin, 3% NaCl and pH 9 each plate had different carbon sources such as cellulose, starch, sorbitol, mannitol and glucose. The maximum zone of clearance of 80mm and 75mm was observed in Glucose and starch respectively. (Fig.7)[20] carried out his work by adding 1% of Glucose as a carbon source in AKS-4 bacterial strain [21] also similarly reported.

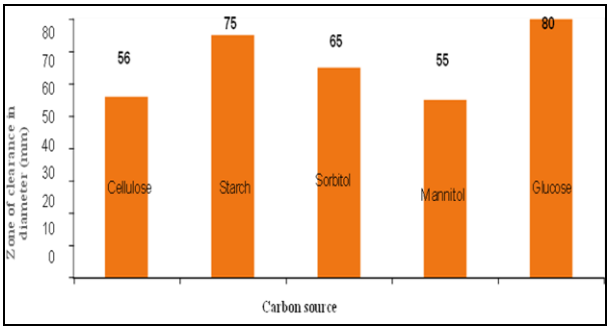

Fig. 7: Optimization of Protease Production of Different carbon source

Optimization of protease production using different nitrogen sources:

The culture was inoculated onto plates with 0.5% gelatin, 3% NaCl, and pH 9, with different nitrogen sources: yeast extract, beef extract, peptone, ammonium sulfate, and Sodium Nitrate. An 80 mm clearance zone was observed with peptone (Fig. 8), indicating it as the best nitrogen source for protease production. Similarly, *N. prasina* HA4 strain [22] and *B. licheniformis* showed maximum production using peptone and yeast extract.

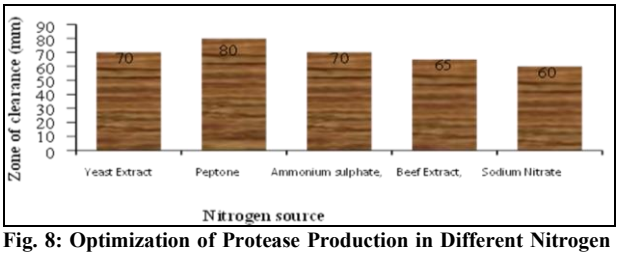

Fig. 8: Optimization of Protease Production in Different Nitrogen source

Partial purification of enzyme:
The optimized medium was prepared, inoculated with 50mL seed culture, and incubated in a shaker for 96 hours. After centrifugation, the supernatant was collected, acetone (1:2 ratio) was added for protease precipitation, and the precipitate was centrifuged. The pellet was dissolved in Tris-HCl buffer and analyzed using qualitative and quantitative assays.

Qualitative Assay:
The enzyme was analyzed by well method by addition of different concentration of samples at (50, 100, 150, 200, and 250µL) into the well of gelatin amended medium showing the clear zone was about 18mm, 20mm, 22mm, 24mm and 26mm, which can be visualized by adding saturated ammonium sulphate in 1N HCL (indicator). (Fig. 9)

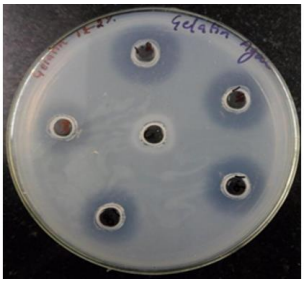

Fig: 9 Qualitative Assay For Protease Enzyme

Estimation of total protein and enzyme activity:
Total protein estimation was carried out by the Bradford method and the reaction mixture contained 1ml of enzyme sample with 5ml of CBB-G 250. The result shows 60ug/ml with 10mg in 1ml of water. The enzyme assay followed by the standard method shows an enzyme activity of 3150 units/ml.

Characterization of protease:
Effect of Different Temperature:
For thermal stability of protease enzyme it was pre-incubated at room temperature between 4˚C, 24˚C, 37˚C, 54˚C, 80˚C and 100˚C from 15 to 60 minutes at optimum pH and the enzyme were stable up to 37ºC. (Fig. 10)[23] investigated the same and found the activity of the enzyme was stable upto 54ºC.

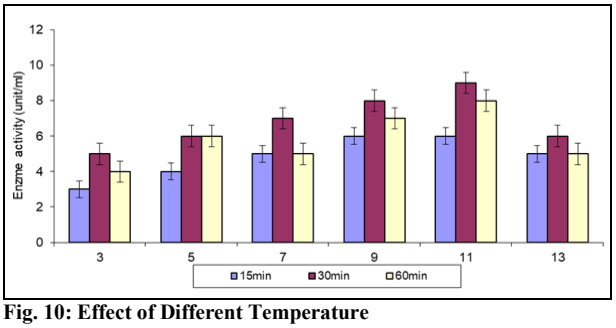

Fig. 10: Effect of Different Temperature

Effect of pH:
The stability of the enzyme ranges from pH 3, 5, 7, 11 and 13, the enzyme activity was stable upto pH 11. (Fig. 11)[24] revealed that the protease enzyme produced from *Bacillus subtilis* S1 are active at higher 5% NaCl concentration and the enzyme from *Bacillus amyloliquefaciens* KSM12 has its highest activity at 10% NaCl concentration.

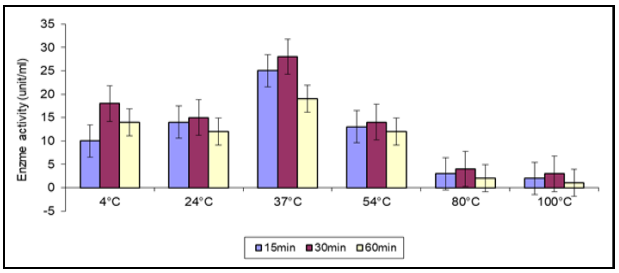

Fig. 11: Effect of pH

Effect of metal ions and inhibitors:
The effects of various metal ions (5mM) on the activity of partially purified proteases were studied at pH 11 and 37°C. Ca^{2+} significantly increased enzyme activity in EBPL0007, while $ZnSO_4$ and $CuSO_4$ inhibited it. The enzyme remained stable in Triton X-100 with a specific activity of 170µg/mL (Fig. 12). Mg^{2+} and Ca^{2+} showed substantial stimulatory effects, increasing relative activity to 125% and 140%, respectively[25]. However, thermostable alkaline proteases are sensitive to solvents, metal ions, and surfactants, limiting their applications in the detergent industry[26,27].

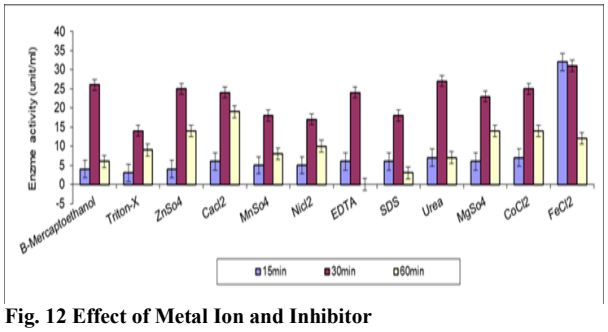

Fig. 12 Effect of Metal Ion and Inhibitor

Application of protease enzyme:
Washing test with protease:
The partially purified enzyme removed the blood, chocolate and Egg stain within ten minutes. Thus, the alkaline protease enzyme produced from the Halophilic Bacterium *Bacillus licheniformis* EBPL0007 showed that it has high prospective additive to be used in the detergent Industry. (Fig.13). The result of the study is similar to the study by[28] showed that protease produced from *Bacillus megaterium*, 2020 showed better washing performance with blood-stained fabric cloths.

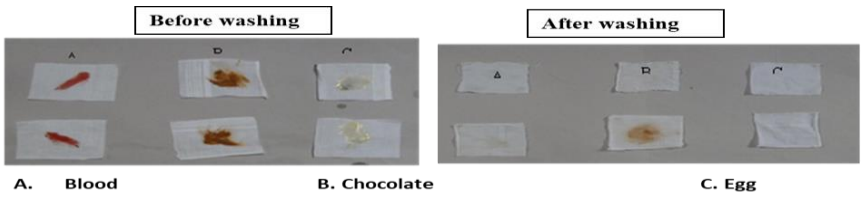

Fig. 13: Washing Test Portease

CONCLUSION:

Alkaline protease produced by bacteria is vital for pharmaceutical, food, and detergent industries, with growing future applications. In this study, the halophilic bacterium EBPL007, identified as *Bacillus licheniformis*, was a promising source of industrial protease. Optimization revealed glucose and peptone as the best carbon and nitrogen sources, with 0.05% gelatin as a substrate, 3% NaCl, pH 9, and 36°C as optimal conditions. The partially purified enzyme showed 3150 units/mL activity, with optimal performance at pH 10, 6% NaCl, and 54°C. It effectively removed stains within 10 minutes, demonstrating its potential for the detergent industry due to its stability and high activity in extreme conditions.

REFERENCE:

1. Espoui AH, Larimi SG, Darzi GN. Optimization of protease production process using bran waste and Bacillus licheniformis. Korean J Chem Eng. 2022; 39: 674-683. https://doi.org/10.1007/s11814-021-0965-3.
2. Brown AD. Microbial water stress. Bacteriol Rev. 1976; 40: 803-846.
3. Cui H, Wang L, Yu Y. Production and characterization of alkaline protease from a high-yielding and moderately halophilic strain of SD11 marine bacteria. J Chem. 2015; 1-8.
4. Mackay MA, Norton RS, Borowitzka LJ. Organic osmoregulatory solutes in cyanobacteria. Microbiology. 1984; 130: 2177-2191.
5. Chu WH. Optimization of extracellular alkaline protease production from species of Bacillus. J Ind Microbiol Biotechnol. 2007; 34: 241-245.
6. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol. 2002; 59: 15-32.
7. Cheong C, Chun SS, Kim YH. Production and properties of an alkaline protease from Pseudomonas sp. SJ-320. Korean Biochem J. 1993; 26: 479-484.
8. Joe HS, Kumar CG, Park GC, Kim KT, Paul SR, Chang CS. Optimization of the production of an extracellular alkaline protease from Bacillus horikoshii. Process Biochem. 2002; 38: 155-159.
9. Contesini FJ, Rodrigues de Melo R, Sato HH. An overview of Bacillus proteases: from production to application. Crit Rev Biotechnol. 2017. https://doi.org/10.1080/07388551.2017.1354354.
10. Suthindhiran K, Jayasri MA, Dipali D, Prasar A. Screening and characterization of protease-producing actinomycetes from marine saltern. J Basic Microbiol. 2014; 54: 1098-1109.
11. Bradford, M.M. A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry.1976; 72: 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3
12. Shaikh I, Bhagya T, Malpani J, Goudar SV. Extracellular protease production, optimization, and partial purification from Bacillus nakamurai PL4 and its applications. Int J Biol Macromol. 2023; 35(1): 102429
13. Hashmi S, Iqbal S, Ahmed I, Janjua HA. Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated Bacillus subtilis S1 and Bacillus amyloliquefaciens KSM12. Processes. 2022; 10(6): 1050.
14. Gaurav P, Anil P, Pavani JVP, Sayantan B, Devirama GVNS, Ajay K, Mitali P, Ravi GP. Production, optimization and partial purification of protease from Bacillus subtilis. J Taibah Univ Sci. 2015; 9: 50-55.
15. Campos C, Dias DC, Valle JS, Colauto NB, Linde GA. Produção de biomassa, protease e exopolissacarídeos por Pleurotus ostreatus em cultivo líquido. Arquivos de Ciência Veterinária e Zoologia. 2010; 13(1): 19-24.
16. Arcus VL, Prentice EJ, Hobbs JK, Mulholland AJ, Van der Kamp MW, Pudney CR, Parker EJ, Schipper LA. On the temperature dependence of enzyme-catalyzed rates. Biochemistry. 2016; 55(12): 1681-1688
17. Jessika GS, Ruann JC, Helia HS. Alkaline protease production by Bacillus licheniformis LBA 46 in a bench reactor: Effect of temperature and agitation. Braz J Chem Eng. 2019;36(2):615-625.
18. Wang Y, Zhang L, Xu T, Ding K. Influence of initial anolyte pH and temperature on hydrogen production through simultaneous saccharification and fermentation of lignocellulose in microbial electrolysis cell. Int J Hydrogen Energy. 2017; 42(36): 22663-22670.
19. Sankaralingam S, Harinathan B, Palpperumal S, Kathiresan D, Rajendran S, Shankar T, Prabhu D, Sivakumar N. Optimization of culture conditions for the production of halophilic protease by newly isolated Bacillus sonorensis. Am Eur J Agric Environ Sci. 2017; 17(4): 293-299.
20. Sharma KM, Kumar R, Panwar S, Kumar A. Microbial alkaline proteases: optimization of production parameters and their properties. J Genet Eng Biotechnol. 2017; 15(1): 115-126.
21. Rozanov SV, Shekhovtsov NV, Bogacheva EG, Pershina AV, Ryapolova DS, Bytyak SE, Peltek SE. Production of subtilisin proteases in bacteria and yeast. Vavilovskii Zhurnal Genet Selektsii. 2021; 25(1): 125-134
22. Ningthoujam DS, Kshetri P, Sanasam S, Nimaichand S. Proteases from extremophiles and their potential applications. World Appl Sci J. 2009; 7: 907-916.
23. Ramesh S, Rajesh M, Mathivanan N. Characterization of a thermostable alkaline protease produced by marine Streptomyces fungicidicus MML1614. Bioprocess Biosyst Eng. 2009; 32: 791-800.
24. Kalwasińska A, Jankiewicz U, Felföldi T, Burkowska-But A, Brzezińska MS. Alkaline and halophilic protease production by Bacillus luteus H11 and its potential industrial applications. Food Technol Biotechnol. 2018; 56(4): 553-561.
25. Ullah N, Rehman MU, Sarwar A, Nadeem M, Nelofer R, Shakir HA, et al. Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus cereus straain. Fermentation. 2022; 8: 628.
26. Oztas EG, Gormez A. Characterization and biotechnological application of protease from thermophilic Thermomonas haemolytica. Arch Microbiol. 2020; 202: 153-159.
27. Suthindhiran K, Jayasri MA, Dipali D, Prasar A. Screening and characterization of protease-producing actinomycetes from marine saltern. J Basic Microbiol. 2014; 54: 1098-1109.
28. Manavalan T, Manavalan A, Ramachandran S, Heese K. Identification of a novel thermostable alkaline protease from Bacillus megaterium-TK1 for the detergent and leather industry. Biology. 2020; 9(12): 472.