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Screening Production and Application of Alkaline Protease Enzyme Producing *Bacillus Cereus* from Fish

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Abstract

Alkaline protease are one of the most important enzymes in the biological world. Microbial production of alkaline protease is getting more attention from researchers due to their unique properties and substantial activity. The present study was to isolate protease producing bacteria from contaminated sardine fish sample collected from local habitat of Ernakulam. The isolation was done by serial dilution and plating methods. All the isolates were screened for proteolytic activity on Skim milk agar plate at 37˚C for 48hrs. Protease activities were determined by the formation of clear zone around the colonies on SMA medium. Protease activity was compared using protease assay. The selected isolate was identified as *B. cereus* using standard identification parameters such as Gram staining followed by molecular analysis. The16srDNA of the selected organism showed 100% similarity in the BLAST search with *B. cereus*. The activity of enzyme in purified sample and supernatant was measured by tyrosine standard curve and it was found to be as 0.48 U/ml/min respectively. This result showed that *B. cereus* is a good producer of extra cellular protease and it can be used as biocleaner or in detergent manufacture.

Keywords: Alkaline protease, *B. cereus*, skim milk agar, proteolysis, protease assay, tyrosine standard curve, detergent

Introduction

Protease are a group of enzymes that have been found in several microorganisms like bacteria and fungi which are involved in the breakdown of complex protein molecules into simple polypeptide chains (Absida, 1985). Alkaline proteases are one of the most important groups of microbial enzymes that find varied uses in various industrial sectors such as leather, detergents, textile, food and feed etc. Industrially important alkaline proteases from bacterial sources have been studied extensively, of which Bacillus sp. was most reported (Singhal *et al*., 2012) [20]. Currently, a large Proportion of commercially available proteases are derived from Bacillus strain (Sevine N *et al*., 2011). Prominent bacterial producers of this enzyme are Pseudomonas sp., Bacillus sp., Staphylococcus sp., and Aeromonas sp. (Saha and Masi 2017). Traditionally, digestion is described as the process by which food in the gastrointestinal (GI) tract is split into simpler absorbable compounds performed primarily by the digestive enzymes (Kapoor *et al*., 1975). Prior to the discussion of the contribution of the gut microbiota in production of digestive enzymes, a brief introduction regarding endogenous enzyme activities in fish seems pertinent. The endogenous digestive enzymes, which are secreted to the lumen of the alimentary canal, originate from the oesophageal, gastric, pyloric caeca and intestinal mucosa and from the pancreas (De Silva & Anderson 1995). Numerous studies have reported diverse microbial communities in the GI tract of carnivorous, herbivorous and omnivorous fish species (Merrifield and Nayak 2010). Austin (2006) and Nayak (2010) presented some information on studies of exogenous enzyme activity in fish, but a more comprehensive review is needed as the GI microbiota of fish have been reported to produce a wide range of enzymes; amylase, cellulase, lipase, proteases, chitinase and phytase. Furthermore, the role of enzyme-producing fish gut bacteria as probiotics in enhancement of food digestibility and their effect on gut enzyme activity has been evaluated through several investigations (Ray and Ghosh 2012).

Materials and Methods

A. Collection of Sample

Gut of the sardine fish samples were collected from the local habitat of South kalamassery Kochi, Kerala, India with the aid of sterile spatula. The samples were stored under sterile conditions under dark place in an airtight container. Date and time were labelled on it.

B. Isolation of Bacteria from Gut of the Sardine Fish

Serial dilution is a procedure of stepwise dilution of substance in a solution. To minimise the microbial load, the serial dilution approach was used .5gm gut of sardine fish sample was dissolved in the 50 ml of distilled water taken as a first tube which has a dilution of 10-1 and serially diluted up to 10-9 dilution. The target bacteria were isolated using serial

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dilution and spread plate techniques. Spread plate technique is employed to plate the liquid sample for the purpose of isolating the bacteria present in it. A countable number of bacterial cells were evenly distributed on an agar plate.0.1 ml of diluted sample from 10-5dilutions were spread on to the plates using an L-rod. The plates were kept at the incubated at 30°c for 24hours.

C. Primary Screening of Potential Alkaline Protease-Producing Bacterial Isolates

Primary screening of bacterial isolates was made to screen alkaline protease producers using skim milk agar, 2.6 g of nutrient agar was mixed with 80 ml of distilled water by autoclaving. Another 20 ml of distilled water, 2.8g of skim milk powder was dissolved by heating at 50 ºC. The milk solution was cooled and then combined with the agar in one conical flask. The solution was poured into the petri plates and allowed it to solidify under the laminar air flow chamber and maintaining pH 8.5 by using pH paper strips. The isolates are spotted on SMA plates and kept inside the incubator at 37 ºC for 24 hours at inverted position.

D. Identification of Bacteria

The identification of bacteria was carried out by morphological studies i.e. Gram staining. Cultural characterization on agar plates like colony morphology that is shape, size, margin, elevation, opacity, texture and pigmentation.

E. Production of Protease Enzyme

To isolate was inoculated in to the medium (0.1% peptone, 0.5% glucose, 0.05% NaCl (w/v), and 0.01% MgSO4.7H2O) and put in shaking incubator (150 rpm, 48h). Te culture-pellet was centrifuged (10,000 rpm; 150 min at 4 °C), then added to it the 500 µL of 1% casein in 50 mM phosphate bufer (pH 7) and 200 µl of cell-free supernatant, then incubated in a water bath (40 °C, 20 min), and the reaction was terminated with the addition of 1mL of 10% TCA, subsequently kept at room temperature (15 min). Te unreacted casein was separated by centrifuging the mixture, fnally the supernatant was added with Na2Co3 (2.5 m ML) and Folin-Ciocalteu phenol and incubated in dark room (30 min). Te OD was taken at 660 nm, using the standard. One unit of protease is defned as the amount of enzyme that releases 1 µg/ml/min of tyrosine.

F. Molecular Identification of Selected Isolate

The isolate was identified using the molecular technique. The genomic DNA of the isolate was extracted by using the Bacterial Genomic DNA extraction kit according to the manufacturer protocol (QIAGEN, QIAamp DNA Mini Kit) with some modification. The isolated DNA was then amplified using the following PCR mix: 1 µl of bacterial universal 16S rRNA primers forward (5'-AGAGTTTGATCMTGG-3') and 1 µl of reverse primer (5'-ACCTTGTTACGACTT-3'), 2 µl of genomic DNA and 6 µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to the NCBI database and NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbors of the isolates.

G. Application Study

Removal of Stain (Washing Test)

This experiment was done to evaluate how protease enzyme works as a bio-cleaner by their washing performance on the blood stain. A clean piece of cloth was cut into 4 pieces and stained with blood. Then cotton fabrics was treated with Distilled water, Crude enzyme (protease), and Crude enzyme with detergent at room temperature for 30 minutes. After incubation cloth was rinsed with water for 5 minutes and then dried. The same procedure was done with control (detergent).

Result

A. Sample Collection

The sardine fish sample was collected from a local fish market in kalamassery town, Kochi, India.

Fig 1: Sardine fish sample

B. Spread Plate Method

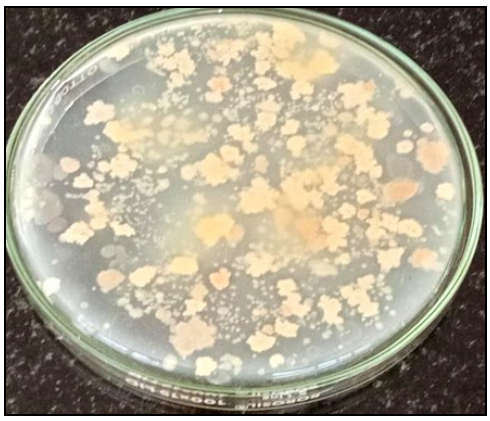

Fig 2: Sub culturing of fish sample

In the Nutrient agar plates inoculated with the serially diluted fish sample, growth was observed after 24 hours of incubation.

Plate Screening

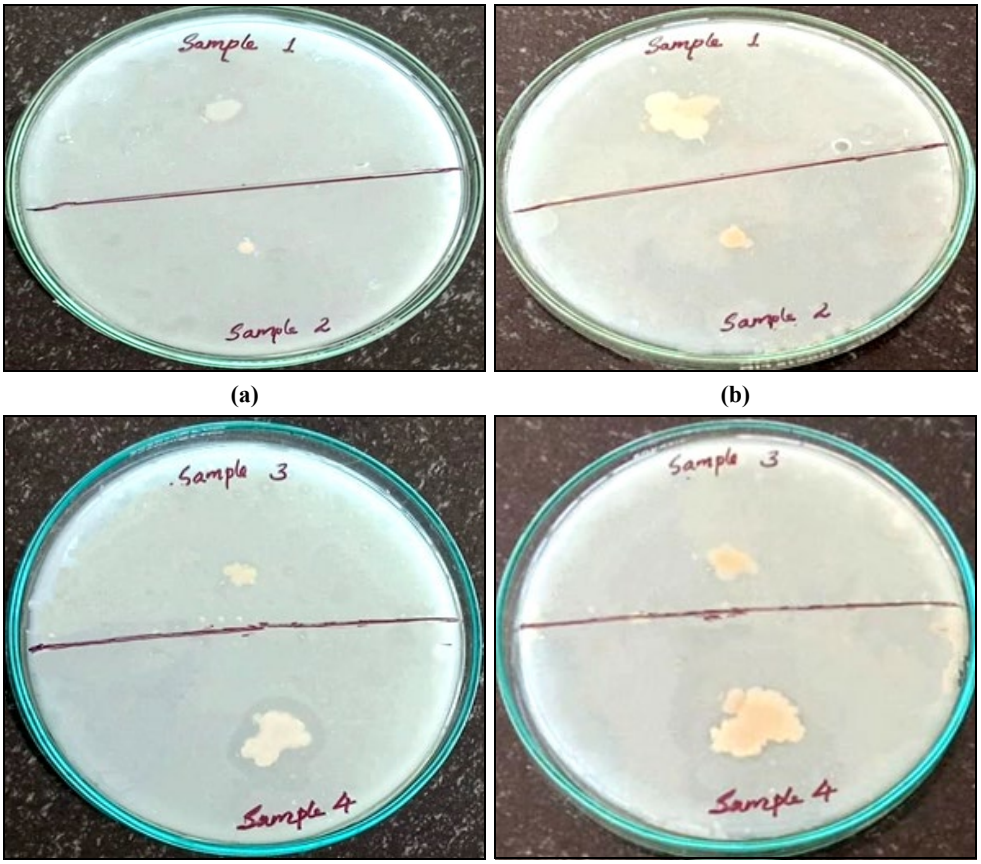

Fig 3: Screening after 24 hours and 48 hours of incubation

C. Skim Milk Agar Plating

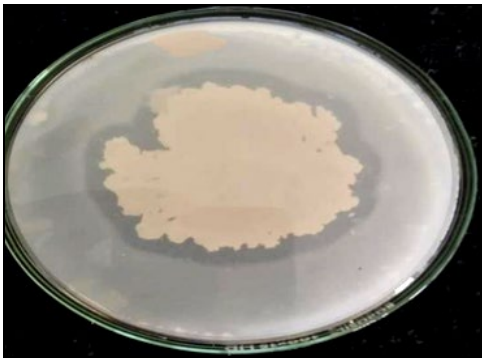

Fig 4: Zone formation analysis on skim milk agar

D. Gram Staining

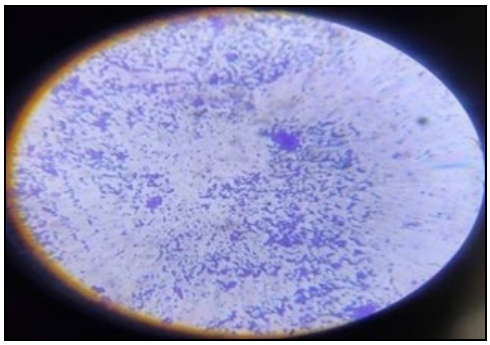

Fig 5: Morphology analysis of culture by gram staining
Gram staining showed the presence of gram positive bacteria

E. Enzyme assay

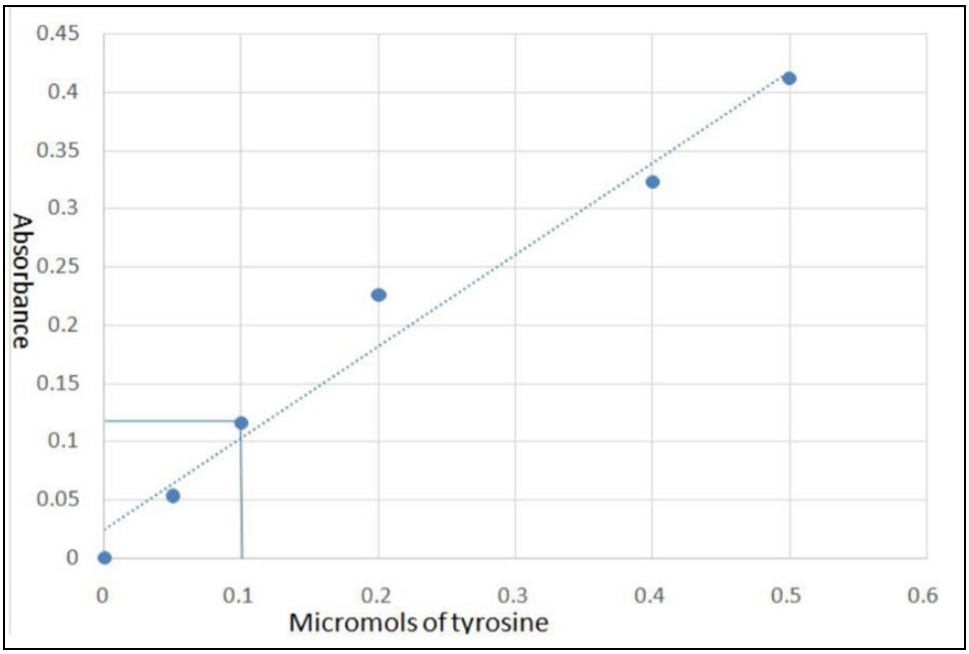

Fig 7: Enzyme Essay

$$\text{Activity of Enzyme (Unit/ml)} = \frac{\text{Tyrosine equivalent released X total assay}}{\text{Time in assay X Vol enzyme used X vol in calorimeter}}$$

F. Isolation of DNA

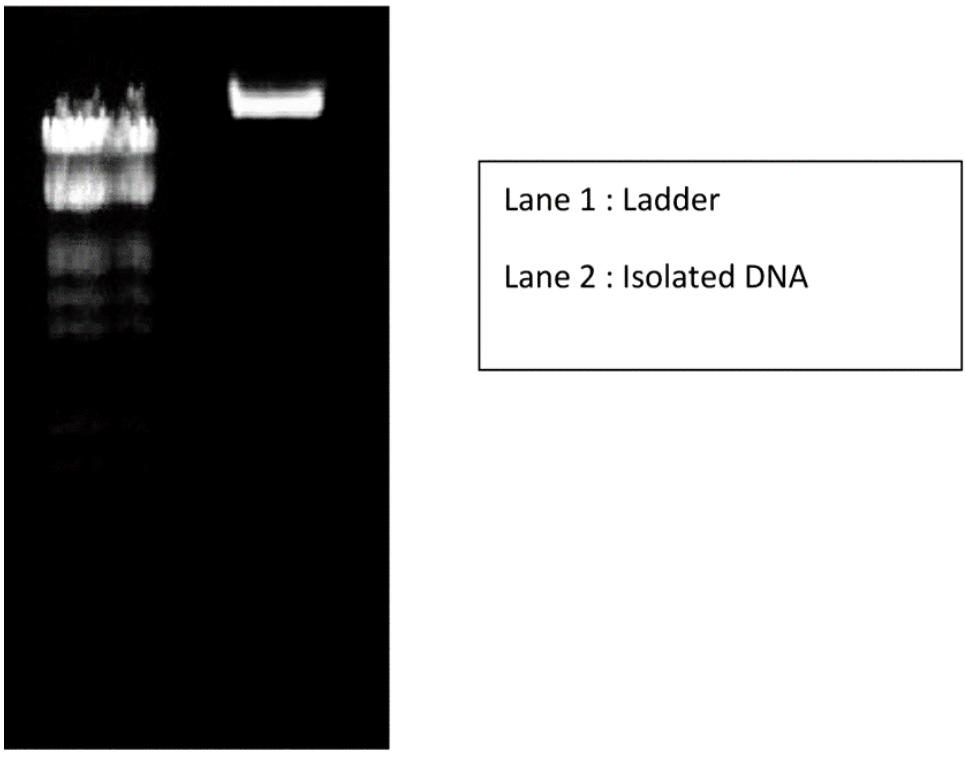

Fig 8: Agarose gel image

G. PCR

Fig 9: PCR bands

H. Sequencing

Bacillus Cereus

TATGAGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCCATAAGACTGGGATAA CTCCGGGAA

ACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGG CTGTCACT

TATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGAT GCGTAGCCG

ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCA GCAGTAGGG

AATCTTCCGCAATGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAGGCTTTCGGG TCGTAAAAC

TCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAA
GCTGGCACCTTGACGGTACCTAACCA GAAAGCCA

CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATT GGGCGTAA

I. Blast

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus partial 16S rRNA gene, strain S...	Bacillus ...	905	905	100%	0.0	100.00%	1262	LT844658.1
Bacillus thuringiensis strain NSK-KAU 16S ribo...	Bacillus t...	896	896	100%	0.0	99.59%	767	MT509428.1
Bacillus cereus strain >SR 1572-4 16S riboso...	Bacillus ...	896	896	100%	0.0	99.59%	1279	MN473189.1
Bacillus sp. (in: Bacteria) 042 gene for 16S rib...	Bacillus ...	896	896	100%	0.0	99.59%	1277	LC474016.1
Bacillus cereus strain K3 16S ribosomal RNA ...	Bacillus ...	896	896	100%	0.0	99.59%	1225	MK530096.1

Fig 10: BLAST analysis

The DNA sequence obtained is tested, for finding the region of local similarity between sequences, in the BLAST software. The results showed that Bacillus bacteria gives 100% result for Bacillus cereus.

J. Application Study
Removal of Strain

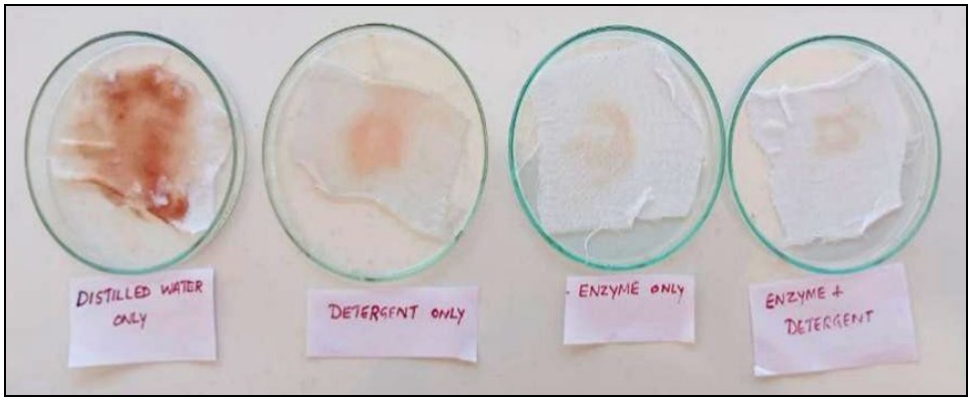

Fig 11: Blood stain removal assay using the extracted enzyme

Discussion

Protease producing organisms are generally isolated from gut of the sardine fish and most of the work is focused on alkaline protease. Therefore the present study deals with the isolation of protease producing bacteria from fish. Members of the genus Bacillus produce a large variety of extracellular enzymes of which protease are of particularly significant industrial importance. A major commercial use is the addition of microbial protease to domestic detergents for the digestion of proteinacious stains of fabrics (Sharma *et al*., 1980). Isolation of protease producing bacteria was performed by the serial dilution and spread plate method. Similar method has been used by Clark *et al*., 1958. Identification of selected Bacillus strain was identified on the basis of standard morphological tests according to the method described in Bergeys's Manual of Determinative Bacteriology. The selection of organism was based on the zone of clearance it showed in the skim milk agar plate. The protease enzyme produced by the organism showed a clear zone around the colony as the protein near the colonies was utilized. These colonies were taken for gram staining and it was found out that they were rod shaped and gram positive. Weiss and Ollis, 1980 also reported that B. licheniforms produced very narrow zone of hydrolysis on casein agar despite giving very good protease production in submerged condition. For quantitative screening casein yeast extract dextrose broth is used and inoculated with isolated Bacillus sp. which was incubated at 37˚C for 24hrs. Abo-Abe *et al*., (2006) produced alkaline protease from Bacillus circulance, B. alvei, B. sphaericus and B. pumilus using Luria-Bertanibroth, Luria-Bertani agar milk. Atalo *et al*., (1993) showed that yeast extract and peptone can induce the alkaline protease production in growth medium. On the place of peptone we prefer casein, which have high protein concentration. Protease activity was compared using protease assay. The activity of enzyme in purified sample and supernatant was measured by tyrosine standard curve and it was found to be as 0.2191 U/ml/min and 0.186285 U/ml/min respectively. The potential of removing blood stains was tested by Bacillus cereus. It was observed that crude enzyme removed stains completely as compared to water and detergent without hampering the texture of the cloth, this potential of Bacillus spp makes it ideal to be used as biocleaner or in a detergent manufacture

References

1. Abdul Razzaq, SadiaShamsi, Arfan Ali, Qurban Ali, Muhammad Sajjad, Arif Malik and Muhammad Ashra. Frontiers Microbial Proteases Applications Bioengineering and Biotechnology, 2019.
2. Mayuri Sharma, YogeshGae, ShaliniArya, Vikaskumar, Anil Panghal and Ashwanikumar. Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches Industrial Biotechnology, 2019.
3. Mehak Baweja, Rameshwar Tiwari, PuneetK. Singh,Lata Nain and Pratyoosh Shukla. Frontiers an Alkaline Protease from Bacillus pumilus MP 27: Functional Analysis of Its Binding Model toward Its Applications As Detergent Additive Microbiology, 2016.
4. MA Khan, N Ahmad, AUZafar, IA Nasir, MA Qadir. Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme *African Journal of Biotechnology*, 2011.
5. Janos András Mótyán, Ferenc Tóth, and József Tőzsé. Research Applications of Proteolytic Enzymes in Molecular Biology, 2013.
6. Gupta R, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of microbial alkaline proteases-PubMed, 2002.
7. Wei-Hua Chu. Optimization of extracellular alkaline protease production from species of Bacillus Journal of Industrial Microbiology and Biotechnology Oxford Academic. *Journal of Industrial Microbiology and Biotechnology,* 2007.
8. Koki Horikoshi. Production of Alkaline Enzymes by Alkalophilic Microorganisms: Part I. Alkaline Protease Produced by Bacillus No.221. Agricultural and Biological Chemistry. 1971; 35(9):1407-1414.
9. M Kalpana Devi, A RasheedhaBanu, GR Gnanaprabhal, BV Pradeep, M Palaniswam. Purification, characterization of alkaline protease enzyme from native isolate Aspergillus niger and its compatibility with commercial detergents. *Indian journal of science and technology,* 2008.
10. Muhammad Nadeem, Javed Iqbal Qazi, Quratulain Syed, Muhammad Gulsher. Purification and characterization of an alkaline protease from Bacillus licheniformis UV-9 for detergent formulations *J Sci. Technol. 2*013; 35(2):187-195, 2013.
11. Ami D Varia, Viral Y Shukla, Devayani R Tipre. Research Scholar, Department of Microbiology and Biotechnology, School of Sciences, Gujarat University, Ahmedabad 380009, Gujarat, India. Department of Microbiology, L. *J Institute of Applied Sciences-Alkaline protease-A Versatile Enzyme*, 2019.

12. Adinarayana K, Ellaiah P. Response surface optimization of the critical medium components for the production of alkaline protease by newly isolated Bacillus sp. *J. Pharma Sci*. 2002; 5(3):272-278.
13. Das G, Prasad MP. Isolation, purification & mass production of protease enzyme from Bacillus subtilis. *Int. Res. J. Microbiol*. 2010; 1(2):026-031.
14. Payen A, J Persoz. Me ´moiresur la Diastase, les principaux Produits de ses Re ´actions, et leurs applications aux arts industriels. Ann. Chim. Phys. 1833; 53(2):73-92.
15. Gupta R, Beg QK. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enzyme and Microbial Technology. 2003; 32(2): 294-304.
16. Bayoudh A, Gharsallah, Chamkha M, Dhouib A, Ammar S, Asri M. Purification and characterization of an alkaline protease from Pseudomonas aeruginosa MNI. J. of Industrial Microbiology &Biotechnology. 2000; 24:291-295.
17. Deng A, WU J, Zhang Y, Zhang G and Wen T: Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresour. Technol. 2010; 101:7100-7116.
18. Gouda MK. Optimization and purification of alkaline proteases produced by marine Bacillus sp. MIG newly isolated from eastern harbour of Alexandria. *Polish J Microbiol*. 2006; 55:119-126.199.
19. Ferrari E, Jarnagin AS and Schmidt BF: Commercial production of extracellular enzymes. In: Bacillus subtilis and other Gram positive bacteria. American Society for Microbiology Washington, 1993, 917-938.
20. Singhal P, Nigam VK, Vidyarthi AS. Studies on production, characterization and applications of microbial alkaline proteases. *Journal of Advanced Biotechnology*, 2012; 23(4):653-669.