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SCREENING AND BIO-CHEMICAL CHARACTERIZATION OF PROTEASE PRODUCER *ESCHERICHIA COLI* AND INDUSTRIAL APPLICATION IN PLASTIC DEGRADATION

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ABSTRACT

A protease enzyme was produced from *Escherichia coli* that was isolated from sewage water and was biochemically identified and studied. To induce the protease enzyme, *E.coli* was grown in a shake flask containing tryptic soy broth. Ammonium sulphate precipitation served to partly purify the protease enzyme. The ability of the bacteria to produce protease enzyme was screened on skim milk plate agar, and the pH, temperature, and incubation time were optimised to determine the enzyme's maximal output. The purified protease has the maximum activity at pH 6, with an inoculum concentration of 1.5108 CFU/ml at 37°C after 24 hours of continuous agitation at 150 rpm, the protease enzyme is commercially exploitable. Additionally, *E.coli* bacteria are used in degrading polythene and plastic bottles. The biodegradation of plastic polythene and plastic bottles was analysed after 15 and 30 days of incubation in nutrient broth, respectively. Comparatively, more polythene is used than plastic bottles degraded by biodegradation. In a month, the degradation of plastic polythene increased to 4.08% and that of plastic bottles to 1.02%.

KEYWORD: *E. coli*, Optimisation, Plastic, Polythene, Protease, Sewage.

INTRODUCTION

Kuhne first used the word "enzyme" in 1876. An enzyme is a protein that accelerates a chemical process by acting as a catalyst; it is not used up during the chemical reaction but is recycled and used repeatedly, all enzymes are proteins. Protease is one of the three main classes of industrial enzymes, accounting for 60% of all enzyme sales globally (Godfrey and West 1996; Madhavi et al., 2011) and predicted to achieve a sales value of $ 2 billion. Proteases are enzymes that catalyse the breaking of the CO-NH peptide link in proteins, releasing peptides, which are short sequences of amino acids (Barrett and McDonald, 1986). Proteases are found in all forms of life and are produced by microorganisms, a wide range of plants, and mammals. Plant and animal proteases are unable to keep up with demand and are more labour-intensive to produce than microbial production (Gupta et al., 2002; Kalaiarasi and Sunitha, 2009). Which has led to interest in microbial proteases. The most popular protease enzymes, however, come from microbial sources because of their extensive commercial use. They are one of the biggest groups of industrial enzymes and have a wide range of uses in the detergent (Barindra et al., 2006), leather, pharmaceutical, food, and textile industries, as well as in the recovery of silver (Singh et al., 2016) and the bioremediation process.

Proteases are broadly classified as endopeptidases or exopeptidases enzymes (Otin and Bond 2008) on the basis of their site of action on substrate. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate; further, they are classified as aminopeptidases and cabroxypetidases based on the site of action at the N or C terminus. Endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, endopeptidases are further classified into four major groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metallo-proteases.

There are several reports in the literature on the secretion of proteins by fungi and Gram-positive bacteria, but there is relatively little research on the secretion of proteins by Gram negative bacteria, particularly *E.coli* (Sandkvist 2001, A; Sandkvist 2001, B). *E.coli* are generally classified as non-secretors of protein. In contrast, non-pathogenic E. coli are traditionally thought not to produce protein; nonetheless, the secretory process of a variety of virulence factors in pathogenic *E.coli* has been investigated in some depth (Sandkvist 2001, A; Sandkvist 2001, B; Pugsley 1993; Pugsley 1996).

The pharmaceutical business, the leather industry, the production of protein hydrolyses, the food industry, and the waste processing sector are just a few of the industries that use these enzymes (Saha et al., 2011; Younes et al., 2011; Vadlamani and Parcha 2011; Josephine et al., 2012).

METHOD AND MATERIALS

Collecting samples and isolating the bacteria

The bacteria used in this study are *E.coli* isolated from sewage water. The bacterium strain was cultured for 24 hours on a Skim milk agar plate (Riffel and Brandelli, 2006). A single bacterium colony with the greatest casein hydrolysis rate on a Skim milk agar plate after 24 hours was taken for subculture. On a nutrient agar plate and nutrient broth, a single colony of bacteria was subcultured.

Bacteria identification

Subculturing was done for single-colony isolation. From an overnight sample, a loopful of seed culture was streaked onto a nutrient agar plate and cultured for 24 hours at 37°C. The bacterial isolate was identified by a variety of biochemical studies.

Qualitative estimation of protease enzyme

To detect the ability of bacteria to produce the alkaline protease enzyme, skim milk agar (Riffel and Brandelli, 2006; Harely and Prescott, 1996; Usharani and Muthura 2010) was used. A loopful of bacterial culture was plated onto a pre-sterilised skim milk agar plate and incubated at 37°C for 24 hours to examine the proteolytic activity. Enzyme activity was seen as a clear zone on the plate.

Production of protease in shake flask fermentation

The bacteria strain (10%) was cultured in a 25-ml conical flask containing Tryptic soy broth (Jorgensen et al., 2021) and the following ingredients: Tryptone (0.42g), soytone (0.07g), glucose (0.06), NaCl (0.12), K_2HPO_4 (0.0 g), and pH 7.3 were incubated at 37°C for 48 hrs in a shaking incubator (150 rpm), then centrifuged at 5000 rpm for 20 min at 4°C. collected culture supernatant (a crude enzyme) to assay protease and measure the amount of protein concentration.

Purification of enzymes

Ammonium sulphate precipitation (Lakshmi and Prasad, 2015) at 70% (47.6 g $(NH_2)2SO_4$ /100 mL crude enzyme) was used to purify the culture supernatant. Weighing the ammonium sulphate, the enzyme is gently mixed with a stirrer over the course of an overnight period at 4°C until homogenous. Following a 30-minute centrifugation procedure at a speed of 3,500 rpm and 4°C and dissolved in a minimum quantity of glycine-NaOH buffer (pH 10.0). This preparation was treated as a partially purified enzyme. The pellets and supernatant are separated to fit in distinct tubes once the centrifugation procedure is finished in order to check the protein content using the ninhydrate test.

Measurement of protease activity

The following steps were used to assess protease activity (originally described by Murachi, 1970; Brock et al., 1982; modified by Senior, 1999):

Added 0.2 ml of culture supernatant to 1.8 ml of casein solution 2%, the mixture was incubated in a water bath at 37°C for 10 min, then 3 ml of TCA 5% was added to this mixture and placed at 4°C for 10 min to form precipitate, then it was centrifuged at 5000 rpm for 15 min also the blank was prepared using the same steps apart from TCA (5%) being added before the addition of the crude enzyme. Absorbance was measured at 280 nm, and the amino acids generated by the breakdown of casein. The protease activity was determined by using the following equation:

$$\text{Protease Activity Unit/ml} = \frac{\text{Absorbance at 280nm}}{0.01 \times 10 \text{ mins} \times 0.2\text{ml}}$$

Protein concentration measurement

Using the Lowry technique (Lowry *et al.,* 1951), the protein content was calculated.

- Developed four standards, T1, T2, T3, and T4, to create the Bovin Serum Albumin (BSA) (1 mg/ml) standard curve.
- Pipette out 30µl, 60µi, 120µl and 240µl of the working BSA standard into each test tube, accordingly.
- Add distilled water to each test tube to bring the volume up to 1 ml.
- In each test tube, add 4.5 ml of Reagent 1 (an alkaline copper solution). Incubate for 10 minutes.
- Now fill each test tube with 0.5 ml of Reagent 2 (Folin's reagent).
- Prepare the blank by adding 1 ml of distilled water, Reagent 1, and leaving out BSA incubate for 10 minutes, then add Reagent 2.
- Incubate both the BSA standard and the blank at room temperature in the dark for 30 minutes.
- The same procedures were followed to estimate an unknown protein, except that instead of BSA, 25µl of the unknown protein sample was added.
- Now compare the absorbance of each sample and standard at 660 nm to a blank.
- Plotted the absorbance of the standard of their concentration and calculated the concentrations of unknown samples.
- Calculated enzyme-specific activity using the following equation:

$$\text{Specific activity unit/mg protein} = \frac{\text{Enzyme activity unit/ml}}{\text{Protein concentration mg/ml}}$$

Determination of an optimised initial medium pH for protease production

To examine their impact on the production of proteases, varied starting pH values of 5, 6, 7, and 8 were adjusted to medium tryptic soy broth. The cultures were cultured in a shaking incubator for 24 hours after being inoculated with 1% ml of an optimised inoculum concentration.

analysed after centrifugation to measure protein content and protease activity.

Determination of an optimised incubation temperature for protease production

A 1% ml of the optimised inoculum was added to the tryptic soy broth medium before it was cultured for 24 hours at various temperatures (25, 37, 45, 4, and -20°C). Then it was centrifuged and assayed for protease activity and protein concentration.

Determination of an optimised incubation period for protease production

The optimised protease production medium, tryptic soy broth, was inoculated with 1% of the optimal inoculum concentration and incubated for varying amounts of time (24, 48, 72, and 96 hours). The culture solution obtained was used to assay protease activity and protein concentration.

RESULT AND DISCUSSION

Based on morphological and biochemical characteristics and a biochemical test (Table 1), the isolated bacterium was identified as E. coli. The zone of hydrolysis on skim milk agar is depicted in Figure 1. E. coli clearing the cloudy agar (zone of proteolysis) indicates the positive result of proteolytic activity, and by qualitative and quantitative protease assays, these isolated enzymes are further studied for protease optimisation.Ammonium sulphate precipitation at 70% purified the crude enzyme.

Different impacts of various pH levels (5, 6, 7, 8, and 9) on the production of proteases were studied. The ideal pH for the synthesis of proteases was discovered to be pH 6 (Abed et al., 2016), with a specific activity of 3.115 units/mg. Which is similar to acidic protease.

Table 1: Biochemical characterisation for isolated bacteria.

Gram staining	-
Rod shaped	+
Mobility	+
Indole production	+
Methyl red	+
Voges Prokauer	-
Nitrate reduction	+
Citrate	+
Urease	+
Catalase	+
Sucrose	+
Dextrose	+
Lactose	+
Maltose	+
Mannitol	+
Sorbitol	+
Starch	-

(+ Positive - Negative)

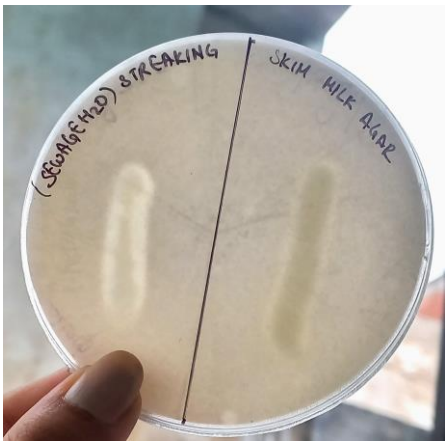

Fig. 1. (Left) Zone of hydrolysis by *E.coli* on skim milk agar. (Right) (Unknown bacteria) No zone of hydrolysis on skim milk agar.

The influence of temperature on protease synthesis was studied at various temperatures (-20, 4, 25, 37, and 45°C). The highest levels of protease production were found at 37°C (Ahmad et al,. 2014), where the specific activity was measured at 2.823 unit/mg of protease.

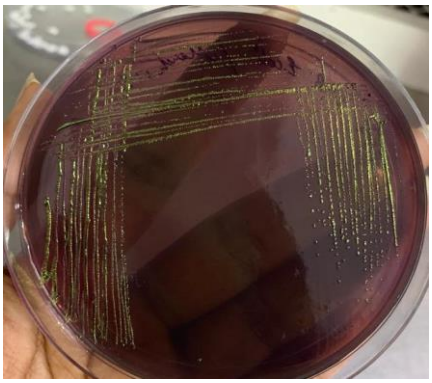

Fig. 2. *E.coli* streaked on EMB media, showing a green metallic colour.

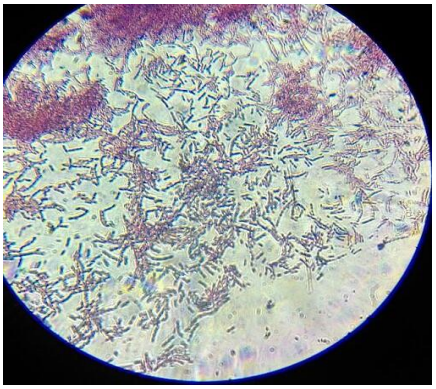

Fig. 3. Gram negative staining of *E.coli*.

Different incubation times (24, 48, 72, and 96 hours) were studied to determine the optimum protease production. A high level of protease production was recorded at 24 hours (Suganthi et al., 2013) at 2.662 units/mg, and the production of protease decreased over the longer incubation times, reaching 1.354 unit/mg during 96 hours. This could be because of autolysis or a change in the culture conditions.

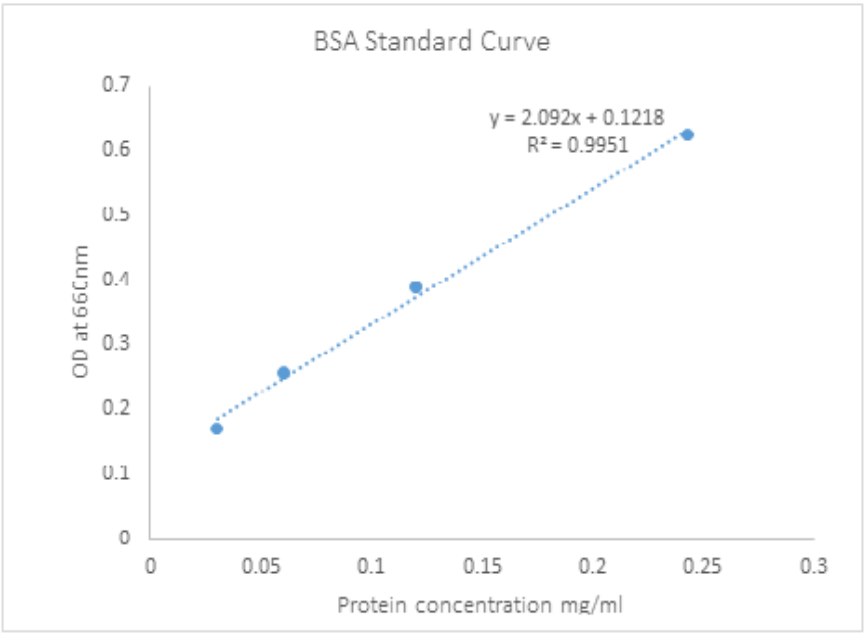

Figure 2: Standard curve of Bovin Serum Albumin (BSA)

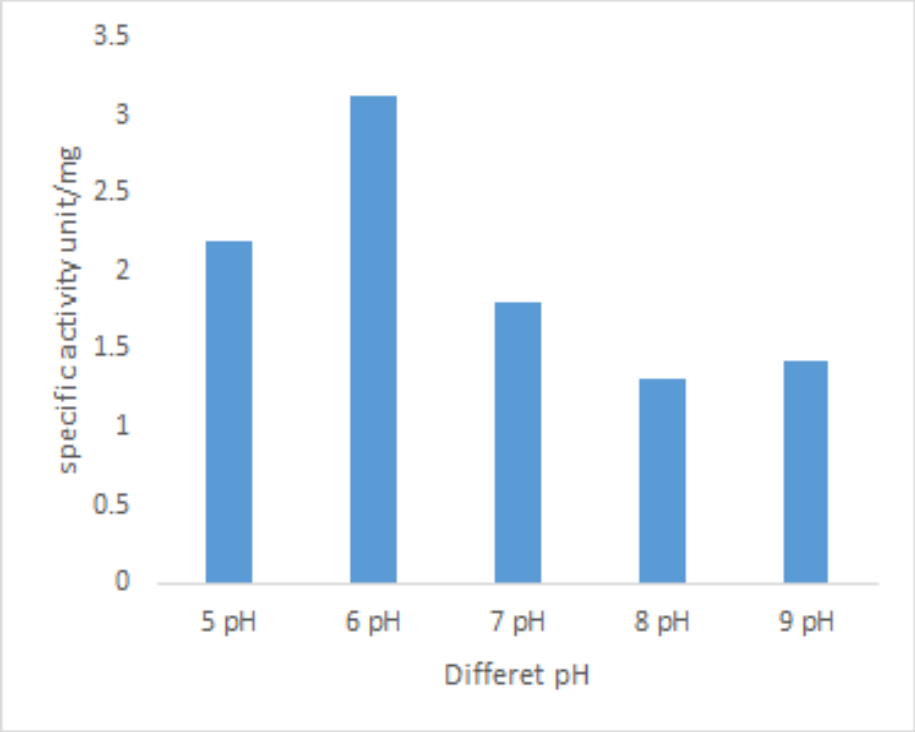

Figure 3: The optimised pH for protease produced from.

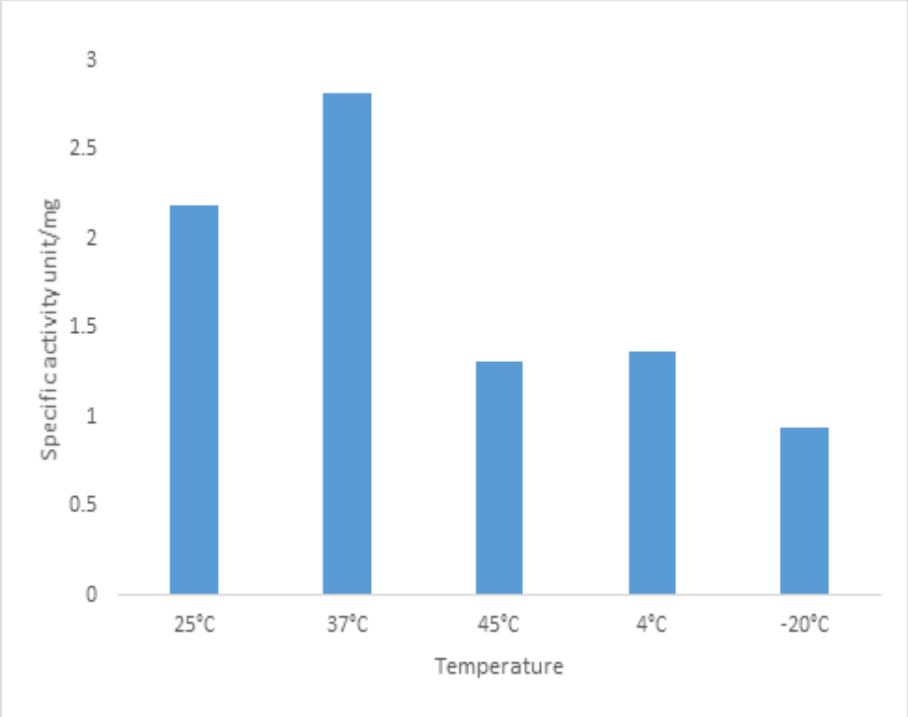

Figure 4: The optimised incubation temperature for protease produced from *E.coli*.

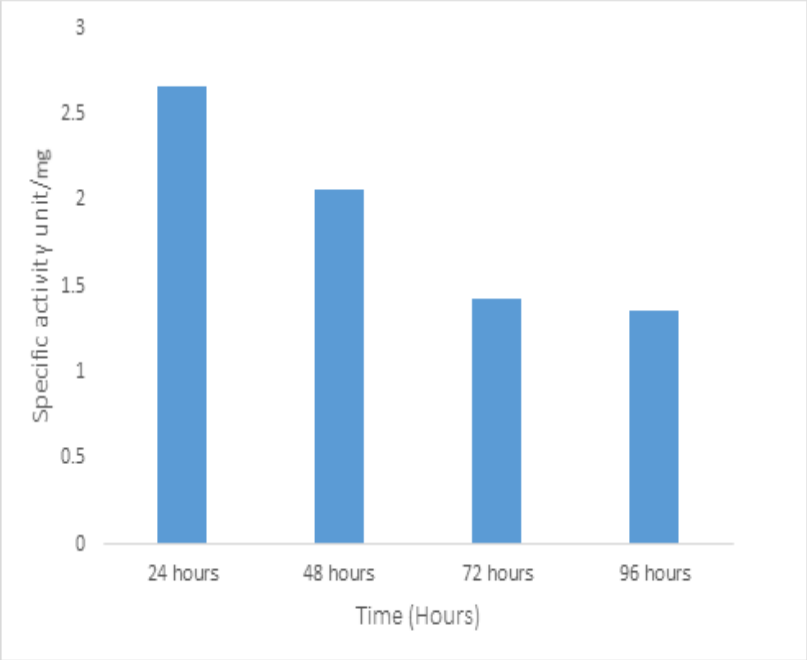

Figure 5: The optimised incubation period for protease enzyme produced from *E.coli*.

APPLICATION

In every aspect of our daily lives, plastic has become more prevalent. Plastic is helpful since it is sturdy, lightweight, and long-lasting (Ilyas et al., 2018).

However, utilising plastic has drawbacks since it doesn't biodegrade easily and causes pollution, which is harmful for both humans and animals and the environment (Zalasiewicz et al., 2016). The most prevalent form of non-biodegradable solid trash that has lately been identified as posing a serious hazard to marine life is polythene. Fish, birds, and marine animals may occasionally get intestinal obstructions due to polythene (Jamieson et al., 2019).

The biodegradation of polythene bags and plastic bottles (Kathiresan 2003) was examined after 15 and 30 days of incubation in nutrient broth contaminated with the bacterium E. coli. This material was shaken vigorously to ensure even dispersion at 150rpm rpm at 30°C.

Plastics breakdown by microbes in a lab setting

Pre-weights of sterilised plastic polythene and plastic bottles were individually infected with E. coli bacteria and aseptically transferred to a conical flask containing 25 ml of culture broth media. The medium for bacteria was nutrient broth. Two flasks were maintained for each treatment and left in a shaker. After 15 and 30 days of shaking, respectively, the plastic materials were collected, carefully cleaned with distilled water, dried in the shade, and then weighed to determine their ultimate weight. The initial weights of plastic and polythene were 0.98g and 0.99 g, respectively, and the final weights of plastic and polythene were 0.97g and 0.95 g, respectively, within a month. We computed the weight loss of the plastic and polythene bags using the data we had obtained.

Table 2: Degradation of Plastic and Polythene incubated with *E.coli* in shaker culture under laboratory condition.

Days of analysis	Biodegradation (% weight loss)	
	Plastic	Polythene
15	0	0
30	1.02	4.08

This experiment has proven that these microbes cause degradation of plastic and polythene up to 1.02% and 4.08%, respectively, within a month**.**

CONCLUTION

In the current study, *E.coli* was isolated from sewage water, and the optimum pH, temperature, and incubation

time were observed at pH 6, 37 °C, and 24 hours, respectively, with an inoculum concentration 1.5×10^8 CFU/ml. Plastic and polythene degradation were studied for 15 and 30 days, respectively. At 30 days, plastic and polythene were degraded by 1.02% and 4.08%, respectively. These studies demonstrated that the breakdown rate would have greatly increased if the plastic and polythene were incubated for a longer length of time, over 6 months. *E.coli coli is* capable of degrading plastic and polythene, which could contribute towards solving environmental pollution.

Hence, further studies on microbial enzymes will pave the way for the development of technologies for industrial applications.

REFERENCE

1. Alessandro Riffel, Adriano Brandelli. (Keratinolytic Bacteria isolated from Feather Waste). [Braz J Microbiol,] 2006; 37: 399-395.
2. Barindra S, Debashish G, Malay S, Joydeep M. (Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma Proteobacterium isolated from the marine environment of the Sundarbans). Process Biochem, 2006; 41: 215-208.
3. Barrett AJ, McDonald JK (Nomenclature: protease, proteinase and peptidase). Biochem J, 1986; 237: 935.
4. Basima Khalid Abed, Sawsan Hassan Authman, Khadim Hashim Yassein. (Optimization of extracellular protease extracted from *Escherichia coli*). Eur J Pharmaceu Med Res, 2016; 3(2): 118-113.
5. Brock FM, Forsberg CW, Buchanan SG. (Proteolytic activity of rumen microorganisms and effect of proteinase inhibitors). J App Environ Microbiol, 1982; 44: 561-569.
6. Suganthi C, Mageswari A, Karthikeyan S, Anbalagan M, Sivakumar, Gothandam AK. (Screening and optimization of protease production from a halotolerant Bacillus licheniformis isolated from saltern sediments). J Gen Eng Biotec, 2013; 11: 52-47.
7. Carlos López-Otin and Judith S Bond. (Proteases: Multifunctional Enzymes in Life and Diseases). J Biol Chem, 2008; 283(45): 30437-30433.
8. Lakshmi G, Prasad NN. (Purification and characterization of alkaline protease from a mutant *Bacillus licheniformis* Bl8). Adv Biol Res, 2015; 9(1): 23-15.
9. Godfrey T and West S. Introduction to industrial enzymology. In: Godfrey T and West S eds., Industrial Enzymology, 2nd ed. London; 1996; 1–8.
10. Gupta, R., Beg, Q. K. and Lorenz, P. (Bacterial alkaline proteases: molecular approaches and industrial applications). Apl Microbiol Biotech, 2002; 59: 32-15.
11. Harley PJ and Prescott ML. Laboratory exercises in microbiology. 5th ed., The McGraw-Hill Companies: 2005.
12. Ilyas M, Ahmad W, Khan H, Yousaf S, Khan K, Nazir S. (Plastic waste as a significant threat to environment – a systematic literature review). Rev. Environ. Health, 2018; 33: 406-383.
13. Jamieson AJ, Brooks LSR, Reid, WDK, Piertney SB, Narayanaswamy BE, and Linley TD. (Microplastics and synthetic particles ingested by deep-sea amphipods in six of the deepest marine ecosystems on earth). R Soc Open Sci, 2019; 6: 180667.
14. Josephine FS, Ramya VS, Devi N, Suresh BG, Siddalingeshwara KG, Venugopal N and Vishwanatha T (Isolation, production and characterization of protease from *Bacillus Sp*. isolated from soil sample). J Microbiol Biotech Res, 2012; 2: 168-163.
15. Kalaiarasi K, Sunitha PU. (Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil). African Journal of Biotechnology, 2009; 8(24): 7041-7035.
16. Kathiresan K. (Polythene and Plastics-degrading microbes from the mangrove soil). Rev biol trop, 2003; 51: 4-3.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (Protein measurement with the Folin phenol reagent). J Bio Che, 1951; 193(1): 275–65.
18. Madhavi J, Srilakshmi J, Raghavendr VM, Rao KRSS. (Efficient leather dehairing by bacterial thermostable protease). Int J Bio-sc Bio-tec, 2011; 3: 26-11.
19. Michael Bording-Jorgensen, Hannah Tyrrell, Colin Lloyd 1 and Linda Chui. (Comparison of Common Enrichment Broths Used in Diagnostic Laboratories for Shiga Toxin—Producing *Escherichia coli.* Microrganism, 2021; *9*(3): 503.
20. Murachi T. Bromelain enzymes. In: Methods in Enzymology (eds). Perlman GE and Lorand L) Academic press, New York., 1970; 19: 273-284.
21. Pugsley A. (The complete general secretory pathway in Gram – negative bacteria). Microbial. Rev. J, 1993; 57: 108-50.
22. Pugsley FOA. (The cryptic general secretory pathway (gsp) operon of Escherichia coli K12 encods function proteins). J. Bacteriol, 1996; 176: 3549-3544.
23. Rajendra Singh, Anshumali Mittal, Manoj Kumar, Praveen Kumar Mehta. (Microbial Proteases in Commercial Applications). J Pharm Chem Biol Sci, 2016; 4(3): 374-365.
24. Saha ML, Begum KJMH, Khan MR, Gomes DJ. (Bacteria associated with the tannery effluent and their alkaline protease activities). Plt Tis Cul Biotech, 2011; 21: 61-53.
25. Sandkvist M. (Biology of type II secretion). Mol Microbiol J, 2001; 40: 283-271.

26. Sandkvist M. (Type II secretion and pathogenesis. Infect). Immun J, 2001; 69: 3535-3523.
27. Senior BW. (Investigation of the types and characteristics of the proteolytic proteus spp. Bacterial pathogencity). J. Med. Microbiol, 1999; 48: 628-623.
28. Usharani B, Muthura M. (Production and characterization of protease enzyme from *Bacillus laterosporus*). Afri J Microbiol Res, 2010; 4(11): 1063-1057.
29. Vadlamani S Parcha SR. (Studies on industrially important alkaline protease production from locally isolated superior microbial strain from soil microorganisms). Int J Biotech Appli, 2011; 3: 105-102.
30. Varish Ahmad, Azhar Kamal, Khurshid Ahmad, and Mohd Sajid Khan. (Protease characteristics of bacteriocin producing *Lysinibacilli*, isolated from fruits and vegetable waste). Bioifo, 2014; 10(1): 18-13.
31. Younes G, Sara R, Alireza E, Aboozar K, Maryam S, Najme T. (Screening and isolation of extracellular protease producing bacteria from the Maharloo salt Lake). Iran J Phamceu Sci, 2011; 7: 180-175.
32. Zalasiewicz J, Waters CN, Ivar do Sul J, Corcoran PL, Barnosky AD, Cearreta A, et al., (The geological cycle of plastics and their use as a stratigraphic indicator of the anthropocene). Anthropocene, 2016; 13: 17-4.