The effectiveness of Proteolytic bacteria isolated from effluent of Modjo tannery for their application in the leather and detergent industry

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Abstract

Protease also called proteinase or peptidase is a digestive enzyme that is categorized under proteolytic enzymes and it has great potential in industrial application. Extracellular proteases are used in a variety of industries because they exhibit practically all of the characteristics needed for biotech applications such as detergent, bioremediation, food, and leather processing.In the synthesis of all three major types of acidic, neutral, and alkaline proteases, microbial sources have dominated an unbeatable area. Alkaline proteases are a large group of industrial enzymes formed by a wide variety of species, including animals, fungi, and bacteria. The fermentation method serves to make bacteria, fungi, and yeast alkaline proteases. Proteases are produced in large quantities by Gram-positive bacteria, especially those belonging to the *Bacillus genus*. Following standard procedures, the bacterial isolates PMOJ-01 and PMOJ-05 with the prominent zone of clearance and efficient enzyme development were further characterized to the genus level. Moreover, the growth conditions for the highest protease production were optimized with different pH, temperatures, and NaCl concentrations, in the results of PMOJ-01 and PMOJ-05 pH (7 and 8), temperatures 45^{o}C, and 1% NaCl Concentrations both cases respectively. The proteases activities from PMOJ-01, *Pseudomonas aeruginosa,* and PMOJ-05, *Bacillus subtilis* were most active at pH7.0 and pH8.0 and temperature at 35^{o}C and 45^{o}C, respectively. The enzyme activity and the total solid protease sample of the crude enzyme of *Pseudomonas aeruginosa* and *Bacillus subtilis* were 0.299 U/ mL and 0.289 U/ mL, 1.37±0.14 U/mg, and 1.199 U/mg respectively. The effect on dehairing, distaining, and scum removal revealed that the purified protease enzyme of PMOJ-01 and PMOJ-05 can be used in detergent and leather industries.

Keywords: Bioremediation; concentration;dehairing; detergent; extracellular proteases.

1. Introduction

Enzyme technology is well established and used in many industrial applications in the modern era of biotechnology. A better understanding of and functionality of enzymes suggests numerous new applications for these catalytic activities and a constant discovery with new R&D properties

(Amar, 2001; Najafi *et al*.,2005). Enzymes are used in many environmentally-friendly industries such as food, dairy, leather, etc. since they are efficient, selective, accelerate and accelerate reactions through the forming with its substrate of transition state complexes, which reduce the energy of activation of the reaction (Bayoudh *et al.,* 2000; Madan *et al.,*2000). Proteases or proteolytic enzymes catalyze the cleavage of peptide bonds in proteins. Protease is one of the most common types of industrial enzymes, accounting for more than 65 percent of all industrial enzymes (Pastor *et al.,* 2001; Belma *et al.,* 2002). In contrast to their behavior, the wide variety of proteases has drawn international attention and concentrated on exploiting physiological and biotechnologies (Rao *et al.,* 1998;Chandran *et al.,* 2014a; Hatem *et al.,* 2018). Proteases are also expected to play an important role in the advancement of environmental innovations and bioremediation processes (Singh *et al.,* 2001; Masi *et al.,* 2014b). In a large number of industries, one of thebiggest industrial enzymes (Protease) is commonly used:- in detergent, leather manufacturing, meat processing, milk product, organic fertilizer preparation, digestive aid, silk industry, and silver recovery from X-ray films used (Sudha *et al.,* 2010; Chandran *et al.,* 2014b).

Microbial origins have dominated an invincible domain in the development of all three major acidic, neutral, and alkaline protease forms. Alkaline proteases, a broad group of industrial enzymes, are generated by a wide variety of species, including animals, fungi, and bacteria. Alkaline proteases are produced by bacteria, fungi, and yeast using the fermentation process (Takami *et al.,* 1989; Masi *et al.,* 2014c). Gram-positive bacteria are considered a major commercial producer of proteases, in particular the *Bacillus* genus (Thangam *et al* 2002;Masi *et al.,* 2015). Gupta *et al.,* (2002) reported *Bacillus licheniformis, Bacillussubtilis, Bacillus acidophilus, Bacillus lentus*, etc., are important industrial alkaline protease producers. Alkaline proteases are one of the most essential classes of enzymes used as detergents, pharmaceutical products, leather, meat-treating products, protein hydrolysates, dairy products, and even waste processing products in various industrial processes (Masi *et al.,*2016a; Thangam *et al.,* 2016).

Alkaline proteases have a large share of the enzyme market, despite having just a two-thirds share of the detergent industry. They're also used in industries like leather, food, textiles, organic synthesis, and waste-water treatment (Wang *et al.,* 2007: Masi *et al.,* 2016b). Microbial proteases account for nearly 60% of total global enzyme sales, with 25% of total global alkaline protease enzyme sales (Madan *et al.,*2000; Masi *et al.,* 2014b). The important applications of the enzymes have been authenticated in this present study.Protease is usually produced from bacteria such as Teredinobacterturnirae, *Bacillus sp., Pseudomonas sp*. and also from other fungal species (Isiaka & Agbaje, 2016;Masi *et al.,* 2021). Bacterial species can be isolated from various industries' effluents(Sudha *et al.,* 2010; Masi *et al.,* 2017). Protease production conditions are optimized and the highest protease producing environments with optimized nutrient sources, pH, and temperature were determined to get maximum protease from each bacterium (Takami *et al.,* 1989; Masi *et al.,* 2018). Bacterial species can be isolated from the effluents by the serial dilution method. A normal agar medium can be used to isolate various numbers of colonies. Each species have a different potential for producing protease. By comparing the protease activities of different strains that were isolated from the effluents; the maximum protease yielding bacteria can be obtained (Gopinath,2002; Thangam *et al.,* 2002).

Bacterial proteases have recently been noted as a viable alternative to the bioremediation and use of tannery-rich protein waste for rawhide treatment by substituting dangerous chemicals that are particularly involved in the soaking, dehairing, and tanning of hides before tanning, for quality leather production without pollution (Takami *et al.,* 1989;Masi *et al.,* 2018). Even though proteases have been identified in several areas and many have been used in industry and biotechnology, the proteases now in use are insufficient to suit all biotechnological needs. As a result, the goal of this research was to identify and characterize possible proteolytic bacteria from Modjo Tannery Waste, as well as to assess their potential for use in detergent and leather industries.

2. Materials and methods

2.1 Screening for protease production

A total of six samples were taken from more than 50 years of accumulated sludge. The samples were transported to the Microbiology Laboratory at Addis Ababa Science and Technology University, where 1.0 g was transferred into 10 mL sterilized normal distilled water in a 250 mL conical flask and agitated at 100 rpm in a water bath shaker at 37^{o}C for 15 min. Nutrient agar was used to dilute and spread the soil suspension. After 24-hour incubation in 37^{o}C and using separate bacterial colonies, the capacity of their proteolytic enzyme was screened on Petri plates containing skim milk agar medium (Skin Milk Powder 2.8 g, Casinenzymichydrolysates 500 mg, Yeast extract 250 mg, Dextrose 100 mg, and agar 1.5 g powder with 100 mL of distilled water to maintaining pH 8).The inoculated plate was then incubated for 48 hours at 37°C, looking for clearance zones that indicated bacterial isolate proteolytic activity.Five isolates with the potential to produce protease in the primary screening were chosen based on the diameter of the clearance zone, and they were further screened to find the most potent isolates among them based on incubation time, clearance zone size, and production (the ratio of the clearance zone diameter to the colony diameter) (Belma *et al* 2002;Chandran *et al.,* 2014a).

2.2 Proteolytic isolates identification

The proteolytic isolates were identified based on biochemically (citrate utilization, triple iron sugar test, Urease, VP tests, catalase, indole starch hydrolysis, and oxygen requirements) and morphological (Gram staining, motility test, and endospore staining) features and then the identified isolates were subjected to the methods suggested in Bergey's Manual, which showed prominent clearances in the sky-milk agar medium. (Masi *et al.,* 2014c).

2.3 Characterization of protease producing isolates

By inoculating the species to nutrient broth supplemented with skim milk agar, the effects of NaCl concentration, pH, and temperature on bacterial growth and activity were investigated.

2.4 Protease assay

For protease assay, the method adopted by Takami *et al.,* (1989) was used. To establish a standard curve, a 0.2 mg/mL L-tyrosine standard solution was prepared. Five test tubes containing 0.2, 0.4, 0.6, 0.8, and 1.0 mL of standard solution, but no standard blank, were prepared. Then, using

distilled water and a blank, it was rendered up to 2mL. Following the addition of 5mL of Na_2CO_3, 0.5mL of Folin and Ciocalteu's reagent was applied to each vial. The blue color produced was measured at A660 nm using a UV-visible spectrophotometer after 30 min of incubation at 37ºC. Casein acts as a substratum in this test. When we test the protease for digester casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin&Ciocalteo Phenol interacts mainly with free tyrosine to create blue-colored chromospheres, which can be quantified and calculated as an absorbent value on a spectrophotometer at 660nm.

2.5 Estimation of total protein

The total protein content of the samples was measured using a slightly modified method of Lowry's process. Lowery *et al.,*(1951) used Egg Albumin as a protein standard. In the test tube, different dilutions of egg albumin solutions were made by mixing one mg of egg albumin and one ml of water. Each of the test tubes had a final volume of 5ml. The solution's concentration ranged from 0.05 to 1 mg per mL. A 0.5ml protein solution was taken from each of these dilutions and 5 ml of lowery reagent D was applied to each test tube. This solution was incubated for 10 minutes at room temperature. After that, each tube received 0.5 mL of FolinCiocalteu reagent (responsible solutions) and was incubated for 30 minutes.

2.6 Purification of protease enzyme

Around 10 ml of solution-free cell enzymes (supernatants) were taken from parent isolates by and adding 80 percent saturation of ammonium sulfate. The solutions of the enzyme were agitated for 1 hour at 4ºC after the ammonium sulfate interacted with protein to form precipitous. The precipitated proteins were extracted by centrifugation at 6,000 rpm for 30 min at 4°C. To obtain a concentrated suspension of the injectable enzyme, the pellets were re-dissolved in 10ml Tris-Hcl buffer, vol. 0.05M, pH 7.5 (Masi *et al.,* 2015).

2.7 Characterization of protease activity

The two test isolates were subjected to different temperatures (25°C, 35°C, 45°C, 55°C,and 65°C) and pH levels (4 to 12) to determine the optimal conditions for protease activity.

2.8 Experiments to evaluate commercial applications of proteases

Removing a bloodstainrequired soaking a clean piece of cloth in blood and allowing the blood cloth to dry. The cloth was cut into equal pieces and incubated with the purified protease for 5 min at 45°C. The control underwent the same process as the experimental group, except for incubation with the enzyme solution. Using enzymes as a detergent ingredient to remove egg albumin protein stain: On pieces of white cotton cloth (5 x 5 cm) stained with egg yolk, the use of enzymes as a detergent additive was investigated (rich in protein).Skin dehairing: - sheepskin was sliced into 5cm 2 pieces and treated in 50 mMTris-HCl (pH 8) at 50ºC with the purified protease. At different incubation times, the skin was examined for hair eradication (Najafi*et al.,* 2005).

2.9 Analytical Statistics:

Origin 2019 was used to draw the graph and conduct analyses of the data at which the optimum point were calculated

3. Results

3.1 Isolation, screening, and identification

The sludge samples obtained from the Modjo Tannery industry yielded a total of 20 distinct bacteria. Only 12 of them were effective in forming clear zones in 24 hours on 1.0 percent skim milk agar. Only two protease-producing isolates were chosen after primary and secondary screening based on their incubation time, clearance zone size (Figures 1 and 2), and efficiency (the ratio of the diameter of the clearance zone to colony diameter) in 24 hours (Table 1). These were PMOJ-01 and PMOJ-05 and were subjected to further study (P- Protease, MO - Modjo Tannery industry, J - Jimma University).

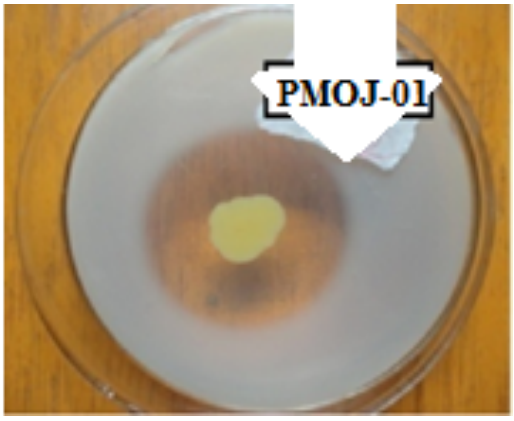

Fig. 1. Clear zone (Protease producing bacteria) formed by isolate PMOJ-01

Fig. 2. Clear zone (Protease producing bacteria)formed by isolate PMOJ-05

Table 1. Screening of protease producing bacterial isolates using zone of inhibition methods

Isolates	The efficiency of Diameter (in mm)
PMOJ-01	22.3
PMOJ-02	8.6
PMOJ-03	9.15
PMOJ-04	2.1
PMOJ-05	14.05

Further species identification (Biochemical Test and microscopic examination) of isolates was performed at the microbiology lab at AddisAbaba Science and Technology and isolate in PMOJ-01 was identified as *Pseudomonas aeruginosa* while PMOJ-05 was identifiedas *Bacillus subtilis* (Table 2).

Table 2. Microscopic examination and biochemical characterization of growing colonies of the isolate PMOJ-01 and PMOJ-05

	Isolate PMOJ-01	Isolate PMOJ-05
Pigment	Yellow	White
Margin	Convex with an entire margin	Circular form and flat elevation
Gram reaction	-	+
Spore	-	+
Motility	-	+
Catalase	++	++
Oxygen requirement	Facultative	facultative
Triple sugar iron test	+ (very less)	+
MR	+	-
VP	-	+
Indole	-	-
Citrate	-	+
Mannitol utilization	-	-
Starch hydrolysis	-	+
Nitrate test	+ (less)	+(less)
Growth in 0%NaCl	+	+
Growth in 2%NaCl	+	+
Growth in 5%NaCl	-	+
Growth at 0^0C	-	-
Growth at 20^0C	+	+
Growth at 37^0C	++	++
Growth at 65^0C	-	+(less)
Urease test	+	-
Identified as	*Pseudomonas aeruginosa*	*Bacillus subtilis*

3.2 Characterization of bacterial isolates

Maximum cell density for *Pseudomonas aeruginosa* (PMOJ-01) and *Bacillus subtilis*(PMOJ-05) was observed when growth temperature was 45^{0}C with the highest Optical Density (OD) measurement of 0.429±0.001 and 0.488±0.004, and the least cell density was recorded at 5^{0}C with mean OD 0.142±0.002 and 0.127±0.007 respectively (Figure 3). The highest cell growth was recorded at pH 7 with 0.659±0.013 OD and pH 8 with 0.694±0.001 OD of PMOJ-01 (*Pseudomonas aeruginosa*), and PMOJ-05 (*Bacillus subtilis)* respectively (Figure 4). The maximum growth of PMOJ-01(*Pseudomonas aeruginosa*), was obtained at 1% NaCl with cell density (OD) of 1.31±0.035 but isolated PMOJ-05(*Bacillus subtilis)* also its OD measurement of 0.71±0.035 was obtained at 1 % concentrations of NaCl as shown high (Figure 5).

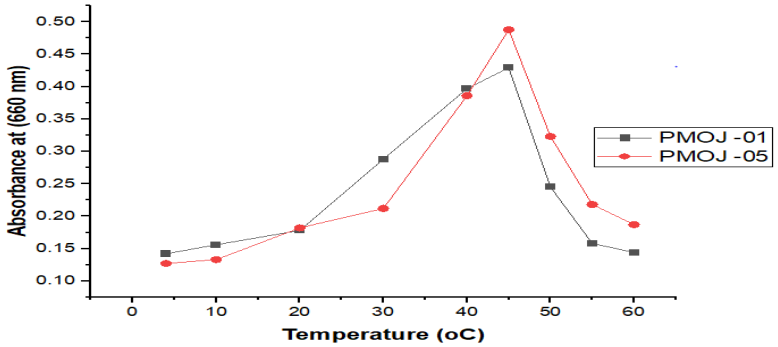

Fig.3. Effect of temperature on microbial growth of the selected two isolates

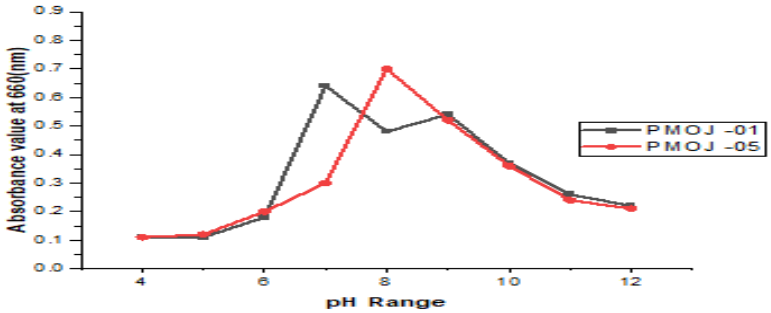

Fig. 4. Effect of pH on the growth pattern of the selected two isolates at 37ºC

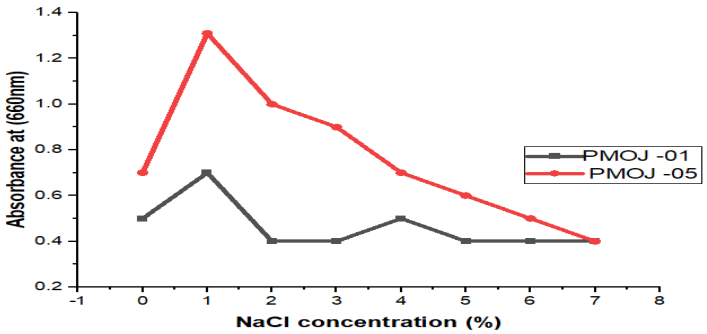

Fig. 5.Growth pattern of the selected two potent isolates under differentsalt concentrations at 37ºC

3.3 Protease assay

The concentration for PMOJ-01 (*Pseudomonas aeruginosa*) and PMOJ-05 (*Bacillus subtilis*) was 0.544µmole and 0.526 µmole and crude enzyme activity was calculated as 0.299 U/ mL and 0.289 U/mL, respectively. Furthermore, the enzyme activity of the partially purified enzyme of PMOJ-01(*Pseudomonas aeruginosa*) and PMOJ-05(*Bacillus subtilis*) was 0.242 U/ mL and 0.231 U/ mL, respectively. The total solid protease sample was 1.37 U/mg for PMOJ-01 (*Pseudomonas aeruginosa)* whereas 1.199 U/mg PMOJ-05 (*Bacillus subtilis)*. The total solid protease of a partially purified enzyme of PMOJ-01 and PMOJ-05 was 2.05 U/mg and 1.55 U/mg respectively. One unit of proteolytic enzyme activity is classified as the amount of enzyme that generated an absorbance difference per milliliter of crude or distilled extract solution after a 10-min incubation at 37°C.

3.4 Characterization of protease enzyme

Protease production increased in both species as pH increased (Figure 6). *Pseudomonasaeruginosa*and *Bacillussubtilis* produced the most proteases at pH 7 and 8, respectively. *Pseudomonas aeruginosa*produced more protease when the temperature was raised to 35ºC. (PMOJ-01) But, the protease production started decreasing then after till the end of work with the lowest protease production obtained at the temperature of 65ºC. But the protease production of *Bacillus subtilis* (PMOJ-05) is maximum growingat 45ºC (Figure 7).

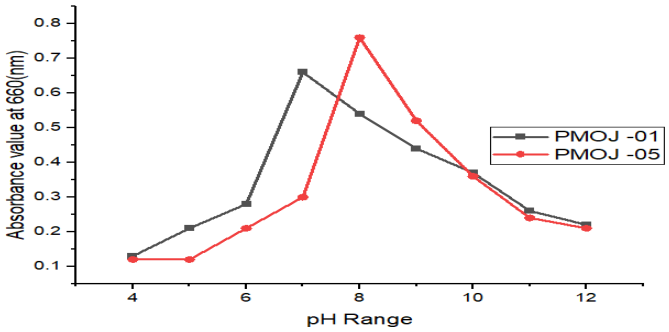

Fig. 6. Effect of different pH on Proteases activities produced by *Pseudomonas aeruginosa* - PMOJ-01 and *Bacillus subtilis*- PMOJ-05

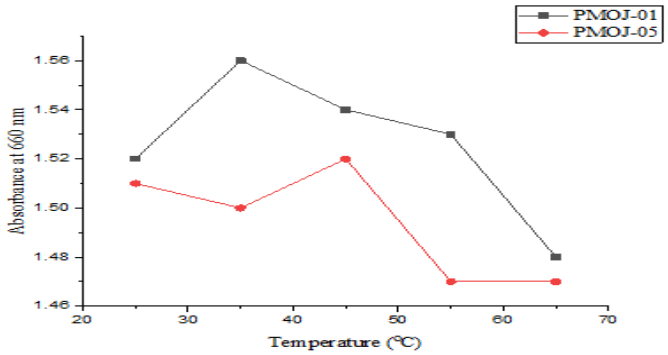

Fig. 7. Effect of temperature on Protease activities produced by *Pseudomonas aeruginosa* - PMOJ-01 and *Bacillus subtilis*- PMOJ-05

3.5 Application of protease in industries

The result of the bloodstain removal study (Figure 8) and egg albumin removal study (Figure 9) revealed that protease from isolate PMOJ-05(*Bacillus subtilis)* is a promising additive for the detergent industry as such applications are well established by the related study conducted. Thus, the enzyme produced by our isolate could be a choice for the commercially available enzymes.In the present dehairing study, incubation of protease with sheepskin for hair (Figure 10) and 12 hrs.showed removal scum (Figure 11) very easily without affecting the skin quality as compared to skin treated with buffer only. However, the skin quality was damaged in the chemically treated skin. Under similar conditions, the control skin incubated in distilled water showed no signs of Scum removal.

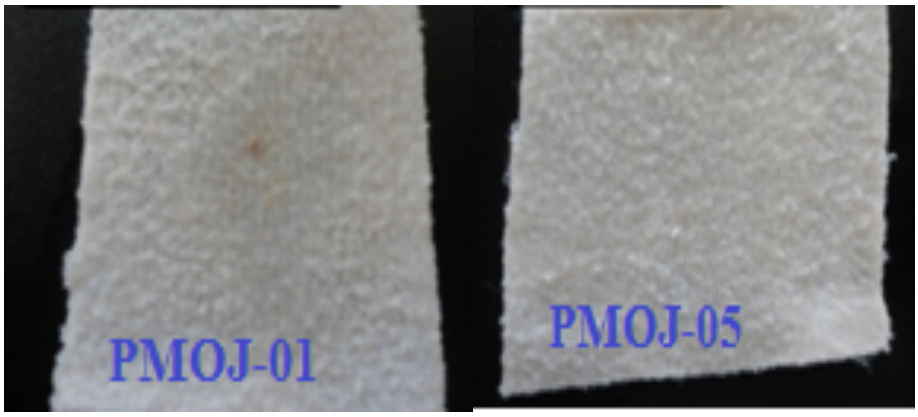

Fig. 8. Effects of distilled water, Alcohol, Enzyme of PMOJ-01(*Pseudomonas aeruginosa)*, Enzyme of PMOJ-05 *(Bacillus subtilis)* on blood-stained clothes after 30 minutes incubation.

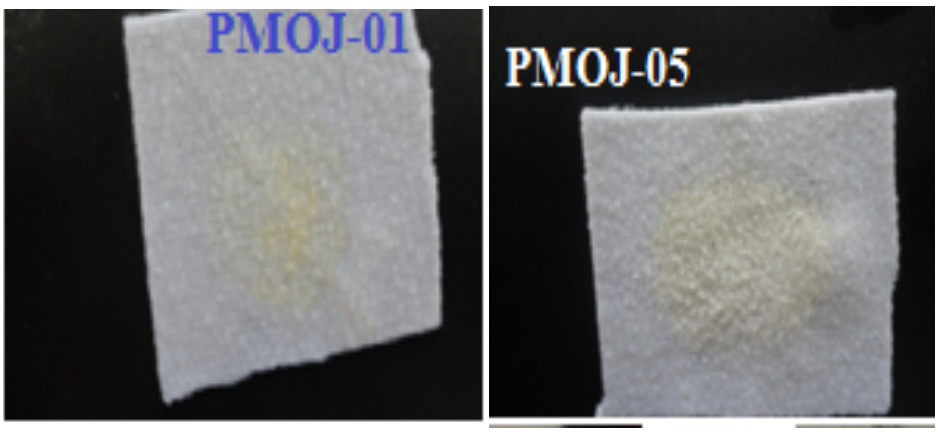

Fig. 9. Effects of distilled water, Alcohol, Enzyme of PMOJ-01(*Pseudomonas aeruginosa)*, Enzyme of PMOJ-05*(Bacillus subtilis)* on prepared egg-stained clothes after 30 minutes incubation.

Fig. 10. De-hairing effect PMOJ-01(*Pseudomonas aeruginosa)* enzyme and PMOJ-05 *(Bacillus subtilis)*enzyme after 12 hours incubation

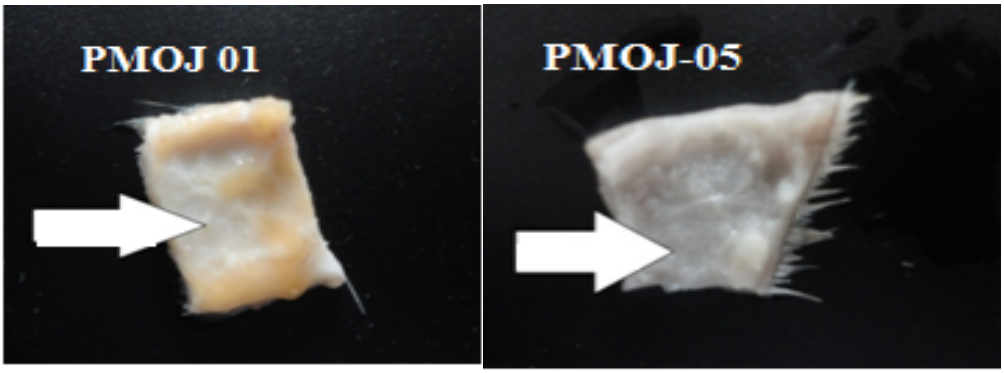

Fig. 11. Scud removal effect of PMOJ-01(*Pseudomonas aeruginosa)* enzyme and PMOJ-05*(Bacillus subtilis)* enzyme after 12 hours incubation

4. Discussion

During the screening, isolation, and production of protease from marine microbes, Guravaiah *et al.,*(2010) reported that bacteria that form a large clear zone on skim milk agar were found in the highly proteolytic zone. After the evaluation of the entire morphological and biochemical test, the two most promising isolates in this study were identified as *Pseudomonas aeroginosa* and *Bacillus subtillis*. High-yielding isolates such as *Bacillus sp*., *Alcaligenes faecalis*, *Pseudomonas fluorescent*, and *Aeromonashydrophilia* contain microbial proteases (Isiaka & Agbaje, 2016;Yu *et al.,* 2017). *Bacillus sp*., for example, is one of the most common classes of bacteria used in the enzyme industry, and this bacterium is also known to produce efficient proteolytic enzymes (Chandran *et al.,* 2014b). As per the observation by Gopinath (2002), the predominant bacterial genera in tannery sludge were *Pseudomonas, Vibrio, Bacillus,* and*Micrococcus*.

The most commercially important proteases come from *Bacillus species*. Their ability to secrete large amounts of proteases with high proteolytic activity and stability at extremely high pH and temperatures is well known (Gupta *et al.,* 2002). Another research looked at the development of alkaline protease from soil samples and found that 27 of the 40 bacteria isolated belonged to the genus *Bacillus* (Guravaiah *et al.,* 2010). In the current study, the predominant isolates were *Bacillus*, followed by *pseudomonas.*When Amar (2001) looked at the microflora in tannery effluent, he

discovered that *Bacillus* was the most common species, followed by Coryneformes, Vibrio, Micrococcus, *Pseudomonas*, and *Acinetobacter*. Furthermore, the importance of this enzyme has been emphasized by various researchers due to the rapid growth rate and improved genetic manipulation production in *Bacillus species* (Morita *et al.,* 1998).

The enzyme activity of the partially purified enzyme of PMOJ-01 and PMOJ-05 was 0.242U/ mL and 0.231 U/ mL and the total solid protease of the partially purified enzyme of PMOJ-01 and PMOJ-05 was 2.05 U/mg and 11.55 U/mg, respectively. According to Kumar (2002),*Bacillus pumilus* had a maximum enzyme activity of 0.065 U/ml, and *Staphylococcus auricularis* had a maximum enzyme activity of 0.038 U/ml at optimum temperature, pH, and nutrient availability. *Bacillus pumilus* and *Staphylococcus auricularis* enzymes were found to have high activity in leather processing and biofilm degradation.About this, enzymes from the current isolates are much better in their activities.

The effect of pH on protease activities of our isolates was consistent with (Gupta *et al.,* 2002) protease development findings, in which the optimum temperature and pH of partially purified *Bacillus species* protease were found to be 40°C and pH 7 respectively. In a Flavobacterium population, the optimal pH for protease development was 7.4(Masi *et al.,* 2014a; Dewi *et al.,* 2018). In *Bacillus polymyxa*, however, protease development was best at pH 9. In different *Bacillus species*, the optimal pH for protease development was between 7 and 10 (Holt *et al.,* 1994). *Pseudomonas* protease development is best at pH8 (Thangam *et al.,* 2002).

The leather industry generates many pollutants that are environmentally detrimental, including sulfides and chromate. There have been many reports of goat/beef dehairing using cleansed and semi-cleansed protease (Nadeem *et al.,* 2006). The spark removal of protease activity has generated the interest of researchers as well as the improvement of leather quality as a chemical methodology due to a significant reduction in toxicity (Najafi *et al.,* 2005; Masi *et al.,* 2014a). In support of the above observations, the current study confirmed the scum removal activity of protease from our isolates with promising potential applications in the leather industry (Longo *et al.,* 1999). Furthermore, blood removing study revealed that protease from PMOJ-05 (*Bacillus. sp)* was a promising additive for the detergent industry in agreement with the well-established practices reported by many authors including Nadeem *et al.,* 2006. They studied the high capacity of bloodstain removal by *Bacillus licheniformis*N-2 and *Bacillus subtilis.*Surprisingly, our findings revealed that the isolates' proteolytic enzyme performed better than some commercially available enzymes. Protein stains, such as grass, blood, egg, and human sweat, are easily removed with proteases, which are commonly used in the detergent industry (Isiaka & Agbaje,2016; Masi *et al.,* 2021).

5. Conclusion

The PMOJ-01(*Pseudomonas aeruginosa)* and PMOJ-05 (*Bacillus subtilis)* bacterial strains are very powerful sources for the production of protease enzymes. Leather making can produce leather with the most softness with the aid of protease enzymes and eliminate the use of pollutant substances such as sodium, lime, and solvents. It is, therefore, reasonable to assume the promising nature of

this enzyme for commercial applications in the detergent industry since they can degrade animal blood and egg albumin in 30 min. so these two bacterial strains (*Pseudomonas aeruginosa*and *Bacillus subtilis)* are very potential protease enzyme-producing. These two bacteria strain references to detergent and leather industries.

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