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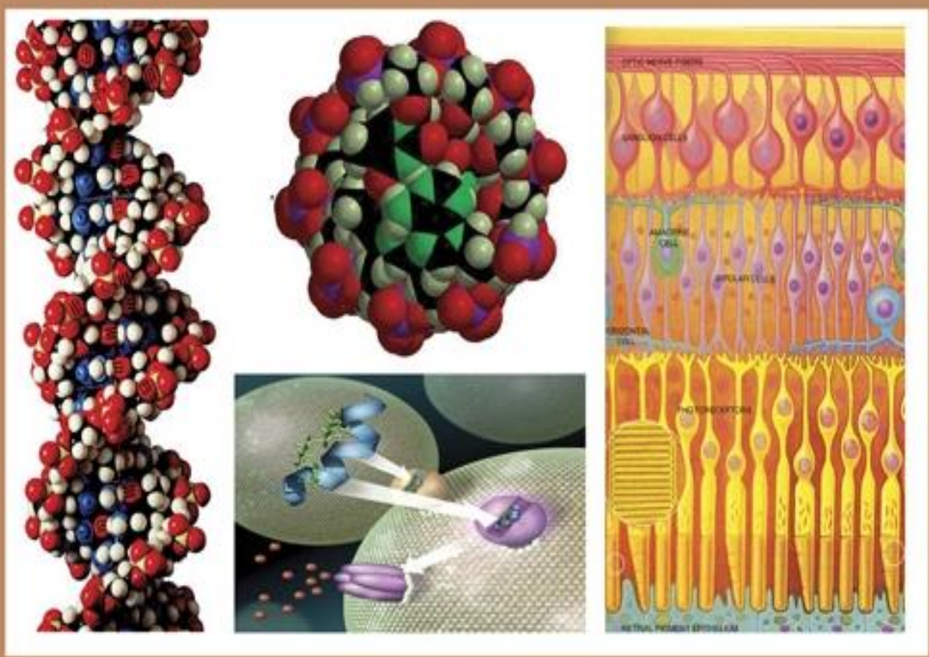

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# Screening and Optimization of Alkaline Protease Production from Bacteria Isolated from Seawater in The North West of Algeria

**Nourine, Zeyneb[1*]; El Kadi Fatima Zohra[1]; Didaoui, Hayat [2]; Kanoun, Khedoudja[1*]; Bouchouicha, Sara[1]; Bouyakoub, Nesrine[1]; Benine, Mohamed Lamine[3]; Mouffok, Benali[3]; Harir, Noria[1] ;Abbouni, Bouziane [1] and Megharbi, Aicha[4]**

1- Molecular Microbiology Health and Proteomics Laboratory, Biology Department, Natural Sciences and Life Faculty. Djillali Liabes University of Sidi-Bel-Abbes, BP N°. 89 Sidi-Bel-Abbès 22000 Algeria.
2- Biology Department, Science Faculty. Dr Moulay Tahar University of Saida Algeria.
3- Synthesis of Environmental Information Laboratory, Djillali Liabes University of Sidi Bel Abbes. Algeria.
4- Valorisation of Phytoresources and Eco-Development of Spaces Laboratory, Environnement Department, Natural Sciences and Life Faculty. Djillali Liabes University of Sidi-Bel-Abbès, BP N°. 89 Sidi-Bel-Abbès 22000 Algeria.

*E. Mail: zeyneb.nourine@univ-sba.dz /khedoudja.kanoun@univ-sba.dz

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**ABSTRACT**

Bacteria are attracting the interest of worldwide investors; their use is interesting in several industrial fields, this organism produces a wide variety of extracellular enzymes, including proteases. The objective of this study was to evaluate the production of protease by different bacterial strains isolated from local marine samples collected from the Cap Rousseau beach in the Oran city, northwestern Algeria, 44 bacterial isolates were tested for protease production by cultivating them on skim milk agar medium. The proteolytic activities of all strains were tested using skim milk agar and gelatin agar. Among the 14 isolates that showed a significant hydrolysis diameter, two bacterial strains $EC2_3$ and $EC2S_3$ demonstrated the highest potential for protease production and they were selected for further studies. In addition, the extracellular protease was examined using the fermentation production medium at 30°C for 48h, with a constant agitation of 150 rpm. The enzyme activity was determined under varying conditions of pH, incubation temperature, and salt concentration, using Sigma's Universal Protease Activity Assay. The enzyme from $EC2_3$ strain showed higher activity in all cases than the $EC2S_3$ strain, which indicated that it was the most ideal organism for enzyme production.

## INTRODUCTION

Waters with a salinity of 3% or above are referred to as saline waters (De Decker, 1983). Saline habitats are frequently populated by an abundance of microbial communities, which are adapted to these ecosystems. Among the microorganisms, bacteria play a major role as important and dominant inhabitants of saline and hyper-saline environments (Zahran, 1997; Spring *et al.*, 2019).

Due to their unique properties, halophilic microorganisms have been explored for their biotechnological potential in different fields (Selvam and Riya, 2013).

The global industrial enzymes market was estimated to be about $4.2 billion in 2014, and it was expected to reach about $6.2 billion by 2020 (Thakur *et al.,* 2018). Proteases are one of the most important groups of industrial enzymes, representing about 60% of the enzyme market, and microorganisms are the main source of protease production (Shaikh and Dixit, 2018). The proteases (EC 3.4.16) are a group of hydrolytic enzymes that catalyze protein hydrolysis by cleaving peptide bonds to produce short peptides and free amino acid residues (Sawant and Nagendran, 2014).

The proteases are an enzyme complex group that occupies a central position among different enzyme classes, due to their physiological and commercial roles. They are ubiquitous in nature, and present in plants, animals and microorganisms (Barrett and McDonald, 1986; Bach *et al.,* 2012). Microorganisms, especially bacteria, are considered the main source of enzyme production, as they have several advantages, including rapid and easy production processes, high yield, and genetic manipulation opportunities (Breithaupt, 2001).

Among all protease types, alkaline proteases represent about 89% of the protease market (Sharma *et al.,* 2016; Dorra *et al.,* 2018). Their industrial application is restricted by their limited activity and stability at high temperatures, extreme pH, detergent ingredients addition and organic solvents (Olajuyigbe and Falade, 2014). One of the main applications of alkaline proteases is in the detergent formulation industry, constituting about 30% of the total alkaline protease application. In addition, the alkaline proteases have important roles in several industries, including leather, food, peptide synthesis, beverages, silver extraction from X-ray films, and pharmaceutical and food industries (Akolkar and Desai, 2010; Banerjee *et al.,* 2016; Marathe *et al.,* 2018). Therefore, the microbial production of highly active alkaline proteases with novel properties has attracted the research's interest (Ahmad *et al.,* 2020; Gulmus and Gormez, 2020; AL Hakim *et al.,* 2018; Harer *et al.,* 2018; Lakshmi *et al.,* 2018; Ramkumar *et al.,* 2018; Zheng *et al.,* 2020).

## MATERIALS AND METHODS

**Samples Source:**

The seawater and sediment samples were collected in sterile containers at different depths surface, (3 meters to 10 meters) from different areas of Cap Rousseau beach in the Oran city in northwestern Algeria (35.1786636, -0.6180867) **(**Fig. 1).

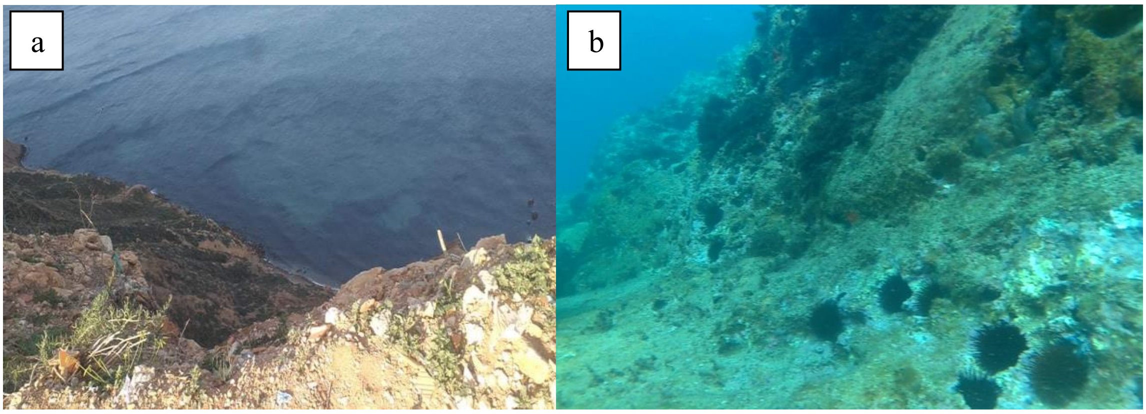


**Fig. 1:** a) A photo of the seawater in the Cap Rousseau area. b) A photo showing the sediment at a depth of 8 meters.

**Isolation of Protease-Producing Bacteria:**

The protease-producing bacteria were isolated using a serial dilution of up to $10^{-3}$ in sterile saline water after enrichment in a nutrient broth prepared with seawater. Dilutions were inoculated on nutrient agar medium dishes, supplemented with 10% of skim milk, prepared with seawater, and incubated at 30°C for 48 to 72 hours (Uyar *et al.,* 2011). The positive strains colonies, which presented a lightening halo around their borders, were selected on the basis of their divergence in morphology, size, and color; that's how they were purified by repetitive streaking on an agar nutrient medium and maintained systematically at +4°C in culture.

**Screening of the Best Protease-Producing Strain:**

The proteolytic activity of the isolated pure cultures was screened using two specific media prepared with seawater and adjusted to pH=8:

- The skim milk agar (10%), with some modifications that consisted of skim milk 10%, peptone -0.5%, yeast extract - 0.25%, agar - 1.5%.
- Gelatin medium containing $K_2HPO_4$ 0.2%, glucose 0.1%, peptone 0.5%, gelatin 1.5%, and agar 1.5%.

In order to estimate the protease enzyme produced by the selected bacteria, the cleared zone diameters around the inoculated colony; were measured after 24h of incubation at 30°C, and the hydrolysis zone diameters on the gelatin medium, were then measured by inundating the dishes with mercuric chloride solution ($HgC_{l2}$), this method was qualified as gelatin clear zone method (Abdel Galil, 1992). The microorganism with the largest clear zone and with the production of the maximum protease was selected, identified and maintained at +4°C.

**Identification of the Selected Protease-Producing Bacteria:**

The isolated and purified cultures with high protease productivity were identified by morphological examination such as macroscopic and microscopic observation after Gram staining and biochemical characterization according to the methods **by (Buchanan and Gibbons, 1974).**

**Bacteria Culture in The Fermentation Broth Medium and Crude Enzyme Production:**

The isolated protease-producing bacterial strains were inoculated into separated flasks containing protease-producing broth medium which was composed of Horikoshi alkaline medium, with some modifications (Horikoshi, 1999). The medium (pH 8) contained (g/l): glucose -10g, peptone-5g, yeast extract -5g, $KH_2PO_4$-1g, $MgSO_4$ 7 $H_2O$-0.2g, NaCl - 30g, $Na_2CO_3$ -2g. The glucose and the sodium carbonate were autoclaved separately, then added aseptically to the remaining autoclaved medium and incubated at 30°C for 48h with continuous agitation of 150 rpm. After incubation, bacterial growth was observed in the liquid medium. The crude enzyme extract was collected by centrifugation of the inoculated bacterial strain in the fermentation broth at 10000 rpm for 10 minutes at +4°C. The supernatant was then separated and used for further analysis.

**Protease Activity Test:**

The protease production was evaluated according to the observed protease activity using casein as a substrate (Saxena and Singh, 2010). One unit of protease activity was defined as the enzyme amount required to release 1 μg/mL of tyrosine in 1 min under the experimental conditions.

Protease activity was estimated by the Sigma non-specific protease activity test. To start this test, for each enzyme isolated from each organism, 4 vials containing 15 ml were required. One vial was used as a blank, and three others were used to test the protease activity of three dilutions. In all tubes, 5 ml of 0.65% casein solution was added. After allowing them to equilibrate in a 37°C water bath for approximately 5 minutes, varying volumes (<1 mL) of enzyme solution were added for testing in three of the vial samples, except for the blank, and the tubes were agitated to uniformly mix the enzyme and the substrate. The tubes were then incubated at 37 °C for 15 to 20 minutes. After incubation, 5 mL of 110 mM TCA was added to all tubes to stop the reaction. Then, an appropriate volume of enzyme solution was added to all tubes, including the blank, so that the final volume of enzyme solution in each tube was 1 mL, to accommodate the absorbance value of the enzyme itself and to provide an equal final volume in each tube. The tubes were then incubated at 37 °C for 30 minutes. After incubation, 2 mL of each test and the blank solution were obtained by centrifugation at 10000 rpm for 5 min, and the supernatant was transferred to new, unused tubes. Then 5 mL of sodium carbonate was added to all tubes and 1 mL of Folin's reagent was added immediately. The tubes were incubated again in the dark at 37 °C for 30 min. After incubation, 3-4 mL of solution from each tube was obtained by filtration and used to check the absorbance at 660 nm (Cupp-Enyard, 2008).

Standard preparation: a 1.1 mM Tyrosine standard solution was prepared. Six test tubes were collected, and in the first tube, 50 µL of standard solution (corresponding to 0.055 µM Tyrosine) was added, followed by 100 µL (0.111 µM Tyrosine), 200 µL (0.221 µM Tyrosine), 400 µL (0.442 µM Tyrosine), and 500 µL (0.553 µM Tyrosine), respectively, to the other tubes, except for the blank. Then, to each tube, including the blank, the distilled water was added so that the total volume reached 2 mL in all tubes. The tubes were kept apart for incubation at 37 °C for 30 minutes. Then, 5 mL of sodium carbonate was added to all tubes and 1 mL of Folin's reagent was added immediately. The tubes were incubated again at 37 °C for 30 minutes. After incubation, 3-4 ml of solution from each tube was obtained by filtration and used to test the absorbance at 660 nm. These values were then used to draw the standard graph. The enzyme activity was calculated using the following formula (Cupp-Enyard, 2008):

$$\text{Protease activity}\left(\frac{U}{mL}\right)=\frac{\mu\text{Moles of tyrosine equivalents released}\times\text{Total volume of assay(mL)}\times\text{Dilution factor}}{\text{Total volume of enzyme used in the assay(mL)}\times\text{Time of assay(min)}\times\text{Volume in cuvette(mL)}}$$

**pH Effect on Alkaline Protease Activity:**

To explore the pH value effect on the protease activity, the enzyme from each organism was tested with different pH values. The enzyme was pre-incubated with the corresponding buffer for 20 minutes at 37 °C. The buffers used for the test were the potassium phosphate buffer (pH 6-7) and the borate buffer (pH 8-11). After incubation, the protease activity was tested according to Sigma's non-specific protease activity assay using 0.65% of casein as substrate.

**Temperature Effect on Alkaline Protease Activity:**

To study the temperature effect on protease activity, the enzyme was diluted using a buffer with an optimum pH. The protease activity was tested according to Sigma's non-specific protease activity assay using 0.65% of casein as substrate. The enzyme-substrate mixture incubation time was maintained for 20 minutes but at different incubation temperatures. The temperatures for which the enzyme activity was tested were 20 °C, 25 °C, 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 60 °C and 70 °C.

**Salt Concentration Effect on Alkaline Protease Activity:**

As the organisms were obtained from seawater, the salt concentration effect on the enzyme activity was tested by Sigma's non-specific protease activity assay using 0.65% of casein as substrate. Different enzyme production media were prepared for each organism, and these different media included a NaCl concentrations range (1-10%). The culture flasks were then incubated at 37 °C for 48 hours with an agitation of 150 rpm. The obtained enzyme was then separated and tested for protease activity (Marathe *et al.*, 2018).

## RESULTS AND DISCUSSION

### Best Protease-Producing Strain Isolation and Selection:

Various bacteria were isolated from seawater and sediments to determine if they produced a protease. From the 44 positive strains, which showed a clearance zone around their borders as illustrated in (Fig.2), 13 isolates were selected after screening for a qualitative protease test based on the clearance zone using a skim milk agar and gelatin agar (Table 1) (Figs. 3 and 4).


**Fig. 2:** Casein hydrolysis manifested by a clearance zone around the colonies on skim milk agar.

**Table 1:** Hydrolysis zones measured on skim milk agar and gelatin agar.

| Isolate | Hydrolysis zone on skim milk agar (mm) | Hydrolysis zone on gelatin agar (mm) |
|---|---|---|
| EC2”$_1$ | 26 | 77 |
| EC2S$_4$ | 43 | 72 |
| EC2$_{12}$ | 52 | 76 |
| EC2’$_2$ | 16 | 13 |
| EC2S$_2$ | 50 | 75 |
| EC2”$_3$ | 35 | 55 |
| EC2”$_5$ | 29 | 70 |
| EC2”$_{11}$ | 37 | 18 |
| EC2”$_9$ | 70 | 16 |
| EC2$_3$ | 90 | 75 |
| EC2S$_3$ | 76 | 75 |
| EC2”$_7$ | 32 | 78 |
| EC2’$_4$ | 55 | 35 |
| EC2S$_1$ | 26 | 58 |

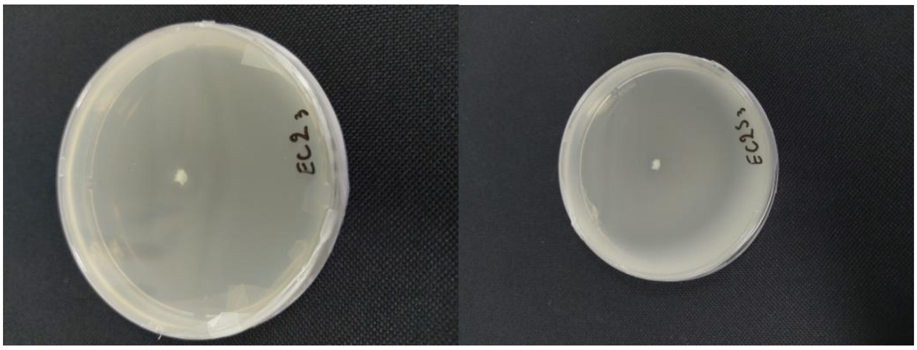


**Fig. 3:** The inhibition zone of $EC2S_3$ and $EC2_3$ isolates on skim milk agar.

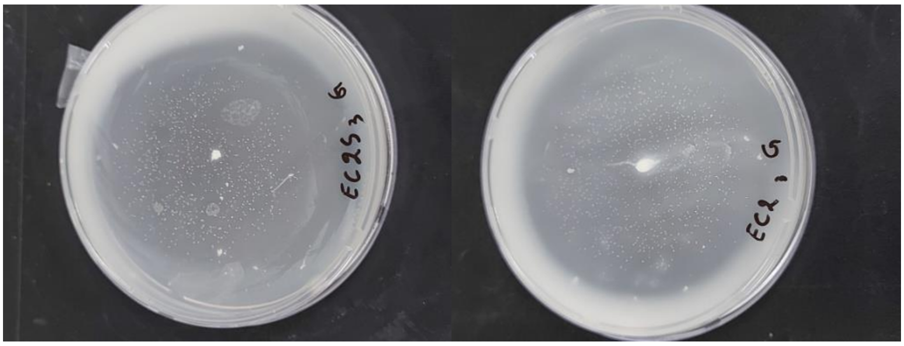

**Fig. 4:** The inhibition zone of $EC2S_3$ and $EC2_3$ isolates on gelatin agar.

From the 13 isolates, $EC2S_3$ and $EC2_3$ were determined to possess a higher protease activity and were selected for further studies. The $EC2S_3$ and $EC2_3$ isolates identification was determined by the morphological observation and biochemical test (Table. 2) (Fig. 5).

**Table 2:** Morphological and biochemical characterization of $EC2S_3$ and $EC2_3$ strains isolated from marine samples

| Characteristics | $EC2S_3$ strain | $EC2_3$ strain |
|---|---|---|
| Colony morphology | Medium colony, circular, slightly domed, smooth, shiny, opaque, yellowish, creamy with a regular contour. | Medium colony, irregular, flat, filiform, rough, opaque, creamy, matted |
| Gram staining | - | + |
| Motility | + | + |
| Mannitol | + | + |
| Catalase | + | + |
| Oxidase | - | - |
| Voges-Proskauer | + | + |
| Citrate | + | - |
| Urease | - | - |
| Nitrate test | + | + |
| $H_2S$ production | - | - |
| Starch | - | - |
| Gelatin liquefaction | + | + |
| TSI agar (Slant/Butt) | + | + |
| Glucose fermentation | + | + |
| Indole | + | + |
| Cellulase | + | + |
| Lipase | + | - |

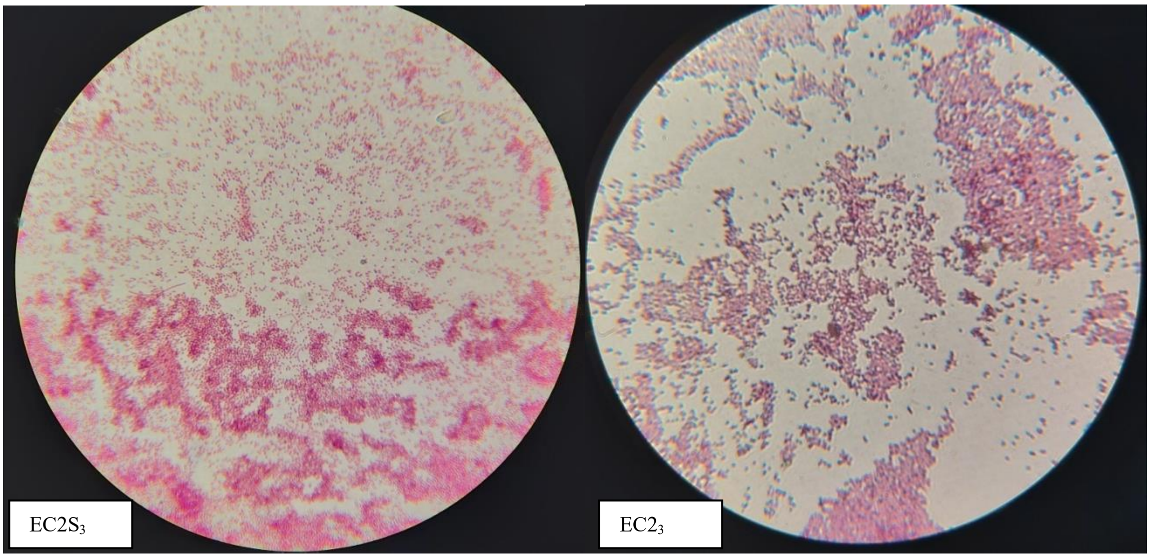


**Fig. 5:** Bacterial strains (EC$2_3$, EC2$S_3$) Microscopic observation, after Gram staining, at x100 magnification.

The samples taken allowed the isolation of Gram-positive and negative bacteria, able of producing proteases. Many works carried out are largely focused on enzyme production from Gram-positive bacteria, of which species belonging to the bacillus genus are the most targeted for their significant and high proteolytic activity.

**Extracellular Protease Quantitative Analysis:**

The current study's objective was to select bacterial strains with a high level of protease production capacity. In the first screening, we selected two isolates that were subjected to a quantitative extracellular protease test in a liquid medium after 48h.

**pH Effect on Protease Activity:**

To explore the pH value effect on the protease production by the EC$2_3$ and EC2$S_3$ strains, the enzyme crude extract was studied at different pH, and incubated in the casein presence at a temperature of 37°C. The protease isolated from strain EC$2_3$ registered a maximum enzyme activity of 271 U/ml at pH 10, while the enzyme isolated from EC2$S_3$ strain reported a maximum enzyme activity of 87 U/ml at pH 9 (Fig. 6). These results were comparable to those in the literature; (Marathe *et al.*, 2018), which indicate that Bacillus protease production has an optimal pH of 10, while *Alcaligenes* and *Pseudomonas* have an optimal pH of 9 and (Ibrahim *et al.,* 2015), which show that the strain *Bacillus* sp NPST-AK15 could grow and produce alkaline protease over a wide pH range from 7 to 12, with maximal enzyme production at pH 11. While according to the literature; (AL-Shehri *et al.,* 2004), alkaline proteases useful for detergent applications were mostly active in pH range of 8-12. In another literature, for eight isolates studied for protease production, it was found that the optimum pH for growth was 9 for the majority of the isolates, while the optimum pH with regard to enzyme secretion varied between pH 8-10 (Dodia *et al*., 2006). A pH 9 has been reported as optimal for protease production by *Bacillus* sp (Prakasham *et al*., 2006), *Bacillus* sp. Strain APP1 (Chu, 2007), *Bacillus proteolyticus* CFR3001 (Bhaskar *et al*., 2007) and *Pseudomonas fluorescens* (Kalaiarasi and Sunitha, 2009). While a pH of 8 has been reported as optimal for protease production by *Bacillus licheniformis* isolated from saltern sediments (Suganthi *et al*., 2013).

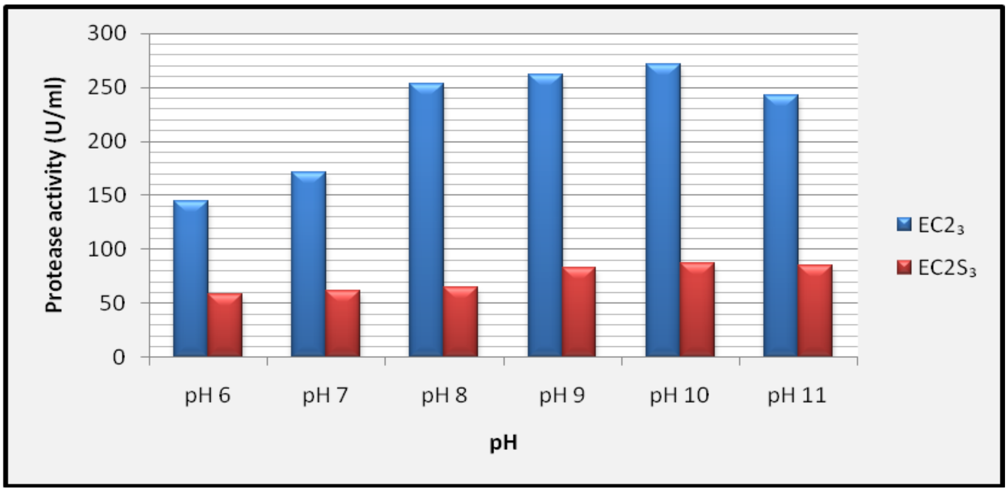


**Fig. 6:** pH effect on alkaline protease activity (U/ml) produced by isolates $EC2_3$ and $EC2S_3$.

**Temperature Effect on Protease Activity:**

Temperature is a critical parameter that must be controlled and that varies from organism to organism. It influences extracellular enzyme secretion by modifying the cell membrane's physical properties. To explore the temperature effect on the protease production by the selected strains $EC2_3$ and $EC2S_3$, the crude enzyme extract was incubated in the casein presence at different temperatures. The results obtained for temperature optimization for enzyme production indicated that $EC2_3$ and $EC2S_3$ isolates produce protease in a wide range of temperatures, the optimal temperature for protease enzyme production was 60°C, $EC2_3$ strain registered the highest activity of 326 U/ml, while the $EC2S_3$ strain reported the highest activity of 120 U/ml (Fig. 7). These results are similar to the literature; (Marathe *et al.*, 2018), which indicates that *Bacillus* and *Alcaligenes* protease production has an optimal temperature of 55°C, while the *Pseudomona*s has an optimal temperature of 50°C and the literature; (Ibrahim *et al.*, 2015), which report that the strain *Bacillus* sp NPST-AK15 has a maximal growth and alkaline protease production at 40°C. While according to AL-Shehri *et al.*, 2004, alkaline proteases useful for detergent applications were mostly active at temperatures between 50°C–70°C. Interestingly, the enzyme from an alkalophilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85°C (Nilegaonkar *et al*., 1998). In another literature high optimum temperature of 50°C has been reported for *Bacillus* sp. Strain APP1 (Chu, 2007).

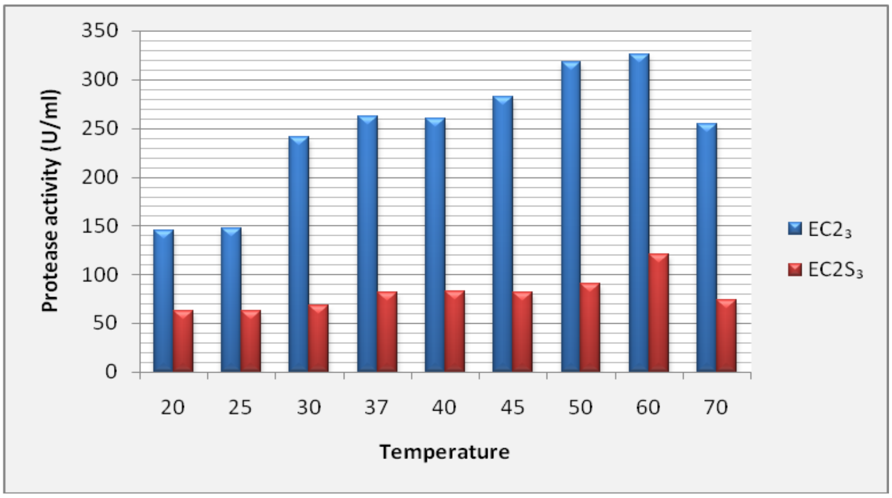


**Fig. 7:** Temperature effect on alkaline protease activity (U/ml) produced by isolates $EC2_3$ and $EC2S_3$.

**Effect of Salt Concentration on Protease Activity:**

In the case of salt concentration ranging from 0% to 10%, it was observed that both isolates $EC2_3$ and $EC2S_3$ protease demonstrated a significant protease activity and salt tolerance up to 10%, but it was observed that the $EC2_3$ optimal activity was reported as the highest activity of 241 U/ml in the presence of 4% NaCl, while the enzyme isolated from $EC2S_3$ strain recorded the highest enzyme activity of 83 U/ml in the presence of 3% NaCl, which is shown in (Fig. 8). These results are compared to the literature; (Ibrahim *et al*., 2015), which revealed that the strain *Bacillus* sp NPST-AK15 can grow over a wide range of NaCl concentrations from 0 to 20%. However, maximal growth and alkaline protease production were seen in a medium that contained 0-5% of NaCl.

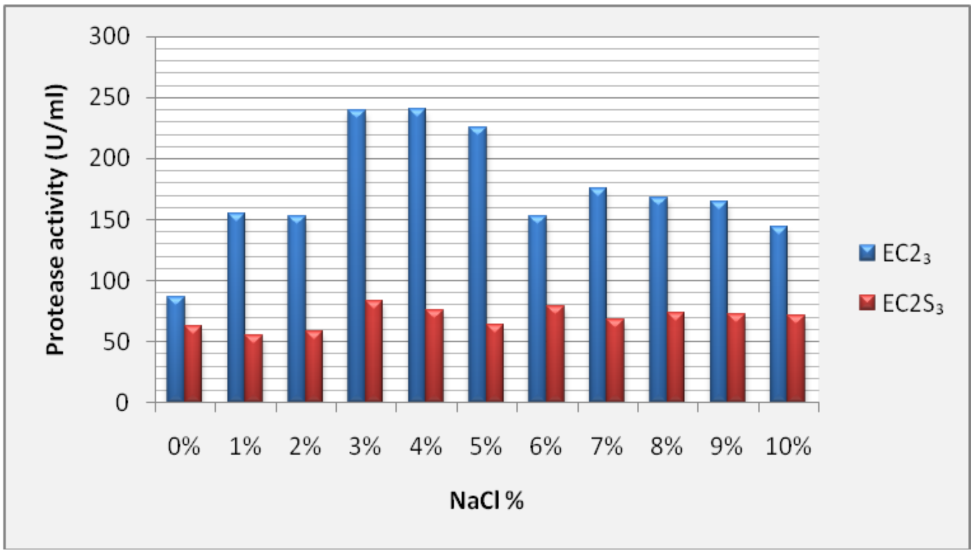


**Fig. 8:** Effect of salt concentration on alkaline protease activity (U/ml) produced by isolates $EC2_3$ and $EC2S_3$.

## CONCLUSION

Protease is one of the most widely used enzymes in industrial processes. The microorganism field is currently attracting interest from global investors, and technological progress is being made rapidly. Currently, the production cost is high, which is driving the development towards value-added markets. The microbial enzymes application has allowed the replacement of chemicals used in industries (paper bleaching) or at home (chemical-based cleaning solution), which prevented the release of toxic substances in the environment. The current study is focused on the search for microbial species with a high-capacity level for producing proteases that have potential applicability in various industries, as well as the improvement of the microbial biodiversity knowledge in the ecosystems will conduct to the use of this biodiversity in various industrial processes or in the biotechnological valorization of interest enzymes. Our study showed that the isolates are *Bacillus* bacteria producing alkaline proteases; it is interesting to search for the enzyme production new sources, the isolated strain $EC2_3$ was shown to be an excellent protease producer with a maximum enzyme yield of 326 U/ml. Therefore, further enzyme characterization studies, such as extraction and purification, will be required.

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