ISOLATION AND CHARACTERIZATION OF BACTERIAL PROTEASE ENZYME OF LEATHER WASTE

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The objectives of this study were to isolate and characterize bacteria which produced protease enzyme from tannery solid waste. A solid leather waste sample was used for bacterial isolation, taken from different waste warehouses (solid waste in unhairing phase). Several bacterial strains were isolated from the cultures in Petri dishes, after the growth of the colonies. These strains were characterized in terms of the production of proteolytic enzymes, by a method of screening on the media with casein, which allows the determination of proteolytic indices of microorganisms, the colony diameter, diameter of clear zone, proteolytic index, and enzymatic activities characterization on difference of pH and temperature.

Keywords: leather waste, enzymes, proteolytic bacteria

INTRODUCTION

The versatility and abundance of proteases are unmatchable and hence have drawn the attention of many researchers for use in various industrial processes (Naveed *et al*., 2021). The leather industry has started using proteases in various processes to combat pollution-related issues. Protease enzyme can be produced from animals, plants and microorganism products. When the enzyme derived from plant and animal products is used, it may have drawbacks (Thanikaivelan *et al*., 2004). This is because the plant tissues contain hazardous materials such as phenolic compounds. Protease enzymes used in the industry are generally produced from microorganisms (Baehaki *et al*., 2011). The use of microorganisms to produce the protease enzyme has several advantages. It can be easily produced on a large scale, it has relatively short production time, and it can be produced in a sustainable manner with a relatively low cost (Noble *et al*., 2009, Kanagaraj *et al*., 2015, Fernandez *et al*., 2019).

For a time, numerous natural enzymes have been attempted to investigate in leather processing to replace chemicals, although complete chemical substitution by enzymes has yet to be done (Calin *et al*., 2019). Degradation of unwanted protein by a simple eco-friendly and inexpensive method is one of the central requirements in several industries, especially the leather industry. Enzymes are employed as an auxiliary during liming to speed up the processing stages.

Proteases are fundamental industrial enzymes having wide applications in biochemical and chemical processes because they can break peptide bonds present in proteins. The major advantages of using proteases are specificity, biodegradability, the opportunity of producing natural products and activity under mild reaction conditions (Hamza *et al*., 2017).

Proteases are the most important extensively utilized enzymes for dehairing hides and skins. Some researchers have utilized them in rehydration, pickling, soaking, and chrome tanning (Nyakundi *et al*., 2021). Proteases differ in terms of substrate selectivity, working pH, catalytic activity, working temperature, active site specificity,

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stability profiles, and other characteristics (Kumar *et al*., 1999). There are 259 different families of proteolytic enzymes that have been indexed in the MEROPS peptidase database so far, grouped according to amino acid sequence similarity. It is known that proteases from microbial sources account for around 40–60% of the total global enzyme sales (Kumar *et al*., 2020).

Recently, several proteolytic bacteria of the genus *Bacillus* have been tested for waste treatment in the leather industry, due to their properties for the synthesis of hydrolytic enzymes, especially collagenases and keratinases, as well as resistance to environmental factors (Li *et al*., 2021; Ferdes *et al*., 2020)

The objectives of this study were to isolate and characterise the bacteria producing protease enzyme from solid waste of tannery.

The protease is characterized for enzymatic activities. Results of this study are proposed as an alternate source of protease enzyme contributing to the tanning industry, especially in the unhairing phase (Zouboulis *et al*., 2004; Ferdes *et al*., 2020).

MATERIALS AND METHODS

The studied samples were mainly protein waste from different phases of leather processing in tanneries. In order to determine the pH value of the samples, fragments of 1 cm^2 were taken from each protein waste, which were suspended in Erlenmeyer flasks with 25 ml physiological serum. Also, sample specimens with the same surface area were suspended in Erlenmeyer flasks with 25 ml nutrient broth and incubated at 28°C for 72 hours. The resulting culture liquids were used to make serial decimal dilutions. These were seeded by embedding in Petri plates with nutrient agar, in three repetitions, in order to obtain isolated colonies and determine the number of viable cells. The plates were incubated for 24 hours in a bacteriological thermostat, at 28°C and then the number of isolated colonies was estimated by calculating the average of the values obtained in the three repetitions performed for each dilution. In order to obtain pure cultures, the isolated colonies were transplanted onto tubes with the solidified nutrient medium, which were incubated for 24 hours. After this interval, the bacterial strains were examined in terms of colonial morphological characteristics, Gram staining, the presence of endospores, the ability to hydrolyze proteins. The study of the morphological characteristics of the isolated microorganisms was carried out by optical microscopy observations on smears stained by the Gram staining method, which were examined under a microscope with a 100x immersion objective.

The proteolytic activity of isolated microorganisms was determined by a semi-quantitative screening method in Petri dishes, on an agar medium containing 0.25% casein as sole carbon source. Due to the hydrolysis of casein (which causes opacification of the agar medium), a transparent area appears around the colonies producing proteolytic enzymes.

After the development of the colonies (3 days for bacteria) the diameters of the colonies and of the hydrolysis zone were measured. The proteolytic index was determined as the ratio between the 2 diameters:

$$I_P = \frac{Diameter\ of\ hydrolysis\ zone}{Diameter\ of\ colony} \quad (1)$$

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RESULTS AND DISCUSSION

The determination of the pH value highlighted the pH change of the physiological serum after the immersion of the leather samples, from pH 6.4 to values between 6.6-8.6.

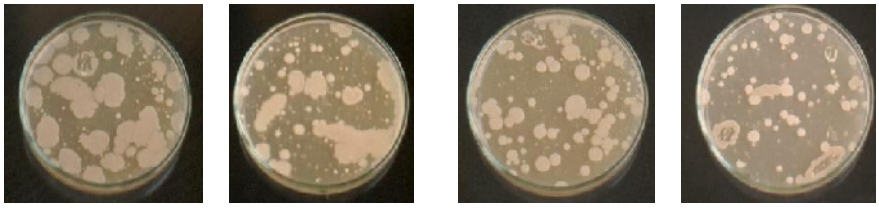
The calculation of the number of viable cells obtained on plates with agar nutrient medium led to values between $2 \cdot 10^7$ in the case of skin samples P_1 and P_2, and $4 \cdot 10^7$ in sample P_3 while, sample P_4 presented a relatively lower number of bacterial cells - $2 \cdot 10^5$ / cm^2 (Table 1).

Table 1. Determining the number of cells obtained on plates with agar nutrient medium

No.	Sample	pH	Number of bacteria / cm^2 sample	Number of isolated strains
1.	P 1	6.82	$2 \cdot 10^7$	12
2.	P 2	6.80	$2 \cdot 10^7$	9
3.	P 3	6.66	$4 \cdot 10^7$	8
4.	P 4	8.69	$2 \cdot 10^5$	11

From the four samples analysed, a total number of 40 bacterial strains were isolated using the method of serial decimal dilutions, which were seeded on nutrient agar. Among the strains, the majority presented white-beige colonies, with a matte, rough surface and irregular edges. In the case of sample P_3, the strains that formed spherical, white colonies on nutrient agar predominated, with a smooth surface and whole edges. Strains $P_{4/10}$ and $P_{4/11}$ were differentiated by the color and appearance of the colonies formed, which were flat, semi-glossy, dark beige, with a smooth surface and whole edges.

Examination by optical microscopy of the smears stained by the Gram method enabled checking the purity of the cultures and highlighted the fact that most of the strains were represented by Gram-positive bacilli, with rounded ends, arranged in short chains of 2-4 cells or in irregular agglomerations.



P1 P2 P3 P4

Figure 1. Isolation results of tannery solid samples obtained from tanning industry

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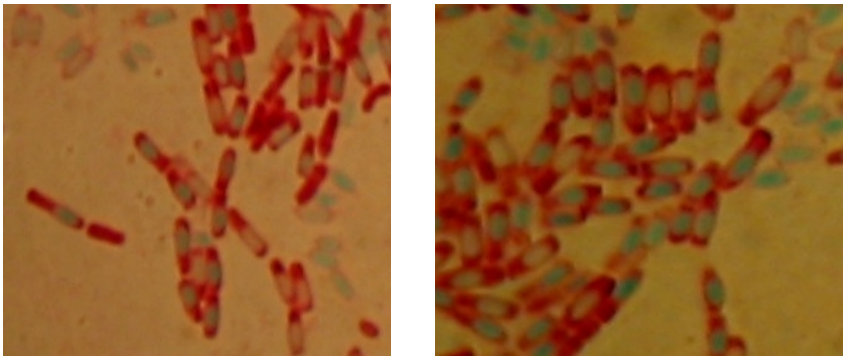

Figure 2. Microscopic highlighting of bacterial endospores in strains P 4/1 (left) and P 4/5 (right). Green spores are observed in the red vegetative cells

Gelatin hydrolysis testing highlighted the fact that out of the 40 isolated strains, 38 determined the complete liquefaction of the culture medium, while two strains P 4/10 and P 4/11 (isolated from sample P 4) did not present the ability to hydrolyze gelatin.

Testing the casein hydrolysis capacity led to similar results in the case of both experimental variants. Thus, all the strains tested determined the hydrolysis of the substrate and the clarification of the culture medium around the areas of bacterial growth. The determination of the hydrolysis zone size led to the observation of some differences between the tested microorganisms so that, in 17 strains (42%) the diameter of the hydrolysis zone was between 6-12 mm, while 23 strains (58%) showed superior activity of casein hydrolysis, the diameter of the clear zone exceeding 12 mm (Fig. 3).

Figure 3. Testing the ability of the isolated strains to hydrolyze the casein present in the sample plate (left) compared to the control plate (right)

CONCLUSION

The microbiological analysis of leather waste samples revealed the presence of bacteria and the density of bacteria developed on the leather samples was estimated at $2 \cdot 10^7$ cells/cm^2 in the case of samples P 1 – P 3 and $2 \cdot 10^5$ cells/cm^2 in the case of P 4.

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The vast majority of isolated colonies have a morphology characteristic of *Bacillus* species, with irregular, flat "R"-type shapes. This suggests specific colonization and restricted species diversity. Microscopic observations revealed the presence of bacillary, sporulated, Gram-positive cell forms.

Following the isolation work in pure cultures, 40 strains of bacteria were obtained which constitute a first collection of bacteria that contaminate the skin samples. The proteolytic capacity of the tested strains was highlighted by the gelatine and casein hydrolysis test.

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