ENZYMATIC BIOTECHNOLOGY APPLIED TO PELT WASTE

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ABSTRACT. Enzymes are the substances that speeds up a chemical reaction suitable for green chemistry and can be used to achieve ecological industrial processing in order to reduce the effects of industrial pollution. Microbiological degradation of pelt waste is amongst the permanent concerns of tanners. Several enzymes have been used in the leather industry to substitute the conventional process, mainly proteases. The objectives of this study were to isolate and identify bacteria which produced protease enzyme from tannery solid waste. The selected bacterial strains showed increased protease biosynthesis capacity, which had a significant hydrolytic action on pelt waste. The results obtained in this study demonstrated the ability of strains belonging to the *Bacillus* genus to synthesize increased amounts of proteolytic enzymes and to degrade pelt waste, as well as the possibility of using these microorganisms as a source of protease in various biotechnological processes.
KEY WORDS: leather waste, isolation of bacteria, protease enzyme

BIOTEHNOLOGIE ENZIMATICĂ APLICATĂ DEȘEURILOR DE PIELE GELATINĂ

REZUMAT. Enzimele sunt substanțele care accelerează o reacție chimică în acord cu chimia verde și pot fi utilizate în procese industriale ecologice pentru a reduce efectele poluării industriale. Degradarea microbiologică a deșeurilor de piele gelatină se numără printre preocupările permanente ale tăbăcarilor. Mai multe enzime au fost folosite în industria de pielărie pentru a înlocui procesul convențional, în principal proteaze. Obiectivele acestui studiu au fost de a izola și identifica bacteriile care produc enzima protează din deșeurile solide din tăbăcărie. Tulpinile bacteriene selectate au prezentat o capacitate crescută de biosinteză a proteazei, care a avut o acțiune hidrolitică semnificativă asupra deșeurilor de piele gelatină. Rezultatele obținute în acest studiu au demonstrat capacitatea tulpinilor aparținând genului *Bacillus* de a sintetiza cantități crescute de enzime proteolitice și de a degrada deșeurile de piele gelatină, precum și posibilitatea utilizării acestor microorganisme ca sursă de protează în diferite procese biotehnologice.
CUVINTE CHEIE: deșeuri de piele, izolarea bacteriilor, enzimă protează

LA BIOTECHNOLOGIE ENZYMATIQUE APPLIQUÉE AUX DÉCHETS DE PEAU EN TRIPE

RÉSUMÉ. Les enzymes sont les substances qui accélèrent une réaction chimique conformément à la chimie verte et peuvent être utilisées pour réaliser un traitement industriel écologique afin de réduire les effets de la pollution industrielle. La dégradation microbiologique des déchets de peau en tripe fait partie des préoccupations permanentes des tanneurs. Plusieurs enzymes ont été utilisées dans l'industrie du cuir pour remplacer le procédé conventionnel, principalement des protéases. Les objectifs de cette étude étaient d'isoler et d'identifier les bactéries qui produisent l'enzyme protéase à partir des déchets solides de la tannerie. Les souches bactériennes sélectionnées ont montré une capacité accrue de biosynthèse des protéases, qui a eu une action hydrolytique significative sur les déchets de peau en tripe. Les résultats obtenus dans cette étude ont démontré la capacité des souches appartenant au genre *Bacillus* à synthétiser des quantités accrues d'enzymes protéolytiques et à dégrader les déchets de peau en tripe, ainsi que la possibilité d'utiliser ces micro-organismes comme source de protéase dans divers procédés biotechnologiques.
MOTS CLÉS : déchets de cuir, isolement de bactéries, enzyme protéase

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INTRODUCTION

The leather processing industry generates a huge highly polluting quantum of solid wastes relative to the main product – leather, while processing animal hides for leather making. The pollution effect caused by the present inefficient disposal of these solid wastes impedes the industry's path towards sustainable growth. In order to comply with pollution and discharge restrictions imposed by environmental regulatory agencies, leather industries around the world are shifting to cleaner and milder technology, such as enzyme biotechnology [1]. Current technology allows for the isolation, immobilization and purification of the specific enzymes required for the desired function. Proteases can be used in several biotechnological processes including leather, food and detergent industries. In addition to facilitating the tanning process, the enzymes can also replace chemicals, which means a reduction in the amount of chemical waste [2, 3]. Therefore, the production of enzymes is needed for the leather tanning process.

In recent years, there has been an increased interest in the use of biological degradation of pelt waste. Leather has a complex composition comprising collagen, keratin, elastin, albumins and globulins [4, 5]. Each of these compounds can be degraded under certain environmental conditions (pH, temperature, substrate selectivity, humidity) under the action of enzyme complexes synthesized by a variety of microorganisms (bacteria and molds). Pelt waste degradation occurs by means of proteolytic enzymes [6]. Microbiological degradation of pelt waste is amongst the permanent concerns of leather processing units. Microorganisms (bacteria and molds) play an important role in solving these problems [7].

Among various enzymes proteases have long been used in the bating stage of leather processing, because of their ability to execute reactions with excellent efficiency and selectivity; as a result, enzyme has emerged as a popular tool in green processing applied in the leather sector [8-10].

Protease enzyme can be produced from animals, plants and microorganism products. However, the use of enzymes derived from animal and plant products may have drawbacks. Protease enzymes used in the industry are generally produced from microorganisms [11]. They can be easily produced on a large scale, they have a relatively short production time, and they can be produced in a sustainable manner with a relatively low cost.

Several microorganisms that had been known as protease-producers in commercial applications were *Bacillus cereus*, *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa*, *Aspergillus oryzae* and *Aspergillus flavipes* [12-14]. There are several types of *Bacillus* bacteria capable of producing protease [15, 16].

The objectives of this research were to identify and isolate the bacteria producing protease enzyme from tannery solid waste. The protease is characterized for enzymatic activities. Results of this study are proposed as alternate source of protease enzyme contributing to tanner industry, as well as the possibility of using these microorganisms as a source of protease in various biotechnological processes. The studies have shown that employing enzymes in leather manufacturing can result in higher quality leather.

EXPERIMENTAL

Materials and Methods

Samples

Ground pieces of grey-yellowish pelt waste of hard, slightly wet and gelatinous consistency were used in the experiments (Figure 1).

Figure 1. The macroscopic appearance of pelt waste samples used in degradation experiments

Microorganisms

Three strains with high proteolytical activity were used: P_1, P_2, P_3, strains of the *Bacillus* genus.

To obtain a preinoculum, strains were transplanted in tubes with inclined solidified nutrient medium (agar), which were incubated in a thermostat at 28°C for 24 hours. Subsequently, from the exponentially growing cultures, the bacterial inoculum was made in Erlenmeyer flasks with nutrient broth, which were incubated in a thermostat at 28°C for 24 hours.

Growth Conditions

In order to obtain the bacterial cultures used in pelt degradation experiments, the inoculum was then seeded in a 1/10 ratio in nutrient broth with different pH values (5, 7, 9) in Erlenmeyer flasks which were incubated in a thermostat at 20, 28 and 37°C, respectively, under static conditions for 72 hours.

After this interval, ground samples of pelt, with a known weight of 2.02-2.05 g, were added to the bacterial cultures developed in liquid medium. The Erlenmeyer flasks containing the pelt sample and the bacterial cultures with high proteolytic activity were incubated in a thermostat at different temperature values (20, 28, 37°C), in static conditions for 21 days.

In order to determine the proteolytic activity of the selected strains, culture fluids were harvested at intervals of 7, 14 and 21 days from bacterial cultures developed on a nutrient medium with different pH values and incubated at different temperatures (20, 28 and 37°C).

Enzymatic activity was determined spectrophotometrically at 280 nm and expressed in mg casein/ml.

The decomposition of pelt waste was assessed by macroscopic observations of samples from bacterial cultures and gravimetric determination of their weight, after 21 days of incubation, for each experimental variant.

RESULTS AND DISCUSSION

The obtained results showed that the synthesis of proteases by bacterial strains P_1, P_2, P_3 intensified during the incubation period, the maximum values of enzymatic activity being determined in all experimental variants after 21 days of contact with pelt samples (Figures 1-9).

Bacterial strain P_1 showed maximum proteolytic activity under incubation conditions at 37°C at all pH values of culture media (5, 7, 9), but the highest enzymatic activity was found in the nutrient medium with pH=5 (5.798 mg casein/ml). It was also found that the biosynthesis activity of protease decreased with increasing pH of the culture medium to 7 and 9, respectively (5.295 and 5.123 mg casein/ml) (Figures 1, 2, 3).

Macroscopic observations made after 21 days of culture of the bacterial strain P_1 in the presence of pelt samples indicated a very good development in the incubation conditions at higher temperatures (28 and 37°) at all pH values of the culture media.

It was also found that strain P_1 caused complete degradation of pelt samples in culture

media with pH=5 at 28°C, pH=7 at 28 and 37°C and pH=9 at all three temperature values tested. In these experimental variants, the pelt samples lost their structural integrity, resulting in colloidal solutions, with a cloudy appearance, containing bacterial biomass and particles of different sizes (Table 1).

In comparison, in the case of cultures developed in the medium with pH=5 incubated at 20 and 37°C and of the culture in the medium with pH=7 incubated at 20°C, partial degradation of the pelt samples was observed after 21 days.

Table 1: Pelt waste degradation by fungal treatment

Strain code	pH and temperature (°C)	Initial weight (g)	Weight loss (g)
P_1	pH 5, 20°C	2.03	0.69
	pH 5, 28°C	2.04	-
	pH 5, 37°C	2.04	0.83
P_2	pH 5, 20°C	2.05	remnants
	pH 5, 28°C	2.03	remnants
	pH 5, 37°C	2.04	-
P_3	pH 5, 20°C	2.05	remnants
	pH 5, 28°C	2.03	-
	pH 5, 37°C	2.04	-
P_1	pH 7, 20°C	2.04	0.46
	pH 7, 28°C	2.02	-
	pH 7, 37°C	2.02	-
P_2	pH 7, 20°C	2.02	-
	pH 7, 28°C	2.02	-
	pH 7, 37°C	2.03	remnants
P_3	pH 7, 20°C	2.04	0.24
	pH 7, 28°C	2.03	-
	pH 7, 37°C	2.03	-
P_1	pH 9, 20°C	2.03	remnants
	pH 9, 28°C	2.03	remnants
	pH 9, 37°C	2.04	-
P_2	pH 9, 20°C	2.03	-
	pH 9, 28°C	2.04	-
	pH 9, 37°C	2.04	remnants
P_3	pH 9, 20°C	2.04	remnants
	pH 9, 28°C	2.04	remnants
	pH 9, 37°C	2.03	remnants

Thus, in these experimental variants, colloidal solutions with a cloudy appearance were obtained, made up of bacterial biomass, different particles, as well as pelt residues with small dimensions and wet weight of 0.69 g, 0.83 g and 0.46 g, respectively (Table 1).

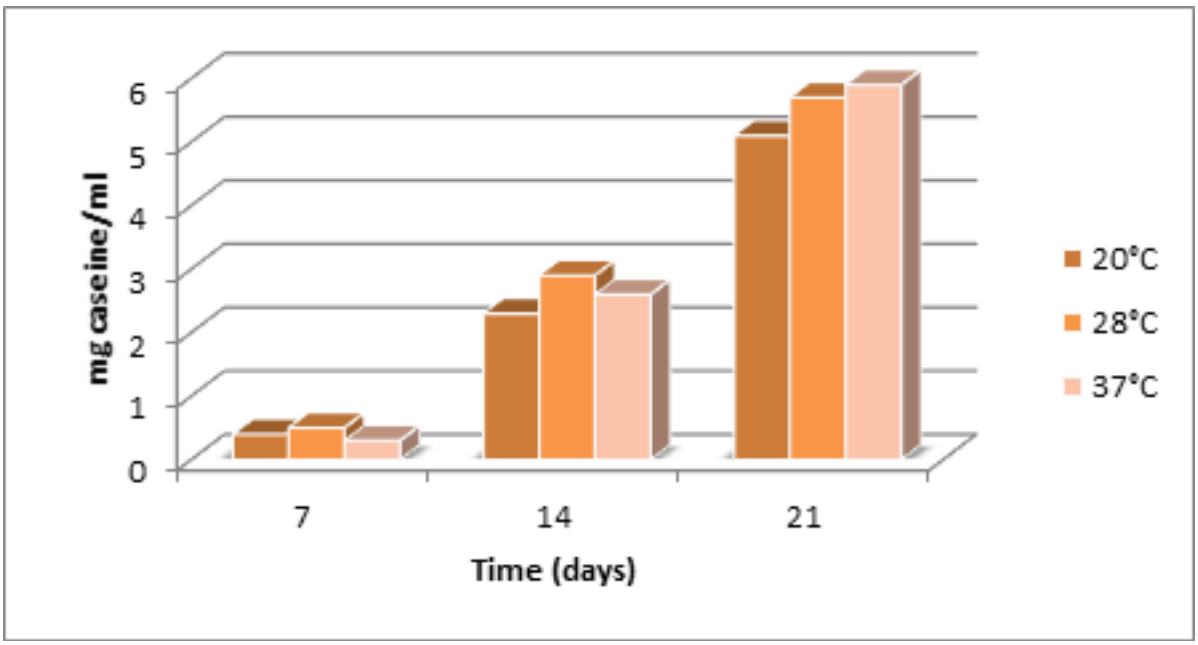

Figure 1. Dynamics of proteolytic activity of bacterial strain P_1 in the culture medium with pH=5, under different temperature conditions

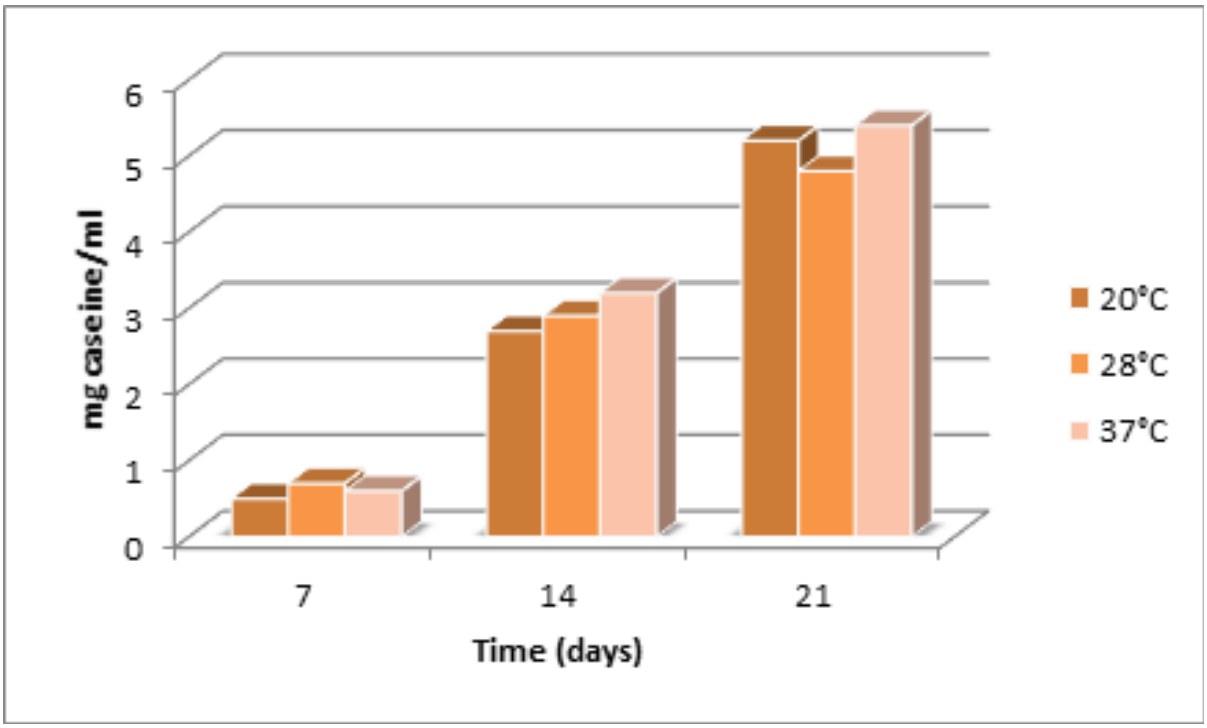

Figure 2. Dynamics of proteolytic activity of bacterial strain P_1 in the culture medium with pH=7, under different temperature conditions

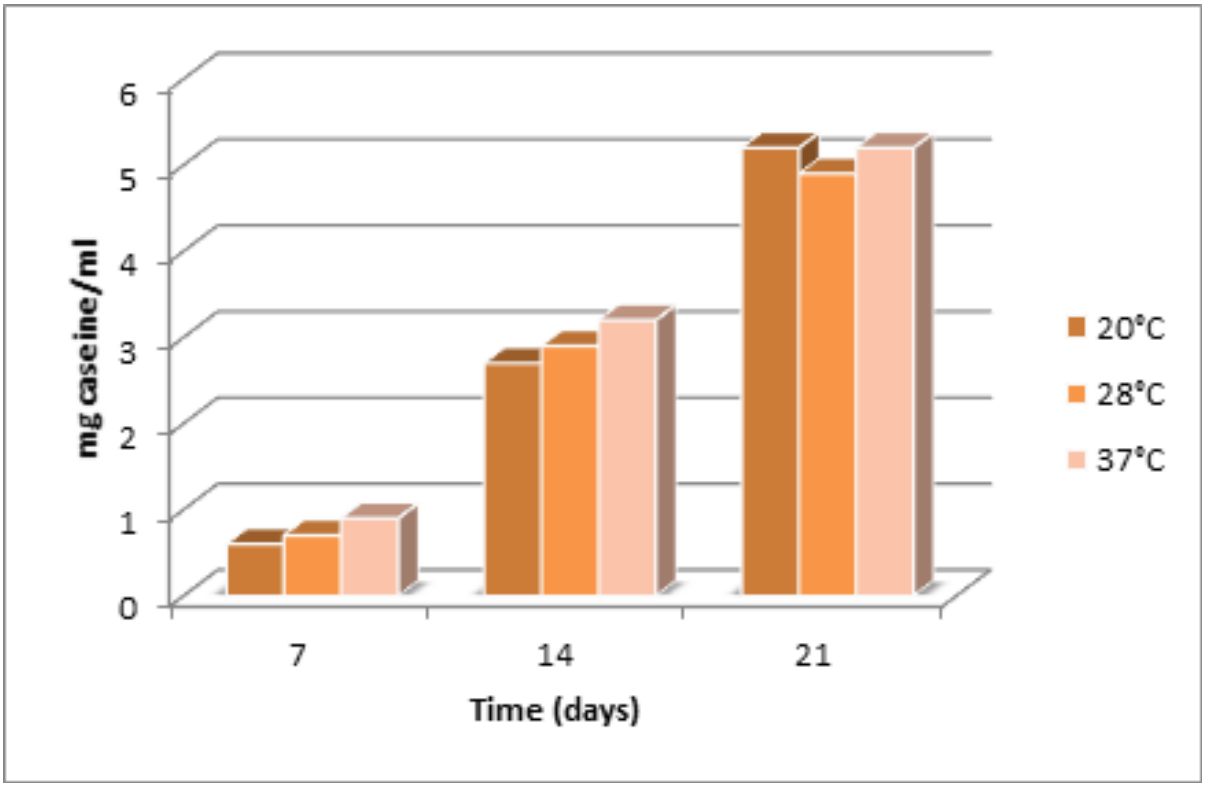

Figure 3. Dynamics of proteolytic activity of bacterial strain P_1 in the culture medium with pH=9, under different temperature conditions

The bacterial strain P_2 showed maximum proteolytic activity under the conditions of incubation at 20°C in the medium with pH=9 (5.397 mg casein/ml), but close values were also determined at the temperature of 28°C in the medium with pH=5 (5.41 mg casein/ml) and in the medium at 20°C with pH=7 (5.312 mg casein/ml) (Figures 4, 5, 6).

Macroscopic observations made after 21 days of culturing the bacterial strain $P_{3/1}$ in the presence of pelt samples indicated a very good development of the culture media in all pH conditions and at all temperature values tested.

It was also found that the very good development of the $P_{3/1}$ strain was correlated with the complete degradation of the pelt samples in culture media with pH=5, 7, and 9 at temperatures of 20, 28, and 37°C. Thus, in all experimental variants, strain $P_{3/1}$ showed high proteolytic activity, causing the loss of structural integrity of pelt samples, their decomposition and obtaining colloidal solutions, with cloudy appearance, containing bacterial biomass and particles of different sizes (Table 1).

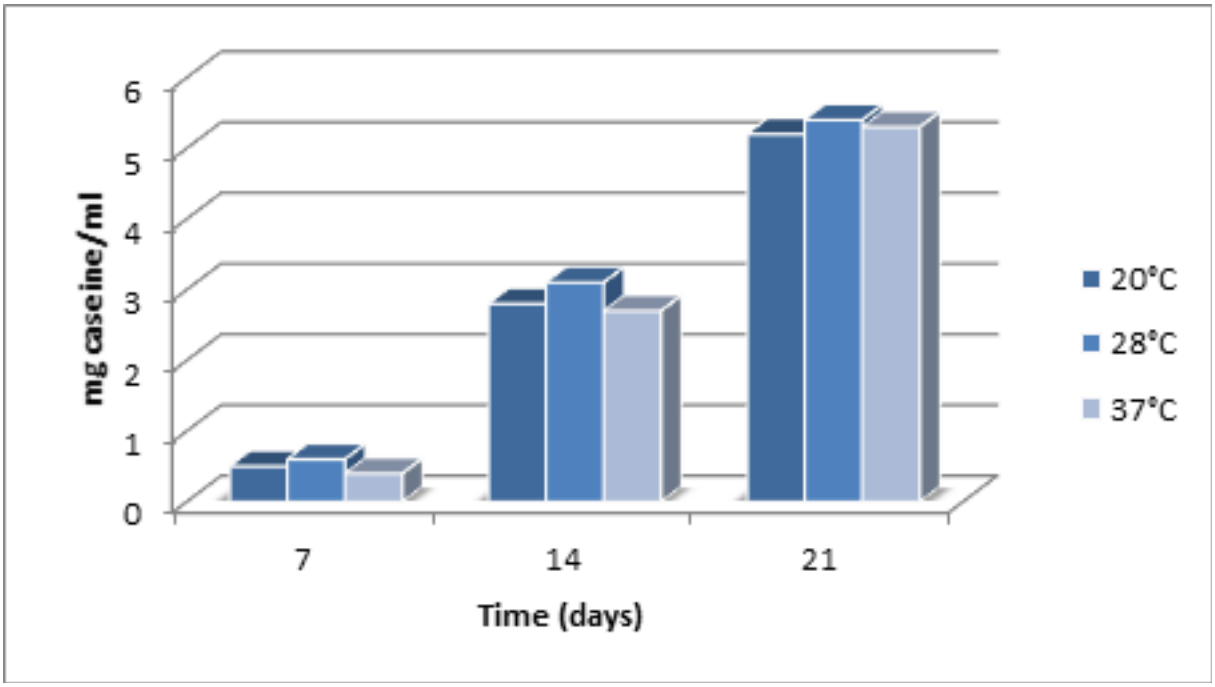

Figure 4. Dynamics of proteolytic activity of bacterial strain P_2 in the culture medium with pH=5, under different temperature conditions

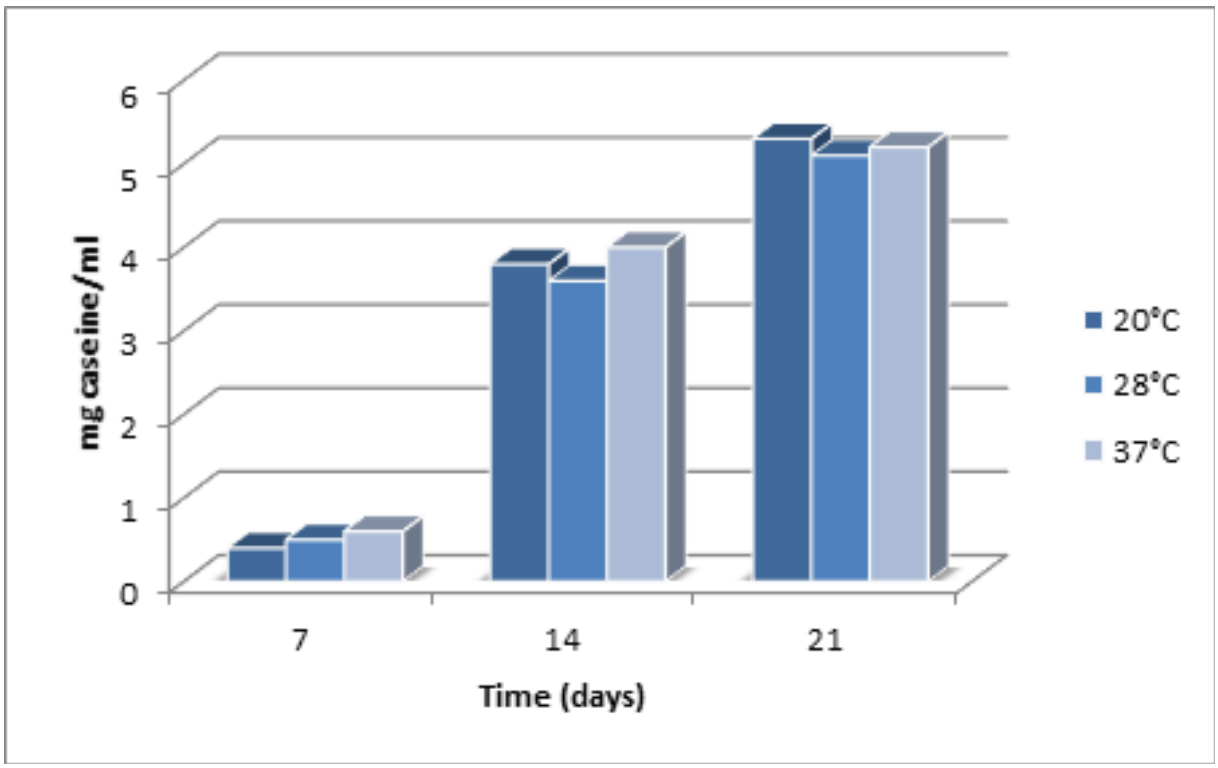

Figure 5. Dynamics of proteolytic activity of bacterial strain P_2 in the culture medium with pH=7, under different temperature conditions

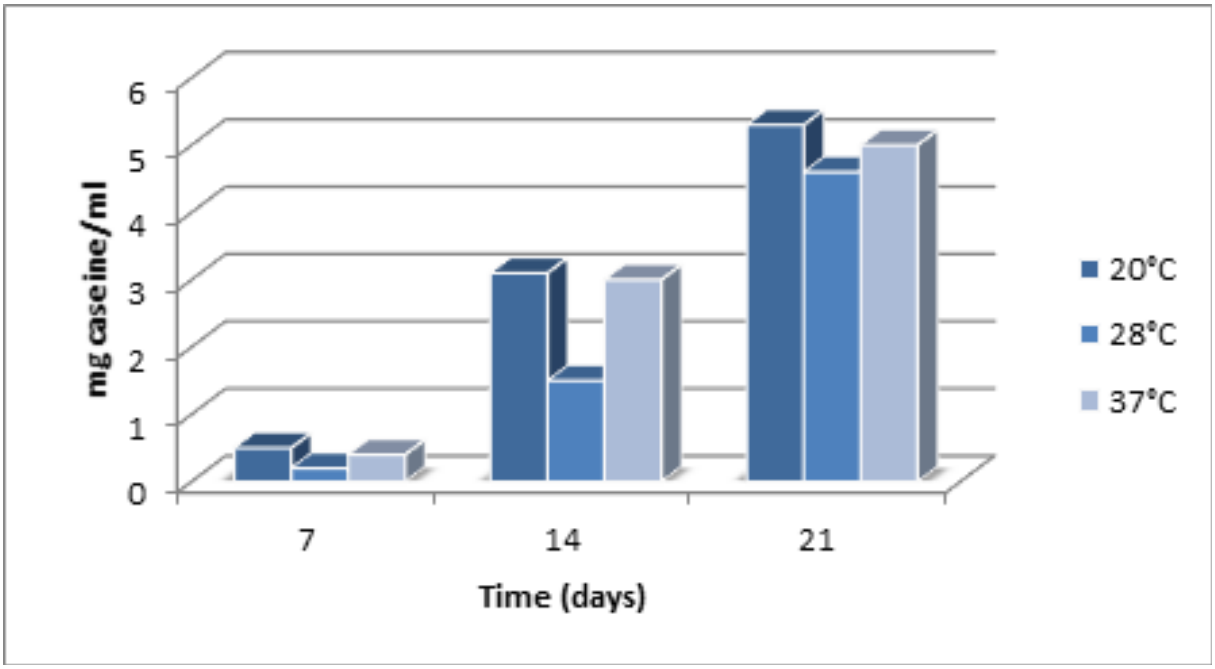

Figure 6. Dynamics of proteolytic activity of bacterial strain P_2 in the culture medium with pH=9, under different temperature conditions

Bacterial strain P_3 showed maximum proteolytic activity under incubation conditions at 37°C, in culture medium with pH=5 (5.5745 mg casein/ml), but high enzymatic activity at the same temperature was also achieved by the culture developed in the nutrient medium with pH=9 (5.4808 mg casein/ml).

In comparison, in the case of culturing the P_3 strain in the medium with pH=7, the highest activity of protease biosynthesis was found in the variant incubated at 20°C (5.3132 mg casein/ml) (Figures 7, 8, 9).

Macroscopic observations made after 21 days of culturing the $P_{4/1}$ strain in the presence of pelt samples indicated a very good development in all experimental variants, which was correlated with a high proteolytic activity, which determined the complete degradation of the samples, losing their structural integrity. Thus, at the end of the incubation period, colloidal solutions were obtained, with a cloudy appearance, which contained bacterial biomass and particles of different sizes (Table 1).

An exception was noticed in the cultivation of strain P_3 on medium with pH=7 and incubated at 20°C, in which partial degradation of the pelt samples was observed after 21 days and a cloudy colloidal solution consisting of bacterial biomass, various particles, as well as small-sized pelt debris and wet weight of 0.25 g was obtained (Table 1).

The partial degradation of the pelt samples in the case of this experimental variant may be due to a decreased enzymatic activity, determined by the low temperature value at which the bacterial culture was incubated with the pelt sample.

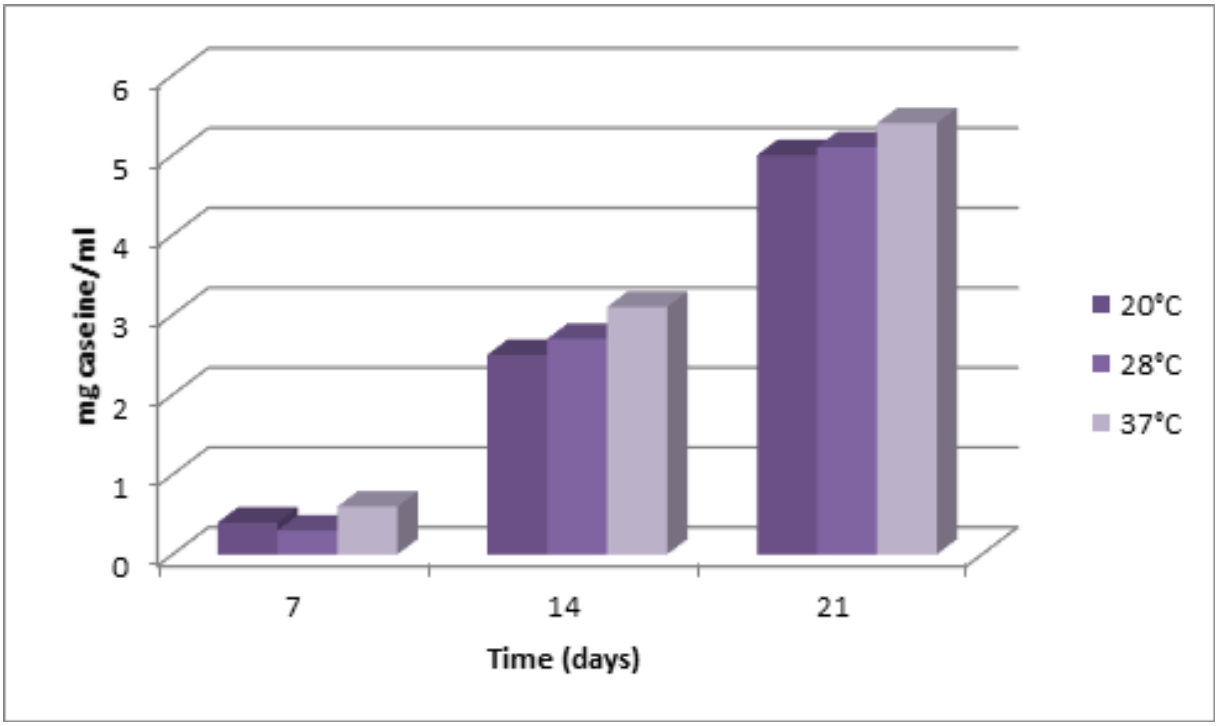

Figure 7. Dynamics of proteolytic activity of bacterial strain P_3 in the culture medium with pH=5, under different temperature conditions

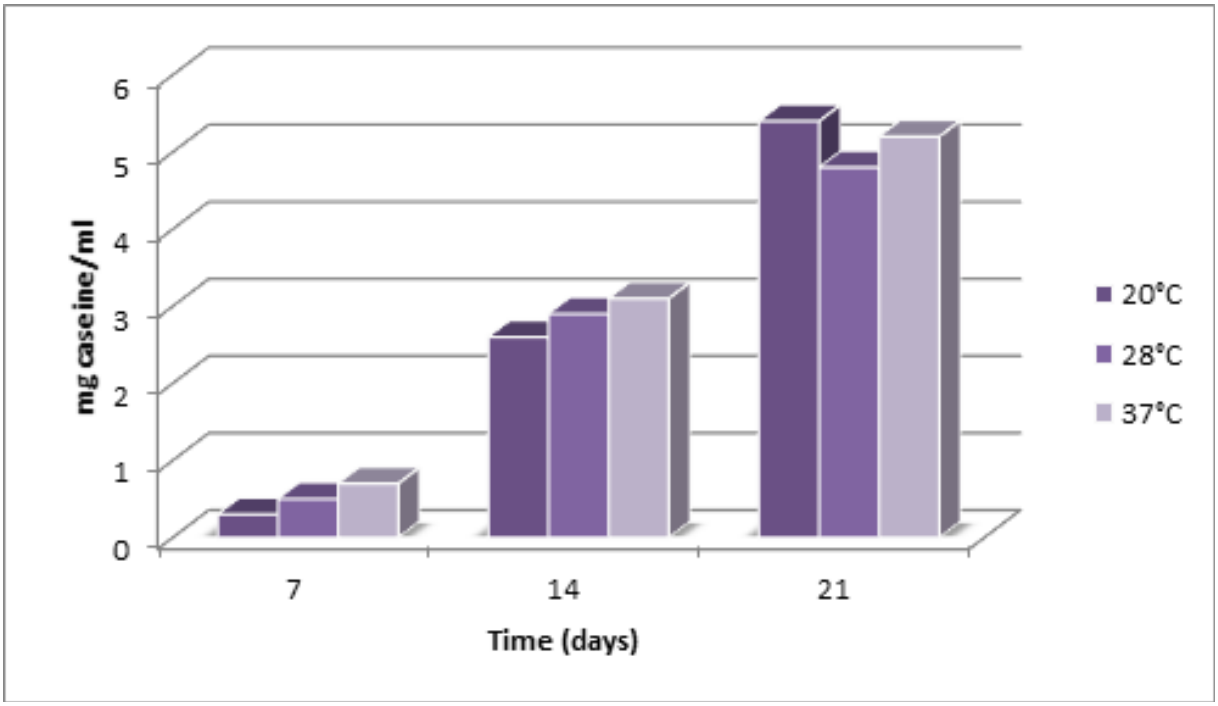

Figure 8. Dynamics of proteolytic activity of bacterial strain P_3 in the culture medium with pH=7, under different temperature conditions

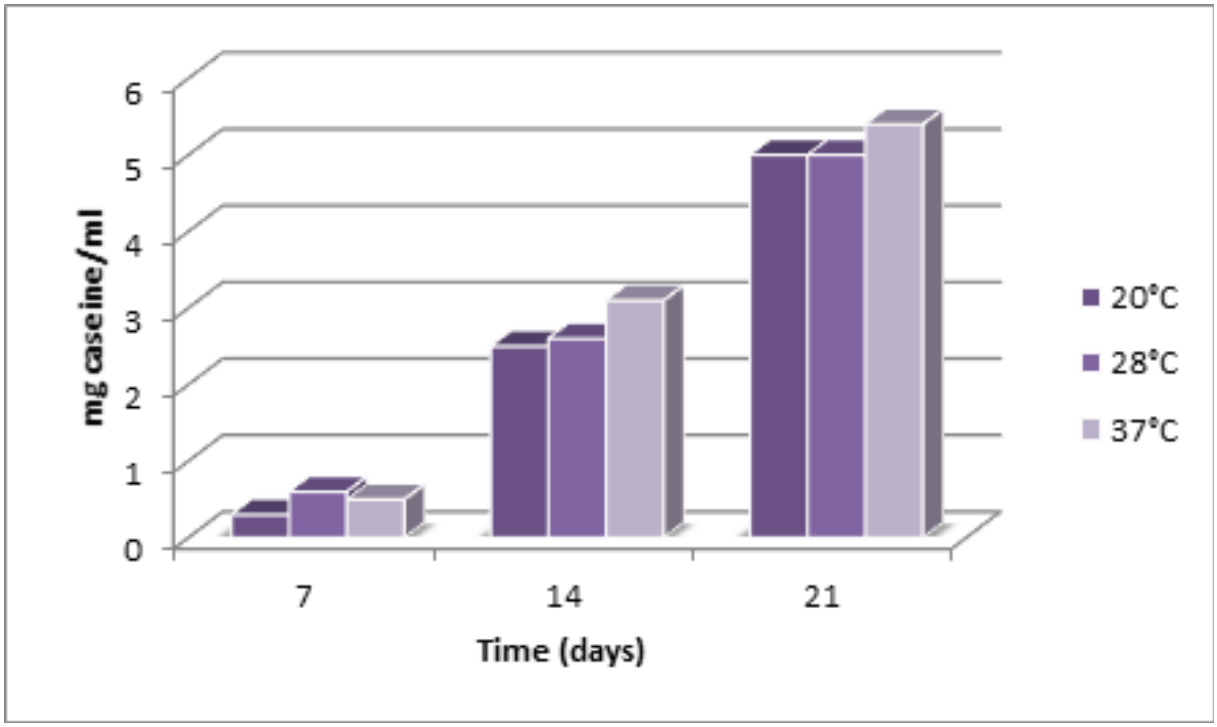

Figure 9. Dynamics of proteolytic activity of bacterial strain P_3 in the culture medium with pH=9, under different temperature conditions

CONCLUSIONS

The selected bacterial strains showed increased protease biosynthesis capacity, which had a significant hydrolytic action on pelt waste.

The quantitative determination of the enzymatic activity showed that the proteolytic activity on the pelt waste medium was specific for each of the three bacterial strains tested. Also, in the case of each strain there were differences depending on the pH value of the culture medium and the incubation temperature. Thus, strains P_1 and P_3 showed maximum protease synthesis activity on the medium with pH=5, under the conditions of incubation at 37°C. In comparison, in the case of P_2 strain the highest proteolytic activity was determined at lower temperatures (20°C) and at a higher pH value of the culture medium (9).

Some differences were found among the bacterial strains tested for the degradation capacity of the pelt samples. Thus, in the case of the bacterial strain $P_{1/1}$, the complete degradation of the pelt samples was obtained in the environment variant with a higher pH, at all the tested temperature values. In contrast, strains P_2 and P_3 showed superior ability to degrade pelt waste, causing loss of structural integrity of the samples and their decomposition at all pH values of the culture media and at all incubation temperatures tested.

The synthesis of proteases by the tested bacterial strains intensified during the incubation period, the maximum values of the enzymatic activity being determined in all experimental variants after 21 days of contact with the pelt samples.

The results obtained in this study demonstrated the ability of strains belonging to the *Bacillus* genus to synthesize increased amounts of proteolytic enzymes and to degrade pelt waste, as well as the possibility of using these microorganisms as a source of protease in various biotechnological processes.

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