**G&BM**
**Tome IV**
**Iaşi, 2003**

# RESEARCH STUDIES ON PROTEASES PRODUCING *BACILLUS SP.* STRAINS

**ERICA NIMITAN[1*], OCTAVITA AILIESEI[1], SIMONA DUNCA[1], MARIUS STEFAN[1]**

**Key words:** *Bacillus*, proteases, qualitative, semi-quantitative, quantitative, screening.

**Abstract:** The present paper is focused on the isolation of a number of proteolytic enzymes-producing bacterial strains belonging to the *Bacillus* genus from various natural media. The investigations included as well the qualitative and quantitative examination of the protease activity of the bacterial strains with a high exoenzyme productive capacity.

## INTRODUCTION

The ever increasing demand of proteolytic enzymes in the industry and medicine have stimulated in many countries extensive research studies in the attempt to discover new producers and to generate highly efficient technologies.

A large number of bacterial species are known nowadays to be rich sources of proteases, such as, for example the bacteria of the genera *Bacillus, Lactobacillus, Streptococcus, Streptomyces, Clostridium, Micrococcus* and *Actinomyces.*

The proteases produced by the species of the *Bacillus* genus have been most extensively investigated due to their numerous applications, activity related characteristics and the possibility to be obtained on industrial scale.

## THE AIM OF INVESTIGATIONS

The paper aimed to elucidate three aspects, as follows:

1. the isolation of highly proteases-producing bacterial strains from various natural media;
2. the selection of proteolytic bacterial cultures;
3. qualitative and quantitative screening of protease activity of the selected bacterial strains.

## MATERIAL AND METHOD

The proteolytic bacteria were isolated from various types of soil, manure, industrial waste waters, hay and other plant materials. In order to isolate strains belonging to *Bacillus* genus the collected samples were subjected to a thermal pre-treatment (kept at $80^0$C for 10 minutes).

The suspensions – dilutions of the samples to be examined – were inoculated in Petri dishes on casein agar medium. After a 24 h incubation at $37^0$ C, the colonies exhibiting a clear, transparent surrounding zone (due to casein enzymatic hydrolysis) were recorded and the diameter of the zone was measured in mm (qualitative screening). The colonies with appropriate lysis zones were isolated as pure cultures; the isolated strains were stored at $4^0$ C on casein medium to be characterised morphologically, biochemically and culturally.

The semi-quantitative screening was carried out by calculating the proteolysis ratio by the formula:

Rp = Dzc/Dc, where Rp = proteolysis ratio; Dzc = diameter of casein hydrolysis zone; Dc = diameter of colony.

The quantitative screening was carried out by determining protease activity using a variant of Anson method (qtd. Artenie and Tanase, 1981), the results obtained being expressed as U/ml.

The capacity to produce proteases was tested by growing the examined bacteria in submerged stationary and stirred cultures at $28^0$ C for 96 hours, using a liquid medium pH 7.5 containing 1% casein and enriched with 0.2% $CaCl_2$.

The specific activity was also determined by calculating the ratio of the enzymatic activity to the quantity of protein determined by Lowry method.

## RESULTS AND DISCUSSIONS

A number of 52 strains belonging to the *Bacillus* genus were isolated as pure cultures and marked with the initials P1 – P52. Based on the various diameters of the hydrolysis zone they exhibited (Fig. 1), they were classified into three categories (Fig. 2):

- highly proteases – producing (Rp>3.5) – 5 strains (9.6%);
- moderately proteases – producing (Rp=2.0-3.5) – 18 strains (34.61%);
- low proteases – producing (Rp≤2,0) – 29 strains (55.76%).

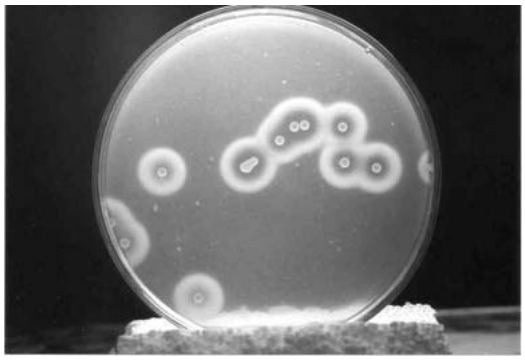

**Fig. 1 - Casein hydrolysis zones at the strain P8**

In order to assay protease activity the 5 highly proteases – producing strains were selected to be examined.

A series of experimental trials were carried out before the actual testing, with a view to establishing the optimal conditions appropriate to the maximum capacity of the examined strains to biosynthesize proteases. For this purpose, the influence of aeration level of the bacterial cultures was studied.

The protease activity of the strains was determined both in the broth originated from the culture stirred on the rotary shaker and in the broth originated from the stationary culture. These two alternatives were selected based on the fact that bacteria's behavior depends on the amount of oxygen in the medium.

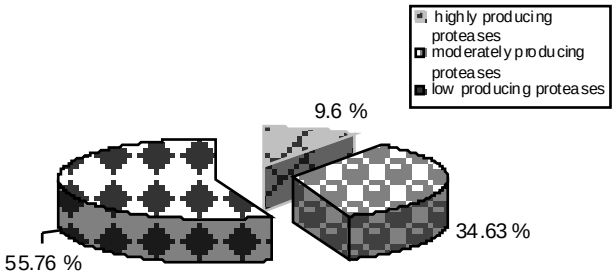


**Fig. 2 – Percentage representation of the proteases producing strains**

Upon testing (Table 1) the strongly aerated (stirred) cultures proved a higher biosynthesis capacity of proteases than the stationary cultures. As a result, all the values of the specific activity in the stationary cultures are higher than the corresponding ones for the stationary cultures.

**Table 1 – Quantitative protease activity of the examined strains**

| Strain | Total protein (mg/ml) | | Proteolytic activity (U/ml) | | Specific proteolytic activity (U/mg protein) | |
|---|---|---|---|---|---|---|
| | stationary | stirred | stationary | stirred | stationary | stirred |
| $P_8$ | 0,795 | 0,552 | 1,475 | 1,089 | 1,855 | 1,972 |
| $P_{29}$ | 0,645 | 0,750 | 0,310 | 2,063 | 0,480 | 2,750 |
| $P_{45}$ | 0,787 | 0,750 | 0,192 | 3,894 | 0,273 | 5,192 |
| $P_7$ | 0,687 | 0,512 | 0,182 | 2,336 | 0,264 | 2,876 |
| $P_{21}$ | 0,635 | 0,493 | 1,250 | 6,300 | 1,968 | 12,778 |

The results of the tests showed that the specific protease activity varies not only from one strain to the other but also at the same strain, depending on the growth conditions. Thus, the values of the specific protease activity vary depending on the strain between 0.264 and 1.968 U/mg protein in the stationary cultures and between 1.972 and 12.778 U/mg protein in the stirred ones.

The results of our research studies regarding bacterial protease activity are similar to those presented in literature. Thus, Valentina Dan and collab. (1998), examining high potential cultures of *Bacillus subtilis* MIUG 9.61 in a stationary cultivation system, on semi-solid media, but industrial ones (2 types) determined an average protease activity between 1.79 and 17.07.

Simona Dunca and collab. (2000) examining thermophilic actinomycetes identified strains having the specific protease activity between 0.155 and 8.929 for the stirred cultures and between 0.045 – 2.714 for the stationary ones.

Upon morphological, biochemical and physiological examination of the 5 highly proteases-producing strains, 4 (P8, P29, P7, P21) were taxonomically classified as belonging to the species *Bacillus subtilis* and 1 (P45) to the species *Bacillus pumilus*.

## CONCLUSIONS

From various natural media a number of 52 bacterial strains belonging to the genus *Bacillus* exhibiting protease activity was selected.

Upon the quantitative screening 5 strains, which proved the best proteases producing were selected.

From the taxonomic point of view, by morphological, biochemical and physiological examinations 4 strains (P8, P29, P7, P21) were found to belong to the species *Bacillus subtilis* and 1 (P45) to the species *Bacillus pumilus*.

The *Bacillus* strains isolated and selected by our team constitute a valuable biologic material which, following a more thorough research, may be successfully used in the biosynthesis processes.

## BIBLIOGRAPHY

**Artenie, V., Tanase Elvira, 1988.** *Practicum de biochimie generala*, Ed. Univ. "Al. I. Cuza" Iasi, pp.228-230

**Dan Valentina, Gabriela Bahrim, Anca Nicolau, 1998.** Conditii de obtinere a complexului amilazo-proteazic prin cultivarea tulpinii B. subtilis MIUG 9.61 pe medii semisolide, Noutati in microbiologie si biotehnologie, Ed. Corson, Iasi, pp.37-44

**Duncа Simona, Octavita Ailiesei, Erica Nimitan, Rodica Pasa, 2000.** Study of several proteases-producing thermoactinomycete strains, Analele Stiintifice ale Univ. "Al. I. Cuza" Iasi, T XLVI, s a-II-a, Biologie vegetala, pp. 151-155

**Nimitan Erica, Octavita Ailiesei, Simona Dunca, Comanescu Stefan, 1998.** *Metode si tehnici de microbiologie*, Ed. Univ. "Al. I. Cuza" Iasi, pp. 167-169

**Topala, N.D., Artenie, V., Erica Nimitan, Dolcea, C., Bontas, I., 1977.** Studiul unor tulpini bacteriene producatoare de proteaze izolate din sol si alte medii naturale, Lucrarile Primului Simpozion de Microbiologie Industriala, dec. 1976, pp. 144-151

[1] "Al.I.Cuza" University of Iaşi", B-dul Carol I, 11A 6600, Iaşi-România
* Author to whom may adress correspondance/Corresponding author: Prof. PhD Erica Nimitan (e-mail: enimitan@uaic.ro)