INFLUENCE OF CULTURE MEDIUM COMPOSITION ON THE PROTEOLYTIC ACTIVITY OF THE HALOTOLERANT BACTERIAL STRAIN *SALINIVIBRIO COSTICOLA* SUBSP. *ALKALIPHILUS* IBB 37

ALINA BADEA (CIOACĂ)[1], SIMONA NEAGU[2],
MĂDĂLIN ENACHE[23], VERONICA LAZĂR[1]

Abstract: Microbial proteases are important biocatalysts with broad industrial and environmental applications, and halophilic strains offer robust enzymes capable of functioning under extreme conditions. This study investigated the influence of culture medium pH and glucose concentration on the production and activity of an extracellular protease synthesized by the halotolerant bacterium *Salinivibrio costicola* subsp. *alkaliphilus* IBB37, isolated from a mud sample. The strain was cultivated in a medium containing 10% NaCl at varying pH values (5, 6, 7, and 8) and supplemented with either 5% or 10% glucose and 1% casein as the substrate. Results showed that alkaline conditions greatly enhanced protease yield: the highest proteolytic activity was obtained at pH 8 with 5% glucose, whereas lower pH or higher glucose (10%) led to reduced enzyme activity. Under the optimal pH 8 and 5% glucose medium, the protease's specific activity increased approximately nine-fold after purification compared to the crude extract. Efficient production and purification of this halotolerant protease underscore its potential for biotechnological applications, such as in high-salinity bioprocesses, waste management, or agricultural soil amendments, where robust salt-tolerant enzymes are required.

Keywords: moderately halophilic bacteria, proteolytic activity, pH, glucose concentration.

INTRODUCTION

Proteases are among the most important classes of hydrolytic enzymes, responsible for the degradation of proteins into peptides and amino acids (Razzaq

[1] Faculty of Biology, University of Bucharest, 91–95 Splaiul Independentei 050095, Bucharest, Romania
[2] Institute of Biology Bucharest of Romanian Academy, 296 Splaiul Independentei, 060031, Bucharest, Romania
[3] Corresponding author e-mail: madalin_enache@yahoo.com

ROM. J. BIOL. – PLANT BIOL., VOLUME **70**, Nos. 1–2, P. 67–74, BUCHAREST, 2025
DOI: 10.59277/RJB-PB.2025.1-2.05

et al., 2019). These enzymes are widely distributed across various biological systems, being naturally produced by microorganisms, plants, and animals (Gupta *et al.*, 2002; Romero-Garay *et al.*, 2020; Adentuji *et al.*, 2023). Microorganisms in particular are known to produce proteases in abundance, making them highly valuable in biotechnology.

Microbial proteases account for a significant share of the global enzyme market (more than 40% of worldwide enzyme production)(Adentuji *et al.*, 2023) and are used in diverse industries such as food processing, detergents, leather, and pharmaceuticals (Kumar *et al.*, 1999; Ellajah *et al.*, 2002; Sawant *et al.*, 2014; Singh *et al.*, 2016; Razzaq *et al.*, 2019; Song *et al.*, 2023).

The broad utility of these enzymes results from their versatility and ability to function under a wide range of conditions, including extremes temperatures, pH, and salinity. The expression and activity of microbial proteases are influenced by environmental parameters. Nutrient composition and physicochemical factors (*e.g.* pH, salinity, and temperature) can regulate enzyme production (Sharma *et al.*, 2017). In many bacteria, the presence of metabolizable carbon sources like glucose can repress protease synthesis through catabolite repression mechanisms (Sharma *et al.*, 2017). pH is another important factor influencing enzyme production. Thus, most proteases have a characteristic pH range for stability and activity, and the ambient pH can induce or inhibit the expression of specific protease genes. Optimizing these factors is therefore crucial for maximizing microbial proteolytic activity.

Halophilic bacteria are attractive sources of extracellular hydrolytic enzymes that remain active under harsh conditions (Oren, 2008). In these microorganisms, which thrive in environments with high salt concentrations, protease production depends on the composition of the culture medium. The activity of salt-tolerant enzymes varies with salinity, pH, temperature, and carbon source (e.g., glucose) (Chuprom *et al.*, 2015). Such adaptations are of great interest for industrial applications that require enzymes stable in harsh process conditions.

The genus *Salinivibrio* is represented by halophilic bacteria with significant proteolytic activity. Members of this genus, represented by only five recognized species, include moderately halophilic Gram-negative rods, commonly isolated from saline lakes and other hypersaline habitats. Many strains of *Salinivibrio* are also haloalkaliphilic, thriving in high salt (up to 25 % NaCl) and high pH conditions (de la Haba *et al.*, 2019).

The present study aimed to evaluate the influence of two key culture parameters – pH and glucose concentration – on the proteolytic activity of *Salinivibrio costicola* subsp. *alkaliphilus* IBB_{37}, a halotolerant bacterial strain isolated from Movila Miresii Salt Lake, Brăila County, Romania.

MATERIALS AND METHODS

BACTERIAL STRAINS, GROWTH CONDITIONS AND CULTURE MEDIUM

The strain *Salinivibrio costicola* subsp. *alkaliphilus* IBB37, previously characterized by Badea *et al.* (2023), was selected as the protease-producing organism for this study. The halotolerant bacterium was isolated from a mud sample collected from Movila Miresii Salt Lake, Brăila County, Romania, and was stored at −80 °C in MH broth supplemented with 25% (v/v) glycerol. The composition of MH culture medium was (g/L): yeast extract, 10; proteose peptone, 5; glucose, 1; $MgCl_2 \cdot 6H_2O$, 7; $MgSO_4 \cdot 7H_2O$, 9.6; $CaCl_2 \cdot 2H_2O$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.026; and NaCl, 100 (Ventosa *et al.*, 1982). To obtain a fresh culture, the bacterium was inoculated into MH broth and incubated at 30 °C for 48 hours.

EXTRACELLULAR PROTEASES BIOSYNTHESIS

The MH liquid culture medium was modified to promote proteolytic enzyme synthesis by eliminating proteose peptone and glucose, reducing the yeast extract concentration to 5 g/L, and supplementing with 1% casein as a substrate, as previously described (Badea *et al.*, 2023). Biosynthesis was performed by incubating the freshly inoculated medium at 30 °C and pH 8, under agitation at 150 rpm, for 72 hours.

ENZYME ACTIVITY

Protease activity in the cell-free culture filtrate and in the partially purified preparations obtained by 50% and 80% acetone precipitation was measured according to the Anson method (Karbalaei-Heidari *et al.*, 2006), as previously reported (Badea *et al.*, 2023). The protein concentration was determined using the Lowry method, based on a standard curve prepared with bovine serum albumin (0–100 mg/mL), and expressed as mg protein/mL liquid (Lowry *et al.*, 1951).

THE INFLUENCE OF PH AND GLUCOSE CONCENTRATION ON PROTEOLYTIC ACTIVITY

To evaluate the effect of pH on protease activity, different assay buffers were used: 0.05 M phosphate buffer for pH 5.0, 6.0, and 7.0, and 100 mM Tris-HCl buffer for pH 8.0. The effect of carbon sources, such as glucose, on protease activity was assessed by cultivating the bacterial strain in MH media supplemented with 5% or 10% glucose and 1% casein as sole carbon sources. All experiments were performed in duplicate.

RESULTS AND DISCUSSIONS

EFFECT OF CULTURE PH ON PROTEASE ACTIVITY

In our previous study (Badea *et al.*, 2024), the halotolerant strain *Salinivibrio costicola* subsp. *alkaliphilus* IBB37 was isolated and characterized, demonstrating high proteolytic potential. Furthermore, the proteolytic enzyme produced by this strain was successfully employed in the development of a hybrid enzyme/nanostructured oxide material designed to enhance soil biodegradation. As a continuation of that research, the present study investigates the influence of pH and glucose concentration on the proteolytic activity of the enzyme secreted by *S. costicola.* To this end, enzyme production and purification were assessed under varying culture conditions, and the following results describe the effects observed at different pH values and glucose concentrations.

The halotolerant bacterium, grown in MH medium supplemented with 5% glucose and a proteolytic substrate, exhibited a strong pH dependence in terms of protease production and purification efficiency (Figures 1,2). At pH 5, the crude culture filtrate had a specific activity of 0.17 nmol/min/mg.

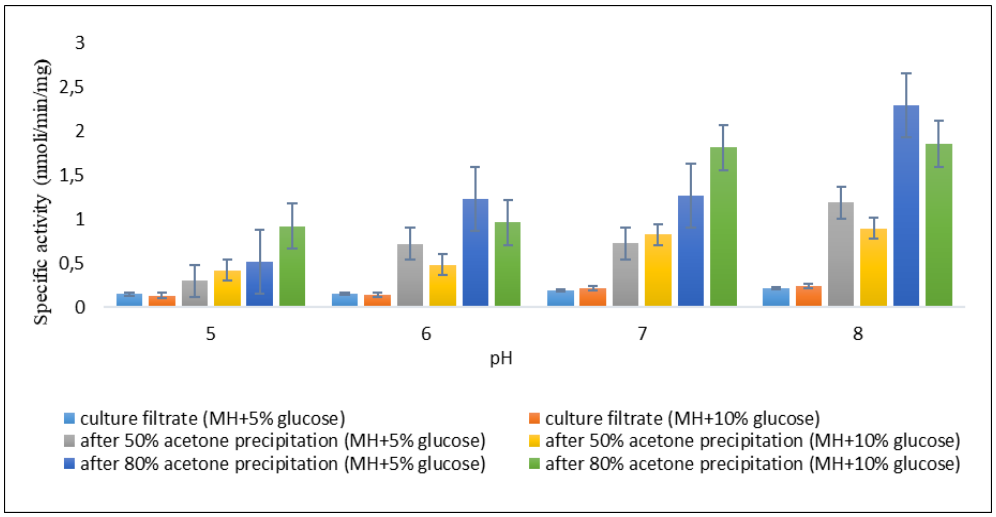

Figure 1. Specific protease activity of *S. costicola* IBB37 at different pH values in media supplemented with 5% and 10% glucose. The columns in the histogram represent the mean ± standard deviation of two replicates.

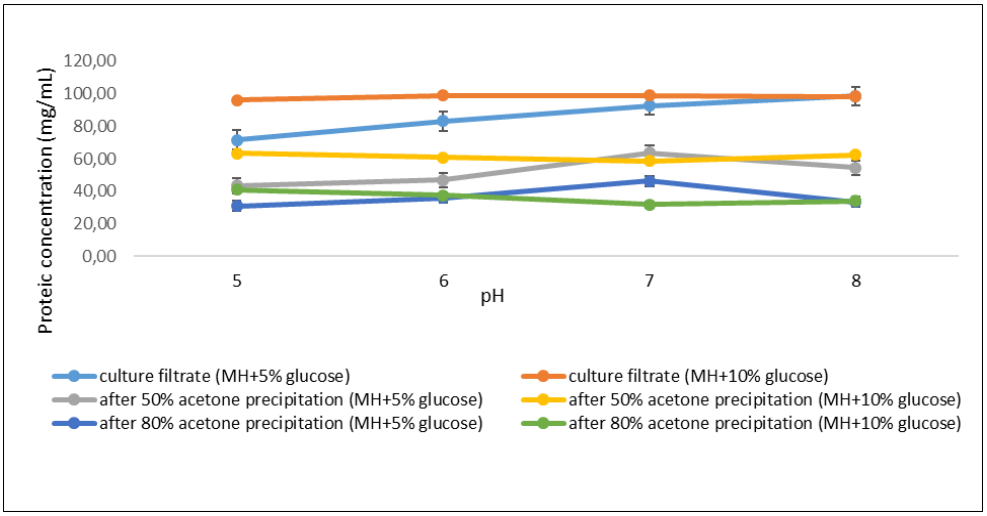

Figure 2. Protein concentration across purification steps.

At pH 8, an increase in the specific protease activity was observed, reaching 0.24 nmol/min/mg. This trend suggests that an alkaline environment strongly favors extracellular protease production by the halotolerant strain. In fact, halophilic and haloalkaliphilic microorganisms often exhibit maximal protease secretion at neutral to alkaline pH values (around 8–10) (Dodia *et al.*, 2006; Nwankwo *et al.*, 2023; Patel *et al.*, 2006).

Stepwise precipitation with acetone (50% and 80% v/v) successfully concentrated and partially purified the protease at each pH, as evidenced by the increase in specific activity. At pH 5, in the filtrate obtained after 80% acetone precipitation, the specific activity increased from 0.15 to 0.52 nmol/min/mg. This corresponds to an overall purification of the enzyme of ~3.2-fold. At pH 8, the enrichment was even more pronounced. Thus, the specific activity increased from 0.22 in the crude extract to 1.7 nmol/min/mg after 50% acetone and finally to 2.29 nmol/min/mg in the 80% acetone fraction. The ~9.5-fold increase in specific activity at pH 8 indicates substantial removal of other proteins and concentration of the target protease. Similar fold-enrichments were reported in other studies involving halophilic proteases purified by acetone precipitation, confirming the efficiency of this approach for initial enzyme concentration (Nwankwo *et al.*, 2023).

A similar pH-dependent trend was observed when the strain was grown in medium supplemented with 10% glucose and a proteolytic substrate. Thus, at pH 5 it was 0.14 nmol/min/mg, while at pH 8 it reached 0.24 nmol/min/mg. These values were comparable to those recorded in 5% glucose cultures, confirming that alkaline pH supports protease synthesis regardless of glucose concentration. At pH 7–8, the initial specific activities in 10% glucose cultures were similar to those in

5% glucose, suggesting that under near-optimal pH conditions, the strain was able to utilize the additional glucose to maintain a proportional level of protease synthesis. Fractional precipitation with 50% and 80% acetone significantly enhanced the specific activities at all pH levels. For instance, at pH 5, the specific activity increased from 0.14 nmol/min/mg in the crude extract to 0.92 nmol/min/mg in the 80% acetone fraction, corresponding to a 6-fold increase. At pH 7, the specific activity increased from 0.22 to 0.83 nmol/min/mg after 50% acetone and reached 1.82 nmol/min/mg after 80% precipitation, indicating ~8-fold purification. The highest specific activity obtained in the 10% glucose condition was 1.86 nmol/min/mg at pH 8, but did not exceed the value obtained at pH 8 in the 5% glucose condition (2.29 nmol/min/mg). These findings reinforce the conclusion that culture pH is a critical determinant of protease yield prior to purification.

Varying the glucose concentration (5% vs 10%) in the growth medium had a noticeable impact on protease production and purification, although the effect was somewhat complex and pH-dependent. In general, providing a higher glucose level increased the total protein yield in the culture filtrate, especially at suboptimal pH values. For example, at pH 5, the 10% glucose culture had approximately 38% more protein in the filtrate than the 5% glucose culture. Similar increases were observed at pH 6 and 7. This suggests that the additional carbon source stimulated cell growth and overall protein secretion, but not enzyme production. Such a result aligns with the phenomenon of catabolic repression, where microbial cells preferentially utilize abundant nutrients for growth and repress secondary metabolism (such as enzyme synthesis) when energy is readily available (Habicher *et al.*, 2021). It is well documented that high concentrations of rapidly metabolizable carbon (glucose) can suppress protease production in bacteria. In their study, Habicher reported that, in *Bacillus* species, excess glucose triggers regulatory pathways that reduce protease gene expression, limiting enzyme yield until the preferred carbon source is depleted. These results suggest that excess glucose may be counterproductive for protease purity when baseline production is already high (as at pH 8), possibly due to multiple contaminating proteins or suboptimal induction of enzyme synthesis. A study of a haloalkaliphilic *Bacillus* species also found that protease production was strongly inhibited above 1% glucose, even though that strain thrived at pH 8 (Patel *et al.*, 2006).

Cultivation of the halophilic strain *S. costicola* IBB37 at pH 7–8, particularly in the presence of 5% glucose, resulted in a culture broth with a high protease content and specific activity. This significantly facilitated the downstream purification process. Under these optimal conditions (pH 8, 5% glucose), acetone fractionation led to a more than nine-fold increase in specific activity, reflecting both high enzyme yield and effective removal of non-enzymatic proteins. In contrast, cultivation at suboptimal pH or in the presence of excess glucose resulted in lower enzyme concentrations and required more extensive purification to achieve comparable enrichment.

CONCLUSIONS

This study demonstrates that optimizing culture conditions, particularly pH and carbon source concentration, can significantly improve both the yield and purity of extracellular proteases produced by *Salinivibrio costicola* subsp. *alkaliphilus* IBB37. The ability of this halotolerant strain to synthesize active proteases under alkaline and saline conditions highlights its potential for biotechnological applications, especially in processes that require enzymes stable in extreme environments, such as bioremediation, waste treatment, and industrial catalysis.

ACKNOWLEDGEMENTS

The study was funded by project no. RO1567-IBB05/2025 from the Institute of Biology Bucharest of Romanian Academy.

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