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Protease Production And Purification In Bacillus Cohnii Isolated From Vegetable Market Waste

Senthil Kumar Swaminathan*,
Puthupalayam Thangavelu Kalaichelvan
Cas In Botany, University Of Madras, Chennai - 600 025, India
Corresponding author: srssenthil.bros@gmail.com

ABSTRACT
Using skim milk agar plates, proteolytic bacteria were identified from vegetable waste dumping soil. Bergey's handbook was used to select and define the isolate with the highest activity. It showed the most resemblance to Bacillus cohnii in 16S rDNA study, and the sequence was deposited in Genbank. The strain SSK1 was cultivated on a modified production medium with sludge as a substrate to produce protease. For maximum enzyme synthesis, many culture factors were tuned. With 1% inoculums, peak proteolytic activity was reported at pH-8.5 and temperature of 50°C. Tween-80, SDS, and Tween-20 had the strongest inhibitory action, followed by Cetrimide. Q-Sepharose was used to homogenise the protease, revealing a molecular weight of 35 kDa. At pH-9 and 55°C, the enzyme was more active and stable.
Key Words: Proteases, pH, Temperature, and Inoculum Concentration in Vegetable Waste Soil

INTRODUCTION

Proteases are the most significant industrial enzymes, performing a wide range of actions and serving a number of biotechnological purposes [1]. They account for at least a quarter of total worldwide enzyme output and two-thirds of total enzymes used in various sectors [2]. Though alkaline proteases are produced by a variety of microorganisms including bacteria, fungi, yeast, plants, and mammalian tissues [3, 4], with increasing industrial demand for proteases, hyperactive strains are expected to emerge, and the enzymes produced by new exotic microbial strains could be used as biocatalysts in the current biotechnological era. Microbial proteases, among the different proteases, play an essential part in biotechnological processes. Produced alkaline proteases are of particular interest because they could be utilised to make detergents, food, medicines, and leather [5, 6].

The majority of alkaline proteases used in industry have two drawbacks: first, many of them have limited activity and stability over a wide pH and temperature range, and second, the cost of growing medium is estimated to account for 30–40% of the total cost of commercial enzymes [7]. The amount of enzyme generated is known to be highly dependent on the strain and growing circumstances. For the manufacture of alkaline proteases from an alkalophilic *Bacillus* sp., it is very necessary to choose a cost-effective growing medium [8]. *Bacillus* sp. is a microorganism that produces extracellular alkaline proteases [9].

Microbial proteases, particularly those from Bacillus species, have long had a prominent portion of the global industrial enzyme industry, with major applications in detergent compositions [10]. Members of the genus Bacillus, primarily strains *B. subtilis* and *B. licheniformis*, have been discovered to be a significant and prolific source of alkaline proteases in several bacteria that have been evaluated for usage in diverse industrial applications [11]. As a result, new Bacillus strains that

produce proteolytic enzymes with unique features are needed, as well as the development of low-cost medium. In light of this, the current work looked at the development of alkaline protease from *Bacillus* sp., as well as the purification of the alkaline Bacillus protease using protein separation techniques.

MATERIALS AND METHODS

Collection of Samples

Koyambedu Market in Chennai, Tamil Nadu, provided samples of vegetable trash dumping soil.

Isolation of Protease Producing Bacteria

Each soil sample was serially diluted and inoculated into the screening medium (g/l) (skim milk powder–10, yeast extract–3.0, ammonium sulphate–6.7, NaCl–0.5, K_2HPO_4–0.7, $MgSO_4H_2O$–0.5) [12] and incubated at 55°C for 48 hours [13]. The creation of a clear zone around the colonies indicates that protease is being produced. As enzyme producers, the selective strains colonies that had created a clear zone around their edges were chosen. The positive isolates were next evaluated for improved enzyme production using a protease activity assay [14] in liquid culture with casein as the substrate at 55°C. The strain with the highest level of activity was chosen for further investigation.

Bacterial Characterization and Molecular Identification

Bergey's manual of systemic bacteriology [15] was used to do preliminary characterisation of the isolated strain. Sequence analysis of the 16S rDNA gene was used to determine the isolate's identification. The DNA was isolated from the overnight grown bacterial cells using the phenol:chloroform (1:1) extraction method reported by Wilson [16]. PCR primers BcF (GGATTAAGAGCTTGCTCTTAT) and BtF (GGATTAAGAGCTTGCTCTTAT) were used to amplify the 16S rDNA (GATTGAGAGCTTGCTCTCAATA). After that, the amplified area was sequenced and analysed using BLAST to determine its phylogeny [17].

Protease Production Medium

Protease production was carried out in a modified production medium. The vegetable waste was used to substitute the natural substrates in Chenel et al[18]. One millilitre of overnight grown bacterial strain SSK1 was injected into 100 millilitres of modified production medium comprising (g/l): Vegetable waste –15, KH_2PO_4–1, $MgSO_4$–$7H_2O$–0.3, $FeSO_4$–$7H_2O$–0.2, $ZnSO_4$–$7H_2O$–0.2, $CaCO_3$–1, pH-9.0. The medium was centrifuged at 10,000 g for 10 minutes after incubation. The crude enzyme was obtained from the supernatant (culture filtrate).

Assay for Protease

Beg and Gupta [14] established a method for measuring protease activity. In a glycine–NaOH buffer with a pH of 10.5 and a millilitre of culture filtrate, 1ml of 1% (w/v) casein solution was added and incubated for 10 minutes at 60°C. The reaction was halted by adding 4 mL of 5% trichloroacetic acid to the mixture. The reaction mixture was centrifuged at 3000 g for 10 minutes, after which 5 ml of 0.4 M Na_2CO_3 and 0.5 ml Folins–Ciocalteus reagent were added to 1 ml of the supernatant. Against an enzyme blank, the amount of tyrosine released was measured spectrophotometrically at

660 nm. Under conventional test conditions, one unit of protease activity was equal to the quantity of enzyme necessary to liberate 1 g of tyrosine/ml/min.

Total Protein Content

Using Bovine Serum Albumin (BSA) as a standard, the amount of protein was calculated using the Bradford technique [19].

Determination of Biomass

Bacteria biomass was evaluated using a 600 nm absorbance measurement [20].

The Influence of the Inoculum Concentration

For optimal protease production, the concentration of the inoculum and its effect were investigated. Experiments were conducted using a 1–5% inoculum volume containing an OD of 1.0. The flasks containing the production medium (pH-8.5) were inoculated and incubated for 48 hours at 50 degrees Celsius.

The Influence of the Incubation Period

Protease activity was measured after each 12 hour incubation period (12, 24, 36, 48, 72, 96, and 120) to find the best incubation period for maximum extracellular protease synthesis.

The Influence of pH Values and Temperature at the Start

The influence of starting pH on protease synthesis was investigated using 1 M NaOH and 1 M HCl solutions to modify the pH of the production medium (8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0). The flasks with the production medium were inoculated and incubated at various temperatures ranging from 45 to 60ºC for 24 hours to evaluate the influence of incubation temperature on protease production.

Surface-active Agents Impact

The influence of several surfactants on protease synthesis was investigated, including Tween-20, Tween-80, SDS, and Centrimide. Bacteria were injected into flasks containing the production medium (pH-8.5) and incubated at 50°C.

Purification of the Protease

Continuous stirring was used to precipitate 85 percent ammonium sulphate from the culture filtrate. At 4 degrees Celsius, the saturated solution was left undisturbed overnight. The precipitate was dissolved in a tiny volume of 50 mM glycine–NaOH buffer (pH-10.5) and dialyzed for 24 hours after centrifugation. A Q-sepharose column was filled with two ml of dialyzed protein and pre-equilibrated with glycine–NaOH buffer. The flow rate was set at 30 ml/h, and 2 ml fractions were

collected and tested for protease activity. The most active fractions were pooled, concentrated, and employed as a source of protease for further enzyme characterisation [14].

Electrophoresis

The purity of the protease was determined utilising reducing conditions and a 10% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide is a kind of polymer. The Laemmli method [21] was used to make the gel. PAGE was used to perform the Zymogram Analysis, and the staining was done with Commassie brilliant blue-R250 [22].

The Influence of pH Stability

Using phosphate Tris- or Glycine buffers, the purified enzyme was incubated at several pH values, including 6, 7, 8, 9, 9.5, 10, and 11. The tubes were then incubated at 55°C for 18 hours to assess the relative activity.

Temperature Stability Impact

The purified enzymes were incubated for 2 hours at various temperatures, including 50, 55, 60, 65, 70, 75, 80, 85, and 90 degrees Celsius. To evaluate relative enzyme activity, the replicate tubes were cooled and analysed for each purified enzyme at the end.

RESULTS AND DISCUSSION

One of the potential bacteria-bearing areas is vegetable waste dumping. The sludge environment is ideal for the growth of proteolytic bacteria. The screening medium was used to screen for bacteria generating proteases in vegetable waste soil samples at 55°C in this study. Protease production was established by the creation of clear zones around the colonies. In a skim milk agar plate, nine isolates revealed a clear zone. An isolate that exhibited an intense proteolytic zone was chosen as the best strain SSK1 and used for further identification out of nine isolates.

The strain SSK1 was identified as *Bacillus cohnii* based on microscopic appearance and biochemical assays, with further confirmation provided by 16S rDNA sequence analysis. After utilising particular primers to amplify the 16S rDNA sequence, a 1402 bp amplified product was generated, which was then sequenced and compared to the Genbank databases using the BLASTN tool. With 97 percent identity, the isolate's 16S rDNA sequence demonstrated a close relationship to *Bacillus cohnii*. As a result, *Bacillus cohnii* was confirmed as the strain, and the sequence was submitted to Genbank.

At a concentration of 2% inoculum, maximum protease production was obtained. The activity of the enzyme steadily reduces from 3% to 5%. (Fig.1). These findings are consistent with those of Elibol and Moreira et al. [23], who found that a 2.5 percent inoculum level produces the most protease.

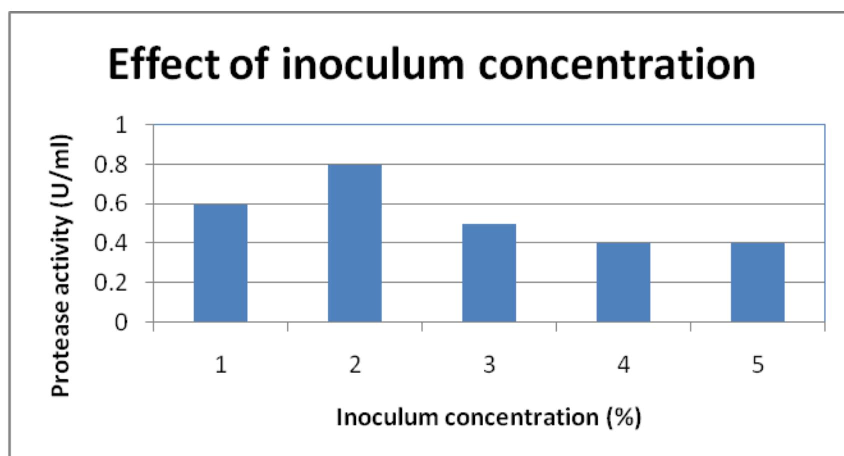

Fig. 1: Effect of inoculum concentration on *Bacillus cohnii* protease production

Because organisms show significant variance at different incubation times, determining the optimum incubation time at which an organism exhibits the maximum enzyme activity is critical (Yossan *et al.,* 2006). It was discovered that after 48 hours of incubation, a high level of protease activity was produced. At 96 hours of incubation, high biomass content was observed (Fig. 2). The findings are consistent with those of Shumi *et al.* [25], who found strong proteolytic activity in *Bacillus cohnii* after 48 hours of incubation.

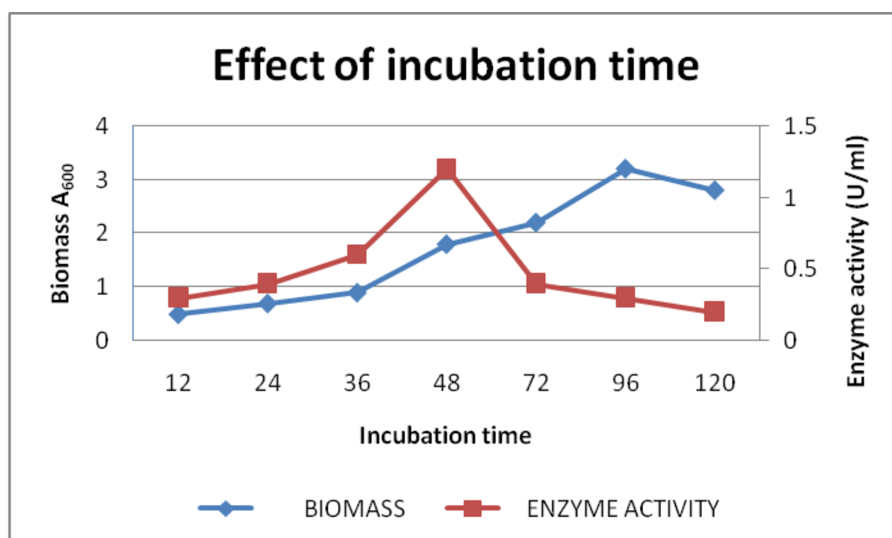

Fig. 2.*Bacillus cohnii* biomass and protease production as a function of incubation time

Many enzymatic processes and chemical transport across the cell membrane are influenced by the pH of the culture. *Bacillus cohnii* was able to produce the most protease at pH-9.0 (Fig. 3). Protease synthesis increased as the pH of the medium was raised, peaking at pH-9.0. There was a decrease in enzyme synthesis after pH-9.0. At alkaline pH, enzyme production is stimulated, according to the findings. The acquired results are consistent with Kumar *et al.* [2], who found that Bacillus sp. strain S4 produces a lot of protease at pH-9.

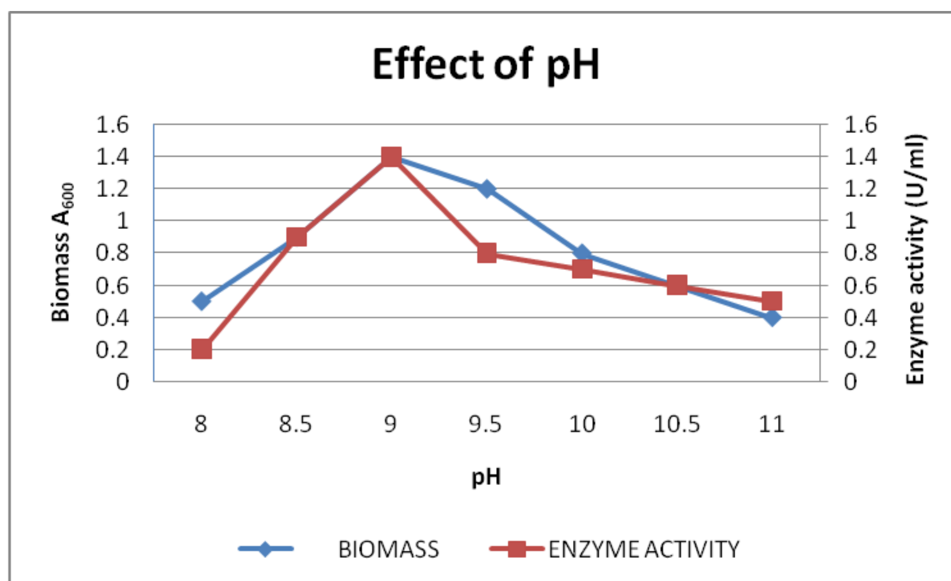

Fig. 3.Effect of initial pH on protease synthesis

Different temperatures (40–60ºC) were employed in the study to investigate the influence of different temperatures on growth and protease synthesis. It was discovered that high protease production was found at 55°C, showing that the released enzyme is thermotolerant (Fig. 4). Several researchers looked into the effect of incubation temperature on bacterial alkaline protease synthesis [26–28]. For all of the test isolates, 50 °C was determined to be the best incubation temperature for protease synthesis. *B. licheniformis* produces protease best at 45 °C [29], and *B. subtilis* at 50 °C, but both are inactive from 55–90 °C. They also discovered that the optimal temperature for protease action is 50 degrees Celsius.

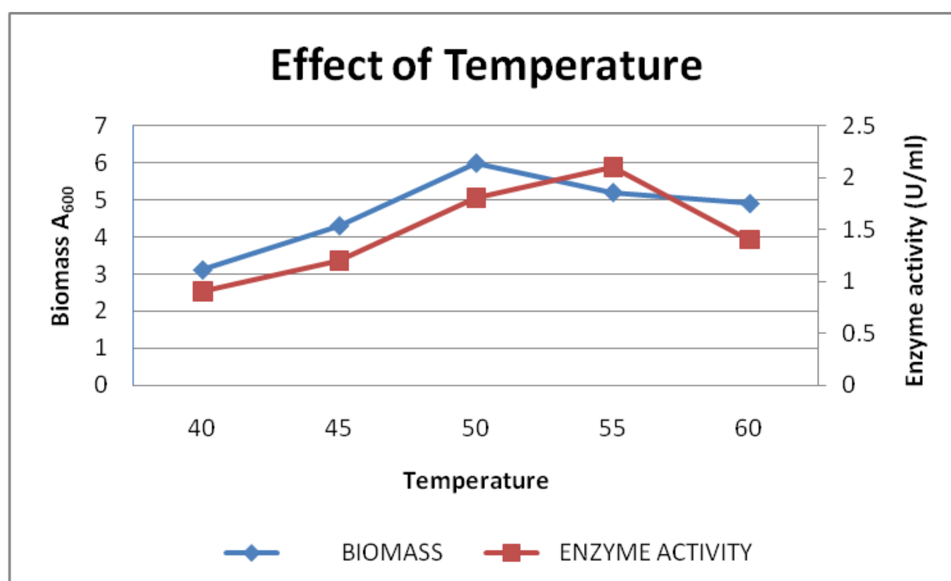

Fig 4: Protease production and temperature.

The influence of different surfactants on the formation of alkaline protease was investigated. Surfactants change microorganism cell permeability, resulting in enhanced protein secretion or surface effects on cell-bound enzymes [30]. At a concentration of 0.05 percent, all of the surfactants

reduced protease synthesis. Tween-80, SDS, and Tween-20 had the strongest inhibitory action, followed by Cetrimide (Table 1).

Table 1: Effect of surfactants on protease activity

1 mM	Residual activity (%)
Control	100
Tween-20	73.6
Tween-80	57.3
Cetrimide	48.7
SDS	67.5

The crude extracts from various fermentation techniques contain a variety of proteins as well as unwanted compounds such as organic acids and other metabolites. As a result, multiple purification processes must be used to purify the desired favourable product. In most enzyme purifications, the crude total proteins are precipitated after the culture is separated from the fermentation broth by filtering or centrifugation, and the culture supernatant is concentrated by using solid ammonium sulphate as a precipitation agent [2]. Another purification method, such as ion exchange and gel filtration chromatography, follows. The precipitation agent utilised in this investigation was ammonium sulphate, which produced 1048.5 U mg 1 of specific activity and 3512 U mg 1 after Q-sepharose chromatography (Table 2).

Table 2: Purification scheme for protease from *Bacillus cohnii*

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})
Crude ultrafiltrate	2309	887,800	378
Ammonium sulphate perception	510	616,000	1048.5
Q-sepharose	85	323,000	3512

SDS-PAGE was utilised by a number of researchers to determine the homogeneity and molecular weight of proteases generated by bacteria and actinomycetes [31]. The homogeneity of the isolated protease from *Bacillus cohnii* was determined using an SDS-polyacrylamide gel, which revealed only one band (Fig. 5), indicating that the purified protease was homogeneous and had a molecular weight of 24,000 Da.

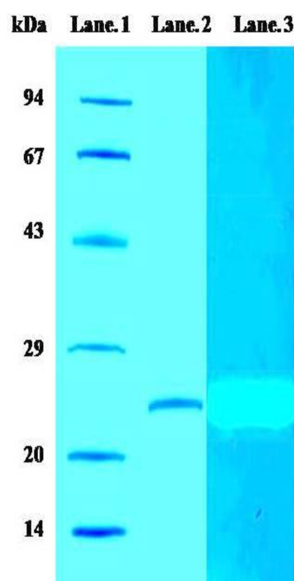

Fig. 5 Photographic depiction of SDS-PAGE Gel (Lane 1, Protein marker; Lane 2, protease single band after Q-sepharose, Lane 3, Zymogram of the purified protease activity on PAGE incorporated with gelatin)

The pH stability of the protease activity was tested using several acceptable buffers and evaluating the enzyme activity at different pH values ranging from 6, 7, 8, 9, 9.5, 10, and 11. The enzyme activity was shown to be steady and highest between pHs 8 and 9.5, with further increases in pH resulting in lower enzyme activity (Fig. 6). The thermal stability of the isolated enzyme was determined by incubating it at various temperatures (50–90°C). These temperature ranges were used in the investigation because the enzyme had an ideal temperature of 55°C in the early phases of the trials. The enzyme's highest activity was determined to be between 55 and 65 degrees Celsius, demonstrating the purified protease's thermotolerance (Fig. 7).

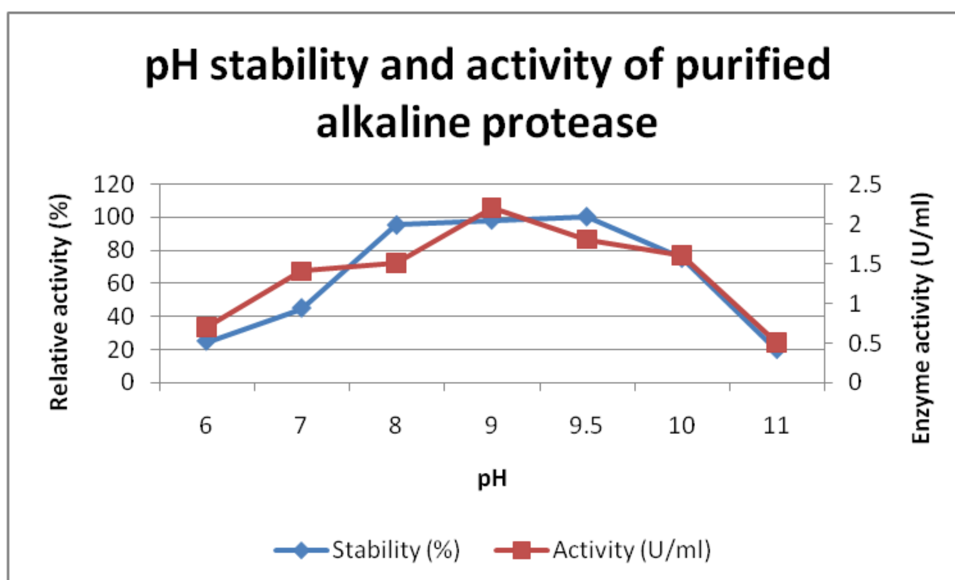

Fig 6: The effect of pH on the purified enzyme's activity and stability.

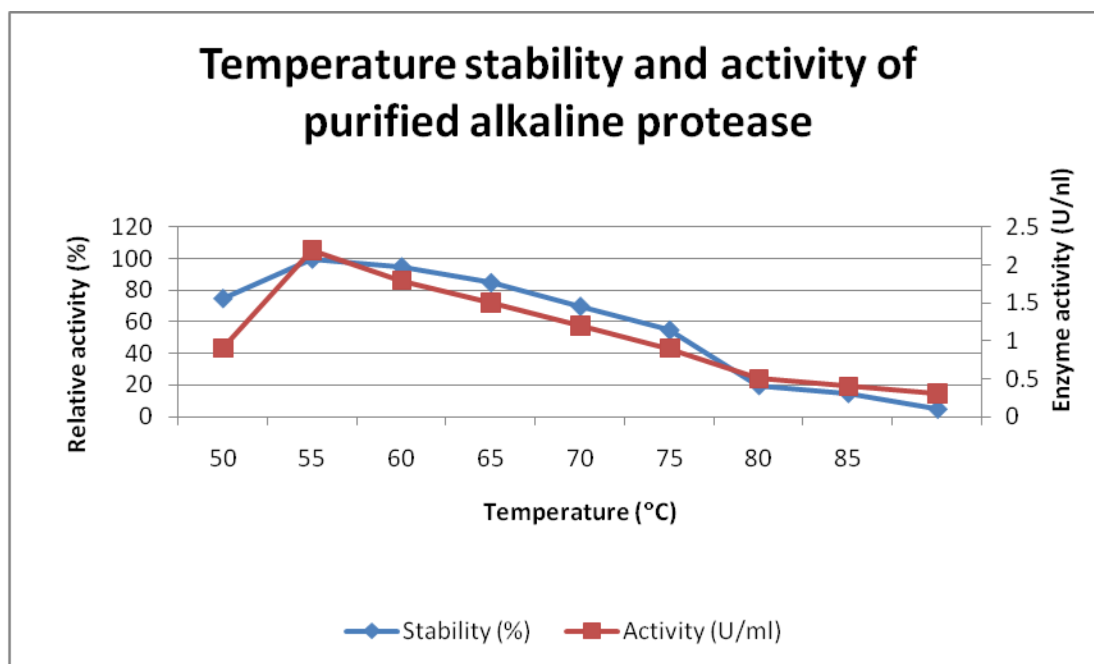

Fig 7: The influence of temperature on the purified enzyme's activity and stability.

The findings of this study demonstrated that value-added goods such as proteolytic enzymes may be made from vegetable waste. By repurposing vegetable waste and sludge as enzyme substrates, it is possible to reduce production costs while also increasing the production of valuable bio-products.

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