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Bharat Pokhrel
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Ankit Pandeya
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Seema Gurung
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Govinda Bista
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Sunil Kandel
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Ram Chandra Kandel
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Rubin Thapa Magar
*Department of biochemistry,
Universal Science College
Chakupat, Lalitpur, Nepal*

Screening and optimization of extra cellular protease from bacteria isolated from sewage

Bharat Pokhrel, Ankit Pandeya, Seema Gurung, Govinda Bista, Sunil Kandel, Ram Chandra Kandel, Rubin Thapa Magar.

Abstract

This study was conducted to screen for strains with proteolytic activity and to examine the effects of pH, temperature and incubation time on the activity of isolated crude enzymes. Bacterial isolates were isolated from the sewage site present in different river banks of Kathmandu valley. Isolated samples were screened for production of protease. One of the isolates was found positive and designated as SAG-P2. Based on the biochemical characterization and staining organism was identified as *Corynebacterium* sp. This isolate was taken for further studies. The optimum pH for enzyme activity was found to be at pH 6.6 and the optimum temperature for the activity was found to be at 50 °C

Keywords: Protease, enzyme activity, pH, temperature

1. Introduction

At present context, enzymes are of great importance and they have dragged the world's attention due to their wide range of application in various fields. Enzymes have been very much important in replacing the use of harsh chemicals in various industrial processes.^[1] Proteases constitute one of the most important group of industrial enzymes and have established role in detergent, food, pharmaceutical, leather, waste processing industries and silk industries.^[2] Protease can be defined as any enzyme that performs proteolysis i.e. begins protein catabolism by hydrolysis of peptide bonds that link amino acids together in the polypeptide chain forming the protein^[11].

Proteases useful for industrial application are influenced by different temperature, incubation period, quantity of inoculum, medium pH, NaCl concentration and composition or type of media.^[5] Depending upon the optimal pH, protease can be acidic, neutral or alkaline. Alkaliphilic protease plays an important role in the detergent industry, whereas acidophilic protease have significant role in leather tanning process, food industry and X-ray films (for removal of silver)^[4, 8].

Protease shares nearly 60% of the total world-wide enzyme market^[6, 7, 9]. Among these, detergent industry is the largest market for protease. Microorganisms are largely responsible for the production of protease. Also microbial proteases are more preferred due to the broad biochemical diversity, rapid growth, limited space required for cell cultivation and ease with which the enzymes can be genetically manipulated to generate new enzymes for various applications^[5]. Both fungi and bacteria can be used for the production of protease. Fungi like *Aspergillus*, *Mucor*, *Rhizopus* sp. and bacteria like *Clostridium*, *Bacillus*, *Pseudomonas* sp. produce extracellular protease^[3].

The presently available proteases are not sufficient to meet industrial demands. Hence, there is continuous search for new proteases with novel characteristics for industrial application from diverse bacterial isolates. Microbes from varied habitats have been examined by many researchers to obtain the industrially suitable proteases. So the main objective of this study was to isolate and characterize protease producing bacteria and to optimize the isolated enzyme.

2. Materials Required:

- Reagents: Casein agar, staining reagents (Gram staining, capsule staining, endospore staining, acid fast staining), SIM agar, OF glucose medium, paraffin, tryptone broth,

Kovac's reagent, agar Simmon's citrate, MR-VP broth, H₂O₂, 0.1M phosphate buffer, 0.4M trichloroacetic acid (TCA), 0.5M sodium carbonate, FC reagent, distilled water, 70% ethanol.

- Apparatus: Laminar air flow chamber, petri plate, inoculating loop, test tubes, incubator, centrifuge, autoclave, hot air oven, spectrophotometer, micropipette, burner, gloves, zip-lock plastic, forceps, spatula, conical flask, measuring cylinder, beaker, glass slides.

3. Methodology:

3.1 Sample collection:

Soil samples were collected from sewage site present in different river banks of Kathmandu valley. Zip-lock plastic bag was used for taking the sample which was taken from 4-5cm deep from the surface by the help of spatula. Both zip-lock plastic and spatula were autoclaved before used and sterile gloves were used for handling the equipments. Date and time were also noted during collection of each sample.

3.2 Isolation of protease producing bacteria:

1gm of obtained soil sample was taken and serial dilution was done to produce 10⁻¹-10⁻⁸ dilutions. 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁸ dilutions from each sample were grown in casein agar medium (containing 1% casein) by spread plate technique. The plates were incubated at 37 °C for 24 hours and zone of hydrolysis were observed. For further study the strain showing largest zone of hydrolysis was taken and designated as SAG-P2. The isolated colony of the strain SAG-P2 was obtained by repeated streaking on nutrient agar plate and thus obtained isolated colony was grown in nutrient agar slant and stored at 4 °C.

3.3 Characterization:

3.3.1 Staining:

- Gram staining:
- Capsule staining:
- Endospore staining:
- Acid fast staining:

3.3.2 Biochemical tests:

- H₂S production test:
- OF test:
- Indole production test:
- MR-VP test:
- Citrate utilization test:
- Catalase test:
- Starch hydrolysis test:

4. Assay of Enzyme Activity

4.1 Proteolytic Activity:

4.1.1 Preparation of casein solution:

Proteolytic activity was measured with soluble casein (1%, w/v) as substrate. Soluble casein was prepared from alkali soluble casein. 0.1gm of casein was weighed and dissolved in 10 ml distilled water. The insoluble portion was dissolved by addition of the alkali. The pH was adjusted to 8.0 with 0.1M Sodium hydroxide.

4.1.2 Crude Enzyme Preparation

For the production of enzyme, selected bacterial colony

after its preliminary identification and characterization was inoculated into casein broth medium. It was then incubated at 37 °C for seven days. The cultured medium then was filtered through WhatmanNo.1 filter paper aseptically in laminar air flow chamber. The filtrate was then subjected to centrifugation at 10,000 rpm for 10 minutes to remove the unwanted particles. The supernatant was then referred as crude enzyme and this crude enzyme was used for further enzyme assay and characterization.

4.2 Protease activity assay:

For proteolytic activity culture media was centrifuged and supernatant was used as enzyme source. However, 1% casein (in 0.1 M phosphate buffer and pH 7.0) was used as substrate. 1 ml each of enzyme and substrate was incubated at 50 °C for 60 min. The reaction was terminated by adding 3 ml of Trichloroacetic acid (TCA). One unit of protease activity was defined as the increase of 0.1 unit optic density at 1 h incubation period. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using Spectrophotometer [14]. The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity

$$\text{Enzyme activity} = \frac{\text{Concentration of tyrosine degraded}}{\text{Mol.wt of tyrosine} \times \text{vol. of enzyme (ml)} \times \text{Incubation time}}$$

4.3 Effect of pH on protease activity:

In order to assess the maximum enzyme activity, phosphate buffers with a range of pH (5.8-8) are been tested. Casein solutions are prepared individually in phosphate buffers with above mentioned pH. Enzyme activity was determined by standard enzyme assay.

4.4 Effect of temperature on protease activity: Effect of temperature on protease activity:

With the optimum pH of the crude enzyme from previous experiment, the optimum temperature was standardized by incubating the enzymes with the substrate (casein solution prepared according to optimum pH) at varying temperature range from 25 to 100 °C. The experiment on the effect of temperature on enzyme stability was carried out by incubating the enzyme solution at temperature ranges of 25-100 °C The enzyme activity was determined by the standard enzyme assay.

International units (IU)

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions.

5. Results and Discussion

5.1 Screening of Proteolytic activity

Various isolates were screened for protease activity on the Casein agar plates. Protease activity was observed from the zone of hydrolysis observed on agar surface. For further study the strain showing largest zone of hydrolysis was

taken and designated as SAG-P2 and maintained by repeated sub culturing.

5.2 Characterization of the organism

5.2.1 Morphological Characterization

The colonies were observed to be large, round, irregular, mucoid and fast growing. The bacteria were found to be Non spore forming and appeared Gram negative rods. The result of acid fast staining showed that it was non-acid fast. The results of all the staining done is shown in table 1.

Table 1: Results of staining of strain SAG-P2

S.N.	Type of staining	Result
1.	Gram staining	Negative
2.	Capsule staining	Capsule non-forming
3.	Endospore staining	Non Spore forming
4.	Acid fast staining	Non-acid fast

5.2.2 Biochemical Characterization

Various biochemical tests were done whose results are shown in table 2. Among the tests done the bacteria showed positive results for citrate utilization test, VP test and catalase test whereas negative results was shown for H₂S production, indole production, starch hydrolysis and MR test. The bacteria showed fermentative type of metabolism. The results of all the biochemical test done is shown in table 2.

Table 2: Biochemical activity of strain SAG-P2

S.N.	Name of biochemical test	Result
1.	H ₂ S production test	Negative
2.	Indole production test	Negative
3.	OF test	Fermentative
4.	M.R. test	Negative
5.	V.P. test	Positive
7.	Citrate utilization test	Positive
8.	Catalase and oxidase test	Positive
9.	Starch hydrolysis test	Negative

5.3 Protease Assay

5.3.1 Tyrosine standard curve

Tyrosine standard curve is been plotted to measure the enzyme activities of protease and keratinase. A standard solution was prepared by using 0.5M of Na₂CO₃, 50mM of Glycine buffer, pH 10.00, 1:1 diluted Folin reagent and 200 µg/ml of Tyrosine stock solution. A required amount of buffer and Tyrosine were added to each test tube. 2.5 ml of 0.5M Na₂CO₃ was added in each test tube subsequently, the mixtures were kept at room temperature for 10 min. After incubation 500µl of Folin reagent was added and further incubated for 30 min at room temperature. A solution of glycine buffer (pH 10), 2.5ml of Na₂CO₃ and 0.5ml of Folin reagent were added in a test tube which served as a blank. The optical density (OD) was measured at 660nm using spectrophotometer and the standard curve was plotted.

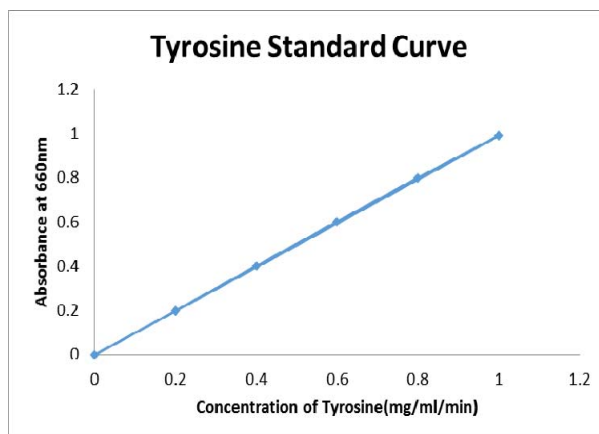


Fig 1: Tyrosine Standard Curve

5.3.2 Assay of Protease Enzyme Activity

The maximum enzyme activity was observed on 60 minutes of incubation. As the incubation time increase within the range of 20 minutes to 60 minutes enzyme activity increased.

At 60 minutes of incubation the protease shows 0.36IU of enzyme activity.

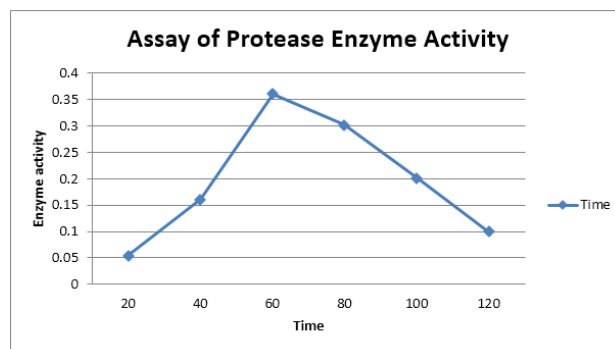


Fig 2: Enzyme activity of protease

5.4 Effect of pH on protease activity:

A pH range between 5.8 and 8 was used to study the effect of pH on protease activity (Fig. 3). Optimum pH was found to be 6.6 and it shows 0.031 IU at pH 5.8 only 0.005 IU of the enzyme activity was found

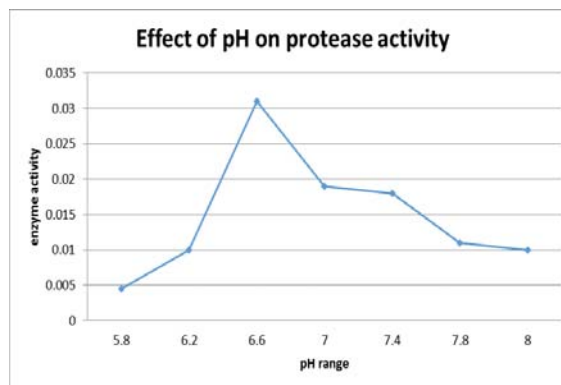


Fig 3: Effect of pH on protease activity.

5.5 Effect of temperature on protease activity

A temperature range between 25 °C and 100 °C was used to study the effect of pH on protease activity. As shown in Figure 4, the highest protease production level was obtained at 50 °C. Enzyme activity increased with temperature within the range of 37 °C to 50 °C. A reduction in enzyme activity was observed at values above 50 °C. The optimum temperature of this protease was 50 °C. At 50 °C the protease shows 0.078IU of enzyme activity.

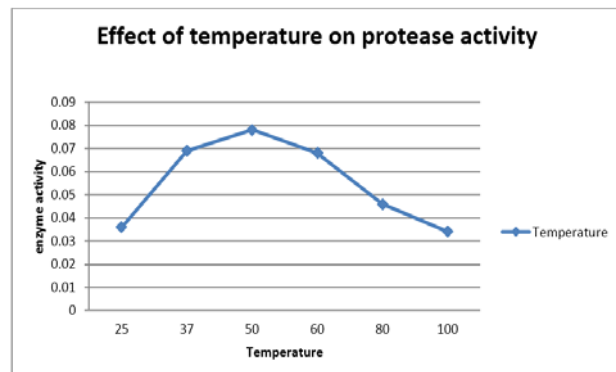


Fig 4: Effect of temperature on protease activity

6. Conclusion

Proteolytic bacteria are widespread in nature and are able to grow under various growth conditions, such as different temperatures, pH and ionic strength. A protein degrading bacterium was isolated from sewage, proteolytic property of the enzyme helps to convert proteinaceous wastes in to more nutritionally available animal feed stuff. It can be used in detergent industry for removing protein stains, leather industry for dehairing, textile industry for silk degumming, photographic industry for silver recovery, food and feed industry, chemical industry for peptide synthesis. The organism is rod shaped, Endospore forming, catalase positive and gram negative. Based on the biochemical characterization and staining organism was identified as *Corynebacterium* sp. Enzyme is highly active at optimum temperature of 50 °C and pH 6.6. It is also active in broad pH and a temperature range which was primary and essential step for the production of adequate amount of such industrially important enzyme.

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