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# SCREENING, OPTIMIZATION AND PARTIAL PURIFICATION OF PROTEASE ENZYME FROM DIFFERENT PULSES

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## ABSTRACT

*Enzymes are the protein molecules which are useful in detergent industry. The production of industrially important enzyme protease from different pulse varieties such as pigeon pea, green gram, Bengal gram, black chickpeas, white chickpeas, have been studied. The protease producing media for pulses have been studied. The crude enzyme is tested for protease activity by Anson method. The maximum protease activity was showed at higher rate from pigeon pea on the gelatine substrate when compared to the other samples. It was partially purified by dialysis method and molecular weight of the protein was calculated by SDS-PAGE.*

## KEY WORDS

*Protease enzyme, optimization, Anson method, dialysis, SDS-PAGE, blood stain removal.*

## INTRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells [1]. Enzymes are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries [2]. Pulses are valuable source of dietary protein, carbohydrates, fibre, and essential vitamins and minerals. Their nutritional characteristics have been associated with a reduction in the incidence of various cancers, HDL cholesterol, type-2 diabetes and heart disease. The use of plants for the production of proteases is dependent on the availability of land for agriculture and certain climatic conditions. Papain, bromelain, keratinases are some of the well-known proteases of plant origin.

## MATERIALS AND METHODS

### Collection and storage of samples

Totally 5 different pulses (pigeon pea, green gram, Bengal gram, black chickpeas, white chickpeas) were collected from the market at Guindy, Chennai, Tamilnadu, India. Sample was stored in zip lock cover at room temperature for further use.

### Processing and preparation of samples

Ten grams of each pulses were taken and washed. After 30 min pulses were grounded with 0.2 M phosphate buffer of pH-7. Then the supernatants were centrifuged at 5000 rpm for 15 min. The clear supernatant obtained after centrifugation was used for the determination of protease enzyme.

### Qualitative screening

Screening of protease producing pulses was identified using 3 different substrates. viz Medium A (1%casein +agar), Medium B (1% skim milk + agar), Medium C (1% gelatine + agar) in distilled water. The media was autoclaved at 121°C for 15 min [3].

### Effect of pH

The effect of pH on protease production was determined by applying the samples in to the wells of production media with different pH (5.8 ,6 ,6.2 ,6.4 ,6.5 ,6.6, 6.8, 7 ,7.2 ,7.4) of 0.2 M of phosphate buffer.

### Effect of temperature

Samples were kept at different temperatures such as (-4$^{o}$C, -20$^{o}$C, 45$^{o}$C, 80$^{o}$C) as well as at UV then the samples were added in to the wells of protease production

media and incubated for overnight at room temperature.

**Effect of time**

Samples were boiled at different time interval from 1 min to 5 min at 80$^{o}$C were incubated for overnight at room temperature.

**Effect of substrate concentration**

Protease production medium was prepared with different substrate concentrations of gelatine. i.e., (0.6%, 0.8%, 1.2%, 1.4%)

**Quantitative analysis (Anson, 1938)**

The assay was carried out routinely in a mixture containing 0.5 ml of crude enzyme solution and 0.5 ml of (gelatine) 1% of substrate (pH 7) solution were mixed. The mixture was incubated for 1 hour at RT. After incubation, the reaction was terminated by addition of 1 ml of 10% Trichloro acetic acid (TCA) solution. After 10 min the mixture was centrifuged at 5000 rpm for 20 min at 4$^{o}$C. An aliquot of 0.5 ml of supernatant was mixed with 2.5 ml of alkaline solution (2.9% $Na_2CO_3$ in 0.3N NaOH) and 0.75 ml of 1:3 Folin-Ciocalteu's phenol solution in distilled water and kept for 20 min at room temperature. The optical densities of the solutions were determined with respect to sample blanks at 660 nm. One unit of enzyme activity is defined as µM of tyrosine released from 1 ml crude enzyme in 1-hour incubation.

**Standard tyrosine assay for protease activity**

The standard assay for protease activity was performed using tyrosine as standard. The stock solution used for this assay was 50 mg of Tyrosine which was dissolved in distilled water to make 100 ml in a volumetric flask, which resulted in 500 µg/ml.

The assay was performed by test tubes containing 1 to 5 ml of standard stock solution of tyrosine and make up to 10 ml by distilled water. The solution was mixed well and the optical density was measured at 660 nm after developing the blue colour against a reagent blank prepared in same manner. A standard curve was constructed taking concentration of Tyrosine µg/ml on X-axis and corresponding optical density on Y-axis.

**Protein estimation**

Protein estimation was performed by Bradford method. 100 µl of supernatant was added to 900 µl of distilled water, and it was mixed thoroughly. To that 5 ml of coomassie brilliant blue (CBB) was added, incubated at room temperature for 5 min. And absorbance was measured at 595 nm [4].

**Partial purification of protease**

**Precipitation**

The crude enzyme extracted from *cajanus cajan* was subjected to acetone precipitation. 70% of ice cold acetone was slowly added to the supernatant. The mixture was kept in magnetic stirrer for 1 hr. then it was incubated overnight in refrigerator at 4$^{o}$C [5].

**Dialysis**

Dialysis bag was prepared by following method; 8- 10 cm 0f dialysis membrane was dipped in to the boiled distilled water for 10 min with slow stirring. Then the membrane was decanted and placed in 100 ml of 2% sodium carbonate solution [6].

Then the membrane was taken and tied with thread tightly one side of the membrane then the enzyme to be purified is injected into the dialysis bag with the help of syringe. Then the experimental set was kept in magnetic stirrer for 8 hrs in phosphate buffer [7].

**SDS – page**

The purity and molecular mass of protease were determined by sodium dodecyl sulphate-poly acryl amide gel electrophoresis (SDS-PAGE) performed on 12% poly acryl amide slab gel under reducing conditions using method of Laemmli. 50 microliters of protein sample was loaded onto gel composed of 5% stacking gel and 12% resolving gel. The electrophoresis was carried out at constant 100V current using Mini-Protean apparatus (Bio-Rad). After electrophoresis, gel was stained with 0.1% coomassie Brilliant blue R-250 of water: methanol: acetic acid in 50:40:10 for 1 h and de stained with solution of water: methanol: acetic acid in 50:40:10 overnight. The molecular weight was estimated by comparing with Puregene pre-stained Protein Ladder, Broad Range (kDa) [7].

**Destaining activity of protease on blood stain**

When protease was applied on a white cotton cloth, the blood stain was removed by incubating the cloth with partially purified protease. After 35 min of incubation the blood was completely destained by protease enzyme [7]. It was observed that the partially purified protease from *cajanus cajan* had high capability of removing the blood stain, which indicated that the protease enzyme has potential application in detergent industries [8].

## RESULTS

On screening of different substrates, it was found that gelatine was the best substrate for protease production and will be used for further studies.As *Cajanus cajan*

has the highest zone of clearance , the sample was checked for its activity when it is boiled and processed. And optimal conditions for protease production were shown in **Fig 1,2,3,4.**

**Optimization of protease production**

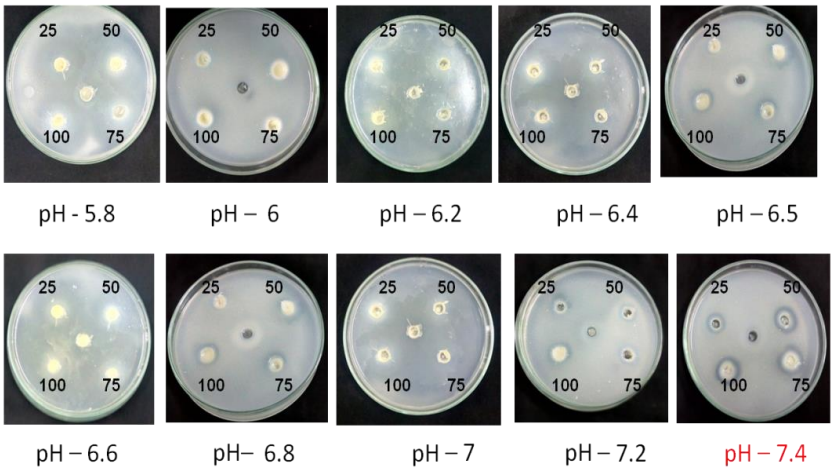


**Fig. 1: Effect of pH on protease production**

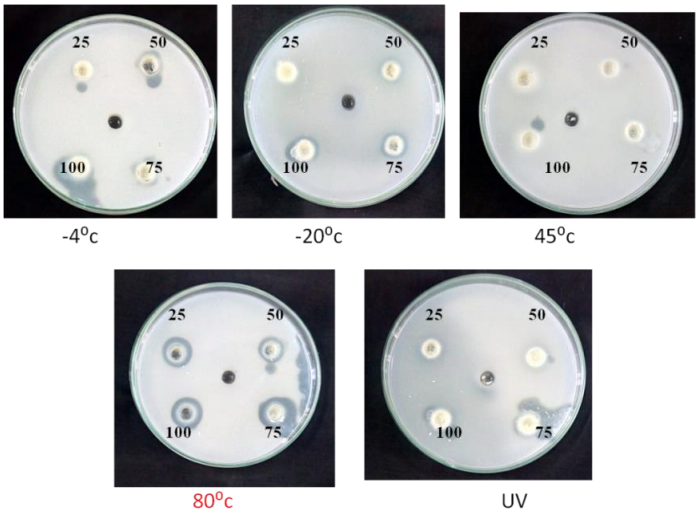


**Fig. 2: Effect of temperature on protease production**

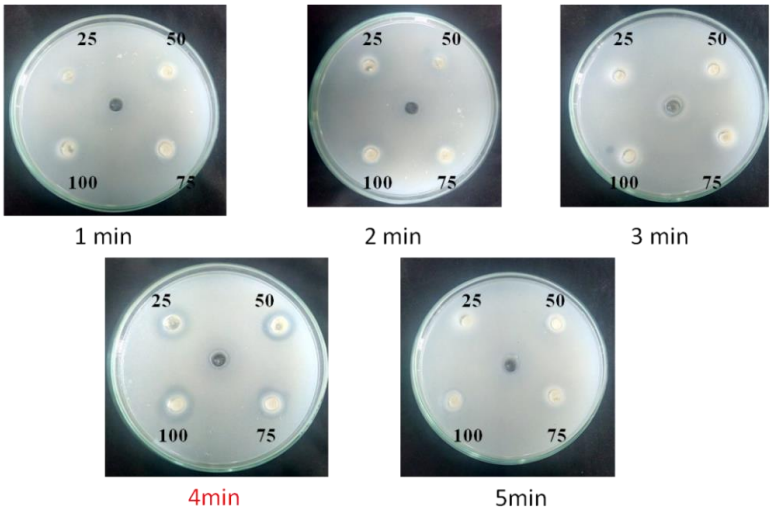


**Fig. 3: Effect of time on protease production**

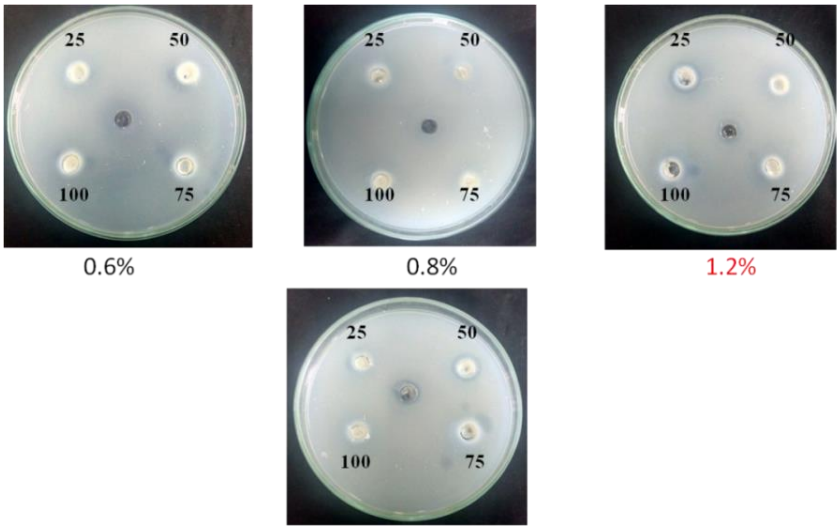


**Fig. 4: Effect of substrate concentration on protease production**

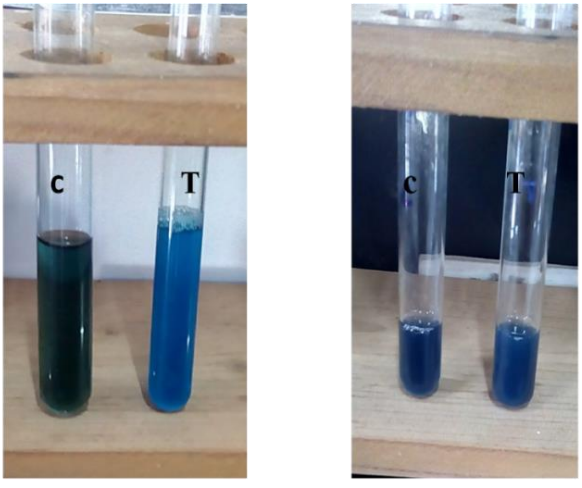


**Fig. 5: protease activity**

| | CONTROL | TEST |
|---|---|---|
| **OD at 650 nm** | 0.599 | 1.095 |

**Amount of enzyme present is 0.08 enzyme units / ml.**

**Protein estimation by Bradford**

Standard value for protein BSA = 60

OD value for *Cajanus cajan* = 0.622

Amount of protein = 60 × OD value

= 60 × 2.501 = 150.06 mg/ml

**Enzyme activity by Anson method**

Tyrosine standard value = 0.999

$$\text{Units / ml of enzyme} = \frac{\mu \text{ moles of tyrosine} \times \text{total vol. Used in assay (ml)}}{\text{Vol.of enzyme (ml)} \times \text{time (min)} \times \text{vol.Used for colorimetric}}$$

$$= \frac{0.999 \times 5.75}{0.5 \times 80 \times 2}$$

= 0.071 Enzyme units

OD value for *Cajanus cajan* = 2.501
= 2.501 × 0.071
= 0.18 Enzyme units / ml

**Molecular weight determination**

Purity and molecular weight of protease enzyme was determined. Hence the molecular weight of protein was 55 kDa. Shown in **Fig 6.**

**SDS – PAGE**

**( molecular weight determination)**

**Fig. 6: molecular weight determination**

**Destaining activity of protease on blood stain**

The blood stain was removed by incubating the cloth with partially purified protease. After 15 min of incubation the blood was completely destained by protease enzyme. It was observed that the partially purified protease from *cajanus cajan* had high capability of removing the blood stain shown in **Fig 7.**

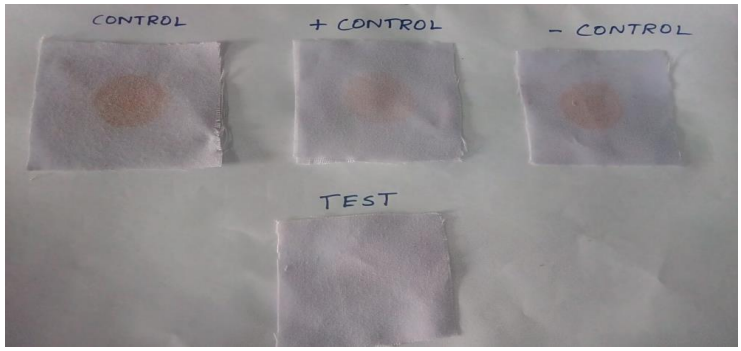


**Fig. 7: Destaining activity of protease enzyme on blood stain**
**Ctrl - 20ml distilled water**
**(+) ctrl - 20ml distilled water + 1ml detergent**
**(-) ctrl - 20ml phosphate buffer (Ph-7.4)**
**Test - 20ml distilled water + 1ml enzyme solution**

## DISCUSSION

In this we conclude that screening of different pulses on different substrates gives maximum zone of clearance in Cajanus *cajan* (pigeon pea) on gelatine as substrate. The maximum zone of clearance was found to be 17 mm. further estimations was done with specified sample (*Cajanus cajan* ) will be quantified and the total amount of protein was estimated by brad ford method was 150.06 mg/ml .

From the present study it was revealed that *cajanus cajan was* capable of producing a considerable quantity of the protease enzyme and it was subjected to acetone precipitation followed by dialysis.

The protease enzyme was partially purified molecular weight 55 kilo Dalton. The partially purified protease has

optimum pH at 7.4 and temperature of 80$^{o}$C for 4 min and substrate concentration of 1.2% which showed a positive result in the removal of blood stains from the cloths.

Therefore, this enzyme can be extensively used in the industrial applications in pilot scale.

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