

# Studies On Optimization Of Protease Production Using Bacterial Isolate Clri Strain 5468 And Its Application In Dehairing And Hydrolysis Of Tannery Fleshings (Solid Waste Management)

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**ABSTRACT:** The strain which produces protease was originally isolated, characterized in Biotechnology laboratory at CLRI and was maintained. The microorganism was grown on several proteolytic media and the maximum activity was observed. The characterization of enzyme was analysed for different pH, temperature, size of inoculum, inhibitors, age of the culture. Then the enzyme was observed for the unhairing of skin and the disadvantage in chemical treatment was studied. The conformation of unhairing was studied using histology studies. The tannery waste (solid fleshings) as it is cannot be directly disposed off to the environment. It was treated with the microbial proteases. The hydrolysis of waste was done using proteases. The solid waste was converted to protein, fat and the salt matter. Future work is to optimize the cheap media for the production of the enzyme for large scale applications in various industries.

**KEY WORDS:** Protease, unhairing, tannery, hydrolysis, solid waste management

## INTRODUCTION:

Proteases constitute one of the most important groups of enzyme and have applications in different industries including detergent, food, pharmaceutical, silk and leather industries. Proteases do not refer to a single enzyme but a mixture of enzymes including proteinases, peptidases, and amidases. Among various proteases, proteases from *Bacillus* sp. are the most significant, compared to animal and fungal proteases[3]. They produce two groups of proteases, alkaline and neutral. Alkaline proteases are active in narrow pH range (8-12). This property is advantageous for controlling their activity during the production of food hydroxylates with low degree of hydrolysis[9].

## MATERIALS AND METHODS:

### REVIVAL OF THE CULTURE

The strain which produces protease was originally isolated and characterized in Biotechnology laboratory at CLRI and was maintained in a lyophilized vial. The vial was then revived and put in to use. The glass vial containing the lyophilized culture was heated slightly in the flame[6]. The vial was then cut opened using sterile lancet. A loop full of culture was transferred in to nutrient agar plate and the remaining culture was inoculated in to the liquid medium. They were kept at 37 °C incubator for 24-48 hrs. Sub culturing was done after every 24 hrs for three days[20]. Identification of bacteria was followed by the method described in Bergey's Manual Of Systemic Bacteriology.

## PROTEOLYTIC ACTIVITY CONFORMATION

Qualitative assay rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. Both the parent and the UV exposed cultures were tested for the proteolytic activity on Gelatin-Agar plates.[12] 5µl of the bacterial suspension was inoculated on to the hole made using gel puncture on the surface of the gelatin-agar plates. These plates were incubated for 48hrs at 30°C. After 48hrs, the plates were flooded with 10% tannic acid solution for 5 minutes. Protease secretion was detected by observing the zone of hydrolysis around the hole in agar gelatin plates.[19]. Both the parent and UV irradiated culture were streaked on skim milk agar plates and stored at 4°C. These plates can be used as master plates and sub cultured further.

## ALKALINE PROTEASE PRODUCTION IN SUBMERGED FERMENTATION

SMF is the technique employed to grow microorganisms on liquid media. The used medium is nutrient broth with 1% gelatin solution. About 6% (v/v) of the culture was used to inoculate production flasks. Incubate it in orbital shaker (200rpm) for 24hrs at 37°C. Then the protease assay has been carried out

## Enzyme Extraction

After 24hrs of incubation the enzyme from the fermented broth was extracted by simple centrifugation at 10,000 rpm for 10 mins at 4°C. The resultant supernatant was used as a source of enzyme. The proteolytic activity of the enzyme preparation was determined by Anson modified by Kunitz method (1938) using casein as substrate. By the enzyme activity determined, the production was continued for the further application.

## Characterization of enzyme

The growth profile of the organisms were observed. The enzyme was characterized at different pH, Temperature, different substrates, period of incubation, different carbon and nitrogen sources, SDS Page and Zymogram.

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**APPLICATION**

**Dehairing of the goat skin using protease**

The goat skin was taken and cleaned properly with water until the waste materials are washed off. Then it was cut in to 4 equal parts. The upper parts were used for the studies. As the hair root was longer in the backbone region. 300grams of skin was taken. The 15 % (v/w) enzyme was applied on the inner side of the skin and kept at room temperature, then observed for the hair removal after 18 hours. The other part of the skin was treated with the chemical treatment.

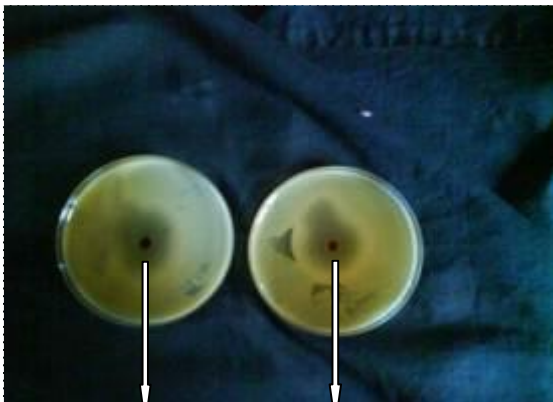
**Hydrolysis of Tannery waste**

About 3 kg of waste flesh was collected from tannery and minced it properly using mincer. The initial pH was measured and adjusted the pH using H<sub>2</sub>SO<sub>4</sub>. Incubate the above mixture at 65-85°C for half an hour with proper mixing at regular interval.[8] Then cool the substrate to room temperature. Add enzyme to the mixture in the ratio of 10-15% V/W. Mix it thoroughly and incubate it at 37°C for 2-5hours rotating at 900rpm. Take the aliquots from the reaction mixture periodically at every one hour including an aliquot at 0<sup>th</sup> hour after the addition of the enzyme.[17]

**RESULTS**

**CONFORMATION OF PROTEASE PRODUCTION**

The proteolytic activity of the bacteria was confirmed in gelatin-agar plates were a circular zone of clearence is formed around the hole to which the inoculation was done. The zone appear only after the treatment with 10% tannic acid.[3]



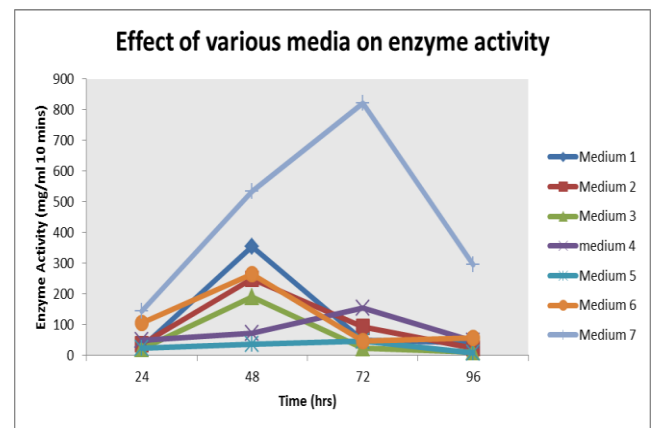
Zone of clearence in 1% GELATIN Nutrient agar medium confirms the presence of protease

**5.3. BIOCHEMICAL CHART FOR IDENTIFICATION OF BACTERIA. [18]**

S.NO	CHARACTERISATION	RESULT
1	Shape	Rod
2	Gram staining	-ve
3	Motility	+ve
4	Growth temperature	37°C
5	Reduction of nitrate	+ve
6	Production of indole	-ve
7	Oxidase	+ve
8	Catalase	+ve
9	Urease	+ve
11	Voges Proskauer test	-ve
12	Citrate utilization test	+ve
13	Triple sugar iron test	+ve
14	Casein hydrolysis	+ve

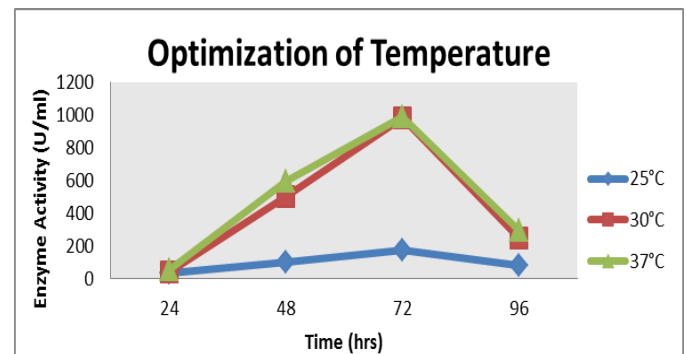
**5.4. OPTIMIZATION OF CULTURE CONDITIONS FOR ENZYME PRODUCTION**

**Effect of various medium on enzyme activity[25]**



**Effect of incubation time and temperature on protease production (conditions; pH- 8.0, temperature-37°C).**

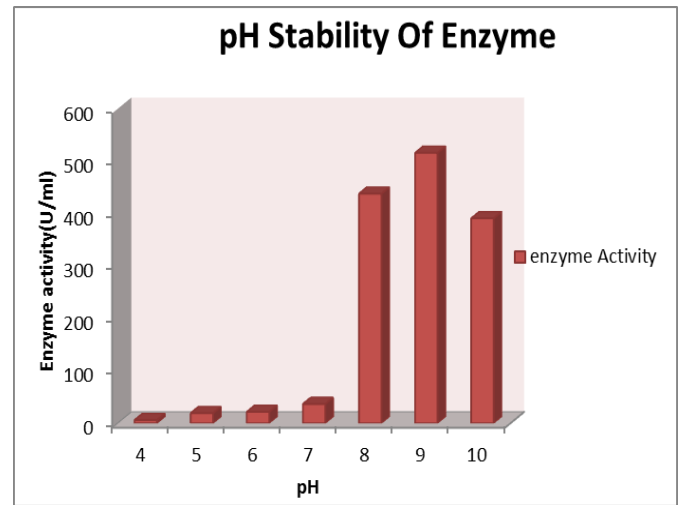
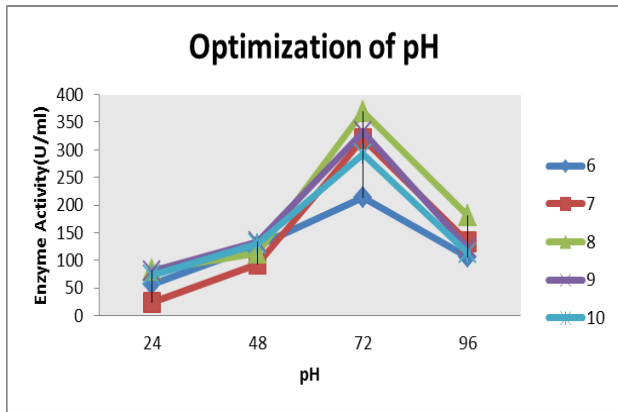
**Temperature Optimization[25]**



**OPTIMIZATION OF pH**

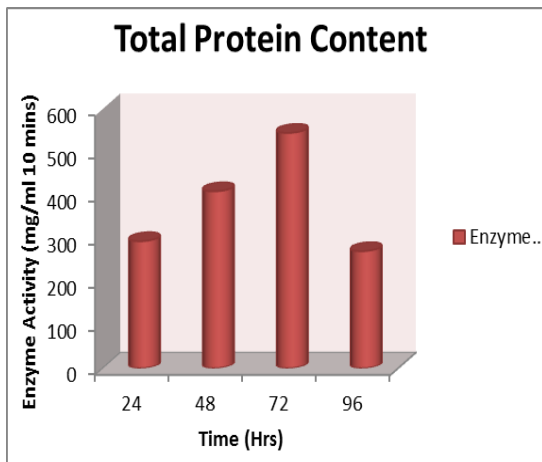
**Effect of pH on protease production (conditions: temperature-37°C).**

**pH Optimization[25]**

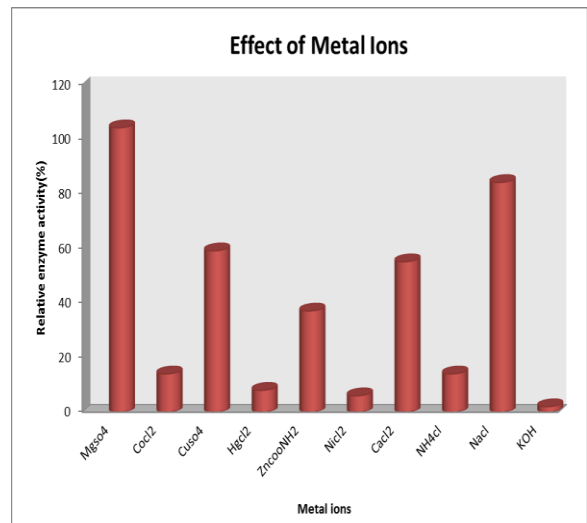


**DETERMINATION OF PROTEIN CONTENT IN THE ENZYME EXTRACT[8]**

**Total Protein Content**

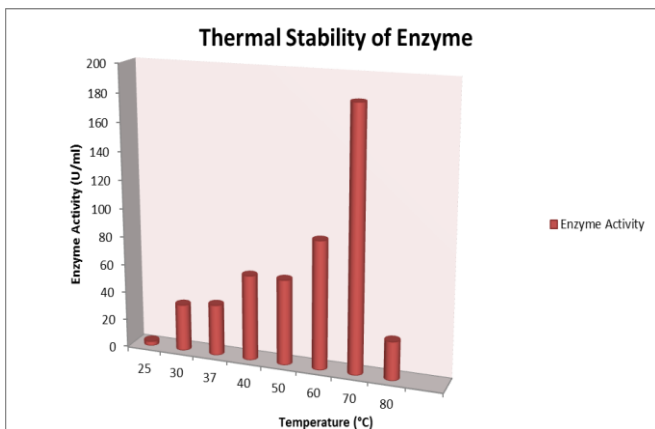


**Effect of Metal Ions on Protease Enzyme (conditions: pH- 8.0, temperature-37°C)**



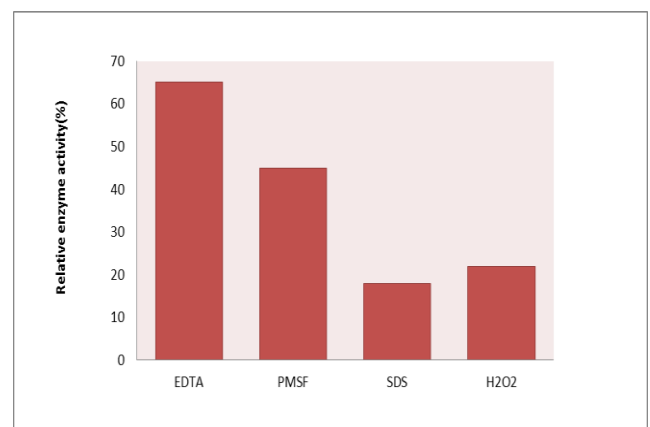
**Thermal stability of enzymes (conditions: pH- 8.0).**

**Effect of temperature on enzyme stability[25]**



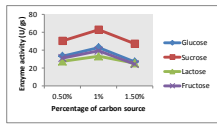
**Effect of Inhibitor, Detergent and oxidizing agent on protease enzyme (conditions; pH- 8.0, temperature-37°C).**

**Effect of Inhibitor,detergent and oxidizing agent on enzyme**

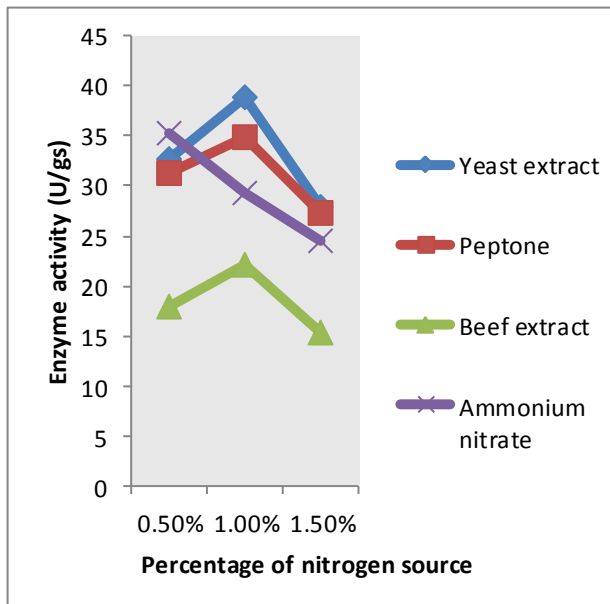


**Effect of pH on enzyme stability (conditions: temperature-37°C). [25]**

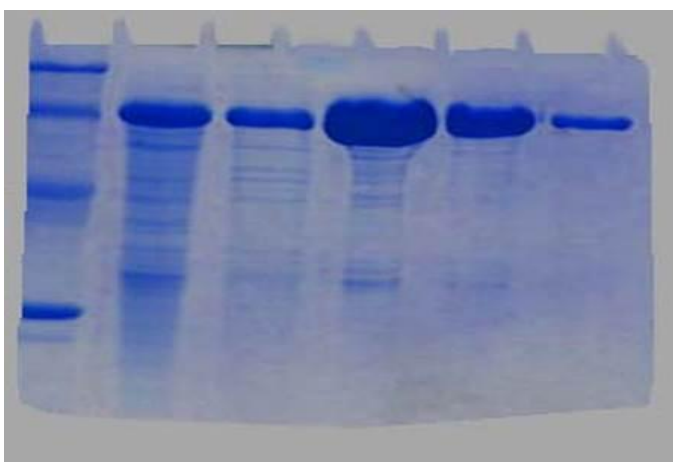
**Effect of Carbon sources on alkaline protease enzyme activity (conditions; pH- 8.0, temperature-37°C)**



**Effect of Nitrogen sources on alkaline protease enzyme activity (conditions; pH- 8.0, temperature-37°C)**



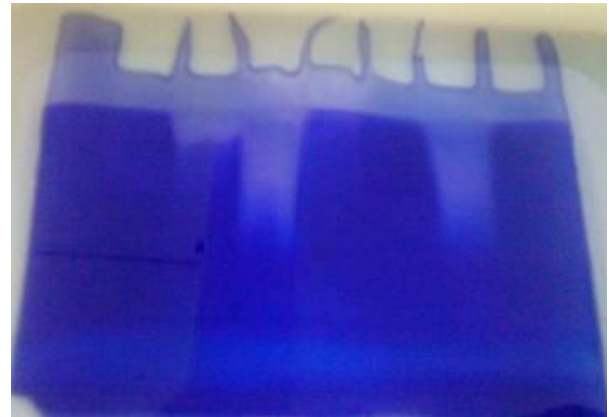
**SDS- PAGE**



**Fig 5.20.** SDS- Page of protease enzyme

**5.5.6. ZYMOGRAPHY**

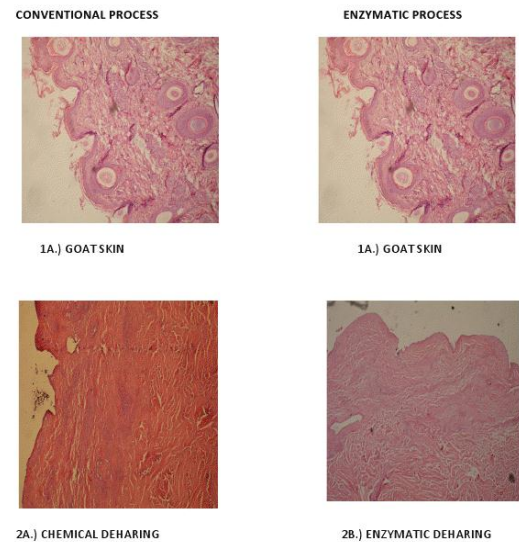
**Fig 5.21.** Zymography of protease enzyme



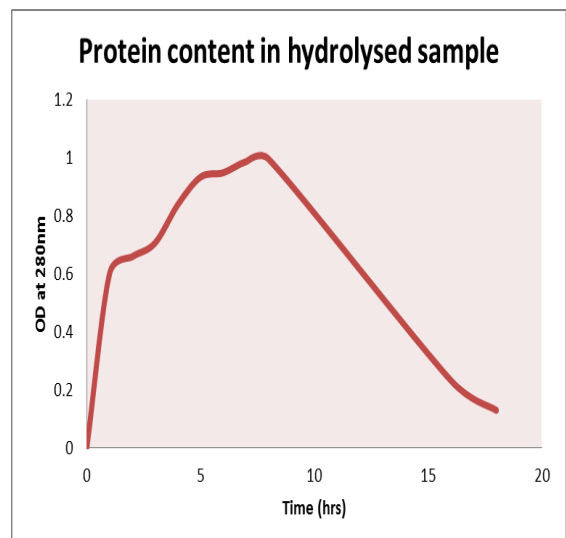
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**PLICATION STUDIES**

**HISTOLOGICAL CONFIRMATION OF UNHAIRING**



**5.6.4 Protein content in hydrolysed sample**



**PERCENTAGE RECOVERY OF HYDROLYSED PRODUCTS**

HYDROLYSED PRODUCTS	Batch I (%)	Batch II (%)
Protein	3	6.6
Fat	3.5	5.06
SALT CaSO <sub>4</sub> Ca(OH) <sub>2</sub>	8	11.6
	4.25	6.348
Water	81.25	76.67

**CONCLUSION:**

The Bacteria- derived proteases were considered as major industrial workhorses because of their high production capacities and activities. The Organism isolated was found to be fermentative bacteria. Protease production by this bacterial species was shown to be affected by various environmental and nutritional conditions. The Organism produced an extracellular alkaline protease with optimum pH 9.0 and optimum Temperature around 30°C. Based on the optimization studies fructose and skim milk was the most effective Carbon and Nitrogen sources for protease production. The crude enzyme seemed to contain three enzymatically active proteins in the molecular weight range of 66 to 205 KDa. This was confirmed by Zymogram. The crude enzyme exhibited dehairing activity on goat skin without chemical assistance and without hydrolyzing fibrous proteins. Due to these properties, the enzyme could be potentially useful in leather industry for dehairing of goat skin without damaging collagen layer resulting in a better quality production and avoiding the pollution problem associated with the use of chemicals. The Enzyme was also studied for the hydrolysis of solid waste.

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