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**Research Article**

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# Production of extracellular *protease* using bacillus species from the red soil and optimization of *protease* activity

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**ABSTRACT**

*Enzymes plays a major role in industries like textile, leather, sugar, paper, rubber, tea, etc., microbes plays a major role in enzyme production due to their fast growth rate, easy to manipulate for getting highly stable enzymes through genetic engineering and requires shorter time for production and purification steps.in our present study, we concentrated on the enzymes used in leather industries. Recently many novel enzymes that prove to be efficient in the process of dehairing, soaking, bating and solubilizing the chrome shaving have been developed for leather industries. hence, we tried to isolate the proteolytic bacteria from red soil and produced the enzyme protease using specific media. The isolated bacterial species was identified as bacillus species by morphological and biochemical analysis. Further the protease activity was analyzed and the parameters like pH, temperature, substrate concentration and incubation time were also optimized.*

**Key words:** *Bacillus species*, Incubation time, $P^H$, *Protease*, Substrate concentration and Temperature.

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## INTRODUCTION

Enzymes are proteins that have catalytic functions indispensable to maintain the activity of life. All chemical reactions occurring in a living organism is dependent on the catalytic action of enzymes and this is why enzymes whose action are well known.

**Role of enzymes in industries**

In recent years, investigations to search for biocatalysts that can cope with the conditions of industrial process have been continuously increasing. Organic chemical synthesis is characterized by high precision and purity of products, but it may sometimes impose a hazardous effect on the environment. Enzymes, though act as biocatalysts, on the other hand, often have better chemical precision, which can lead to a more efficient production of single stereoisomer, decreasing the number of secondary reactions, and with a lower environmental impact. The majority of important industrial processes are conducted under specific parameters, pH, ionic strength, temperature etc., but some of these enzymes cannot exhibit all the times their optimal activities.

**Enzymes in leather processing**

The emphasis on the use of enzyme has come about because of the unique properties of the enzyme. The most important properties are the catalysis of chemical reaction at high rate under mild environmental conditions of pH, temperature and pressure, specificity of reaction, minimal side reaction, simple operations, non-toxic nature and non-polluting effluent generation. The leather industry in world over is coming under consideration from

environment regulations to with the pollution and discharge legislation. The current activity in the area of leather processing is shifting towards the design and utilization of cleaner and softer technology like enzymatically enhanced processes. The enzyme are successfully employed for the better quality leather production with less pollution impact and also for the treatment of waste discharged from the industry.

**Leather processing**

Leather processing operation is categorized into three main categories i.e.,

- Pretanning
- Tanning
- Post tanning

Prior to Pretanning, the raw animal skin and hides are preserved by application of salt that restrains microbial attack[1]. Soaking, liming, deliming, bating, degreasing and pickling are the pre-tanning process. The main objective of soaking is to rehydrate the skin and also to open up the contracted fiber structure of the skin. Similarly the main objective of liming is to remove lime, bating is to split the fiber into fibril, degreasing is to remove the fat and pickling is to reduce the pH of the skin from 8 to 2.8-5.0.Tanning renders permanent stability to the skin /hide [2]. The post-tanning operation includes retanning, dyeing and fat liquoring.

**Depilation / dehairing**

Depilation stage is where the root of the hair is to be attacked by a selective proteolytic action. The mechanism of enzymatic dehairing is based on the capacity to break the number of peptide bonds that surrounds the basement membrane of the hair bulb that constitutes mainly of proteoglycans such as Glycosaminoglycans and Dermatansulphate, thereby, removing hair very safely.

Enzymatic dehairing with alkali pre-treatment is effective in depilation of skin. This includes protease with a narrower range of specificities may be sufficient to induce depilation. Cleavage of Proteoglycans and protein denaturation in strongly alkaline conditions would result in the exposure of more peptide bonds, facilitating proteases with narrow ranges of specificities to disrupt the integrity of proteins [3].

**Enzyme application in leather processing**

**Table 3-Enzymatic function and its involvement at different leather processing stages**

| Stages | Enzymes Involved | Function Of Enzymes |
|---|---|---|
| Curing | Enzyme are directly not involved | To preserve hides and skins |
| Soaking | Alkaline & pancreatic proteases | To remove non fibrillar proteins |
| Dehairing | Alkaline & neutral proteases | To improve the waste water quality |
| Degreasing | Lipases & proteases | To remove fats |
| **Stages** | **Enzymes Involved** | **Function Of Enzymes** |
| Bating | Trypsin & alkaline proteases | To makes soft, supple and pliable |
| Tanning | Enzyme are directly not involved | To influence the quality of tanning |
| Waste processing | Trypsin &proteolytic enzymes | Chrome-tanned-waste processing |

**Proteases**

Proteases are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields. Proteases are also known as peptidyl–peptide hydrolases and are industrially useful enzymes which catalyse the hydrolysis of peptide bond from protein molecule.

They are commercially important and isolated from various living sources such as plants, animals, bacteria and Fungi. With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry.

To meet the current largely increase demand, studies on cost effective production of industrially important enzymes have become the need of today. Proteases from microbial sources are preferred over the enzymes from plant or animal sources since they possess all most all the characteristics desired for their biotechnological applications.

## EXPERIMENTAL SECTION

**Sample collection**
The Red soil was collected from the locality of Avoor, Trichy. It was further used for isolation of proteolytic bacteria mainly Bacillus species.

**Isolation and screening of proteolytic bacteria**
1g of the collected red soil was taken in 100ml of sterile water and agitated for 45 minutes. From this 0.2ml of sample was spreaded on nutrient agar plates. Plates were incubated at 37°C for 2 days. From colonies enriched sample was placed on nutrient agar medium containing 0.4% gelatin. Plates were incubated at 37°C for 24 hours. The specialized medium with gelatin will facilitate the growth of proteolytic bacteria. After 24 hours of incubation the plates were flooded with 0.1% tannic acid. Based on the size of zone of clearance colonies were selected for the production of protease enzyme.

**Identification of *bacillus species***
The selected colonies were analysed by gram staining method and by biochemical tests for the identification of the bacteria at species level.

**Gram Staining Method**
A smear was prepared by taking and spreading loop full of organism in a slide and it was heat fixed using a sprit lamp. Then the smear was flooded with Crystal Violet solution and allowed to stand for 1 minute. The slide was rinsed with tap water and then the smear was flooded with Gram's Iodine solution for 1 minute. The rinsed with tap water. Then drain the water droplets completely. Then the smear was added with Decolorizer (95% Ethanol or Acetone) for 5- 10 sec. Then it was washed with water. At last counter staining was done using Safranin for45 sec. The slide was washed completely with water and air dried. Then it was analysed using a microscope for the identification of the morphology of the organism.

**Biochemical Tests**
**Methyl Red – Voges Proskauer Test**
The bacteria was inoculated in glucose phosphate broth and incubated at 37ºC for 48 hr.

**For Methyl Red Test**
After incubation the pH of the medium was tested by the addition of 5 drops of methyl red reagent (+ve red colour - ve yellow colour).

**For VP Test**
After incubation 0.6ml of Barrit's Reagent A was added to the broth and shaken. 0.2ml of Barrit's Reagent B was added to the broth and shaken. Then the tube was allowed to stand for 15 minutes. Then the results were observed. (+ve ,- Red)

**Casein Hydrolysis Test**
The selected bacterial colony was inoculated in casein agar plates and incubated at 37° C for 24 hrs and observed for the results.
***Catalase* Test**
A single colony of the bacteria was picked and placed over clean glass slide. One drop of 3% Hydrogen peroxide was added over the colony. The colonies were not allowed to mix with each other. The results were observed.

**Estimation of protein**
The amount of protein present in the partially purified *protease* extract was estimated spectrophotometrically by taking OD at 280nm in UV – Visible Spectrophotometer.

**Assay for protease activity**
The water bath was set to 37°C. For each sample, three test tubes were kept and marked as Test1 (T1), Test2 (T2) & Test Blank (TB). 1ml of casein substrate was added to all the test tubes. All these test tubes were pre-warmed in the water bath for 2 minutes. 1ml ofcrude enzyme extract was added to T1 & T2 tubes alone at suitable time interval and mixed well. After exactly 20 minutes at the same interval as that recorded for the inoculation, 3ml of 5% tri-

chloro acetic acid was added to all the test tubes and mixed well. After taking the tubes from the water bath 1ml of diluted enzyme was added to the blank. Then the reaction mixtures were centrifuged separately at 10, 000rpm for 5 minutes to precipitate all the unreacted protein. The supernatant was further used to analyse the amount of tyrosine released by Folin et al method colorimetically.

**Folin's method**

Various concentrations of standard test tubes were maintained, ranging from 5µg to 45µg of tyrosine as per mentioned in the table 1 for tyrosine standard preparations. For standard blank (SB) test tube with 1 ml of distilled water was maintained. Similarly 1ml from each of the supernatant of T1, T2 & TB was also maintained. All the tubes were added with 2ml of 0.5N Sodium hydroxide and 0.6ml of Folin's reagent. Then optical density was read at 580nm colorimetrically.

**Assay for *protease* activity with various pH**

The above said assay was repeated by using Borate buffers of various pH 7, 8, 10, 11. The other parameters maintained were Temperature - 37°C, Substrate concentration – 2%, Incubation Time – 20 minutes.

**Assay for *protease* activity with various temperature**

The 4 more reaction sets were prepared and maintained in water bath for varying temperatures 33°C, 35°C, 39°C, 41°C for 20 minutes. The other parameters maintained were pH- 9, Substrate concentration – 2%, Incubation Time – 20 minutes.

**Assay for *protease* activity with various substrate concentration**

Various concentrations of substrate casein ranging from 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% were prepared and assayed as per the above said procedure. The other parameters maintained were Temperature - 37°C, Incubation Time – 20 minutes, pH - 9.

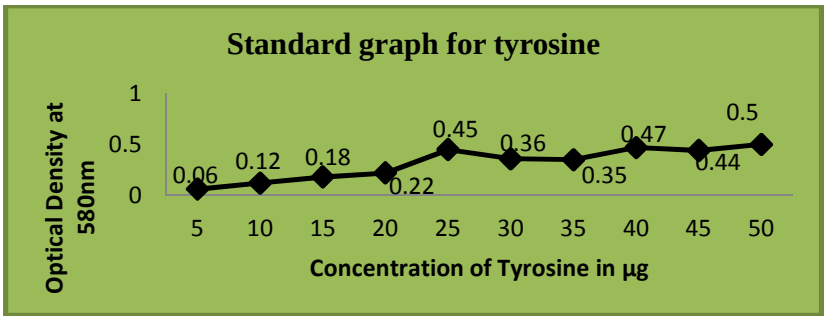
**Assay for *protease* activity with various incubation period**

In the similar way 6 more reaction sets were maintained and each set is maintained in water bath for different incubation periods of 5, 10, 15, 20, 25, 30 minutes. The other parameters maintained were Temperature - 37°C, substrate concentration – 2%, pH - 9.

## RESULTS

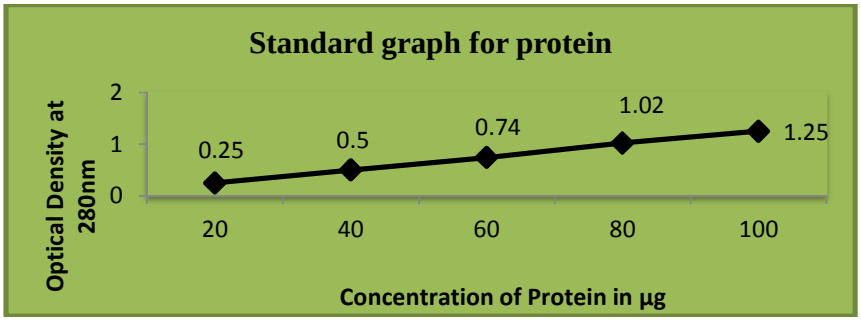
The isolated colonies from the Nutrient Agar plates are further streaked on gelatin Agar plates for screening of proteolytic bacteria. These colonies are used for further analysis.

The graph 1 is the standard graph for estimation of tyrosine concentration. From this graph, the standard OD (0.12) and standard concentration (10µg) for the calculation of amount of tyrosine released has been taken.




**Graph 1**

The graph 2 is the standard graph for the estimation of protein concentration. From this graph, the amount of protein present in 1ml of the crude enzyme extract has been derived as 0.5mg (Test OD – 1.25) and this is used in the specific activity calculation.



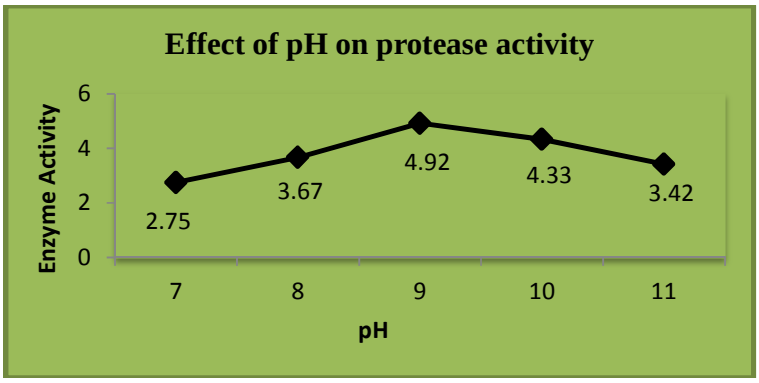

**Graph 2**

The graph 3 shows the effect of pH (7 to 11) on the activity of protease. As increasing the pH, the activity is also increasing. But after reaching a particular pH, the activity starts decreasing. Thus the optimum pH for getting maximum enzyme activity is found to be 9.

**Effect of pH on the activity of *protease***

**Table 5 - Effect of pH on *protease* activity**

| S. No. | pH of the borate buffer | OD at 580nm | Amount of Tyrosine released in µg | Specific activity of the enzyme in units (µg of tyrosine produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 7 | 0.33 | 27.50 | 2.75 |
| 2 | 8 | 0.44 | 36.67 | 3.67 |
| 3 | 9 | 0.59 | 49.17 | 4.92 |
| 4 | 10 | 0.52 | 43.33 | 4.33 |
| 5 | 11 | 0.41 | 34.17 | 3.42 |



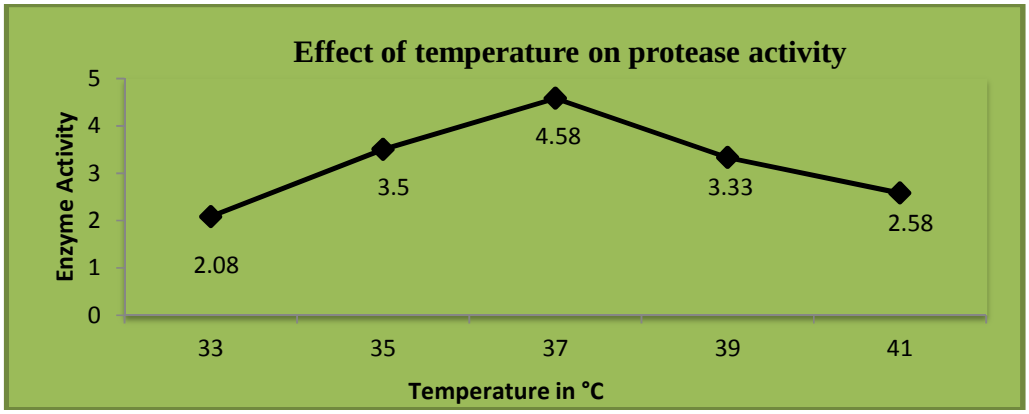

**Graph 3**

The graph-4 shows the effect of Temperature (33 to 41°C) on the activity of protease. As increasing the temperature, the activity is also increasing. But after reaching a particular temperature, the activity starts decreasing. Thus the optimum temperature for getting maximum enzyme activity is found to be 37°C.

**Effect of Temperature on the activity of *Protease***

**Table 6 - Effect of Temperature on protease activity**

| S. No. | Incubation Temperature in (°C) | OD at 580nm | Amount of Tyrosine released in µg | Specific activity of the enzyme in units (µg of tyrosine produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 33 | 0.25 | 20.83 | 2.08 |
| 2 | 35 | 0.42 | 35.00 | 3.50 |
| 3 | 37 | 0.55 | 45.83 | 4.58 |
| 4 | 39 | 0.40 | 33.33 | 3.33 |
| 5 | 41 | 0.31 | 25.83 | 2.58 |



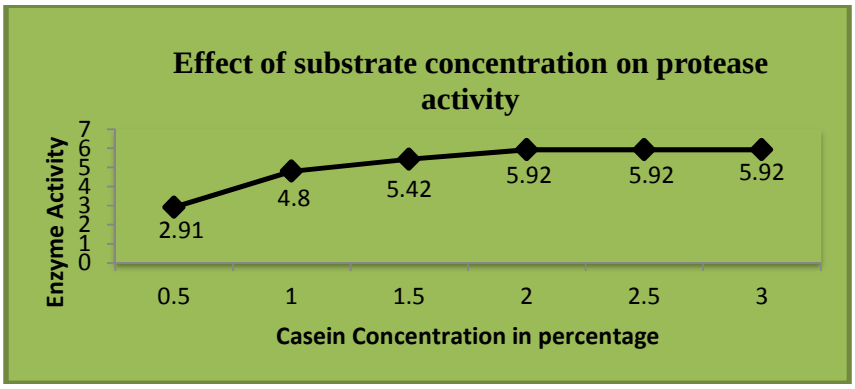

**Graph 4**

The graph 5 shows the effect of casein concentration (0.5 to 3 %) on the activity of protease. As increasing the concentration of casein, the activity is also increasing. After reaching a particular concentration, it does not change even though increasing the casein concentration. Thus the optimum concentration of Casein for getting maximum enzyme activity is found to be 2%.

**Effect of Substrate Concentration on *Protease* Activity**

**Table 5 - Effect of substrate concentration on *protease* activity**

| S. No. | Concentration of Casein (Substrate) in % | OD at 580nm | Amount of Tyrosine released in µg | Specific activity of the enzyme in units (µg of tyrosine produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 0.5 | 0.35 | 29.16 | 2.91 units |
| 2 | 1.0 | 0.48 | 48.00 | 4.80 units |
| 3 | 1.5 | 0.65 | 54.16 | 5.42 units |
| 4 | 2.0 | 0.71 | 59.16 | 5.92 units |
| 5 | 2.5 | 0.71 | 59.16 | 5.92 units |
| 6 | 3.0 | 0.71 | 59.16 | 5.92 units |



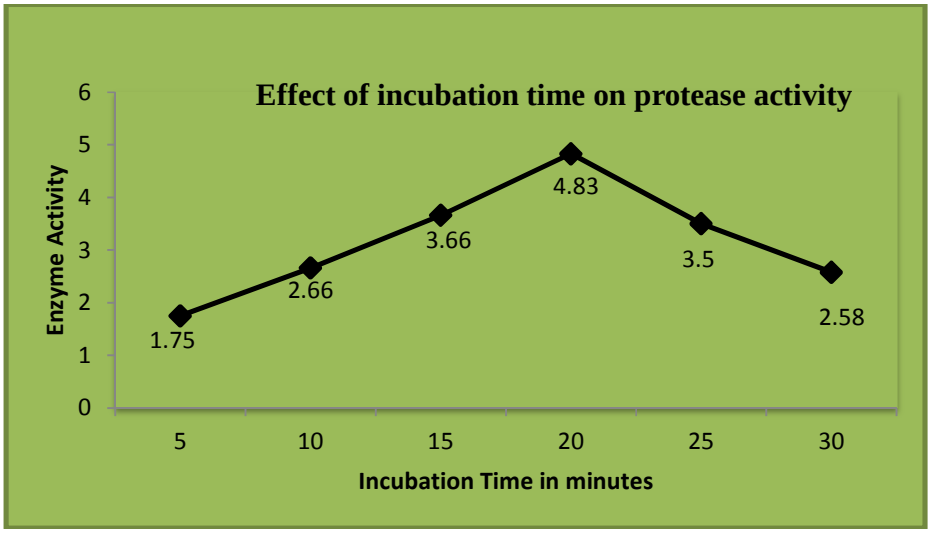

**Graph 5**

The graph 6 shows the effect of Incubation Time (5 to 30 minutes) on the activity of protease. As increasing the incubation time, the activity is also increases. But after reaching a particular time period, the activity starts decreasing. Thus the optimum Incubation Time for getting maximum enzyme activity is found to be 20 minutes.

**Effect of Incubation Period on the activity of *Protease***

**Table 6 - Effect of Incubation Time on *protease* activity**

| S. No. | Incubation Period (in minutes) | OD at 580nm | Amount of Tyrosine released in µg | Specific activity of the enzyme in units (µg of tyrosine produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 5 | 0.21 | 17.50 | 1.75 units |
| 2 | 10 | 0.32 | 26.60 | 2.66 units |
| 3 | 15 | 0.44 | 36.60 | 3.66 units |
| 4 | 20 | 0.58 | 48.30 | 4.83 units |
| 5 | 25 | 0.42 | 35.00 | 3.50 units |
| 6 | 30 | 0.31 | 25.83 | 2.58 units |




**Graph 6**

## DISCUSSION

Advancements in the various areas of biotechnology have overcome most of the hurdles and currently, the place of development of enzyme technology is quite rapid. The advancements in the techniques of genetic engineering which permit the manipulation of cellular DNA, have led to the opening up of a new field called protein engineering. The structurally altered enzyme thus obtained has different physico chemical properties which distinguishes it from its normal cellular component. The physico chemical differences engineered into the enzyme would, of course, depend on the requirements of the relevant industry.

It has also been possible to increase manifold production of microbial enzyme by inserting extra copies of the gene responsible for producing the enzymes. Capability has now been developed to make use of microbes to express important enzymes of animal and plant origin.

Leather industries have enormous potential for the wide range of applications of several industrial enzymes such as *protease* (alkaline, natural and acidic), lipases, amylases, pepsin, Trypsin, rennin and *glutaminase* etc.,[4].The parameters involved in the enzyme application are the parameters like enzyme and substrate concentration, pH and temperature. Microbial *proteases* have a number of commercial applications in industries like food, leather, meat processing and cheese making. It has been reported that the production of extra cellular proteases by different microorganisms can be strongly influenced by the culture conditions.

## CONCLUSION

The *protease* assay with varying substrate casein concentration, Incubation time, pH and temperature was also performed for optimization. It was found out that the optimum substrate concentration was 2%, optimum incubation time was 20 minutes, optimum pH was 9 and optimal temperature was 37°C.Proteolytic bacteria can be isolated

from red soil by plating on Gelatin Agar plates and sub culturing in specific protease production medium which belongs to *Bacillus species* and the produced extra cellular protease will have the optimum pH of 7, optimum Substrate concentration of 2%, optimum incubation time of 20 minutes and optimum temperature of 37°C.

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