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An inexpensive substrate for the production of alkaline protease by *Bacillus* sp and its application studies of *Manihot esculenta*

V. N. Kalpana, N. Sravani, T. Vigneshwari and V. Devi Rajeswari*

Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, India

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ABSTRACT

Protease executes a large variety of functions and it has many biotechnological applications. It represents as one of the third largest group of industrial enzyme and finds its applications in detergents, leather industry, pharmaceutical industry etc. Among the different vegetable waste products, peel of Manihot esculenta (Tapioca/ cassava) was used as substrates for the production of protease by Bacillus sp. The aim of our study is to utilize the waste of Tapioca peel as input for protease production using Bacillus sp. The protease produced by Bacillus sp from M. esculenta showed a pH of 10 and temperature of about 30°C. Protease produced was tested for possible industrial applications. This enzyme shows high capacity in removing the blood stain, dehairing the animal skin, digestion of natural proteins and bioprocessing of used X-ray film. The wash performance analysis of blood stains on cotton fabrics showed that blood stains were completely removed within 15 minutes of incubation of fabrics along with enzyme. Complete hair removal of goat skin by the protease achieved within 12 hours of incubation at 30°C. This enzyme has the ability to dissolve the blood clot and coagulated egg within 20 minutes of incubation. Enymatic hydrolysis of gelatin from waste X-ray films was also investigated. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean. Gelatin hydrolysis was monitored by measuring increase in turbidity. Gelatin layer was removed completely within 10 minutes of addition of enzyme. The protease produced exhibits high antibacterial activity against Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa. The present study was a humble attempt towards the production of protease and to check their efficiency in industrial applications. These properties indicate the possibilities of enzyme usage in various industries and it can be exploited commercially in future.

Key words: *Manihot esculenta*; Goat Skin; X- ray film; Gelatin Hydrolysis.

INTRODUCTION

Globally**,** 50 million tonnes of vegetable wastes are generated every year. The agro wastes are being dumped into the environment and causing foul odour and makes unhygiene to the residents [1]. Abundant amount of waste are produced mainly by vegetable and fruit peels, which pose a considerable disposal problems and ultimately leads to pollution [2]. There are many varieties of microorganisms which are exploited for the degradation of waste material. Thus, the waste is a valuable raw material and it can be converted into many useful products by enzyme biotechnology [3]. Enzymes are the biological substances that act as a catalyst and have the ability to carry out a large number of chemical reactions. Commercially available enzymes are not economically comparable against the naturally synthesized enzymes. Many Enzymes are commercially exploited in various industries. Proteases are one of the most important enzymes that hydrolyze proteins which accounts for nearly 60% of the total enzyme sale [4].

A proteolytic enzyme plays an important role in the metabolism of almost of all organisms such as plants, animals, fungi, bacteria and viruses. Due to their immense physiological importance and wide application, investigations about protease have become a central issue in enzymology [2]. Proteases generally have wide applications in various industrial segments such as laundry detergents, food industries, Protein hydrolyzates, leather industry, meat processing industry, cheese making industry, silver recovery from X - ray films, pharmaceutical industry, and Silk industry and even in the waste processing industry. New sources of protease have been explored by biotechnologists due to the increasing demand of proteases [5]. Microbes serve as a best source of enzyme and they share two-third of commercial protease production in the world. Many microbial sources are available for producing protease but only a few were recognized as commercial producers [6]. In general, proteases produced by microbes are extracellular in nature that breaks down proteins into amino acids [7]. A large proportion of the enzyme proteases are derived from *Bacillus* strains. Most commercial proteases mainly such as neutral and alkaline protease are produced by microorganisms belonging to the genus *Bacillus* includes *B. sterothermophilus*, *B.cereus*, *B.mojavensis*, *B.megaterium*, *B. subtilis* etc [6]. The aim of the present study is to evaluate the utilization of waste peel of *Manihot esculenta* (Tapioca/ Cassava) (Fig.1) for the production of protease by *Bacillus* sp. Cassava peels is one of the main food waste in developing nation. High number of harvest of cassava results in high waste of cassava peels. One kilogram of cassava results in 15% - 20% of peel which is considered as waste and thrown away [8]. By this virtue, the present study utilizes cassava peels to produce protease. The result of this study could be used as consideration that peels from *M. esculenta* could be used as substrate to fulfill the requirement of industries.

Fig.1: *Manihot esculenta*

MATERIALS AND METHODS

Substrate

The waste peel of *Manihot esculenta* were collected, washed, air dried and milled into powder. It was sieved to get fine powder (Fig.2)

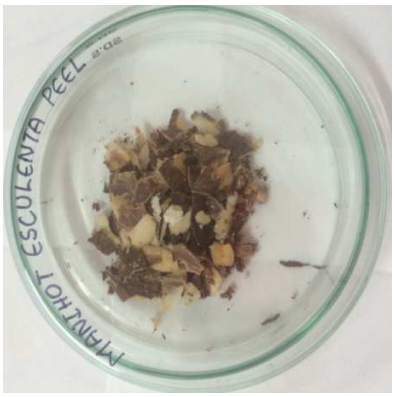


Fig.2: Substrate – Waste Peel of *Manihot esculenta*

Maintenance of Culture
The microorganism used in this study is *Bacillus* sp, which was previously isolated and maintained in our laboratory.

Screening for protease producers
Skim milk agar medium (Skim milk powder 0.1% and 0.2% agar) was used to detect the protease activity. The isolates were streaked on skim milk agar plates and the plates were incubated for 48 hours and examined for zone of clearance [9].

Inoculam preparation
The isolated bacterial culture was inoculated into fresh nutrient broth. Then, the culture was incubated at 37°C for 24 hours.

Cultural characterization
Microscopic, macroscopic, biochemical and physiological characteristics of the potent protease producer were determined by following these microbiological methods.

Microscopic Analysis
Grams Staining
A loop full of overnight culture was smeared on a glass slide. The heat fixed smear was stained with crystal violet for 1 minute. Rinse the slide with water. Add few drops of Gram's iodine and allow them to react for 60 seconds. The slide was again rinsed with water. Now, the slide was deocolourized with 95% ethanol for 30 seconds. Again the slide was restained with safranin for a minute. Rinsed with water. It was blot dried and examined under the light microscope [2].

Motility Determination
Vaseline was placed at the corner of cover glass. Loop full of the culture was placed at the centre of cover glass. A depression slide was pressed over the cover glass and quickly inverted. It is now observed under the microscope [2].

Biochemical Analysis
Indole production
The isolated organism was inoculated into peptone water broth. Then, it is incubated at 37^0C for 24 hours. After incubation, 0.5ml of Kovac's reagent was added and shaken gently to determine the positive or negative isolates [2].

Methyl red test
The organism was inoculated in glucose phosphate broth. Incubated at 37^0C for 24-48 hours. After incubation, the methyl red indicator was added to determine the positive or negative isolates [2].

Voges proskauer test
The organism was inoculated in 5ml glucose phosphate broth and it is incubated at 35^0C for 48 hrs. Then**,** 1ml of 40% potassium hydroxide containing 0.3% creatine and 3ml of 5% solution of alpha-napthol mixed in absolute alcohol was added to determine the positive and negative isolates [2].

Citrate utilization test
The colony was inoculated directly on Simmon's Citrate agar and incubated at 37^0C for 24- 48 hrs [10].

Urease production test
The isolates were inoculated onto Christensen's urease agar slants and incubated at 37 °C for 18-24 hours. When the colour of the medium changed to pink, it is considered as positive test result [10].

Triple sugar iron agar (TSI) test
The isolates were inoculated onto triple sugar iron agar test and incubated at 37 °C for 18-24 hours. When the colour of the medium changes to yellow or pink, it is considered to be positive [10]

Catalase
3% hydrogen per oxide were added onto the colonies. Vigorous formation of air bubbles indicates the presence of catalase enzyme [2]

Oxidase test
A drop of freshly prepared 1% solution of oxidase reagent (Tetramethyl – p – phenylenediamine) was added on a piece of filter paper. With a sterile loop a test colony was picked up and rubbed on the filter paper impregnated with oxidase reagent [2].

Physiological Analysis
Starch hydrolysis test
The isolated colonies were streaked on sterile starch agar plates and incubated for 48 hours at 37°C. After incubation, Iodine was added on to the culture plates to determine the starch hydrolysis activity [2].

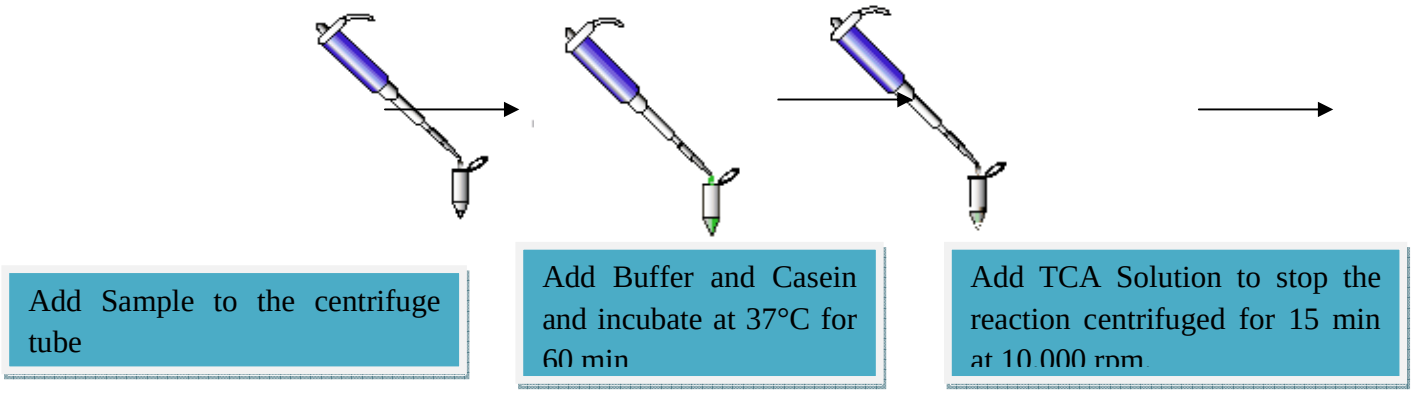
Casein hydrolysis test
Culture was inoculated and incubated for 24-48 hours at room temperature. The clear zone surrounding the microbial growth in casein milk powder indicates the protease activity [2]

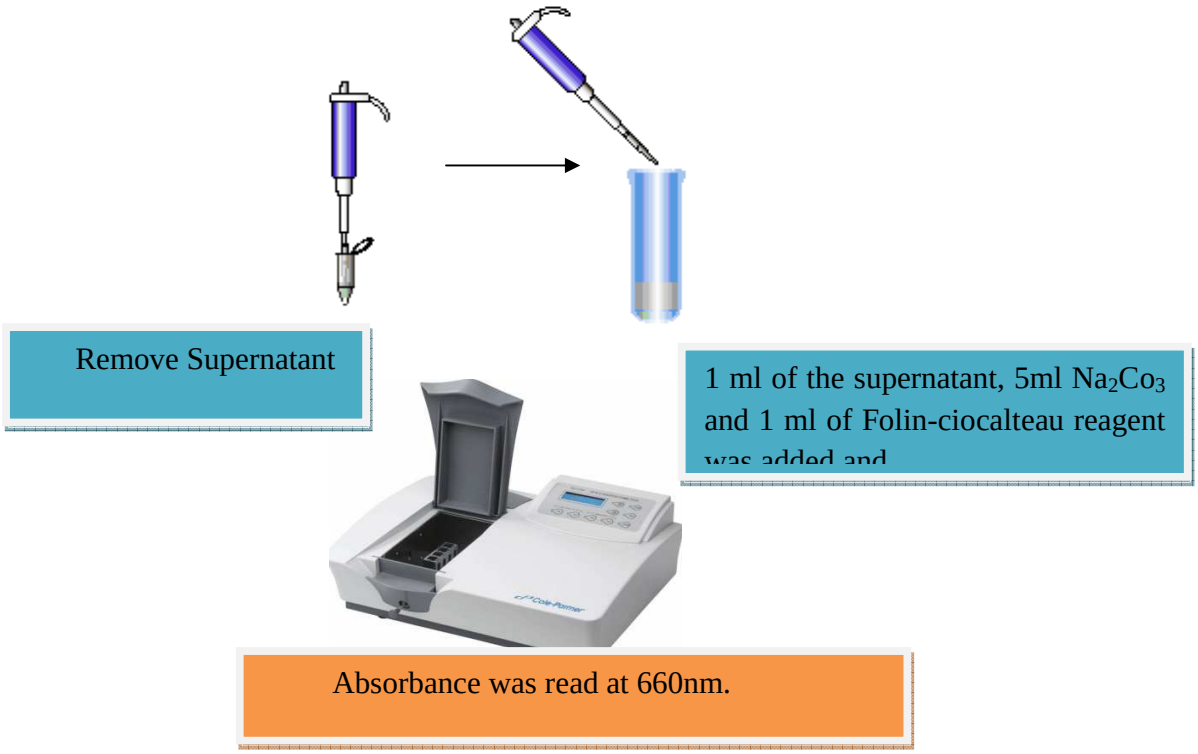
Gelatin hydrolysis
The isolates were inoculated into gelatin deep tubes by stab inoculation. It was then incubated at 37°C for about 48 hours. Then the tubes were placed in refrigerator at 4ºC for 30 minutes [2]

Enzyme Production
Production of protease from *Bacillus* sp was done by using the following medium: peptone – 0.75% (Wt/Vol), Glucose- 0.5% (Wt/Vol), Salt solution 5% [$MgSO_4$. $7H_20$ 0.5% (Wt/Vol), KH_2PO_4 0.5% (Wt/Vol) and $FeSO_4.7H_2O$ 0.01% (Wt/Vol)] [11]and supplemented with 10 grams of waste peel from *Manihot esculenta*. The pH of the medium was adjusted to 7.0 with 1N NaOH or 1N HCL. The flask was sterilized and cooled at room temperature and inoculated with *Bacillus* sp and incubated at 37°C with continuous shaking. Sampling was done every 24 hours of incubation and assayed for protease activity.

Protease Assay
Protease activity was assayed by the modified method of [12]. 1 ml of enzyme was added to 2 ml of 1% casein and the mixture was incubated for 15 min at 60°C. The reaction was terminated by adding 3ml of 10% Trichloroacetic acid and it is centrifuged for 15 min at 10,000 rpm. Then 1 ml of the filtrate was mixed with 5ml Na_2Co_3 and 1 ml of Folin-ciocalteau reagent was added and incubated at room temperature for 30 minutes and the absorbance was read at 660nm. Similarly, blank was carried out by replacing enzyme with distilled water. (Fig.3). One unit of enzyme activity was defined as the amount of enzyme that releases 1µg of tyrosine per ml per min under the assay conditions. Tyrosine was used as standard reference.

Fig.3: Protease Assay





Optimization of culture Parameters

Effect of Incubation Period

The effect of incubation period on protease was determined by incubating the production medium for different incubation periods viz 24, 48, 72, 96 and 120 hrs.

Effect of Temperature

The inoculated substrates were incubated at different temperatures viz 20, 25, 30, 35, 40, 45°C to find the effect of temperature on protease production.

Effect of pH

Different levels of pH 6, 7, 8, 9, 10, 11 were evaluated for protease production.

Application Studies

Removal of Blood Stain

The effectiveness of enzyme protease in removal of stain from the piece of cloth stained with human blood was determined by following the methods of [13, 14]. A piece of white cotton cloth was stained with human blood and allowed to dry at 80°C for 5 min in hot air oven. Detergent solution having 7 mg/ml was prepared and placed in water bath for 60 minutes at 100°C. Controls were washed only with distilled water. Test 1 was washed with only detergent solution. Test 2 was washed with only enzyme solution. Test 3 was washed by combining both enzyme and detergent solutions. After washing, the cloth was rinsed well with distilled water to remove excess of detergent / enzyme, and then it is dried and visualized.

Digestion of natural proteins

The enzyme was incubated with blood clot and the coagulated egg white in 20mM Tris-HCl (pH 8) at 37°C.Substrates were monitored at different time of incubation [14]**.**

Dehairing of skin

Two set of goat skin were first washed with distilled water and then with preservatives to remove dirt, germs and blood. One set serves as a control and placed in distilled water and the other serve as a test , which is incubated with

an protease enzyme along with 50mM Tris-HCl (pH 8). The skin was monitored for removal of hair at different incubation times [15]

Antimicrobial activity
The protease isolated was tested for the ability to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*. After incubation, the plates were observed for zone of clearance around the colonies [14].

Decomposition of Gelatin layer from Used X – Ray films
Used X ray films were washed with distilled water and it is wiped with cotton impregnated with ethanol. The washed film was dried in an hot air oven at 40°C for 30 minutes. X ray film was made into small pieces (i.e. 2X2 cm). 1 gram of X – ray film was then incubated at 40°C in a water bath with protease, in such a way that all the pieces were completely immersed with the enzyme. The progress of hydrolysis (i.e.) the turbidity was monitored by measuring the absorbance at 660 nm. Samples were removed at 5 minute interval and time required for complete removal of gelatin layer was observed. The hydrolysis time was defined as the time in which turbidity (OD_{660}) attained its maximum value. Visual examination of films after complete hydrolysis was also performed [16.]

Repeated utilization of enzyme protease for gelatin hydrolysis from waste X-ray film
Repeated utilization of enzyme was studied by changing the X ray films once every 30 minutes. Every 30 minutes change of X ray sheets was recorded as one run and in total, four runs were carried out. The weight of X ray films before and after each run was noted. The extent and time of decomposition was also noted [17].

RESULTS AND DISCUSSION

Bacteria are well known for their ability to excrete enzymes into the environment. *Bacillus* sp being industrially important organisms, they produces a wide variety of extracellular enzymes including proteases [18]. The extracellular proteases are of commercial value and finds its applications in various sectors [6].

Screening for protease producers
The proteolytic activities of *Bacillus* strain were assayed using skim milk agar. The results revealed that the isolates were capable of producing a clear zone around the colony growth. (Fig.**4**).

Fig.4: Screening for protease producers

Cultural characterization
Microscopic, macroscopic, biochemical and physiological characteristics of the potent protease producer were determined and the results were tabulated (Table.**1**)

Table 1: Cultural characterization of Bacillus sp

S.No	**Macroscopic Analysis**	
1.	Colony Morphology	Filamentous bacteria
2.	Colour	White
Microscopic Analysis		
3.	Gram Stain	Gram Positive Rods
4.	Motility	Motile
Biochemical Analysis		
5.	Indole Production	Negative
6.	Methyl Red	Negative
7.	Voges Proskuer	Positive
8.	Citrate	Positive
9.	Urease	Negative
10.	TSI	Alkaline slant/ Acid Butt
11.	Catalase	Negative
12.	Oxidase	Positive
Physiological Analysis		
13.	Starch hydrolysis	Positive
14.	Casein hydrolysis	Positive
15.	Gelatin Hydrolysis	Positive

Protease Assay

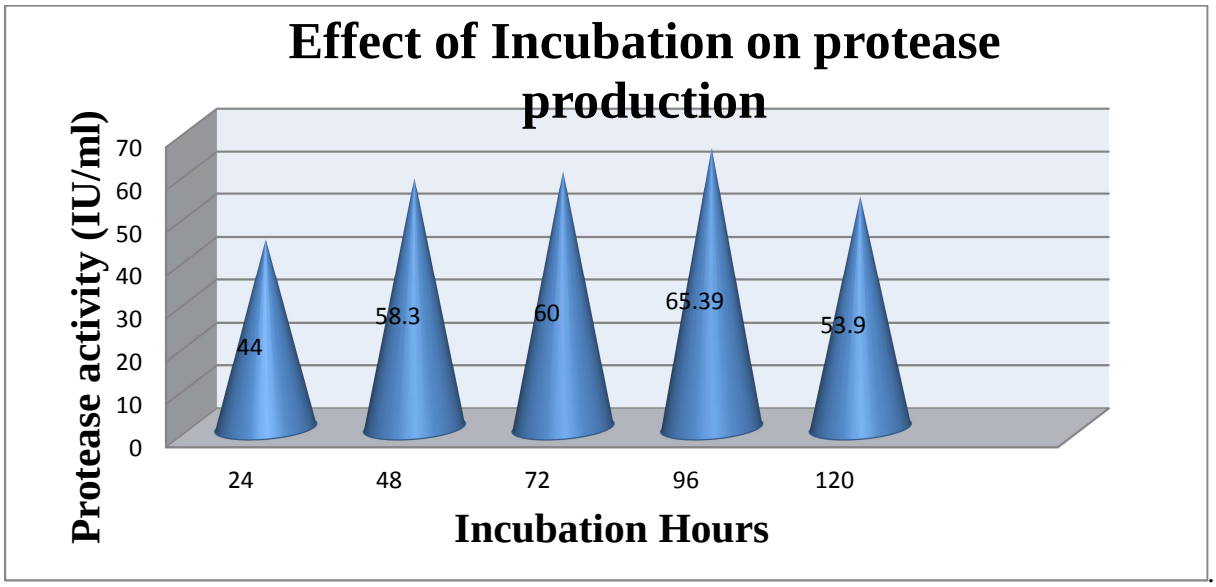
Enzyme production by microorganisms is greatly influenced by different media components, especially carbon and nitrogen sources and different physical factors such as Temperature, pH and incubation time. It is also important to produce the enzyme in an inexpensive medium on a large scale for the process to be commercially viable [19]. Hence, the studies on the influence of various parameters such as Temperature, pH and incubation period was carried out.

Effect of culture parameters

Effect of Incubation Period

The fermentation medium was inoculated with *Bacillus* sp and incubated for various time intervals (24, 48, 72, 96,120 hours). The enzyme production was gradually increased at 96 hours of incubation and decreases down as it prolongs. This is because the growth of microorganism is significantly affected. At the end of 96 hours, the substrate delivered maximum enzyme activity. The highest of 65.39 IU/ml was estimated which is shown in Fig.5.

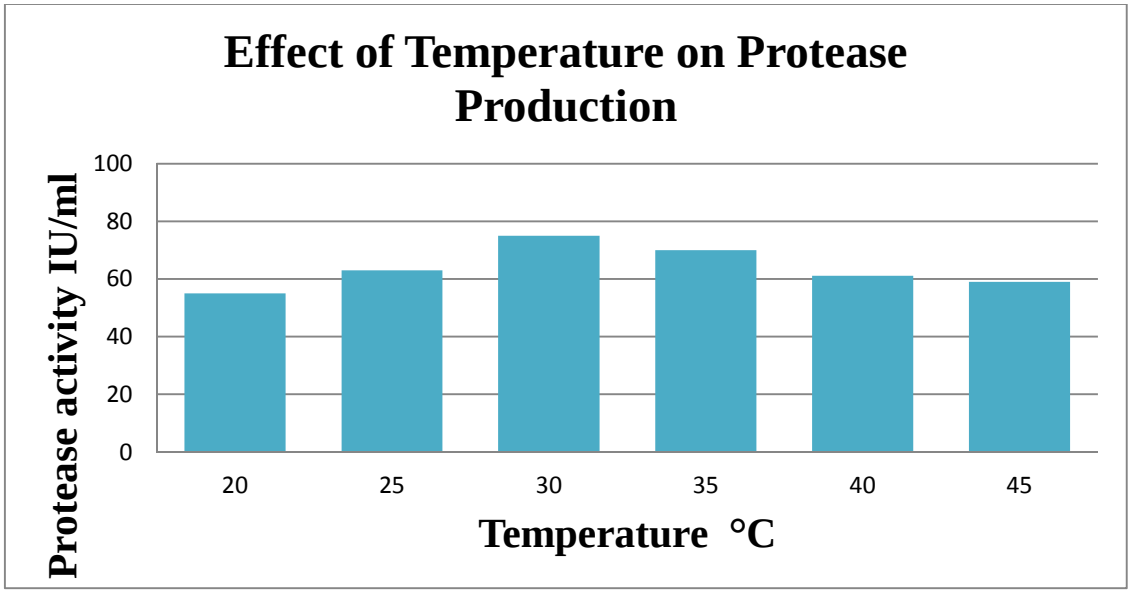
Fig.5



Effect of temperature

The maximum production of enzyme was obtained at 30°C (75 IU/ml) which is shown in Fig.6. The production of enzyme falls as the temperature increases. Therefore the optimum incubation temperature for the production of protease was found to be 30°C.

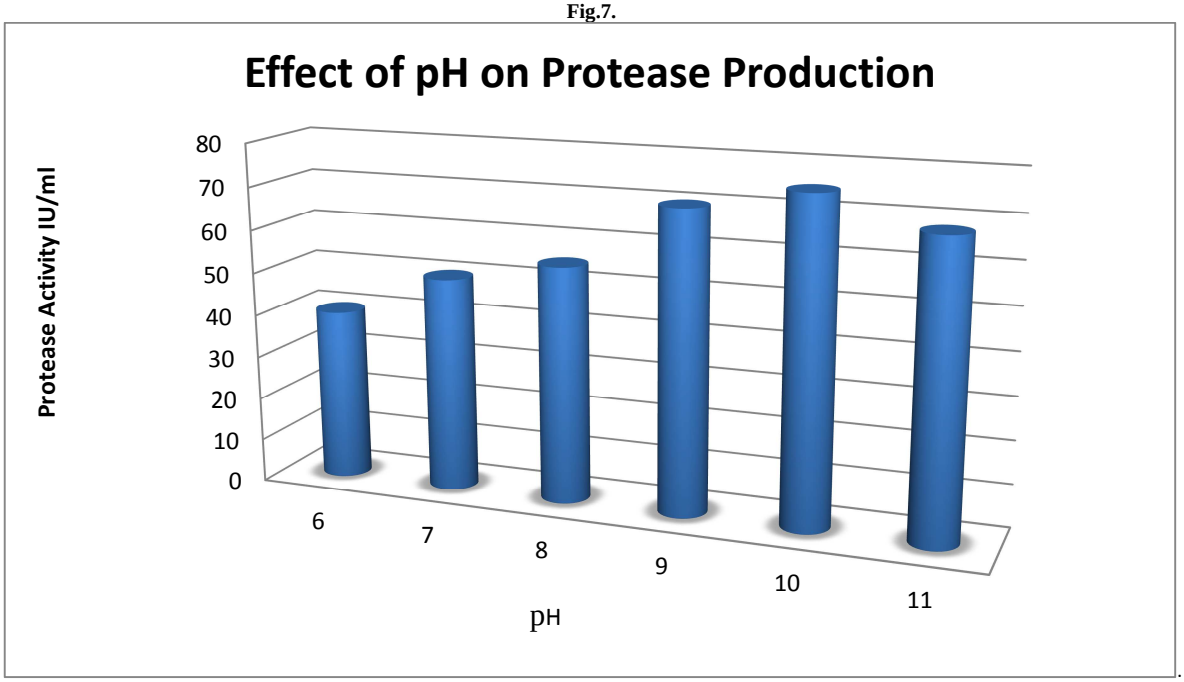
Fig.6



Effect of pH

Production of the enzyme from *Bacillus* sp greatly depends on pH of the production medium. Therefore the effect of pH (6.0 to 11.0) was studied for the production of protease. Maximum enzyme production was observed at the pH 10 i.e. 75 IU/ml. which is represented in Fig.7.

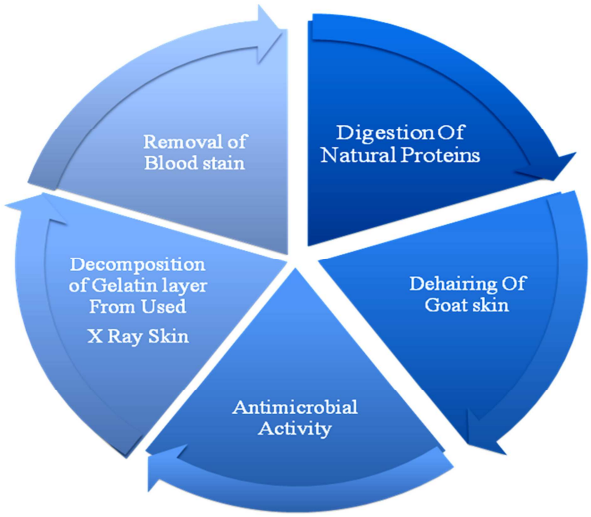
Fig.7.



Application Studies

Proteases produced have been applied for number of purposes which is shown in Fig.8

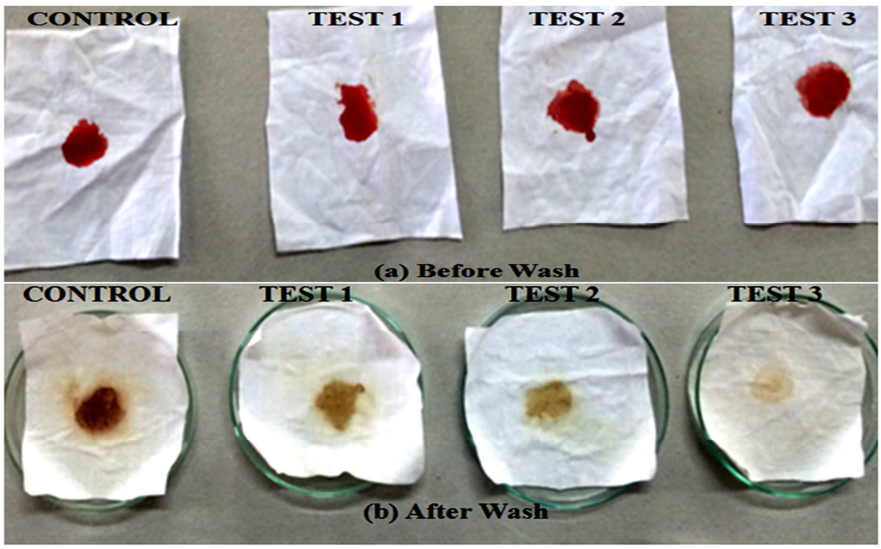
Fig.8: Application Studies of Protease



Removal of Blood Stain

The ability of proteases in removing blood stains can be measured performing wash test [20]. Anwar and Saleemuddin [21] reported the effectiveness of protease on removal of blood stain from cloth in the presence and absence of detergents. It was noticed that Protease produced from waste peel of *Manihot esculenta* has the capacity of removing blood stains from fabric cloth (Fig.**9**) which indicates its potential in detergent industries. Similar studies were performed by various authors**.** Hema [9] studied the blood stain removal by *Bacillus clausii* sm3. Devi [14] reported the blood stain removal in fabrics from a newly isolated *Virgibacillus dokdonensis* VIT P14.

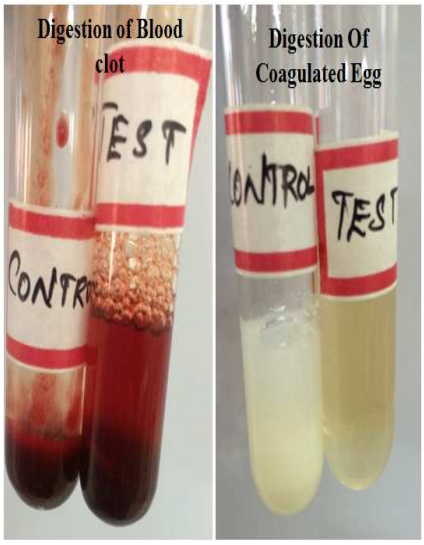
Fig.9: Removal of Blood Stain



Digestion of natural proteins

The blood clot and the coagulated egg white was incubated with protease and it is observed for digestion of natural proteins [14] . The insoluble forms are converted to soluble form within 20 minutes of incubation. (Fig.**10**)

Fig.10: Digestion of natural proteins



Dehairing of skin

Proteases play a major role in leather processing starting from soaking of hides to finished products [21, 22]. Protease produced in this study revealed the dehairing activity. Incubation of the protease with goat skin for dehairing showed complete dehairing occurs within 12 hours of incubation [23]. Studies of Sivasubramanian [24] stated that there is not much published literature available for concerning enzymatic dehairing process. Thus these results suggest that this enzyme can be a better option for dehairing applications. (Fig.**11**)

Fig.11: Dehairing of skin

Determination of Antimicrobial activity

The protease produced had tested for its ability to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Among the tested bacterial isolates, the enzyme showed maximum inhibition against *E.coli* followed by *S.aureus* and *P. aeruginosa.*

The zones of partially purified protease are presented in Table.**2**. This result suggests that the enzyme have the potential against several bacteria species and possess various applications [14]

Table 2 Determination of Antimicrobial activity

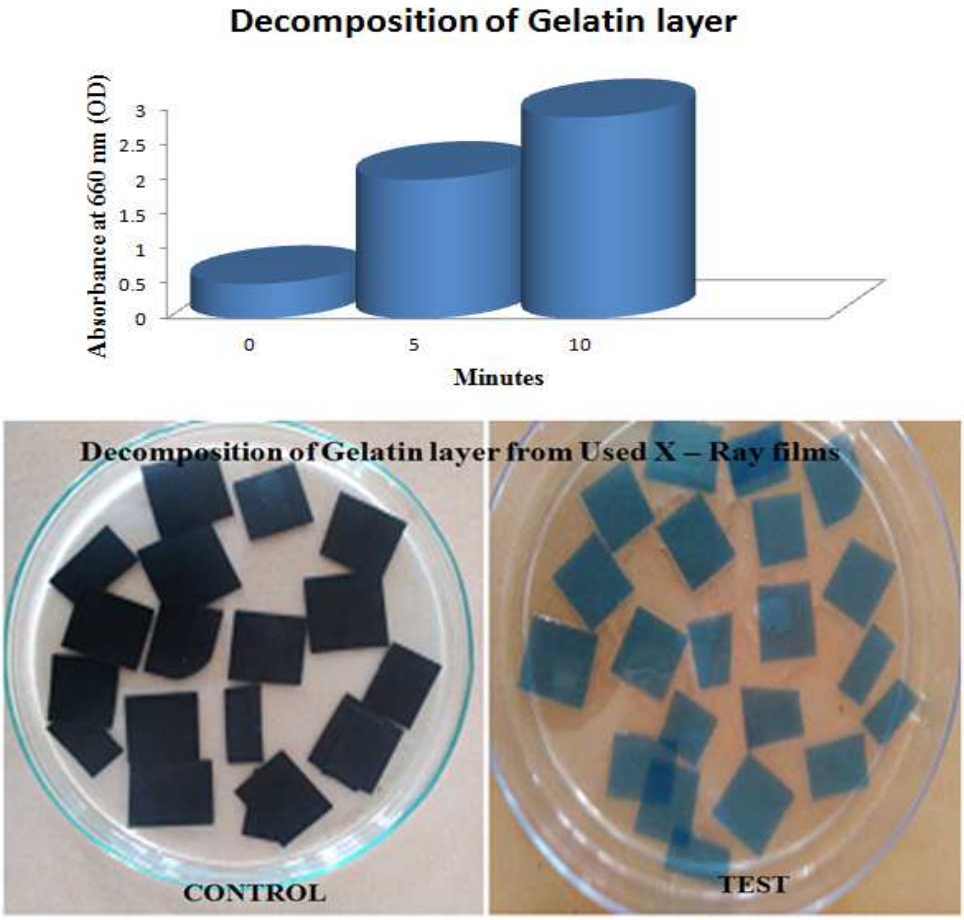
Microorganism	Zone of inhibition (mm)
Escherichia coli	14
Staphylococcus aureus	13
Pseudomonas aeruginosa	9

Decomposition of Gelatin layer from Used X – Ray films

Visual examination of the films clearly revealed that protease from *Bacillus* sp has the ability of protease to hydrolyse the gelatin in X-ray films leaving behind a clear polyester sheet. (Fig.**12**).

Nakiboglu [25] reported that the protease from *Bacillus subtilis* ATCC 6633 takes less than 15 min to decompose the gelatin layers at 50˚C. Masui [26] reported that the time required for complete gelatin hydrolysis from X-ray film by *Bacillus* B21-2 protease (at the enzyme to film ration of 5.6 x 10-7g cm-3) to be temperature dependent and was between 8-10 min at 50˚C**,** pH 10.5. Singh [27] reported complete gelatin degradation in 24 min by 10 U ml-1 of alkaline protease.

Fig.12: Decomposition of Gelatin layer from Used X – Ray films



Repeated utilization of enzyme protease for gelatin hydrolysis from waste X-ray film

The ability of the crude enzyme to retain its activity for repeated use makes it possible for industrial application. Repeated utilization of enzyme was studied by changing the X-ray films once every 30 minutes. Each 30 minutes change of X-ray sheets was recorded as one run and in total 5 runs were carried out. The first three cycle showed

complete decomposition of gelatin layer and the fourth cycle required 40 minutes for complete hydrolysis and the fifth cycle required more than 1 hour. (Table **3**)

Table 3: Repeated utilization of protease for gelatin hydrolysis from waste X-ray film

Enzyme	Time required for complete gelatin removal (min)				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Protease	20 Minutes	20 Minutes	30 Minutes	40 Minutes	More than 1 hour

CONCLUSION

From the results, it is suggested that the waste peel of *Manihot esculenta* was more useful for the production of protease enzyme by solid state fermentation. Nowadays, natural mineral resources are getting depleted hence reuse and recycling, remains the most feasible option to slow down this exhaustion as well as the environment pollution. This study reported that protease produced by *Bacillus* sp using peel of *Manihot esculenta* has the capacity of removing blood stains, dehairing the animal skin, digestion of natural proteins and bioprocessing of used X-ray film. Thus, it can be concluded from the present study that the production of protease can be made cost effective by using agro residues.

Further work is recommended to purify and characterize the protease and study the effect of this enzyme on other industrial applications. Hence this investigation may be used for commercial production of economically valuable proteases by utilizing agro-industrial wastes in near future.

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