

Research Article

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Corresponding Author:

*** Hemalatha N**

Applied Microbiology Laboratory,
Department of Microbiology, School of
Biosciences, Periyar University,
Periyar Palkalai Nagar, Salem – 636
011,
Tamil Nadu, India.



e-mail: siddhanabi11@gmail.com

Characterization and Optimization of Thermostable Alkaline Protease Producing *Pseudomonas aeruginosa* from Tannery effluent

Siddharthan N, Rajaprabu M, Janani P R, Poongothai E,
Hemalatha N*

ABSTRACT

Proteases are being an industrial candidate, which are widely used in bakery, beverages and detergent industry. The leather industry deals with proteinous skin material for the conversion of leather and this generates huge amount of solid and liquid wastes giving rise to pollution that need to be overcome by introducing sustainable cleaner technologies. This study is a search for potential protease producing strain from tannery industry effluent. Different isolates were screened for the ability to produce protease. Then the potential organisms were tested using various biochemical tests, which lead to their identification as *Pseudomonas aeruginosa*. The effects of various environmental factors on the production of protease were studied. The organism showed the maximum growth and enzyme production at pH 8.0 and temperature at 40°C. The protease enzyme was purified using ammonium sulphate (70%) precipitation. Further purification was done by membrane dialysis. The crude and partially purified protease was preliminary characterized and used for dehairing process and destaining process at lab scale level in which the dialyzed fraction showed comparatively significant effect. The protease obtained from these strains showed good stability and activity in pH ranges of 8 and at temperature of 40°C suggesting the possibility of using the enzyme in leather, detergent, food tenderization and many such industries.

Key-words: Protease, Tannery, *Pseudomonas aeruginosa*, Leather, Detergent, Dehairing, Destaining and Biological liquification.

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INTRODUCTION

Enzymes are considered as nature's catalysts (Fariha *et al.*, 2006). Proteases are the enzymes of utmost biotechnological interest and are present in all living organisms but bacterial proteases are the most preferred group of industrial enzymes as compared to animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement, faster growth rate, higher productivity and low production cost. Among various types of proteases, alkaline proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation (Tuhina and Vandana, 2013).

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolases and are responsible for hydrolysis of peptide bonds in a protein molecule (Habib *et al.*, 2012). Proteases are one of the most important groups of industrial enzymes accounting for more than 60% of the total enzyme sales (Gupta *et al.*, 2005). A large proportion of commercially available proteases are currently derived from *Bacillus* strains, although potential use of fungal proteases is being increasingly realized (Seeta *et al.*, 2005). The microbes which produce protease include *Bacillus subtilis*, *Bacillus cereus*, *Bacillus lichieniformis*, *Bacillus pumilis*, *Serratia marcescens*, *Acinetobacter calcoaceticus*, *Lactobacillus plantarum*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas fragi* and fungi such as *Penicillium restrictum* (Mukesh *et al.*, 2012). Thirty to forty percent of the production cost of industrial enzymes is estimated to be the cost of the growth medium. Therefore it is important to optimize the conditions for cost efficient enzyme production (Joo *et al.*, 2005).

However, this fact implies the production of another solid by-product to be managed and treated. Additionally, conventional dehairing processes use a large amount of fresh water, alkaline substances and sodium hydrosulfide that increases the chemical and biochemical oxygen demand and the dissolved solids in effluents during the process (Juliana *et al.*, 2014). Regarding the management of hair wastes, previous research has effectively tested co-composting techniques to biodegradable hair waste and sludge (Vijayaraghavan *et al.*, 2014).

MATERIALS AND METHODS

Collection of sample

The tannery effluent wastewater collected from Senkulam, Dindigul district. The untreated effluent sample were collected in sterile bottle and transported to the laboratory maintaining temperature around 4°C as early as possible for analysis.

Physiochemical parameters of sample

The effluent samples were analysed physiochemical parameters such as colour, pH, electrical conductivity (EC), total dissolved solids (TDS), total solid suspensions (TSS), total hardness, biological oxygen demand (BOD), chemical oxygen demand (COD), chloride, sodium, calcium and heavy metals were estimated by standard methods.

Isolation and screening

The sample was serially diluted with sterile distilled water and the bacteria were isolated on the skim milk agar plates by the pour plate technique. Plates were then incubated at 37°C for 24-36 h. Colonies forming transparent zones around the bacterial colony due to hydrolysis of milk casein.

Six morphologically distinct bacterial colonies showing the clear zone were selected and re streaked several times on the same medium to obtain pure isolates.

Estimation of enzyme activity of isolates

Enzyme production

Pure culture of each isolate was inoculated into the production medium and incubated at 30°C for 48-72 hours on shaker. At the end of fermentation period, the culture medium was centrifuged at 10000 rpm for 15 minutes to obtain the crude extract which was used as enzyme source and the protease activity was assayed.

Protease assay

Different aliquots of standard solution (tyrosine-2mg/ml) ranging from 0.2-1ml were pipetted in different test tubes and the volumes were made up to 1ml with distilled water. 5ml of alkaline reagent (0.44M Na₂CO₃) was added to all the tubes. They were incubated at room temperature for 10 minutes. 0.5ml of FCR was added to all the

tubes and incubated at room temperature for 20 minutes. OD was measured at 660nm against a suitable blank containing distilled water, alkaline reagent and FCR. A standard graph was plotted by taking conc. of standard along X-axis and OD value along Y-axis.

A 0.5ml of 2% casein (in 0.1M phosphate buffer of pH 10.0) solution was added to test tubes labelled appropriately. Then 0.5ml of enzyme extract was added to the test and incubated at 40°C for 10 minutes. After incubation, the reaction was terminated by the addition of 1ml 10% TCA and 0.5ml of heat killed enzyme was added to the control tube. It was centrifuged at 10000 rpm for 10 minutes and 1ml of supernatant from test and control tubes were transferred to fresh tubes. To 1ml of supernatant, 5ml of 0.44M Na₂CO₃ and 1ml of FCR were added and the tubes were incubated at room temperature for 30°C in dark. The OD was measured at 660nm. One unit of enzyme activity was defined as the amount of enzyme that released one µg tyrosine/ml/min.

Identification of efficient protease producing bacteria

Morphological characterization

Macroscopic and microscopic characterizations were carried out based on colony, pigmentation, gram stain and hanging drop method.

Biochemical tests

Biochemical characterization was carried out based on IMViC tests, catalase test, nitrate reductase, carbohydrate fermentation, urease test, gelatin liquification, H₂S production, starch hydrolysis, casein hydrolysis and lipid hydrolysis.

Optimization of cultural conditions

Optimization of temperature

The production medium (pH 7) was inoculated with the test strain and incubated at different temperatures (20°C, 30°C, 40°C, 50°C and 70°C) in a incubator for 48-72 hrs and assayed for enzyme activity.

Optimization of pH

The pH of the medium was optimized by varying the pH of the medium (pH 4, 5, 7, 9 and 10) keeping the temperature (30°C) and incubation period (48 hrs) constant and assayed for enzyme activity.

Optimization of incubation period

It was determined by subjecting the inoculum to varying incubation periods such as 0, 24, 48 and 72 hours while keeping other parameters such as pH and temperature constant and assayed enzyme activity.

Optimization of inoculums size

As production of enzyme is very well correlated with the concentration of inoculums provided in the assay medium, therefore the effect of inoculum size on protease production was carried out. Varied inoculum concentrations of 0.5ml, 1 ml, 1.5ml, 2 ml and 2.5 ml of the isolate was inoculated into the assay medium for protease production and the protease assay was carried out.

Effect of different Carbon and Nitrogen sources

The best carbon and nitrogen source for protein production was determined by substituting different carbon sources such as fructose, sucrose, lactose, starch and glucose and different nitrogen sources such as beef extract, yeast extract, casein, soybean and skim milk were substituted for peptone and the activity of the enzyme was determined spectrophotometrically. During this process, the other conditions such as temperature, pH and incubation period were kept constant.

Partial purification of enzyme

The culture broth, after 48 h of incubation, was centrifuged at 10,000 rpm for 20 minutes. The saturate ammonium sulphate was added to the culture supernatant and incubated at 4°C for 3h. The concentration of ammonium sulphate was increased in 20% increments from 20% to 80% saturation in order to precipitate enzyme. The resulted precipitate was separated and dissolved in 20mM Tris hydrochloride (pH 8) and dialysed against the same buffer at 4°C for 24 h by continuous stirring.

Stability of enzyme

pH stability of protease

The pH stability of the isolated enzyme were investigated by using different buffers: Sodium acetate (pH 5 and 6), Phosphate buffer (pH 7), Tris HCl (pH 8 and 9). Two ml of enzyme was mixed with 2ml of the 0.1M buffer solutions (Sodium acetate (pH 5 and 6), Phosphate buffer (pH 7), Tris HCl (pH 8 and 9) and incubated at 37°C for 2 h. The activity of the enzymes was studied by using plate assay (skim milk agar).

Thermal stability of protease

Thermal stability of protease was determined by pre-incubating the enzymes at different temperatures ranging from (30°C to 70°C) for 30 minutes. The activity was measured using plate assay (skim milk agar).

Dehairing of goat skin

Common salt preserved goat skins procured from the local market were cut into two pieces along the backbone. The one piece was chosen for dehairing using lime-sulphide to serve as control in the study and the other piece was taken for enzyme application trials. The skins were soaked with three changes of water till they are free from dirt, dung, blood and other contaminating materials and also free from sodium chloride as checked by silver nitrate solution. After soaking, the skins were piled to drain water for about an hour before the application. The soaked weight of the skins was noted separately for the two pieces. The weight of the chemicals/enzymes used in the trials was based on this weight. One piece was applied on the flesh side uniformly with a paste of lime (10%) and 3% sodium sulphide of 60% purity and 10% water. Another piece of skin was applied on the flesh side with 2% enzyme and 10% water. After application, pieces were piled separately with flesh and covered with a gunny cloth and left overnight. Next day (after about 18 h), the skins were dehaired manually on a wooden beam using a knife in tune with the commercial practice in the leather industry and the dehairing efficacy of the enzyme assessed by an experienced leather technologist subjectively in comparison with the control process based on lime-sulphide system.

Destaining of dirty motor oil and blood stain

The compatibility of produced protease with a local detergent (surf excel) was studied. The detergent was diluted with distilled water (7mg/ml) and incubated with the crude enzyme for 15 min at 60°C and the residual activity was determined. Application of protease as a detergent was studied on white cloth pieces stained with blood and grease. The stained cloths were taken in separate flask. The following sets were prepared and studied as follows:

1. Flask with distilled water (100ml) + stained cloth (cloth stained with blood and grease) in separate flask.
2. Flask with distilled water (100ml) + stained cloth (cloth stained with blood and grease) + 1ml surf excel detergent (7mg/ml).
3. Flask with distilled water (100ml) + stained cloth (cloth stained with blood and grease) + 1ml surf excel detergent (7mg/ml) + 5ml of enzyme.
4. Flask with distilled water (100ml) + stained cloth (cloth stained with blood and grease) + 5ml of enzyme.

The above flasks were incubated at 60°C for 15 minutes. After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of blood and grease stains. Untreated white cloth with blood stain and grease stain were taken as control.

Biological liquification

Freshly cut meat of chicken and mutton were taken in 20g capacity of four small bottles. One ml of the enzyme poured into the two bottles, containing one with chicken and one with mutton and another two considered as control. The bottles with chicken incubated for 24 hrs at room temperature and the bottle with mutton incubate for 48 hrs at room temperature. After the incubation the liquification of meat were noticed in the bottle with crude enzyme.

RESULT AND DISCUSSION

Physiochemical Parameters of tannery effluent sample

Physiochemical parameters of tannery effluent samples were analyzed by Omega Laboratories (Analytical testing & Research centre), Namakkal. The tannery effluent was dark brown color and high alkalinity. The physiochemical

characters are listed on Table.1. According to Noorjahan (2014), reported the pH ranged from 7.14 ± 0.0187 to 7.27 ± 0.0187 , indicated the pH of the tannery effluent was found to be alkaline.

Table.1: Physiochemical parameters of tannery effluent sample

S.No.	Parameters	Results
1.	Color (Hazen units)	Dark Brown
2.	pH Value at 25°C	8.52
3.	Turbidity (NTU)	Less than 80
4.	Electrical/Specific Conductivity	5230 $\mu\text{mho/cm}$
5.	Total Dissolved Solids	2920 mg/l
6.	Total Suspended Solids	973 mg/l
7.	Total Hardness	1550 mg/l
8.	Chloride as Cl ⁻	940 mg/l
9.	Sulphate as SO ₄ ⁻	310 mg/l
10.	Calcium as Ca	450 mg/l
11.	Magnesium as Mg	103 mg/l
12.	Sodium as Na	56 mg/l
13.	Residual free Chlorine	24 mg/l
14.	BOD for 48 hours at 20°C	128 mg/l
15.	COD for 24 hours	340 mg/l
16.	Total Nitrogen	2.6 mg/l
17.	Total Phosphorus as PO ₄	1.5 mg/l
18.	Oil and Grease	2.2 mg/l
19.	Phenolic Compounds	1.8 mg/l
20.	Fluorides	2.5 mg/l

Isolation and screening protease producing bacteria

About 13 isolates were recovered from liquid samples collected from tannery waste water. Isolated bacterial strains were screened for protease producing ability on skim milk agar. Among 13 isolates (P1 to P13) only 6 isolates (P1, P3, P6, P8, P9 and P10) showed maximum zone around the colonies on skim milk agar. The zone formation around the bacterial colony indicated the protease positive strain which may be due to hydrolysis of casein (Fig.1). Habib *et al.*, (2012) have used skim milk agar media for the isolation of thermostable extracellular alkaline protease producing bacteria from tannery effluents.

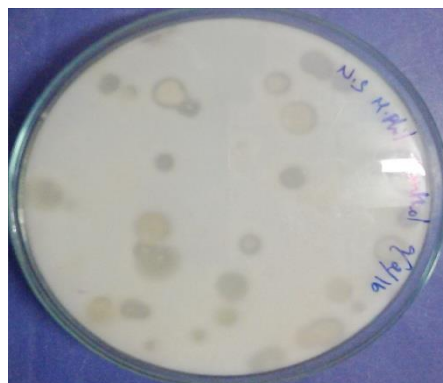


Fig.1: Proteolytic bacterial isolates on skim milk agar plate

Estimation of protease activity

The objective of present investigation was to select the bacterial strains with high level of protease producing ability (Fig.2). In order to achieve the aims, we have selected during the initial screening, a total of 6 different bacterial strains were isolated on skim milk agar medium. The six isolates were checked for quantitative test of protease in liquid medium. The maximum protease activity was attained after 48 h by isolate P3. It was

found that maximum production occurred at end of exponential phase. Other each samples three strains showed high production of protease, isolates P1, P6, P9. The lowest enzyme activity was observed by isolates P8, P10 with enzyme activity respectively. Hence the maximum protease activity strain was identified as a protease producer and it was taken for further experimental studies and biochemical test. Similarly Hanan (2012) suggested the six isolates were checked for quantitative test of extracellular protease in liquid medium and the maximum protease activity was attained after 72 h.

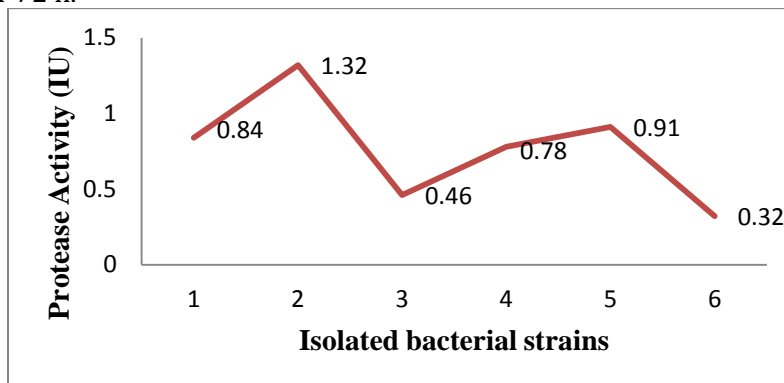


Fig.2: Protease activity of isolated bacterial strains

Identification of bacterial isolates

The identification of selected isolate were characterized based on their morphological, cultural, physiological and biochemical properties and is presented in proteolytic bacterial isolates respectively. The isolated P3 strain was gram negative and this strain showed positive result on motility, catalase, nitrate reduction, gelatin and casein hydrolysis (Table.2). The isolated proteolytic strain identified, was *Pseudomonas aeruginosa* based on analysis. Confirmed *Pseudomonas aeruginosa* to produced greenish blue pigmentation on cetrimide agar medium (Fig.3). In Soumya (2012) were isolated the 42 protease producing bacteria out of 6 different samples, among 42 isolates 20 isolates were identified as *Pseudomonas* sp.



Fig.3: *Pseudomonas aeruginosa* on cetrimide agar plate

Table.2: Biochemical characterization of the isolated bacterial strain

S.No.	Characteristics	Results
1.	Gram Staining	-ve
2.	Shape	Short Rod
3.	Motility	Motile
4.	Spore formation	Non-spore
5.	Oxygen requirement	Aerobic
6.	Indole test	-ve
7.	Methyl red test	-ve
8.	Voges-Proskaur test	-ve
9.	Citrate test	+ve
10.	Catalase test	+e

11.	Nitrate reductase	+ve
12.	Casein hydrolysis	+ve
13.	Gelatin hydrolysis	+ve
14.	Starch hydrolysis	+ve

Effect of temperature on protease production

Temperature is one of the most important factors affecting the enzyme production. The results referred to a positive relationship between protease production and incubation temperature up to 40°C, while the activity decreased rapidly above 50°C. Previously Tuhina and Vandana (2013) reported the optimum temperature for maximal enzyme activity between 30-50°C indicated its thermostable nature of alkaline protease of TVP-9.

Effect of pH on protease production

The growth and enzyme production was studied at pH 5.0 – 9.0. It was optimum at pH 8.0. Increased alkalinity was not favourable up pH 9.0, as both growth and enzyme production were reduced. Further, the reduction in enzymatic level was more pronounced than that in growth pattern at lower pH. The *Pseudomonas* sp was highest protease activity at pH 7 recorded by Soumya (2012).

Effect of inoculums size

Results from the present study showed that optimum inoculums size of the bacterial isolate for protease production was 1 ml from overnight culture broth. The less protease production in small inoculums sizes of 0.1 ml and 0.5 ml may be due to insufficient number of bacteria, which lead to reduced amount of secreted protease and the decrease even though luxurious growth was observed in highest inoculums size of 2 ml. Similarly inoculum size 2.0 ml obtained the highest protease activity reported by Sai Smita *et al.*, (2012).

Effect of incubation period

The growth and enzyme production was studied at various incubation periods like 0 to 72 h. It was found that isolated strain showed maximum enzyme activity at 48 h. In Sharmin *et al.*, (2005) conclude the highest protease activity at 48 h of incubation periods but highest biomass yield was recorded at 96 h.

Effect of carbon and nitrogen sources

On the basis enzyme activity, it was concluded that the medium amended with glucose was suitable for the production of protease. In the same way sucrose was also found to be a good source for the enzyme production.

Nitrogen sources act as a secondary energy source for the growth and enzyme production of any organisms. The skim milk and casein influenced the production of protease, followed by soya bean meal. Nazenin *et al.*, (2015) reported the carbon source like lactose and nitrogen source like yeast extract produce the maximum protease activity.

Purification of enzyme

The crude protease sample was precipitated with 70% saturation of ammonium sulfate. The precipitate is dissolved in 0.1M phosphate buffer and this ammonium sulfate precipitate method only partially purified. Then next step of purified involved in dialysis. The 50kDa dialyses pack used to the dialysis and determined the protease activity. Earlier reported the purification of the enzyme used ammonium sulphate precipitation, dialysis and followed by SDS gel electrophoresis reported the Nazenin *et al.*, (2015).

Stability of enzyme

Thermal stability

Thermal stability of partially purified enzyme was determined by pre-incubating the cell free extract at different temperatures for 30 minutes. Protease enzymes showed thermal stability for a range of 30-50°C and the maximum stability was found to be at 50°C.

pH stability

The stability of the partially purified enzyme was determined by pre-incubation of the enzyme in various buffers at different pH for 30 minutes. The enzymes were stable over a pH range of 6-9 and the maximum stability was found at the pH 7. Folasade (2013) reported the thermal stability of enzyme obtained 60°C and pH stability obtained pH 7.

Applications of isolated protease enzyme

Dehairing of goat skin

Protease from isolated strain was found to be effective in leather processing. To evaluate the effect of protease on goat hide, it was incubated with a crude protease sample for 18 h at room temperature. The enzyme produced by *Pseudomonas* sp. revealed its activity on goat hides and removed fine hairs. The protease from this organism effectively dehaired the goat hide within 18 h of incubation (Fig.4). Additionally we also used scanning electron microscopy to study the effect of enzyme treatment on goat skin. Electron micrographs on enzyme treated, chemical treated and untreated goat skin (Fig.5). The enzyme treated goat skin was fully dehaired and smooth surface comparatively chemical treated goat skin. This enzyme was non-kertinolytic and non collagenolytic in nature. Thus these results indicate that the enzyme can be a better option for dehairing applications. Previously reported the dehairing activity of the alkaline protease from *Bacillus cereus* reported the Shakilanishi *et al.*, (2011).

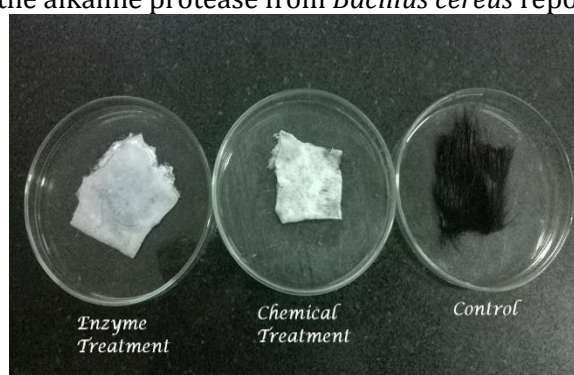


Fig.4: Dehairing of goat skin

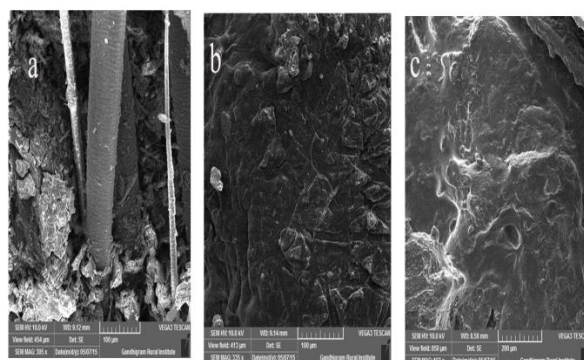


Fig.5: Electron micrographs of dehaired goat skins. (a) untreated goat skin, (b) chemical treated goat skin and (c) enzyme treated goat skin

Destaining of dirty motor oil and blood stain

In removing the blood stain and the grease from the stain cloth, the enzyme showed greater efficiency (Fig.6). The experiment proves that the addition of enzyme to the detergent showed its efficiency in removal of blood and grease stains. The crude extract contains protease, amylase and lipase which can be used as additives in detergents since these enzymes are found to be very effective in the removal of blood stains with or without the presence of detergent. Mukesh kumar *et al.*, (2012) earlier reported the destainig capability protease from *Serratia marcescens*.



Fig 6: Destaining of Blood and Dirty motor oil greese stain

Biological liquification

The bottles with chicken and mutton meats were incubated for 24 hrs and 48 hrs respectively. After the incubation period the meats were found liquefied in which protease enzyme added (Fig.7). The meat without addition of protease enzyme remained the same which is considered as control. Gerelt (2000) investigated the meat tenderization by dipping the meat cut in a solution containing proteolytic enzymes after contact-osmotic dehydration. After the dehydration of each piece of meat from culled cow for 18 h by contact-dehydration sheet, each sample was dipped for 3 h in a solution containing papain or protease from *Aspergillus* traditionally used for soysauce production in Japan.

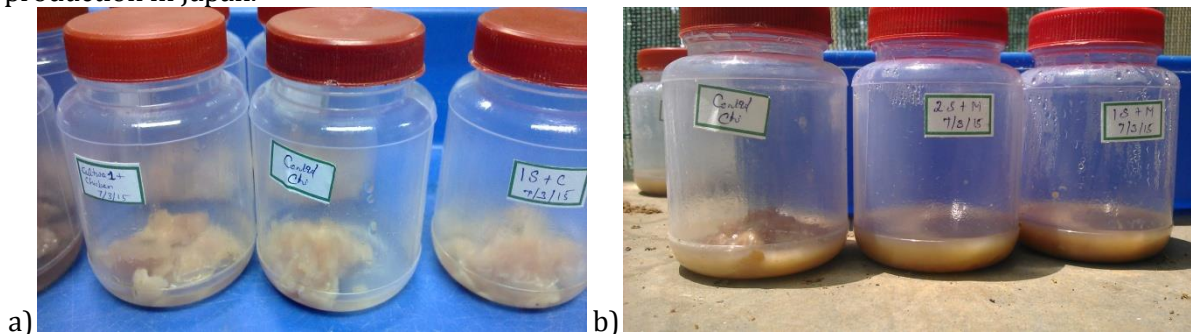


Fig.7: Biological liquification. (a) before enzyme treatment; (b) after enzymatic treatment

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