
Isolation of Protease Producing Bacteria from Soil for Polyester and Silver Recovery from Waste X-ray Film

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Abstract: Proteases are multipurpose group of enzymes which mainly produced from microorganism and used in various industries such as detergent, silver recovery, food, pharmaceutical, leather and textile industries. This work aimed to isolate protease producing bacteria from local environment for use as silver recovery and reuse of polyester from waste X-ray film. Isolation of protease producer was undertaken using skim milk agar medium. A total of 36 protease positive bacteria were isolated from the study area (Arba Minch fish processing site). Five isolates were screened which had relatively higher clear zone and further examined gelatin removal efficiency of their crude enzymes. One isolate designated as *Bacillus sp.* THZ14 was selected on the basis of gelatin removal efficiency. The crude enzyme from this strain removed gelatin on waste X-ray film at pH 9, 40°C in 40 min. These properties suggest that protease from *Bacillus sp.* THZ14 could find potential application in silver recovery process and reuse of polyester sheet which have an economic implication.

Keywords: *Bacillus sp.* THZ14, Enzyme, Gelatin Hydrolysis, Protease, X-ray Films

1. Introduction

Almost All form of life (animal, plant and microbes) on earth constituent's protease enzyme for their physiological function. Microbes are ubiquitous and live in familiar setting such as Soil, water, food, and animal intestines, as well as in more extreme setting such as rocks, glaciers, hot springs, and deep-sea vents. The beneficial microbes are fascinating, versatile and carry out extremely useful processes that can't be achieved by other physical and chemical means. Proteolytic enzymes from microbial sources are preferred over the enzymes derived from plant and animals due to their susceptibility for genetic manipulation [1]. Currently, microbial proteases represent one of the largest groups of industrial enzymes and account for approximately 60% of the total industrial enzyme sale in the world.

Microbial proteases are one of the most important groups of enzymes, used in various industrial processes as food, pharmaceutical and detergent industries, as well as in the preparation of leather, textile and wool, among others [2, 3, 4, 5]. It has also promising application in medical usage and management of industrial and household waste. The use of microbial system (enzymes) is the best alternative for

generation of pollution free industries [6].

Recovery of silver and polyester sheet from waste X-ray film is possible through biological treatment. The conventional method for recovery of silver is burning and chemical treatment of the films directly. This generates undesirable foul smell and environmental pollution. In addition polyester film on which emulsion of silver and gelatin is coated cannot be recovered. Microbial proteases are being used as best alternatives to the burning and chemical methods of silver recovery from waste X-ray films. This not only helps in extracting silver, but also the polyester film base can be recycled [7, 8, 9]. Microbial protease has a significant potential for hydrolysis of gelatin layer and as result releases silver from waste photographic film [10, 11, 12]. Bearing in mind this critical factor, effort should be directed to search microorganisms which produce proteases with high stability in the environment of silver recovery process. These all contribute toward the idea of clean technology and environmentally friendly approach for the welfare human being.

Considering these vital and unlimited industrial applications of microbial product, there is a need to effort by investigating new microorganisms because they are the major

sources of all commercially important enzymes. In this regard, microorganisms have still potential to produce enzymes, even though production cost of the enzyme is the critical issue.

In fact, Ethiopia have broad microbial diversity, however protease producing bacteria for silver recovery have not yet been explored. Therefore a research project has been initiated with the objectives of isolating protease producing bacteria from local environment for silver recovery and reuse of polyester from waste x-ray sheet.

2. Materials and Methods

2.1. Sample Collection and Isolation of Proteolytic Bacteria

Soil samples were collected from fish processing area (Southern Nation Nationalities of people Region, Gamo Gofa Zone, Arba Minch). Each sample was kept in clean sterile sample bottles sealed and transferred to the laboratory and stored at 7°C.

The soil samples were suspended in water by vigorous vortexing and serial dilutions were made and appropriate dilution were spread on skim milk agar plate containing peptone (0.1%), NaCl (0.5%), Agar (2%) and skimmed milk (10%) at pH 9 and incubated at 40°C for three days. Clear zone forming (proteolytic) bacteria were screened and purified by streaking on skim milk agar. The cultures were subsequently sub-cultured and used regularly.

2.2. Screening of Potential Isolate

The potential bacterial strains were screened for the quality of enzyme produced under submerged condition. The supernatant from each isolate was used as crude enzyme for observing individual for gelatin hydrolysis performance. Single potential strain was select on the basis of gelatin removal efficiency from used X-ray sheet. Identification of the selected potential isolate was performed according to Bergey's manual of determinative bacteriology [13].

2.3. Morphological Characterization

Potential isolate was characterized by colony (shape, size, elevation, colour, margin and configuration); cell (Gram stain, determination of the presence of spore).

2.4. Biochemical Characterization

Potential isolates were characterized by different biochemical methods catalase test, citrate utilization test and MR-VP test.

2.5. Enzyme Activity

Protease production media was prepared according to the method described by Das and Prasad, 2010) [14]. The pH of the medium was adjusted to 9 and bacterial isolate was inoculated with 1% of inoculum into 100 ml production medium followed by incubation at 40°C for 48 hours in water bath shaker at 150 rpm. Culture filtrates were used as

crude enzyme source for quantitative enzyme assay. Protease activity in the culture supernatant was determined using casein as a substrate with some modification of the method described by Gaur *et al.* (2010) [15]. The enzyme activity was expressed in units (U) and calculated by using tyrosine standard calibration curve. One unit of enzyme was defined as the amount of enzyme that releases 1 μ mol of tyrosine per ml of crude extract per minute under standard conditions at 40°C.

2.6. Recovery of Silver and Reuse of Waste X-ray Film

Removal of gelatinous coating from used X-ray film: Removal of gelatin from used X-Ray film was done as described in Shankar *et al.* (2010) [16] with some modification. Used X-ray films (0.213 g each) were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in oven at 40°C for 30 min. X-ray film (2 x 3 cm pieces) was incubated with 20 ml of crude enzyme (film completely immersed in solution) at 40°C with continuous shaking at 150rpm until complete removal of gelatin from films. Optimum pH for hydrolysis of gelatin was determined at 40°C and pH from 7.0 to 12.0.

Weight loss of X-ray film: Weight loss of the film was analyzed by measuring the weight of used X-ray film before and after complete removal of gelatin layer.

Reuse of enzyme for hydrolysis: Reuse of enzyme for gelatin hydrolysis was evaluated at 40°C, pH 9 and 150 rpm in a rotary shaker with continuous shaking until the gelatin layer was completely removed. After complete removal of gelatin, a new film was incubated in the reaction mixture up to six turns.

Recovery and detection of silver: The presence of silver in the reaction slurry was confirmed by nitric acid test [12]. After complete gelatin removal, reaction slurry was digested with nitric acid and analyzed by Atomic Absorption Spectrophotometer at wavelength of 328.1nm. The concentration of silver metal was determined by using generated silver standard curve with regression coefficient of $R^2=0.9935$.



Figure 1. Zone of hydrolysis by strain C14 in milk agar plate after 18 h incubation at 40°C.

2.7. Data Analysis

Average values of duplicate experiments were taken. Microsoft office Excel worksheet 2010 was used for data analysis and presentation.

3. Results

Isolation of Proteolytic Bacteria



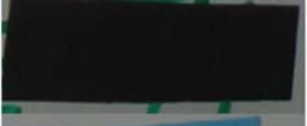
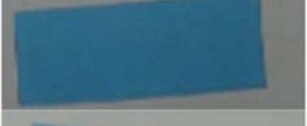


Thirty six protease positive isolates were obtained from the study area. Hence the strains were identified as a protease

producer since the formation of clear zone around the colony due to hydrolysis of skimmed milk protein in the medium (Figure 1). Out of 36 protease positive bacterial isolates, 5 isolates with relatively higher clear zones were further examined.

Selection of Potential Isolate

The five isolates were compared for gelatin removal efficiency with respect to time and isolate C14 was selected for further study. The results are summarized in Table 1.

Table 1. Results of gelatin removal efficiency of five selected isolates at pH 8.

S. No	Strain code	Gelatin hydrolysis ability from used x-ray	Time taken (Minute)
0	Control		-
1	C5		70
2	C10		120
3	C16		80
4	C33		120
5	C14		45

Morphological and Biochemical Characteristics of Isolate C14.

Table 2 and Figure 2 show results of morphological and biochemical tests of the selected isolate. Motile, long

irregular and rod shaped cells were observed under light microscope. Based on this the isolate was designated as *Bacillus sp.* THZ14.

Table 2. Results of the morphological and biochemical tests of isolate C14.

Morphological characteristics	Results	Biochemical characteristics	Results
Colour	Creamy white	Nitrate reduction	Negative
Size	Large	Indole tests	Positive
Margin	Irregular	MR-VP test	Negative
Elevation	Flat	Citrate utilization	Negative
Configuration	Irregular and spread	Casein hydrolysis	Positive
Morphology	Rod		
Gram reaction	Positive		
Endospore	Elliptical		
Motility test	positive		

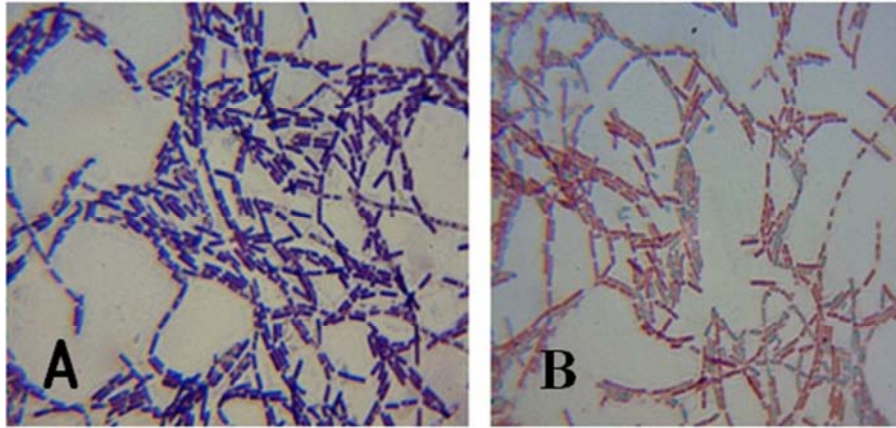


Figure 2. Gram reaction result (+ve) (a) and Endospore of *Bacillus sp. THZ14* (b) at 40°C and 24 h on milk agar.

Silver and Polyester Recovery from Waste X-ray Film Sheet

Effect of pH on Gelatin Hydrolysis

Gelatin removal was observed over a pH range of 7 to 10 with maximum hydrolysis at pH 8 and 9. Complete gelatin removal was observed within 40 min at pH 9 (See Figure 3).

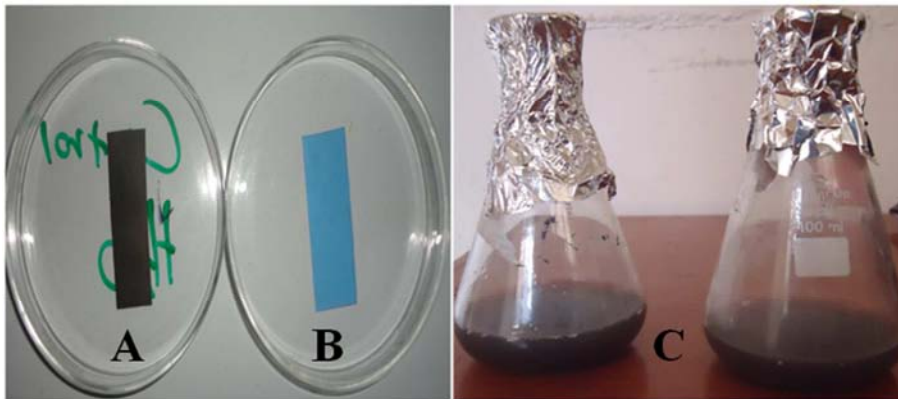


Figure 3. Hydrolysis of used X-ray film by crude enzyme produced from *Bacillus sp. THZ14*. Sample pictures: (A) control (20ml of Bicarbonate buffer) and (B) gelatin layer removal from the film after 40 min incubation at 40°C and pH 9 with crude enzyme. (C) Reaction slurry after 5th cycle of gelatin removal.

The hydrolytic efficiency of the enzyme increased from pH 7 to 9 and declined above pH 9 (Figure 4). Desired hydrolysis was observed at pH ranges from 8.0-10.0. Turbidity of the reaction mixture was compared with respect to their pH after 20 minute incubation.

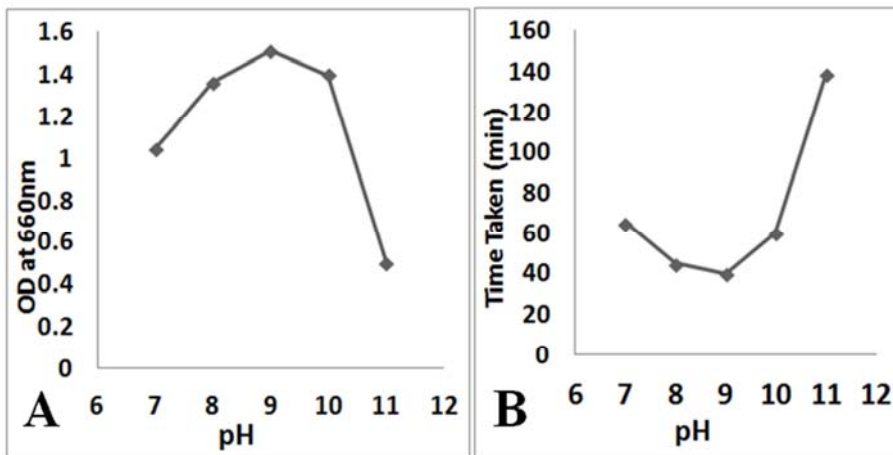


Figure 4. Effect of pH on gelatin hydrolysis with crude enzyme at pH 7-11, 150 rpm and 40°C (A) Optical density of the reaction slurry after 20 min incubation (B) Time course for complete gelatin removal.

Reusability of Enzyme for Gelatin Hydrolysis

The crude enzyme removed gelatin from used X-ray films for 4 successive cycles in comparable time intervals. The removal took excessive time after the fourth cycle (Table 3).

Table 3. Reusability of the enzyme at 40°C.

Cycle	1	2	3	4	5
Time for complete removal (min)	40	54	65	119	Take excessive time

Weight Loss of Used X-Ray Film after Gelatin Removal.

Used X-ray films were treated with crude enzyme and resulted in stripping off the silver bound with gelatin into the reaction mixture and clean plastic film was recovered (Figure 3). As a result, weight loss of the film was noticed after cleaning the used X-ray film. The average weight of the X-ray film before incubation was 0.213g (2cm x 3cm). After gelatin and other components of the film were removed, the weight of the film became 0.201g. Therefore, the weight loss was calculated as 0.012g. The value indicates that 5.6% of the film weight was lost after the film was treated with crude enzyme.

Recovery and Detection of Silver

The presence of silver in the slurry was detected by nitric acid test. The result of the nitric acid test is shown in Figure 5. It was confirmed by atomic absorption spectrophotometry and the concentration determined as 0.042 M.



Figure 5. Nitric acid test (reaction slurry with a drop of nitric acid (A); Control (B)).

4. Discussion

Isolation and identification of bacteria which have vital ability to secrete extracellular protease enzyme to be used for silver recovery and reuse of polyester from waste X-ray film is one of the main concerns of this study. Accordingly, 36 bacterial strains were isolated from study area. Formation of clear zone around the bacterial colony indicated the protease positive strains hydrolysed the skim milk present in the

media (Figure 1). The use of skim milk agar medium for the isolation of protease producing bacteria has earlier been reported by some workers [17, 18, 19].

Five isolates had larger clear zone are screened. Out of these isolates, *Bacillus sp.* THZ14 exhibited high capability of removing gelatin from used X-ray films within short period of time when compared to others (Table 1).

As shown in Figure 3 and 4, used X-Ray films treated with protease produced from *Bacillus sp.* THZ14 resulted in the silver bound with gelatin being stripped off in to the reaction mixture after 40 min at pH (9) and 40°C. On the other hand, no gelatin removal was also observed at all when the experiment was conducted without incorporation of enzyme in the reaction mixture (Bicarbonate buffer only at pH of 9) at 40°C for 40 min. This implies protease produced in the present study has appreciable application in the process of silver recovery and reuse of polyester from used X-ray film. Similar results were reported by some researchers [8, 9, 11, 12].

Colour change was observed when a drop of nitric acid was added into the reaction slurry (Figure 5). The presence of silver in the mixture was confirmed by the color change observed. Similar finding was reported [12]. The concentration of silver found in the mixture was 0.042M. This implies that it is possible to recover silver from used X-ray film using the protease produced from *Bacillus sp.* THZ14. Similar result was reported by Shankar *et al.* (2010) [16].

Gelatin removal efficiency of the enzyme required relatively the same time up to the fifth cycle and required more time at the 5th cycle reuse. This shows the reusability of the produced enzyme. Masui *et al.* (1999) [9] reported that a protease spent 60 min and >120 min for removal of gelatin from used X-ray film in the 1st and 2nd cycles, respectively at 50°C. Hence, comparing the experiment conducted in this study, which requires 40 min and 54 min to remove gelatin in the 1st and 2nd cycle (Table 3), respectively the present enzyme saves approximately two and four fold time for the same process, respectively. This indicates that, the enzyme produced by *Bacillus sp.* THZ14 is good candidate for silver recovery and reuse of polyester from used X-ray film.

When the X-ray films treated with crude enzyme for about 30 min and the result indicates that used X-ray film could loss its weight by 5.6% based on initial weight of the film. Results are almost similar with that investigated by Shankar *et al.* (2010) [16] and loss in weight after enzyme treatment was around 5% (w/w).

In general the use of microorganisms to produce enzymes has a number of technical and economic advantages and in

recent years it has become the predominant mode of enzyme production [20]. The exploration of protease producing bacteria is still underway to achieve high quality industrial grade enzyme to produce goods [21].

5. Conclusions

As the method described in this investigation for removal of gelatin from used X-ray film undergoes the process without any additive of hazardous chemicals, it would have minimum impact on the environment as compared to conventional chemical and burning methods. The recovered pure polyester film after gelatin removal can also be reused that helps minimize the production of large number of polyester films. Further, silver is one of the precious metals, so recovering it from the film will have economic implication.

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