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Isolation and Screening of Protease Producing Bacteria from Local Habitat for Polyester Recovery from Waste Photographic Films

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Abstract: **Microbes are living organisms, they carry out extremely useful processes and they are the best preferable sources of proteases with compared to plant and animal. Proteases are physiologically necessary for almost all living organisms such as bacteria, fungi and viruses. It is 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 habitat for use as polyester recovery from waste X-ray film. Isolation of protease producer was undertaken using skim milk agar medium. A total of 47 protease positive bacteria were isolated from the study area. Ten isolates were screened for gelatin hydrolysis test and out of ten; two potential isolates (Abc2 and Abc5) were selected based on gelatin removal efficiency. All ten isolates were found to be positive for Gram reaction, citrate utilization, catalase; motile and rod shaped morphology. However, they found to be negative for methyl blue test. The crude enzyme from isolate Abc2 and Abc5 were digested gelatin layer on waste X-ray film at 30°C in 120 and 140 min respectively. Used X-ray film was lost 5.6% of its weight after treatment of film with crude enzyme. These properties suggest that protease enzyme produced in this study could find potential application in silver recovery process and reuse of polyester sheet which have an economic implication besides mitigating the risk of our environment.**

Keywords: **Polyester Recovery, X-Ray Film, Gelatin, Protease, Hydrolysis.**

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, 2, 3]. 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 [4, 5, 6]. 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 [7, 8, 9].

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 [10, 11]. This not only helps in extracting silver, but also the polyester film base can be recycled. Microbial protease has a significant potential for hydrolysis of gelatin layer and as result releases silver from waste photographic film [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 [12, 13, 14].

Silver is a valuable metal used in photographic and X-ray film, which is considered as an important source of silver metal after recycling of used films compared to other types of films. X-ray films contain about 1.5 to 2% ratio of silver in gelatin-coated film made from polyester layer. And it can restore this quantity of silver by dissolving gelatin layer in alkaline protease to be used for other purposes. X-ray film is a rich source of silver, which is distributed in the gelatin layer [6]. Proteases represent one of the three largest groups of industrial enzymes. The most important applications of protease are used in laundry detergent, leather processing, brewing, food and pharmaceutical industries [2].

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 polyester recovery from waste X-ray sheet. The output of the work help for re-use of polyester from waste X-ray film which minimize the foreign currency beside protection of the environment from pollution.

2. METHODOLOGY

2.1. Description of the Study Area:

Arba Minch located in the Gamo Gofa zone of the southern nations, nationality, and people region. About 500 kilo meter south of Addis Ababa at an elevation of 1285 meter above sea level. It is the largest town in Gamo Gofa zone and the second town in SNNPR next to Hawasa. Is located to the west lake of Abaya the annual rare fall in Arba Minch regions from 623.5mm to 1061 mm and the temperature is 32^{o}C the altitude ranges from 2200 to 1400m above sea level the most common vegetation type grow in Arba Minch are mango tree, banana and acacia plants [15].

Arba Minch University (AMU) is one of the well-established universities found in the Southern Nations, Nationalities and People's Region (SNNPR). It is located at Arba Minch town. The main campus of the university is situated at the eastern foot of Gamo mountain ranges and 3.2. adjacent to the vast low land stretching towards Lake Abaya and Lake Chamo which form part of the East African Rift Valley [15]. This study was conducted in Arba Minch University particularly Abaya campus. The study was focus on the isolation and screening of protease producing bacteria from local environment for silver recovery from waste x-ray film. This study not only conducts in Abaya campus by collecting soil samples from Arba Minch main campus abattoir, water from Chemo Lake and another soil sample from Abaya campus cafeteria waste disposal. In this research was used primary source of data by gathering through doing experiment in Arab Minch University Abaya campus.

2.2. Sample Collection and Isolation of Proteolytic Bacteria:

Soil and water samples were collected aseptically from three different environments around Arba Minch town like abattoir, Abaya campus and Lake Chamo using sterilized bottle. Each sample was kept in clean sterile sample bottles sealed and transferred to the plant biotechnology laboratory and stored at 4^{0}C.

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Figure 1 Sample collected Area: (A) abattoir waste disposal; (B) Abaya campus cafeteria waste and (C) Lake Chamo

2.3. Isolation and Screening of Protease Producing Bacteria:

2.3.1. Isolation of Protease Producing Bacteria:

The soil samples were suspended in water by vigorous vortexing and serial dilutions were made up to 10^{-6} in sterile distilled water. 0.1 ml of appropriate dilution were spread on skim milk agar plate (see table1) at pH 8 and incubated at 37^0C for 48 hrs. Clear zone forming (proteolytic) bacteria were screened and purified by streaking on skim milk agar. The cultures were subsequently sub-cultured and used regularly. Appearance of clear zone around bacterial colonies indicates hydrolysis of skimmed milk due to secretion of protease by the organism [5].

Table 1 Composition of milk agar Medium required for isolation of protease producing bacteria

S.No	Component	Concentration in (g /l)
1	Peptone	10
2	Meat extract	5
3	NaCl	20
4	Agar	20
5	Skimmed milk	100

2.3.2. Screening of Potential Isolates:

The bacterial strains were screened based on the size of clear zone formed on the skimmed milk agar plate. A bacterial strain with larger clear zone was screened and selected for further study from each site. Finally various biochemical studies were undertaken according to Bergey's manual of determinative bacteriology for identification of the selected potential isolates.

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. Two potential strains were select on the basis of gelatin removal from used X-ray films.

2.4. Characterization of Potential Isolates:

2.4.1. Morphological Characterization:

Potential isolate was characterized by colony (shape, size, elevation, colour, margin and configuration); cell (Gram stain, determination of the presence of spore) [16].

Gram staining: This was carried out by using standard techniques with a step-wise application of Crystal violet solution, iodine solution, ethanol (95%) and Safranin solution.

Motility test: Bacterial motility was observed directly by microscope. A drop of bacterial suspension was placed in to glass slide and put coverslip in the center [17].

2.4.2. Biochemical Characterization:

Potential isolates were characterized by different biochemical methods catalase test, citrate utilization test, starch hydrolysis, Urease and methyl blue [16, 18].

Catalase test: Catalase test was carried out by using 3% hydrogen peroxide. The cells from a culture were mixed with a drop of hydrogen peroxide on a clean slide using the inoculating loop. The presence and absence of bubbles was recorded as positive and negative, respectively [16].

Starch hydrolysis: This test was carried out by dividing starch agar plate into two equal sectors using a marker. After labeling the organism's name, the test organisms were spot inoculated and incubated for 24h [16]. Zone of hydrolysis of starch was detected as a brownish clear zone in a blue black background after flooding the starch agar plate with iodine solution. The presence of zone of hydrolysis on the plate indicated the ability of the test organism to metabolize starch.

Urease taste: Urease test was carried out by preparing urea agar containing phenol red as pH indicator. After inoculating the agar with the test isolate and incubating the culture for 24 h, color change of the agar from red to pink was observed and recorded as a positive result for urease test [16].

2.5. Inoculums Preparation for Production of Protease:

A single bacterial colony from potential isolate were inoculated in to 10 ml of nutrient broth under aseptic condition and incubated at 37°C for 24 h. After 1 day of incubation, growth (turbidity) was appeared in inoculated broth and this preparation was directly used as a source of inoculums [19].

2.6. Production of Protease in Shake Flask Fermentation:

The production medium containing (g/l): glucose (1.0), peptone (10.0), yeast extracts (0.2), casein (10), $CaCl_2$ (0.1), K_2HPO_4 (0.5) and $MgSO_4$ (0.1) was prepared for production. The bacterial isolate was inoculated with 1ml of inoculums into 250 ml Erlenmeyer flasks containing 100 ml production medium followed by incubation at 30°C for 48 hours in rotary shaker at 100 revolutions per minute (rpm). Culture filtrates were separated by centrifugation at 5000 revolution per minute (rpm) for 20 min and the supernatants were used as crude enzyme source for gelatin hydrolysis test [19].

2.7. Degradation of Gelatin Layer and Polyester Recovery:

The used X-ray films were cut into 2 × 2 cm pieces, washed with distilled water and wiping with cotton, impregnate with ethanol. After drying in an oven at 40°C for 30 minutes each of the film was submerged in series 10 ml of crud enzyme extract. The solution along with submerged film was stirred in shaker until the gelatin-silver layer is stripped completely. Required numbers of films were stripped and obtained slurry was dried. During the smelting ammonium chloride was added for getting the white color to the silver. After the completion of the smelting process the dried powder were washed with the water and the white colored crystals of the silver was collected [12].

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.

3.9. Data Analysis:

The data were analyzed and organized using basic statistical parameters like table.

3. RESULTS

3.1. Isolation and Screening of Protease Producing Bacteria:

Forty seven (47) protease positive isolates were obtained from three sample areas. Among 47 isolates, 10 potent clear zone forming colonies were screened 6, 3 and 1 from abattoir, Abaya campus cafeteria waste and Lake Chamo sample respectively. These 10 protease positive bacterial isolates with relatively higher clear zones were further examined.

3.2. Characterization of Potential Isolate:

The selected organisms were characterized on the basis Gram stain, motility, Simon citrate, starch hydrolysis, Urease, catalase and methyl. The result of this was show in table 2 and fig 2.

Table 2: Results of Biochemical Characterization of potential isolates

Isolates	Biochemical Tests							
	Cell shape	Gram stain	Motility	Starch hydrolysis	Citrate utilization	Catalase	Urease	Methyl blue
Ab-c_2	Rod	+	+	+	++	+	+	-
Ab-c_3	Rod	+	+	+	+++	+	+	-
Ab-c_5	Rod	+	+	+	++	+	+	-
Ab-c_8	Rod	+	+	+	++	+	-	-
Ab-c_9	Rod	+	+	+	++	+	+	-
Ab-c_{10}	Rod	+	+	+	+++	+	+	-
Ac-c_3	Rod	+	+	+	+	+	+	-
Ac-c_4	Rod	+	+	+	+++	+	+	-
Ac-c_9	Rod	+	+	+	+++	+	-	-
W-c_4	Rod	+	+	+	+	+	+	-

Key: +++ (highly positive); ++ (medium positive); +(positive); - (negative), Ab (Abattoir), Ac (Abaya campus), W (water), C_n (colonies number)

Figure 2: Result of some biochemical tests: (A) Positive citrate utilization test; (B) positve urease test and (C) negative urease test result

3.3. Enzyme Production:

The crude enzymes were produced from ten potential bacterial strains under submerged condition. Culture filtrates were separated by centrifugation at 5000 rpm for 15 min and the supernatants were used as crude enzyme source for gelatin hydrolysis and for silver recovery (fig 3). Cell pellet was discarded.

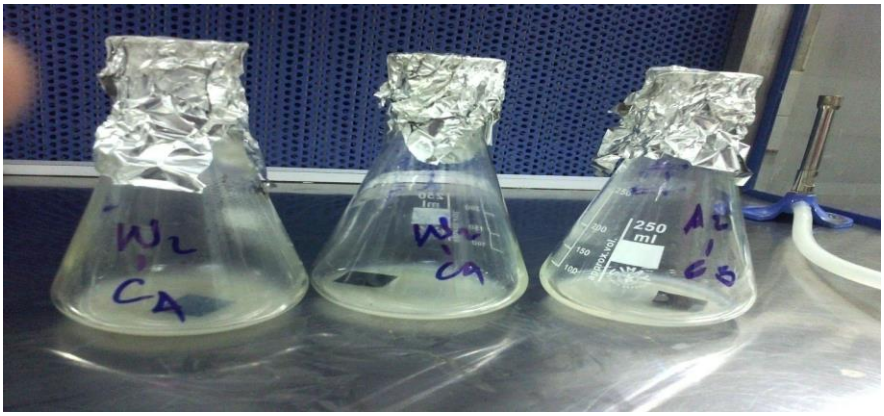

Figure 3: West X-ray in immersed in 10 ml of crude enzyme during gelatine hydrolaysis

3.4. Removal of Gelatinous Coating from Used X-Ray Film:

Visual examination of the films clearly revealed the ability of proteases to hydrolyze the gelatin cover in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysates. Crud enzymes of each isolates were not very efficient in gelatin hydrolysis from waste X-ray film. Under the obtained conditions from ten isolates two isolates were effectively hydrolysis of gelatin from waste X-ray film (table 3). Treatment of X-ray films with protease resulted in the sliver bound with gelatin being stripped off in to the reaction mixture and clean plastic film (polyester sheet) was recovered (fig 4).

Table 3 Length of Time for Gelatin Hydrolysis and Weight Loss

Potential isolates	Time taken (minutes)	Temperature (oC)	Initial weight of x-ray sheet in gram (2×2)cm	Weight of X-ray film after treatment	weight loss in gram
Abc_2	120	Room	0.213	0. 201	0.012
Abc_5	140	Room	0.213	0. 201	0.012

3.5. Weight Loss of the Film during Gelatin Removal from Used X-Ray Film:

Used X-ray films were treated with crude enzyme and resulted in stripping off the sliver bound with gelatin into the reaction mixture and clean plastic film was recovered (Fig 4). 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 2cm). 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.

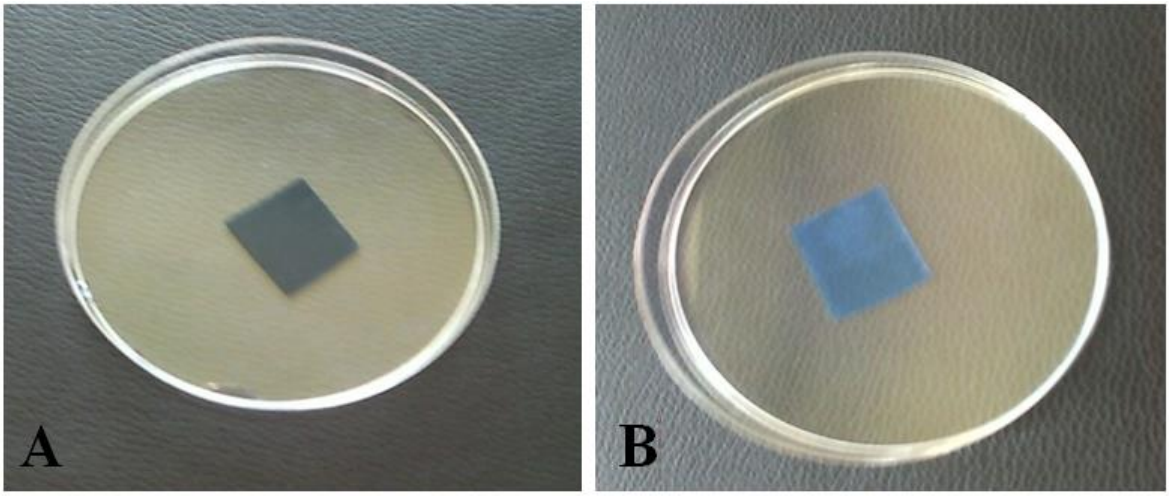

Figure 4: Hydrolysis of used X-ray film by crude enzyme produced from Abc2 and Abc5. Sample pictures: (A) control (10ml of bicarbonate buffer) and (B) gelatin layer removal from the film after 120 min incubation at 30°C with crude enzyme

4. DISCCUSION

Isolation and identification of bacteria which have vital ability to secret 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, 47 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. The use of skim milk agar medium for the isolation of protease producing bacteria has earlier been reported by some workers [20, 21]. As shown in the result, abattoir is rich in protease producing bacteria. This indicates that waste from abattoir has potential for screening of bacterial strain which produce industrially important enzymes.

Ten isolates had larger clear zone are screened. Out of these isolates, two isolates, Abc2 and Abc6 exhibited high capability of removing gelatin from used X-ray films within short period of time when compared to others (table 3). These all imply that this strain has potential for extraction of industrial important products like alkaline protease used as polyester recovery process.

Table 2 shows results of morphological and biochemical tests of the screened isolate. All the isolate were found to be Gram positive, citrate utilization positive and catalase positive. Motile and rod shaped cells of all potential isolate were observed under light microscope with 1000X magnification. The strain was able to hydrolyze starch and casein.

As shown in fig 4, used X-Ray films treated with protease produced from Abc2 and Abc5 isolate resulted in the silver bound with gelatin being stripped off in to the reaction mixture after 120 and 140 min respectively, at 30^{o}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 (10ml of bicarbonate buffer only) at the same condition. 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 researchers [22, 23, 24, 25].

When the X-ray films treated with crude enzyme for about 120 and 140 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 researcher [26] and loss in weight after enzyme treatment was around 5% (w/w).

5. CONCLUSIONS

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.

REFERENCES

[1] Amandeep K., Seema A. and Geetika S. (2013). Isolation of Protease Producing Bacteria from Soil and Genomic Library Construction of Protease Gene. *Journal of Chemical, Biological and Physical Sciences*. 4(1):216-219.

[2] Onkar N.T., Thiyam B. Devi, Kangjam S.D., Gunapati O., Thingujam I., Keithellakpam O., Oinam A. and Lakreiphy N. (2015). Isolation and optimization of alkaline protease producing Bacteria from undisturbed soil of NE-region of India falling under Indo-Burma biodiversity hotspots. *Journal of Applied Biology & Biotechnology*. 3(4): 025-031

[3] Srinivas L.N., Dr.kopuri A., Sreevennela P. and–Venkataramanadevi Ch.D. (2013). Isolation and Biochemical characterization of protease isolated from *Bacillus sp* SVN12. *International Journal of Research in Pure and Applied Microbiology*. 3(3): 94-101

[4] Soundra J. F., Ramya V. S., Neelam D., Suresh B.G., Siddalingeshwara K. G., Venugopal N. and Vishwanatha T. (2012). Isolation, production and characterization of protease from Bacillus Spisolated from soil sample. *Journal of Microbiology and Biotechnology Research*. 2 (1):163-168

[5] Abebe B., Abrham S., Genet A., Hiwot G., Paulo s., Melese A. (2014).Isolation, optimization and characterization of protease producing bacteria from soil and water in Gondar town, North West Ethiopia. *International Journal of Bacteriology, Virology and Immunology*. 1(3): 020-024

[6] Amira H.A. and Eida M.A. (2016).Recovery of silver from used X-ray film using alkaline protease from Bacillus subtilissub sp. Subtilis. *African Journal of Biotechnology*. 15(26):1413-1416

[7] Bholay A.D., More S.Y., Patil V.B. and patiln I. (2012). Bacterial Extracellular Alkaline Proteases and its Industrial Applications. *International Research Journal of Biological Sciences*. 1 (7):1-5,

[8] Singha l. P., Nigam V. K., Vidyarthi A. S. (2012). Student on production, characterization and application of microbial protease. *International Journal of Advanced Biotechnology and Research*. 3 (3): 653-669

[9] Mohammed Seid (2011). Production, characterization, and potential application of akeratinolytic alkaline protease produced by alkaliphlic cvibriosp. Msc. Thesis. Addis Abeba, Ethiopia.

[10] Aishwarya R., Nallusamysivas k. and Reginald F. (2016). A brief review waste-potential low cost substrate for Bacterial Protease Production. *The Open Biotechnology Journal*.10: 335-341.

[11] Jayant P. P., Pallavi S. P., PatilI D. and Umesh B. D. (2015). Extraction of Silver from waste x-ray films using protease enzyme. *International Journal of Advanced Biotechnology and Research*. 6: 220-226.

[12] Temam A. H. (2017). Bacterial Protease Enzyme: Safe and Good Alternative for Industrial and Commercial Use International Journal of Chemical and Biomolecular Science. 3(1): 1-10

[13] Vaishali C. (2012). Production, isolation and characterization of alkaline protease from Aspergillus versicolor PF/F/107. *Journal Academic Industrial Research*. 1(5):272-277

[14] Ismail H. and Ahmed M. (2010). Isolation of protease producing bacterial strain from Jazan province and study of effect of the temperature on the production of enzyme. Msc Thesis. Jazan University, Saud Arebia.

[15] https://en.m.wikipedia.org/wikipedia.org/wiki/Arba _Minch, on January, 16,2017.

[16] Bergey, D.H. and Holt, J.G. (1994). Bergey's manual of determinative bacteriology, 9th edition Williams and Wilkins, Baltimore.

[17] Daniel Y., Melaku A., Ameha K. (2016). Production and characterization of bacterial protease from isolates of soil and agroindustrial wastes. *IJIPSR* .4 (9):977-993

[18] Ekhlas Md., Uddin, Pulak .M, Hossain .Md. F,Firoz .Md .A (2014). Isolation and characterization of proteases enzyme from locally isolated *Bacillus sp*.2(6): 338-344.

[19] Daniel Y. L. (2017). Optimization and Partial Characterization of *Bacillus* Protease Isolated from Soil and Agro-industrial Wastes . *International Journal of Nutrition and Food Sciences*. 6(1): 31-38

[20] Boominadhan, U., Rajakumar, R., Karrpaga, P.V.S. and Joe, M.M. (2009). Optimization of protease enzyme production using *Bacillus sp*. Isolated from different wastes. *Botany Research International*, 2:83-87.

[21] Haile, G. and Gessesse, A. (2012). Properties of alkaline protease C45 produced by alkaliphilic *Bacillus sp*. isolated from Chitu, Ethiopian Soda Lake. *Journal of Biotechnology and Biomatter*, 2:1-4.

[22] Masui, A., Fujiwara, N., Takagi, M. and Imanaka, T. (1999). Feasibility study for decomposition of gelatin layers on X-ray films by thermostable alkaline protease from alkaliphilic *Bacillus sp*. *Biotechnological Techniques*, 13:813-815.

[23] Nakiboglu, N., Toscali, D. and Yasa, I. (2001). Silver recovery from waste photographic films by an enzymatic method. *Turkish Journal of Chemistry*, 25:349-353.

[24] Pathak, A.P. and Deshmukh, K.B. (2012). Alkaline protease production, extraction and characterization from alkaliphilic *Bacillus licheniformis* KBDL4: A Lonar Soda Lake isolate. *Indian Journal of Experimental Biology*, 50:569-576

[25] Annapurna, S.A., Singh, A., Garg, S., Kumar, A. and Kumar, H. (2012). Screening, isolation and characterization of protease producing moderately halophilic microorganisms. *Asian Journal of Microbiology, Biotechnology and Environmental Science*, 14:603-612.

[26] Shankar, S., More, S.V. and Seeta, L.R. (2010). Recovery of silver from waste x-ray film by alkaline protease from *Conidiobolus coronatus*. *Kathmandu University Journal of Science, Engineering and Technology*, 6:60-69.