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ALKALINE PROTEASE OF *BACILLUS ALTITUDINIS* GVC11 FOR GELATIN HYDROLYSIS AND SILVER RECOVERY FROM USED X-RAY FILMS

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ABSTRACT

Silver is a precious metal used in photographic/X-ray film industry. Around 18-20% of the world's silver needs are supplied by recycling of photographic waste. Emulsion layer on X-ray film contains silver and gelatin, which can possibly be broken down using proteases and release the silver into the solution. In the present study alkaline protease from *Bacillus altitudinis* GVC11 was studied for silver recovery from used X-ray films. Various parameters such as pH, temperature, enzyme concentration, silver recovery, reutilization of enzyme were studied. 1000 U/mL of alkaline protease removed gelatin completely within 30 min at pH 10 and 40°C. Enzyme was reutilized 6 times effectively for gelatin removal and recovery of silver from X-ray films. Loss in weight of X-ray film after treatment with enzyme was 4% and 10% of silver recovered in hydrolyzate per cycle based on the initial weight of the film.

KEY WORDS: alkaline protease, X-ray film, silver recovery, gelatin, *Bacillus altitudinis* GVC11.

INTRODUCTION

Silver is precious metal used for many purposes particularly in photographic industry. X-ray films are poly-ethylene terephthalate sheets coated by radioactive material on both sides.X-ray films consist of halides of silver crystals like silver chloride in emulsion layer.They are used for the purpose of medical diagnosis such as chest X-rays, CT scans, mammograms etc.[1, 2] X-ray films contain 1.5 -2% (w/w) of silver in its gelatin layer.[3,4,5] X-ray waste includes of used X-ray films spent fixer, spent developer, water rinse and chemical vapors. Used X-ray films and spent fixers are considered as hazardous waste due to high silver content.[6]

With increasing demand for silver, secondary sources (scraps and other forms of wastes) have been focused for silver recovery. Used X-ray films are good secondary sources for silver recovery than other types of films due to presence of considerable amount of silver in them.[7] Nearly 55-65% of metallic silver is still present in used X-ray films.[6] Nearly 25% of world silver demand is met by recycling. Out of this 75% of silver recycled is obtained from used X-ray films[3]. Various methods have been adapted for silver recovery from used X-ray films. But four methods are commonly used to leach silver from used X-ray films. These are burning the film directly, oxidation of the metallic silver following electrolysis, chemical treatment and stripping up of gelatin-silver layer by enzymatic methods.[8]

Physical and chemical treatment methods recover only low quantity of silver. Silver recovery by burning of X-ray film is conventional method which generates foul smell, cause environmental pollution and film is not recovered.[5] In chemical methods such as acid leaching process, X- ray films are submerged into the chemical solution in order to extract the gelatin and silver. Physical and chemical methods for silver recovery from X-ray films are very expensive, time consuming and cause pollution.[1] Due to these reasons, methods applied for processing of used X-ray films should be eco-friendly and cost effective. Biological methods are alternative approaches applied to recover silver from used X-ray films and have minimal impact on the environment. Enzymatic methods specifically hydrolyze the gelatin layer where silver is recovered into solution within short period of time.[9,10] In the present study alkaline protease from *Bacillus altitudinis* GVC11 is used for silver recovery from used X-ray films and appears to be better than earlier reports.

MATERIALS AND METHODS

Processing of used X-ray films

Used X-ray films were collected from local hospitals, washed with distilled water and dried at 40^0C.

Microorganism and enzyme production

Alkaline protease production was carried out in 250 mL conical flasks containing 100 mL mineral salts medium with fish scales as substrate. Flasks were inoculated with

2% (v/v) actively growing broth culture of *Bacillus altitudinis* GVC11 (containing 10^8 cells/ml) and incubated at 37°C, 200 rpm in a rotary shaker.[11] All the experiments were carried out in triplicates, thrice at different occasions and results presented are mean values of the same.

Alkaline protease extraction and assay: The fermented broth was centrifuged at 15,000 rpm for 15 min at 4°C. Supernatant obtained was used as enzyme source. Alkaline protease activity was determined with alkali soluble casein as substrate according to the method described previously.[11] One unit (U) of alkaline protease is defined as the amount of enzyme which releases l µg tyrosine per minute under standard assay conditions.

Effect of temperature and pH on enzyme for silver recovery from used X-ray films: Determination of optimum temperature and pH for hydrolysis of gelatin was studied at temperature ranging from 30 to 50°C and pH ranging from 8 to 11. Alkaline protease hydrolyses gelatin layer and removes from X-ray film, which results in increased turbidity. Turbidity of the reaction mixture (hydrolysate) increased with time (as the hydrolysis progressed) and no further increase in turbidity was observed when hydrolysis was complete. Hence, progress of hydrolysis i.e. increase in turbidity was monitored by measuring the absorbance at 660 nm. Samples were removed at regular intervals and time required for complete removal of gelatin layer was noted.

Effect of enzyme concentration on silver recovery from used X-ray films: Effect of enzyme concentration on gelatin removal was measured by incubating 0.2 g of X-ray film (2x2 cm) with 10mL of enzyme in glycine-NaOH buffer at 40°C and pH 10 with varying concentrations of enzyme 200-1000 U/mL. Samples were removed at regular intervals and absorbance was measured at 660 nm. Reaction was carried out until the absorbance was stable which indicates complete removal of gelatin from the X-ray film and time required was noted.

Effect of enzyme diluents on recovery of silver
Enzyme was diluted in tap water, distilled water and glycine-NaOH buffer, removal of gelatin from X-ray film was observed at 40°C and pH 10. Time required for complete removal of gelatin layer or silver recovery was noted in each case.

Recycling use of enzyme for gelatin removal
Alkaline protease was used repeatedly for removal of gelatin from the X-ray film at 40°C, pH 10 and 200 rpm on shaker incubator for 30 min. After the hydrolysis of gelatin from the X-ray film, they were removed from the flask and new films were added to same enzyme, then further reaction was carried out for reuse of the enzyme. After treatment the X-ray film was dried and reduction in its weight was recorded in each case.

Silver recovery: 0.2 g of X-ray film (2x2 cm) was treated with 10mL enzyme (1000 U/mL) for 30 min at 40°C, pH 10 and 200 rpm on shaker incubator. After complete removal of gelatin layer from X-ray film, reaction mixture was used for determination of silver concentration by AAS.[12]

RESULTS AND DISCUSSION

Table 1- Gelatin removal and silver recovery from X-ray film by repeated use of alkaline protease from *Bacillus altitudinis* GVC11

Run	Loss in weight after 30 min treatment based on the initial weight of the film (w/w)	Observation
1	4%	Gelatin layer completely removed and clean X-ray films obtained
2	4%	Gelatin layer completely removed and clean X-ray films obtained
3	4%	Gelatin layer completely removed and clean X-ray films obtained
4	4%	Gelatin layer completely removed and clean X-ray films obtained
5	4%	Gelatin layer completely removed and clean X-ray films obtained
6	3%	Gelatin layer completely removed and clean X-ray films obtained
7	3%	Almost clean X-ray films obtained

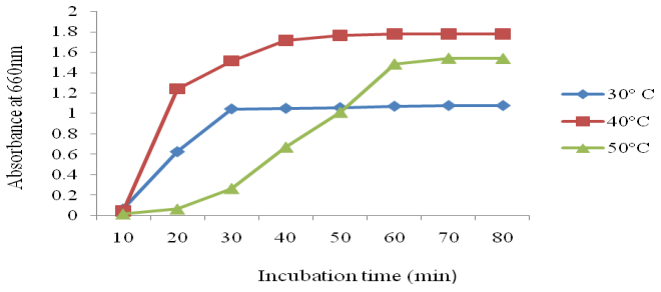


Fig. 1 Effect of temperature on silver recovery from X-ray films by alkaline protease from *Bacillus altitudinis* GVC11

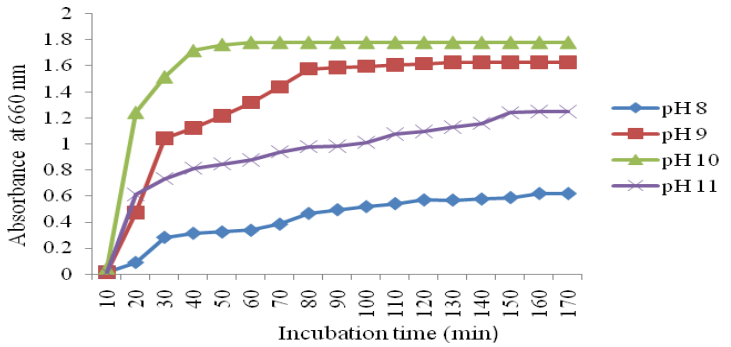


Fig. 2 Effect of pH on silver recovery from X-ray films by alkaline protease from *Bacillus altitudinis* GVC11

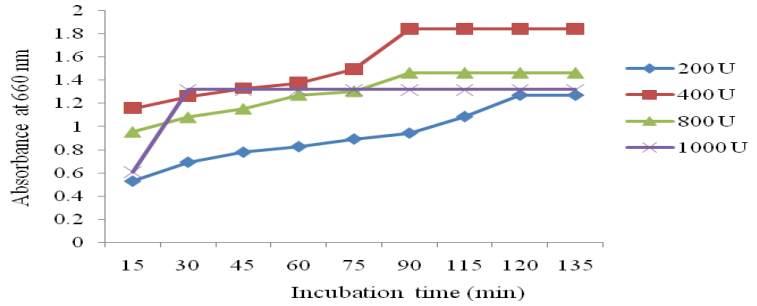


Fig. 3 Effect of enzyme concentration on silver recovery from X-ray films by alkaline protease from *Bacillus altitudinis* GVC11

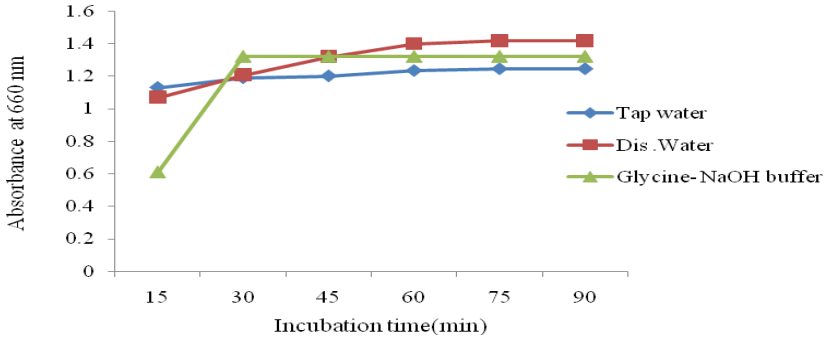
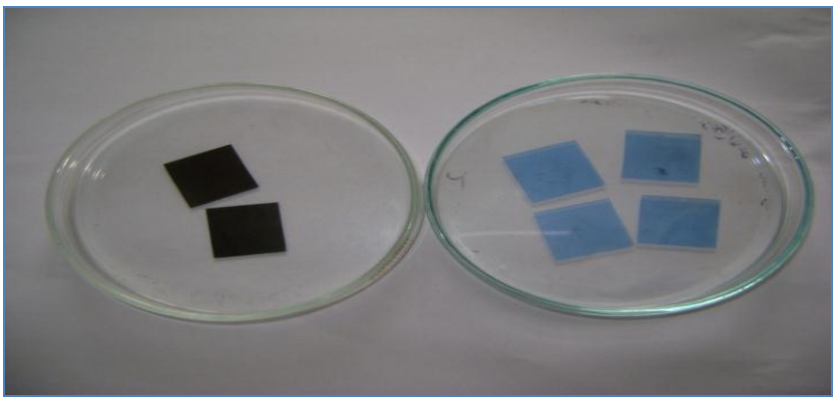


Fig. 4 Effect of dilution on silver recovery from X-ray films by alkaline protease from *Bacillus altitudinis* GVC11



Control (before treatment with enzyme) After treatment with enzyme

Fig. 5 Removal of gelatin from X-ray films by treatment with alkaline protease from *Bacillus altitudinis* GVC11

Effect of pH and Temperature for gelatin removal and silver recovery from used X-ray films

Silver recovery is associated with gelatin removal in X-ray films. Increase in absorbance in enzyme hydrolyzate due to gelatin removal is direct indication for silver recovery. The process of silver recovery from X- ray films was studied at different pH (8-11) and temperature (30-50°C). Gelatin removal and silver recovery were increased with increase in temperature from 30°C to 40°C and further increase resulted in increased time. Maximum gelatin removal was observed at 40°C in 30 min. At higher and lower temperatures complete removal of gelatin took more than 30 min (Fig. 1). pH 10 was observed to be optimum for removal of gelatin from X-ray films (Fig. 2). As gelatin hydrolysis was optimum at 40°C and pH 10, all further experiments were carried out under these conditions. Masui *et al*., (1999) observed that alkaline protease of *Bacillus* sp. A187P mutant removed gelatin layer completely from X-ray film in 45 min at 50°C.[13] Protease of *Purpureocillium lilacinum* LPS # 876 hydrolysed gelatin layer in 33 min at 37°C[1]. *Bacillus thuringiensis* spp. able to hydrolyze the gelatin and silver recovery from X- ray films with in 60 min at 40°C [14]

Effect of enzyme concentration on silver recovery from used X-ray films

Effect of enzyme concentration on silver recovery from X- ray film was studied at 40°C and pH 10. As enzyme concentration increased from 200-1000 U/mL time for complete removal of gelatin decreased. Maximum removal of gelatin and silver recovery was observed with 1000 U/mL enzyme in 30 min (Fig. 3). Shankar *et al*.,(2010) and Ivana*et al.*, (2013) also reported increased concentration of enzyme is enhancing the removal of gelatin layer from X-ray films in shorter time.

Effect of enzyme diluents on silver recovery

Different diluents such as distilled water, tap water and glycine-NaOH buffer were used to observe the gelatin removal and silver recovery using alkaline protease of *Bacillus altitudinis* GVC11. It was observed that removal of gelatin and silver recovery from X-ray film was more with enzyme solution in glycine-NaOH buffer than tap water and distilled water (Fig. 4).Effect of enzyme diluents on silver recovery from X-ray film was studied by Manjusha(2011) and distilled water was found to be the better diluent.[15] The present enzyme is effective in buffered conditions which may be stabilizing the catalytic potential of the enzyme.

Repeated use of enzyme for gelatin layer removal

Alkaline protease was repeatedly used for removing gelatin layer and silver recovery from X-ray films in successive cycles. It is apparent that the alkaline protease of *Bacillus altitudinis* GVC11 is more effective for repeated application in removal of gelatin and silver recovery from used X-ray films. In earlier reports, alkaline protease was active up to 4 cycles as observed by Shankar *et al*., (2010)[3]. Repeated use of alkaline protease from *Vibrio* sp. (V26) was effective up to 3 cycles for removal of gelatin and silver recovery from X-ray films.[15] Relatively alkaline protease from *Purpureocillium lilacinum* LPS # 876 has been efficiently reutilized up to 3 successive runs[1]. Masui etal. (1999) and Shankar et al. (2010) observed that the treatment time was increased after every reuse of the enzyme. It was noted that first run took 60 min for complete hydrolysis of gelatin and the second run required more than 2h.[3] In the present study, reuse of alkaline protease removed gelatin and recovered silver within 30 min till 6 cycles and further cycles took more than 30 min (Table 1). It is a significant observation as compared to earlier reports.

Recovery of silver

X-ray films treated with alkaline protease result in removal of gelatin and silver into the reaction mixture which resulted in clean plastic film (Fig. 5). The loss in weight of X-ray film after the treatment was found to be 4% (w/w) based on the X-ray film weight and 10% of silver was recovered per cycle. Silver from the hydrolyzate was recovered either as metallic silver or as silver halide which can be reutilized. Recovery of silver using alkaline protease of *Bacillus altitudinis* GVC11 may be an ideal approach for large scale application.

CONCLUSION

In this study we report the silver recovery from used X-ray films using alkaline protease by alkaliphilic *Bacillus altitudinis* GVC11. Above observations show that this alkaline protease can be repeatedly used for gelatin removal and silver recovery from used X-ray films. At 40°C and pH 10 enzyme was reused for 6 cycles efficiently for silver recovery in 30 min. This enzyme can become an ideal biocatalyst for recovery of silver from used X-ray films in large scale.

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