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RECOVERY OF SILVER FROM WASTE X-RAY FILM BY ALKALINE PROTEASE FROM *CONIDIOBOLUS CORONATUS*

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ABSTRACT
The waste X-ray/ photographic films contain 1.5 - 2 % (w/w) black metallic silver which is recovered and reused. Around 18-20% of the world's silver needs are supplied by recycling photographic waste. Since silver is linked to gelatin in the emulsion layer, it is possible to break the same and release the silver using proteolytic enzymes. Alkaline protease from *Conidiobolus coronatus* was investigated for enzymatic hydrolysis of gelatin from waste X-ray films. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean and silver was recovered in the hydrolysate, both of which can be reused. Various parameters such as pH, temperature, enzyme concentration, time etc on silver removal from the film were studied. Gelatin hydrolysis was monitored by measuring increase in turbidity in the hydrolysate, which was accompanied by release of protein and hydroxyproline. Gelatin layer was stripped completely within 6 min with 1.35 U ml^{-1} of protease at 40˚C, pH 10. Rate of gelatin hydrolysis increased with increased in protease concentration. The enzyme could be effectively reused for four cycles of gelatin hydrolysis. Silver in hydolysate was around 3.87 % (w/w) based on total weight of sludge.

Key words: Silver recovery, X-ray films, gelatin hydrolysis, alkaline protease, *Conidiobolus coronatus*

INTRODUCTION
Silver is one of the precious and noble metals used in large quantities for many purposes, particularly in the photographic industry. The waste X-ray/photographic films containing black metallic silver spread in gelatin are very good source for silver recovery compared to other types of film. The amount of silver in the X-ray film varies between 1.5 and 2.0% (w/w). It has been reported that 25% of the world's silver needs are supplied by recycling out of which 75% is obtained from photographic waste. With an increasing demand for silver in the world, recent attention is focused on X-ray/photographic films as one of the secondary sources of silver owing to the considerable amount of silver present in them.

Various studies have been carried out to recover the silver from photographic/X-ray film wastes and following methods are reported in literature: (a) burning the films directly (b) oxidation of the metallic silver following electrolysis (c) stripping the gelatin-silver layer using different chemical solutions (d) enzymatic hydrolysis of gelatin (Nakiboglu *et al.,* 2003). Recovery of silver by burning the films directly, a conventional method used at present is the most primitive method and generates undesirable foul smell. The method causes environmental pollution and polyester film on

which emulsion of silver and gelatin is coated cannot be recovered. Stripping the gelatin-silver layer by chemical methods using ammonium thiosulphate, sodium thiosulphate, nitric acid or reagents such as sodium cyanide, NaOH, nitric acid or organic compounds cause environmental hazards and are either time consuming or very expensive while the use of NaOH at high temperatures poses a serious industrial safety problem. For this reason, the methods applied to recover silver from X-ray/photographic waste should be cost effective and have minimal impact on environment and enzyme based methods can be an alternative option.

Gelatin is a protein from animal collagen which contains a large number of glycine, proline and 4-hydroxyproline residues. Since the emulsion layer on X-ray film contains silver and gelatin, it is possible to break down the gelatin layer using proteases and release the silver (Nakiboglu *et al.,* 2001). The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the recovery of the silver, but also the polyester base which can be recycled. Hence in recent years, enzymatic methods using microbial proteases are being explored as alternatives to the burning and oxidation methods of silver recovery from photographic/X-ray films (Singh *et al.,* 1999; Ingale *et al.,* 2002; Nakiboglu *et al.,* 2003*;* Masui *et al.,* 2004). Basically enzymatic processes are more specific and remove gelatin layer from X-ray film in few minutes without damaging the polyester film base. Gelatin molecules are cross linked with hardners and it is difficult for the usual proteases to degrade it in a short time. Most of the proteases used so far for silver recovery are of bacterial origin and there is only one report on use of fungal alkaline protease.

Conidiobolus coronatus ATCC PTA-4132 secretes high levels of alkaline protease in 2-3 days. Optimization and scale up of production of alkaline protease in fermentor is reported earlier (Laxman *et al.,* 2005). This enzyme has been extensively evaluated in tanneries and finds applications in pre-tanning operations in leather manufacture (Laxman *et al.,* 2007). The objective of the present work is to remove the gelatin layer from waste X-ray film and recover both silver as well as the polyester film in an eco-friendly manner. The present paper describes the use of alkaline protease from *Conidiobolus coronatus* for silver removal from X-rays films and its recovery for reuse.

MATERIALS AND METHODS

Malt extract, yeast extract and peptone were obtained from Hi Media Chemicals, India. Hammerstein casein was obtained from Sisco Research Laboratories, India. All other chemicals were of analytical grade. X-ray films were obtained from local medical center.

Cultural conditions and production of enzyme

The organism was routinely sub-cultured and maintained on MGYP (malt extract-3; glucose-10; yeast extract -3; peptone -5, all ingredients in g l^{-1}) agar slants and preserved between 15 to 28˚C. Enzyme production was carried out as described earlier in 500 ml Erlenmeyer flasks containing 100 ml medium (Laxman *et al.,* 2005). After 72 h of growth in MGYP broth containing 2% (w/v) soyabean meal, the cell-free supernatant (obtained by centrifugation at 8000 × *g*, 4˚C, 20 min) was used as source of enzyme for silver recovery studies.

Determination of alkaline protease activity

Protease activity was determined as described earlier at 40˚C, pH 10 (Laxman *et al.*, 2005).

Hydrolysis of gelatin and release of silver

Used X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40˚C for 30 min. One g of X-ray film (cut into 2 x 2 cm pieces) was then incubated with 10 ml of crude protease (such that the film is completely immersed in the solution) at 40˚C, pH 10 in a water bath with continuous shaking. 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. turbidity was monitored by measuring the absorbance at 660 nm. Samples were removed at 1 min intervals and time required for complete removal of gelatin layer was noted.

Effect of temperature & pH

Hydrolysis was carried out as described earlier with 1g film in 10 ml of crude enzyme having protease activity of 1.35 U ml^{-1}. For determination of optimum temperature, gelatin hydrolysis was carried out at pH 10 and temperatures ranging from 30 to 60˚C. Optimum pH for hydrolysis of gelatin was studied at 40˚C and pH values ranging from 7 to 11.

Effect of enzyme concentration & time course of hydrolysis

Effect of enzyme concentration on hydrolysis of gelatin was measured by incubating 1 g film (2 x 2 cm pieces) with 10 ml of enzyme at 40˚C and pH 10 and protease activities ranging from 0 to 2.7 U ml^{-1}. Samples were removed at an interval of 5 min until the gelatin layer was completely stripped off and time required for complete removal was noted. Extent of hydrolysis was expressed as percentage compared to the highest absorbance which was taken as 100%. For time course of gelatin removal, the film was incubated with 10 ml of 0.9 U ml^{-1} of protease at 40˚C and pH 10. Samples were removed at 1 min intervals and increase in turbidity due to gelatin removal was measured at 660 nm.

Release of protein & hydroxyproline during hydrolysis

Hydroxyproline and protein released during gelatin hydrolysis was monitored. Protein was estimated according to Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard. Hydroxyproline was estimated by the method of Neuman and Logan (1950). The purple colour formed was measured at 540 nm and hydroxyproline content was calculated from a standard curve.

Reuse of enzyme for hydrolysis

Reuse of enzyme for gelatin hydrolysis and silver removal was carried out at three enzyme concentrations. One g (2 x 2 cm pieces) of X-ray film was incubated at 40˚C, pH 10 with 10 ml of

protease having 0.18, 0.45 and 0.9 U ml^{-1}. After complete removal of gelatin, old X-ray film was removed and fresh film (1 g) was added to the same enzyme solution and incubation continued till complete removal of gelatin was observed. The process was repeated till gelatin hydrolysis stopped. Time required for complete gelatin removal in each case was noted.

Recovery of silver

Five grams of X-ray film was incubated with 50 ml of 1.35 U ml^{-1} of protease at 40˚C, pH 10, and 200 rpm on a rotary shaker. After complete gelatin removal, reaction slurry containing gelatin and silver was acid digested and used for analysis of metals by AAS-Chemito-201.

RESULTS AND DISCUSSION

Effect of temperature & pH

Effect of temperature and pH was studied at fixed protease concentration of 1.35 U ml^{-1}. Gelatin hydrolysis increased with increase in temperature and reached a maximum at 40˚**C** and decreased thereafter (Fig.1). Removal was complete within 6 min at 40˚**C**, while at higher temperatures it was incomplete possibly due to inactivation of protease. No hydrolysis observed at lower temperatures. Gelatin removal was observed over a wide pH range of 7 to 11 with maximum hydrolysis at pH 10 (Fig. 2). Hydrolysis was complete within 6 min at pH 10 while it was complete in 7 min at other pH values. Singh *et al.* (1999) reported gelatin hydrolysis of X-ray film by a bacterial protease within 8 min at 60˚**C**, pH 11-12. Degradation took longer time at pH 7-10 or at 30˚**C** and 50˚**C**. Complete hydrolysis was achieved by *Conidiobolous coronatus* protease at 40˚**C** where less heating energy would be required. Ishikawa *et al.* (1993) showed that the time required for complete gelatin hydrolysis of X-ray film was the shortest and the rate of hydrolysis was highest at pH 10.5 by protease from *Bacillus* sp. B21-2. Masui *et al.* (1999) reported 60 min for the complete hydrolysis of gelatin layer at 50˚**C**, pH 10.5. Nakiboglu *et al.* (2001) reported that the protease from *Bacillus subtilis* ATCC 6633 takes less than 15 min to decompose the gelatin layers at 50˚**C** while the enzyme was rapidly inactivated at 60˚**C**. As gelatin hydrolysis was optimum at 40˚**C** and pH 10, all further the experiments were carried out under these conditions.

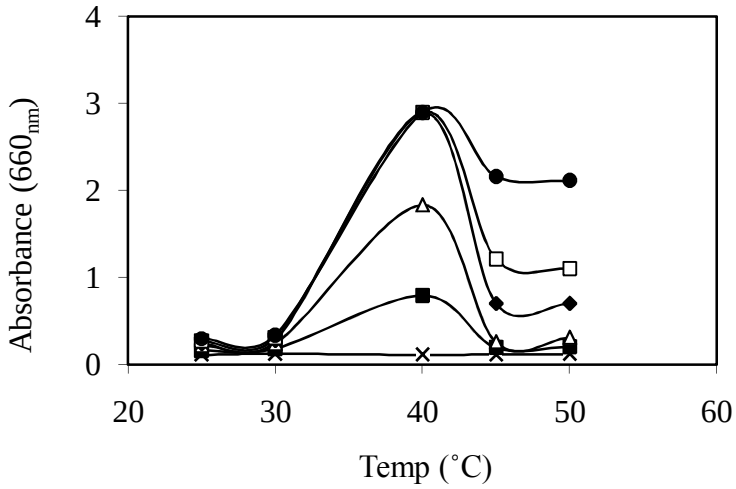

Fig. 1: Effect of temperature on gelatin hydrolysis: 0 min (x), 4 min (■), 5 min (△), 6 min (◆), 7 min (□) and 8 min (●).

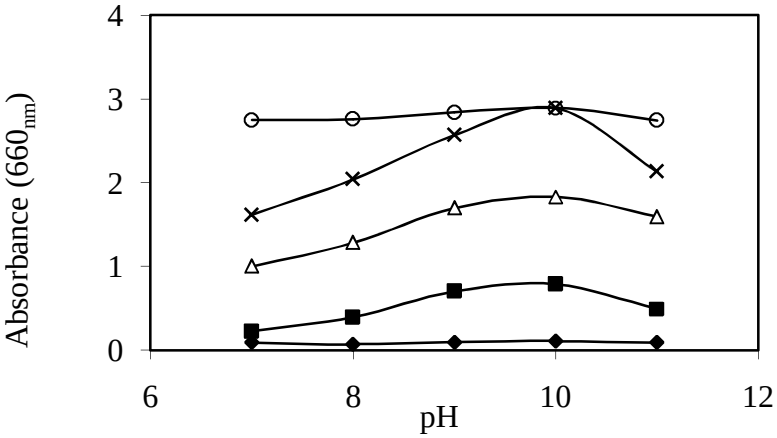

Fig. 2: Effect of pH on gelatin hydrolysis: 0 min (◆), 4 min (■), 5 min (△), 6 min (x) and 7 min (○).

Effect of enzyme concentration

Effect of protease concentration on hydrolysis of gelatin was studied at 40˚**C** and pH 10. Higher the enzyme concentration, greater was the hydrolysis rate. It was observed that gelatin layer was stripped completely within 10 min when enzyme concentration ranged from 0.81 to 2.7 U ml^{-1} (Fig. 3).

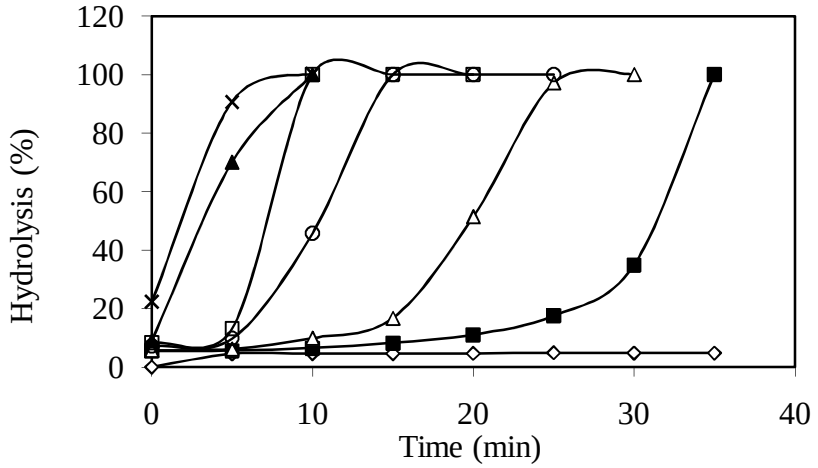

Fig. 3: Effect of enzyme concentration on gelatin hydrolysis: 0 U ml^{-1} (◇), 0.16 U ml^{-1}(■), 0.32 U ml^{-1} (△), 0.49 U ml^{-1} (○), 0.81 U ml^{-1} (□), 1.35 U ml^{-1} (▲) and 2.7 U ml^{-1} (x).

Hydrolysis percentages were 13, 70 and 90 for 0.81, 1.35 and 2.7 U ml^{-1} respectively at the end of 5 min. (Hydrolysis would have been complete in 6 minutes itself with 1.35 and 2.7 U ml^{-1} but not recorded as samples were removed at 5 min intervals). Longer incubations were required for complete removal of gelatin with lower enzyme concentrations. Complete gelatin removal occurred only after 35 min with the lowest enzyme concentration of 0.16 U ml^{-1}. Ingale *et al.* (2002) reported use of 50 U ml^{-1} of protease from *Basidiobolus* for complete hydrolysis. Lower enzymes concentrations of 10 U ml^{-1} required 12 min while degradation was complete within 5 min with higher enzyme concentration of 90 U ml^{-1}. Masui *et al.* (2004) 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^{-7}g cm^{-3}) to be temperature dependent and was between 8-10 min at 50˚**C** , pH 10.5. Singh *et al.* (1999) reported complete gelatin degradation in 24 min by 10 U ml^{-1} of alkaline protease while 8-12 min was required when the enzyme concentration was increased to 25-100 U ml^{-1}.

Time course of hydrolysis and release of protein and hydroxyproline

Nearly more than 80-90% of stripping off of gelatin from X-ray film was achieved in 6-7 min at pH 10 and 40˚**C**. Protein and hydroxyproline in the hydrolysate increased with progress of gelatin removal and the ratio of hydroxyproline to protein was nearly constant (Fig.4a). After complete stripping off of gelatin, hydroxyproline and protein concentrations ranged between 40-45 µg ml^{-1} and 160-170 µg ml^{-1} respectively. In all the experimental runs except in case of high enzyme concentrations, lag periods were observed when the hydrolysis was very slow. This could be due to slow adsorption of protease on to the surface. With time, the hydrolysis rates increased as a result of the increase in the amount of the enzyme adsorbed on the gelatin surface (Fig.4b).

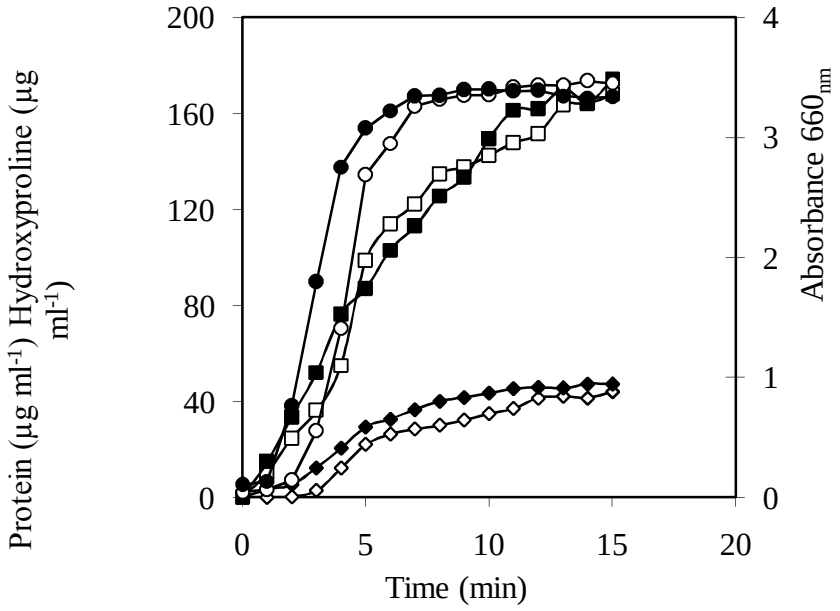

Fig. 4a: Time course of gelatin hydrolysis: hydrolysis (●○), protein (■□) and hydroxyproline (◆◇) released. Closed symbols- 2.7 U ml^{-1}; open symbols-1.35 U ml^{-1}.

Masui *et al.* (1999) studied decomposition of gelatin by proteases from alkalophilic *Bacillus* and its three mutants. They reported 0-17% gelatin degradation in 30 min depending on the enzyme (wild or mutants) while complete degradation was achieved in 45 min for all the enzymes tested at concentration of 5 U ml^{-1} and 50˚C, pH 10.5. They observed that swelling of gelatin on the X-ray film takes place during initial 30 min after which hydrolysis proceeds rapidly from 30-45 min. Fujiwara *et al.* (1989) reported complete breakdown of gelatin by an alkaline protease from alkalophilic *Bacillus* sp. B21-2 in 8 min at 40˚C, pH 10.5 and at enzyme concentration of 100 U ml^{-1} while all the alkaline proteases from the neutrophile *Bacillus subtilis* took more than 20 min to act. Subtilisin BPN, took 30 min to decompose the gelatin layer at 50-60˚C while treatment time increased to 120-180 min at 30˚C.

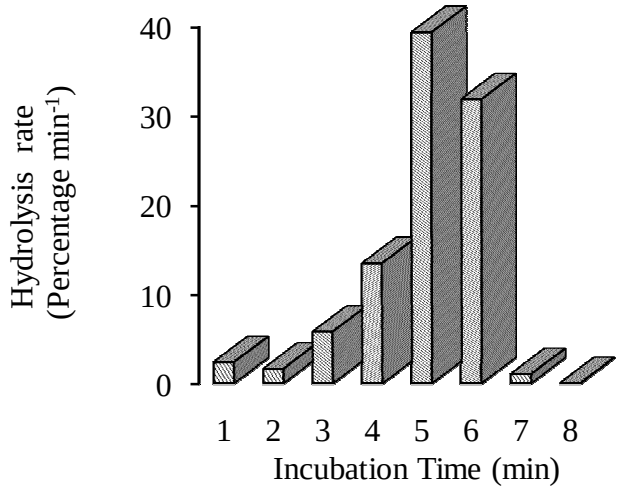

Fig. 4b: Rate of gelatin hydrolysis during the time course.

Reuse of enzyme for gelatin hydrolysis was evaluated at 40˚C, pH 10 and with protease concentrations varying from 0.18 to 0.9 U ml^{-1} in a water bath with continuous shaking until the gelatin layer was completely removed. With 0.9 U ml^{-1}, protease could be reused up to 4 times (Table 1).

Table 1: Reuse of enzyme for gelatin hydrolysis from waste X-ray film

Protease (U ml^{-1})	**Time required for complete gelatin removal (min)**			
	Cycle 1	**Cycle 2**	**Cycle 3**	**Cycle 4**
0.18	40	70	-	-
0.45	8	17	20	-
0.90	6	10	14	20

- Incomplete removal

Lowering the enzyme concentration resulted in longer reaction time for complete gelatin removal and also reduced the number of recycles. Repeated treatment of X-ray films for every 60 min was carried out at 50˚C with 5-20 U ml^{-1} of protease using wild type and A187P enzyme (Masui *et al.*, 1999). They reported that the treatment time increased after every reuse of enzyme. At an enzyme concentration of 5 U ml^{-1}, first decomposition was complete in 60 min while 2^{nd} use required more than 2 h. increasing the enzyme concentration, resulted in increased number of reuses (Masui *et al.*, 1999).

Recovery of silver

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 was recovered. The loss in weight after the treatment was around 5% (w/w) based on initial weight of the film. The silver content in the hydrolysate was determined by atomic absorption and corresponded to 3.87% (w/w) of the solid sludge and 0.2% (w/w) based on the weight of the X-ray film. Ingale *et al.* (2002) reported 0.1% (w/w) of silver recovery from photographic film. Apart from Ag, other metals in the slurry were also analyzed and only Mg was detected in trace amounts while metals like Fe, Cr, Cu, Al, Pb and Ni were not detected. Silver from the hydrolysate was recovered either as metallic silver or as silver chloride. Silver chloride can be used to make photographic paper, as pottery glazes, in photochromic lenses, in stained glass manufacture, in bandages and wound healing products.

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