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Enzymatic Hydrolysis of Gelatin Layers of X-Ray Films and Release of Silver Particles Using Keratinolytic Serine Proteases from *Purpureocillium lilacinum* LPS # 876

Ivana A. Cavello, Roque A. Hours, and Sebastián F. Cavalitto*

Research and Development Center for Industrial Fermentations, CINDEFI (CONICET - La Plata, UNLP), Calle 47 y 115, (B1900ASH), La Plata, Argentina

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**Corresponding author*
Phone: +54-221-483-3794;
Fax: +54-221-483-3794, ext. 103;
E-mail: cavali@biotec.org.ar

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Enzymatic decomposition of gelatin layers on used X-ray films and repeated utilization of the enzyme for potential application in silver recovery were investigated using keratinolytic serine proteases from *Purpureocillium lilacinum* LPS # 876. At pH 9.0, the enzymatic reaction was enhanced by the increase of enzyme concentration or by the increase of the temperature up to 60°C. Under the conditions of 6.9 U/ml, 60°C, and pH 9.0, hydrolysis of the gelatin layers and the resulting release of silver particles were achieved within 6 min. The protective effect of polyols against thermal denaturation was investigated. The presence of glycerol and propylene glycol increased enzyme stability. When the reusability of the enzyme for gelatin hydrolysis was tested, it could be seen that it could be effectively reused for more cycles when glycerol was added, compared with the enzyme without protective agents. The results of these repeated treatments suggested that a continuous process of recycling silver from used X-ray is feasible. Keeping in mind that recycling is (at the present time) needed and imperative, it can be remarked that, in this research, three wastes were successfully used: hair waste in order to produce serine proteases; glycerol in order to enhance enzyme thermal stability; and used X-ray films in order to recover silver and PET films.

Keywords: Silver recovery, serine proteases, X-ray films, thermostability.

Introduction

X-ray film is a poly (ethylene terephthalate) (PET) sheet, coated on both sides by radioactive material, which is sensitive to light. It is used for industrial purposes, and medical and dental services. Around the world, more than 2 billion radiographs are taken each year, including chest X-rays, mammograms, CT scans, *etc*. [9]. The amount of silver in this kind of films varies between 1.5% and 2.0% by weight. According to this, the waste X-ray films (containing black metallic silver spread in gelatin) are a very good source for silver and PET recovery [13]. Several studies have been carried out over a long period of time to recover the silver from wastes radiographic films. Two typical ways to recover silver from used X-ray films are combustion technology and the acid leaching process. Used X-ray films are incinerated at high temperatures and the silver is recovered from ash by smelting and refining processes, whereas in the acid leaching process, they are submerged into a strong acid solution in order to extract the gelatine [8].

Although incineration of the used X-ray films is a conventional method used at the present for the recovery of silver, this method generates an undesirable foul smell, and the PET film cannot be recovered, adding to the environmental pollution caused. Moreover, this is an expensive method to recover silver because of the cost of maintaining the furnace and treating the effluent soot and smoke [15].

Therefore, there is a need to develop cost-effective and environmentally friendly methods to recover both silver and PET from X-ray/photographic waste. Since the emulsion layer on X-ray film contains silver spread in gelatin, it is

possible to break down this protein layer using proteolytic enzymes with the concomitant release of silver [13, 16]. The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the recovery of silver but also the PET base, which can be recycled. In recent years, just a few works using microbial proteases have been reported as an alternative to the burning and oxidation methods in the silver recovery from X-ray films [6, 12, 13, 15, 16]. Most of the proteases used for silver recovery are of bacterial origin; only Shankar *et al*. [15] reported the potential use of a fungal alkaline protease from *Conidiobolus coronatus* for this purpose.

Purpureocillium lilacinum (formerly *Paecilomyces lilacinus*) LPS # 876 was found to produce keratinolytic serine proteases when grown in liquid cultures using hair waste as substrate [3]. Hair waste is recognized as a solid waste generated after a hair-saving unhairing process in leather technology, and it is produced in large amounts in Buenos Aires Province, Argentine. At the present time, the unique option for this solid waste is its disposal, which results in an environmental problem and a loss of money for the tanneries because of the cost of it disposal. Biodegradation of hair waste by keratinolytic microorganisms represents an alternative for the bioconversion of keratin waste and generates value-added products, like enzymes that can even been used for the treatment of other industrial wastes such as X-ray film wastes.

In the present work, the decomposition of gelatin layers on X-ray films and the consequent silver removal were investigated using keratinolytic serine proteases produced by *P. lilacinum*. In addition, several studies were conducted in order to increase the thermostability of the crude enzyme with the aim to enhance the reuse of these enzymes in repeated cycles of gelatin hydrolysis and silver removal from used X-ray films at high temperatures.

Materials and Methods

Microorganism and Culture Conditions

P. lilacinum (formerly *Paecilomyces lilacinus*) LPS # 876 belonging to the Spegazzini Institute fungal culture collection (La Plata National University, Argentina) was used. It was maintained in potato-dextrose agar slants under mineral oil at 4°C. Cultures were performed in 500 ml Erlenmeyer flasks with 100 ml of medium containing (per liter) 10 g hair waste, 496 mg NaH_2PO_4, 2.486 g K_2HPO_4, 0.16 mg $FeCl_3{\cdot}6H_2O$, 0.13 mg $ZnCl_2$, 0.10 mg $MgCl_2$, 0.011 mg $CaCl_2$, 2 g Yeast extract, and 5 g glucose (pH 6.0). The culture flasks were autoclaved at 121°C for 15 min for sterilization and then, after cooling, inoculated with 2×10^7 conidia/ml. The cultures were incubated in an orbital shaker at 200 rpm and 28°C. After 111 h of cultivation, all the contents of each flask were withdrawn and centrifuged at 5,000 ×*g* and 4°C for 20 min in order to precipitate the fungal biomass. The cell-free supernatant was used as a source of enzyme for silver recovery and thermostability studies.

Determination of Proteolytic Activity

Protease activity was measured as described by Liggieri *et al*. [10] using azocasein as substrate, but with some modifications. An aliquot of 0.1 ml of the enzyme preparation, suitably diluted, was mixed with 0.250 ml of Tris-HCl buffer (100 mM, pH 9.0) containing 1% (w/v) azocasein, and incubated for 30 min at 37°C. The reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (10% (w/v)). After a further 15 min at room temperature, the mixture was centrifuged at 5,000 ×*g* for 10 min. A reaction blank was performed with 0.1 ml of heat-inactivated enzyme. One milliliter of 1 M NaOH was then added to 1 ml of the supernatant and the absorbance measured at 440 nm. One unit of protease activity was defined as an increase of 0.1 unit per minute in the absorbance at 440 nm under those experimental conditions. Azocasein was synthesized as described by Riffel *et al*. [14]. Determinations were performed at least in triplicate.

Effect of Temperature on Enzyme Stability of *P. lilacinum* Protease

The thermostability of the crude enzyme was examined by incubating it at different temperatures ranging from 37°C to 60°C for 180 min. Aliquots were withdrawn at desired time intervals, and the remaining protease activity was measured under standard assay conditions. The non-heated crude enzyme was taken as 100%.

The protective effects of propylene glycol (10% (v/v)), glycerol (10% (v/v)), sorbitol (10% (w/v)), and polyethylene glycol 4000 (PEG 4000, 10% (w/v)) on heat inactivation were also studied. The crude enzyme was incubated at 60°C with and without the chemicals mentioned above, and residual protease activity was measured at regular intervals under standard assay conditions. Experiments were performed at least in triplicate and data were expressed as means ± standard deviations.

The first-order rate constants for denaturation (k_d) of the enzyme at 60°C were obtained from Eq. (1) after fitting the residual proteolytic activities with respect to incubation time, using first-order non-linear regression

$$At/A_o = e^{-kdt} \quad (1)$$

where At is the enzyme activity at the time t, Ao is the initial enzyme activity, k_d is the first-order rate constant for denaturation, and t is the time.

The half-life ($t_{1/2}$) of the enzyme was obtained from Eq. (2).

$$t_{(1/2)} = \ln(2)/k_d \quad (2)$$

Recovery of Silver from X-Ray Films

Used X-ray films (cut into 2.5 × 2.5 cm pieces) were washed with distilled water, wiped with cotton impregnated with ethanol,

and dried at 40°C for 30 min. After that, 0.1627 g of X-ray film was incubated with 5 ml of crude enzyme (pH 9.0, 6.9 U/ml), such that the film was completely immersed in the solution, at 37°C in a water bath under continuous shaking. Aliquots were withdrawn periodically to monitor the progress of the reaction by measuring the change in turbidity of the reaction mixture at 660 nm. The hydrolysis time was defined as the time in which turbidity (OD_{660}) attained its maximum value [6, 15]. Measures at 660 nm were performed at least in triplicate and data were expressed as means ± standard deviations.

Effect of Temperature on the Hydrolysis of Gelatin Layers

In order to study the effect of temperature on the hydrolysis of the gelatin layers, several experiments were carried out as described earlier, at pH 9.0 and temperatures ranging from 28°C to 65°C. X-ray films were added to prewarmed crude enzyme, and the reaction was carried out with continuous orbital shaking. Samples were withdrawn at regular times to measure the change in turbidity of the reaction mixture at 660 nm. The extent of hydrolysis was expressed as the percentage compared with the highest absorbance, which was taken as 100%.

Effect of Enzyme Concentration on the Hydrolysis of Gelatin Layers

The effect of enzyme concentration on the hydrolysis of gelatin was measured by incubating 0.1627 g of X-ray film (2.5 × 2.5 cm pieces) with 5 ml of crude enzyme at 60°C and pH 9.0 and protease activity ranging from 1.7 to 34.5 U/ml. Samples were withdrawn at regular intervals until the gelatin layer was completely stripped off, and the time required for complete removal was noted. The extent of hydrolysis was expressed as the percentage compared with the highest absorbance, which was taken as 100%.

Reusability of the Crude Enzyme for Gelatin and Silver Removal from Used X-Ray Film

In order to study the reusability of the crude enzyme (how many times could it be reused) for gelatin hydrolysis and silver removal from the X-ray films, several experiments were performed using 6.9 U/ml of crude enzyme at 60°C. These experiments were also conducted using protective agents against thermal inactivation such as glycerol and propylene glycol (10% (v/v)). After complete removal of the gelatin, the enzyme-treated X-ray film was removed from the reaction mixture and a new untreated one was added to the same crude enzyme solution and the incubation continued until complete removal of gelatin was observed. This procedure was repeated until gelatin hydrolysis stopped. The time required for complete gelatin removal in each cycle was noted [15].

Weight Loss of Used X-Ray Film During Silver Recovery Process and Recovery of Silver

Weight loss of the X-ray films was analyzed by measuring the weight of used X-ray film before and after complete removal of gelatin layer from 2.5 × 2.5 cm of the film. Silver recovery was determined using the gravimetric determination described by Christian [4].

Results and Discussion

Purpureocillium lilacinum LPS # 876, a locally isolated fungus, produced extracellular serine proteases with keratinolytic activity when the microorganism was cultured batchwise in a low-cost process using hair waste as substrate [3]. It was demonstrated that the crude enzyme has potential use in detergent industries, because of it compatibility with several commercial laundry detergents and it also showed a good washing performance [3]. In this paper, the crude enzyme (6.9 U/ml) was assessed for it potential application in the decomposition of gelatin layers and silver recovery from used X-ray films.

Effect of Temperature on Enzyme Stability of *P. lilacinum* Protease

Studies on the thermostability of the enzyme at temperatures ranging from 37°C to 60°C revealed that it was very stable at low temperatures up to 40°C, and for temperatures above 40°C, heat inactivation displayed typical first-order kinetics (Fig. 1A). At 50°C, the enzyme had a half-life ($t_{1/2}$) of 70 min, whereas at 60°C the $t_{1/2}$ was 12 min. Comparing with others reports, *P. lilacinum* protease seems to be more stable than the alkaline protease of *Conidiobolus coronatus* NCL 86.8.20 that was completely inactivated at 50°C after 1 h of incubation [2] or more stable than the proteases reported by Nakiboğlu *et al*. [13] (at 60°C, these enzymes were rapidly inactivated).

It was observed that there was a noteworthy increase in the protease half-life ($t_{1/2}$) at 60°C when polyols were added. Compared with the control, the $t_{1/2}$ was increased around 2.1- and 1.5-fold when glycerol and propylene glycol were added, respectively. PEG 4000 and sorbitol slightly enhanced the enzyme half-life ($t_{1/2}$) (Fig. 1B).

As can be seen in Table 1, the k_d value for crude enzyme was significantly higher without polyols than in the presence of glycerol or of propylene glycol, suggesting a protective effect of these polyols against thermal inactivation, whereas the k_d in the presence of sorbitol or PEG 4000 was slightly lower. Another thermal inactivation parameter that is generally used to represent a first-order reaction is the D value, which is the time needed for a 10-fold reduction of the initial activity at a given temperature, and it is obtained by plotting activity values on a log scale against the corresponding inactivation times. The higher D values in the presence of glycerol or propylene glycol represent the

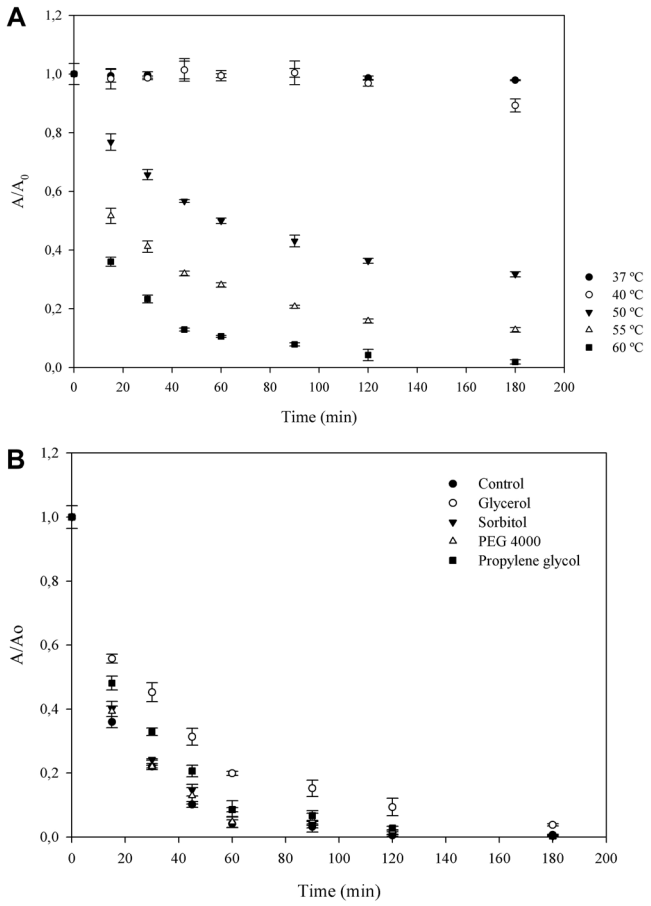


Fig. 1. Temperature and polyols effects on *Purpureocillium lilacinum* LPS # 876 protease activity.

(**A**) Residual activity (A/A_0) of *P. lilacinum* protease at different temperatures: (●) 37°C, (○) 40°C, (▼) 50°C, (△) 55°C, and (■) 60°C. (**B**) Residual activity (A/A_0) of *P. lilacinum* protease submitted to heating at 60 °C, fitted to first-order model, in the presence or absence of polyols: (●) Control, (○) Glycerol, (▼) Sorbitol, (△) PEG 4000, (■) Propylene glycol.

enhanced stability of *P. lilacinum* protease. It is well kwon that water plays an important role in influencing the thermostability of enzymes. Sugars or polyols modify the structure of water or strengthen hydrophobic interactions among nonpolar amino acids, thereby making them resistant to unfolding and thermal denaturation [2]. Ghorbel *et al*. [7] studied the protective effect of several polyols at 60°C, finding that BG1 protease has a half-life of 180 and 45 min in the presence or absence of sorbitol, respectively. Similar studies concerning the protective effects of polyols against thermal inactivation were reported by Cobos and Estrada [5] for a xylanase from *Trichoderma reesei* QM 9414; by Bhosale *et al*. [2] for an alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20); and by Bayoudh *et al*. [1] for an alkaline protease from *Pseudomonas aeruginosa* MN1.

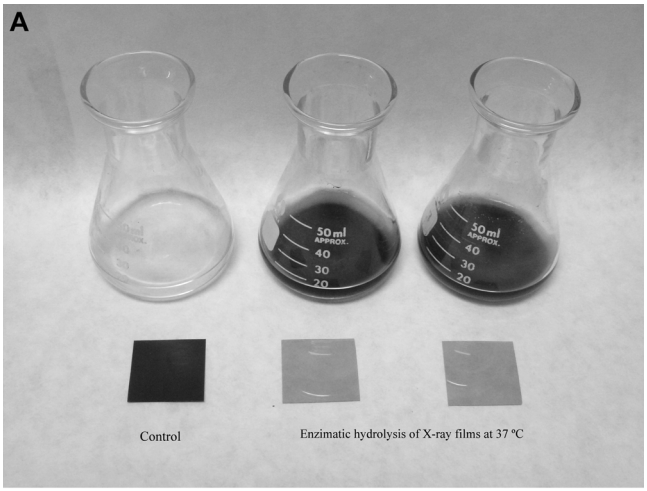
Table 1. Thermal inactivation parameters of *P. lilacinum* proteases in the presence of various polyols.

	k_d (min^{-1})	$t_{1/2}$ (min)	*D* (min)
Control	0.058	12.0	39.6
Propylene glycol	0.039	17.7	60.0
Glycerol	0.027	25.6	85.3
Sorbitol	0.050	13.8	46.9
PEG 4000	0.054	12.8	42.5

With the aim to enhance the reusability of the *P. lilacinum* enzymes in repeated cycles of gelatin hydrolysis and silver removal from used X-ray film at high temperatures, glycerol and propylene glycol (10% (v/v)) were selected for enhancement of enzyme stability.

Recovery of Silver from X-Ray Films: Effect of Temperature on the Hydrolysis of Gelatin Layers

First, enzymatic decomposition of the gelatin layers was carried out by using crude enzyme (6.9 U/ml) at 37°C. Crude enzyme protease decomposed 41.0% of the gelatin layers after 15 min, and complete breakdown of the gelatin layer was achieved after 33 min (Fig. 2A). After that, the effect of temperature on the time course of the gelatin hydrolysis was tested (Fig. 2B). Because of the heterogeneous nature of the reaction, a lag phase was observed in the hydrolysis reaction owing to the difficulty of moving the enzyme from the bulk of the solution to the gelatin layer because of mass transfer resistant. During the time course of the reaction, the amount of the enzyme adsorbed to the film increased, resulting in an augmentation of the hydrolysis rate [12]. As can be seen in Fig. 2B, the reaction rate of the gelatin hydrolysis and resulting release of silver particles improved significantly when the temperature was raised, and the maximum rate was observed at 60°C. The hydrolysis time for complete decomposition at 50°C was 20 min, whereas at 60°C it was 6 min. With a further raise in temperature to 65°C, the rate dropped owing to thermal denaturation of the enzyme. Masui *et al*. [11] reported that at 50°C, *Bacillus* sp. B21-2 protease took about 45 min for the complete hydrolysis, whereas Nakiboğlu *et al*. [13] reported that *Bacillus subtilis* ATCC 663 protease took less than 15 min at 50°C, but at 60°C the enzyme was rapidly inactivated. Complete hydrolysis was achieved within 6 min at 40°C by *C. coronatus* protease [15]. However, the fungal enzymes of the latter mentioned study [15] showed hydrolytic action only at 40°C, unlike the protease of this study, which can act over a broad temperature range (37–65°C).



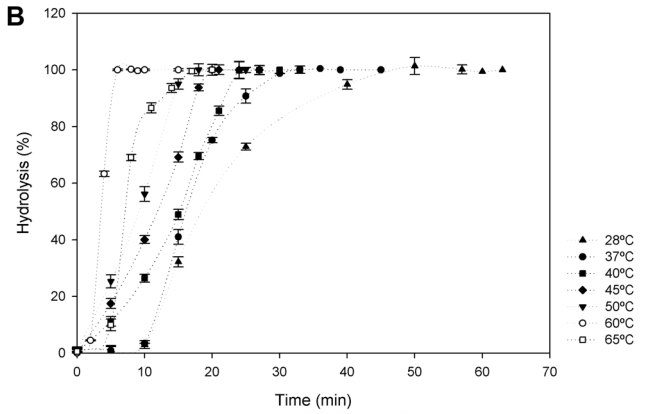


Fig. 2. Enzyme hydrolysis of used X-ray film and gelatin.
(**A**) Enzymatic hydrolysis of used X-ray films at 37ºC. Control solution remains clear whereas the enzyme solution turns turbid owing to the release of black metallic silver. Clean PET films were obtained after enzyme treatment of X-ray films, whereas the control film remains unchanged. (**B**) Effect of temperature on time courses of gelatin hydrolysis: (▲) 28ºC, (●) 37ºC, (■) 40ºC, (◆) 45ºC, (▼) 50ºC, (○) 60ºC, and (□) 65ºC. Enzyme concentration was 6.9 U/ml.

Effect of Enzyme Concentration on the Hydrolysis of Gelatin Layers

The effect of enzyme activity on the reaction was studied in the range of 1.7 to 34.5 U/ml (pH 9.0) at 60ºC (Fig. 3). Hydrolysis was slow at low protease activity. The time for complete hydrolysis was 27 min at 1.7 U/ml and varied from 18 to 5 min in the activity range from 3.45 to 34.5 U/ml, showing that a saturation of the gelatin layer was achieved where the available surface (not the enzyme) limits the reaction rate. These results seem to be better than those reported by Singh *et al*. [16] for *B. sphaericus* protease. This protease took around 24 min to complete hydrolysis at

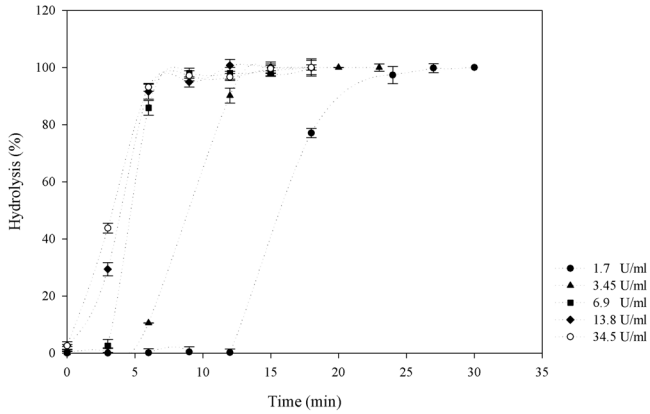


Fig. 3. Effect of enzyme concentration on time courses of gelatin hydrolysis at 60ºC.
(●) 1.7, (▲) 3.4, (□) 6.9, (◆) 13.8, and (○) 34.5 U/ml.

10 U/ml, and varied from 12 to 8 min when the activity of protease enzyme varied from 25 to 100 U/ml (using azocasein as substrate for protease activity determination). Since processing at low cost is attractive from an industrial standpoint, 6.9 U/ml was found to be most suitable for processing used X-ray films in 6 min.

Reusability of the Crude Enzyme for Gelatin and Silver Removal from Used X-Ray Film

The ability of the crude enzyme to retain its activity for repeated use makes it suitable for industrial application. It was found that proteases from *P. lilacinum* could be reused for three cycles without the addition of protective agents and in the presence of propylene glycol; but in the presence of glycerol, *P. lilacinum* protease could be even reused for seven cycles. It can be seen that the decomposition time increased with the cycles (Table 2). After the second cycle of hydrolysis, the enzyme in the presence of polyols took less hydrolysis time than enzyme without them. Masui *et al*. [11] and Shankar *et al*. [15] noted that the treatment time increased after every reuse of the enzyme, and the first cycle took 60 min for complete decomposition and the second one required more than 2 h. It is worth to mention that, although Fujiwara *et al*. [6] and Singh *et al*. [16] studied the protective effect of Ca^{2+} against thermal denaturation, none of them studied the potential reuse of the enzyme nor the potential use of the ion in the reuse cycles. When compared with the proteases reported by Masui *et al*. [11] and Shankar *et al*. [15], *P. lilacinum* protease seems to be more efficient, as it was capable of withstanding reuse to a greater extent, using glycerol as a protective agent against thermal denaturation.

Table 2. Reuse of *P. lilacinum* protease for gelatin hydrolysis from used X-ray films.

	Time required for complete gelatin removal (min)							
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7	Cycle 8
Protease	6	15	23	Ir	-	-	-	-
Propylene glycol	6	12	25	Ir	-	-	-	-
Glycerol	6	9	15	20	25	28	32	Ir

Ir, incomplete removal after 45 min.

Weight Loss of Used X-Ray Film During Silver Recovery Process and Recovery of Silver

The treatment of used X-ray films with *P. lilacinum* protease resulted in silver bound with gelatin being stripped off into the admixture, and a clean PET film was recovered (Fig. 1). The loss in weight after protease treatment was around 4.6 ± 0.1% based on the initial weight of X-ray film. The silver content (as AgCl) in the hydrolysate was determined by the gravimetric method and corresponded to 0.62% based on the weight of X-ray film. Silver chloride can be used to make photographic paper, as pottery glazes, in photochromic lenses, in stained glass manufacture, and in bandages and wound healing products [15].

Nowadays, where natural mineral resources are getting depleted ever faster all over the world, reuse and recycling remains the most feasible option to slow down this exhaustion as well as the environmental pollution. This study reported that *P. lilacinum* LPS # 876 protease, produced using as substrate another pollutant waste (hair waste), has potential application for recycling silver and PET from used X-ray films in an eco-friendly way. In addition, using glycerol (a biodiesel waste product) enhances the repeated use of a single dose of enzyme for silver recovery from used X-ray films. In this work, three wastes were used, which is an environment friendly way of treating them instead of the usual way of disposal.

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