Extraction of silver from waste X-ray films using proteases obtained from halotolerant bacteria *Salinivibrio costicola*

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Abstract

Silver is used in generating photographic or X-ray films. This precious metal may be recovered from used X-ray films with the help of microorganisms to achieve high economic gain additionally reducing the environmental hazard. Therefore, the aim of the present study is to recover silver from used X-ray films with the help of microorganisms. In the present study, halotolerant bacteria were isolated from salt pans in Nahur, Mumbai, India and the crude proteases obtained from two halotolerant bacteria were utilized for silver extraction from waste X-ray films. The isolates were identified by biochemical and 16s rRNA sequencing. The protease production was assayed at various pH and temperature and the recovery of silver was also optimized. The concentration of silver in the slurry was further estimated by 'ICP-OES'.

The protease producers were identified as Salinivibrio costicola and Salinivibrio costicola subspecies vallismortis. The cell-free culture broth with crude proteases was capable of successfully extracting silver at various temperatures (room temperature to 55°C) and pH (4 to 10) within 24 hrs. The extracted silver was estimated to be 2-2.2% w/w in the reaction mixture. The broad range of pH and temperatures of the protease make it useful for industrial purposes. This approach provides an eco-friendly and cost-effective alternative in the recovery of silver from used X-ray films.

Keywords: *Salinivibrio costicola*, Protease, Silver recovery, Halotolerant bacteria.

Introduction

Silver is a precious metal which has great industrial applications in the making of electronic equipment, jewellery, mirrors, dental fillings etc.[3,15] Approximately 8.3% of silver is used in X-ray films and photographic films[2]. X-ray films are made up of gelatine emulsion with radiation-sensitive silver halide crystals. The amount of silver varies between 1.5- 2.0% in X-ray films.[11] Silver-containing materials such as X-ray films are considered as hazardous waste and should not be disposed of without treatment. However, the process of recovering silver from waste X-ray films involves burning of the X-ray film in a furnace which causes the production of soot and foul smell. This process is not only environmentally hazardous, but also expensive due to the requirement of maintaining the furnace.[13]

Hence, there comes a need to obtain silver from waste X-ray films by a process which is not hazardous and time consuming, yet simultaneously cost-effective. One method is that of enzymatic proteolysis[18]. Proteases are enzymes which are secreted by most of microorganisms and have already been of immense use in various industrial applications. The enzymatic breakdown of gelatine not only helps in recovering silver, but also in recycling the polyester film base. Therefore, in recent years the enzymatic approach to deal with the recovery of silver has gained importance.[1,13] Proteases can be used to rip off the gelatine layer consequently releasing silver.

Degradation of the gelatine-emulsion layer of an X-ray film at varied temperatures and pH by usual proteases is not easy to achieve in a short time. So, there is a need to isolate proteases which may carry out proteolytic activities under a wide range of conditions at faster rate. For this purpose, in the present study, isolation of proteases from halotolerant bacteria has been attempted; they work over a wide range of conditions to recover silver from waste X-ray films providing an alternative to chemical methods.

Material and Methods

Enrichment and Isolation: Soil samples were collected from Nahur salt pans (Mumbai, India; 19.15 °N, 72.96 °E) from three different sites at a depth of 10cm with a sterile spatula. Enrichment for halotolerant bacteria was carried out as described by Dodia et al[4] and Khan et al.[9] Briefly, soil samples were inoculated into nutrient broth (HiMedia) containing 3, 6, 12 or 15% salt concentration simultaneously and incubated on a shaker (Neolab) at 37°C, 180rpm for 48h. After second enrichment in the respective media, isolation was carried out for this study from the enriched flasks on nutrient agar (HiMedia) containing 15% salt. Eleven different strains were isolated initially based on their colony morphology.

Screening for the presence of protease producers: To screen for the presence of protease producers, the isolates were spot inoculated onto 10 % milk agar medium (0.5 g casein hydrolysate, 0.3 g yeast extract, 0.1 g glucose, pH-7.0, 100 ml distilled water, 10 ml sterilized milk) and incubated at 37°C for 72 hour. Zone of clearance around the growth of the isolate was taken as positive and reported as a positive result.

Characterization of the Isolates: Two isolates which showed a higher zone of clearance on the Milk Agar medium were further assessed for their colony morphology, gram-staining characteristics and biochemical properties. Identification of the isolates was carried out with sequencing of 16S rDNA using universal primers, 27F and 1492 R.[5] The amplified product was purified using the HiPur Quick Gel Purification (HiMedia) kit. The purified DNA sample was then sent for sequencing with forward and reverse primers. The edited sequence was aligned and compared with the existing sequences in the NCBI database (blastn).

To estimate the salt tolerance potential of the selected isolates, nutrient broths containing NaCl (3-25%) were inoculated with actively growing cultures (OD 0.6 at 600nm) and incubated on a shaker (Neolab) at 37ºC, 180rpm for 24 hour. The optical density of the broths was determined after incubation.

Optimization and estimation of protease activity: The optical density of the culture was adjusted to 0.6 at 600 nm and was used for inoculation of the nutrient broth containing 10 % NaCl at 37ºC, for 72 hr under shaking condition. The protease activity of the isolates at different pH values (6.5, 7.0, 7.5, 8.0, 8.5), temperatures (8ºC, 28ºC, 37ºC, 55ºC) and hours (0 hr, 24hr, 48 hr, 72 hr, 96 hr) was estimated using the protease assay. The broth was centrifuged at 24ºC at 10000 rpm for 10 minutes. The cell-free culture broth was used for the protease estimation.

Protease activity was estimated using the modified Kunitz method.[17] To 0.5 ml of 1 % casein solution (pH 7.5), 0.5 ml of crude enzyme solution i.e. the cell-free broth, was added. The mixture was incubated at 37ºC for one hour. After incubation, 0.5 ml of 5% trichloroacetic acid solution was added to the mixture and the tubes were placed in ice for 30 minutes. The precipitate was centrifuged at 10000rpm for 10 min.

The clear filtrate (0.5 ml) was used for protein estimation with the Folin-Lowry method. The optical density of the samples was read at 600 nm. Appropriate blanks were maintained with the use of tyrosine as a standard. The enzyme activity was determined as tyrosine equivalent (U ml^{-1}).

Optimization of silver recovery from waste X-ray films using crude protease: Silver recovery from X-ray photographic films by hydrolysis of gelatine layer was carried out using crude protease. X-ray films were washed with distilled water and wiped with ethanol soaked cotton balls. The washed films were then set to dry at 37ºC for 30 minutes. 0.1 g of X-ray film was cut into 0.5 cm X 0.5 cm squares and incubated with 1 ml of crude protease (in such a way that the film was completely immersed in the solution). The activity of the protease in terms of hydrolysis of gelatine was assessed at different time intervals (0 hr, 24 hr, 48 hr, 72 hr, 96 hr), temperatures (8ºC, room temperature, 37ºC and 55ºC) and pH values (4.0, 6.0, 8.0 and 10.0). After incubation, the gelatine-free X-ray film was removed. The slurry was then tested for the presence of silver.

Detection and measurement of recovered silver: 5 g of X-ray film was cut into 1 cm^2 pieces and incubated with 50 ml crude protease (such that the film was completely immersed in the solution). The activity of the protease in terms of hydrolysis of gelatine was assessed after 24 and 48 hours. After incubation, the gelatine- free X-ray film was removed. The samples were then qualitatively tested for the presence of silver with HNO_3. For quantitative estimation of silver, the ICP-OES (inductively coupled plasma- optical emission spectrometry) technique was used. For silver estimation, data was monitored at two wavelengths (328.068 nm, 333.29nm) using axial mode in the range, 0.02-20 mg L^{-1}.

Results and Discussion

Although, proteolytic bacteria are widespread in nature, efficient proteases which can tolerate wide range of pH levels, temperatures and salt concentrations, are limited. Hence, there is a constant demand for new proteases which can fulfil various needs. Several studies established the fact that extremophiles are the unexplored sources of various important enzymes.[7,16] Particularly, proteases from the halophilic or halotolerant bacteria can be applied for industrial processes where the harsh conditions would inhibit ordinary proteases.

Therefore, in the present study, eleven halotolerant bacteria from Nahur salt pans, Mumbai, India were screened for their extracellular protease production using Milk Agar plates. Four isolates showed protease production, amongst which two isolates gave significant zones of clearance around colonies. All four isolates were checked for their ability to degrade gelatine coat on used X-ray films.

Only two were capable of degrading the layer and produced clear X-ray films. The two selected isolates were identified using conventional biochemical tests (Table 1) and 16s rDNA sequencing (Supplementary data 1). The isolates were identified to be *Salinivibrio costicola* and *Salinivibrio costicola* subspecies *vallismortis*.

Figure 1 shows the effect of salt on the growth of these two bacteria. Maximum growth was observed in the medium with 3-10% NaCl post 24 hours of incubation. The growth gradually reduced when salt concentration increased up to 25%, for both the strains. A similar observation was also reported by several other groups with halotolerant bacteria.[6,8]

The production of protease was done at different pH levels (6.5, 7.0, 7.5, 8.0, 8.5), temperatures (8ºC, 28ºC, 37ºC, 55ºC) and for different incubation periods (0 hr, 24 hr, 48 hr, 72 hr, 96 hr). The crude protease was estimated from the cell-free broth using the protease assay (Figure 2). The results confirmed that the highest protease production was observed

at pH 8.0, room temperature and after 72 hours of incubation for both the strains.

The cell-free extract obtained from 72 hours of incubation was then used for silver extraction. The activity of the crude protease in terms of hydrolysis of gelatine was assessed at different time points (24 hr, 48 hr, 72 hr and 96 hr). It was observed that a time of 24 hours was sufficient to strip off gelatine layer from the used X-ray films. Then, the same experiment was done at different temperatures (8ºC, room temperature, 37ºC, 55ºC) and pH levels (4.0, 6.0, 7.0, 8.0, 10.0) (Figure 3).

The proteases obtained from the cultures of *Salinivibrio costicola* and *Salinivibrio costicola* subspecies *vallismortis* hydrolyzed gelatine at pH levels of 4, 6, 8 and 10, indicating that they were active over a wide range of pHs. These proteases were also effective at different temperatures - ambient to 55ºC. They were also stable at ambient temperature for long periods (tested for up to 15 days).

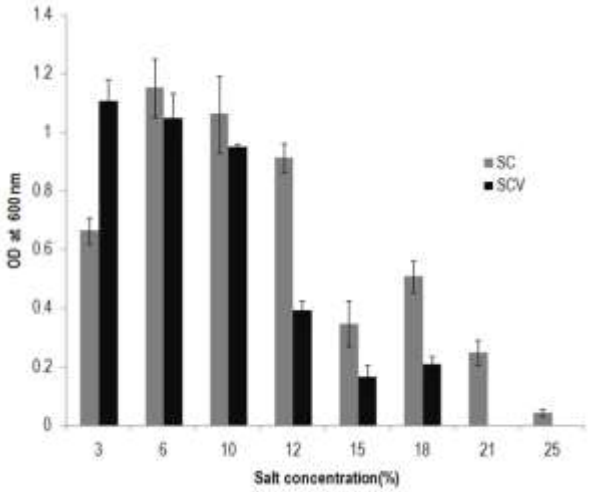

Figure 1: Growth of selected isolates, *Salinivibrio costicola* (SC) and *Salinivibrio costicola* subspecies *vallismortis* (SCV) at different NaCl concentrations (3-25%) in nutrient broth for 24 hour. Each point is the mean of three replicates

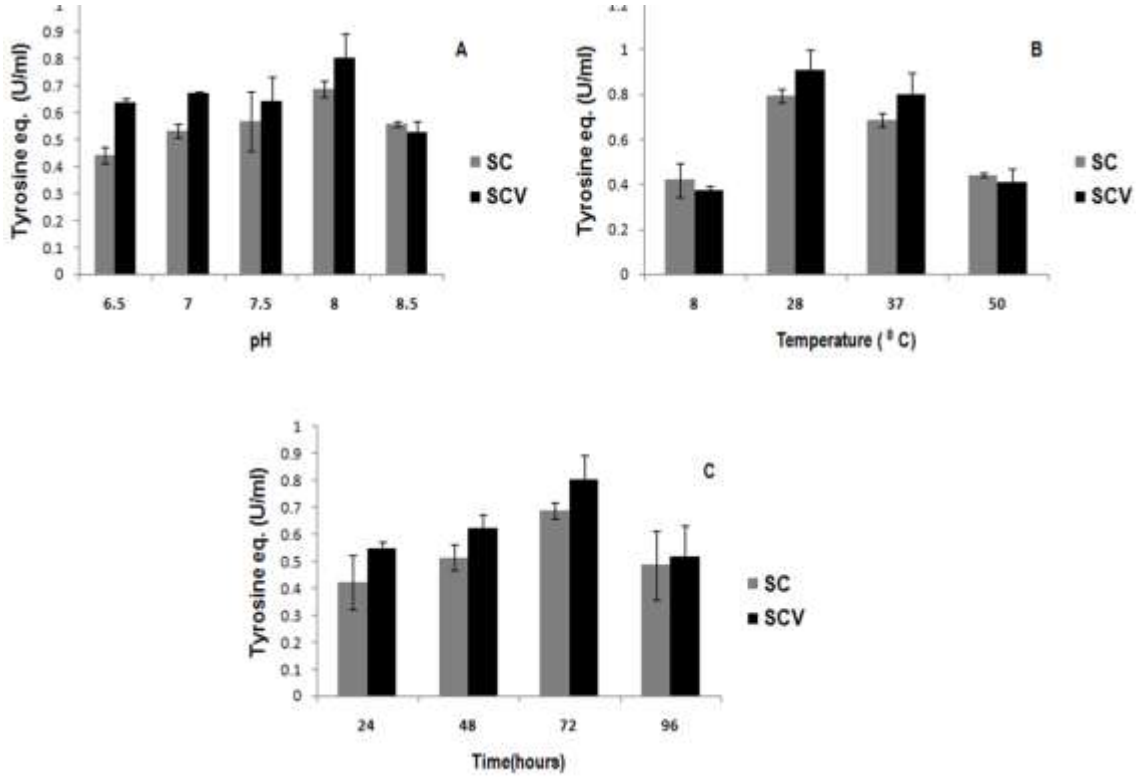

Figure 2: Crude protease production of *Salinivibrio costicola* (SC) and *Salinivibrio costicola* subspecies *vallismortis* (SCV) at (A) various pH levels, (B) temperatures and (C) hours, in cell-free broth was assessed. The protease concentration was expressed as tyrosine equivalent. The experiment was performed in triplicate (n=3)

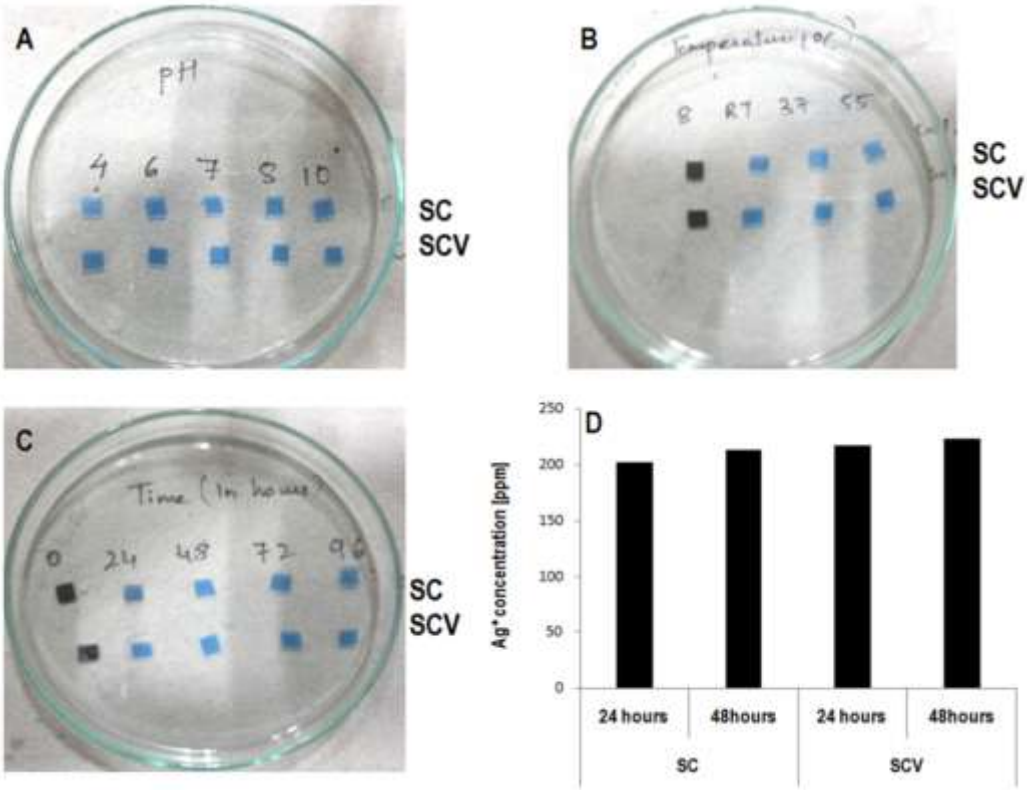

Figure 3: Silver extraction from X-ray film with crude enzyme from *Salinivibrio costicola* (SC) and *Salinivibrio costicola* subspecies *vallismortis* (SCV) at (A) various pH levels, (B) temperatures and (C) hours. The representative pictures are shown. ICP-OES analysis of the extracted silver was performed from the reaction mixture containing crude enzyme and X ray film (D) after 24 and 48 hrs of incubation.

Table 1
Biochemical Characteristics of the selected halotolerant strains

S.N.	Tests	*Salinivibrio costicola*	*Salinivibrio costicola* subspecies *vallismortis*
	Sugar Fermentation		
1	Glucose	Acid	Acid
	Lactose	Acid	-
	Sucrose	-	-
	Maltose	Acid	Acid
	Mannose	-	-
	Xylose	Acid	-
2	Nitrate	-	-
3	Lysine Decarboxylase	-	-
4	Urease	-	-
5	Phenylalanine Deaminase Test	-	-
6	TSI	Acidic butt, alkaline slant, no gas , no H_2S	Acidic butt, alkaline slant, no gas , no H_2S
7	Indole	-	-
	Methyl red	+	-
	VogesPrauskeur	-	+
	Citrate	-	-
8	Gelatin agar	+	-
9	Oxidase test	+	+
10	Catalase test	+	+
11	Gram staining	Gram negative short rods	Gram negative short rods
12	Motility	Motile	Motile

Strain 1 16s rDNA sequence: ***Salinivibrio costicola***

TTTGGGGGGGGGGGTATCGGATTCTGGGCGTAAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAG
CCCGGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTT
CAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGACAAA
GACTGACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCTACTTGGAGGCTGAGGTTTAAGACTTTGGCTTTCGGAGCTAACGCATTAAGTAGACCGCCTGG
GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGT
GCCTTCGGGAGCGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATCTTTGATTGCCTTCCCGTA

Strain 2 16s rDNA sequence: ***Salinivibrio costicola*** **subspecies** ***vallismortis*****.**

CTTGGGGGAGTTATCGGATTACTGGGCGTAAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAAGCCC
GGGGCTCAACCTCGGAACCGCATTTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTTCAG
GTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGACAAAGACT
GACGCTCAGATGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GTCTACTTGGAGGCTGAGGTTTAAGACTTTGGCTTTCGGAGCTAACGCATTAAGTAGACCGCCTGGGGAG
TACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCT
TCGGGAGCGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGAGAAATGTTGGGTTAAGTCCCG
CGACTAGCGCATCCCTTATCTTTGTTTGCATCCAAGTAACGTTGGCAACTCCATGTATACTGTCCGTTGAT
ACTTTCTTTATTA

Supplementary data 1: 16s rDNA sequences for strain identification

A qualitative assay was performed to confirm the presence of silver in the slurry using HNO_3. A cream coloured precipitate of $AgNO_3$ was formed which indicated the presence of silver. The quantitative estimation of silver was performed by a sensitive technique, ICP-OES, after 24 and 48 hours of crude protease treatment which corresponded to 2- 2.2% (w/w) based on the weight of the solid X-ray film (Figure 3D). There was no increase in the concentration of silver after 48 hours of treatment which indicated that crude protease treatment for 24 hours was sufficient to extract silver from the used X-ray films. The estimated concentration of silver was found to be similar to the previous studies.[10]

In literature, most of the proteases used for silver recovery are from bacteria such as *Bacillus subtilis*, *B. sphaericus* and from various fungi like *Aspergillus oryzae* and *Conidiobolus coronatus*.[14,19] The enzymatic extract of *B. subtilis* requires a pH of 8 for stripping of waste X-ray films[11] while some other proteases work well at a pH of 10 and above.[18] The stripping temperature for *B. subtilis* is maintained at 50ºC and as mentioned in most of the reports, these extracellular proteases work only in a narrow range of temperature.

However, the proteases in the present study could specifically act on the gelatine layer at ambient temperature over a wide range of pH levels leaving behind the polyester film base of used X-ray films which could then be recycled. Also, the proteases used for recovering silver were crude proteases which would result in a cost-effective method. Hence, the proteases of the present study are highly advantageous from the industrial point of view.

Conclusion

Precious metals like silver and its impact on the environment over disposal have necessitated their recovery from waste X-ray films. Microbial proteases isolated from the halotolerant organisms of *Salinivibrio* genus have shown potential for its industrial application by functioning at a wide range of pH levels and temperatures.

The crude proteases from *Salinivibrio* genus are reported to provide an alternative, eco-friendly and cost effective option in the recovery and recycling of silver from used X-ray films. The protease activity could be exploited further for other such industrial applications that operate under unfavourable conditions.

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