Bioprocess of silver extraction from used X-ray and MRI films

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ABSTRACT

With the development of science and technology and increasing population, the health facilities are developing very fast. Due to this reason, the consumption of X-ray, MRI and CT scan films are increasing rapidly. Silver is the major cast component which is present as thin coating on these films. Waste prints, negatives and used films are disposed often in municipal waste streams, ending up in landfills or incinerators, which leads to environmental pollution and incineration of used films leads to loss of silver. Microorganisms offer a better alternative method of silver extraction. By employing microbial enzyme like protease, silver is efficiently extracted from films into solution from where it can be recovered by chemical precipitation or by electrolysis. This biotechnological approach has tremendous potential in recycling the extracted silver as well as plastic sheets efficiently. The present work deals with the isolation and characterization of bacterial isolate which showed clearing activity on X-ray and MRI films. Silver extraction by employing Bacillus isolate is reported. Silver recovery was about 90% from the leach out solutions and had a purity of about 0.89 fine quality. An interesting feature of this isolate is its capability of silver nanoparticles synthesis. Recovery of the silver obtained from the used films (leachates) as Nanosilver particles by employing the potential Bacillus isolate is promising and we are making detailed study in this regard.

Keywords: silver, X-ray film, MRI film, nanoparticles

INTRODUCTION

Silver is an important metal and major cast component of the films, used in X-ray, MRI and CT-scans. A significant percentage of silver is consumed for preparation of these films. Demand for this precious metal is never ending. Two-third of the total requirement of silver production is met by mines and the one-third is recovered from the waste chemicals. Hence recovery of silver plays a significant role in terms of economy. Silver is recovered either from used/waste chemicals during film developing or from the used/waste films [1]. The photoactive chemicals present in films and their role is described in table 1. While developing the films, some amount of silver transfers from the image portion to the fixing solution (hypo solution), which can be efficiently recovered by electrolysis. Exposed film material contains image formed by the silver halide crystals dispersed in the emulsion layer. Although some of the silver from the emulsion constituents are extracted from the film during the processing, most of it remains on the film. Silver content present on the films also varies from X-ray film to MRI film (Table 2).

The silver impregnated on the used X-ray and MRI films can be recovered by chemical and biological methods. The chemical methods of extraction are often associated with environmental pollution and also loss of some amount of silver too. Alternatively microorganisms can be used for

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eco-friendly extraction of silver by employing a microbial enzyme, i.e. protease. This biotechnological approach can be used for recycling the extracted silver as well as plastic sheets effectively. The present work deals with the isolation and characterization of bacterial isolate which showed clearing activity on X-ray and MRI films. Silver extraction by employing this isolate is reported. An interesting feature of this isolate is its capability of silver nanoparticles synthesis.

MATERIALS AND METHODS

Screening for proteolytic bacteria: plate based screening

Isolation of the proteolytic bacteria from the soil samples was carried out by serial dilution and plated onto (Saboraud's Dextrose) SBD agar (pH 6.8) with casein and gelatin agar plates [2]. After inoculation the petriplates were incubated at 37°C for 24hrs. The bacterial isolates which exhibited proteolytic activity (clearing around the colonies) were maintained in Saboraud's medium containing 2% casein.

Characterization and identification of isolates

The morphological features of the isolates were studied by standard Gram staining, Capsular staining, Spore staining and Hanging drop methods [3]. The biochemical characterization of the isolates was done by Indole test, Methyl red test, Voges Proskauer test, Citrate Utilization test, Oxidase test and Catalase test [3]. Gelatin agar medium was used to test the proteolytic activity of the microorganisms. Hydrolysis of the medium is tested around the colonies on agar by spraying mercuric chloride solution and tannic acid solution.

Fermentation conditions for the production of Protease

Selected isolates were further tested for protease production by carrying out shake flask fermentation. Erlenmeyer flasks (250ml) containing 50ml of fermentation medium (Saboraud's Dextrose broth with 2% casein) were inoculated with 5ml of the overnight grown culture and incubated at 30°C, in an orbital shaker incubator at 150 rpm.

Enzyme assay

5ml of the fermented medium was centrifuged at 8000rpm for 6 min. The supernatant thus obtained was employed for the enzyme assay. Protease activity was determined by the Kunitz method with slight modifications [4]. The reaction mixture consists of 1ml of 2% casein (suspended in 100ml of the phosphate buffer pH 7.6) and 1ml of the appropriately diluted culture supernatant. The reaction was carried out at 35°C and after 20min by adding 3ml ice cold 0.306M Trichloro Acetic Acid (TCA). Unfermented substrate treated similarly was used as control. 1ml of TCA soluble mixture was mixed with 5ml of 0.4M Sodium Carbonate and 0.5ml of 1N Phenol reagent (Folin Ciocalteau), and the absorbance was measured at 660nm. One protease unit (PU) is defined as the amount of enzyme required to liberate 1μg of tyrosine under standard assay conditions.

Protein assay

Protein was measured by the method of Lowry [5] with Bovine serum albumin as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280nm.

Clearing of films by crude enzyme extracts

Waste films of dimensions 20 × 16 cm were cut into pieces of $8^{1/2} \times 3^{1/2}$ cm size and kept in a glass petriplates. 10ml of each culture supernatant were poured on the films and kept for observation.

Synthesis of silver nanoparticles

Bacterial isolate was inoculated in 100 ml sterile nutrient broth in 250-ml Erlenmeyer flask and incubated on a rotating shaker at 200 rpm for 48 h at room temperature. Culture was centrifuged at 12,000 rpm for 10 min for harvesting biomass and supernatant and used for the synthesis of silver nanoparticles. The supernatant was used for studying extracellular production of silver nanoparticles by mixing it with filter-sterilized $AgNO_3$ solution at 1mM final concentration [6]. Bacterial biomass was taken for intracellular synthesis wherein 2 g of wet biomass was re-suspended in 100 ml of 1 mM aqueous solution of $AgNO_3$ in a 250-ml Erlenmeyer flask. All the reaction mixtures were incubated on rotating shaker at 200 rpm at room temperature for a period of 72 h in light. Control flask was set by incubating heat-killed samples with $AgNO_3$ along with experimental samples.

RESULTS AND DISCUSSION

Soil samples were screened by making serial dilutions and higher dilutions, i.e. 10^{-6}, 10^{-7} and 10^{-8} were plated onto protein rich media, i.e. Saboraud's dextrose agar (SBD) with 2% casein and on gelatin agar. Petriplates were observed for colonies which had a clearance zone around them, after 48hrs of incubation at 37°C. About 10-12 colonies on casein agar plates were observed to be proteolytic. In order to confirm the proteolytic activity of the isolates, these colonies were picked up and inoculated as 4 per plate on casein agar. These colonies differ in their potential in proteolytic activity.

Before standardizing the bioprocess of silver recovery using these isolates; it was pre-requisite to identify and characterize the bacterial cultures by the standard microbial and biochemical tests [3]. Hence the isolates were characterized up to genus level by following standard methods. Colony morphology of the isolates on SBD agar plates revealed large colonies with irregular margins with no pigmentation. Simple staining with crystal violet indicated them as *Bacillus* type wherein the rods are short and slender (Figure 1 and 2). The members of the genus *Bacillus* were characterized as gram positive, rod shaped, aerobic or facultative, endospore forming bacteria. Spore staining was done for these isolates. Isolates were observed to carry an endospore, which is terminal in its location.

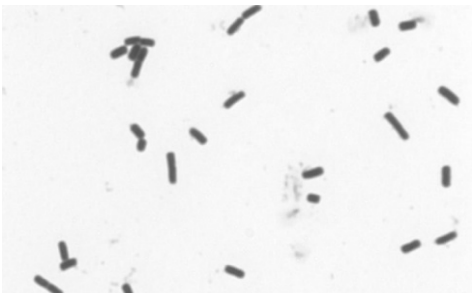

Figure 1. Gram positive Bacilli.

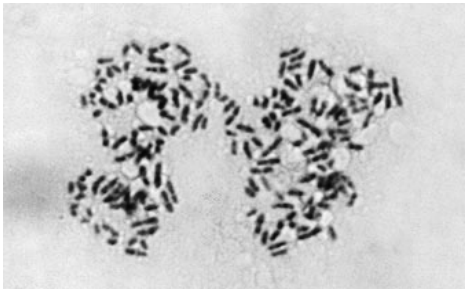

Figure 2. Picture depicting endospores of Bacilli.

Further characterization based on biochemical tests (Table 3) like IMViC indicated that these isolates were Indole negative, Methyl red positive, Voges proskauer test negative and Citrate utilization test positive. The above results indicate that they can ferment glucose and utilize citrate as sole carbon source. These isolates were producing catalase enzyme but not oxidase indicating their aerobic nature for growth. All isolates showed hemolysis when streaked on blood agar plates. When sensitivity towards penicillin was tested, they do differ in their sensitivities, i.e. isolate KSJS 42 was more sensitive than KSJS 51, while isolate KSJS 33 was totally resistant to penicillin. From the above characteristic features it was confirmed that the isolates belongs to the genus *Bacillus*.
Isolates selected from plate based screening were grown in 50ml of SBD broth with 2% casein, in a 250ml Erlenmeyer flask at 30°C on an orbital shaker at 150rpm for 24hrs. Culture supernatants (at 24hrs and 48hrs) were tested for protease activity by modified Kunitz method [4]. The results obtained were depicted in figure 3, which indicates that isolate no. KSJS 51, KSJS 33, KSJS 42 had high enzyme activity than the other isolates tested. Later these isolates were further tested for extraction of silver from used X-ray and MRI films. Films of dimensions 20 x 16 cm were cut into pieces of $8^{1/2} \times 3^{1/2}$ cm size and kept in a glass petriplate. 10ml of each culture supernatant were poured on the films and kept for observation. Within 10-15 minutes the X-ray film was totally cleared which was depicted in figure 4. Table 4 depicts the time taken to clear the X-ray film of $8^{1/2} \times 3^{1/2}$ cm size. From the result it was evident that isolate KSJS 51 would be the ideal choice for further studies as it was producing protease 8000PU/ml.

Table 1. Types of Photochemicals in the Emulsion layer.

Functional groups	Description
Light sensitive agents	Silver halide crystals of different grain size
Color couplers	Form image dyes together with oxidized developer
Correction dyes	Protect light-sensitive films from undesired spectral regions
Spectral sensitizers	Give particular sensitivity to spectral region
Antifogging agents	Prevent the production of non-image silver
Sensitizers	Increase the sensitiveness

For any bioprocess, the condition at which it is operated plays a significant role. Initially the type of the film and the size of the film necessary for the bioprocess were identified randomly by conducting the following experiments. These studies were made in order to recover the silver reproducibly from time to time.

The nature and the thickness of the used film do play a role in the recovery process. In one of the experiment photo negatives, X-ray film, MRI, photo negatives, photo color papers of same size were cut and taken for the enzymatic treatment. Table 4 shows the time required to clear the film by the treatment. Recovery was within 10-15 min for X-ray film, while for the photo negative it was

only 5min, for photo paper the clearance was observed by 30 min and for MRI film it was completed by 1 hr. As the film thickness and the concentration of silver differ from one film to another, there was difference in the time of extraction. The difference in time duration for the extraction of silver could be due to the thickness of the film.

Table 2. General components present in X-ray & MRI films.

Ingredient	Content Cmat ($mg.m^{-2}$) Paper	Content Cmat ($mg.m^{-2}$) Film
Sensitizers	1	25
Photographic stabilizers	5	100
Fungicide	30	150
Silver as Ag	500	12,000
Halides	300	7,000
Split of products masking compounds in color negative films	40	80
Remaining groups of color couplers	80	800
Stabilizers	0	80
Wetting agents	10	300
Filter dyestuffs	50	250

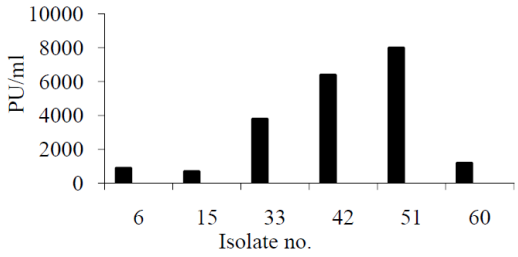

Figure 3. Protease activity of isolates.

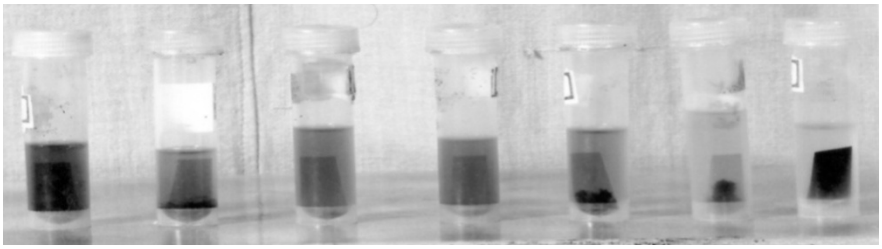

Figure 4. Clearing of films by crude enzyme extracts.

To study the relationship between the times for extraction versus the size of the film for process optimization, various sizes of films like slide size, post card size and 30 × 30 cm size X-ray film were used for silver extraction. With an increase in the size of the X-ray film there was an increase in time for recovery. Even with the same size of X-ray film the difference in company make had a difference in the recovery time. It is advisable to cut the film into small strips of $8^{1/2} \times 3^{1/2}$ cm size for quick recovery. However for an economical viable process the X-ray sheet as such can be utilized for enzymatic treatment (Figure 5) and later the plastic sheet can be recycled into market

through second hand sales. The cleaned X-ray sheets are purchased for making caps, handbags and by the textile industry.

Bacillus isolate KSJS 51 and KSJS 33 was used for this purpose. 48hrs old culture broth was centrifuged at 10,000 rpm for 15min and the supernatant collected was adjusted to pH 9 and used as crude source of alkaline protease. In a plastic tray of $22^{1/2} \times 18$ cm size, used X-ray film of 20×15 cm size was dipped in the solution and incubated for 30 min at room temperature with agitation on an orbital shaker at 100 rpm. The protease works on the gelatinous emulsion layer and releases the metallic silver bound to the film into the solution. The leached out solution was filtered to separate the black silver complex by an ordinary filtration methods. The filtrate can be used twice or thrice for the extraction (up to 3-4 films could be cleaned up by 100ml of crude enzyme preparation). Silver indicator strips assessed the concentration of silver in the solution. The silver rich leach out was then collected into a beaker and aluminium sulphate/sodium sulphate was added in order to precipitate the silver as salt of sulphide. This solution was subjected to further extraction by chemical precipitation method [7,8]. The samples for silver extraction were given out at a commercial recovery centre in Hyderabad wherein the electrolysis was followed for the recovery. Silver recovery was about 90% from the leach out solutions and had a purity of about 0.89 fine quality.

Table 3. Biochemical characteristics of isolates.

Morphological Features	51	33	42
Shape	Bacilli	Bacilli	Bacilli
Size	Medium	Medium	Small
Spores	Terminal	Terminal	Terminal
Gram reaction	+ve	+ve	+ve
Biochemical Reactions			
Indole	-	-	-
Methyl red	+	+	+
Voges proskauer	-	-	-
Citrate utilization	+	+	+
Enzymatic tests			
Catalase	+	+	+
Oxidase	-	-	-
Hemolysis	+	+	+

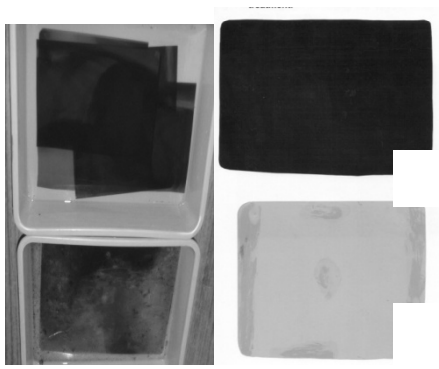

Figure 5. Clearing of films.

Silver extraction by Electrolysis

X-Ray/MRI film

↓

Crude extract of protease

↓

Electrolysis

↓

Silver granules are collected at cathode
(80%-90%)

↓

Granules were collected and ball milled

↓

Transferred to a dilute nitric acid bath which converts
the silver to a silver nitrate solution

↓

Subjected to a second electrolytic process which
produces silver (0.999) fine quality

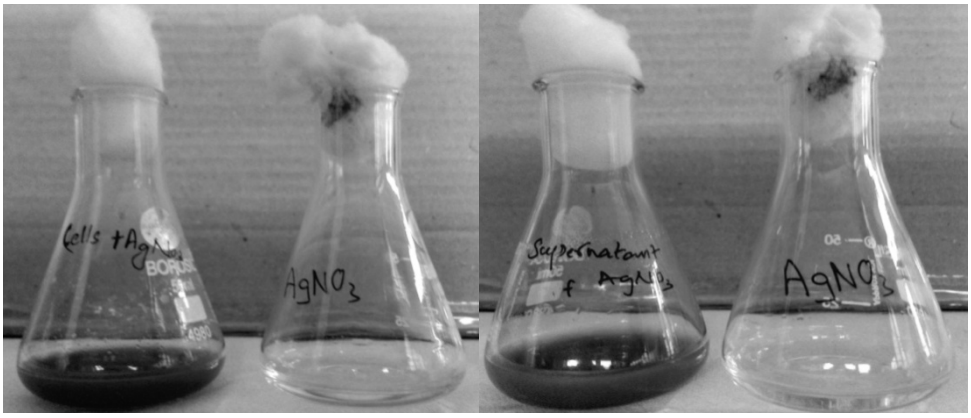

Figure 6. Synthesis of silver nanoparticles by *Bacillus* isolate.

Table 4. Clearing activity of isolates.

Isolate number	X-ray film	MRI	Photo paper	Photo negative
KSJS 51	10–15 min	1 hr	30 min	5 min
KSJS 33	15–30 min	50 min	30 min	5 min
KSJS 42	20–30 min	Nil	Nil	Nil

Nanoparticles are generally produced by either physical or chemical methods which are not ecofriendly. These methods have problems with the stability, controlled crystal growth and

aggregation of the nano size particles. Synthesis of nanoparticles using biological system (bacteria/fungi) has emerged as a novel approach [9]. Microbial synthesis of nanoparticles is eco-friendly and has significant advantages over other processes since it takes place at relatively ambient temperature and pressure [10]. Silver nanoparticles (AgNPs) have application in therapeutics, biomolecular detection, catalysis and also as antimicrobial agents [11]. Microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly [12,13]. Intracellular synthesis of nanoparticles requires additional steps such as ultrasound treatment or reactions with suitable detergents to release the synthesized nanoparticles while extracellular biosynthesis is cheap and requires simple downstream processing [14].

In the present study attempt was made to investigate the ability of Bacillus isolate for the synthesis of nano silver particles. Bacterial isolate KSJS 51 was used for this purpose. Culture was harvested at 24hrs of incubation and used for extracellular and intracellular synthesis of silver nanoparticles. The biosynthesis of silver nanoparticles using both biomass and supernatant were separately investigated primarily through the observation of colour change of the experimental samples in the presence of 1 mM AgNO3. A colour change from pale yellow to brown occurred for both bacterial biomass and supernatant within 24 h of incubation in the presence of light (Figure 6). Color was maintained throughout the 72-h period of observation. No color change was observed in experimental control containing heat-killed biomass or supernatant with silver nitrate. This suggests the colour change observed in the bacterial biomass and the supernatant samples were due to the formation of silver nanoparticles. The colour changes observed were further confirmed by UV–Vis spectral analysis as part of primary confirmation. Silver nanoparticles are known to have an intense absorption peak in UV absorption spectra due to its surface plasmon excitation. An absorption peak at 450 nm was observed which indicates the formation of silver nano particles. After identifying synthesis of extra silver nanoparticles and intracellularly by this isolate, experiments were also conducted with film leachout solution. Our results were encouraging, hence samples would be taken for further characterization like FTIR analysis to know the nanoparticles size and TEM analysis. Recovery of the silver obtained from the used films (leachates) as nanosilver particles by employing the potential Bacillus isolate is promising and we are making detailed study in this regard.

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