Recovery of Silver from Exposed X-ray Film using Alkaline Protease

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A thermophilic alkalophilic organism isolated from the salt pan bed was used for the production of alkaline protease enzyme. The enzyme purification was carried out by using salt precipitation and DEAE cellulose chromatography to yield enzyme with a specific activity of the 225U/mg .The enzyme had a pH and temperature optima of 9.0 and 55°C respectively and was capable of recovering silver from exposed X-ray film at a recovery efficiency of 84.5%.. The recovered silver exhibited 98.10% purity which was better than the 70.5% recovery rate and 97.83% purity of silver recovered by traditional chemical method

Key words: X ray films ,thermoalkalophilic, protease, extraction, Silver.

Silver metal finds varied applications based on its unique properties of hardness, malleability ductility, electrical and thermal conductance, and high reflectance of light besides being bacteriostatic in nature[4]. Anthropogenic sources usually account for the global biogeochemical movements of silver, to release it into the atmosphere, water and and soil ecosystems[2]. Biomedical wastes in the form of discarded X ray plates from the radiology departments of hospitals and nursing homes along with silver thiosulphate complexes from photographic developing solutions amount to a substantial entry of silver ions into ecosystems[2].

The acute toxicity of free ionic silver to aquatic plants and animals is well known[4,5] and varies drastically with respect to its chemical form .Secondary waste water treatment converts most of the silver thiosulphate complex to insoluble silver sulphide and metallic silver which have enhanced toxicity such that free silver ions inhibit 84% sludge respiration when present at a concentration of 100 mg/l while aqueous concentration of 0.5- 4.5 mcg/ liter had adverse growth effects in algae, alms, oysters, snails, daphnids, amphipods and trout.(9). Photographic film plates contain about 1-8 grams of silver/ sq.m. hence recovery of silver from such used photographic film can be a significant means of preventing silver ions accumulation in the ecosystem[5].

Proteolytic enzymes can be used to curtail the entry of silver into the environment. Alkaline proteases found in alkalophilic microorganisms can serve as a potential means to decompose the gelatin layers to release the silver through a method that is fast, cheap, eco-friendly and continuous[1].

Thus this project aims to develop technology for the removal of silver from exposed X-ray films using microbial alkaline protease.

MATERIALS AND METHOD

Sample collection

Soil samples from various salt pan bed and alkaline soil beds in and around Mira road., Mumbai along with effluent samples from the sewage treatment plant at Versova, seawater sample from Shivaji Park and Laundry water sample

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from Dhobi ghat region in Sewri were collected and enriched in Frazier's Alkaline Gelatin broth at 55 C for 7 days

Twice enriched cultures were then isolated on the Frazier's Gelatin agar plate (pH 8.0) and incubated at 55° C/ 48 hrs. Potent isolates showing zone of clearance on flooding with 1% acidified $HgCl_2$ were selected .

Short listing of potent Protease producers

10µl of the cultures adjusted at $O.D_{540nm}$=0.1 were spot inoculated on alkaline gelatin agar plates and incubated at 55 C for 48 hrs. The plates were flooded with 1% acidified $HgCl_2$. and activity ratio (Cx) calculated as the diameter of clearance to colony size The strain having the highest Cx ratio was selected for further analysis.

Production of alkaline protease:

5ml of culture BM-1 adjusted at 0.1 O.D at 540_{nm} was inoculated into three 200ml flasks containing Frazier's Gelatin Broth respectively and incubated for 5 days at 55° C. After incubation the broth was centrifuged and metabolic filtrate pooled and used as crude extract for enzyme assayed by Peek *et al* method[12] using the azogelatin as substrate while protein determination was measured by Lowry *et al* method (08) using BSA as standard.

Ammonium Sulphate precipitation:

Discontinuous ammonium sulphate precipitation was carried out for the purification of the enzyme within the range of 10-80% saturation. The precipitate obtained was collected by centrifugation at 10,000g/10mins and dissolved in 2.5 ml of 0.2 M Tris HCl buffer pH 8.0. The precipitate was then dialyzed against 1:1000 dilution of the same buffer using Himedia dialysis bag. Enzyme activity and protein content was measured to calculate specific activity and purification fold

DEAE-cellulose chromatography:

7 ml of dialysate obtained after ammonium sulphate precipitation at 80% saturation was loaded onto DEAE-cellulose column equilibrated with 0.2 M Tris-HCl pH 8.0 and washed with the same buffer. The enzyme was eluted with a linear gradient of 0-1 M NaCl in 20mM Tris-HCl buffer pH 8.0. Fractions of 5ml each were collected at a flow rate of 30 ml/hr and those exhibiting protease activity were pooled and concentrated using solid polyethylene glycol 6000. Enzyme activity and protein content was measured to calculate specific activity and purification fold.

Effect of pH on enzyme activity

The activity of purified protease was measured at different pH values using buffer- 0.2M Tris HCl buffer (pH 7.0-9.0) and 0.2M Glycine-NaOH buffer pH (8.6-10.6).

Reaction mixtures along with appropriate controls were incubated at 55^{0}C for 30mins and the activity of protease was measured using Peek *et al* method as adapted by Secades[12]

Effect of temperature on enzyme activity:

The activity of purified enzyme was determined by incubating the reaction mixture at different temperature ranging from 30-65^{0}C for 30mins and relative protease activity was measured using Peek *et al* method

Recovery of silver from exposed X-ray film:

The exposed X-ray film collected from K.E.M Hospital Parel was washed with ethanol, and cut into pieces of 2 X 2 cm^2 size. A total of 5 X-ray films of size (20.3 X 25.4cm) and total area of 0.2578 sq. m. containing 1.42 gms of silver were used. (Table 1)

For silver extraction the film pieces were allowed to react with 20 ml of purified enzyme solution at concentration of 3600 units in reaction bath of 500 ml of 0.2M Tris-HCl buffer (pH 9.0) at 55^{0}C for 30mins.

A chemical method of silver recovery was also carried out to compare the efficiency of recovery where an another set of cut X-ray films containing 1.42 g of silver were allowed to react with 500ml of 1.0 N NaOH at 80^{0}C for 30mins. The slurry obtained was again heated in a water bath of 90^{0}C for the formation of black colloidal silver. Both reaction mixtures from enzymatic as well as chemical method were filtered through Whatman no. 1 filter paper and smelted in an incinerator at 100^{0}C for 30mins to recover the precipitated silver which was weighed and assayed for its purity at Venus assaying and metals trading Pvt. Ltd.

RESULTS AND DISCUSSION

Screening and isolation of Proteolytic organisms

Screening of various soil and effluent samples gave a total of 8 thermo-alkalophilic proteolytic micro-organisms consisting of 75% bacteria and 25% actinomycetes, the results of which are shown in Table 2. Most of the isolates were gram +ve, sporulating rods indicating the

unique ability of Gram positive organisms to tolerate the dual thermophilic and alkalophilic conditions prevalent during the enrichment process.

Horikoshi *et al* (1999) suggested that alkalophilic gram positive bacteria contains acidic polymers such as galactouronic acid, glutamic acid, aspartic acid and phosphoric acid whose negative charges gives the cell surface its ability to absorb sodium ion which was an important parameter required for effective solute transport and thus its survival in alkaline conditions (03)

Thangam *et al* (2002) isolated besides a variety of gram positive bacteria , two alkalophilic archea from soda water lake in Tibet which were gram –ve, pleomorphic, flat, non-motile and strictly aerobic with a requirement for at least 12% NaCl and a pH optima of 9.0.

This similar to our results indicating though gram negative protease producers are present, they were not predominant

Preliminary screening of the isolates obtained for their proteolytic activity was determined with respect to its Cx ratio. (Table 3) 5 isolates i.e. 51.25% showed a low Cx activity ranging from 1-2, while 25% of the isolates showed high Cx ratio of >2.5 and only 1 isolate showed a moderate Cx ratio of 2 after 48 hours of incubation. Additionally out of 6 bacterial isolates obtained, 4 isolates showed poor Cx ratio while out of 2 filamentous forms 1 isolate showed poor Cx ratio. Thus, isolate BM-1 having a maximum Cx ratio value, was shortlisted for further studies

Enzyme production and the purification of the enzyme

Isolate BM-1 a Gram +ve, sporulating rod showing a maximum Cx of 2.9 was selected for quantitative estimation of its thermoalkalophilic proteolytic activity. Enzymatic activity was assayed by Peek *et al* method using azogelatin as substrate, the results of which are shown in Table 4

Metabolic filtrate obtained after centrifuging the broth at 10000g/10 mins and used

Table 1. Calculated silver available for recovery in X ray film plates

Length of the X-ray film	20.4 cm
Breadth of the X-ray film	25.4 cm
Area of the X-ray film in sq. m.	0.515 sq.m.
Area of 5 X-ray films in sq.M.	0.2578 sq.m.
Amount of silver present in 1 sq.m. film	5.5 gm
Amount of silver present in 0.2578 sq.m.	1.42 g

Table 2. Screening of the Thermoalkaliphilic proteolytic isolates from various ecosystems

Isolate no.	Sample	Area	pH	Gram nature	Characteristics
BM-1	Salt pan bed	Mira road	7.5	Gram +ve rods	Irregular, transluscent
BM-2	Salt pan bed	Mira road	7.5-8.	Gram +ve rods	Circular, opaque
BM-3	Laundry water sample	Sewree	8.5	Gram +ve rods	Circular, opaque
BM-4	Laundry water sample	Sewree	8.5	Gram +ve rods	Reddish, transluscent
BM-5	Alkaline soil	Mira road	7.5	Gram +ve rods	Circular, opaque
BM-6	Effluent water	Versova effluent pumping station	7.5	Gram +ve rods	Circular, opaque
BM-7	Effluent water	Versova effluent pumping station	7.5	Gram +ve filamentous forms	Dry chalky white
BM-8	Sewage water	Chembur	8.0	Gram +ve filamentous forms	Dry chalky white

as crude enzyme solution gave an enzyme units of 50U/system with specific activity of 13.15 u/mg.

Thangam *et al* (2002) reported the purification of protease from *Alcaligenes faecalis* using modified method of Anson .where in one unit of protease activity was defined in terms of tyrosine residues liberated, resulting in running of tyrosine standards for calculating the enzyme activity[10].

Azogelatin method, used in this study on the other hand, depends on the amount of colour developed due to release of azo dye which is bound to gelatin and thus this method would correlate well with its ability to release silver bound to gelatin from X ray films Hence azogelatin method was selected over modified method of Anson.for analysis

Discontinuous ammonium sulphate precipitation followed by dialysis was attempted for the purification of crude enzyme. The enzyme showed maximum precipitation at 80% saturation with a specific activity of 70 u/mg and a purification fold of 5.32 and an enzyme yield of 12.58%. (Table 4) This was better than the results reported by Thangam *et al* (2002) who purified protease from *Alcaligenes faecalis* using 80% acetone to obtain a specific activity of 64.2 units/mg and the purification fold of 2.2.

The dialysate obtained was further purified using DEAE cellulose chromatography to yield a specific activity of 225 units/ mg with the purification fold of 17.1 and a yield of of 16.17% (Fig 1).

Specific activity of the finally purified enzyme by DEAE cellulose chromatography as reported by Thangam (2002) was found to be 405.3 units / mg with a purification fold of 14.3 and yield of 30.6%. This is contrast to our result wherein though the purification was achieved, loss of the enzyme as indicated by the poor yield was observed.

Effect of temperature on enzyme activity:

Since the isolate was a thermophile, optimization of the effective range under which enzyme shows good activity was undertaken ,the results of which are shown in (Fig2). It was observed that the enzyme was active within the

Table 3. Selection of organism for Protease production based on Cx ratio

Isolate no.	Cx ratio value
BM-1	2.9
BM-2	1.44
BM-3	2.5
BM-4	1.89
BM-5	1.9
BM-6	1.55
BM-7	2.11
BM-8	1.88

Table 4. Purification of BM-1 alkaline protease

Purification step	Volume	Protein content mg/ ml	Total protein	Enzyme Units (units / system)	Total units	Specific activity (units / mg)	Purification fold	Yield %
Metabolic filtrate	445 ml	3.8	1691	50	22,250	13.15	1	
Ammonium sulphate precipitation	20 ml	20	40	140	2800	70	5.32	12.5
DEAE cellulose chromatography	20 ml	0.8	16	180	3600	225	17.1	16.17

Enzyme units = 1U = increase 0.01 O.D. of test over enzyme blank.

Specific activity = Total enzyme units / Total protein content

Purification fold = Specific activity at given step / Specific activity at initial step

% Yield = Total enzyme units at given step / Total enzyme units at initial step

range of 10^0C-65^0C with an optimum temperature of 55 ^{0}C as the enzyme activity was found to decrease as the temperature increased to 65^0 C. This similar to results reported by Thangam *et al* (2002) for the alkaline serine protease enzyme from *Alcaligenes faecalis.* Similar observation were also being made by Ishikawa *et al* (1993) for a protease from alkalophilic *Bacillus spp* KSM-K16 which showed temperature stability upto 50^0 C and loss activity at temperature above 65^0 C. Ohta *et al* (1966) studied the thermo stability of alkaline protease from *Bacillus proteolyticus* and concluded that an abundance of abnormally high tyrosine residues plays an important role in the stability of the enzyme molecule. Additionally hydrogen bonding of the phenolic groups and presence of hydrophobic bonds involving the aromatic ring also enhances its thermostability (02) Thus the thermo stability seems to result from extensive hydrophobic regions in the enzyme, and the enzyme being rich in tyrosine and other amino acids with hydrophobic side chains.

Pantoliano *et al* (1989) reported that six individual amino acid substitution (N218S, G169A, Y217K, M50F, Q206C, N76D) at separate positions in the tertiary structure of Subtilisin BPN' were found to increase the stability of the enzyme to elevated temperature (65^0 C) and extreme alkalinity (pH 12.0). Under these denaturing conditions, the rate of inactivation of the combination variant was 300 times slower than that of the wild type protease Subtilisin BPN'.

Fujiwara *et al* (1991) reported that the introduction of an additional disulphide bond linkage between residues Cys 61 and Cys 98 using protein engineering resulted in the increased thermo stability of protease subtilisin E.

Effect of pH on enzyme activity

The effect of pH on enzyme activity within the range of 7-10 was studied with an aim to determine the optimum activity for protease obtained from isolate BM-1. The enzyme activity increased as the pH increased with an optimum activity at pH 9.0. after which the enzyme activity

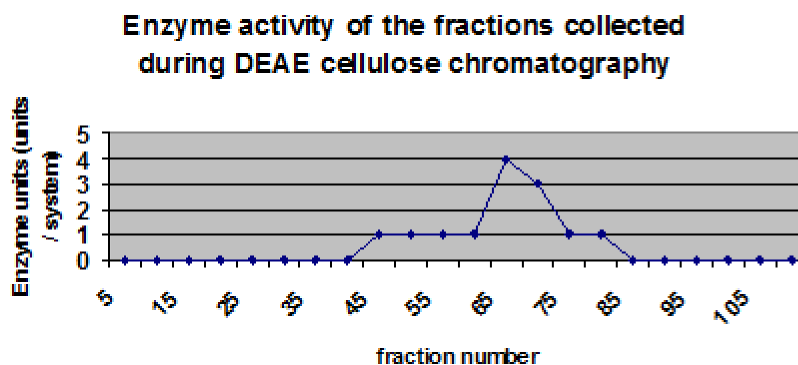

Fig. 1. Purification of protease by DEAE Cellulose chromatography

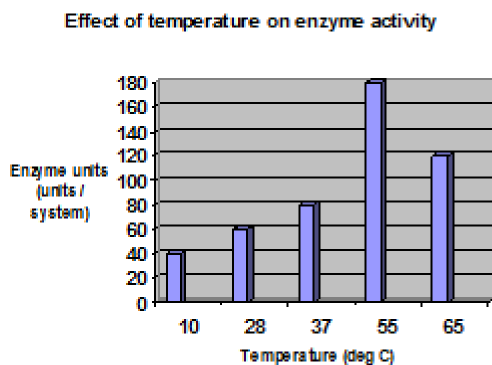

Fig. 2. Effect of temperature on Protease enzyme activity of isolate BM-1

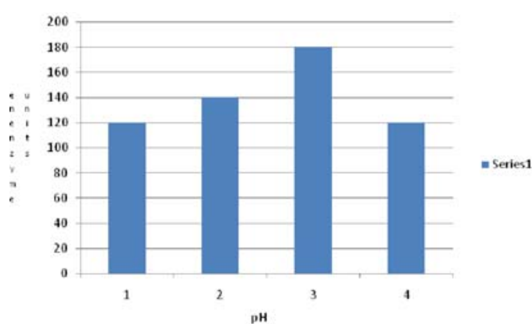

Fig. 3. Effect of pH on protease enzyme activity of isolate BM-1

decreased as the pH was increased to 10.0 (Fig 3). The unsuitability of pH 10.0 for the enzyme activity could be due to the fact that the organism also could not survive that pH.

The crystal structure of M- protease of alkaliphilic *Bacillus spp.* strain KSM-K16 by X-ray analysis to understand the alkaline adaptation mechanism of the enzyme. The analysis revealed a decrease in number of aspartic acid, glutamic acid, and lysine residues and increase in number of arginine, histidine asparagine and glutamine residues during the course of adaptation[7].

Thus a semi purified protease enzyme of 3600 units capable of breaking the protein moiety at high temperature of 55 C and an alkaline pH 9.0 was obtained.

Recovery of silver from the photographic film

Using the alkaline protease of BM-1 recovery of 1.200 g of silver from the X ray films containing 1.42g at an efficiency of 84.5%% was achieved. In contrast the amount of silver recovered by usual chemical method was 1.090 g with a recovery efficiency of 70.5%. indicating that the enzymatic method was better in recovery of silver than chemical method. Additionally the efficiency of recovery by the protease enzyme of isolate BM-1 was better as compared to the results shown by Nakiboglu *et al* (2001) who showed the recovery of just 66 %.using an alkaline protease from *B. subtilus.* Ishakawa *et al* (1993) reported that the time for hydrolysis plays an important role and it can be reduced if the reaction mixture were allowed to react in the stirred tank reactor. The reduction in time required for hydrolysis could be due to the increase in the transfer rate of enzyme throughout the film. In our study the parameter of stirring was not accounted which resulted in the increase in time required for the release of silver. Inspite of the yield of around 84.5% silver extracted using the protease of BM-1 it showed a better luster and had a purity of 98.10% as compared to the silver recovered from the chemical method which had just 97.83% purity.Thus this method can be one of the effective techniques to recover silver from biomedical waste

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