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Novel alkaline protease of *Staphylococcus cohnii* N3 isolated from poultry farm soil used for silver recovery from waste X-ray film

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Abstract

Alkaline proteases are one of the most widely used as industrial enzyme recently explored for its effective degrading ability in bioremediation. In the present study alkaline protease producing bacteria was isolated from soil samples collected from waste dumped area in poultry farm. The isolate was identified as *Staphylococcus cohnii* N3 strain by 16s rRNA sequencing analysis. The effect of pH and temperature on alkaline protease was found that showed maximum activity at pH 9 and at temperature 60 °C (162.U/ml). The isolated *S. cohnii* N3 was further experimented for silver recovery and gelatin hydrolysis. Gelatin hydrolysis was monitored by measuring increase in turbidity in the hydrolysate. Gelatin layer was stripped completely within 20 min at 40 °C, pH 9. Rate of gelatin hydrolysis increased with increased in time shows complete decolorization of X-ray film proves its suitability for silver recovery.

Keywords: alkaline protease, *Staphylococcus*, silver recovery, X-ray films, gelatin hydrolysis

1. Introduction

X-ray films is considered as hazardous waste depends upon the amount of lead aprons and lead dental foil present in the X-ray films are considered as hazardous waste (Galarpe and Leopoldo 2018) ^[1]. Byproduct of X- rays film has lead waste which is held in the topsoil where it can remain for us longer 2000 years. Lead is a deadly neurotoxin is readily pick up by plants, enters our food system and possess threat to environmental and human health (Hossain *et al.*, 2021) ^[2]. The used Xray/photographic films containing black metallic silver spread in gelatin are very rich source for silver recovery. High concentration of silver and compounds containing it led to argyria, which results in a blue-grayish pigmentation of the skin, eyes, and mucous membranes. The fixing and bleaching solution that contains silver thiosulfate with silver cause harmful effect. This waste can be utilized as rich source of silver a valuable noble metal used for many purposes, such in the photographic industry, especially by using X- ray films (Satyanarayana and Chandra, 2020) ^[3].

The conventional method of directly burning the X –ray films to recover the silver, generates the undesirable foul smell, causes the environmental pollution and polyester film which contains emulsion of silver and the coated gelatin cannot be recovered (Guleria *et al.*, 2018) ^[4]. Chemical methods use, nitric acid or reagents such as sodium cyanide, NaOH, nitric acid or organic compounds for performing the process of stripping the gelatin- silver (Erku *et al.*, 2019) ^[5]. Gelatin layer has cross linked molecules which is hardeners and it is difficult for the usual proteases. After enzymatic hydrolysis silver 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. X–ray or photographic films contains 1.5–2% (w/w) of silver in its gelatin layers. Alkaline protease has an important role in the recovery of silver from used X–ray films. The enzyme degrades the gelatin layer embedded with silver in X–ray films. The conventional method of burning X–ray films cause environmental pollution. By recycling the photographic wastes 18–20% of the world’s silver requirement is attained (Sharma *et al.*, 2019) ^[6].

The protease enzyme forms major group of industrially and academically important enzymes that shares 65% percent of annual enzyme market. They have a history of applications in food and detergent industries where the alkaline proteases hold the biggest share of the enzyme market worldwide. Alkaline protease constitutes main ingredient of detergents, have applications in leather industry, medical diagnostics, recovery of silver from X-rays, food and feed industry etc. (Sundus *et al.*, 2016) ^[7].

Enzymes produced from microbial sources are most advantageous comparatively from other sources like animals or vegetables etc. (Varia *et al.*, 2019) ^[8]. Many microbial strains including fungi (*Aspergillus flavus*, *Aspergillus miller*, *Aspergillus niger* and *Penicillium griseofulvin*; *Bacillus* sp. (*Bacillus licheniformis*, *Bacillus firmus*, *Bacillus alcalo*, *Bacillus subtilis* and *Bacillus thuringiensis*) have investigated earlier and optimized for nutrient sources, pH and temperature to get maximum protease (Akshatha *et al.*, 2020) ^[9]. *Bacillus* sp. have wide industrial application and in bioremediation of polluted environment, managing pollutants by detoxification and mineralization. The remediation of recalcitrant pollutants using microbial enzymes is considered environment friendly, cost effective, innovative, and promising (Razzaq *et al.*, 2019) ^[10]. Only few works are available on exploring protease of other microorganisms, hence this work aimed to isolate efficient alkaline protease producing strain from poultry farm waste dumped area. Further the strain was analyzed for gelatin hydrolysis and recovery of silver from used X-ray films.

2. Materials and Methods

2.1 Collection of soil sample

The soil sample was collected from the nearby surrounding of the poultry farm in Sivakasi from waste dumped area. Soil was collected below 4-5 cm depth in sterile container and stored at 4 °C until performing the further experiments.

2.2 Isolation and Screening of Proteolytic bacteria

The soil sample (1g) was serially diluted with the sterile distilled water and spread plated on nutrient agar medium (pH 8) incubated at 37 °C for 24 h. The isolates were screened using skim milk (1% w/v) agar plates and incubated at 37 °C for 48 h.

2.3 Biochemical and Molecular characterization of selected isolate

The isolated colonies were examined microscopically and biochemical characteristics were studied such as Indole test, Methyl red and oxidase test. For further identification of selected bacterial strain at species level, 16s rRNA nucleotide sequence analysis were performed at Exon Biosciences, Vandallur, Tamil Nadu.

2.4 Protease enzyme assay

The culture of proteolytic isolate was inoculated in 20 ml of protease production media with the composition of 1 g casein, 0.2g KH₂PO₄, 0.2 g K₂HPO₄ and 0.1 g MgSO₄·7H₂O in 100 ml of distilled water and incubated for 48 h in a rotatory shaker in 200 rpm at temperature of 37 °C. After incubation, the culture was centrifuged at 10,000 rpm for 20 minutes at 4 °C in the cooling centrifuge and the collected supernatant was used as crude enzyme source for protease assay. For protease assay casein was dissolved in pH buffer and the reaction mixture containing casein and enzyme

solution was incubated for 10 minutes at 37 °C. The reaction was stopped by adding 3 ml of 20% ice cold trichloroacetic acid and the precipitated proteins were removed by centrifugation. The 0.5 ml of the supernatant was mixed with 2.5 ml of 0.5 M Na₂CO₃ and kept for 20 minutes at room temperature. Finally added with the appropriately diluted Folin's phenol reagent. The mixture kept for 10 minutes and the absorbance was measured at 660 nm against the blank sample. One unit of protease activity was calculated as release of one µg tyrosine per ml per minute (Sarkar *et al.*, 2013) ^[11].

2.5 Effect of pH and temperature on enzyme activity

The pH was adjusted using different pH buffer ranging from 7-11 and all tubes were incubated at 37 °C for 30 min and experimented for alkaline protease activity. Effect of temperature was analyzed by maintaining the reaction mix of different tubes at temperature ranging from 30 to 80 °C. All the tubes were incubated for 30 minutes and enzyme activities were determined by standard enzyme assay.

2.6 Recovery of silver and gelatin hydrolysis

Used waste X films were washed with distilled water and wipe with cotton impregnated with ethanol. Then washed X-ray films was dried in hot air oven for 30 minutes. X-ray film (1 g) was cut into 2 × 2 cm pieces was then incubated with 10 ml of crude protease for different time interval at 40 °C pH 9 in a water bath with the continuous shaking. Turbidity of the reaction mixture hydrolysate increased with the time and when no further increase in turbidity was observed, consider hydrolysis was complete. The progress of hydrolysis that Lowry for turbidity was monitored by measuring the absorbance at 660 nm. Protein released during gelatin hydrolysis was monitored following method of *et al.* (1951) ^[12] with bovine serum albumin (BSA) as the standard and used for calculation of percentage of gelatin hydrolysis (Shankar *et al.*, 2010) ^[13].

3. Results and Discussions

3.1 Screening of alkaline proteolytic bacteria

Seven bacterial isolates were recovered from the collected soil sample from poultry farm in the nutrient agar plates. Among four isolated bacteria, the strain N3 showed highest protease activity as 105.05±0.9 U/ml (Fig. 1). Similarly, total six bacterial colonies were screened and *Bacillus* sp. GS-P4 produced highest protease activity (0.0233U/ml) was isolated from soil samples collected from farm soil, garden soil of BIMTS college campus and oil spilled area of Burhanpur (MP), India (Rupali, 2015) ^[14]. Hamdani *et al.* (2019) ^[15] isolated 2 potential proteolytic bacteria from 10 isolates of pig sludge.

3.2 Identification of alkaline proteolytic bacteria

Amongst the isolates the strain with increased proteolytic activity was gram positive, non-spore former, negative for indole and oxidase test and positive for methyl red test. The Blast results of sequence 16s rRNA gene observed that the isolate exhibit the similarity to *Staphylococcus cohnii*. The partial sequence of *Staphylococcus cohnii* N3 was deposited in GenBank under the accession number is MZ013138. Further the phylogenetic tree was constructed based on the 16s sequencing analysis and *Staphylococcus cohnii* N3 showed the close relationship with the *Staphylococcus cohnii* NR03902 (Fig 2). Similarly, among the 12 isolates

screened from sediment samples protease of *Staphylococcus saprophyticus* showed the highest protease production (6.50 ± 0.03 U/ml) (Uttatree *et al.*, 2018) ^[16]. Among the two different proteolytic isolates found in the soil sample which was collected from adjacent areas of sweetshop identified as *Staphylococcus pasteurii* strain produces 1.28 mg/ml of protein (Chakraborty and Karmakar, 2020) ^[17].

3.3 Effect of pH on Protease activity

Staphylococcus cohnii N3 strain produced the highest proteolytic activity at pH 9. Above pH 9 the proteolytic activity declined gradually, however retaining 82.3 % of protease activity at pH 10. Hence the optimum pH for isolate *Staphylococcus cohnii* N3 strain was found to be pH 9 with maximum proteolytic activity of 107.3 ± 0.5 U/ml in with casein as substrate (Fig 3). Whereas, protease of *Geobacillus thermoglucosidasius* SKF4 exhibit maximum protease activity at pH 7 to 8 (Suleiman *et al.*, 2020) ^[18]. *Bacillus isronensis* strain KD3 produced maximum proteases at 42°C and pH 7-8 after 48h of the incubation period (Patil and Kurhekar, 2020) ^[19]. Alkaline protease of *Bacillus* sp. DB14 has maximum activity at pH 10.5 and temperature 60°C (Yigit Sat *et al.*, 2020) ^[20]. Alkaline protease produced by *Bacillus amyloliquefaciens* HM48 showed optimum activity at pH 8 (Mushtaq *et al.*, 2021) ^[21] and *Bacillus stearothermophilus* showed increased activity at pH 10 (Karray *et al.*, 2021) ^[22].

Alkaline protease of *Staphylococcus aureus* S-2 isolated from Chicken Waste have optimal pH and temperature at 8.0 and 50 °C (Akram *et al.*, 2014) ^[23]. Chakrabarty *et al.* (2018) ^[24] investigated increased protease activity of isolate *Bacillus* sp. at neutral and alkaline pH used for digestion of milk and egg proteins, removal of stains.

3.4 Effect of temperature on alkaline protease activity

Proteolytic activity of *Staphylococcus cohnii* N3 strain increased with raise in temperature correlating with increased casein hydrolysis and showed maximum activity at 60 °C (162.3 ± 0.7). It retains 82 % of alkaline protease activity at 80 °C. Hence the optimum temperature for alkaline protease production by isolate *Staphylococcus cohnii* N3 strain was found to be at 60 °C (Fig. 4). This result coincides with alkaline protease of *Bacillus infantis* SKS1 isolated from garden soil that showed increased activity at 60 °C (Saggu *et al.*, 2017) ^[25]. Gopalakrishnan *et al.* (2021) ^[26] observed increase stability of alkaline protease of Endophyte *Brevundimonas diminuta* VKB1 till 40 °C and the activity dropdown at 60 °C.

3.5 Gelatin hydrolysis and Silver recovery

When the X-ray films were treated with the alkaline protease solution of *Staphylococcus cohnii* N3, 90 % gelatin hydrolysis was observed at the end of 15 minutes and complete removal was achieved within 20 min shown by decolourized X-ray film (Fig. 5). Similarly, using protease of *Bacillus* sp. ATP-P5 treated X-ray film 1.5-2% (w/w) metallic silver (Black in color) were recovered (Kumari *et al.*, 2015) ^[27]. With alkaline protease of *Bacillus subtilis* NCIM 2724 complete stripping of gelatin occur within 4 to 6 days with crude protease at 37 °C and pH 8 used for 0.5012 g recovery of silver (Parpalliwar *et al.*, 2015) ^[28]. Al-Abdalall and Al-Khaldi (2016) employed *B. subtilis* protease for recovery of silver within 30 min at 50°C and pH 8. Hamza *et al.* (2017) ^[29] demonstrated recovery of gelatin and silver from waste X-ray film at pH 9, 40°C in 40 min by *Bacillus* sp. THZ14.

Fig 1: Alkaline protease activity of Isolates from Poultry soil

Fig 2: Phylogenetic analysis of identified *Staphylococcus cohnii* N3

Fig 3: Effect of pH on alkaline protease activity of *Staphylococcus cohnii* N3

Fig 4: Effect of Temperature on alkaline protease activity of *Staphylococcus cohnii* N3

Fig. 5: Gelatin hydrolysis (a) and Decolorization of X-ray film (b) for Silver Recovery

4. Conclusion

The present investigation concluded that the increased protease activity of the selected isolate at high pH and temperature emphasis its promising implementation in various industries. In addition, the use of alkaline protease of *Staphylococcus cohnii* N3, an isolate of poultry farm has proven the efficacy of enzymatic method as an ecofriendly, cost effective approach for recovery of valuable silver from waste X-ray film thereby facilitate economical means for pollution.

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