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Recovery of Silver from used X-Ray Films by Protease Isolated from *Bacillus* sp ATP-P5 and its Immobilization

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Protease from *Bacillus* sp ATP-P5 was produced and purified by using ammonium sulphate precipitation and anion exchange chromatography with 1.9 fold of purification. The purified enzyme showed a single band on SDS-PAGE and it was determined as 29 KDa. The purified enzyme was used for silver recovery from used X-Ray film. Silver is a very precious metal used in various purposes such as photographic films, X-Ray, jewelleries and some electronic items. Alkaline protease successfully striped and recovered silver in good yield from the used photographic films. The used X-Ray film contains 1.5-2% (w/w) metallic silver (Black in color) which can be recovered and reused. The purified protease was then immobilized by entrapment method using Ca-Alginate beads. The optimum immobilization conditions such as pH, temperature and incubation time were investigated. After immobilization the pH stability of the protease was improved and there was no change in optimum temperature after immobilization.

Key words: *Bacillus*, Immobilization, X-Ray film, Silver recovery, Ca-Alginate.

Proteases are an important group of industrial enzyme that hydrolyzes peptide bonds of proteins. They can be produced from bacteria, fungi or certain insects, among these, protease produced from bacteria, especially *Bacillus subtilis* are widely used in various industrial processes such as detergents, food manufacturing, leather processing and proteinaceous based bioremediation

(Bayoudh *et al*., 2000) Silver is an important industrial metal used in various sectors such as photographic films, x-rays, jewellries, silver wares and electronic items. There are three reasons as to why silver should be recovered from used X-ray films namely conservation of a precious metal, economic return and environmental concerns. A photographic film/X-ray film uses silver because of its unmatched quality as a light-sensitive material for creating a photographic image recovery of silver by burning the films creates environmental pollution and health hazards. On the other hand, enzyme from microbial source breaks the gelatin layer embedded with silver in films creating pollution free stripping (Chaudhary *et al*., 2013). One of the most required properties for a good industrial catalyst is that enzyme has to be stable under the hardest conditions and for long duration. To overcome these limitations, enzyme can be immobilized on insoluble and solid support (Anwar *et al*., 1998) by immobilization of microbes in different entrapment matrices the enzyme produced can be more stable, pure, and continuous and can be reuse which in turn modulates the enzyme production in an economical manner. Gelatin was found to be best matrix among all with highest enzyme activity (Chatterjee *et al*., 2015). In view of the above facts, alkaline protease produced by *Bacillus sp* ATP-P5 was checked for its

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capability to recover metallic silver from used x-ray films and its immobilization.

MATERIALS AND METHODS

The media components were purchased from Hi Media laboratories (Mumbai, India) casein (Hammarsten) was a product of SISCO research laboratories (Mumbai, India) . All chemicals used were of analytical grade.

Alkaline protease production*:*

Medium containing (g/L) glucose-1.0, casein–0.5, peptone–0.5, yeast extract–0.5, KH_2PO_4–0.4 $MgSO_4$–0.5, $FeSO_4$. $7H_2O$–0.1 was used as production medium. Autoclaved erlenmeyer flasks containing 50 mL of medium was inoculated with 5 mL of seed medium. The flasks were incubated at 50°C for 48 hrs at 120 rpm in rotary shaker. The contents were then harvested by centrifugation and supernatant was used as a source of enzyme. The enzyme activity was determined by the method of (Takami *et al*., 1989) using the optimized reaction conditions. Enzyme assay was carried out using 500 µl enzymes in 50mM Tris-HCl buffer with pH-9.0, 0.5 % casein as substrate and the reaction mixture was incubated at 50°C for10 min. The assayed enzyme with optimum activity was purified by using 70% ammonium sulphate ppt followed by dialysis. Dialysed enzyme was separated by DEAE-Cellulose Anion exchange chromatography further. The separated enzyme was analyzed for purity by SDS-PAGE.

The purified (enzyme) protease from *Bacillus* sp. ATP –P5 was used for hydrolysis of gelatin and release of silver

The used x-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The films were dried in an oven at 50°C for 30 min and were cut into 4X4 cm^2 pieces. Approximately 40 pieces of the films were dipped in 100 mL of crude enzyme diluted in Tris–HCl buffer (pH 9.0) (Shankar *et al*., 2010).The solution containing the films were constantly stirred at 50°C, pH 9.0 in a water bath with continuous shaking until the gelatin-silver layer was stripped completely .The turbidity of the reaction mixture increased with time and the reaction was considered complete as no further turbidity increased. The turbidity was monitored by measuring absorbance at 660 nm. The obtained slurry was washed 2-3 times and filtered to remove the gelatin from the slurry and then dried. To 1 ml of the solution containing silver, 1 ml of conc. HNO_3 was added followed by 1 ml of conc. HCl progressively. The formation of white precipitate indicated the presence of silver. The dried slurry was smelted in the presence of Na_2CO_3 and hard coked at 200°C in a furnace. The silver was settled and the waste was burned out (Chaudhary *et al*., 2013).

Immobilization of purified enzyme

The purified protease enzyme was mixed with sodium alginate solution (2%) in 1:1 ratio. The protease-alginate mixture was added drop wise into calcium chloride (0.2 M) solution with gentle and continuous shaking at 4°C. As soon as the drop of protease-alginate solution mixed with $CaCl_2$ solution, Na^+ ions of Sodium-alginate will be replaced by the Ca^{+2} ions of calcium chloride solution, which formed Ca-alginate beads, in which protease was immobilized. The beads thus formed will be washed 3-4 times with deionized water and finally with 50 mM Tris-HCl buffer of pH 9.0 (Anwar *et al*., 2009).

Optimization of immobilized enzyme

Optimization of immobilized enzyme was done by using different concentration of sodium alginate (2-7 %) and different concentration of calcium chloride on the immobilization of protease. Effect of different pH and temperature was also optimized to determine the stability of the immobilized enzyme.

RESULTS AND DISCUSSION

The isolate was identified as *Bacillus subtilis* based on morphological, biochemical and molecular characterizations. The isolate was able to grow under alkaline conditions at pH 9.0 and a temperature of 50°C. 1.9 fold purification of enzyme following ammonium sulphate precipitation and DEAE-cellulose chromatography was achieved. The molecular weight of the enzyme was estimated to be approximately 29 kDa as shown by electrophoresis. Interestingly Ca^{2+} (5mM) activated enzyme activity, while Mg^{2+}, Mn^{2+} Co^{2+} moderately activated enzyme activity, where as Fe^{2+} and Cu^{2+} inhibited the activity of enzyme (Bholey *et al*., 2012).

Recovery of silver from waste photographic film

For the hydrolysis of gelatin and release of silver the waste x-ray films were used. Approximately 40 pieces of the films were dipped in 100 ml of crude enzyme diluted in Tris-HCl buffer (pH 9.0) (Shankar *et al*., 2010). The solution containing the films were constantly stir at 50ºC, pH 9.0 in a water bath with continuous shaking until the gelatin silver layer was stripped completely (fig 1). Then the slurry was washed and filtered to remove the gelatin. In the solution 1ml HNO_3 was added and 1ml of HCl was added. The white precipitates was formed which indicates the presence of silver (Fig 2). The result revealed that purified protease from *Bacillus* sp ATP-P5 was very efficient in gelatin hydrolysis from waste X-Ray photographic film under the obtained conditions (pH 9.0) and temperature 50°C, 0.054 gm silver was recovered by *Bacillus* sp respectively after 10 min of incubation. Whereas (Bholey *et al*., 2012) had reported the yield of silver was 0.4013gm by *Bacillus pumilus*. According to (Nokoboglu *et al*., 2001) 50°C as the stripping temperature for the enzyme for the hydrolysis was observed in the initial 15min. When nitric acid and hydrochloric acid was added in the solution white precipitates were observed. The white precipitates indicate the presence of silver in the solution.

Immobilization of protease enzyme

Immobilization of biocatalyst helps in their economic reuse and in the development of continuous bioprocesses. Immobilization often stabilize structure of the enzymes thereby allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents, And thus enable their use at high temperatures in non aqueous enzymology.

Effect of sodium alginate concentration

Various concentration of sodium alginate (2-10%) was used to acquire beads with greater stability. The percent entrapped activity was found maximum 35% at 4% (w/v) at sodium alginate concentration (Fig 3). Maximum leakage of enzyme from beads occurred at 2% (w/v) sodium alginate concentration owing to the larger pore size of the less tightly crossed linked fragile Ca-alginate beads while at 5% and 6% (w/v) Sodium alginate concentration the entrapped activity of the enzyme was found comparatively very low which might be due to high viscosity of enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of substrate in the beads. Anwar *et al*.,1998 reported maximum percent entrapped activity that is 45% at 2% (w/v) sodium alginate concentration.

Effect of calcium chloride concentration o immobilization

Concentration of Calcium chloride also varied in order to acquire stable beads capable to secure maximum enzyme and it was found that $CaCl_2$ (0.2 M) retained highest activity of entrapped enzyme and as calcium chloride concentration increased beyond the 0.2 M the activity of the protease decreased (Fig 4). Roig *et al*., 1995 reported a decrease in relative enzyme activity of alkaline protease with increase in the concentration of $CaCl_2$.

Effect of pH and temperature on activity of immobilized protease

Alginate entrapped enzyme was assayed at different temperature and pH, ranging from 40-70° C and pH 7.0-10 respectively and it was observed that optimum temperature of entrapped and it was observed that optimum temperature of entrapped enzyme was 50°C (Fig 5) while optimum

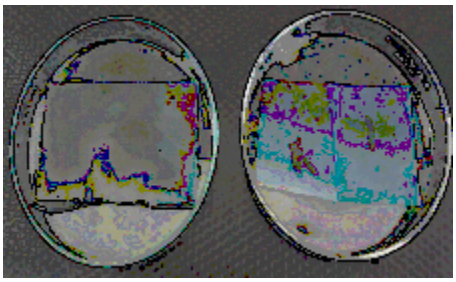

Fig. 1. Shows the removal of gelatin layer from the film

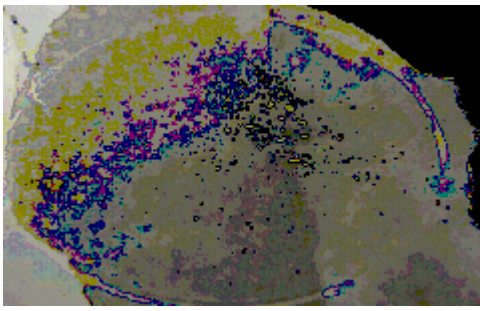

Fig. 2. Shows the presence of white precipitates of silver

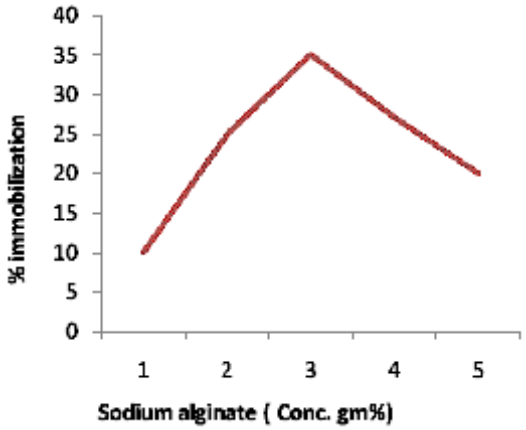

Fig. 3. Percent immobilization of Protease at different concentration of sodium alginate ranging from 1.0% to 6.0%.

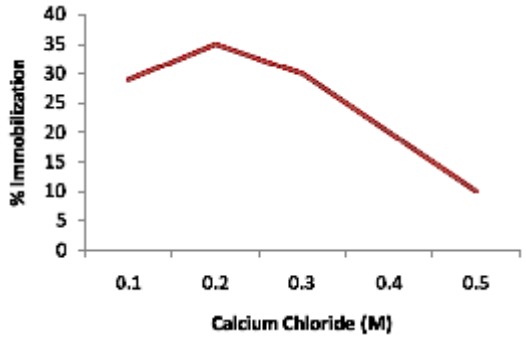

Fig. 4. Effect of calcium chloride concentration on percent immobilization of protease from *Bacillus* sp.

pH was 9.0 (Fig 6). The result showed no change in temperature and pH of protease before and after entrapment. It was reported that the surface of the beads in which the enzyme is localized has a cationic and anionic nature which ultimately affect the nature of the active enzyme protein and the pH of the entrapped enzyme (Norouzian *et al*., 2003). But in the present case the surface of beads at alkaline pH had no effect on the active protease, thus the optimum pH of free and immobilized enzyme remained the same. But when the pH of the immobilized enzyme was increased or decreased from 9.0, a sharp decline in activity was seen. Moreover it was observed that when the temperature was raised above its optimum value (50°C to 70°C) the immobilize enzyme shows decrease in activity. Arya *et al*., 2006 also reported that no change occurred in the optimum

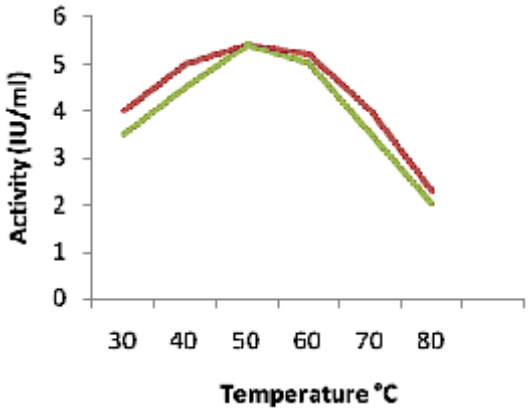

Fig. 5. Effect of different temperature on immobilized and soluble protease activity from *Bacillus* sp ATP-P5

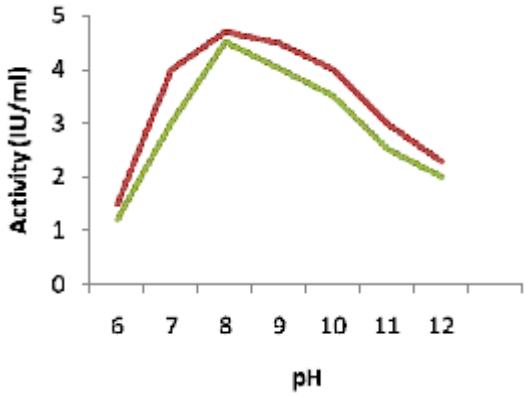

Fig. 6. Effect of pH on immobilized and soluble protease activity from *Bacillus* sp ATP-P5

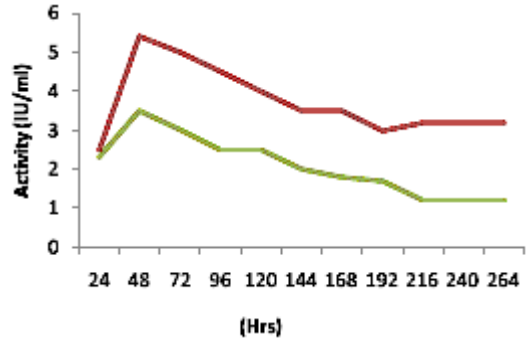

Fig. 7. Effect of incubation time on immobilized enzyme and soluble enzyme

temperature and pH before and after entrapment in Calcium alginate beads.

Storage stability of immobilized enzyme

The immobilized enzyme was stored at 4°C temperature and activity was noted for 12 days to determine the storage stability of entrapped enzymes. Beads stored at 4°C showed 33% loss of activity after 2nd day (48 hrs) and 51 % loss of activity after 10th day of incubation and remained constant after that (Fig 7). Whereas Anwar et al., 2009 reported 35% loss of activity after 2nd day of entrapment of enzyme and 89% loss of activity after 10th day of incubation. Adinarayana *et al*., 2004 reported the stability of alkaline protease up to nine days after entrapment of cells of *Bacillus subtilis* PE-11.

CONCLUSION

Stability and reuse of industrial enzyme is significant in decreasing the cost of enzymes. Breakthroughs in enzyme immobilization have enabled increased enzyme recovery and stability. The present study indicated that purified alkaline protease from *Bacillus* sp ATP-P5 was efficiently immobilized by using 0.2M $CaCl_2$ and 4% sodium alginate. The immobilized enzyme had retained its optimum activity upto 9th day of incubation and was stable at alkaline pH and temperature 50°C. Purified alkaline protease was used for recovery of silver from waste X-Ray films and about 0.054g silver was recovered from X-Ray film. This indicated that alkaline protease from *Bacillus* sp ATP-P5 has a potential of being applied for recovery of silver from waste X-Ray film.

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REFERENCES

1. Adinarayana, K. And Bapi, R. Investigations on alkaline protease production with *Bacillus subtilis* PE-11 immobilized in calcium alginate gel beads. *Proc biochem*., 2004; **39**: 1331-1339.
2. Anwar, A. Quder, S. A .U. Iqbal, S. and Azhar, A. Calcium alginate: A support material for immobilization of protease from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World appli Sci J*. 2009.,**7(10):**1281-1286.
3. Anwar, A. Saleemudin, M. Alkaline protease, A review *Biores Technol*.,1998; **6:**175-183.
4. Arya, S.K. and Srivastava, S.K. Kinetics of immobilized cyclodextrin gluconotransferase produced by *Bacillus marcerans* ATCC 8244. *Enz Microbial Technol*.,2006; **39**:507-510.
5. Bayoudh, A. Gharsallah, N. Chamkha, M. Dhouib, A. Ammar, S. and Nasri, M. Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MN1, *J, Ind Microbiol Biotechnol*.,2006; **24**: 291-295.
6. Bholay, A. D. More, S.Y. Patil, V.B. and Patil, N. Bacterial Extracellular Alkaline Proteases and its Industrial Applications. *Inter Res J,of Biol Sci*.,2012*;* **1(7):**1-5.
7. Chatterjee, S. Production and estimation of alkaline protease by immobilized *Bacillus licheniformis* isolated from poultry from soil of 24 parganas and its reusability, *J ,of adv pharama technol and res*.,2015; **6(1)**: 2-6.
8. Chaudhary, V. Recovery of silver from used X-Ray films by *Aspergillus versicolor* protease. *J, of Academia and ind res*.,2013; **2(1):** 39-41.
9. Kumari. P, Jandaik, S. and Batta, S**.** A Thermotolerant Protease from *Bacillus* sp Isolation, Characterization, Optimization and Purification. *J, of Pure an Appl Microbiol* ., 2014;**8** (5): 3667-3674.
10. Nakiboglu, N. Silver Recovery from Waste Photographic Films by an enzymatic Method *Turkian J of Chem*.,2001; **25**: 349 – 353.
11. Norouzian, D. Review: Enzyme immobilization the state of art in biotechnology. *Ira J of Biotechnol*., 2003; **1**:197-206.
12. Roig, M.G. Rashid, D.L. and Kenndy, J.F. High alkaline protease from *Bacillus* PB92 entrapped in Calcium alginate gel: physicochemical and microscopic studies. *Appl Biochem and Biotechnol*.,1995; **55**: 95-121.
13. Shankar, S.S. Shankar, S.V. More, R. and Laxman, S. Recovery of silver from waste x-ray film by alkaline protease from *conidiobolus coronatus*. Kathmandu university *j of sci, eng and technol* .,2010; **6(1)**: 60-69.
14. Takami, H. Aliba, T. and Horikoshi, K. Production of extremely thermostable alkaline protease from *Bacillus* sp No AH 10, *Appli Microbio and Biotechnol*.,1989; **30**:120-124.