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# Approach towards Submerge Production, Characterization and Application of Extracellular Alkaline Protease from *Pseudomonas aeruginosa* YPVC

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**ABSTRACT: The trend of utilizing eco friendly products is consistently increasing, which results into replacement of several chemical products with microbial formulations. Alkaline protease is an important microbial metabolite having diverse industrial applications. Therefore, in present study, alkaline protease production was carried out from soil dwelling bacterial cultures. Total 66 bacteria amongst 135 were found to be enzyme producers. The isolate VV19 displayed the highest enzyme production with 309.12 ± 4.1 U/ml activity in 24 h under submerge condition. This isolate was identified as *Pseudomonas aeruginosa* YPVC (GenBank accession number MN049574). The optimized culture conditions pH 10, temperature 30°C, inoculum size 7% (v/v), xylose 0.3%, $KNO_3$ 0.2% and NaCl 0.1% resulted into 2.4 fold increment in the alkaline protease productivity. Partial purification of the enzyme resulted into 7.62 purification and 53.51% enzyme yield after dialysis. The enzyme exhibited superior activity at optimum pH and temperature 10 and 50°C respectively with considerable tolerance at elevated pH and temperature. Casein was found to be an ideal substrate with apparent $K_m$ and $V_{max}$ values 12.5 mg/ml and 400 U/ml respectively. The presence of $Na^+$, $K^+$, $Ba^{+2}$, $Cu^{+2}$ and cationic surfactant cetylpyridinium chloride enhanced the enzyme activity. Significant compatibility and stability of the enzyme was observed with Wheel detergent. The enzyme displayed considerable potential in destaining and silver recovery applications.**

**Keywords:** Alkaline protease; enzyme activity, *Pseudomonas aeruginosa* YPVC; optimization; characterization.

## INTRODUCTION

Protease is an important hydrolytic enzyme with catalytic function of peptide bond cleavage in proteins, and holds about 60% occupancy in terms of overall global enzyme production (Thakur *et al*., 2018; Baweja *et al*., 2016). Now days, people are becoming more aware of detrimental effects of products emended with synthetic chemicals. Therefore, several formulations containing environment friendly and cost effective substitutes have become choice of interest for them. Alkaline protease is one of such substitutes with magnified range of applications in detergent, peptide synthesis, food processing, leather, medical diagnosis, pharmaceutical, meat tenderization, silk degumming, baking, brewing, silver recovery, and waste treatment (Razzaq *et al*., 2019; Al-Dhabi *et al*., 2020). Faster growth rate, simple nutrients requirement, less space requirement, ease of acclimatization with different environmental conditions, easy and effective recovery, and simplicity in genetic modification make microorganisms the first choice compared to plant and animal sources for alkaline protease production (Souza *et al*., 2015; Thakur *et al*., 2018). *Bacillus* spp. are the most explored microorganisms for the production of alkaline proteases (Thakur *et al*., 2017; Saggu and Mishra 2017; Patel *et al*., 2018; Masi *et al*., 2021). However, few reports suggested the utilization of *Pseudomonas* spp. for alkaline protease production (Boopathy *et al*., 2013; Cui *et al*., 2015).

Microbial production of alkaline protease is accomplished by solid state and submerged fermentation modes. Nevertheless, major fraction of enzyme production is achieved through submerge fermentation process (Prakasham *et al*., 2006). The microbial production of alkaline protease is greatly influenced by physico-chemical conditions, and hence careful attention is required to optimize such parameters for better enzyme yield (Banerjee *et al*., 1999). Industrial application of alkaline protease demands its considerable activity and stability under diverse antagonistic conditions of extreme pH, temperature, presence of inhibitors, surfactants, oxidizers, and bleaching agents. The alkaline protease produced by microbial sources offers such advantages and therefore largely exploited in detergent industry

(Verma and Pandey 2019; Jellouli *et al*., 2011; Rai *et al*., 2010; Venil and Lakshmanaperumalsamy 2009). Now days the alkaline protease from different microbial sources is utilized for the recovery of silver from X-ray film waste (Al-Abdalall and Al-Khaldi 2016; Choudhary 2013; Seid 2011).

The present study implies the submerged production of alkaline protease from soil isolate *Pseudomonas aeruginosa* YPVC. The study includes optimization of various physico-chemical parameters for hyper production of the enzyme. Moreover, partial purification, characterization, and application of alkaline protease are also incorporated in the present study.

## MATERIALS AND METHODS

***Isolation, screening and identification of alkaline protease producing bacteria***

Soil samples collected from different locations and processed for isolation of bacteria using nutrient agar plate by standard microbiological procedure. Primary screening of alkaline protease production was carried by streaking individual bacterial isolates on casein yeast extract peptone (CYP) agar medium containing (g/l): casein, 10; peptone, 2; yeast extract, 1; $K_2HPO_4$, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; pH, 10 ± 0.2; agar-agar, 30. All the plates were incubated at 28 ± 2°C for 48 h; after that the plates were flooded with Frazier solution ($HgCl_2$, 15 g; concentrated HCl, 20 ml and distilled water, 100 ml). The alkaline protease production was determined by clear zone around bacterial colony. In secondary screening, the bacterial cultures were inoculated into liquid CYP medium, and the activity of alkaline protease was determined from culture supernatants. Primary identification of bacteria was performed on the basis of morphological, cultural and biochemical characteristics according to Bergey's Manuals of Systematic Bacteriology. The most prominent bacterial isolate was identified by 16S rRNA gene sequencing from Gujarat Biotechnology Research Centre (GBRC), Gandhinagar, Gujarat, India.

***Optimization of cultivation parameters for submerged production of alkaline protease***

The submerged production of alkaline protease was carried out in 250 ml Erlenmeyer flasks containing 50 ml CYP medium. The flasks were inoculated with 3% (v/v) overnight growth of active bacterial cultures with uniform cell density ($O.D._{600}$ 1.0) and incubated at 28 ± 2°C and 120 rpm for 48 h. Aliquots (2 ml) were withdrawn from the flasks and centrifuged at 10,000 rpm for 10 min at 4°C. The clear supernatant was processed for alkaline protease activity. The influence of various culture conditions on enzyme production was assessed under static and shaking (120 rpm) conditions, inoculum size (1 - 15% v/v), pH (5 - 12), and temperature (15 - 50°C). In the next step, the effect of different carbon, nitrogen sources, and NaCl on enzyme production was determined.

***Alkaline protease assay***

The alkaline protease activity was determined according to the method described in the reports (Gessesse *et al*., 2003; Kembhavi *et al*., 1993) with minor modification. Briefly, the reaction mixture containing 1 ml suitably diluted culture supernatant and 1 ml casein (1% w/v) in glycine - NaOH buffer (50 mM, 10 pH) was incubated at 50°C for 10 min. After incubation, 2 ml trichloro acetic acid (10% w/v) was added and incubated at room temperature for 10 min. The un-reacted casein was removed by centrifugation at 10,000 rpm for 10 min, and the absorbance of clear supernatant was measured at 280 nm. The tyrosine content was determined using standard graph. One enzyme unit was considered as the amount of enzyme required to liberate 1 µg of tyrosine per minute under standard experimental conditions. For bacterial growth measurement, the cell pellet obtained after centrifugation was re-suspended in 2 ml distilled water, and its absorbance was recorded at 600 nm. Concentration of protein was determined using bovine serum albumin (BSA) as a standard (Lowry *et al*., 1951).

***Partial purification of alkaline protease***

The culture supernatant was precipitated by solid ammonium sulfate to achieve 40 - 90% saturation under cold condition (4°C). The precipitates were separated by centrifugation at 10,000 rpm for 20 min at 4°C and dissolved in a minimal volume of the buffer. The dialysis of enzyme was performed at 4°C using dialysis membrane - 50 with molecular weight cut off 12,000 - 14,000 dalton (HiMedia, India). The sealed dialysis membrane was immersed in the buffer for 24 h with intermittent replacement of fresh buffer at every 4 h interval. The alkaline protease activity and protein concentration of dialysed sample were performed as per methods described earlier.

***Characterization of partially purified alkaline protease***

The activity of partially purified alkaline protease was assessed with different pH (5 - 12), temperature (30 - 90°C), substrates (casein, gelatin, BSA), metal ions, and surfactants. The reaction mixture containing enzyme and substrate was incubated under different conditions for 10 minutes, and the relative activity of the enzyme was measured. The alkaline protease activity of control was considered as 100%.

***Application of partially purified alkaline protease***

***Enzyme compatibility with commercial detergents***

Commercially available detergents like Ariel, Rin, Surf Excel, Tide, Nirma and Wheel were used to determine compatibility of the enzyme. In order to inactivate endogenous enzymes, the detergent solution (0.4%) was heated at 60°C for 1 h. The partially purified alkaline protease (1000 U/ml) was mixed with detergent solution, and incubated at 50°C for 3 h. The residual enzyme activity was determined at every 30 min interval and compared with control.

***Destaining performance test***

The white cotton cloth pieces (4 × 4 cm) stained with human blood were immersed into the solutions containing Wheel detergent (0.4%), enzyme (1000 U/ml), and combination of Wheel detergent and enzyme each for 15 min at 50°C. The cloth pieces after incubation were rinsed with tap water and air dried. Visual examination of cloth pieces was done for comparison of washing efficiency.

***Recovery of silver from X - ray film***

Waste X-ray film was cut into 4 × 4 cm equally sized pieces and dried at 50°C for 30 min. Each piece was soaked into solution containing 1 ml enzyme and 2 ml buffer (50 mM glycine NaOH, pH 10) and incubated at 50°C on the orbital shaker (90 rpm) for different time periods. The progress of hydrolysis was monitored by measuring the absorbance of the reaction mixture (hydrolysate) at 660 nm (Al-Abdalall and Al-Khaldi 2016).

***Data analysis***

All the experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

***Isolation, screening and identification of alkaline protease producing bacteria***

The standard laboratory procedure revealed 135 pure bacterial cultures from 11 soil samples. Primary screening displayed 66 bacterial cultures positive for alkaline protease production. Maximum clear zone (20 ± 0.02 mm) was observed by the isolate VV19 indicating an effective enzyme producer (Fig. 1A). During secondary screening, the isolate VV19 displayed the highest enzyme productivity (309.12 ± 4.1 U/ml) among all the bacterial cultures tested. Owing to superior enzyme yield within shorter incubation period (24 h), the bacterial isolate VV19 was selected as organism of interest, and adopted for further study. The use of solid and liquid media for screening and final selection of alkaline protease producer microorganisms is mentioned in the literatures (Masi *et al*., 2021; Ayantunji *et al*., 2020; Baweja *et al*., 2016). Primary identification of isolate VV19 revealed gram negative, non-sporulated, non-capsulated, and highly motile short rods (Fig. 1B). The growth of isolate VV19 with the formation of water soluble greenish pigment on NA (Fig. 1C), and in liquid medium (Fig. 1D) pointed our findings towards genus *Pseudomonas*. Finally, the partial gene sequence of 1155 bp of 16S rRNA was deposited to the GenBank having accession number MN049574. Upon comparison against NCBI database, the sequence displayed 99.88% identity to the sequences of different strains of *Pseudomonas aeruginosa* (Fig. 1E), and hence the isolate VV19 was designated as *Pseudomonas aeruginosa* YPVC.

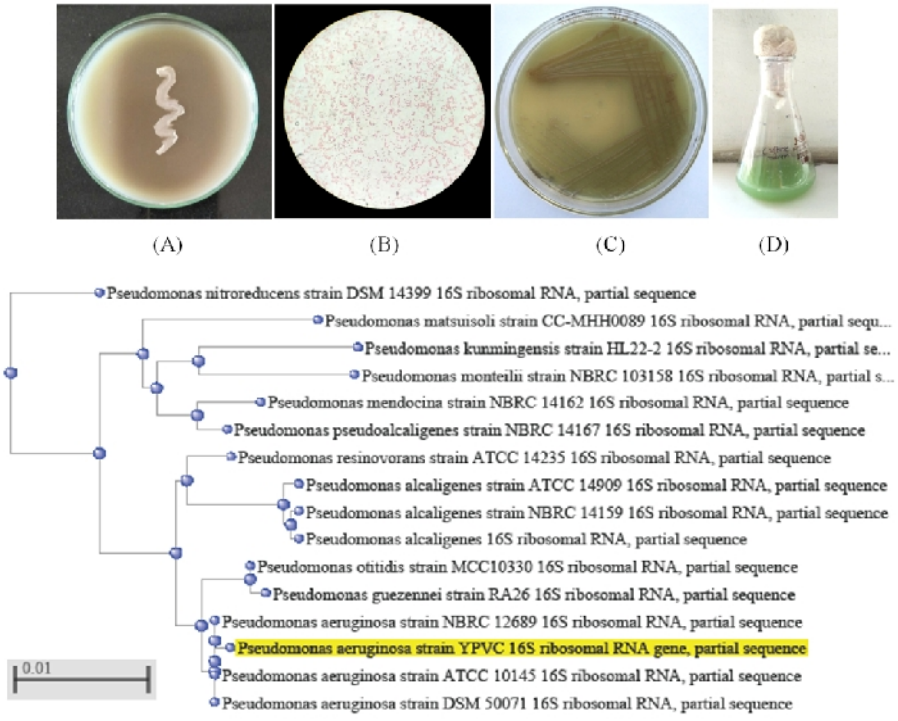



**Fig. 1.** Characteristics of bacterial isolate *Pseudomonas aeruginosa* YPVC: zone of clearance on CYP agar plate (A); gram stain reaction (B); colonial growth on NA plate (C); growth in liquid medium (D); neighbour joining phylogenetic tree with NCBI database bacterial cultures (E).

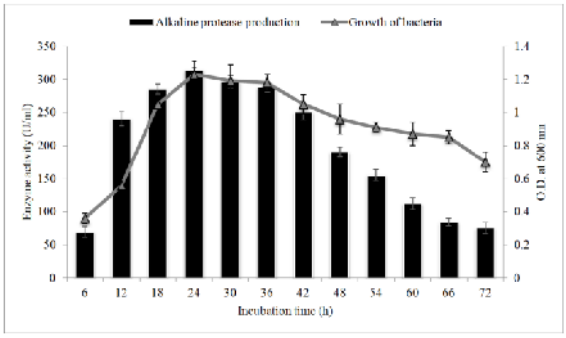
***Optimization of cultivation parameters for hyper production of alkaline protease by Pseudomonas aeruginosa YPVC***

***Time course study of alkaline protease production***

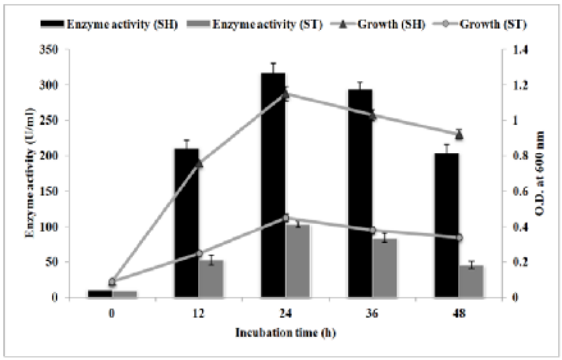
The extracellular enzyme production was found to be increased with concomitant increment in the incubation time from 6 to 24 h. The maximum enzyme productivity of the isolate was observed at 24 h (314.22 ± 6.7 U/ml). The enzyme production then remained fairly constant in the range of 24 - 36 h (Fig. 2A). The diminution of enzyme yield was apparent after 36 h; only 23.88% enzyme production was obtained at the end of 72 h comparative to maximum yield. The growth profile of *Pseudomonas aeruginosa* YPVC revealed steady rise during initial time interval with the highest value at 24 h (1.23 ± 0.08 O.D.). The bacterial growth

entered the stationary phase thereafter up to 36 h. Subsequently, the bacterial biomass production reached to decline phase. This could be correlated with lower enzyme activity at later phase of incubation period. The results displayed quite shorter incubation time for enzyme production by *Pseudomonas aeruginosa* YPVC, as several researchers have mentioned comparatively longer incubation time to achieve desirable enzyme production. For instance, maximum proteolytic activity and biomass production of *Halobacterium* sp. AF1 was obtained at 72 h (Habib *et al*., 2011). In another study, the alkaline protease production by *Bacillus aryabhattai* P1 was the highest (150 U/ml) after 96 h incubation (Pathak *et al*., 2020). It is apparent that longer time for the product formation requires more investment in aeration, agitation and temperature maintenance, which in turn affects overall cost of the fermentation product.

The comparison of incubation conditions (static/shaking) showed that the isolate accomplished higher growth (1.15 ± 0.04 O.D.) with concomitant enzyme productivity (317.61 ± 5.4 U/ml) under shaking condition (Fig. 2B). The resultant enzyme yield and bacterial growth were 3.07 and 2.55 times superior respectively in the shaking condition compared to static incubation. Therefore it was evident that an extracellular enzyme production by *Pseudomonas aeruginosa* YPVC was growth associated; higher the number of active bacterial cells, more the alkaline protease production.




(A)




(B)

**Fig. 2.** Time course profile (A), and effect of static and shaking conditions (B) on alkaline protease production by *Pseudomonas aeruginosa* YPVC under submerged culture condition.

It is obvious that shaking condition facilitates diffusion of higher amount oxygen into culture broth leading to higher concentration of the dissolved oxygen, which is one of the rate limiting factors for submerged fermentation. Our findings are in accordance with previous report, in which the submerged production of alkaline protease and growth of *Bacillus cereus* HP_RZ17, and *Paenibacillus xylanilyticus* HP_RZ19 were highest under shaking condition after 48 h (Jadhav *et al*., 2020).

***Influence of inoculums size, pH and temperature on alkaline protease production***

The enzyme production by *Pseudomonas aeruginosa* YPVC improved steadily with the increase in inoculum size from 1 to 7%. The overall enzyme yield was the highest with 7% inoculum size (Table 1). Further increment in inoculum size led to decline in enzyme productivity of the isolate. Likely, lower inoculums of the isolate had negative effect on the enzyme yield. The alkaline protease production was 29.53 and 74.19% with 1 and 15% inoculums respectively as compared to the highest value. Above finding suggested an importance of initial inoculum size for optimum microbial biomass and associated enzyme production. It is evident that lower inoculum size leads to insufficient microbial biomass for enzyme production; an excess inoculum size on the other hand may have negative impact owing to rapid depletion of dissolved oxygen, competition for available nutrients, and large amount of toxic metabolites in the medium leading to deprived growth and enzyme production capacity of bacteria (Jadhav *et al*., 2020; Suganthi *et al*., 2013). Therefore, appropriate balance between propagating microbial cells and available nutrients is crucial factor in order to achieve maximum enzyme yield. We obtained similar results with the report describing alkaline protease production at different inoculums (1-90%) of *Bacillus infantis* SKS; the enzyme productivity was the highest at 10% inoculum size, and further increase in inoculum size led to declined enzyme yield (Saggu and Mishra 2017).

The *Pseudomonas aeruginosa* YPVC displayed progressive increment in enzyme productivity between pH 3 and 10 with maximum value at pH 10 (Table 1). Nevertheless, compared to the highest value, only 5.3 and 11.08% reduction in enzyme yield was observed at pH 11 and 12 respectively, which suggested considerable pH tolerance of the isolate. Acidic pH showed lesser degree of bacterial growth and enzyme productivity; only 22.33% enzyme production was evident at pH 3. Therefore, owing to superior cellular biomass and enzyme yield, pH 10 was considered as an optimum pH for our study. It is well understood that the pH of growth medium regulates the transfer of nutrients across the cell membrane, which may affect the overall growth and metabolism of bacteria (Moon and Parulekar 1991). Our results are in good agreement with earlier reports, in which the production of alkaline protease by *Citricoccus* sp. (Verma and Pandey 2019), and *Bacillus cereus* (Ayantunji *et al*., 2020) was the highest with culture medium pH 10. In another study, the alkaline protease production by *Bacillus aryabhattai* P1 was maximum when pH of culture medium was in

the range of 9-10 (Pathak *et al*., 2020). The dry biomass and enzyme production by *Bacillus stearothermophilus* were comparatively higher in the pH range 7-11 with the highest values at pH 10 (Karray *et al*., 2021).

The production of alkaline protease by *Pseudomonas aeruginosa* YPVC was pretty lower at 15°C, and reached its highest value at 30°C (Table 1). Nevertheless, any further rise in temperature resulted into declined trend of enzyme production. Upon comparison with optimum temperature (30°C), the alkaline protease production was 26.31 and 5.41% at 45 and 50°C respectively. The bacterial biomass production also followed the same trend with maximum value at 30°C compared to other temperatures. In general, the temperature lower than optimum suppresses the growth of microorganisms by acting as microbiastatic; higher temperature on the other hand acts as microbicidal. The results obtained in our study are similar with earlier report describing maximum production of alkaline protease by *Bacillus cereus* HP_RZ17 and *Paenibacillus xylanilyticus* HP_RZ19 at optimum incubation temperature 30°C (Jadhav *et al*., 2020). However, the bacterial culture *Bacillus subtilis* apr-IBL04 exhibited maximum yield of alkaline protease at 45°C incubation temperature (Shafique *et al*., 2021).

**Table 1: Influence of inoculum size, pH and temperature on alkaline protease production by *Pseudomonas aeruginosa* YPVC.**

| Production parameter | Alkaline protease activity (U/ml) | Bacterial growth (O.D. at 600 nm) |
|---|---|---|
| **Inoculum size (% v/v)** | | |
| 1 | 105.21 ± 4.6 | 0.46 ± 0.13 |
| 2 | 178.13 ± 7.1 | 0.51 ± 0.24 |
| 3 | 202.18 ± 9.9 | 0.87 ± 0.21 |
| 5 | 234.67 ± 4.6 | 1.05 ± 0.13 |
| 7 | 356.27 ± 7.7 | 1.28 ± 0.09 |
| 10 | 303.43 ± 8.9 | 1.08 ± 0.11 |
| 12 | 289.23 ± 5.5 | 0.85 ± 0.14 |
| 15 | 264.32 ± 6.7 | 0.80 ± 0.21 |
| pH* | | |
| 3 | 72.44 ± 7.7 | 0.12 ± 0.08 |
| 4 | 120.82 ± 8.4 | 0.27 ± 0.12 |
| 5 | 144.51 ± 9.1 | 0.31 ± 0.21 |
| 6 | 184.34 ± 6.8 | 0.54 ± 0.14 |
| 7 | 213 ± 4.5 | 0.59 ± 0.09 |
| 8 | 268.22 ± 6.9 | 0.72 ± 0.15 |
| 9 | 313 ± 7.8 | 1.04 ± 0.21 |
| 10 | 324.26 ± 5.3 | 1.30 ± 0.13 |
| 11 | 307 ± 4.7 | 0.95 ± 0.22 |
| 12 | 288.54 ± 7.8 | 0.76 ± 0.14 |
| Temperature (°C)** | | |
| 15 | 66.36 ± 4.2 | 0.34 ± 0.11 |
| 30 | 317.81 ± 8.3 | 1.29 ± 0.08 |
| 37 | 265.48 ± 7.1 | 0.94 ± 0.13 |
| 45 | 83.64 ± 5.9 | 0.46 ± 0.11 |
| 50 | 17.23 ± 3.6 | 0.31 ± 0.08 |

* inoculum size was 7% (v/v)
** inoculum size and pH were 7% (v/v) and 10 respectively
All the flasks were incubated in shaking condition (120 rpm) and enzyme activity was determined after 24 h incubation

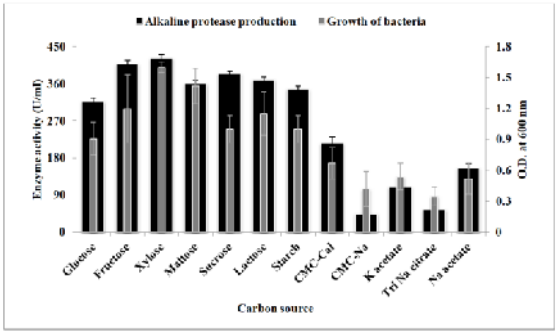
***Influence of carbon, nitrogen source and NaCl on alkaline protease production***

The isolate was able to grow and produce enzyme in the presence of all the carbon sources tested with variable degree of enzyme yield (Fig. 3A). An incorporation of xylose into production medium conferred superior enzyme production (422.61 ± 8.37 U/ml) and biomass (1.6 ± 0.05 O.D.) followed by fructose (408.29 ± 7.77 U/ml). The alkaline protease production was 91.23 - 82.23% in the presence of sucrose, lactose, maltose, and starch. Nonetheless, carboxy methyl cellulose and trisodium citrate were found to be poor carbon sources with 10.7 and 13% enzyme yield respectively. It was peculiar that *Pseudomonas aeruginosa* YPVC preferred xylose over the most common carbon source glucose with 1.33 times higher enzyme yield. The reduction in enzyme productivity with glucose may be correlated with catabolic repression. The production of alkaline protease by *Pseudomonas aeruginosa* YPVC was also evaluated with different concentrations of xylose (0.1 - 2% w/v). The highest recorded growth (1.82 ± 0.04 O.D.) and enzyme productivity (538.54 ± 5.9 U/ml) were obtained at 0.3% xylose concentration. Further increase in xylose concentration led to negative trend of biomass production and enzyme yield. Therefore, above results claimed xylose (0.3%) as an optimum carbon source for biomass and enzyme production. Previous reports suggested fructose as an ideal carbon source for alkaline protease production by *Bacillus* sp. NPST-AK15 (Ibrahim *et al*., 2015), *Bacillus cereus* AG1 (Patel *et al*., 2018), *Bacillus cereus* HP_RZ17, and *Paenibacillus xylanilyticus* HP_RZ19 (Jadhav *et al*., 2020). In another study *Streptomyces* sp. Al-Dhabi-82 was able to produce highest amount of alkaline protease

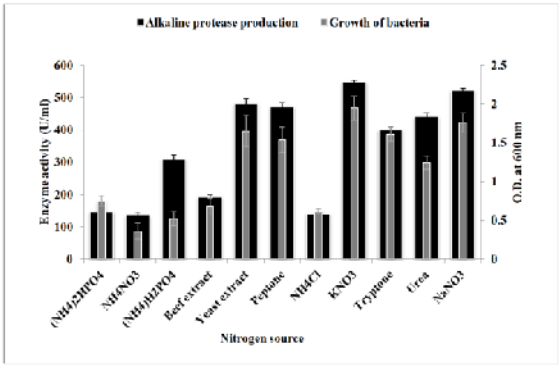
in the presence of maltose; lactose was found to be poor carbon source for enzyme production (Al-Dhabi *et al*., 2020). The production medium supplemented with galactose permitted the highest level of enzyme production by *Bacillus stearothermophilus* (Karray *et al*., 2021).

The isolate displayed variable degree of enzyme productivity in the presence of nitrogen sources tested (Fig. 3B). Organic nitrogen source like yeast extract is considered as ideal nitrogen source, as it contains amino acids, vitamins, growth factors, and carbohydrates required for the bacterial growth and product formation (Hidayat *et al*., 2017). Conversely, the isolate *Pseudomonas aeruginosa* YPVC preferred an inorganic nitrogen source $KNO_3$ for better enzyme production (547.43 ± 5.98 U/ml) followed by $NaNO_3$ (523.21 ± 4.96 U/ml). Organic nitrogen sources like yeast extract, peptone, urea, and tryptone were not as effective as $KNO_3$, and demonstrated 88.12 – 73.13% enzyme yield as compared to maximum output level. Ammonium nitrate and ammonium chloride on the other hand failed to improve enzyme production with 24.86 and 25.59% enzyme yield respectively. The effect of different concentrations of $KNO_3$ (0.1 - 2%) was checked on the alkaline protease production; the highest enzyme productivity (654.48 ± 7.5 U/ml) was observed with 0.2% $KNO_3$ concentration. Later on, the enzyme production declined upon consequent increase in $KNO_3$ concentration. Our findings are in accordance with the previous study, in which the alkaline protease production by *Bacillus subtilis* HB04 was maximum in the presence of inorganic nitrogen source (NH4)2PO4 (Venil and Lakshmanaperumalsamy 2009). Nevertheless, the alkaline protease production by *Pseudomonas aeruginosa* was highest in the presence of organic nitrogen sources like beef extract (Ariole and Ilega 2013).

The results depicted in Fig. 3C revealed that the bacterial biomass (2.12 ± 0.2 O.D.) and enzyme productivity (742.06 ± 6.37 U/ml) was at peak with 0.1% NaCl. Further increase in NaCl concentration to 3% showed relative decrease in biomass and enzyme production. However, 56.06% enzyme yield and 47.62% bacterial growth were obtained at 3% NaCl concentration. The alkaline protease yield was 41.78% at 5% NaCl concentration. Above findings thus revealed halotolerant nature of the isolate, and alkaline protease produced by such bacteria could be explored in detergent industry to work in high salt condition. Nevertheless, decline in bacterial growth and enzyme output was apparent at higher NaCl concentration; the biomass and enzyme yield were 10.48 and 15.09% respectively at 10% NaCl compared to 0.1% concentration. Significant decrease in enzyme yield was obtained at 20% NaCl concentration. It is obvious that higher salt concentration draws water from the cytoplasm resulting into plasmolysis, and this could be the probable reason for very faint growth of *Pseudomonas aeruginosa* YPVC and associated enzyme yield at higher salt concentration. Earlier study showed that the highest enzyme productivity of *Bacillus* sp. strain TD obtained at 0.5% NaCl; an increase in salt concentration to 3% inhibited the enzyme production (Desai and Vyas 2014). After optimizing physico-chemical parameters, the production of alkaline protease by *Pseudomonas aeruginosa* YPVC was improved 2.4 times, this signified the successful optimization of physico-chemical parameters and their positive effect on enzyme production.




(A)

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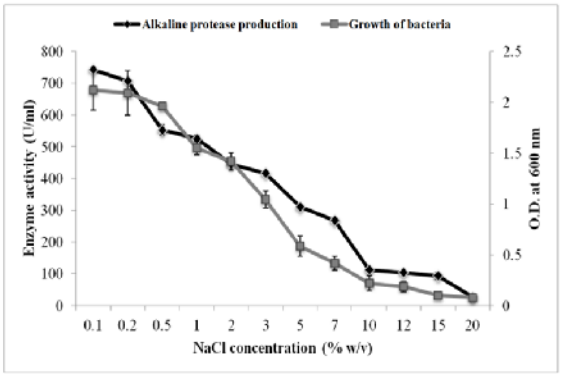


(C)

**Fig. 3.** Effect of carbon source (A), nitrogen source (B), and NaCl (C) on alkaline protease production by *Pseudomonas aeruginosa* YPVC. The experiments conducted in shaking condition (120 rpm) with 7% (v/v) inoculum size, pH 10, and temperature 30°C. The alkaline protease activity was determined after 24 h incubation.

***Partial purification of alkaline protease***

The partial purification of alkaline protease was performed by ammonium sulfate precipitation followed by dialysis. The specific activity in sediments was the highest at 80% saturation with 73.45% yield recovery and 6.69 fold purification (Table 2). The enzyme recovery after dialysis was 53.51% with 7.62 fold purification and specific activity of 644.80 U/mg of protein.

**Table 2: Partial purification scheme for alkaline protease from *Pseudomonas aeruginosa* YPVC.**

| Purification step | Volume (ml) | Total enzyme activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| Crude | 100 | 19100 | 226 | 84.51 | - | 100 |
| 80 % Ammonium sulfate | 20 | 14030 | 24.83 | 565.04 | 6.69 | 73.45 |
| Dialysis | 10 | 10220 | 15.85 | 644.80 | 7.62 | 53.51 |

Values presented are mean of three experiments

***Characterization of partially purified alkaline protease***

***Effect of pH, temperature and substrate on alkaline protease activity***

The activity of alkaline protease increased gradually with the increment of pH, and reached its maximum value at pH 10 (Fig. 4). At lower pH the relative enzyme activities were 9.63% (pH 5) and 22.20% (pH 6). The recorded relative enzyme activities at pH 8 and 9 were 89.57 and 94.62% respectively. Nevertheless, quite considerable relative enzyme activity (82%) was obtained at pH 11; the activity of alkaline protease was 45.42% at pH 12 in comparison to pH 10. We obtained quite significant results, as in earlier report, the highest value of the partially purified alkaline protease from *Pseudomonas aeruginosa* MTCC 10501 was at pH 9, and reduction in enzyme activity was evident at pH 10 (Boopathy *et al*., 2013). Likewise, in another study, the partially purified P1 protease from *Bacillus aryabhattai* P1 displayed highest activity at pH 9, and 90.38, 62.69 and 64.62% enzyme activities were obtained at pH 8, 10 and 11 respectively (Pathak *et al*., 2020). Therefore, compared to above reports, our findings suggested better performance of alkaline protease in the broad range of pH (7 – 11).

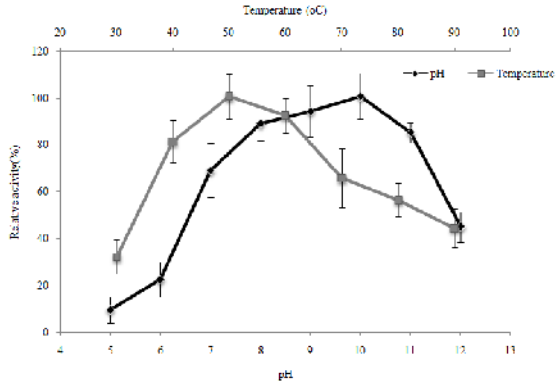


**Fig. 4.** Effect of pH and temperature on catalytic activity of alkaline protease from *Pseudomonas aeruginosa* YPVC.

The optimum enzyme activity and stability at alkaline pH along with broad pH range are the most demanding criteria for the utility of alkaline protease in the detergent industry (Ramkumar *et al*., 2018; Kumar and Takagi 1999).

The temperature optima revealed considerable alkaline protease activity over a broad range of temperature (40 - 70°C), and displayed its highest activity at 50°C (Fig. 4). Conversely, the enzyme retained 92.58% activity at 60°C indicating significant temperature tolerance. At 70 and 80°C, the relative enzyme activities were 65.20 and 48.22% respectively. Above results demonstrated notable performance of alkaline protease at elevated temperature, which implies an important advantage for its industrial applications. Similar result was obtained by Saggu and Mishra (2017) with 50°C temperature as an optimum temperature for alkaline proteases from *Bacillus infantis* SKS1. Compared to our results, the alkaline protease form *Streptomyces* sp. Al-Dhabi-82 displayed maximum activity at lower temperature (40°C) (Al-Dhabi *et al*., 2020).

The substrate specificity is one of the important features of alkaline protease. However, for industrial point of view, the alkaline protease working on various substrates is the most demanding criterion (Kumar and Takagi 1999). The partially purified alkaline protease showed variable degree of activity with different substrates; casein was found to be the best substrate yielding maximum enzyme activity. The enzyme activity with gelatin and BSA was 12.35 and 5.71% respectively compared to casein. Furthermore, the alkaline protease activity was evaluated with different concentrations of casein (2-20 mg/ml). The results indicated that the alkaline protease activity increased linearly with concomitant increment in casein concentration. Under experimental conditions, the correlation between enzyme activity ($V$) and initial casein concentration ($[S]$) was determined by Michaelis-Menten double reciprocal model ($V_{max}[S]$ / ($K_m$+$[S]$). The values of an apparent enzyme activity maxima ($V_{max}$) and Michaelis constant ($K_m$) were 400 U/ml and 12.5 mg/ml respectively (Fig. 5). The kinetic constant ($V_{max}$ / $K_m$) value was 32 U/mg, which indicated that the enzyme worked effectively throughout the range of casein concentrations taken in the study. The hydrolysed products of casein and other protein sources have wide range of industrial applications in the preparation of high protein therapeutic diets, hypoallergenic infant foods, and fortified fruit juices (Ramkumar *et al*., 2018). Number

of reports documented casein as the most suitable substrate for the highest activity of alkaline protease **(**Masi *et al*., 2021; Patel *et al*., 2018; Saggu and Mishra 2017). Nonetheless, the highest affinity of *Halobacterium* sp. AF1 alkaline protease was found towards gelatin as compared to casein and BSA (Habib *et al*., 2011).

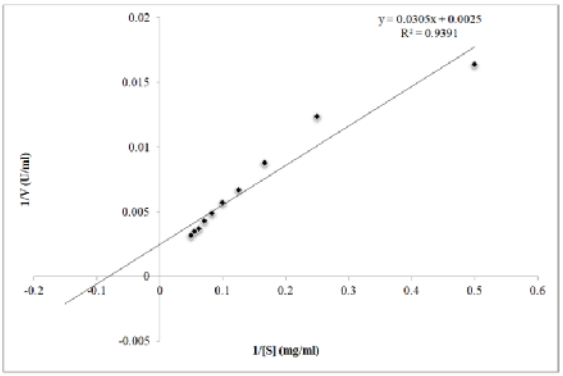


**Fig. 5.** Double reciprocal plot of effect of substrate (casein) concentration on alkaline protease activity.

***Effect of metal ions and surfactants on alkaline protease activity***

The effect of various metal ions at 2, 5 and 10 mM concentrations on alkaline protease activity was evaluated in the present study. The presence of $Na^{+}$, $Cu^{+2}$, $K^{+}$ and $Ba^{+2}$ at 2 mM concentration stimulated alkaline protease activity. However, an inhibitory effect of such metal ions was evident at higher concentrations (Table 3). The presence of $Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ on the other hand inhibited enzyme activity in all concentrations tested. Conversely, $Hg^{+2}$ and $Ag^{+2}$ exhibited significant inhibition of enzyme activity at 2 mM concentration; further increase in concentrations to 5 and 10 mM led to highest inhibition of the enzyme activity. It is explained that number of enzymes contain different metal ions, and the inhibition of enzyme activity is apparent when one of the metal ions is either substituted or displaced with other metal ions having similar size or charge (Lehninger, 1950). Previous reports stated $Hg^{+2}$, $Zn^{2+}$ and $Cu^{+2}$ as the most prominent metal ions displaying inhibitory action on alkaline protease (Al-Dhabi *et al*., 2020; Verma and Pandey 2019; Rai *et al*., 2010). In contrast, stimulation of alkaline protease enzyme activity was reported in the presence of $Cu^{+2}$ and $Hg^{+2}$, suggesting that metal ions exert variable effects on triggering or inhibiting the activity of enzyme.

The alkaline protease from *Pseudomonas aeruginosa* YPVC displayed 98-92% relative activities in the presence of Triton-X-100, Tween-80 and Tween-20 at 0.1% concentration (Table 3). However, these surfactants at higher concentration displayed an inhibitory effect on alkaline protease activity. It was noticeable that cationic surfactant cetylpyridinium chloride stimulated enzyme activity with relative enzyme activities of 116.28 ± 4.6 and 102.14 ± 3.9% at 0.1 and 0.2% respectively. Quite considerable retention of enzyme activity was observed with anionic surfactant SDS (88.93 ± 5.9%) at 0.1% concentration. Moreover, Tween-80 at 0.5% concentration displayed 91.58 ± 5.2% enzyme activity. Since, surfactant like SDS is the most common ingredient of the detergents, the stability of alkaline protease in the presence of different surfactants adds an advantage of its utilization in the preparation of different cleaning and washing formulations. The compatibility of alkaline protease with different surfactants is well documented. For instance, the alkaline protease from *Bacillus licheniformis* UV-9 was quite stable in the presence of Tween-20, Tween-45 and Triton-X-405, but SDS inhibited the enzyme activity (Nadeem *et al*., 2013). Variable degree of stimulatory as well as inhibitory effects of surfactants on alkaline protease activity has reported by many researchers (Pathak *et al*., 2020; Verma and Pandey 2019; Ramkumar *et al*., 2018).

**Table 3: Effect of metal ions and surfactants on alkaline protease activity**.

| Reactant | Relative alkaline protease activity (%) | | |
|---|---|---|---|
| | 2 mM | 5 mM | 10 mM |
| **Control** | **100** | **100** | **100** |
| **Metal ion** | | | |
| $Na^{+}$ (NaCl) | 105.68 ± 6.5 | 90.69 ± 4.2 | 88.21 ± 7.2 |
| $Ca^{+2}$ ($CaCl_2$) | 67.55 ± 7.9 | 53.30 ± 7.1 | 36.49 ± 5.1 |
| $Mg^{+2}$ ($MgCl_2$) | 81.88 ± 5.1 | 64.67 ± 9.1 | 22.77 ± 4.5 |
| $Mn^{+2}$ ($MnCl_2$) | 57.85 ± 4.1 | 47.70 ± 6.4 | 39.52 ± 3.9 |
| $Cu^{+2}$ ($CuCl_2$) | 133.02 ± 8.3 | 73.95 ± 4.7 | 14.34 ± 5.7 |
| $K^{+}$ (KCl) | 127.98 ± 4.4 | 99.40 ± 6.3 | 33.76 ± 6.1 |
| $Zn^{+2}$ ($ZnCl_2$) | 84.40 ± 6.7 | 75.74 ± 4.4 | 19.31 ± 4.8 |
| $Ba^{+2}$ ($BaCl_2$) | 129.04 ± 5.8 | 117.20 ± 7.4 | 68.45 ± 7.6 |
| $Hg^{+2}$ ($HgCl_2$) | 26.92 ± 8.8 | 17.42 ± 5.8 | 5.55 ± 3.5 |
| $Ag^{+2}$ ($AgNO_3$) | 42.20 ± 5.1 | 38.62 ± 4.4 | 11.02 ± 7.1 |
| **Surfactant** | | | |
| | 0.1% (w/v) | 0.2% (w/v) | 0.5% (w/v) |
| Tween-20 | 91.67 ± 4.8 | 82.13 ± 5.1 | 68.26 ± 5.2 |
| Tween-80 | 96.43 ± 4.2 | 95.28 ± 6.2 | 91.58 ± 5.2 |
| SDS | 88.93 ± 5.9 | 81.47 ± 7.1 | 61.10 ± 4.8 |
| Triton-X-100 | 97.81 ± 8.1 | 87.36 ± 5.9 | 54.06 ± 8.5 |
| Cetylpyridinium chloride | 116.28 ± 4.6 | 102.14 ± 3.9 | 59.24 ± 6.4 |

***Application of alkaline protease***

***Compatibility with commercial detergents***

The detergent compatibility of alkaline protease from *Pseudomonas aeruginosa* YPVC showed that the enzyme retained 98% activity with Wheel detergent followed by Rin (83%), Surf Excel (80%), Arial (75%), Tide (63%), and Nirma (60%) after 30 min incubation at 50°C (Table 4). The residual enzyme activity declined with increase in incubation time. However, 80% retention of residual activity was obtained with Wheel detergent after 3 h incubation, which was quite noteworthy. Therefore, in our study, the alkaline protease exhibited compatibility in commercial detergents as Tide < Surf Excel < Nirma < Arial < Rin < Wheel. It is expected that alkaline protease should have good compatibility with commercial detergents along with pH stability, so that it can be explored for the preparation of various washing formulations (Banerjee *et al*., 1999). Many reports have documented detergent compatibility of alkaline protease as an important criterion for its utility as cleaning additive. In one report, the compatibility of alkaline protease from *Pseudomonas aeruginosa* MCM B-327 was evaluated with different commercial detergents; highest enzyme stability was found with Surf Excel, and least stability was obtained with Tide detergent at quite lower temperature (35°C) (Zambare *et al*., 2014). In another study, alkaline protease from *Bacillus licheniformis* NK displayed maximum residual activity with Arial and Bahar (97% each) followed by Tide (95%) and Bonux (92%) at room temperature (Ramkumar *et al*., 2018). Therefore, based on above results, we clearly mention that the alkaline protease from *Pseudomonas aeruginosa* YPVC was quite stable with detergents at higher temperature (50°C) for longer time period. This property of enzyme provides its suitability for the preparation of eco-friendly cleaning and washing formulations.

**Table 4: Compatibility and stability of alkaline protease with commercial detergents at 50°C.**

| Relative residual alkaline protease activity (%) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Time (min)** | **Control** | **Nirma** | **Wheel** | **Surf Excel** | **Tide** | **Arial** | **Rin** |
| 0 | 100 | 100 | **100** | **100** | **100** | **100** | **100** |
| 30 | 89 ± 4.5 | 53 ± 6.5 | 87 ± 5.4 | 71 ± 7.2 | 56 ± 5.2 | 67 ± 4.9 | 74 ± 6.1 |
| 60 | 78 ± 6.2 | 44 ± 2.8 | 68 ± 8.1 | 30 ± 4.6 | 37 ± 5.2 | 55 ± 5.1 | 60 ± 4.7 |
| 90 | 56 ± 4.1 | 35 ± 4.2 | 50 ± 6.1 | 22 ± 5.8 | 20 ± 6.2 | 50 ± 3.4 | 48 ± 3.7 |
| 120 | 44 ± 5.2 | 25 ± 3.7 | 37 ± 4.2 | 14 ± 5.1 | 16 ± 3.9 | 30 ± 6.1 | 33 ± 6.2 |
| 150 | 37 ± 3.7 | 16 ± 5.1 | 31 ± 3.7 | 11 ± 4.2 | 09 ± 4.2 | 17 ± 5.1 | 27 ± 4.4 |
| 180 | 35 ± 3.4 | 04 ± 4.9 | 28 ± 4.7 | 02 ± 3.9 | 01 ± 2.9 | 09 ± 3.7 | 23 ± 7.1 |

***Destaining performance analysis***

The alkaline protease was tested for the removal of blood stains from cotton cloths. After incubation at 50°C for 15 min, qualitative analysis of stain removal clearly indicated that the combination of enzyme and detergent (Wheel) displayed an effective destaining performance than alone (Fig. 6).

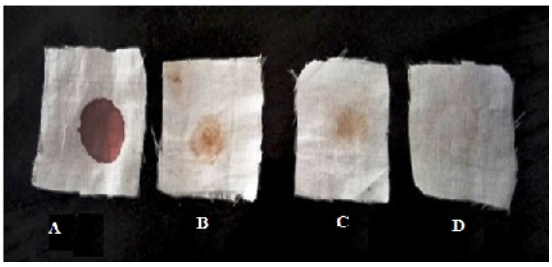


**Fig. 6.** Blood stain removal efficiency of alkaline protease from *Pseudomonas aeruginosa* YPVC: cotton cloth piece stained with blood (A); blood stained cloth washed with enzyme only (B); with detergent only (C); with enzyme and detergent (D).

As alkaline protease acted on blood proteins and hydrolyzed them, it was reflected in terms of removal of blood stains. The detergents on the other hand are surface active agents, the formulation containing enzyme-detergent cocktail proved to be the best for the removal of dirt and proteinaceous stains from the cloths. Now days, the alkaline protease containing preparations are available in the market, and widely used for the cleaning and washing purposes.

In support to our findings, ammonium sulfate precipitated alkaline protease from *Pseudomonas aeruginosa* MCM B-327 showed good compatibility with commercial detergents and blood destaining property (Maruthiah *et al*., 2013). In another study, the destaining performance of alkaline protease from *Bacillus pumilus* MP 27 was better in the combination with Tide detergent (Baweja *et al*., 2016). The destaining of blue ink and tea by the combination of alkaline protease and Ariel detergent was reported in the report (Verma and Pandey 2019).

***Silver recovery from X-ray film***

In general, the X-ray film is composed of polyester sheet having 1.5 - 2% ratio of silver in gelatin-coated film. Therefore, used X-ray films serve as good source of silver. The alkaline protease from *Pseudomonas aeruginosa* YPVC had considerable performance in the gelatin hydrolysis at 50°C. The gelatin layer was completely removed leaving behind clear polyester sheet (Fig. 7). The O.D. of the hydrolysate was the highest during first 30 min indicating an effective gelatin hydrolysis and release of silver from the film. However, no significant difference in the turbidity of the hydrolysate was observed upon subsequent incubation time (Table 5). This suggested complete removal of silver from the X-ray film in 30 min. It is reported that treatment of X-ray film with alkaline protease leads to rapid hydrolysis of gelatin releasing silver (Al-Abdalall and Al-Khaldi 2016; Choudhary 2013). Moreover, an ideal combination of pH and temperature plays an important role in the silver recovery. For instance, 55°C temperature and 10.5 pH

were optimum for rapid recovery of silver using alkaline protease (Seid, 2011). The conventional method of silver recovery includes burning and oxidation by number of toxic chemicals, which intern is more hazardous. Moreover, this procedure is time consuming and costly. The use of alkaline protease facilitates not only the recovery of silver, but also allows the reuse of polyester sheet proving an environment-friendly alternative.

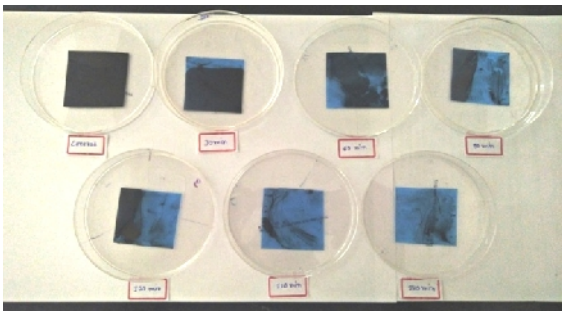

**Fig. 7.** Hydrolysis of silver containing gelatin layer of X-ray film by alkaline protease from *Pseudomonas aeruginosa* YPVC.

**Table 5: Extraction of silver from X-ray film by alkaline protease from *Pseudomonas aeruginosa* YPVC.**

| Time (min) | O.D. at 660 nm | Enzyme activity (U/ml) |
|---|---|---|
| 30 | 1.002 ± 0.06 | 201 ± 7.21 |
| 60 | 0.991 ± 0.04 | 187 ± 8.31 |
| 90 | 0.868 ± 0.03 | 155 ± 5.41 |
| 120 | 0.891 ± 0.05 | 149 ± 7.01 |
| 150 | 0.600 ± 0.06 | 147 ± 4.25 |
| 180 | 0.304 ± 0.04 | 132 ± 2.23 |
| 210 | 0.302 ± 0.04 | 126 ± 3.17 |

## CONCLUSION

The overall study portrays the production of alkaline protease from soil isolate *Pseudomonas aeruginosa* YPVC. Significant improvement in enzyme production was achieved by optimizing physico-chemical parameters under submerged cultivation. The partial purification of alkaline protease along with considerable yield was obtained after dialysis. The enzyme displayed significant stability with wide range of pH, temperature, metal ions, surfactants and detergents. The destaining and silver recovery ability of alkaline protease suggests its potential application in different industrial processes.

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**Conflict of Interest.** None.

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