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# Media Optimization, Production, Purification and Characterization of Alkaline Protease Enzymes from *Pseudomonas aeruginosa.*

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**ABSTRACT**

Industrial production of enzymes gaining its importance on behalf of its applications, of which 80% of enzymes are hydrolytic in nature. Microorganisms are an excellent sources of enzymes than plant and animal due to ease of mass cultivation, and they can able to sustain in acidic, neutral and alkaline condition for the production of enzymes, etc. Protease belongs to the class of important groups of industrial enzymes which account for about 65% of the global market. The present study focuses mainly on the optimization of media, production, purification and characterization of alkaline protease secreted by the isolated bacterial strains of *Pseudomonas aeruginosa*.The *Pseudomonas aeruginosa* species was isolated from dairy effluents of in and around Chennai region.Optimization studies werecarried out with different Carbon sources, Nitrogen sources, Substrates, Metal ions, pH and temperature to determine the optimum conditions required for maximum protease activity. At 48hrs *Pseudomonas aeruginosa* species was found to be producing higher amount (200 ± 2.983U/ml)of alkaline protease. Maximum enzyme activity was observed in glucose and yeast extract medium and the produced crude proteases were purified by the Ammonium sulphate precipitation method, the highest protease activity (180 ± 1.982U/ml) were detected at 60% precipitation. Followed by recovery of enzyme by dialysis process, after dialysis process it was found that the specific activity of enzymewas 1.39U/mg protein with 80% recovery and a purification fold of 1.12, the purified proteases was subjected to SDS-PAGE analysis to detect molecular weight and it was identified as 55 KDa. Further studies on application of enzymes have been carried out.

**Keywords:** *Pseudomonas aeruginosa*, Media optimization,Ammonium sulphate, SDS-PAGE

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## INTRODUCTION

Proteases represent one of the most important groups of industrial enzymes that are currently in use and account for at least a quarter of the total global enzyme production [1,14]. Different species of bacteria produce acidic, neutral and alkaline proteases. Microorganisms are an ideal source for enzymes, since they have the ability to propagate rapidly under optimum condition [18,20,27]. A protease is a member of a very large group of enzymes that have a variety of functions in the body. It is one of the digestive enzyme plays a major role to process protein. Without protease, the body would not be able to digest the protein in food. Some kinds of proteases are involved in the regulation of cellular events like blood clotting. Protease are otherwise called as *proteolytic enzymes* or *proteinases*[26].

Proteases are active at mild conditions, with pH optima in the range of 6 to 8; they are robust and stable[7,12].These properties are quite relevant to use them as catalysts in organic synthesis. This is possible because proteases can not only catalyze the cleavage of peptide bonds but also their formation[8,12], as well as other reactions of relevance for organic synthesis[9]. Chymotrypsin, trypsin and papain have been widely used as proteases in the chemical synthesis of peptides.

Microbial proteases are one of the most important hydrolytic enzymes and have been studied in a fair manner since the introduction of enzymology, for the production of enzymes in a large scale. These enzymes not only play a role in the cellular metabolic processes but also have added some considerable attention in the industrial sector[29]. These enzymes are commonly used in the detergent industry. Microbial proteases, been categorized into 2 parts which are intracellular and/or extracellular [11].

Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover etc. Extracellular proteases are important for the protein hydrolysis in cell-free environments, this process helps the cell to absorb and utilize hydrolytic products[3]. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes [13,19]. For the research purpose, we have taken *Pseudomonas aeruginosa* as a microbial source, were available in our vel tech high tech laboratory as culture, for the production of protease, followed by the isolation and purification of the enzyme.

## MATERIALS AND METHODS

### Media optimization

The medium optimization for the selected bacterial strains was carried out with different Carbon sources, Nitrogen sources, Substrates, Metal ions, Micronutrients, pH and temperature to determine the optimum conditions required for high protease activity. The optimization of carbon and nitrogen sources were carried out in salt medium containing Potassium dihydrogen phosphate (0.42g/L), Dipotassium hydrogen phosphate (0.375g/L), Ammonium sulphate (0.244g/L), Sodium chloride (0.015g/L), Calcium chloride (0.015g/L), Magnesium sulphate (0.015g/L) and Ferric chloride (0.054g/L). The pH of the salt medium was set at 7.

### Optimization of Carbon source

Glucose, Fructose, Sucrose, Lactose, Maltose and Starch were used for the optimization of carbon sources. 6 conical flasks were taken for optimization of the bacterial strains. One gram of Glucose, Fructose, Sucrose, Lactose, Maltose and Starch were added to the conical flasks containing 100 ml of salt medium separately. 0.5 g peptone and 0.2 g of Yeast extract was added. The medium with the carbon sources were sterilized in autoclave at 121°C, 15 lbs. for 15 min. 1 ml of inoculum of the bacterial strains was inoculated to the conical flasks containing sterilized medium with carbon sources and incubated in an orbital incubator shaker at 37°C and the protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Optimization of Nitrogen source**

Yeast extract, Beef extract, Peptone, Ammonium chloride, Ammonium sulphate and Sodium nitrate were used for the optimization of nitrogen source. One gram of Yeast extract, Beef extract, Ammonium chloride, Ammonium sulphate and Sodium nitrate were added to the conical flasks containing 100ml of salt medium separately and 0.1 g of Glucose was added as a source for carbon. The medium with the nitrogen sources were sterilized in autoclave at 121°C, 15 lbs. for 15 min. One ml of inoculum of the bacterial strains was inoculated to the conical flasks containing sterilized medium with nitrogen sources and incubated in an orbital incubator shaker at 37°C and the protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Optimization of pH**

Nutrient broth was used as the medium for bacteria, for the pH optimization in the salt medium. 1.3 g of nutrient broth was added to the conical flasks containing 100 ml of salt medium and the pH was set at 4, 5, 6, 7, 8 and 9 using 0.1 N NaOH and HCl.

The medium with the different pH were sterilized in an autoclave at 121°C, 15 lbs. for 15 min. 1 ml of inoculum of the bacterial strains were inoculated to the conical flasks containing sterilized medium with different pH and incubated in an orbital incubator shaker at 37°C and the protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Optimization of Temperature**

Nutrient broth was used as the nutrient medium for the bacteria, for the temperature optimization in the salt medium. Nutrient broth 1.3 g was added to the conical flasks containing 100 ml of salt medium. The medium were sterilized in an autoclave at 121°C, 15 lbs, for 15 min. 1 ml from inoculum of the bacterial strains were inoculated to the conical flasks containing sterilized medium and incubated at 20°C, 37°C, 45°C, 55°C, and 65°C. The protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Optimization of Substrate**

Casein, Gelatin, Bovine serum albumin, Peptone and Tryptone were used for the substrate optimization. One gram of substrate was separately added to conical flask containing 100 ml of nutrient broth. The medium with the substrate were sterilized in an autoclave at 121°C, 15 lbs, for 15 min. One ml of inoculum of the bacterial strains were inoculated to the conical flasks containing sterilized medium with substrates and incubated in an orbital incubator shaker at 37°C and the protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Optimization Metal ion**

Sodium chloride, Ferric chloride, Magnesium sulphate, Zinc chloride, Calcium chloride was used for the metal ion optimization. One gram of the metal ion was separately added to conical flasks containing 100 ml of nutrient broth. The medium with the substrate were sterilized in an autoclave at 121°C, 15 lbs, for 15 min. One ml of inoculum of the bacterial strains were inoculated to the conical flasks containing sterilized medium with substrates and incubated in an orbital incubator shaker at 37 °C and the protease activity was determined after 24 hrs, 48 hrs, 72 hrs and 96 hrs of incubation by universal protease assay [4].

**Concentration Optimization**

The concentration of the Carbon source, Nitrogen source, Substrate, Metal ions, and Micronutrients was optimized for the bacterial strains. The concentration optimization was carried out with the Carbon source, Nitrogen source, Substrate responsible for highest protease activity of the bacterial strains. The metal ions used for the concentration optimization were Zinc chloride, Sodium chloride, Calcium chloride, Magnesium sulphate, and Ferric chloride. The Micronutrients used were Potassium dihydrogen phosphate and Dipotassium hydrogen phosphate. All the sources of nutrients were varied with 5 different concentrations.

One varying source was added in 5 different concentrations separately and all other sources were added in assigned constant minimal quantities.

For the concentration optimization, 100 ml of distilled water was taken in different conical flasks and the different nutrient sources were added. The different concentrations of Carbon source added were 4 g/L, 8 g/L, 12 g/L, 16 g/L and 20 g/L. The different concentration of Potassium dihydrogen phosphate added were 1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L. The different concentratioon of Dipotassium hydrogen phosphate added were 2.5g/L, 4g/L, 6g/L, 8g/L, and 10g/L. The different concentrations of Nitrogen source added were 4g/L, 8g/L, 12g/L, 16g/L, and 20g/L. The different concentrations of Substrate added were 4g/L, 8g/L, 12g/L, 16g/L, and 20g/L. The different concentrations of Sodium chloride added were 0.25g/L, 0.4g/L, 0.6g/L, 0.8g/L, and 1g/L. The different concentrations of Magnesium sulphate added were 0.4g/L, 0.6g/L, 0.8g/L, 1g/L, and 1.2g/L. The different concentrations of Calcium chloride added were 0.4g/L, 0.6g/L, 0.8g/L, 1g/L and 1.2g/L. The different concentrations of Zinc chloride added were 0.4g/L, 0.6g/L, 0.8g/L, 1g/L and 1.2g/L. The different concentrations of Ferric chloride added were 0.2g/L, 0.3g/L, 0.4g/L, 0.5g/L, and 0.6g/L. One ml inoculum of bacterial strains was inoculated to all the conical flasks containing the different concentrations of nutrient sources and was incubated in an orbital incubator shaker at 37°C. The protease activity was determined after 24 hrs, 48 hrs and 72 hrs of incubation by the universal protease assay [4].

**Mass culture of alkaline protease producing bacteria**

The alkaline proteases are thus produced in a 5L fermenter using the media composed of the contents mentioned in table given below. The bacterial cultures fermented with the optimized medium and optimized conditions were taken for the ammonium sulphate precipitation and purification of protease. After precipitation, the pellets produced were subjected to dialysis. Minimal amount of culture was eluted from the fermenter periodically for the analysis of the enzyme production.

**Purification of Protease**

**Ammonium sulphate precipitation**

The bacterial cultures fermented with the optimized medium and optimized conditions were taken for the Ammonium sulphate (20%, 40%, 60% and 80%) precipitation and partial purification of protease. The filtrate broth (crude protease) was collected and centrifuged at 4000rpm for 15 min at 4$^{o}$C in order to obtain a cell free filtrate (CFF). After performing a test for sterility, 200ml of the cell free filtrate (CFF) containing protease were collected and their proteolytic activities and protein content were determined. 200ml of the crude protease enzyme were first added with 20% (w/v) Ammonium sulphate. The precipitated proteins were regimented by centrifugation for 15 min at 500 rpm. The resulted pellet was dissolved in 5 ml of phosphate buffer at pH 7.0. The left supernatant was precipitated again with Ammonium sulphate to achieve 20%, 40%, 60% and 80% (w/v) saturation. Both enzyme activity and protein content were determined for each separate action[10].

**Dialysis**

The pellets suspended in trisHCl buffer, that were obtained from 20%, 40%, 60%, 80% Ammonium sulphate precipitation were filled in the dialysis bag and suspended over trisHCl buffer till the salts were separated from the dialysis bag. The protein precipitate of 20%, 40%, 60%, and 80% were collected and mixed with trisHCl buffer consisting 20mM trisHCl, with 20mM sodium chloride and 10mM magnesium chloride and followed by centrifugation process. After the centrifugation the supernatant was removed and the pellet containing protease was collected. The trisHCl buffer was removed by dialysis of the partially purified protease with sterilized distilled water. The protease activity of the pellet was measured by universal protease assay (Beg et al 2002). The partially purified protease that has the highest protease activity was taken for SDS-PAGE analysis to estimate its molecular mass.

**SDS-PAGE analysis**

The gel matrix was prepared by pouring the Acrylamide solution for separating gel and stacking gel. The gel was placed in a vertical position in room temperature for polymerization. After the gel was set, the

comb was removed without distorting the shapes of the well. The wells were washed with distilled water to remove unpolymerized Acrylamide by flushing with the pipette. The vertical gel was then placed in the electrophoresis system with running buffer at the bottom reservoir. The running buffer was filled to the top reservoir and bubbles were removed. The samples were prepared using a sample buffer containing stacking gel buffer, SDS, Glycerol, Mercaptoethanol, and Bromophenol blue. The standard marker for protease is also mixed with the sample buffer. The molecular weight of the marker ranges from 14.4 kDa to 97.4 kDa. The standard protease marker was loaded in the first well, along with the samples in the corresponding wells. The position of the wells in the glass plate was noted.

The electrophoresis was started at 100-125V, after the dye had moved into the separating gel, it was increased to 200 V. The power pack was switched off, when the dye reaches the bottom of the gel. The gel plates were then removed using a spatula. The gel was immersed in a staining solution containing Coomassive brilliant blue dye, and was kept overnight with uniform shaking. The gel is then transferred to another container with destaining solution and shaken continuously. The destainer must be changed frequently, until the background of the gel is colourless. Individual bands were obtained and the molecular weight of the protease was determined with the help of the marker used.

**Determination of the specific activity of protease enzyme**

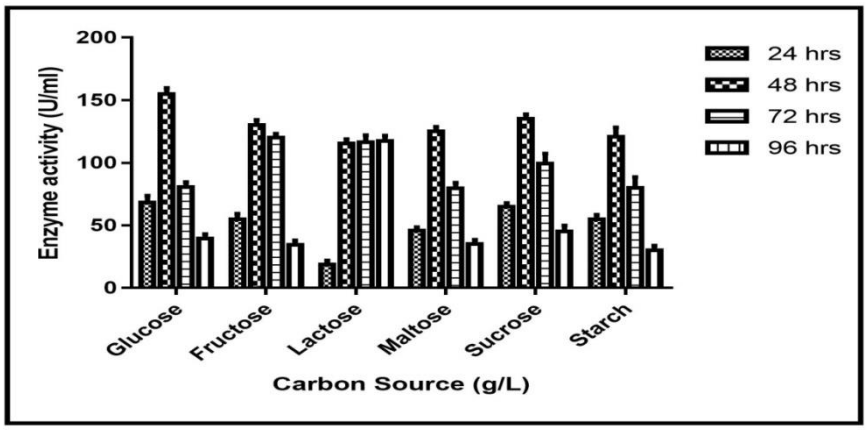
The specific activity of the protease enzyme protein was expressed according to the following equation:

Specific activity = Activity of enzyme is expressed in Units / protein content (mg/ml).

## RESULT AND DISCUSSION

**Optimization of Carbon Sourceof *Pseudomonas aeruginosa***

Maximum production of protease was observed when Glucose was served as the Carbon source with a value of 155 ± 4.761 (U/ml) followed by Sucrose with 135.3 ± 3.5 (U/ml) and Lactose with a least value of 115.3 ± 3.5 (U/ml) at 48$^{th}$ hrs. Krishnaveni et al (2012)[17] reported the production of Alkaline protease in the presence of glucose using *Bacillus subtilis* isolated from dairy effluent. In contrast, starch served as the best carbon source for many species such as *Bacillus licheniformis* Bhunia et al (2012)[6], and Haloalkaliphilic bacterium Joshi et al (2008)[15].




**Optimisation of Nitrogen source of *Pseudomonas aeruginosa***

Among the various nitrogen sources that have been tried, Yeast extract showed maximum enzyme activity of 160 ± 5.164 (U/ml) followed by beef extract with 135.5 ± 3.697 (U/ml) and a minimum enzyme activity of 102.3 ± 5.737 (U/ml) was observed in Ammonium chloride at 48$^{th}$ hrs. Comparatively yeast extract and peptone mixture had a great influence on enzyme production from *Bacillus subtilis* by Vanitha et al(2014)[32].

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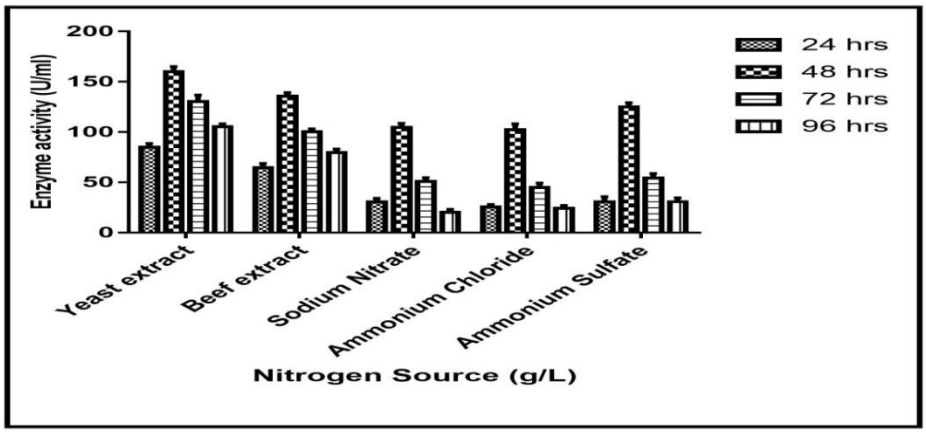
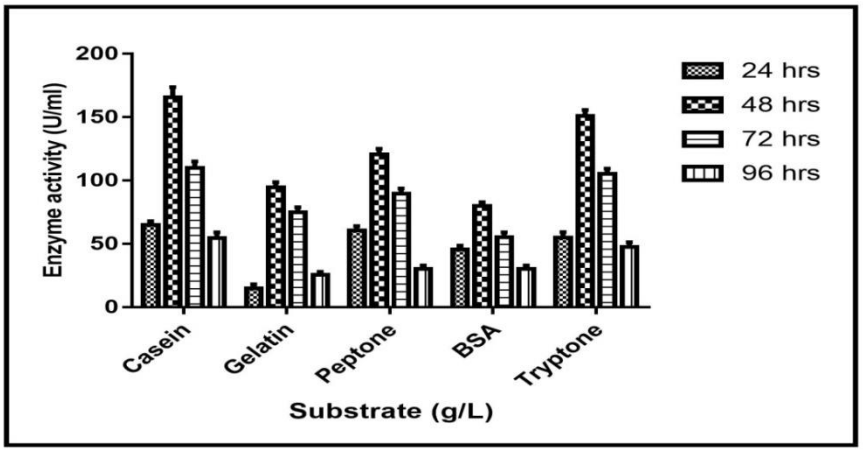
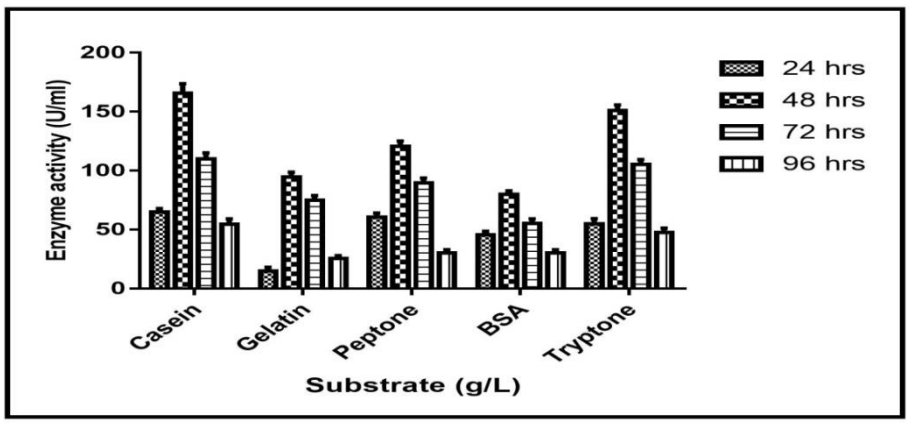



**Optimization of Substrate of *Pseudomonas aeruginosa***

During the 48 hrs incubation period the maximum enzyme activity of 165.5 ± 8.185 (U/ml) was observed in casein, followed by trptone with an enzyme activity of 151 ± 4.69(U/ml) and a minimum enzyme activity of 80 ± 2.944 (U/ml) was observed in BSA.Kathiresan & Manivannan (2007)[16] used casein as a medium for alkaline protease production from coastal mangrove isolate, *Streptomyces sp.* Nurullah& Uyar (2011)[25] and Sumantha et al (2006 )[31] reported the utilization of casein as a source of nitrogen for the production of alkaline protease.




**Optimization of Metal ions of *Pseudomonas aeruginosa***




A maximum protease activity of 164.5 ± 9.183 (U/ml) was found in the presence of $ZnCl_2$ followed by $MgSO_4$ with 140 ± 7.616 (U/ml) and a minimum protease activity of 40 ± 3.651 (U/ml) was found in NaCl when

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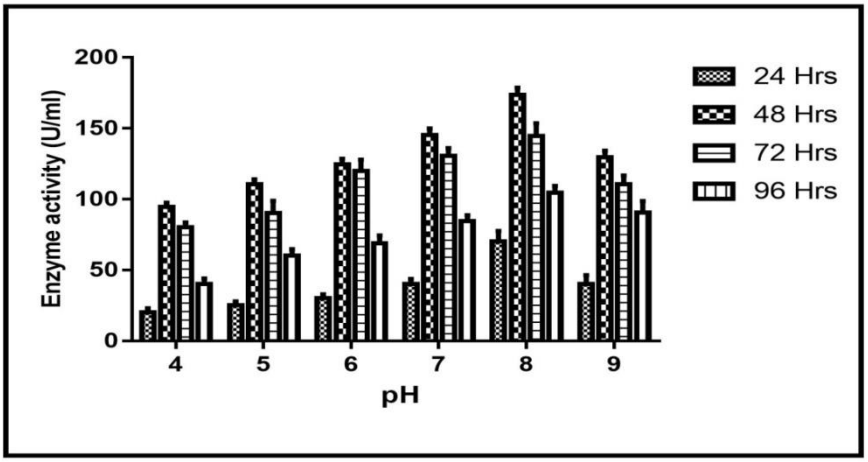
compared with the other metal ions like $FeCl_3$, $CaCl_2$ and $CuSO_4$ . But Nurullah& Uyar (2011)[25], found the maximum protease activity with $MgSO_4$ whereas, $Zn^{2+}$ was found to repress protease production in *B. subtilis.* Similar observation was reported by Mukesh Kumar et al (2012)[23].

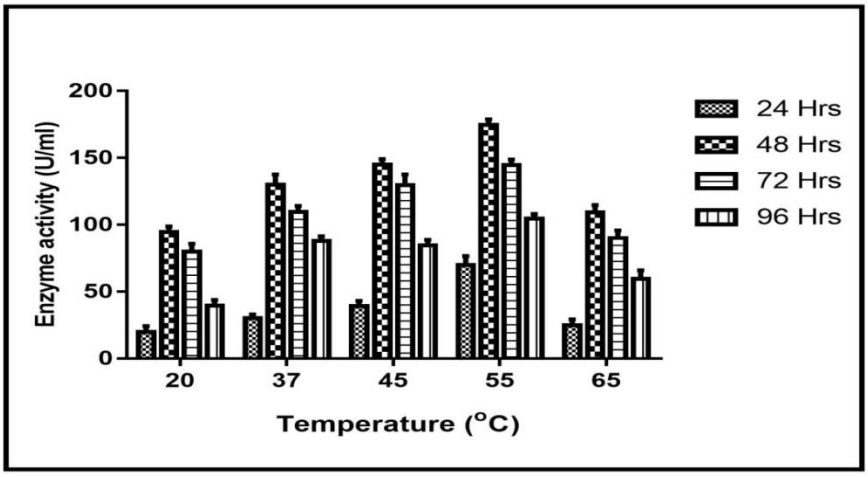
**Optimization of pH of *Pseudomonas aeruginosa***

The results for the activity of ezymes with variable pH indicated a gradual increase in the activity and the optimum activity of 173.8 ± 4.992 (U/ml) was observed at a pH of 8 and 145.3 ± 4.573 (U/ml) at pH of 7. Whereas the activity of the enzyme from *Bacillus pumilus* was found to be high at a pH of 9 Gomaa (2010)[10]. Mukherjee et al (2008)[24], observed an optimum pH range between 8 and 9 in *Bacillus subtilis*. Srinivasan et al (2009)[30] found the optimum activity was at a pH of 8 for *Bacillus sp.*




**Optimization of Temperature of *Pseudomonasaeruginosa***

Among the various temperatures the maximum enzyme activity of 175.8 ± 4.031 (U/ml) was observed at 55°C followed by 145 ± 4.163 (U/ml) at 45°C and a minimum enzyme activity of 94.5 ± 4.203 (U/ml) was observed at 20°C. Protease activity was found to be maximum between 30°C while purified from Bacillus pumilus[10]. Sepahy&Jabalameli (2011)[28] studied range of temperature between 30-70°C in *Bacillus sp.* and observed a maximum enzyme activity at 60°C.Based on the above results obtained, the following media composition can be used to grow the bacterial strain, *Pseudomonas aeruginosa.* The protease production of *Pseudomonas aeruginosa* was found to give a maximum activity of 155 ± 4.761 U/ml with Glucose as carbon source, 160 ± 5.164 U/ml with Yeast extract as nitrogen source, 165.5 ± 8.185 U/ml with Casein as substrate, 164.5 ± 9.183U/ml with Zinc chloride as metal ion, 173.8 ±4.992 U/ml at pH 8, 175.8 ± 4.031 U/ml at 55°C of temperature.



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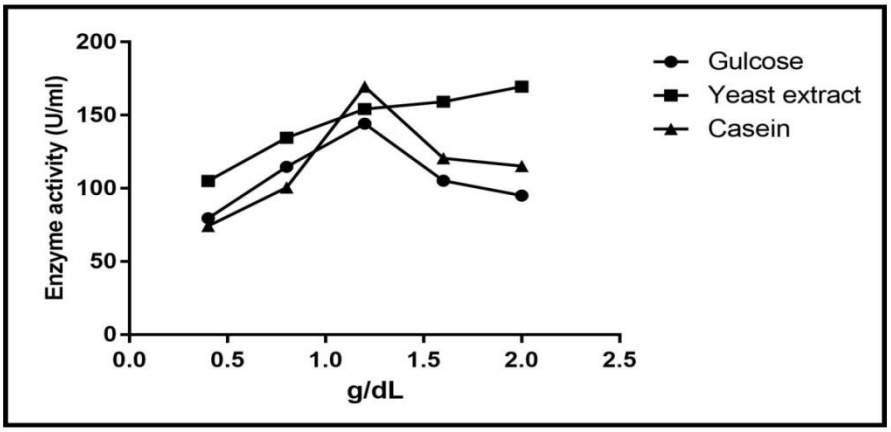
*Pseudomonas aeruginosa* produces maximum amount of protease in a medium containing Glucose (Carbon source), Yeast extract (Nitrogen source), Casein (Substrate), $ZnCl_2$ (Metal ion), pH 8 at 55°C and at 48hrs of incubation.

**Concentration Optimization Method**

The optimized medium for the maximum production of the protease which were analysed by the previous steps were subjected to the concentration optimization in which the different nutrients in the medium will be selected in each trial and it will be analysed for maximum protease production by increased concentration of the selected nutrient with other nutrients at a constant concentration.

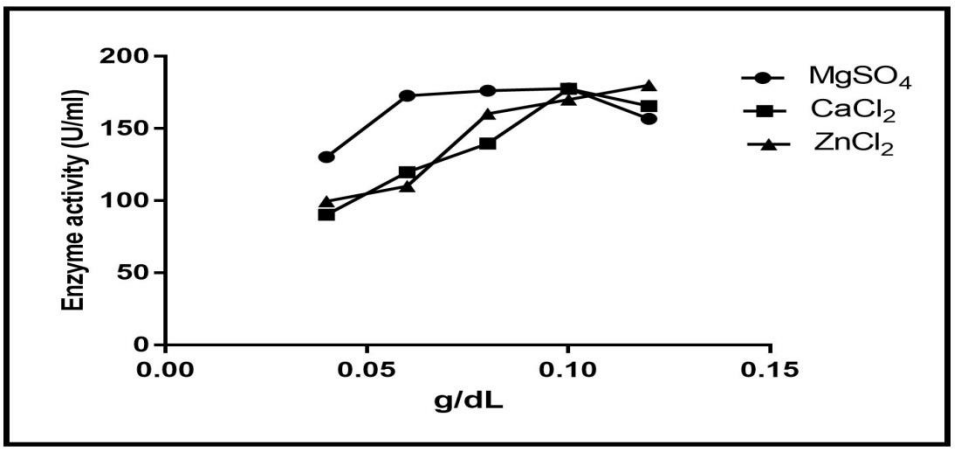
**Concentration optimization of media constituents for maximum production of protease from *Pseudomonas aeruginosa*: Glucose, Yeast extract and Casein**

Among the optimisation of carbon source, nitrogen source and substrate concentrations, it was found that the enzyme activity was high at 12 g/L of glucose, 16 g/L of yeast extract and 12 g/L of casein. Krishnaveni et al (2012)[17] has reported higher enzyme activity in the production of alkaline protease from *Bacillus subtillus* when glucose was used as carbon source.While working with nitrogen sources for production of alkaline protease from a Haloalkaliphilic bacterium yeast was found to be the best according to Joshi et al (2008)[15]. Kathiresan & Manivannan (2007)[16] used casein as a medium for alkaline protease production from coastal mangrove isolate, *Streptomyces sp.*




**Concentration Optimization of media constituents for maximum production of protease: Metal ions**

Among the various metal ions used $MgSO_4$, $CaCl_2$ and $ZnCl_2$ was subjected to concentration optimization and found that the enzyme activity was high at 1 g/L of $MgSO_4$, 1 g/L of $CaCl_2$ and 1.2 g/L of $ZnCl_2$.



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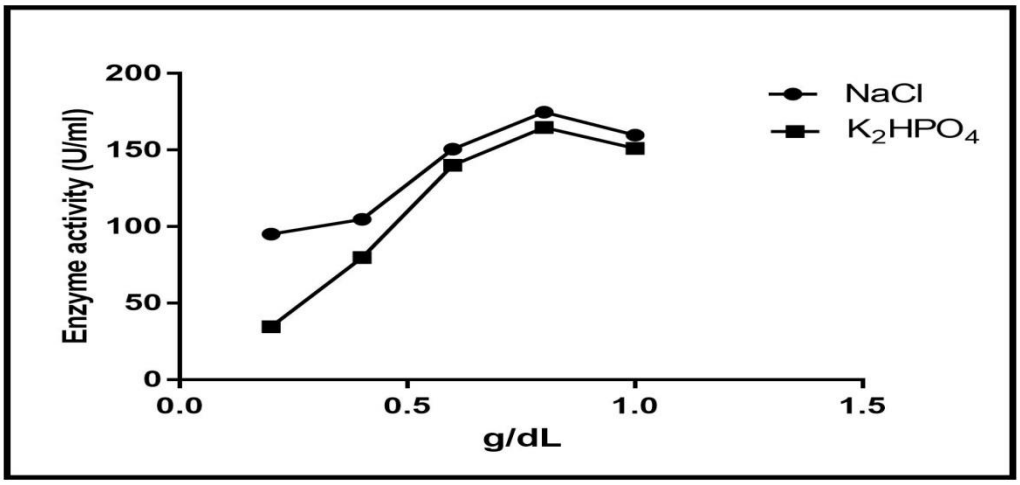
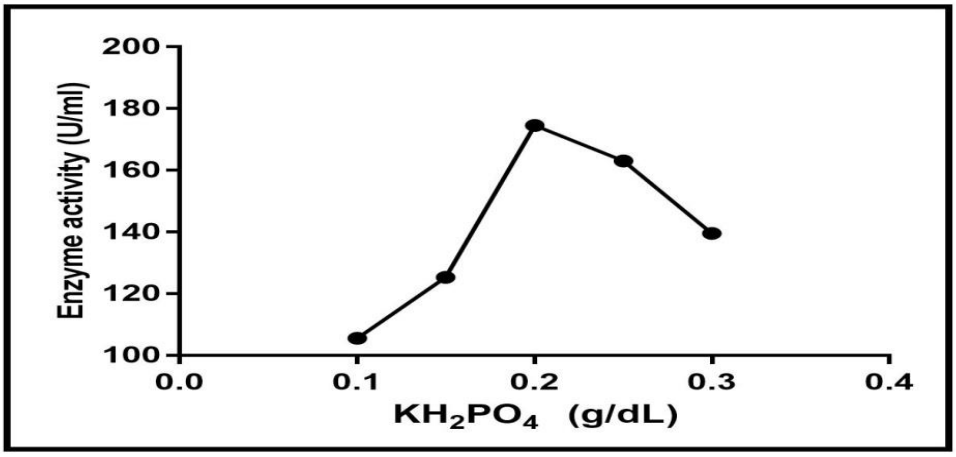
**Concentration optimization of media constituents for maximum production of protease: $MgSO_4$, $CaCl_2$, $ZnCl_2$**



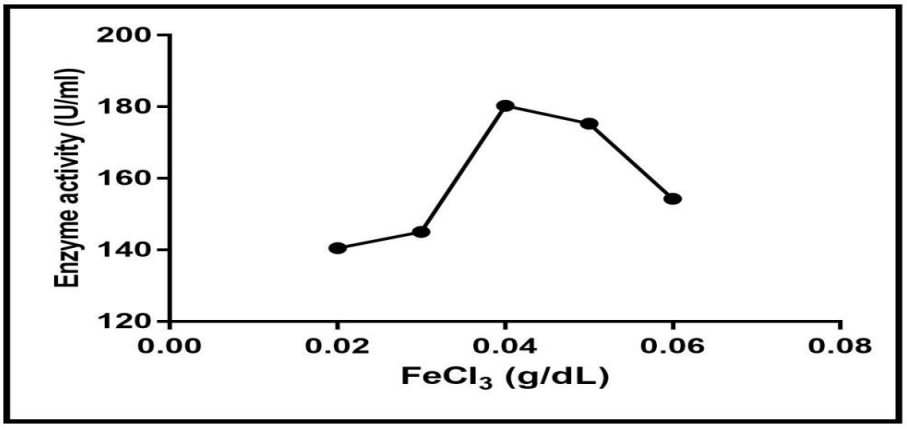

**Concentration optimization of media constituents for maximum production of protease: NaCl, $K_2HPO_4$**

Metal ions NaCl and $K_2HPO_4$ was optimised separately with different concentration levels and the result showed that maximum enzyme activity was obtained at 8 g/L of NaCl and $K_2HPO_4$. Also, the metal ion $KH_2PO_4$ was optimized at different level and the maximum activity was observed at a concentration of 2g/L.




**Concentration optimization of media constituents for maximum production of protease:$KH_2PO_4$**

Among the different concentrations $FeCl_3$, the maximum enzyme activity was observed at 0.4 g/L of $FeCl_3$.



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**Concentration Optimization of media constituents for maximum production of protease: $FeCl_3$**

The maximum protease activity for *Pseudomonas aeruginosa* was found to be 144.3 ± 6.801U/ml at 12 g/L concentration of glucose, 169.5 ±7.724U/ml at 20g/L concentration of yeast extract, 169.5 ±7.141U/ml at 12g/L concentration of casein, 174.8 ±6.238U/ml at 8g/L concentration of NaCl, 164.8 ±6.801U/ml at 8g/L concentration of $K_2HPO_4$, 174.5 ±4.203U/ml at 2 g/L concentration of $KH_2PO_4$, 177.8 ±6.449U/ml at 1g/L concentration of $MgSO_4$, 177.5 ±8.347U/ml at 1g/L concentration of $CaCl_2$, 180 ±7.165 U/ml at 1.2g/L concentration of $ZnCl_2$ and 175.3 ±6.238U/ml at 0.5g/L concentration of $FeCl_3$ in 1000ml of broth.

Overall result of the concentration optimization indicates that the production of protease was maximum with the increased concentration of nitrogen source.

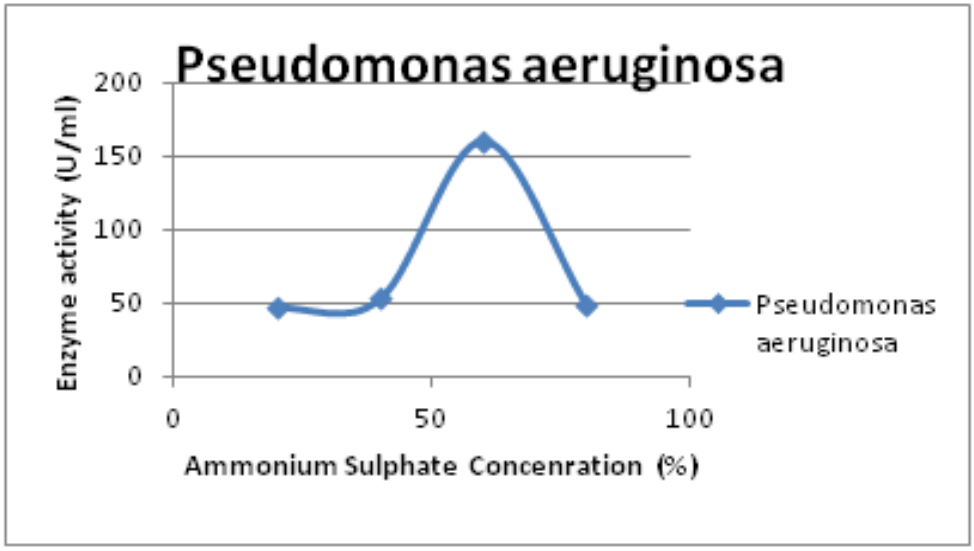
**Optimized media (*Pseudomonas aeruginosa*)**

| ***Pseudomonas aeruginosa*** | | |
|---|---|---|
| **S.No** | **Nutrients** | **Con. (g/L)** |
| 1. | Glucose | 12 |
| 2. | Yeast extract | 20 |
| 3. | Casein | 12 |
| 4. | $ZnCl_2$ | 1.2 |
| 5. | NaCl | 8 |
| 6. | $KH_2PO_4$ | 2 |
| 7. | $K_2HPO_4$ | 8 |
| 8. | $MgSO_4$ | 1 |
| 9. | $CaCl_2$ | 1 |
| 10. | $FeCl_3$ | 0.5 |

**Mass production and partial purification of protease**

**Partial purification**

High protease activity for *Pseudomonas aeruginosa* was found at 60% ammonium sulphate concentration, which was measured by universal protease assay



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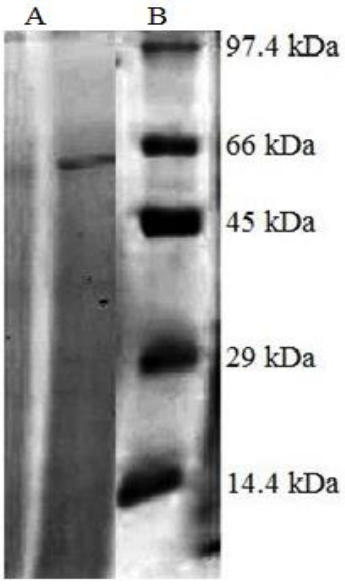
**Molecular weight determination using SDS-PAGE**

The molecular weight of the protease produced from both the bacterial species were determined with the help of the marker. The molecular weight of the protease from *Pseudomonas aeruginosa* showed a prominent band of approximately 55kDa

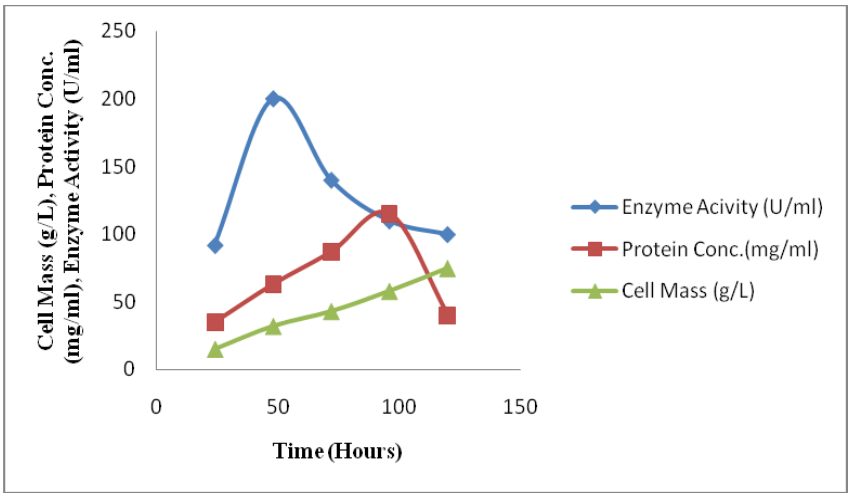
SDS- PAGE: FORMATION OF BANDS IN THE GEL

**A. *Pseudomonass aeruginosa***
**B. Marker (10 to 100 kDa)**




**Enzyme Production**

The bacterial strains (*Pseudomonas aeruginosa*) were subjected to various analyses: Whole cell mass, Alkaline protease activity and Protein concentration. It was observed that the maximum value (75 g/L) of cell mass was observed at 120 hrs, enzyme activity was maximum (200 ± 2.983U/ml) at 48 hrs and Protein concentration was maximum (115 mg/ml) at 96 hrs for *Pseudomonas aeruginosa* .



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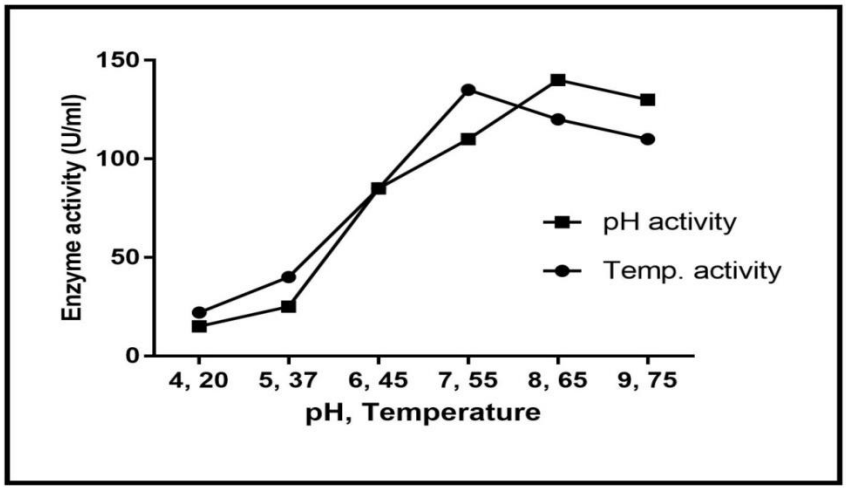
**Enzyme Production (*Pseudomonas aeruginosa*)**

**Purification of alkaline protease for *Pseudomonas aeruginosa***

| *Purification step* | *Total activity (U/ml)* | *Total protein (mg/ml)* | *Specific activity (U/mg)* | *Recovery (%)* | *Purification (fold)* |
|---|---|---|---|---|---|
| Culture filtrate | 200 ±2.983 | 160 | 1.25 | 100 | 1 |
| After Dialysis | 160 ±1.982 | 115 | 1.39 | 80 | 1.12 |

**Effect of temperature and pH on the activity of the protease enzyme**

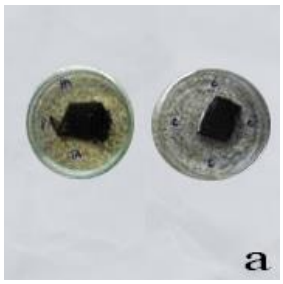



The effect of temperature and pH on the activity of the free protease were calculated and illustrated. *Pseudomonas aeruginosa* showed highest protease activity at a temperature of 50ºC and pH 8.

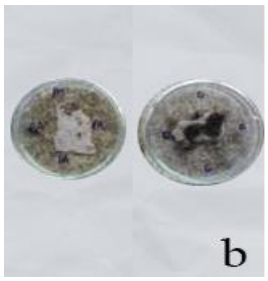
**Applications**

**Dehairing:**The goat skin was observed after incubation for 4 days. It was affirmed that there was a complete depilation of skin, which had undergone protease treatment. The protease produced from *Pseudomonas aeruginosa* showed depilation of skin.

It was also confirmed that there was a partial depilation of skin, which was incubated in distilled water for 4 days . It is in contrast to the depilation done by Mitra et al (2005)[22] where depilation is done at 12 and 22 hrs and complete depilation was also reported in 24 hrs in the experiment done by Anissa et al (2011)[2].




**Dehairing Process without protease enzyme**




**Dehairing Process with protease enzyme**

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**Feather Degradation**

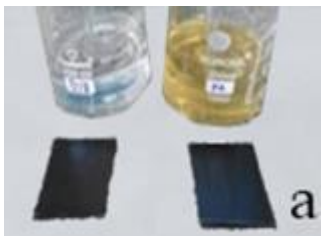
The feathers that were subjected to protease treatment were observed after 30 days. There was a minimal degradation of feathers, which had undergone protease treatment. A bluish-green layer was formed on the surface of the protease from *Pseudomonas aeruginosa* indicating more degradation effect. Anissa et al (2011)[2] has reported complete degradation of chicken feathers after 24 hrs of incubation at 50$^{O}$C and Minghai et al (2012)[21] reported complete degradation of raw feathers after 24 hrs by purified keratinolytic protease at 37$^{o}$C.


**Feather degradation Process without protease enzyme**

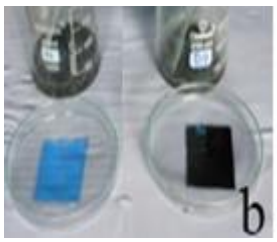

**Feather degradation Process with protease enzyme**

**Recovery of Silver from X - Ray Film**

The pieces of the X-ray film that were treated with protease were observed after an hrs. The gelatin layer in the X-ray film was completely stripped off by the protease from *Pseudomonas aeruginosa*. Shankar et al (2011)[29] and Bholay et al (2012)[5] has reported silver content using protease isolated from different strains.




**Recovery of silver without protease enzyme**




**Recovery of silver using protease enzyme**

**CONCLUSION**

The *Pseudomonas aeruginosa* species produce higher amount of protease with fructose media and beef extract which was optimized by the concentration optimization method. *Pseudomonas aeruginosa* species was found to be producing higher amount (200 ± 2.983U/ml) of alkaline protease at 48hrs.*Pseudomonas aeruginosa* showed the highest protease activity (180 ± 1.982U/ml) at 60% ammonium sulphate precipitation . It was found that the specific activity of enzyme was 1.39U/mg protein with 80% recovery and a purification fold of 1.12, after dialysis process **.**By SDS-PAGE analysis, identified that alkaline protease with a molecular weight of 55kDa. *Pseudomonas aeruginosa* showed highest protease activity at a temperature of 50ºC and pH of 8. *Pseudomonas aeruginosa* can be effectivel used as ideal species for the production of alkaline protease and it can be effective for various industrial applications.

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