

Original Article

Microbial Alkaline Protease: Production, Optimization of its Parameters and Application on Blood Stain Removal

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Abstract

Proteases are one of the most important groups of industrial enzymes used in detergent, protein, meat, photographic, leather, dairy, pharmaceutical and food industry. Proteases are one the groups of enzymes whose catalytic function is to breakdown protein. The protease enzyme was purified by ammonium sulphate precipitation. Further, the protease was confirmed by hydrolyzed zone using skimmed milk agar plate and the activity was analyzed and the parameters like pH, temperature, salt concentration were also optimized. The comparison of the protease production between the *Bacillus thuringiensis* and *Pseudomonas aeruginosa* was studied. The growth conditions for *Bacillus thuringiensis* were optimized by inoculating it into Protease Production Medium under varying pH levels, temperatures, and salt concentrations. It showed proteolytic activity. The protease was purified using 80% ammonium sulfate precipitation, with optimal activity at 37°C, pH 8.0, and a salt concentration of 0.7%. We concluded that the *Bacillus thuringiensis* showed effective result as compared to *Pseudomonas aeruginosa*. This study demonstrates that protease effectively removes blood stains within 25 minutes when used with commercial detergents, highlighting their potential application in the detergent industry.

1. Introduction:

Microorganisms have been historically employed in various biotechnological processes, starting as early as 6000 BC with yeast fermentation [1]. The term "enzyme" was introduced in 1876, defining proteinaceous biological catalysts, with the exception of ribozymes [1]. Microbial enzymes have gained global recognition for

industrial applications due to their efficiency, low energy requirements, and eco-friendliness [1]. The term "protease" has long been established to describe enzymes hydrolyzing peptide bonds [2].

In 1928, Grassman & Deckerhoff identified two distinct types: one acting on intact proteins, and the other on small peptides. Barrett and McDonald further refined this by introducing the terms "endopeptidase"

and "exopeptidase" based on action sites. Proteases are abundant across plants, animals, and microbes [2].

The industrial application of plant-derived proteases, such as papain and bromelain, is influenced by the feasibility of large-scale cultivation and extraction [3]. Animal proteases like trypsin, chymotrypsin, and pepsin play key roles in digestion and are extracted for commercial use [3]. However, rising demand has shifted focus to microbial sources, notably bacteria (*Bacillus* spp.), fungi (*Aspergillus*, *Trichoderma*), and even viruses, which produce highly specific proteases of biomedical interest [3].

Proteases are classified by catalytic action, pH preference (acidic, neutral, alkaline), and active site chemistry of serine, cysteine, aspartic, metalloproteases [3]. Their widespread applications are available in food processing, detergents, leather treatment, medicine (thrombolytics), and pharmaceuticals where serine proteases are used as digestive aids and fungal proteases serve for protein hydrolysis and antioxidant generation [4]. In this work, attempts were made to isolate and screen alkaline protease-producing bacteria, purify the protease enzyme from the isolated bacteria, estimate its activity, and optimize various growth parameters.

2. Materials and Methods

2.1 Collection of Sample

Pseudomonas aeruginosa was collected from the Rural Hospital in Rui, Taluka Baramati, District Pune, Maharashtra, India. *Bacillus thuringiensis* was obtained from the Bio-inoculum Unit of Vidya Pratishthan's School of Biotechnology, Baramati, Vidyanaigari, Taluka Baramati, District Pune, Maharashtra, India.

2.2 Screening of Alkaline Proteases Producing Strains

Bacillus thuringiensis, *Pseudomonas aeruginosa* were spot inoculated onto skimmed milk agar plate containing beef extract (3 g/L), peptone (10 g/L), NaCl (10 g/L), skimmed milk powder (10 g/L), and 18 g/L agar with pH adjusted to 8.5 and plate was incubated for 24 hours at 37°C [5].

2.3 Fermentations and Crude Enzyme Preparation

Bacillus thuringiensis, *Pseudomonas aeruginosa* were grown in fermented in protease production medium

consisting of beef extract (3 g/L), peptone (10 g/L), and NaCl (7 g/L), with pH 8.5. The medium was autoclaved for 20 minutes at 121°C. Fermentation was conducted in 250 mL Erlenmeyer flasks containing a 50 mL working volume, incubated at 37 °C for 24 hours on a rotary shaker that operated at 150 rpm.

The centrifuge was used to separate the biomass produced at 4°C for 10 minutes at 10,000 rpm. Then, the supernatant was collected [5].

2.4 Purification of Protease by Ammonium Sulfate Precipitation (table 1, table 2)

24 hours grown broths of *Bacillus thuringiensis* and *Pseudomonas aeruginosa* were used for the study. The cultures were centrifuged at 10,000 rpm for 10 minutes at 4°C to separate the biomass. The resulting supernatant with the enzyme extract was carefully collected. The supernatant was then transferred into sterile tubes, with one portion reserved for homogenate preparation [5].

A total of 100 ml of Protease Production Medium was taken in a flask, and 20 ml of the previously obtained enzyme supernatant was added [5]. Ammonium sulfate was added to reach final saturation levels of 40%, 60%, 80%, and 100% [Figure 8]. The mixture was incubated at 4°C for 20 minutes. The next day, the precipitate was collected by centrifugation at 10,000 rpm for 20 minutes. The resulting fractions were dissolved in 10 ml of Phosphate Buffer Saline [5]. Enzyme activity was assessed using a protease assay [5], and protein concentration was estimated using the Folin-Lowry method [6].

2.5 Determination of Enzyme Activity

The activity of the protease (caseinolytic) was assayed using a modified method of Kunitz [5].

Blank Sample Preparation: 1ml of the test sample was incubated at 40°C for 2 minutes. Subsequently, 2 ml of trichloroacetic acid solution was added and mixed thoroughly. The mixture was then incubated at 40°C for 10 minutes. After incubation, 1 ml of casein solution was added and mixed well. The solution was left undisturbed for 10 minutes. It was then filtered using Whatman filter paper. From the filtrate, 1 ml was taken and mixed with 5 ml of sodium carbonate solution.

Then, 1 ml of the working solution of Folin's reagent was added. The mixture was incubated at 40°C for 20 minutes, and the absorbance was measured at 660 nm using a 10 mm cuvette [7].

Test Sample Preparation: 1 ml of the test sample was incubated at 40°C for 2 minutes. Then, 1 ml of casein solution was added and mixed thoroughly. The mixture was left to stand for 10 minutes. After this, 2 ml of trichloroacetic acid solution was added and mixed well. The mixture was further incubated at 40°C for 10 minutes. It was then filtered using Whatman filter paper. From the filtrate, 1 ml was taken and combined with 5 ml of sodium carbonate solution. Subsequently, 1 ml of the working solution of Folin's reagent was added. The mixture was incubated at 40°C for 20 minutes, and the absorbance was measured at 660 nm using a 10 mm cuvette [7].

2.5.1 Standard Curve Preparation of Tyrosine (100 µg/mL)

To create a standard graph for protease activity, a tyrosine standard curve was typically used. Protease enzymes, such as caseinase, facilitate the hydrolysis of casein, leading to the release of free amino acids, notably tyrosine. These amino acids react with Folin's reagent, producing a measurable color change.

To prepare the standard solution, 0.1 g of L-tyrosine was weighed and dissolved in 60 ml of 1 mol/L hydrochloric acid. The solution was then transferred to a 100 ml volumetric flask, and the volume was made up to 100 ml with 1 mol/L hydrochloric acid, resulting in an L-tyrosine stock solution with a concentration of 1 mg/ml. From this stock, 10 ml of the solution was taken and mixed with 0.1 mol/L hydrochloric acid in another 100 ml volumetric flask. The volume was adjusted to 100 ml using 0.1 mol/L hydrochloric acid, yielding a standard L-tyrosine solution of 100 µg/ml [7].

2.5.2 Calculations

The protease activity was calculated using the following equation [7]:

$$\text{Unit/ml} = (\mu\text{mole tyrosine equivalent released} \times \text{total volume}) / (\text{protease volume} \times \text{Time} \times \text{colorimetric Assay volume})$$

2.6 Determination of Protein concentration

Folin Lowry method was used to measure the protein concentration. The bovine serum albumin was selected as a standard. Casein, the test protein, was obtained from HiMedia Laboratories Private Limited.

Protein estimation: A set of test tubes was labeled for blank, standards containing 0.2, 0.4, 0.6, 0.8, and 1.0 mL of Bovine Serum Albumin solution, and test containing 0.2, 0.4, 0.6, 0.8, and 1.0 mL casein solution. The Bovine Serum Albumin was pipetted into the standard tubes, and casein was added to the test tube. Distilled water was added to adjust the total volume to 1.0 mL in all tubes, except for the test tube if it already contained 1.0 mL. Subsequently, 5.0 mL of alkaline copper solution was added to each tube. The contents were mixed thoroughly, and the tubes were allowed to stand at room temperature for 10 minutes. Then, 0.5 mL of diluted Folin-Ciocalteu reagent was rapidly added to each tube and mixed immediately. The tubes were incubated in the dark at room temperature for 30 minutes. Finally, the absorbance was measured at 660 nm using a spectrophotometer [8].

2.7 Optimization of parameters for Alkaline Protease Production

2.7.1 Determination of Optimum pH

50 mL of the protease production medium was taken into three Erlenmeyer flasks, and the pH was adjusted to 8, 10, and 12, respectively, while keeping all other variables constant. The flasks were inoculated with 5% inoculum of *Bacillus thuringiensis* and *Pseudomonas aeruginosa*, and incubated at 37°C for 24 hours. After incubation, the alkaline protease activity was estimated [9].

2.7.2 Determination of Optimum Temperature

50 mL of the protease production medium was taken into three flasks. Each flask was inoculated with 5% inoculum of *Bacillus thuringiensis* and *Pseudomonas aeruginosa*, and incubated at different temperatures: 37°C, 55°C for 24 hours. After incubation, the alkaline protease activity was estimated [9].

2.7.3 Determination of Optimum Salt Concentration

50 ml of the protease production medium was taken into three flasks, and the NaCl concentration was

adjusted to 0.6%, 0.7%, and 0.8% respectively. Each flask was inoculated with 5% inoculum of *Bacillus thuringiensis* and *Pseudomonas aeruginosa*, and incubated at 37°C for 24 hours. After incubation, the alkaline protease activity was estimated [9].

2.8 Application on Blood Stain Removal

For this application, white cotton cloth pieces (5cm × 5cm) were stained with human blood and oven-dried at 95–100°C for 5 minutes. The dried blood stained cloth pieces were then placed in separate trays. The following sets were prepared and studied [10]:

- a. A tray containing 100 mL of distilled water and a blood-stained cloth (control).
 - b. A tray containing 100 mL of distilled water, a blood-stained cloth, and 2 mL of protease enzyme.
 - c. Tray with distilled water (100 mL) + blood-stained cloth + 2ml of commercial detergent-Tide-5mg/mL+ 1ml of protease enzyme.
 - d. A tray containing 100 mL of distilled water, a blood-stained cloth, and 2 mL of commercial detergent (Tide 5 mg/mL).
 - e. A tray containing 100 mL of distilled water, a blood-stained cloth, 2mL of commercial detergent (Tide 5 mg/mL) and 2 mL of protease enzyme.
- All trays were incubated at 55°C for 10 minutes. After incubation, the cloth pieces were rinsed with water, dried, and examined visually [10].

3. Results:

3.1 Screening of Alkaline Protease Producing Strains:

Bacillus thuringiensis and *Pseudomonas aeruginosa* were spot-inoculated onto skimmed milk agar plates with the pH adjusted to 8.5, and the plates were incubated at 37°C for 24 hours (Figure 1 and 2). The production of protease by the screened bacteria was confirmed by the appearance of clear proteolytic zones around the colonies [5].

3.2 Fermentation for Protease Enzyme Production:

Bacillus thuringiensis and *Pseudomonas aeruginosa* were fermented in protease production medium. The media were autoclaved for 20 minutes at 121°C. Fermentation processes were completed on a rotary shaker at 37°C for 24 hours at 150 rpm

[5]. 50ml volume of microbial culture was added in 250 ml Protease producing Medium in flask. The centrifugal machine was used to separate the produced biomass at 4°C for 10 minutes at 10,000 rpm [5].

3.3 Media Optimization for Alkaline Protease Production:

From the graph (figure 12, figure 13, figure 14) depicting the effect of pH (8, 10, and 12) using the ammonium sulfate precipitation method, protease activity was observed in *Bacillus thuringiensis* at 80% and 100% saturations, and in *Pseudomonas aeruginosa* at 60%, 80%, and 100% saturations. Upon comparison of both organisms, the optimum pH for maximum protease activity was found to be **pH 8 at 80% saturation** for *Bacillus thuringiensis* (table 1 and table 2).

From the graph (figure 15, figure 16, figure 17) showing the effect of temperatures (37°C and 55°C) using the ammonium sulfate precipitation method, protease activity was observed in *Bacillus thuringiensis* at 80% and 100% saturations, and in *Pseudomonas aeruginosa* at 60%, 80%, and 100% saturation. Upon comparison of both organisms, the optimum temperature for maximum protease activity was found to be **37°C at 80% saturation** for *Bacillus thuringiensis* (table 1 and table 2).

Similarly, from the graph (figure 18, figure 19, figure 20) showing the effect of salt concentration (0.6, 0.7, and 0.8) using the ammonium sulfate precipitation method, protease activity was observed in *Bacillus thuringiensis* at 80% and 100% saturation, and in *Pseudomonas aeruginosa* at 60%, 80%, and 100% saturation. Based on the comparison, the optimum salt concentration for maximum protease activity was determined as **0.7% at 80% saturation** for *Bacillus thuringiensis* (table 1 and table 2).

3.4 Application of Protease on Blood stain removal:

In the case of blood stain removal from cloth, it was observed that the protease was able to remove blood stains effectively even without addition of any detergent. The protease demonstrated a strong capability to break down proteins and remove stains from fabric, suggesting its potential use as an alkaline protease in detergent powders or liquid cleaning formulations. Its stability and activity in the presence of solvents and detergents can be exploited for such applications [14].

Blood stains were removed from five cloth pieces

placed in separate Petri dishes. The present study was conducted to evaluate the application of the crude enzyme extract as a cleansing additive for blood stain removal. The results showed that blood stains were successfully removed within 25 minutes when a combination of detergent (5 mg/mL) and protease enzyme extract was used (Figure 21e). In contrast, no stain removal was observed when cloth pieces were rinsed with only distilled water and crude enzyme extract (Figures 21 b, c, d), or with detergent alone. The control cloth (Figure 21a) also showed no change. Thus, the results clearly indicated that the addition of the protease enzyme extract to a commercial detergent significantly enhanced the removal of blood stains from cotton cloth. These findings support its potential application in the formulation of cleaning agents for the detergent industry [14].

4. Discussion

The present study aimed to evaluate the protease-producing potential of *Bacillus thuringiensis* and *Pseudomonas aeruginosa* under varying laboratory conditions. The findings clearly demonstrated that *Bacillus thuringiensis* exhibited superior proteolytic activity as compared to *Pseudomonas aeruginosa*. The consistently elevated enzyme production observed in *Bacillus thuringiensis* highlighted its potential as a promising candidate for industrial applications where microbial proteases are required.

Both bacteria produced distinct zones of hydrolysis on skim milk agar, confirming their ability to degrade casein. These results were consistent with earlier reports by Agasthya et al. [13], who noted similar protease activity in these microorganisms. Notably, the performance of *Bacillus thuringiensis* improved significantly under optimized conditions, particularly at pH 8.0, temperature 37°C, and 80% salt concentration. These optimal conditions aligned with previous studies by Gupta et al. [4] and Kumar and Takagi [14], which demonstrated the favorable growth and enzymatic activity of *Bacillus* species in moderately alkaline environments.

In contrast, *Pseudomonas aeruginosa* displayed enhanced protease activity at elevated temperatures, with maximum activity recorded at 55°C. This thermotolerant behavior corroborates the findings of Zambare et al. [24], who also observed high-temperature enzyme expression in *Pseudomonas aeruginosa*. However, despite its temperature

adaptability, the overall enzyme yield from *Pseudomonas aeruginosa* was comparatively lower than that of *Bacillus thuringiensis*. This difference in enzyme production may be attributed to variations in the regulatory mechanisms governing protease expression in response to environmental stressors such as pH and salinity.

Further purification of the crude enzyme extracts using ammonium sulfate precipitation revealed that 80% saturation was optimal for *Bacillus thuringiensis*, resulting in a marked enhancement of enzyme activity. This method proved effective for partial purification and may serve as a foundation for further downstream processing.

The functional efficacy of the produced protease was also evaluated in a practical application involving the removal of dried blood stains from cotton fabric. The enzyme, when combined with a commercial detergent (Tide), significantly improved stain removal efficacy within 25 minutes compared to the detergent or enzyme used alone. These findings supported the observations made by Mothe et al. [14], who highlighted the potential of microbial proteases in detergent formulations for domestic and industrial cleaning purposes.

Overall, *Bacillus thuringiensis* demonstrated greater efficiency in the production of alkaline proteases under the tested conditions, with maximum activity observed at pH 8.0, temperature 37°C, and salt concentration of 80% saturation. Its consistent enzyme activity and adaptability to moderately alkaline environment, and application potential in fabric stain removal underscore its industrial relevance. Future work should focus on enhancing enzyme recovery through techniques such as immobilization, and evaluating performance in pilot-scale trials to assess commercial viability.

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Competing interest: Authors declare that no conflict of interest exists.

Ethical Statement: This work does not violate ethical guidelines.

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Appendices

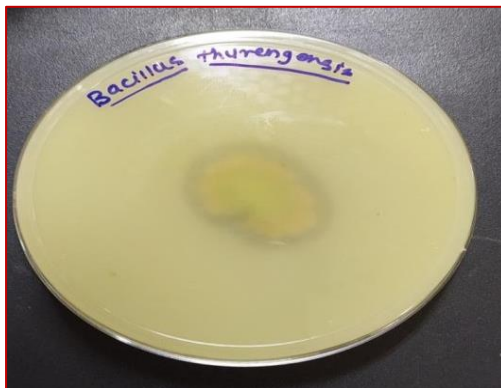


Figure 1: *Bacillus thuringiensis* showing zone of clearance on skim milk agar plate.



Figure 2: *Pseudomonas aeruginosa* showing zone of clearance on Skim milk agar plate.



Figure 3: Protease Production Medium broth inoculated with *Pseudomonas aeruginosa* (left) and *Bacillus thuringiensis* (right) for protease estimation.

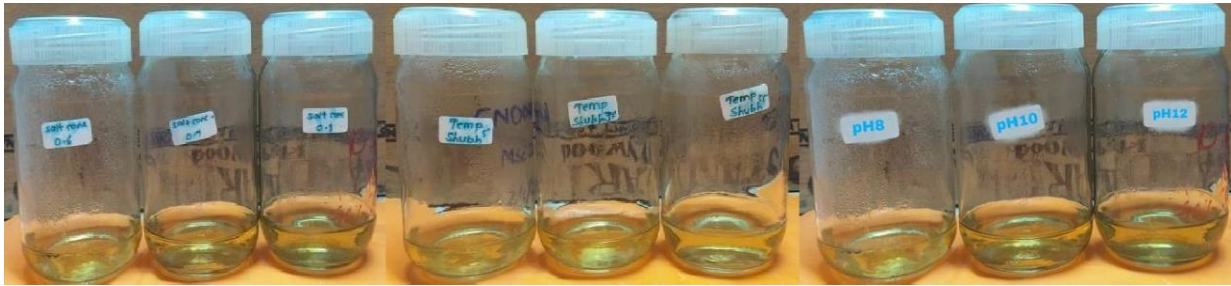


Figure 4: Protease Production Medium with *Bacillus thuringiensis* studied for optimization of pH, temperature, salt concentration before incubation.

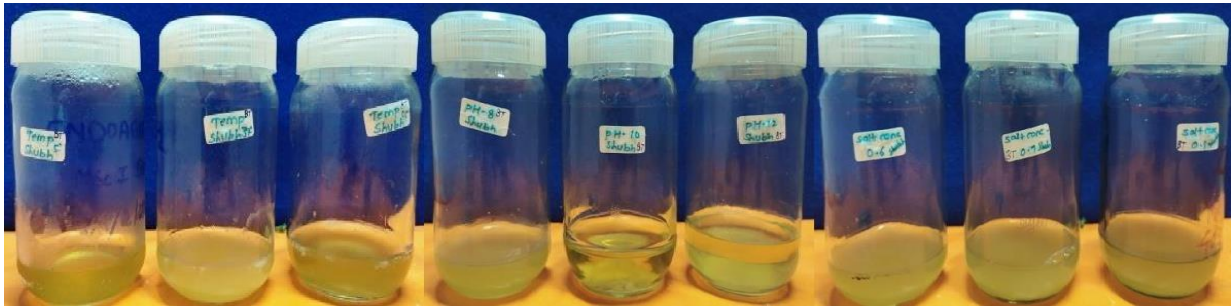


Figure 5: Protease Production Medium with *Bacillus thuringiensis* studied for optimization of pH, temperature, salt concentration after incubation.



Figure 6: Protease Production Medium with *Pseudomonas aeruginosa* for optimization of pH, temperature, salt concentration before incubation.



Figure 7: Protease Production Medium with *Pseudomonas aeruginosa* for optimization of pH, temperature, salt concentration after incubation.

Starting percent saturation	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Figure 8: Ammonium sulfate precipitation chart [25].

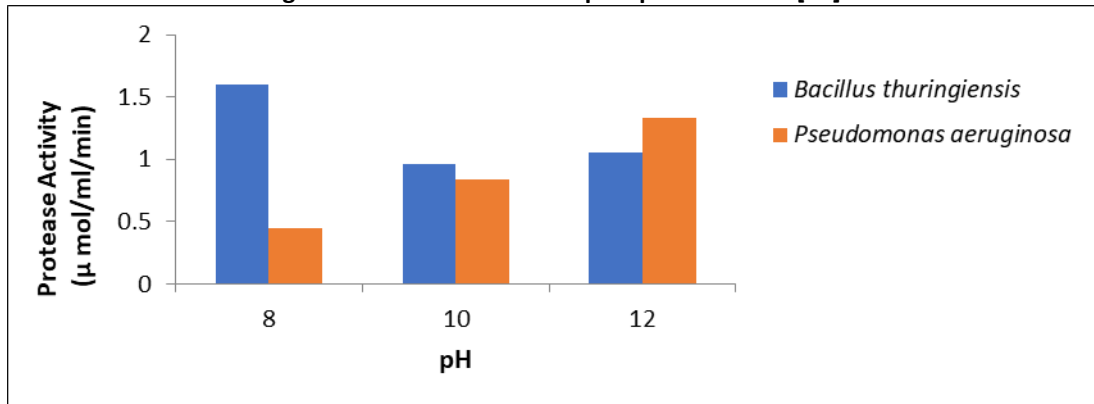


Figure 9: Effect of pH on crude protease activity.

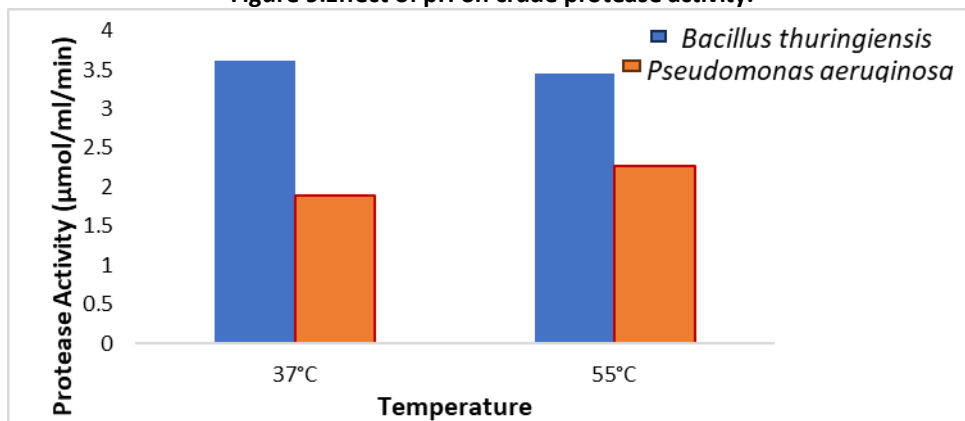


Figure 10: Effect of temperature on crude protease activity

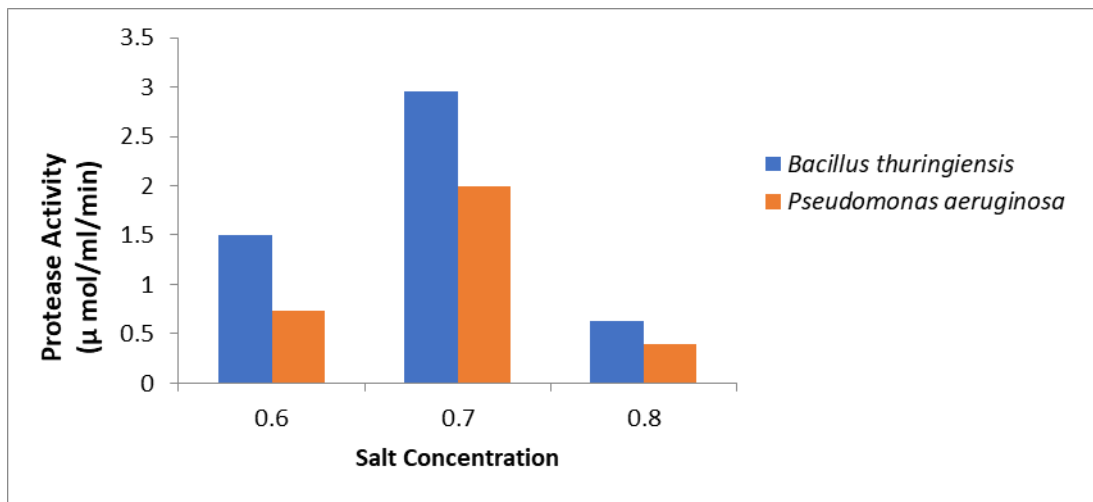


Figure 11: Effect of salt concentration on crude protease activity.

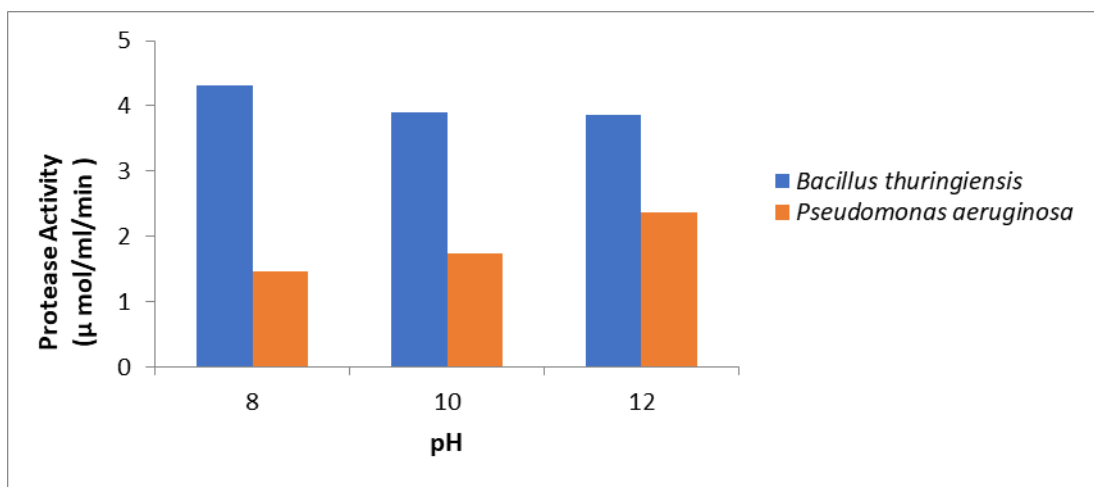


Figure 12: Effect of pH on Protease Activity at 80% Ammonium Sulfate Saturation.

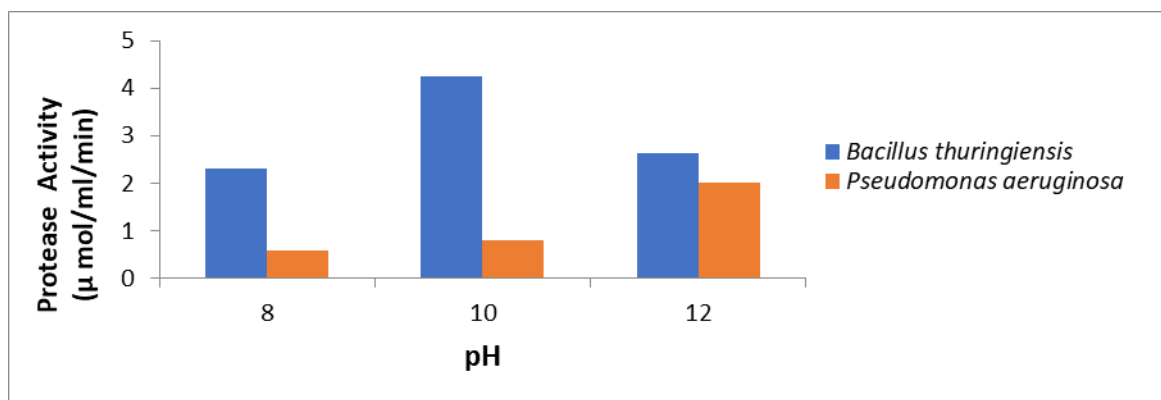


Figure 13: Effect of pH on Protease Activity at 100% Ammonium Sulfate Saturation.

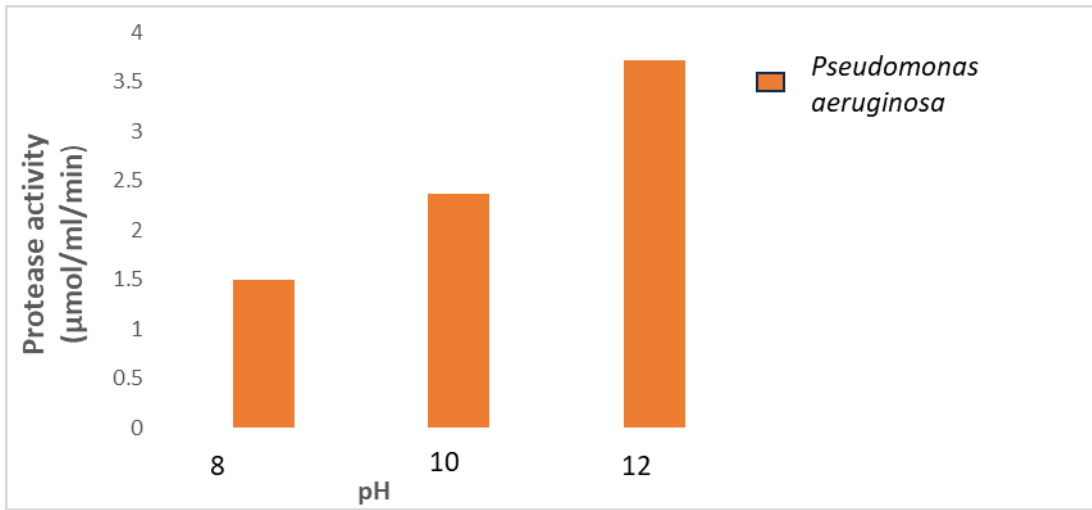


Figure 14:Effect of pH on Protease Activity at 60% Ammonium Sulfate Saturation

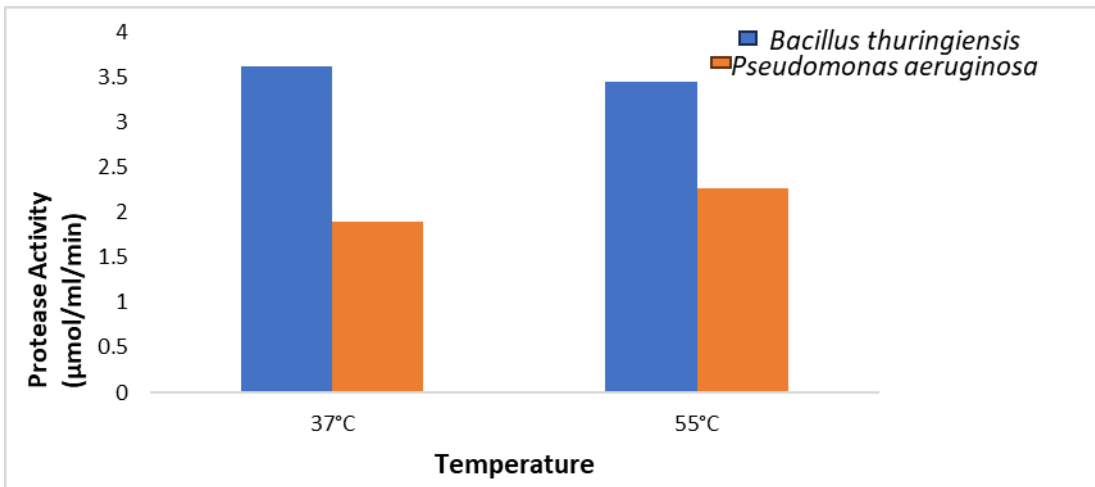


Figure 15:Effect of Temperature on Protease Activity at 80% Ammonium Sulfate Saturation

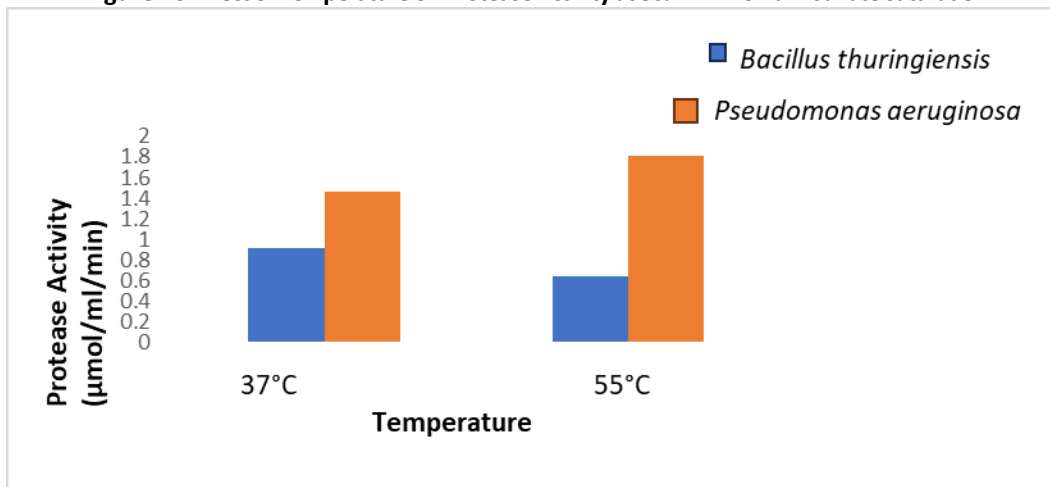


Figure 16:Effect of Temperature on Protease Activity at 100% Ammonium Sulfate Saturation.

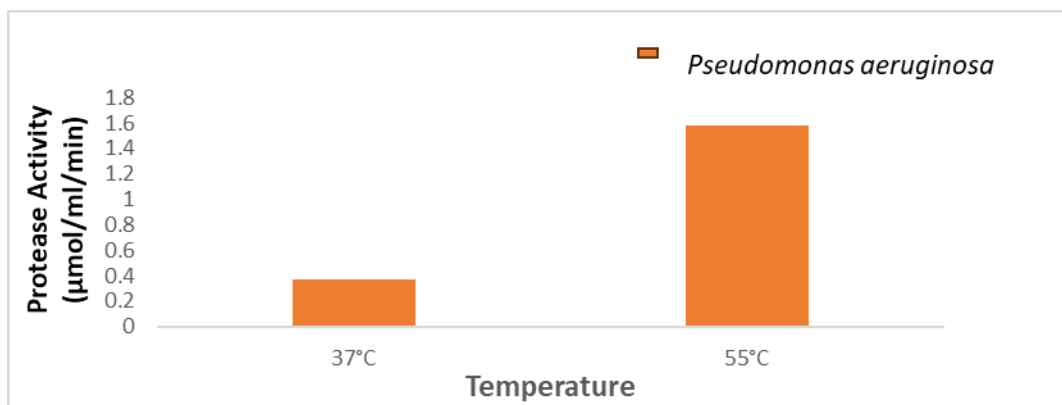


Figure 17: Effect of Temperature on Protease Activity at 60% Ammonium Sulfate Saturation.

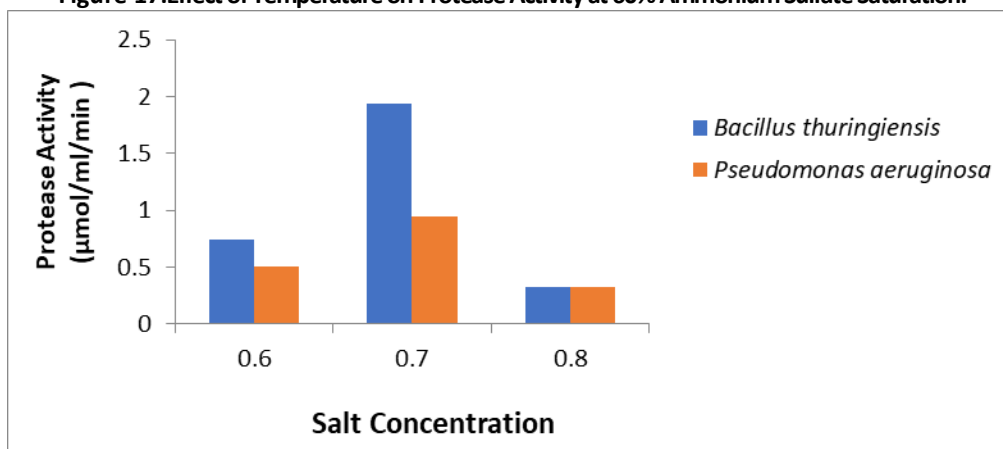


Figure 18: Effect of salt concentration on Protease Activity at 80% Ammonium Sulfate Saturation.

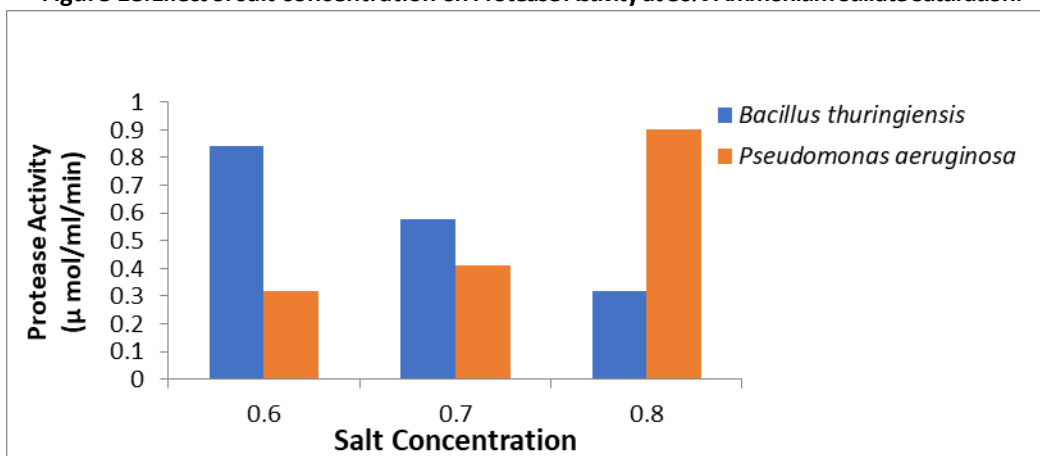


Figure 19: Effect of salt concentration on Protease Activity at 100% Ammonium Sulfate Saturation.

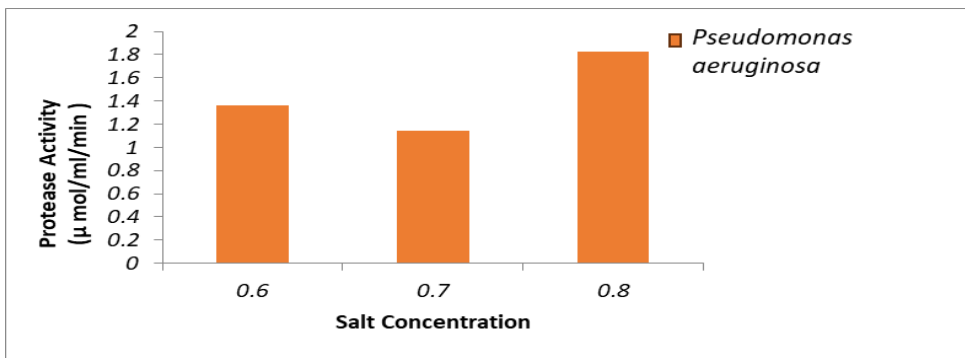


Figure 20: Effect of salt concentration on Protease Activity at 60% Ammonium Sulfate Saturation.

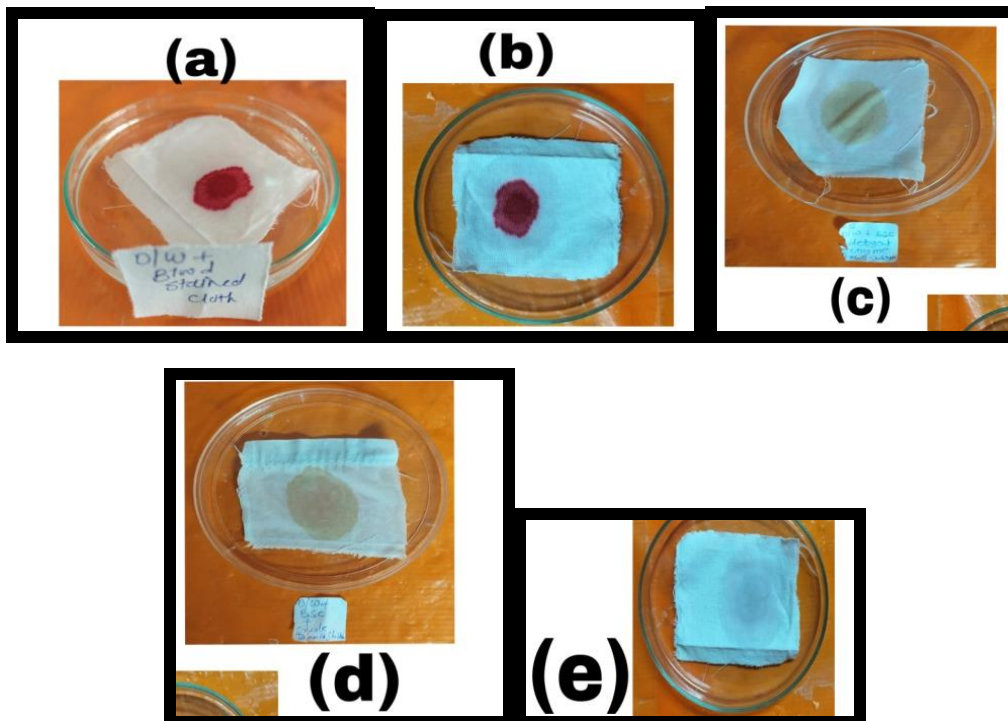


Figure 21: Blood stain removal activity of protease from *Bacillus thuringiensis*. a. A tray containing 100 mL of distilled water and a blood-stained cloth (control). b. A tray containing 100 mL of distilled water, a blood-stained cloth, and 2 mL of protease enzyme. c. Tray with distilled water (100 mL) + blood-stained cloth + 2 mL of commercial detergent-Tide-5 mg/mL + 1 mL of protease enzyme. d. A tray containing 100 mL of distilled water, a blood-stained cloth, and 2 mL of commercial detergent (Tide 5 mg/mL). e. A tray containing 100 mL of distilled water, a blood-stained cloth, 2 mL of commercial detergent (Tide 5 mg/mL) and 2 mL of protease enzyme.

Table 1: Purification table for the protease from *Pseudomonas aeruginosa*.

Purification step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield (%)	Fold Purification
Salt Optimization							
Ammonium Sulfate salt (grams)	Ammonium Sulfate Saturation (%)						
0.8	60%	50 ml	12	21.98	1.83	0.083	3.51
0.8	80%	50 ml	37	52.77	1.42	0.026	2.73
0.6	100%	50 ml	34	10.99	0.32	0.029	0.61
0.7	60%	50 ml	23	26.38	1.14	0.043	2.19
0.7	80%	50 ml	46	43.97	0.95	0.021	1.82
0.7	100%	50 ml	64	26.38	0.41	0.015	0.78
0.6	60%	50 ml	21	28.58	1.36	0.047	2.61
0.6	80%	50 ml	26	13.19	0.50	0.037	0.96
0.8	100%	50 ml	51	46.17	0.90	0.019	1.73

Purification Step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity(Units/mg)	Purification yield(%)	Fold Purification
pH Optimization							
pH	Ammonium Sulfate Saturation (%)						
8	60%	50 ml	49	61.56	1.25	0.02	2.4
8	80%	50 ml	58	85.75	1.47	0.017	2.82
8	100%	50 ml	67	39.58	0.59	0.014	1.13
10	60%	50 ml	39	92.35	2.36	0.025	4.53
10	80%	50 ml	52	90.15	1.73	0.019	3.32
10	100%	50 ml	65	52.77	0.81	0.015	1.55
12	60%	50 ml	23	85.75	3.72	0.043	7.15
12	80%	50 ml	26	61.56	2.36	0.038	4.53
12	100%	50 ml	37	74.76	2.02	0.027	3.88

Purification Step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield(%)	Fold Purification
Temperature Optimization							
Temperature	Ammonium Sulfate Saturation (%)						
37°C	60%	50 ml	65	24.18	0.37	0.015	0.71
37°C	80%	50 ml	52	10.99	0.21	0.019	0.40
37°C	100%	50 ml	24	35.18	1.46	0.041	2.80
55°C	60%	50 ml	61	96.75	1.58	0.016	0.03
55°C	80%	50 ml	29	39.58	1.36	0.034	2.61
55°C	100%	50 ml	17	30.78	1.81	0.058	3.48

Purification step	Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield(%)	Fold Purification
Optimization of the crude enzyme						
pH 8	50 ml	39	17.59	0.45	0.025	0.86
pH 10	50 ml	52	44.15	0.84	0.019	1.61
pH 12	50 ml	23	30.78	1.33	0.043	2.55
Temperature 37°C	50 ml	43	81.35	1.89	0.023	3.63
Temperature 55°C	50 ml	31	70.36	0.46	0.032	4.34
Salt concentration 0.6%	50 ml	42	30.78	0.73	0.023	1.40
Salt concentration 0.7%	50 ml	45	90.15	2.00	0.022	3.84
Salt concentration 0.8%	50 ml	28	10.99	0.39	0.035	0.75

Table 2: Purification table for the protease from *Bacillus thuringiensis*.

Purification Step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity(Units/mg)	Purification yield(%)	Fold purification
Salt Optimization							
Ammonium Sulfate salt (grams)	Ammonium Sulfate Saturation (%)						
0.8	100%	50 ml	68	22.07	0.324	0.014	0.17
0.8	80%	50 ml	72	24.28	0.337	0.013	0.176
0.7	100%	50 ml	75	44.15	0.588	0.013	0.31
0.7	80%	50 ml	25	48.56	1.94	0.039	1.037
0.6	100%	50 ml	52	44.15	0.84	0.019	0.44
0.6	80%	50 ml	86	64.02	0.74	0.011	0.39

Purification Step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield(%)	Fold purification
pH Optimization							
pH	Ammonium Sulfate Saturation (%)						
8	80%	50 ml	23	99.34	4.31	0.043	2.30
8	100%	50 ml	19	44.15	2.3	0.052	1.22
10	80%	50 ml	22	86.09	3.91	0.045	2.09
10	100%	50 ml	14	59.6	4.25	0.071	2.27
12	80%	50 ml	16	61.81	3.86	0.062	2.06
12	100%	50 ml	20	52.98	2.64	0.049	1.41

Purification Step		Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield(%)	Fold purification
Temperature Optimization							
Temperature	Ammonium Sulfate Saturation (%)						
37°C	80%	50 ml	23	85.75	3.7	0.043	1.97
37°C	100%	50 ml	29	26.49	0.91	0.034	0.48
55°C	80%	50 ml	37	74.76	2.02	0.027	1.08
55°C	100%	50 ml	31	19.86	0.64	0.032	0.34

Purification step	Volume (ml)	Total Protein (mg)	Total activity	Specific activity (Units/mg)	Purification yield (%)	Fold Purification
Optimization of the crude enzyme						
pH 8	50 ml	11	17.66	1.6	0.09	0.85
pH 10	50 ml	25	24.24	0.96	0.037	0.51
pH 12	50 ml	23	24.24	1.05	0.043	0.56
Salt concentration 0.6%	50 ml	22	33.11	1.5	0.045	0.80
Salt concentration 0.7%	50 ml	29	86.09	2.96	0.034	1.58
Salt concentration 0.8%	50 ml	28	17.66	0.63	0.035	0.33
Temperature 55°C	50 ml	23	79.47	3.45	0.043	1.84
Temperature 37°C	50 ml	14	50.77	3.62	0.071	1.93