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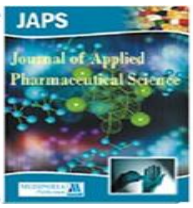

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Characterization and Purification of Protease enzyme

K. Shanti Naidu

K. Shanti Naidu
Department of Biotechnology
School of Biotechnology and Health Sciences, Karunya University, Coimbatore, India

***For Correspondence**:
K. Shanti Naidu
Lecturer
Department of Biotechnology
School of Biotechnology and Health Sciences
Karunya University, Karunya Nagar
Coimbatore – 641114
Tamilnadu, India
E-mail : shantinaidu@karunya.edu

ABSTRACT

Protein is one of the three major food groups needed for proper nutrition. Proteolytic enzymes or proteinases are the group of enzymes whose catalytic function is to hydrolyze (breakdown) proteins. Production and partial purification of protease enzyme by Bacillus subtilis was the aim of this study. Bacillus subtilis was allowed to grow in shake flask broth culture, 3.5L and 7L fermenters for purpose of inducing protease enzyme. We are finding out the effect of minerals which are useful for the production of protease by Plackett Burman design. Minerals such as magnesium sulphate, potassium dihydrogen phosphate and manganese sulphate were showing the results for the production of protease. The protease enzyme was purified by ultra filtration, ammonium sulphate precipitation, dialysis, and lyophilization.The activity of protease was increased as there was increase in the enzyme concentration. Purified protease enzyme had a maximum activity at pH 9.0 of carbonate buffer and the optimum incubation time was 48hr. The protease assay is done for the crude enzyme at different temperature. It showed greater activity at 50 °C but after that it started decreasing the activity so, we had selected the temperature at 40 °C for the good activity..

Key words: Bacillus subtilis, Placket Burman, Ultra Filtration, Ammonium Sulphate Precipitation, Dialysis, Lyophilisation, Fermenters.

INTRODUCTION

Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Proteolytic enzymes are very important in digestion as they breakdown the peptide bonds in the protein foods to liberate the amino acids needed by the body (Ogino et al., 2008). Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is notable based on several clinical studies indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation. Parasites, fungal forms, and bacteria are protein (Coelho et al., 1978). Viruses are cell parasites consisting of nucleic acids covered by a protein film. Enzymes can break down undigested protein, cellular debris, and toxins in the blood, sparing the immune system this task. The immune system can then concentrate its full action on the bacterial or parasitic invasion. Protease also has an ability to digest unwanted debris in the blood including certain bacteria and viruses. Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. Subtilis.*

Probably the largest application of proteases is in laundry detergents, where they help removing protein based stains from clothing (Jeong et al., 1968). Proteases have been used in the hidedehairing process, where dehairing is carried out at pH values between 8 and 10. In this paper we aimed to purify protease and to study the factors affecting the activity to present potential application of the protease for industrial proposes.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade from Himedia laboratories, India, S.D. Fine chemicals, India, Fischer chemicals, India. Other chemicals were used in this study were of reagent grade and were commercially available. The different concentration of minerals designed for several experiments is shown in table 1.

Table 1: Plackett Burman Design

Expt No.	NaCl (ml)	$MgSO_4$ (ml)	$CaCl_2$ (ml)	$FeSO_4$ (ml)	$MnSO_4$ (ml)	KH_2PO_4 (ml)	$ZnSO_4$ (ml)
1	1.0	1.0	1.0	0.0	1.0	0.5	0.0
2	0.5	1.0	1.0	1.0	0.5	1.0	0.0
3	0.5	0.5	1.0	1.0	1.0	0.5	1.0
4	1.0	0.5	0.5	1.0	1.0	1.0	0.0
5	0.5	1.0	0.5	0.0	1.0	1.0	1.0
6	1.0	0.5	1.0	0.0	0.5	1.0	1.0
7	1.0	1.0	0.5	1.0	0.5	0.5	1.0
8	0.5	0.5	0.5	0.0	0.5	0.5	0.0

Table 2: Stock solutions

Salt	Concentration (%)	Volume (ml)
NaCl	2.5	30
$MgSO_4$	1.25	30
$CaCl_2$	1.25	30
$FeSO_4$	1.25	30
$MnSO_4$	1.25	30
KH_2PO_4	1.25	30
$ZnSO_4$	1.25	30

a. Stock Solution Preparation

Procedure
Prepare the stock solutions
Take 8 flasks in duplicates (for production)
↓
Now add distilled water, soya flour (1%)
↓
Add all the inorganic to the test tubes as the compositions given above
Triss buffer and mineral nutrients were also prepared separately and autoclaved
↓
All the flask were inoculated with 1% of 24th hour old Inoculum
↓
Samples were collected at 38th and 48th hour and checked for the protease production

b. **Inoculum Preparation**

Procedure
Sub-cultures (slants)
↓
Inoculum preparation (50 ml) Luria-Bertani media
↓
Control Medium was prepared (50 ml) and autoclaved
(Maltodextrin 0.25 gm, Yeast Extract 0.25, Skim milk 0.25)
↓
Triss buffer and minerals were added to the flasks separately
↓
Each flask contains 42 ml water + 5 ml triss buffer + 3 ml mineral
(After autoclave add triss buffer and mineral in aseptic condition)

c. **Protease assay**

d. **Protein estimation (**Lowry *et al.*, 1951)

e. Protease enzyme production by 3.7 L and 7L fermenters

f. Effect of temperature on enzyme activity:

- The protease assay is done for the crude enzyme at different temperatures to find out the activity of enzyme.

g. Effect of pH on enzyme activity

- The effect of pH on enzyme activity was found by conducting assay for the crude enzyme samples with the different pH buffers.
- These pH buffers were used for the preparation of substrate casein solution.

h. Effect of temperature on enzyme stability

- 5 ml of crude enzyme sample was taken in 5 different sterilized test tubes.
- All the test tubes were maintained at different temperatures (4°C, 20°C, 30°C, 40°C, and 50°C).
- The initial activity of the enzyme was checked.
- Then the enzyme kept at the different temperatures (4°C, 20°C, 30°C, 40°C, 50°C) was checked for the activity at 2nd hr, 24th hr and 48th day.

i. Effect of pH on enzyme stability

- The effect of pH on enzyme stability was found by using three different buffers.
- Casein was dissolved in acetate, phosphate and carbonate buffer solution.
- Then routine assay was done at 2nd hr and 24th hr to check the activity of the enzyme.

j. Purification of protease enzyme

- Enzyme purification was done by using the following techniques; Ammonium Sulphate Precipitation, Dialysis, Ultra filtration and Lyophilization.

RESULTS AND DISCUSSION

Comparative study of protease production in 250 ml flask, 3.7L and 7L fermenter

The production of protease started after 12th hour of inoculation where the growth constantly increased and reached a constant phase towards the 48th hour. After 48th hour in 54th hour we can see the decrease in the activity (Table 3). Hence, we prefer 48th hour for the production of protease.

Table 3: Protease Activity in 250 ml flask, 3.7L & 7L Fermenter

S.No.	NaCl	$MgS0_4$	$CaCl_2$	$FeS0_4$	$MnS0_4$	KH_2P0_4	$ZnS0_4$	response
1	1.0	1.0	1.0	0.0	1.0	0.5	0.0	11.42
2	0.5	1.0	1.0	1.0	0.5	1.0	0.0	12
3	0.5	0.5	1.0	1.0	1.0	0.5	1.0	0.6
4	1.0	0.5	0.5	1.0	1.0	1.0	0.0	11
5	0.5	1.0	0.5	0.0	1.0	1.0	1.0	6.6
6	1.0	0.5	1.0	0.0	0.5	1.0	1.0	0.9
7	1.0	1.0	0.5	1.0	0.5	0.5	1.0	0.6
8	0.5	0.5	0.5	0.0	0.5	0.5	0.0	11.6

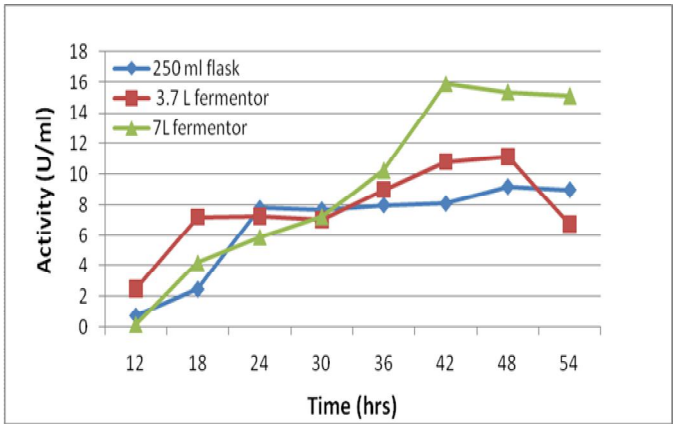

Fig 1: Production of Protease in 250 ml, 3.7 L & 7L Fermenters

The graph (figure 1) shows comparative study of 250 ml flask, 3.7 L fermenter and 7 L fermenter. The growth of 7 L fermenter is giving greater activity at 42nd hour.

3.7 L Fermenter

The maximum protease activity (99 U/ml) was obtained at 48th hour of cultivation, when cells were in the stationary phase. When compared to shaken flask experiments previously reported, the enzyme production was now 1.8-fold higher, but occurred 24 h later. Similar results were obtained (Boyer et al., 1968) who observed that the maximum protease production by *Streptomyces peucetius* occurred after 100 h of cultivation at the stationary phase growth. The nitrogen and sugar contents in the medium decreased slowly and where not completely consumed along all growth.

7L Fermenter

The compositions mentioned in table 4 were chosen for the production of protease. The values were obtained by software when the minerals of high and low values were inserted in the software.

The greater activity of protease was found to be at 38th hour (table 5). The activity decreased by 48th hour in case of Plackett Burman design.

The high values of NaCl were added up and noted in ∑H (Table 6); similarly the low values of NaCl were added and noted in ∑L. The differences between high and low values were taken. To find the effect the square of difference values divided by four should be done where we can see the effect of minerals which are useful for the production of protease. Magnesium sulphate, potassium dihydrogen phosphate and manganese sulphate were showing the results for the production of protease.

Characterization of protease:

The protease assay is done for the crude enzyme at different temperatures to find out the activity of enzyme (table 7). It showed greater activity at 50 °C but after that it started decreasing the activity (figure 2). Activity was checked in duplicates (2 sets) at different temperatures to find out the temperature suited for the production of protease.

To investigate the temperature effect, the protease assay was performed at the temperature range from 30 to 100 degree at pH 7.0. The influence of pH was investigated using 50mM buffer solutions ranging from pH 5.0 to 10.0 (sodium citrate buffer for pH 5.0 and 6.0; potassium phosphate buffer for pH 6.0–8.0; Tris–HCl buffer for pH 8.0 and 9.0; and glycine–NaOH buffer for 9.0 and 10.0) at the optimum temperature previously determined (Bascaran Vet al.,1990).

When the activity was checked for different pH starting from 8 to 11 (table 8) and the casein was diluted with different pH to check which pH is exactly required for the production. The best productivity was found at 9.5. Their was decrease in productivity when the activity was taken for pH 10. Hence the graph (figure 3)describes increase of protease at 9.5 after that it shows decrease in productivity.

The crude enzyme was incubated at temperatures ranging from 30 to 70°C, and also at low temperatures (−20 and 4°C), in a 50mM phosphate buffer pH 6.0. After 1 h incubation, the reaction mixtures were assayed, and the residual enzymatic activity determined (Aretz et al., 1989). But it didn't show much activity at pH 6 and phosphate buffer. As phosphate buffer doesn't show much stability we prefer for carbonate buffer.

Hence, maintaining the crude enzyme at different temperature was checking the stability of enzyme. Initially the activity of crude was checked and then after maintaining at different temperatures the activity was checked (table 9). It showed stable at 30 degree (figure 4). Finally at 50 degree it didn't show any activity of enzyme.

The enzyme stability was checked at different pH (table 10). The initial activity of crude was taken then the activity was checked with the different buffer. It showed the best stability of enzyme with carbonate buffer. The graph (figure 5) explains that after 1day the activity was less. Activity increased after 2 days. So after 2 days the activity starts increasing. Hence the activity was checked after 2 days.

Table 4: Compositions of inorganic for the production of protease

S. No.	NaCl	$MgS0_4$	$CaCl_2$	$FeS0_4$	$MnS0_4$	KH_2P0_4	$ZnS0_4$	response
1	1.0	1.0	1.0	0.0	1.0	0.5	0.0	11.42
2	0.5	1.0	1.0	1.0	0.5	1.0	0.0	12
3	0.5	0.5	1.0	1.0	1.0	0.5	1.0	0.6
4	1.0	0.5	0.5	1.0	1.0	1.0	0.0	11
5	0.5	1.0	0.5	0.0	1.0	1.0	1.0	6.6
6	1.0	0.5	1.0	0.0	0.5	1.0	1.0	0.9
7	1.0	1.0	0.5	1.0	0.5	0.5	1.0	0.6
8	0.5	0.5	0.5	0.0	0.5	0.5	0.0	11.6

For protease purification, the basal medium (100 ml in 500 ml Erlenmeyer flask) was inoculated with 1% of a stationary-phase culture and incubated at 32 ºC with shaking at 220 rpm for 40 h. Supernatant obtained by centrifugation (8000×*g* for 20 min at 4 ºC) of strain AF-2004 culture broth was dialysed against 20mM Tris–HCl, pH 8.5 containing 50mM NaCl and 0.5mM $CaCl_2$ (buffer A) and used as the crude enzyme solution (Cherdyntseva et al., 1982). All the purification steps were performed at 4 ºC. At the first step, pre-chilled acetone was added slowly to the solution up to 50% saturation and after discarding the precipitate; further acetone was added to the solution up to 80% saturation with gentle stirring and left for 1 h. The precipitate formed was collected by centrifugation at 12,000×*g* for 20 min, dissolved in a minimum amount of buffer A and dialysed against the same buffer for 24 h with two buffer changes (Ellaiah et al., 1996).

A protease from *Bacillus subtilus*, was partially purified by a method involving ammonium sulfate precipitation, ultrafiltration, lyophilization and dialysis. Fold purification of 0.98 was obtained for Lyophilization; the enzyme with 92.7 % recovery of activity was achieved (table 11). Similarly for ultrafiltration 93.6 % of yield, for dialysis 86.8 % and for ammonium sulphate precipitation 82 % of yield was achieved (table 12). Enzyme stability and activity were checked and found at pH 9 and temperature 40 degree was optimum for the protease.

Table 5: Protease activity at 38^{th} and 48^{th} hr

Expt. No.	Activity(U/ml)	
	38^{th} hr	48^{th} hr
1	11.42	9.3
2	12	9.7
3	0.6	1.2
4	11	8.7
5	6.6	5.9
6	0.9	0.5
7	0.6	2.9
8	11.6	9.2

SUMMARY AND CONCLUSION

Proteases are one of the most important groups of industrial enzymes, with considerable application in the food industry. A protease was successfully purified from the *bacillus subtilis*. The optimum temperature and pH of protease ranged from 40C to 50C and pH 8, respectively. Pre-incubation at temperatures above 50C resulted in a decrease of enzyme activity, indicating that protease A is a thermally unstable enzyme. The optimal pH for protease A was pH 8, and the enzyme activity was stable over a broad range of pH 3.5^10.4, through which it retained at least 80% of its original activity. *Bacillus* sp. was purified from culture supernatant. The protease hydrolyzed casein, gelatine, and especially leather powder effectively under alkaline conditions. Proteases are enzymes that hydrolyze peptide bonds of proteins.

Table 6: Results obtained from Plackett Burman experiment

	NaCl	$MgSO_4$	$CaCl_2$	$FeSO_4$	$MnSO_4$	KH_2PO_4	$ZnSO_4$
∑H	23.72	30.62	24.92	24.2	29.62	30.05	10.5
∑L	30.8	24.1	29.8	30.52	25.1	24.22	46.02
Difference	-7.08	6.52	-4.88	-6.32	4.52	6.28	-35.52
Effect	-1.77	1.63	-1.22	-1.58	1.13	1.57	-8.88
Mean square	0.392	0.332	0.186	0.312	0.159	0.308	9.856

Some proteins have very specific structures or amino acid sequences. Therefore, it is very difficult for usual proteases to decompose such proteins. Purification techniques were done to obtain the pure enzyme after the production of protease. Techniques like ammonium sulphate precipitation, ultra filtration, lyophilisation and dialysis were done. Collagen is a typical protein which has a very specific structure. It has a very specific amino acid sequence and a three-helical chain structure, each chain consisting of a peptide chain. In each chain, glycine appears in third amino acid in the sequence. In mammals, approximately 30% of the total protein consists of collagen as a structural protein.Comparitive study of protease in 250 ml flask, 3.5 L and 7 L fermenter was done where it shows the highest activity in 7 L fermenter and showed less activity in flask because the maintainence in flasks are risky. They may get contaminated. In case of screening of minerals we found the greater activity in 38^{th} hour. The high values of NaCl were added up and noted in ∑H; similarly the low values of NaCl were added and noted in ∑L. The differences between high and low values were taken. To find the effect the square of difference values divided by four should be done where we can see the effect of minerals which are useful for the production of protease. Magnesium sulphate, potassium dihydrogen phosphate and manganese sulphate were showing the results for the production of protease. The protease assay is done for the crude enzyme at different temperatures to find out the activity of enzyme. It showed greater activity at 50 °C but after that it started decreasing the activity. Activity was checked in duplicates 2 sets were taken and at different temperatures the activity was checked to find out the temperature suited for the production of protease. The activity was checked for different pH starting from 8 to 11. The casein was diluted with different pH to check which pH is exactly required for the production. We found the best productivity at 9.5. Their was decrease in productivity when we took the activity for pH 10. Hence the graph describes increase of protease at 9.5 after that it shows decrease in productivity. The stability of enzyme was being checked by

maintaining the crude enzyme at different temperature. Initially the activity of crude was checked and then after maintaining at different temperatures the activity was checked. It showed stable at 30 degree. Finally at 50 degree it didn't show any activity of enzyme. In this three different buffers at different pH were taken. The initial activity of crude was taken then the activity was checked with the different buffer. It showed the best stability of enzyme with carbonate buffer. The graph explains that after 1day the activity was less. Activity increased after 2 days. So after 2 days the activity starts increasing. Hence we prefer checking the activity after 2 days.

Table 7: Effect of temperature on enzyme activity

Temperature (°C)	Activity (U/ml) 1st set	Activity (U/ml) 2nd set	Activity (U/ml) Average
20	1.1	0.9	1.0
30	3.14	3.33	3.23
40	8.0	8.2	8.1
50	13.71	14.3	14.0
60	15.0	15.8	15.4
70	3.9	4.3	4.1

A protease from *Bacillus subtilus*, was partially purified by a method involving ammonium sulphate precipitation, ultra filtration, lyophilisation and dialysis. Fold purification of 0.98 was obtained for Lyophilization; the enzyme with 92.7 % recovery of activity was achieved. Similarly for ultrafiltration 93.6 % of yield, for dialysis 86.8 % and for ammonium sulphate precipitation 82 % of yield was achieved. Enzyme stability and activity were checked and found at pH 9 and temperature 40 degree was optimum for the protease.

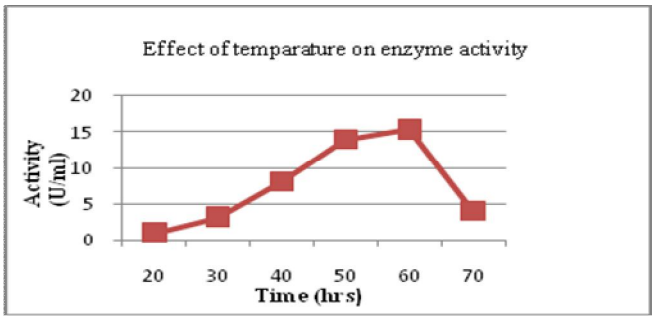

Fig 2: Effect of temperature on enzyme activity

Table 8: Effect of pH on enzyme activity

pH of casein solution	Activity (U/ml) 1st set	Activity (U/ml) 2nd set	Activity (U/ml) Average
8	5.2	5.5	5.35
8.5	6.5	6.8	6.65
9	7.9	8.0	7.9
9.5	8.1	8.3	8.2
10	7.5	8.0	7.75
10.5	6.2	5.9	6.05
11	4.3	4.1	4.2

Table 9: Effect of temperature on enzyme stability

Temperature (°C)	Initial activity	Activity after 2 hr (U/ml)	Activity after 1 day (U/ml)	Activity after 2 days (U/ml)
4	10.8	11.3	13.1	13.0
20		11.0	9.2	8.2
30		10.7	9.8	6.2
40		6.2	1.2	0.0
50		0.8	--	--

Table 10: Effect of pH on enzyme stability

Type of Buffer	pH of Buffer	Initial activity	Activity after 2 hr (U/ml)	Activity after 1 day (U/ml)
Acetate	4	34	12.0	0.0
Acetate	5		18.2	4.3
Phosphate	6		20.2	8
Phosphate	7		26.4	16
Phosphate	8		29.0	21.3
Carbonate	9		33.8	30.2
Carbonate	10		33	29.7
Carbonate	11		32	28.6

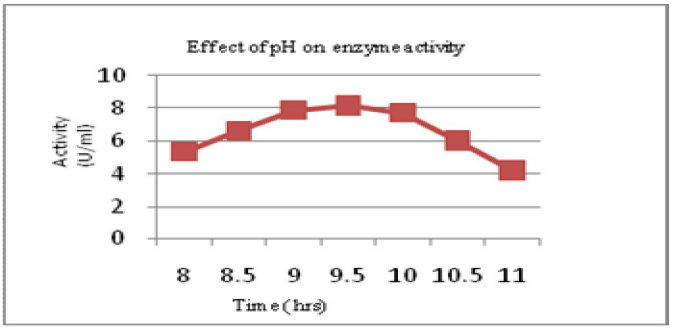

Fig 3: Effect of pH on enzyme activity

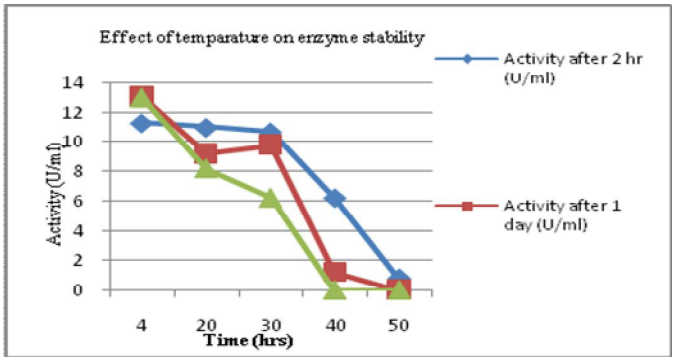

Fig 4: Effect of temperature on enzyme stability

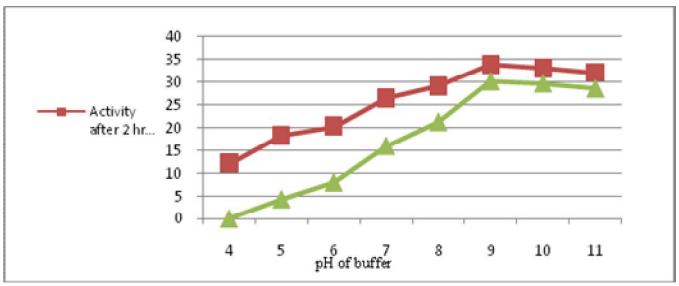

Fig 5: Effect of pH on enzyme stability

Table 11: Purification of protease by different techniques

S. No	Fraction	Volume (ml)	Protease activity (U/ml)	Total activity (U/ml)	Total protein (mg)	Specific activity(U/mg)	Yield (%)	Purification fold
1	Crude	20	7.4	148	32	4.62	92.7	0.94
	Lyophilization	4.5	30.5	137.25	31.92	4.3		
2	Crude	1500	8.01	12015	2860.7	4.2	93.6	2.74
	Ultrafiltration	310	36.3	11253	978.5	11.5		
3	Precipitated enzyme	2	131.75	263.5	41.9	6.29	86.8	1.26
	Dialysis	4	57.2	228.8	29.0	7.9		

Table 12: Ammonium sulphate purification

S. No	Fraction	Volume (ml)	Protease activity (U/ml)	Total activity (U/ml)	Total protein (mg)	Specific activity(U/mg)	Yield (%)	Purification fold
1	Crude	300	10.7	3210	984	3.26	100	1
2	20% Salt	10	16	160	56.11	2.85	4.98	0.87
3	40% Salt	10	102.5	1025	294.54	3.48	31.9	1.06
4	60% Salt	20	112	2240	484.85	4.62	69.8	1.42
5	80% Salt	20	131.75	2635	418.92	6.29	82	1.92

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