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Partial purification and characterization of protease enzyme from soil borne bacteria

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ABSTRACT

Protease enzyme from bacterial isolates AKS-4 and AKS-6 was successfully purified by ammonium sulfate precipitation. Purified enzyme fractionate obtained from 80% salt saturation showed highest protease activity. Protease activity of crude lysate was increased after purification with 80% salt concentration. Purified protease fractions (80% salt saturation) obtained from bacterial isolate-AKS-4 and AKS-6 were named as P-1 and P-2, respectively with protease activity of 35.07 U/mL and 34.46 U/mL. Extracellular protease from isolate AKS-4 was purified to 9.29 fold by $(NH_4)_2SO_4$. Enzyme fractionates P-1 and P-2 showed increased activity at neutral and alkaline pH range while enzyme activity was reduced under acidic pH. P-1 and P-2 demonstrated increased protease activity at higher temperature range (50 ºC, 60 ºC). The impact of different divalent metal ions and organic solvents on protease activity of both enzyme fractions was investigated. Highest protease activity by both enzyme fractions was obtained with calcium chloride and n-butanol.

Key words: Bacterial isolates, purified enzyme, ammonium sulfate precipitation, crude lysate, temperature.

INTRODUCTION

Proteases are group of enzymes, catalyze hydrolysis of polypeptide chain into smaller peptide chain or free amino acids (Abirami et al., 2011).

Proteases are of the most imperative industrial enzymes synthesize and produced by a wide range of microorganisms, including fungi,

bacteria, yeasts, molds, and are also found in different animal tissues and in plants. Bacterial proteases are generally extracellular, active at broad pH range, and easily produced in large quantity (Walsh et al., 1970). *Bacillus* produces a broad range of extra-cellular enzymes, including proteases. Several *Bacillus* species were known as protease producers i.e., *Bacillus mojavensis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus sterothermophilus* (Asker et al., 2013).

A submerged fermentation in which nutrients are present in solubilized form in large quantity of water with good aeration rate and agitation speed in a fermenter. In this method environmental factors such as temperature and pH can be controlled very well. Impeller of fermenter control mixing and allows equal distribution of microbial cells, products, toxic waste products, nutrients and oxygen throught fermentation broth. This method is recommended for the production of extracellular enzymes from microbes (Gervais et al., 1996). Protease has application in laundry industry, where they are used to remove protein based stains from clothing (Asker et al., 2013). About 50% of the total industrial enzymes is contributed by proteases so they are very important enzyme (Rao et al., 1998). They occupy an important position due to their broad applications in leather, pharmaceutical, food, detergent, laundry, photography and agriculture industries. They are also used in brewing, peptide synthesis, meat tenderization, cheese making, bioremediation and medical diagnosis and as treatment for wound and inflammation (Anwar and Saleemuddin, 1998; Gupta et al., 2002). The exploitation of an enzyme for various industrial purposes depends on its distinctive characteristics such as optimum pH, temperature, enzyme stability in presence of various organic solvents, metal ions, mode of action of enzyme, etc. (Zhang et al., 2011). Therefore, the aim of present study was to purify and characterize the protease enzyme from soil borne bacteria.

MATERIALS AND METHODS

2.1. Isolation, screening and identification of proteolytic bacteria from soil

Soil samples 1 and 2 were collected from botanical garden and medicinal plant garden of Banasthli University, respectively. Bacteria were isolated by serial dilution agar plate technique earlier described by Kader et al. (1999). In order to identify protease producers, bacteria were inoculated in gelatin agar medium plates followed by incubation of plates at 37ÚC for 2 days. Protease producers were identified based on formation of clear zone around their colonies after flooding the plates with $HgCl_2$ solution ($HgCl_2$ 5.0 gm, concentrated HCl 20 mL and distilled water 100 mL). $HgCl_2$ reacted with unhydrolyzed gelatin to produce opacity making the clear zones easier to see (Abdel Galil, 1992).

2.2. Protease production

One mL of 24 h old Luria broth culture was inoculated in 100 mL of protease production medium containing (% w/v): $CaCl_2$ 0.01; K_2HPO_4, 0.05; yeast extract 0.02; peptone 1.0; $MgSO_4$ 0.01; glucose 0.1; pH 7.0 (Quadar et al., 2009). Crude enzyme extract was prepared by centrifugation of fermentation broth at 8000 rpm, 4 °C for 15 minutes.

2.3. Measurement of enzyme activity

Protease activity in the crude enzyme extract was determined according to the method of Carrie Cupp-Enyard (2008) by using casein as substrate.

2.4. Partial purification of protease

Crude enzyme extracts were used for purification. Different concentration of ammonium sulfate (30%, 70% and 80%) was taken for partial purification of protease. For 30% salt saturation, solid ammonium sulfate was added to enzyme extract with stirring and it was left for 3 hours at 4 ÚC. The precipitate was removed by centrifugation at 10,000 rpm at 4ºC for 15 minutes. The pellet was reconstituted in minimum amount of 50 mM potassium phosphate buffer (pH 7.5) and protease activity in pellet was determined. Additional ammonium sulfate was added to the supernatant in order to bring the saturation to 70% and it was left for 3 hrs at 4 ÚC. Again it was centrifuged to obtain pellet. Enzyme activity was measured in pellet and additional ammonium sulfate was added to the supernatant in order to bring the saturation to 80% and enzyme activity at 80 % saturation was determined. Fold purification and percentage yield was determined using following formula.

$$\text{Fold purification} = \frac{\text{Specific protease activity of each fraction}}{\text{Specific protease activity of crude protein extract}}$$

$$\text{Percent yield} = \frac{\text{Protease activity of each fraction}}{\text{Protease activity of crude protein extract}} \times 100$$

2.5. Measurement of activity of partially purified protease

The reaction mixture was containing 1 mL of 0.65% case in solution and 0.2 mL of crude enzyme extract were placed at 37 ˚C for 30 minutes thereafter 1mL of Trichloroacetic acid solution was added to stop the enzymatic reaction. After standing for 15 minutes for precipitation reaction to occur, it was filtered using Whatmann's No 1 filter paper. 0.4 mL of filtrate was added in 1 mL of sodium carbonate solution followed by addition of 0.2 mL of 2 fold diluted Follin Ciocalteus phenol reagent. Above mixture was incubated in dark at room temperature for 30 minutes for the development of blue color. The concentration of tyrosine released from casein by protease action was measured at 660 nm against a reagent blank using tyrosine standard. Tyrosine was taken in following concentration range for the preparation of standard curve: 27.5 µM, 55 µM, 110 µM, 220 µM and 275 µM. One protease unit was defined as the amount of enzyme required to releases 1 µM of tyrosine per minute per mL at 37ºC, pH 7.5 (Mohapatra et al., 2003). All the experiments were done in triplicates and mean values are presented.

The enzyme activity (U/mL) was calculated by following formula

$$\text{Enzyme activity (Units/mL)} = \frac{\text{µmole tyrosine equivalent releases} \times 2.2 \text{ (Total volume of assay)}}{\text{Volume of enzyme taken (0.2 mL)} \times \text{Incubation time (30)} \times 1.6}$$

2.2= Total volume of assay (in milliliters).
0.2= Volume of enzyme used (in milliliters).
1.6= Volume of sample taken in cuvette for absorbance.

Specific activity is the enzyme activity in one mg of total protein (expressed in µmol/min/mg).

The specific enzyme activity (U/mg) was calculated by following formula

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein content (mg/mL)}}$$

2.6. Characterization of partially purified enzyme

Partially purified enzyme from both bacterial isolates (AKS-4 and AKS-6) obtained after 80 % salt saturation was used for characterization study. The effect of various parameters on enzyme activity and stability was measured.

2.6.1. Effect of pH on protease activity

The impact of pH on the activity of purified enzyme was studied by incubating reaction mixture at different pH range (4-9) of potassium phosphate buffer (50 mM) at 37 ºC. pH of buffer was adjusted using 1N HCl and 1N NaOH. Upon completion of incubation period enzyme activity was determined.

2.6.2. Effect of temperature on protease activity

The impact of temperature on the activity of purified enzyme was studied by incubating the reaction mixture at different temperature range (40 ºC, 50 ºC, 60 ºC and 70 ºC). Upon completion of incubation period enzyme activity was measured.

2.6.3. Effect of metal ions on enzyme stability

The impact of various metal ions at concentration of 5mM on protease stability was studied by pre-incubation of enzyme preparation for 2 hrs at 37 ºC in the presence of divalent metal ion solution (Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Cu^{2+}, Li^{2+} and Zn^{2+}). Thereafter, protease assay was performed at 50 ºC and pH 8.0. The enzyme activity was determined without adding metallic ions, considered as the control for determining relative enzyme activity. Relative enzyme activity was determined using following formula (Sharma et al., 2014).

$$\text{Relative enzyme activity (U/mL)} = \frac{\text{Enzyme activity of control (without metal ion)}}{\text{Enzyme activity with metal ion}}$$

2.6.4. Effect of organic solvents on enzyme stability

The impact of various organic solvents (10%, v/v) on protease stability was studied by pre-incubation of enzyme preparation for 2 hrs at 37 ºC in the presence of organic solvent solution. Thereafter, protease assay was performed at at 50 ºC and pH 8.0. Relative enzyme activity was determined by considering the activity of the enzyme without any organic solvents as 100%.

RESULT AND DISCUSSION

3.1. Isolation, screening and production of protease

From a total of 2 collected soil samples, 12 bacterial colonies were isolated. Among 12 isolates, 4 isolates from soil sample 1 and 4 isolates from soil sample 2 were shown clear zone around streaked lines. Then these 8 bacterial isolates were selected for protease production in fermentation broth. Four bacterial isolates from soil sample 1 were named as AKS-1, AKS-2, AKS-3 and AKS-4 respectively. Four bacterial isolates from soil sample 2 were named as AKS-5, AKS-6, AKS-7 and AKS-8 respectively. Isolates AKS-4 and AKS-6 was showing highest protease activity so protease from these isolates were selected for purification and characterization study.

3.2. Partial purification of enzyme

Results presented in Table 1 showed that $(NH_4)_2SO_4$ concentration of 80% totally precipitated protease enzyme. In the crude enzyme preparation, the enzyme extracted from bacterial isolates AKS-4 and AKS-6 was demonstrated 24.46 U/mL and 33.61 U/mL enzyme activity, respectively. Protease activity was increased by enzyme fractions of bacterial isolates AKS-4 and AKS-6 obtained after purification with 80% $(NH_4)_2SO_4$ concentration. It was 35.07 U/mL and 34.46 U/mL, respectively. Partially purified enzyme fractions obtained from 80% $(NH_4)_2SO_4$ concentration from bacterial isolate-AKS-4 and AKS-6 was named as P-1 and P-2.

Similar purification process was done by Abirami et al. (2011). Umayaparvathi et al. (2013) reported purification of protease from *Bacillus cereus* SU12 with ammonium sulfate precipitation. Highest specific activity was obtained after 80% salt concentration. Oke and Onilude, 2014 reported purification of protease by $(NH_4)_2SO_4$ precipitation, gel filtration chromatography and ion exchange chromatography. Sharma et al. (2014) purified protease by ammonium sulfate and reported that 6.65-fold purification was achieved from initial crude lysate during $(NH_4)_2SO_4$ precipitation (50-70%).

Table 1: Summary of Partial purification of protease extracted from bacterial isolates AKS-4 and AKS-6.

Percentage saturation of $(NH_4)_2SO_4$	Absorbance at 660nm		Protease activity (U/mL)		Protein content (mg/mL)		Specific activity (U/mg)		Fold purification		Percentage yield	
	AKS-4	AKS-6	AKS-4	AKS-6	AKS-4	AKS-6	AKS-4	AKS-6	AKS-4	AKS-6	AKS-4	AKS-6
Crude enzyme extract	0.80	1.10	24.46	33.61	4.99	4.33	4.90	7.66	1.0	1.0	100	100
30%	0.53	0.206	16.31	6.28	0.22	0.54	74.13	11.62	15.12	1.51	66.68	18.68
70%	0.632	0.215	19.27	6.55	0.78	0.25	24.70	26.2	5.04	3.42	78.78	19.48
80%	1.15	1.13	35.07	34.46	0.77	0.08	45.54	430.75	9.29	56.23	143.37	102.52

3.3. Effect of pH on enzyme activity

Results presented in Table 2 showed that the optimum pH for protease activity of enzyme fractions P-1 and P-2 was 8.0. The enzyme activity for P-1 and P-2 was 21.76 U/mL and 12.50 U/mL, respectively. Enzyme activity was increased at neutral and alkaline pH while protease activity was reduced under acidic pH. Alkaline nature of protease indicates it's suitability in alkaline environment of various industries.

Balachandran et al. (2012) reported protease activity over a wide pH range and it was found that enzyme was most active at pH 8-9. Asker et al. (2013) reported bacterial protease was 78% active in the pH range of 7.0-8.0. Joo et al. (2003) reported that *Bacillus clausii* protease was stable in the broad pH range of 4.0-12.0 while maximum activity was obtained at pH 12.0. Abirami et al. (2011) reported optimum pH for highest protease activity from *Penicillium janthinellum*. It was 6.5.

3.4. Effect of temperature on enzyme activity

Table 3 demonstrated results of impact of temperature on protease activity of purified enzyme fractions (P-1 and P-2). Both fractions P-1 and P-2 showed highest protease activity when incubation temperature of protease assay was 50 ºC. The protease activity was increased at higher temperature range. The enzyme

Table 2: Effect of pH on the activity of partially purified enzyme fractions (P-1 and P-2).

pH	Absorbance at 660nm		Concentration of liberated tyrosine (µM)		Protease activity (U/mL)	
	P-1	P-2	P-1	P-2	P-1	P-2
4	0.16	0.07	26.66	11.66	4.88	2.13
5	0.21	0.13	36.16	21.66	6.61	3.96
7	0.68	0.18	114.66	31.00	20.98	5.67
8	0.71	0.41	118.66	68.33	21.76	12.50
9	0.55	0.21	92.33	36.33	16.89	6.64

activity was found in following descending order: 50 ºC > 60 ºC > 70 ºC > 40 ºC.

Balachandran et al. (2012) reported protease activity over a broad range of temperature from 4 to 50 ºC. Highest activity was obtained at 4-8 ºC. Enzyme activity was completely lost at 37 ºC. Bajaj et al. (2011) reported that protease activity was tested over a wide range of temperature 60-90 ºC. Asker et al. (2013) investigated effect of different temperature on *Bacillus megaterium* protease activity and It was found that protease activity was highest when incubation temperature was 50 ºC. Abirami et al. (2011) reported highest protease activity at temperature between 30-40 ºC.

3.5. Effect of metal ions on enzyme stability

Table 3: Effect of temperature on the activity of partially purified enzyme fractions (P-1 and P-2).

Temperature (ºC)	Absorbance at 660nm		Concentration of liberated tyrosine (µM)		Protease activity (U/mL)	
	P-1	P-2	P-1	P-2	P-1	P-2
40	0.2	0.11	34.00	19.00	6.22	3.47
50	1.96	1.04	326.66	174.83	59.78	31.99
60	1.85	0.42	309.33	70.00	56.60	12.81
70	1.62	0.33	271.33	56.00	49.65	10.24

Table 4 revealed results of impact of various metal ions on protease stability of purified enzyme fractions (P-1 and P-2). Protease activity from P-1 was increased in presence of following divalent metal ions Ca^{2+}, Mg^{2+}, Mn^{2+} and Li^{2+}. A decrease in the protease activity was measured when enzyme was preincubated with Ba^{2+}, Cu^{2+} and Zn^{2+}. Highest enzyme activity by both fractions (P-1 and P-2) was observed in presence of Ca^{2+}. It was 59.78 U/mL for P-1 and 47.33 U/mL for P-2, respectively.

Sharma et al. (2014) reported that Ca^{2+}, Mn^{2+} and Mg^{2+} increased enzyme activity by 36%, 22% and 8%, respectively while other metal ions Cu^{2+}, Zn^{2+}, Na^{+} and K^{+} had a negative effect on protease activity. Uddin et al. (2015) reported that $MgSO_4$ increased the protease activity while β-mercaptoethanol decreased enzyme activity. NaCl did not change the enzyme activity. Asker et al. (2013) reported that Mg^{2+}, Ca^{2+} and Cu^{2+} increased protease activity from purified bacterial enzyme fractionate by 110, 112 and 109%, respectively.

3.6. Effect of organic solvents on enzyme stability

Table 4: Stability of partially purified enzyme fractions (P-1 and P-2) under various divalent metal ions.

Metal ions	Absorbance at 660nm		Concentration of liberated tyrosine (µM)		Protease activity (U/mL)		Percentage relative enzyme activity	
	P-1	P-2	P-1	P-2	P-1	P-2	P-1	P-2
Control	0.9	0.88	150	146.66	27.45	26.84	100	100
$CaCI_2$	1.96	1.55	326.66	258.66	59.78	47.33	217.77	176.36
$MgSO_4$	0.98	0.75	163.33	125	29.89	22.87	108.88	85.22
$MnCI_2$	1.18	1.45	196.66	241.66	35.99	44.22	131.11	164.77
$BaCI_2$	0.85	0.88	142.66	147.5	26.10	26.99	95.11	100.56
$CuCI_2$	0.85	0.87	141.66	145	25.92	26.53	94.44	98.86
$LiSO_4$	0.99	0.99	166	165	30.37	30.19	110.66	112.5
$ZnSO_4$	0.77	0.70	128	116.66	23.48	21.35	85.55	79.54

Results presented in Table 5 showed that highest enzyme activity by both fractions (P-1 and P-2) was obtained in presence of n-butanol. Table 5 revealed that protease was found stable in various organic solvents. Methanol, ethanol and n-butanol increased protease activity by both protein fractions (P-1 and P-2) while decrease in the enzyme activity was measured in presence of acetone. Karbalaei-Heidari et al. (2013) reported that purified protease was completely stable in ethyl acetate, toluene, n-hexane and chloroform at 10% and 50% (v/v) and moderate stable in ethanol and DMSO at 50% (v/v). Rahman et al. (2006) reported stability of protease in different organic solvents. Solvents such as n-dodecane, n-decane, isooctane and n-hexadecane (25%, v/v) increased protease activity by 85%, 83%, 78% and 59%, respectively after 30 minutes preincubation.

Table 5: Stability of partially purified enzyme fractions (P-1 and P-2) under various organic solvents.

Organic solvents	Absorbance at 660nm		Concentration of liberated tyrosine (µM)		Protease activity (U/mL)		Percentage relative enzyme activity	
	P-1	P-2	P-1	P-2	P-1	P-2	P-1	P-2
Control	0.35	0.33	58.33	55.66	10.67	10.18	100	100
Methanol	0.31	0.46	51.66	76.66	9.45	14.03	88.61	137.81
Ethanol	0.43	0.55	71.66	92.33	13.11	16.89	122.91	165.98
n-butanol	0.99	0.86	166.33	143.33	30.43	26.23	285.27	257.66
Acetone	0.25	0.28	43	46.66	7.86	8.54	73.74	83.88

CONCLUSION

From the present study, it is concluded that protease extracted from soil borne bacterial isolates AKS-4 and AKS-6 was purified by ammonium sulfate precipitation. The characterization of partially purified protease was done. Protease activity was maximum at pH 8.0 of reaction mixture, indicates alkaline nature of protease. Purified protease is highly active at temperature near 50 ºC. Enzyme activity from protein fractionate P-1 and P-2 was increased in presence of calcium chloride. Methanol, ethanol and n-butanol have positive impact on protease activity. Further characterization of protease can be carried out

to determine its suitability in various industrial processes. The purified protease can be used for various purposes in food, pharmaceutical and detergent industry.

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