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AN PRAGMATIC STUDY OF OPTIMIZATION OF PROTEASE PRODUCTION FROM BACTERIA

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ABSTRACT

Microbial proteases contribute nearly 40% of the total worldwide enzyme market. Hence, with the view of this significance, objective of the present study was to isolate and optimize the protease production from soil borne bacteria. Soil is the ideal habitat for many extracellular enzyme producing bacteria therefore soil samples were collected from two different sites of Rajasthan University and bacteria were isolated by serial dilution agar plate technique. Isolated bacteria were screened on gelatin agar medium for proteolytic activity. Based on the results of primary screening 4 bacterial isolates from soil sample 1 and 4 bacterial isolates from soil sample 2 were selected for protease production in production medium. 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. Isolate AKS-4 exhibited maximum protease activity (44.89 U/mL) after 48 h of incubation and isolate AKS-2 was producing minimum protease activity (2.37 U/mL) after 72 h of incubation. Isolate AKS-6 exhibited maximum protease activity (37.94 U/mL) after 3 days of incubation and isolate AKS-8 was producing minimum protease activity (1.22 U/mL) after 3 days of incubation. Hyper producing isolates (AKS-4 and AKS-6) from both soil samples were selected for optimization study. The optimum conditions for protease production by isolate AKS-4 were found to be at pH 8.5 after 5 days of incubation using glucose as carbon source and casein as nitrogen source. Maximum yield of enzyme was obtained by isolate AKS-6 at pH 10.5 after 5 days of incubation using starch as carbon source and casein as nitrogen source. Among all studied bacterial isolates, the maximum protease activity (52.29 U/mL) was recorded in isolate AKS-4 after optimization of culture conditions.

1. INTRODUCTION

Proteases are group of enzymes which catalyze hydrolysis of peptide bonds in proteins. They are also called as peptidases or proteinases or proteolytic enzymes. Based on their structure and properties of the catalytic site, proteases are classified into several types such as carboxyl-, metallo-, serine-, neutral-acidic-, and alkaline proteases. Proteases are obtained from various living organisms such as plants, animals, bacteria and archaea[i]. Proteases of microbial origin are preferred over enzymes of plant or animal origin because microbes require very little space for their growth and cultivation. Microbes grow very rapidly and they can be easily genetically altered to obtain the enzyme with desired modified properties to

fulfill various applications of biotechnology based industries[ii]. Several microbial strains including bacterial *(Bacillus subtilis, Bacillus licheniformis, Bacillus alcalo, Bacillus thuringiensis and Bacillus firmus)* and fungi *(Aspergillus miller, Aspergillus flavus, Aspergillus niger and Penicillium griseofulvin)* have identified as protease producers[iii]. Proteases perform a large variety of functions and have important applications in biotechnology based industries such as food, leather, detergent, pharmaceutical industries and bioremediation process[iv]. In general, microbial proteases are directly secreted into the production medium because they are extracellular in nature, thus simplifying purification of the enzyme as compared to proteases obtained from plants and animals[v]. Hence, the aim of present investigation was to isolate the protease producing bacteria from soil. Various physiochemical parameters influence the yield of extracellular enzymes. Hence, physiochemical parameters were optimized for the maximum productivity. This study presents effect of different cultural conditions on protease production from bacteria isolated from soil.

2. MATERIAL AND METHODS

2.1. Isolation and Screening of proteolytic bacteria

Soil samples 1 and 2 were collected from botanical garden and medicinal plant garden of Banasthli University, respectively. One gm of each soil sample was added to 9 ml of sterile distilled water and serially diluted up to 10^{-6} under aseptic environment of laminar air flow chamber. One mL of each dilution was spread on nutrient agar medium and plates were incubated at 37ºC for 2 days. Bacterial colonies appeared on agar plates were screened for evaluating their proteolytic potential by inoculating them in gelatin agar medium containing (g/L): peptone 5, gelatin 120, beef extract 3, agar 20, pH 7.0. Plates were placed at 37˚C for 2 days. Appearance of clear zone around bacterial colonies indicates hydrolysis of gelatin due to secretion of protease by the organism. For better visualization of zone of hydrolysis, gelatin agar plates were flooded 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[vi].

2.2. Inoculum preparation

Bacterial isolates from their respective slants were inoculated in 100 mL of Luria broth under aseptic condition. Flasks were placed in a shaker incubator at 37ºC, 120 rpm for 24 h. After 1 day of incubation, growth (turbidity) was appeared in inoculated broth and this preparation was directly used as a source of inoculum.

2.3. Production of protease in shake flask fermentation

From Luria broth culture, 1 mL of inoculum was transferred in 100 mL of production medium containing (% w/v): $CaCl_2$-0.01, K_2HPO_4,-0.05, yeast extract-0.02, peptone-1, $MgSO_4$-0.01, glucose-0.1, pH 7.0[vii]. Flasks were placed in a shaker incubator at 37ºC, 120 rpm for 24, 48 and 72 h.

2.4. Extraction of enzyme from bacterial fermentation broth

Two mL of fermentation broth was taken into a centrifuge tube at the end of 24 h, 48 h and 72 h of incubation and it was centrifuged at 8000 rpm, 4 ºC for 15 minutes. After centrifugation, bacterial cell pellet was removed and bacteria free supernatant was used as a

source of crude enzyme for protease assay and protein estimation.

2.5. Demonstration of enzyme activity

Protease activity in the crude enzyme extract was determined according to the method of Carrie CuppEnyard[viii] by using casein as substrate.

Two test tubes were taken and labeled as test (T) and blank (B). Five mL of 0.65% casein solution (prepared in 50 mM potassium phosphate buffer, pH 7.5) was added in both the test tubes. One mL of crude enzyme extract was added in T-test tube. Enzyme was not added in blank test tube. Both test tubes were placed at 37 ºC for 30 minutes for enzymatic reaction to occur. Five mL of Trichloroacetic acid solution (110 mM) was added in both test tubes to stop the enzymatic reaction.

One mL of crude enzyme extract was added to blank test tube to bring the total volume to 11 mL. Solution from both test tubes was filtered using Whatmann's No 1 filter paper. Two mL of each filtrate (test and blank) was taken in two new test tubes.

Five ml of sodium carbonate (500 mM) was added in both test tubes followed by addition of 1 ml of 2 fold diluted Follin Ciocalteus phenol reagent. The resulting solutions in both test tubes were placed in dark at room temperature for 30 minutes for the development of blue color. The unknown concentration of tyrosine liberated after enzymatic reaction in T-test tube was measured at 660 nm against a reagent blank using tyrosine standard. Standard curve of tyrosine was prepared by taking tyrosine in following range: 27.5 µM to 275 µM/mL. One protease unit was defined as the amount of enzyme that releases 1 µM of tyrosine per minute at 37ºC, pH 7.5[ix]. All the experiments were done in triplicates and mean values are presented.

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

$$\text{Enzyme activity (Units/mL)} = \frac{\text{µmole tyrosine equivalent releases x 11 (Total volume of assay)}}{\text{Volume of enzyme taken (1 mL) x Incubation time (30) x (2)}} \quad (1)$$

11= Total volume of assay (in milliliters).
1= Volume of enzyme used (in milliliters).
2= Volume of sample taken in cuvette for absorbance.

Specific activity is the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg). It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins.

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

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein content (mg/mL)}} \quad (2)$$

2.6. Protein assay

Protein concentration in crude enzyme extract was determined by Folin- Lowry method using Bovine Serum Albumin (BSA) as standard[x].

2.7. Optimization of culture conditions for maximum productivity

Hyper producing bacterial isolates (AKS-4 and AKS-6) were selected for optimization study.

2.7.1. Optimization of protease production using different carbon sources

The effect of different carbon sources such as starch, glucose, fructose and sucrose on protease production by bacterial isolates was investigated. These carbon sources were added

individually in the fermentation broth at a constant concentration (1%, w/v). The carbon source of original production medium was replaced by alternative carbon sources. One mL of 24 h old bacterial culture was transferred in 100 mL of production medium. Flasks were placed in a shaker incubator at 37ºC, 120 rpm for 24, 48 and 72 h. At the end of incubation protease activity and total protein content in cell free supernatant was determined.

2.7.2. Optimization of protease production using different nitrogen sources

The effect of different nitrogen sources such as casein, ammonium sulphate, yeast extract, beef extract and peptone on protease production by bacterial isolates was investigated. These nitrogen sources were added individually in the fermentation broth at a constant concentration (1%, w/v) using previously optimized carbon source. Protease activity and protein concentration in cell free supernatant was determined.

2.7.3. Optimization of protease production at different pH

Fermentation broth for each isolate was formulated using previously optimized carbon source and nitrogen source. To study the effect of various pH on productivity of protease, pH of production medium was adjusted in the range of 6, 8, 8.5, 9.5 and 10.5 using 1 N HCl and 1 N NaOH. Bacterial isolates were inoculated in fermentation broth with different pH. Inoculated flasks were placed in a shaker incubator at 37ºC, 120 rpm for 24 h. Protease activity and protein concentration in cell free supernatant was determined.

2.7.4. Optimization of protease production at different incubation period

Fermentation broth for each isolate was formulated using optimized carbon source, nitrogen source and pH. To investigate the effect of incubation period on productivity of protease, each isolate was inoculated in fermentation broth and flasks were incubated for 24 h, 48 h, 72 h, 96 h and 120 h at 37ºC, 120 rpm shaking speed. At the end of each incubation period protease activity and protein concentration in cell free supernatant was determined.

3. RESULT AND DISCUSSION

3.1. Isolation and screening of proteolytic bacteria

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.

3.2. Protease production by bacterial isolates

Protease production and specific activity by bacterial isolates of soil sample 1 and 2 are represented by Table 1 and 2, respectively. Table 1 showed that among the 4 isolates of sample 1, maximum protease activity (44.89 U/mL) was obtained from isolate AKS-4 after 2 days of incubation. Isolate AKS-4 showed maximum specific activity (74.19 U/mg).

Table 1 Protease production and specific activity by bacteria isolated from soil sample 1

Bacterial Isolates	Absorbance at 660 nm			Protease activity (µ/mL)			Total protein content (mg/mL)			Specific activity (µ/mg)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
AKS-1	0.163	0.677	0.909	4.97±0.7	20.64±1.92	27.72±1.32	1.42	1.12	2.39	3.6±0.46	19.09±1.95	15.51±2.18
AKS-2	0.237	0.903	0.078	7.22±0.83	27.54±0.88	2.37±1.65	0.835	0.84	1.25	8.69±0.5	32.39±0.56	1.88±1.29
AKS-3	1.126	1.314	1.24	34.34±0.43	40.07±0.4	37.82±1.22	0.63	0.59	0.9	54.45±1.81	67.76±1.76	41.76±0.01
AKS-4	1.216	1.472	1.33	37.08±0.25	44.89±2.73	40.59±0.34	0.6	0.6	0.69	61.5±0.55	74.19±1.81	58.3±2.10

Table 2 demonstrated that among the 4 isolates of sample 2 maximum protease yield (44.89 U/mL) was obtained from isolate AKS-6 after 3 days of incubation. From the above results, two hyper producing isolates such as AKS-4 and AKS-6 were selected for further optimization study.

Table 2 Protease production and specific activity by bacteria isolated from soil sample 2.

Bacterial Isolates	Absorbance at 660 nm			Protease activity (µ/mL)			Total protein content (mg/mL)			Specific activity (µ/mg)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
AKS-5	0.36	1.02	1.152	10.98±1.97	31.24±1.15	35.13±0.37	2.5	1.04	1.53	4.3±2.22	30.73±1.87	24.19±5.7
AKS-6	0.08	1.176	1.244	2.47±0.03	35.86±1.38	37.94±0.15	2.02	0.86	0.99	1.21±0.02	41.95±2.26	38.42±2.43
AKS-7	0.156	1.196	1.178	4.75±0.03	36.47±1.59	35.92±0.31	1.01	0.62	0.66	4.7±0.07	59.34±1.83	53.63±3.35
AKS-8	0.04	0.812	1.23	1.22±0.06	24.76±0.06	37.51±0.49	0.84	0.41	0.57	1.54±0.43	62.03±14.36	65.59±2.18

Alnahdi[xi] reported protease activity by 2 isolates of Bacillus sp. (No. 2 and No. 3) selected on the basis of primary screening in gelatin agar medium. Without optimization the highest protease activity (243 U/mL) was recorded by isolate No. 2.

3.3. Effect of carbon sources

Results are presented in Table 3. Table 3 showed that glucose was found to be the best carbon source that enhanced the protease production by AKS-4 isolate when compared to other carbon sources. Protease production was 59.10 U/mL with glucose as carbon source in fermentation broth. The effect of different carbon sources on protease production efficiency of isolate AKS-4 were found to be in the descending order: glucose (59.10 U/mL) > sucrose (54.42 U/mL) > fructose (51.83 U/mL) > starch (45.56 U/mL).

The highest protease activity (48.76 U/mL) was obtained by isolate AKS-6 when starch was used as a carbon source (Table 3). The protease production by isolate AKS-6 was affected by carbon sources in the descending order: starch (48.76 U/mL) > sucrose (47.50 U/mL) > glucose (47.45 U/mL) > fructose (34.96 U/mL).

Table 3 Optimization of carbon sources for protease production by bacterial isolates: AKS-4 and AKS-6

Carbon sources	Absorbance at 660 nm		Protease activity (U/mL)		Total protein content (mg/mL)		Specific activity (U/mg)	
	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6
Glucose	1.938	1.556	59.1±0.92	47.45±1.96	1.07	0.94	54.62±2.31	50.4±2.01
Starch	1.489	1.599	45.56±0.22	48.76±0.55	1.485	0.85	30.63±0.04	57.08±0.94
Fructose	1.699	1.146	51.83±1.36	34.96±0.05	0.64	0.68	79.79±0.19	51.27±0.79
Sucrose	1.784	1.55	54.42±0.54	47.5±2.58	0.63	0.59	85.68±1.88	79.28±2.82

Sevinc and Demirkan replaced glucose in basal medium by different sugars (sucrose, maltose, glycerol, fructose and starch) and reported that among all carbon sources used, fructose was found to be the best carbon source for highest protease production (125 U/mL) by *Bacillus* sp. N-40.

Boominadhan et al[xii] reported maximum protease from Bacillus sp. using glucose as carbon source. Another author reported sucrose as a best carbon source for extracellular protease production[xiii]. Sangeetha et al[xiv] reported glucose as best carbon source and casein, yeast extract as best nitrogen source for maximum protease activity by Bacillus pumilus SG 2. Shafee et al[xv] observed maximum protease production by Bacillus cereus strain 146 using glucose as carbon source.

3.4. Effect of nitrogen sources

Results are given in Table 4. Among the various nitrogen sources tested, casein was found to be the excellent nitrogen source for highest protease activity by both isolates. The effect of different nitrogen sources on protease production by isolate AKS-4 were found to be in the descending order: casein (49.77 U/mL) > peptone (39.31 U/mL) > yeast extract (28.54 U/mL) > beef extract (12.96 U/mL) > ammonium sulphate (0.51 U/mL). Isolate AKS-6 exhibited highest protease production (36.03 U/mL) with casein as nitrogen source. The protease production by isolate AKS-6 was affected by nitrogen sources in the descending order: casein (36.03 U/mL) > ammonium sulphate (17.59 U/mL) > beef extract (16.07 U/mL) > peptone (7.41 U/mL) > yeast extract (2.08 U/mL).

Table 4 Optimization of nitrogen sources for protease production by bacterial isolates: AKS-4 and AKS-6.

Nitrogen sources	Absorbance at 660 nm		Protease activity (U/mL)		Total protein content (mg/mL)		Specific activity (U/mg)	
	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6
$(NH_4)_2SO_4$	0.089	0.089	0.51±0.03	17.59±0.34	0.06	0.22	7.69±2.29	100.63±2.28
Casein	1.294	1.294	49.77±2.11	36.03±0.35	1.03	1.61	47.76±3.21	22.29±0.68
Yeast extract	0.083	0.083	28.54±3.11	2.08±0.42	0.145	0.06	191.37±2.88	35.6±2.21
Beef extract	1.324	1.324	12.96±0.15	16.07±0.27	0.185	0.82	68.01±1.47	19.41±0.3
Peptone	1.385	1.385	39.31±0.88	7.41±0.3	0.309	0.69	126.3±0.11	10.7±0.62

Puri et al[xvi]; Sangeetha et al reported that casein, peptone, yeast extract and skim milk enhance protease production by Bacillus sp. Naidu and Devi[xvii] reported beef extract as optimum nitrogen source for protease production by Bacillus sp. K-30.

Another author reported that maximum protease activity was obtained with NH4NO3 as nitrogen source[xviii].Safey and Abdul-Raouf[xix] reported (NH4)2SO4 as best nitrogen source for extracellular protease production. Jayasree et al[xx] reported maximum protease production with casein as nitrogen source in production medium.

3.5. Effect of pH

Table 5 revealed that highest protease activity was observed in production medium of pH 8.5 in case of isolate AKS-4 and pH 10.5 in case of isolate AKS-6. The enzyme activity by isolate AKS-4 in production medium at pH 6, 8, 8.5, 9.5, 10.5 were observed as 22.4 U/mL, 32.28 U/mL, 52.29 U/mL, 20.99 U/mL, 1.49 U/mL, respectively. Similarly, enzyme activity by isolate AKS-6 at pH 6, 8, 8.5, 9.5, 10.5 were observed as 23.02 U/mL, 20.14 U/mL, 25.74 U/mL, 26.1 U/mL, 28.65 U/mL, respectively. It is clear from Table 5 that alkaline medium was more suitable for growth and protease production by both isolates.

Table 5 Optimization of pH for protease production by bacterial isolates: AKS-4 and AKS-6

pH	**Absorbance at 660 nm**		**Protease activity (U/mL)**		**Total protein content (mg/mL)**		**Specific activity (U/mg)**	
	AKS- 4	**AKS -6**	**AKS- 4**	**AKS -6**	**AKS- 4**	**AKS -6**	**AKS- 4**	**AKS -6**
6	0.734	0.755	22.4±0.78	23.02±0.89	1.03	0.96	21.68±1.38	24.13±2.15
8	1.058	0.66	32.28±0.56	20.14±0.29	0.79	0.62	40.44±1.94	32.79±4.02
8.5	1.714	0.844	52.29±1.81	25.74±0.55	0.65	0.64	80.2±9. 6	39.87±0.38
9.5	0.688	0.856	20.99±0.17	26.1±0.19	0.69	0.55	30.35±0.71	47.68±5.84
10.5	0.049	0.939	1.49±0.03	28.65±0.47	0.49	0.59	3.02±0.14	48.38±0.69

Sinha et al[xxi] reported maximum protease production (124 U/mL) by Bacillus sp. SP5 at pH 9 after 24 h of incubation using yeast extract as nitrogen source and sucrose as carbon source. Johnvesly and Naik[xxii] reported that the maximum enzyme activity in the pH range 6.5 to 12.0 indicating its better use in industrial purpose where enzyme activity over wide pH ranges is required. Josephine et al reported maximum protease production (87 U/mL) by *Bacillus* SNR01 strain at pH 7.0. Nascimento and Martins[xxiii] reported maximum protease activity by *Bacillus* sp. at pH 8.0 to 9.0 of fermentation broth. Smita et al[xxiv] reported maximum production of protease (74.3 IU/mL) by bacterial isolates at pH of 9.

3.6. Effect of incubation period

Highest protease activity by both isolates was observed after 5 days of incubation at 37ºC (Table 6). It was 45.21 U/mL, 27.57 U/mL with isolate AKS-4 and AKS-6, respectively. The enzyme activity by isolate AKS-4 was gradually increased from 24 h to 120 h incubation and the activity were 6.2 U/mL, 13.48 U/mL, 39.08 U/mL, 42.91 U/mL, 45.21 U/mL, respectively. It is clear from Table 6 that protease activity by isolate AKS-6 was gradually increased from 24 h to 120 h incubation and the activity were 0.22 U/mL, 15.31 U/mL, 18.66 U/mL, 27.12

U/mL, 27.57 U/mL, respectively.

Table 6 Optimization of incubation period for protease production by bacterial isolates: AKS-4 and AKS-6

Incubation time	Absorbance at 660 nm		Protease activity (U/mL)		Total protein content (mg/mL)		Specific activity (U/mg)	
	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6	AKS- 4	AKS -6
Day1	0.203	0.007	6.2±0.11	0.22±0.02	2.38	0.09	2.6±0.04	7.31±0.19
Day2	0.442	0.502	13.48±1.28	15.31±0.48	0.90	0.77	15.06±2.51	19.84±0.15
Day3	1.281	0.612	39.08±2.06	18.66±1.68	0.96	0.61	39.45±3.18	31.03±6.46
Day4	1.407	0.889	42.91±0.49	27.12±1.12	0.92	0.43	46.4±1.67	64.56±3.27
Day5	1.482	0.904	45.21±0.69	27.57±0.12	0.8	0.40	56.44±0.15	67.34±2.41

Lakshmi et al[xxv] reported highest protease production (174.30 U/mL) by *B. cereus* strain S8 after 72 h of incubation.

4. CONCLUSION

The first goal of this study was to optimize the protease production from bacteria isolated from local soil sample. The fermentation conditions (carbon source, nitrogen source, pH and incubation time) were optimized in order to enhance protease production and activity by 2 bacterial isolates (AKS-4, AKS-6). The results of present study showed that protease production by isolate AKS-4 was increased under optimized conditions with a medium containing glucose as carbon source and casein as nitrogen source at pH 8.5 of fermentation broth. Among all evaluated isolates, highest protease activity (52.29 U/mL) was found in isolate AKS-4. These bacterial isolates with considerable protease activity can be further identified and characterized for their use in various industrial purposes. The optimum pH for highest protease production by both isolates was found in alkaline range (8.5, 10.5) therefore these isolates can be alternative source of proteases in biotechnology based industries where protease activity over wide alkaline range of pH is needed.

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