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Screening of thermophilic bacillus cereus strains and optimization of growth conditions for mass production of alkaline protease enzyme

R Vidhya Pallavi, T Kuberan and S Arumugam, PW Jebamalar

Post Graduate and Research Dept of Microbiology, Sengunthar Arts and Science College, Trichengode - 637 205
Post Graduate Dept of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi - 626 124

ABSTRACT

Several strains of Bacillus cereus were isolated from five soil samples collected from leather tanneries in Salem, India. The strains positive on skim milk agar (1%) were selected as protease producing strains and biochemically characterized. The strains were found capable of growth at temperature >40ºC and in wide pH range of 7.0-12.0.The enzyme assay of strains revealed maximum activity at 50ºC and pH 10. The enzyme production was carried out at 37ºC for 48 hr in fermenters containing 1 L medium having pH 8.0. The molecular weight of enzyme determined through SDS-PAGE, was 6000 kDa. The study revealed that Bacillus cereus strain, which is a good producer of protease and this, may prove beneficial to the industries.

Keywords: Bacillus cereus, protease enzyme, screening, tannery,

Corresponding author

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INTRODUCTION

Proteases are the class of enzymes which occupy a pivotal position due to their wide-spread application in detergent, pharmaceutical, photography, leather, laundry, food and agricultural industries. These enzymes are also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, bioremediation process and as treatment against inflammation and virulent wounds and in unhairing of sheepskins [1, 6]. Among the various proteases, bacterial proteases are most significant compared to animal and fungal proteases. Amongst bacteria, Bacillus species are specialized producers of extracellular proteases. The global requirements of thermo-stable biocatalysts are far greater than those of derived from mesophiles which proteases contribute two thirds [2]. Thermo-stable proteases are advantageous in several applications because higher processing temperatures results in faster reaction rates, increase in solubility of non-gaseous reactants, products and reduces the incidence of microbial contamination by mesophiles. Thermophilic bacteria from hot springs produce unique thermo-stable enzymes [7]. Bacillus cereus is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of amylase and protease enzymes. The present study was aimed to isolate and characterize these thermophilic bacterial strains which may be potent producer of extra-cellular alkaline protease as well as to optimize the culture conditions required for optimum enzyme production.

MATERIALS AND METHODS

Isolation and characterization of bacteria:

The five samples were collected from five leather tanneries in and around the Salem, and after proper packing and labeling were brought to the laboratory. The soil samples were serially diluted (10^{-2} to 10^{-9}) and spread onto nutrient agar media. The Petri plates were incubated at 35°C for 48 hr. The bacteria were sub-cultured on skim milk agar (1%) and the isolates, which produced clear zone on skim milk agar after 24 hrs incubation were maintained on nutrient agar plates. The selected bacteria were identified on the basis of their morphological, cultural and biochemical characteristic [3].

Assay for protease production:

For protease enzyme production, the selected strains were grown on glucose casein yeast extract medium (GCYE) comprising of 1% glucose (w/v), 0.5% casein, 0.55 % yeast extract, 0.2% KH_2PO_4, 1% Na_2CO_3 and 0.2% $MgSO_4.7H_2O$ with pH of the medium adjusted to 8.0.

Effect of pH:

To assess the effect of pH on alkaline protease production, the strains were inoculated to GCYE medium having variable pH values ranging from 7 to 12 in flasks. The protease

production was assayed after 24 hrs incubation at 35°C and the reading was noted spectrophotometrically at 660 nm [4].

Effect of temperature:

To assess the effect of temperature on protease production, the strains were grown on GCYE medium at various temperatures like 40°C, 50°C and 55°C and protease assay done after 24 hr incubation.

Enzyme activity in UV mutated culture:

The culture broth was diluted from 10^{-2} -10^{-8} dilution. The diluted sample (0.1 ml) was placed on skimmed milk agar (1%) and exposed to ultra violet radiations for 2 minutes and incubated at 35°C for 24 hrs and results were noted.

Protection of enzyme in agitation mode:

For protection of enzyme in agitation mode GCYE broth (100 ml) was inoculated and kept 200 rpm on shaker at 35°C for 24 hrs and enzyme activity was assayed and result were recorded.

Mass production of alkaline protease enzyme:

The medium used for mass production of protease enzyme, in 1L capacity fermentor comprised 1% dextrose (w/v), 0.5% peptone, 0.2% KH_2PO_4, 0.2% $MgSO_4.7H_2O$ and 1% casein with pH adjusted to 8.0. The medium was inoculated with Bacillus cereus (1 ml of culture) and fermentation carried out at 35°C for 48 hr on a shaking incubator revolving at 200 rpm. At the end of each fermentation period, the whole culture broth was centrifuged at 10,000 rpm for 15 minute to remove the cellular debris and the clear supernatant was used as enzyme preparation [5].

Assay of protease activity:

The assay system consisted of following ingredients: 1.25 ml Tris buffer (pH 7.2), 0.5 ml 1% aqueous casein solution and 0.25 ml culture supernatant. Approximate controls (presence of assay ingredients but absence of culture supernatant) were also maintained. The mixture (1.25 ml Tris buffer (pH 7.2), 0.5 ml 1% aqueous casein solution and 0.25 ml of culture supernatant) was incubated for 30 min at 30^0C. Then 3 ml 5%TCA was added to this mixture and kept at 4^0C for 10 min. to form precipitate. It was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken to which 2.5 ml 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. Then 0.5 ml Folin's phenol reagent was added to it and absorbance measured spectrophotometerically at 660 nm. The amount of protease produced was expressed in terms of µg tyrosine released by 1 ml enzyme in 30 min. at 30^0C on tyrosine

equivalent [4].Purification Alkaline protease secreted by the organism was fractionated by solid ammonium sulphate.

Purification of protease:

Ammonium sulphate fraction:

After 48 hrs the bacterial culture was centrifuged at 10,000 rpm for 10 min. Solid ammonium sulphate was added slowly to the crude extract with constant stirring till the percentages 27,36,42,44 and 56% (g%) of the enzyme were obtained. The samples were stored at 4^0C for 4 hrs. The pellet recovered by centrifugation at 10,000 rpm for 10 min was resuspended in cold double distilled water (three changes, final change against buffer (Tris buffer, pH 8.0,50 mM). It was stored at 2 to 8 ^{0}C for further studies.

Purification of protease by gel filtration chromatography method:

5 g of sephadex G-75 (Amersham Biosciences, Sweden) was weighed and transferred into 100 ml of 50mM Tris buffer (pH 8.0) to swell the matrix. It was kept for 24 hrs at room temperature. Tris buffer (50mM at pH 8.0) was poured to the half of the glass column (0.4*x50 cm). The swelled slurry was applied carefully through the edge of the column and aloe to settle at its own gravitational force.

SDS PAGE:

The molecular weight of purified thermophilic alkaline protease enzyme was determined by using SDS-PAGE method [8].

RESULTS AND DISCUSSION

Fifteen isolate of Bacillus cereus were obtained from the soil of leather tanneries. These were screened for protease producing ability on skim milk agar. The bacterial isolates which formed zone around the colonies were considered to be protease positive strain. The clear zone formation may be attributed to the hydrolysis of casein. Three B. cereus isolates (namely HS1, HS2 and HS3) showing higher enzyme activities were taken for further studies and characterized on morphological-biochemical basis (Table 1). These isolates were assayed for their growth conditions at different pH and temperature on production media. These isolates inoculated into GCYE broth were centrifuged after sufficient incubation time. The crude enzyme preparations were processed. The optimum pH and temperature for production of thermophilic alkaline protease was pH 10 and 50^{o}C, respectively. The pH of medium strongly affected enzymatic process and transport of compounds across the membrane. Majority of the thermophilic Bacilli are grown at pH 7 to 12 and 50-55^{o}C. The mechanism of temperature control in enzyme action is not well understood. A link exists between enzyme synthesis energy and metabolism in Bacillus species which is controlled by temperature and O_2. By growing these

cultures at optimum pH and temperature the activity of protease was estimated at different intervals of growth. The maximum protease production was recorded after 96 hrs of incubation at 50^{o}C and pH 10 (Table 2A & 2B). The maximum protease production was recorded in mutant strains with aerated at 50^{o}C (Table 3) compare other wild strain with aerated and static condition. Bacillus species usually produces extracellular protease during late exponential phase. The specific activity of the enzyme after UV mutation was also performed at different variety of B. cereus showed maximum ADU when compared to wild one (table 4) . The appreciable high enzyme activity and stability at high temperature and pH suggest that the three isolated noted as HS1, HS2, HS3 can be a potential producer of alkaline proteases. They can be used for the degradation of protenaceous waste into useful biomass. Based on the result SDS-PAGE, the appreciate molecular weight of the protease extracted from the isolates were in the order of 6000 kDa (Fig.1). In conclusion the alkaline protease produced from Bacillus cereus is a alkaline protease having high thermo stability. The alkaline protease produced during exponential phase may play an important role in food industry for making cheese. Further studies are in progress in the application of alkaline protease in commercial field.

Table.1. Morphological and Biochemical characteristics of the Bacillus cereus

Morphological & Biochemical tests	Bacillus cereus HS1	Bacillus cereus HS2	Bacillus cereus HS3
Morphological identification	Large, mucoid, orange colour colonies	Small, white, dried, rinkler colonies	Large, dried, white, raised, rinkled colony
Gram's staining	Gram Positive	Gram Positive	Gram Positive
Endospore staining	Sub-terminal spores	Sub-terminal spores	Sub-terminal spores
Motility	+	+	+
Carbohydrate fermentation test			
a. D-glucose	+	+	+
b. Mannitol	-	-	-
c. Lactose	-	-	-
d. Sucrose	+	+	+
Indole Production	-	-	-
Methyl red test	-	-	-
Voges – Proskauer test	+	+	+
Citrate utilization test	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Urease test	-	-	-
Oxidase	-	-	-
Nitrate utilization test	+	+	+

+ : Positive Results : - Negative Results

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Table 2: Protease activity of wild and mutant strains of Bacillus cereus under well-aerated and agitated growth conditions at three variable temperatures (OD VALUES)

Strains	40°C				50°C				55°C			
	Wild		Mutant		Wild		Mutant		Wild		Mutant	
	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated
HS1	0.604	0.482	0.480	0.474	0.472	0.470	0.469	0.468	0.721	0.720	0.719	0.716
HS2	0.721	0.603	0.560	0.559	0.554	0.550	0.548	0.544	0.796	0.794	0.792	0.790
HS3	0.824	0.820	0.810	0.557	0.556	0.549	0.546	0.542	0.770	0.769	0.765	0.764

(0.4OD = 1000ADU/g)

Table 2b: ADU value of Bacillus cereus strains at variable pH levels (temperature 50°C)

Strains	pH levels									
	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	12.0
HS1	6578.94	6993.00	7042.25	7299.27	8333.33	9803.92	17857.14	16393.44	12345.67	12195.12
HS2	6711.40	6756.75	6993.00	7194.24	8000	1000.00	21739.13	15151.51	11627.90	11494.25
HS3	6451.61	6451.61	7142.85	77633.58	8771.92	10309.27	19607.84	14084.50	10869.56	10638.29

(0.4OD = 1000ADU/g)

Table 3: Thermophilic protease activity of Bacillus cereus strains at variable pH levels (temperature 50°C)

Strains	pH levels (OD VALUES)									
	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	12.0
HS1	0.152	0.143	0.142	0.137	0.120	0.102	0.102	0.061	0.081	0.082
HS2	0.149	0.148	0.143	0.139	0.125	0.100	0.100	0.066	0.086	0.087
HS3	0.155	0.155	0.140	0.131	0.114	0.097	0.097	0.071	0.092	0.094

(0.4OD = 1000ADU/g)

Table 4: ADU value of wild and mutant strains of Bacillus cereus under well-aerated and agitated growth conditions at three variable temperatures

Strains	40°C				50°C				55°C			
	Wild		Mutant		Wild		Mutant		Wild		Mutant	
	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated	Static	Aerated
HS1	1655.62	2074.68	2083.33	2109.70	2118.64	2127.65	2132.19	2136.75	1386.96	1388.88	1390.82	1396.64
HS2	1386.96	1658.37	1785.71	1788.90	1805.05	1818.18	1824.81	1838.23	1256.28	1259.44	1262.62	1265.82
HS3	1213.59	1219.51	1234.56	1795.33	1798.56	1821.49	1831.50	1845.01	1298.70	1300.39	1307.18	1308.90

(0.4OD = 1000ADU/g)

Purification of alkaline protease by sephadex G-75 gel filtration chromatography:

The fractionated protease was purified by gel filtration chromatography. The column was packed and equilibrated with Tris buffer (pH 8.0,50mM) and the protein was eluted with same buffer. Different fractions were observed in fraction 8,9,13,14,15,16. The maximum activity was observed in fraction 8.

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Purification of alkaline protease by DEAE chromatography:

The fractions obtained in gel filtration was pooled and concentrated. This sample was loaded into DEAE cellulose chromatography. The column was packed and equilibrated with Tris buffer (pH 8.0,25mM) the protein was eluted with buffer containing 0-1.0 M of NaCl. One major peak was obtained fraction no.8 which showed the highest enzyme activity. This sample was loaded to check the purity of enzyme.

Analysis of alkaline protease by SDS-PAGE:

SDS-PAGE analysis revealed that the molecular weight of the bacillus cereus alkaline protease was 6000 kDa (fig.1).

CONCLUSION

In alkaline protease produced from Bacillus cereus is a alkaline protease having high thermo stability. The alkaline protease produced during exponential phase may play an important role in food industry for making cheese. Further studies are in progress in the application of alkaline protease in commercial field.

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