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Research Article

ISOLATION AND SCREENING OF ALKALINE PROTEASE PRODUCING BACTERIA FROM FERMENTED FOODS

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Catalase, oxidase and citrate tests were positive.

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Isolated strain AP13 was good source of alkaline protease it was found to be It was found to be gram positive. Catalase, oxidase and citrate tests were positive. Test such as methyl red, indole and VP was found to be negative. It showed clear zone on casein hydrolyzed media. The strain AP13 produced amount of proteolytic enzyme of 498.345 units/mg after the incubation of 24hrs at 37°C. As the time of incubation increased the proteolytic activity got decreased

INTRODUCTION

Microorganisms are known to be highly versatile in producing a wide range of enzymes, with varied patterns of activity. Due to the physiological and biochemical properties of microorganisms, they are considered as the most common source of commercial enzymes. Several microbial strains including fungi (*Aspergillusflavus, Aspergillus miller, Aspergillusniger and Penicilliumgriseofulvin*) and Bacterial (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalo, Bacillus subtilis and Bacillus thuringiensis*) have reported to produce proteases [1]. Production of these biocatalyst using agro-biotech substrate under solid state fermentation and conditions provide several advantages in producing, cost effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization. Bacterial species are either isolated from effluents of industries or been bought from institutes like MTCC[2].

Protease production conditions are optimized and highest protease producing environments with optimized nutrient sources, pH and temperature were determined to get maximum protease from each bacterium. In the present study, alkaline producing strains were isolated from various sources and identified. Further the proteases were extracted and tested for various commercial applications.

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Proteases constitute one of the most important groups of industrial enzymes, accounting for at least a quarter of the total global enzyme production sales [3].

Proteases are by far the most important group of enzymes produced commercially and are used in the detergent, protein, brewing, meat, photographic, leather and dairy industries [4]. Fibrous proteins, such as horn, feather, nails and hair, are abundantly available in nature as wastes, but these can be converted to useful biomass, protein concentrate or amino acids using proteases derived from certain micro- organisms. Other successful commercial applicationsof alkaline proteases include the possibility of using them to catalyse peptide synthesis and to resolve racemic mixtures of amino acids [5].

The chemical biocatalysts used in industrial processes could be replaced by enzymes to increase efficiency and ensuring ecological and economic sustainability of theprocess. Proteases arethe mostdominant group of enzymes constituting sixty percent of the entire enzyme industry [6] and hydrolyse peptide bond between protein with paramount application in industrial as well as pharmaceutical sector[7]. They have wide applications across various industries such as detergent, food, bakery, leather, infant formulas etc. due to their attractive features like ease in production, thermo tolerance and ability to perform at varied pH range [8].

A large number of microorganisms have been reported to produce alkaline proteases under various physiochemical and

nutritional conditions e.g. Bacillus, Micrococcus, Pseudomonas and Streptomyces etc in last twenty years [9].

Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases perform best at pH range of 2.0-5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin. While proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases. Alkaline proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. [10]. The genus "Bacillus" is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production [11]. They are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. Isolation of alkaline protease producing Bacillus spp. has been reported from a variety of sources including soils characterized by high pH and/or the presence of detergent contamination [12], dried fish, sand soil, milk processing plant, slaughterhouses. Screening of alkaline proteases producing bacillus spp. from different ecological environments can result in isolation of new alkaline proteases[13].

Review of Literature

Isolation of alkaline protease producing Bacteria

Soil samples were collected from different habitats including, tanneries, soap factory, garden soil, soil composite and waste dumping areas. One gram of soil sample was added to a glass tube containing 10 mL sterilized distilled water. The test tube containing the soil suspension was placed in a water bath at 80°C for 15 min. After 15 min they were immediately cooled in ice-cold water. Samples of 50 to 100 µL were spread on the S.K agar plate (Bactotryptone 1%, NaCl 0.5%, BactoYeast Extract 0.5% and Skim milk[2].

Screening of protease producing microbes and characterization

The samples were streaked on skimmed milk agar medium (5% milk powder and 3% agar) and incubated at 37°C for 72hrs, Proteolytic activity i.e. the hydrolysis of casein, is seen by a zone of clearance around the colony. Only colonies showed the zone formation are chosen for further experiments because zone indicates the presence of proteolytic enzyme. The isolates were subjected to morphological and biochemical studies. Standard Biochemical tests included Indole, Methyl red, VogesProskauer and Citrate Test[1].

Gram staining of Bacterial Isolates

The Gram stain technique is a differential staining technique since it reacts differently with different types of bacteria based on their cell wall structure. Bacteria with a thick, highly cross-linked layer of peptidoglycan (20 to 80 nm) trap the primary stain-mordant complex and stain purple, and are designated Gram positive. Those bacteria that have a thin layer of peptidoglycan (1 to 3 nm) with a lower percentage of cross-linkage, followed by a thin second layer called the outer membrane (7 to 8 nm), do not retain the primary stain-mordant complex upon alcohol treatment. These bacteria with a thin cell wall are counterstained with safranin and are labelled as the Gram-negative bacteria. Both Gram-positive and Gram-negative staining of bacteria reveal the overall cell morphology sothat the cells can also be further labelled as rods or cocci. This method is useful for taxonomic purposes, preliminary diagnostic examination of patient specimens, and assessment of culture purity[15].

The Bradford Method for Protein Quantifiation

The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. Detailed studies indicate that the free dye can exist in four different ionic forms for which the pKa values are 1.15, 1.82, and 12.4 . Of the three charged forms of the dye that predominate in the acidic assay reagent solution, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively. In contrast, the more anionic blue form of the dye, which binds to protein, has an absorbance maximum at 590 nm. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm[16].

Inoculum preparation for enzymatic assay

A loopful culture of bacterial isolates was inoculated into 10ml of Luria Bertani Broth medium till a culture density (OD600) of 0.8 was obtained. These 24 hrs old culture suspensions were inoculated in 200ml of Production medium (Glucose-0.20gm, Peptone-0.30gm, MgSO4-0.5gm, KH2PO4-0.2gm, FeSO4-0.004gm, Distilled water-200ml) The flasks were incubated at 37°C for 72 hrs. It was further centrifuged at 10,000 rpm for 10 minutes. The supernatant (crude enzyme) was used for further protease assay[17].

Protease activity of the isolates

The protease enzyme activity of the bacterial isolates was determined with the moles of tyrosine released. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions. Enzyme units were measured using slope obtained from tyrosine as standard[18].

Objectives

1. Isolation of alkaline protease bacteria from fermented foods.
2. Screening of bacteria for alkaline protease activity.
3. Identification of bacterial isolates by microscopic and macroscopic method including biochemical test.
4. Quantification of alkaline protease activity produced by the screened bacterial isolates.

MATERIALS AND METHODS

Sampling

Fermented food samples such as milk, curd, pickle,batter and butter milk were collected from super market, Bangalore.

Isolation and purification

Isolation of bacteria from soil carried out by serial dilution method and isolated bacterial colonies were
Purified by sub culturing and stored as slants at 4±20C.

Characterization of bacterial culture

Various biochemical tests were performed for the identification and characterization of isolated bacteria viz-

Gram staining, Catalase test, oxidase test, methyl-red test, vogesproskauer test, indole test, citrate test

Enzymatic study

Qualitative Screening of Bacteria (for Protease)

The bacteria were streaked on casein hydrolysed medium and plates were incubated at 35±20C for 24 hours. A clear zone around the growth indicates proteolytic activity of the strains and visible difference in the extent of zone of clearing was recorded for proteolytic activity

Preparation of Standard Graph of L-tyrosine

L-tyrosine standard solution of concentration 1mg/ml was prepared. Aliquots of the standard ranging from 100µg/ml to 1000 µg/ml were prepared. To the tubes 10 ml of casein was added and the mixture was incubated for 15 minutes at 37ºC. To this 10 mlof TCA solution was added. The mixture was incubated for 30 minutes at 37ºC and then filtered with 42Whattman filter paper. The absorbance of the filterate was measured at 275nm and the graph of OD versus concentration was plotted to determine the curve

Quantitative Determination (Proteolytic Activity)

Enzyme assay was determined by the modified method of Sharma *et al*., 2015 as followed by the optimized media of components peptone 0.5%, dextrose 1%, KH2PO4 0.1%, K2HPO4 0.1%, MgSO4 0.1%, casein 0.5%, yeast extract 0.5%pH was adjusted to 8.

Protease Assay

The method followed for analyzing the enzyme activity was that of FCC (food chemical codex) edition V, with slight modifications. 24 hours fresh culture was inoculated into the production media and incubated at 37ºC. Sample at regular intervals of 12 hours, 24 hours and 36 hours was collected, centrifuged at 5000rpm for 15 minutes and the supernatant was used for the assay.1:10 dilution of samples were prepared. The reaction mixture consisting of 10ml of casein substrate and 2 ml of cell free supernatant was vortexes and incubated for 30 minutes at 37ºC. To this 10 ml of TCA solution was added. Individual blank solutions were maintained for each enzyme sample , wherein the enzyme solution was added after stopping the reaction with TCA solution to the blank tubes. The mixture was incubated at 37ºC for 30 minutes and filtered with 42Whatmann filter paper . The absorbance of the filtrate was measured at 275nm . The protease activity was determined considering L-tyrosine (1mg/ml) as standard using the equation .

Determination of Specific Activity of the Bacterial Isolates

The protein content of the supernatant was determined by bradford assay using bsa(0.1mg/ml) as standard.1 ml of the diluted (1:5) supernatant was dispensed into a test tube. To this 5ml of bradford reagent was added. The mixture was incubated in dark condition for 5 minutes. 1ml of phosphate buffer of ph 7.0 was considered for the blank. The absorbance of the mixture was read at 595nm. The protein content of the sample was calculated using the equation.

$$Concentration\ of\ the\ protein\ \left(\frac{\mathrm{mg}}{\mathrm{ml}}\right) = \frac{(\mathrm{T}-\mathrm{c})*\mathrm{d.f}}{\mathrm{slope}}$$

Preparation of standard graph of BSA*:* BSA standard of 0.1mg/ml was prepared using ph buffer. Aliquots ranging from 20 µg/ml to 100 µg/ml were prepared and to each 5ml of bradford reagent was added. The mixture was vortexed and incubated for 5 minutes at dark condition. The absorbance of the mixture was read at 595nm. A graph of od versus concentration of protein was plotted to determine the slope.

The specific activity of the enzyme was then calculated using the equation.

$$Specific\ activity\left(\frac{uints}{mg}\right) = \frac{protease\ activity}{protein\ concentration}$$

RESULTS

Isolation and Screening for Protease Producing Bacteria

The Protease secreting bacterial isolates were initially screened using SMA plates. A total of 40 bacterial cultures were isolated from 11 samples. Based on the clear zone exhibited on the plates, 16 of them were considered for the further experiments In order to confirm the caseinolytic activity of the bacteria, a single streak of each bacterial isolates was made on SMA plates and the zone of clearance was observed.

Bacterial isolates with its casenolytic activity

Sl	Sample	Culture code	Zone of clearance
1.	Pickle	AP01	No zone of clearance
2.	Pickle	AP04(1W)	Zone of clearance was observed
3.	Pickle	AP04(1G)	Zone of clearance was observed
4.	Pickle	AP04(2)	Zone of clearance was observed
5.	Pickle	AP05	No zone of clearance
6.	Curd	AP07	Zone of clearance was observed
7.	Milk	AP13	Zone of clearance was observed
8.	Milk	AP15	Zone of clearance was observed
9.	Milk	AP17	No zone of clearance
10.	Butter Milk	AP19	No zone of clearance
11.	Butter Milk	AP22	Zone of clearance was observed
12.	Brown chick peas Batter	BFL21	Zone of clearance was observed
13.	Green gram batter	BFL22	Zone of clearance was observed
14.	Butter Milk	BFL24	Zone of clearance was observed
15.	Butter Milk	BFL25	Zone of clearance was observed
16.	Butter Milk	BFL27	Zone of clearance was observed
17.	Cottage cheese	BFL32	Zone of clearance was observed
18.	Butter Milk	BFL33	Zone of clearance was observed
19.	Topioca sago batter	BFL35	Zone of clearance was observed
20.	Toddy	BFL36	No zone of clearance
21.	Jeevani	BFL40	Zone of clearance was observed

Identification of Bacteria

Staining was performed to study the cell morphology of bacterial isolates. The stained smear was observed under 100X objective of a light microscope. Gram staining was done to determine the nature of bacterial cell wall. All the cultures were found to be Gram positive as recorded in table

Morphology Characteristics of Bacterial Isolates

Sl	Culture code	Morphology	Cell arangement	Gram reaction
1	AP04(1W)	Short rod	Chain	Positive

2.	AP04(1G)	Short rod	Chain	Positive
3	AP04(2)	Long rod	Chain	Positive
4	AP07	Short rod	Single	Positive
5	AP13	Short rod	Single	Positive
6	AP15	Long rod	Chain	Positive
7	AP22	Short rod	Single	Positive
8	BFL21	Short rod	Single	Positive
9	BFL22	Short rod	Single	Positive
10	BFL24	Short rod	Single	Positive
11	BFL25	Short rod	Single	Positive
12	BFL27	Short rod	Single	Positive
13	BFL32	Short rod	Single	Positive
14	BFL33	Short rod	Single	Positive
15	BFL35	Long rod	Single	Positive
16	BFL40	Short rod	Single	Positive

Biochemical test such as Catalase, Oxidase, Methyl red, Vogesproskauer, Indole and Citrate were performed to charecterise the bacterial isolates and results are tabulated in table 6.

Biochemical test for Charecterisation of Bacterial Isolates

Culture code	Catalase	Oxidase	Methyl red	Voges proskauer	Indole	Citrate
AP04(1W)	+	+	+	+	-	-
AP04(1G)	+	+	+	-	-	-
AP04(2)	+	+	+	+	-	+
AP07	+	+	-	+	-	+
AP13	+	+	-	-	-	+
AP15	+	+	+	+	-	+
AP22	+	+	-	-	-	+
BFL21	+	+	+	+	-	+
BFL22	+	+	-	-	-	+
BFL24	+	+	-	+	-	-
BFL25	+	+	+	+	-	-
BFL27	+	+	-	+	-	-
BFL32	+	+	-	-	-	+
BFL33	+	+	-	+	-	-
BFL35	+	+	-	-	-	+
BFL40	+	+	-	+	-	-

Enzyme Activit Assay

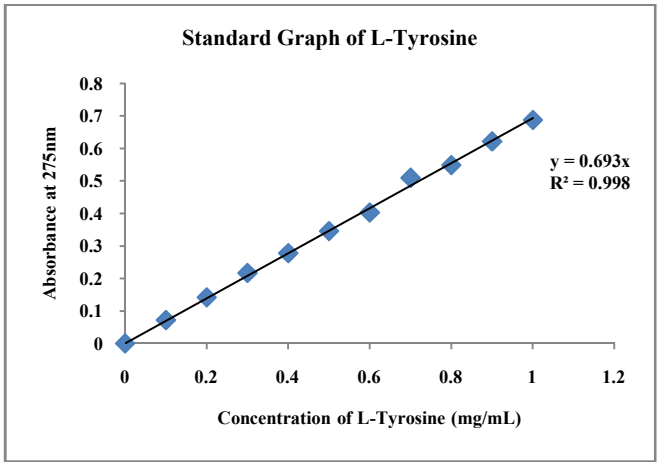
The bacterial isolates were incubated in production media at 37°C andsanple was collected at time interval of 12, 24 and 36 hours in order to check for the activity of protease.

Standard Graph of L-Tyrosine

A standard graph for the enzyme assay was generated using L-Tyrosinr (1mg/ml) as the standard and processing it under the assay conditions. The slope of the graph was found to be 0.0105.

Absorbance of L-Tyrosine at 275nm

Standard	Conc. Of L-Tyrosine (mg/mL)	A_{275}
1	0	0
2	0.1	0.028
3	0.2	0.128
4	0.3	0.228
5	0.4	0.328
6	0.5	0.428
7	0.6	0.528
8	0.7	0.628
9	0.8	0.728
10	0.9	0.828
11	1	0.928



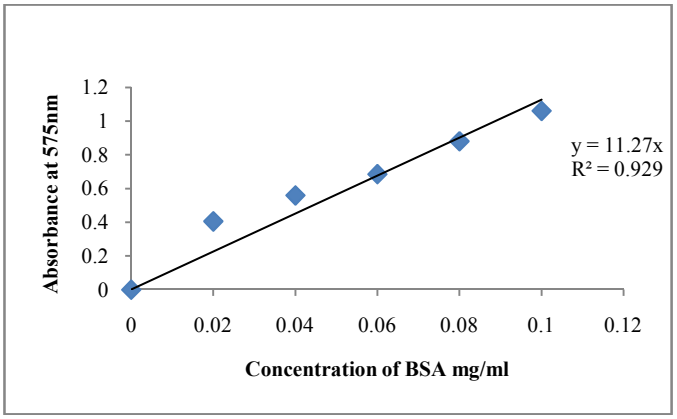
Graph 1 Standard graph of L-Tyrosine

Standard Graph of Bsa

A standard graph of BSA (0.1mg/ml) was generated for Bradford assay and the slope of the graph was found to be 0.011.

Absorbance of BSA at 595nm.

Standard	Concentration of BSA(mg/ml)	OD at 595nm
1	0	0
2	0.02	0.405
3	0.04	0.559
4	0.06	0.685
5	0.08	0.88
6	0.1	1.06



Graph 2 Standard graph of BSA.

Enzyme Assay and Bradford Assay

The protease enzyme activity was analysed by using casein as substrate. The activity of the isolates were recored as in table 9, table 10 and table 11.

Table 9 The enzyme activity of the bacterial isolates at 12 hours.

Sample Code	Protease Activity (units/ml)	Protein Concentration (mg/ml)	Specfic Activity (units/mg)
AP04(1W)	56	0.0782	716.2791
AP04(1G)	-85	0.0777	-1093.5673
AP04(2)	1	0.0986	10.1382
AP07	-19	0.1073	-177.1186
AP13	-12	0.0918	-130.6931
AP15	-18	0.0800	-225.0000
AP22	-22	0.0986	-223.0415
BFL21	-36	0.1191	-302.2901
BFL22	-51	0.1564	-326.1628
BFL24	33	0.1255	263.0435
BFL25	-51	0.1418	-359.6154
BFL27	-26	0.1168	-222.5681
BFL32	-24	0.0986	-243.3180
BFL33	-2	0.0491	-40.7407
BFL35	-5	0.1286	-38.8693

BFL40	-24	0.1705	-140.8000

Table 10 The enzyme activity of the bacterial isolates at 24 hours

Sample Code	Protease Activity (units/ml)	Protein Concentration (mg/ml)	Specfic Activity (units/mg)
AP04(1W)	0	0.07227	0.0000
AP04(1G)	14	0.07864	178.0347
AP04(2)	25	0.0909	275.0000
AP07	22	0.13045	168.6411
AP13	41	0.0823	498.3425
AP15	10	0.06545	152.7778
AP22	11	0.04455	246.9388
BFL21	7	0.12136	57.6779
BFL22	13	0.10591	122.7468
BFL24	115	0.12909	890.8451
BFL25	21	0.14500	144.8276
BFL27	12	0.10955	109.5436
BFL32	21	0.1064	197.4359
BFL33	80	0.1073	745.7627
BFL35	69	0.09000	766.6667
BFL40	47	0.17318	271.3911

able 11 The enzyme activity of the bacterial isolates at 36 hours

Sample Code	Protease Activity (units/ml)	Protein Concentration (mg/ml)	Specfic Activity (units/mg)
AP04(1W)	14	0.0814	172.0670
AP04(1G)	3	0.1159	25.8824
AP04(2)	-19	0.1086	-174.8954
AP07	43	0.1209	355.6391
AP13	17	0.1577	107.7810
AP15	10	0.0809	123.5955
AP22	16	0.0773	207.0588
BFL21	15	0.1409	106.4516
BFL22	-1	0.2305	-4.3393
BFL24	92	0.1345	683.7838
BFL25	6	0.1491	40.2439
BFL27	10	0.1464	68.3230
BFL32	56	0.1377	406.6007
BFL33	41	0.1882	217.8744
BFL35	22	0.0882	249.4845
BFL40	88	0.2514	350.0904

The specific activity for the bacterial isolates were calculate at 12, 24 and 36 hours. Most of the bacterial strains had the maximum activity at 24 hours. AP04(1W) had the maximum activity at 12 hours and all the other isolates activity was found to be negative at 12 hours. Among the isolated cultures AP13 has the greater specific activity (498.3425 units/mg) at 24 hours. BFL24 among the screened cultures has the greatest specific activity (890.8451 units/mg) at 24 hours. The specific activity of the bacterial isolates at 36 hours has found to be decreased compared to 12 and 24 hours culture.

CONCLUSION

The work so far carried out describes the isolation and screening of alkaline protease producing bacteria from fermented foods. 40 isolates were obtained from 11 samples and based on the activity on the skimmed milk agar plate 16 of them were considered for the further experiments. Out of the bacterial isolates AP13 has maximum specific activity at 24 hours. Microscopic and macroscopic techniques were performed for the identification of the bacteria. It was found to be gram positive. Catalase, oxidase and citrate tests were positive. Test such as methyl red,indole and VP was found to be negative.

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