Isolation and Identification of A Novel Strain *Bacillus stratosphericus* DF Producing Alkaline Protease and Optimization of Enzyme Production

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Abstract: An extracellular alkaline protease producing novel bacterial strain was isolated from soil sample collected at dump yard of dairy farm industry, Visakhapatnam and identified as *Bacillus stratosphericus* DF by morphological, physiological, biochemical tests and 16S rRNA gene sequence analysis. Protease production was enhanced 2.3 fold by optimizing the culture conditions. The nutritional factors such as carbon and nitrogen sources and also physical factors like pH, temperature, agitation speed, inoculum level and incubation period were optimized for the maximum yield of protease. Studies on the effect of different carbon and nitrogen sources revealed that lactose and combination of yeast extract and soya bean meal enhances the enzyme production. The bacterium produced the maximum amount of enzyme when allowed to grow for 48 hrs at 35°C and pH 10. The present study is the first report on the production of alkaline protease from *Bacillus stratosphericus*, which is a rare species usually, found 20 miles above the Earth and is believed to have been brought to the surface by atmospheric cycling process.

Keywords: Alkaline protease, *Bacillus stratosphericus*, Dairy farm industry, lactose, Optimization, Soya bean meal, 16S rRNA gene sequence.

1 Introduction

Proteases have weaved their own niche as an indispensible biocatalysts in industrial sectors of detergent, leather, pharmaceuticals, food, textile, silk, bakery, soy processing, meat tendering, brewery, protein processing, peptide synthesis, ultra filtration membrane cleaning, extraction of silver from used x-ray films as well as in basic research [1]. They represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world and they are the leaders of the industrial enzyme market worldwide [2], [3].

Although 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 [4]. Of all proteases, alkaline proteases produced by *Bacillus species* are of significant importance in detergent industry due to their high thermal and pH stability. Isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process of enzyme production for industrial use [5].

In the present study we report the isolation, screening and identification of alkaline protease producing *Bacillus stratosphericus* strain DF and on optimizing the production of extracellular protease under various environmental and nutritional factors.

2 Materials and Methods

2.1 Chemicals:

Nutrient broth, skim milk agar medium, all carbon and nitrogen sources (99% purity) used in this study was purchased from Hi-Media Laboratories (Mumbai, India).

2.2 Microorganism

The bacterial strain used in this study for alkaline protease production was isolated from soil samples collected at the dump yard of dairy farm industry, Visakhapatnam and it was identified as *Bacillus stratosphericus* DF. This strain was deposited in BIOTEC Culture Collection (BCC), Thailand and Microbial Collection Centre (MCC), Pune, India as *Bacillus sp*. under the accession number BCC 64300 and MCC 2230 respectively. It was maintained by monthly sub culturing at 37°C and stored at 4°C.

2.3 Sample collection and Isolation of bacterial strain

Soil sample was collected in sterile plastic bags from dump yard of dairy farm industry, Visakhapatnam and was processed within 24 hrs. Collected sample was serially diluted in sterile distilled water and the dilutions were plated in nutrient agar plates and incubated at 37°C for 24h. Well isolated colonies were picked and further purified by repeated streaking on nutrient agar plates.

2.4 Screening of protease producing bacteria

The isolated pure colonies were screened for proteolytic activities on skim milk agar medium at pH 7.0±0.2. The inoculated plates were incubated at 37°C for 24 hrs and a clear zone of hydrolysis gave an indication of protease producing organisms. The isolates which showed maximum hydrolysis zone were selected for further work.

2.5 Protease production

For protease production, the selected strains were cultured in 250 ml of Erlenmeyer flasks containing 100 ml culture medium (TDYE), which consists of 1g tryptone, 0.1g of dextrose, 0.25 g of yeast extract, 0.1g of KH_2PO_4[6]. The inoculated medium was placed in a thermostatic orbital shaker for 24 hrs at 37°C with 120 rpm. The culture was centrifuged at 10,000 rpm for 15 min to obtain crude enzyme.

Protease assay

Alkaline protease activity was determined with a modification of the method [7]. 1ml of suitable diluted enzyme solution was added to 1 ml 1% (w/v) casein solution (dissolved in 0.1 M Glycine-NaOH buffer with pH of 10) and incubated at 37°C for 20 min. The reaction was terminated with 4 ml of 10% (w/v) trichloroacetic acid and the mixture filtrated through a filter paper. The filtrate absorbance was determined using Lowry method and extrapolated against a tyrosine standard curve. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the experimental conditions.

Protein determination

Protein concentration of enzyme samples in each step was determined by the method of Lowry *et al*., with bovine serum albumin (BSA) as the standard [8].

2.6 Identification of the selected strain

Phenotypic characterization

Phenotypic characterization of the selected isolate was studied based on different morphological, physiological and biochemical characteristics. The data was compared with standard description given in Bergey's Manual of Determinative Bacteriology [9].

Molecular characterization

The genomic DNA was isolated according to the procedure of Weisburg *et al.,* [10]. About 50-100 ng of the purified DNA was used for sequencing by PCR using Big Dye® Terminator v3.1 Cycle Sequencing Kits [Applied Biosystems] with two degenerate primers–forward: 5´-AGAGTTTGATCHYGGYTYAG-3´; and reverse: 5´-ACGGCTACCTTGTTACGACTT-3´. Cycling conditions for sequencing PCR were as follows: denaturation at 96°C for 10s, annealing at 55°C for 10s and extension at 60°C for 4 min. After 35 cycles, the templates were purified by Ethanol/EDTA precipitation method and sequenced on ABI 3730xls Genetic Analyzer (Applied Biosystems). The nucleotide sequence obtained was deposited in GenBank with the accession number KC866366**.** The deduced sequence was subjected to homology search using basic local alignment search tool (BLAST) programme of the National Centre for Biotechnology Information (NCBI) [11]. Representative sequences of the most similar neighbors were retrieved and aligned using CLUSTAL W for multiple alignments. The multiple alignment file was then used to create neighbor- joining tree using MEGA version 4 software [12].

2.7 Optimization Parameters

Effect of initial pH, temperature, agitation speed, inoculum level and incubation period on protease production

The effect of various physical parameters on protease production was assessed by growing bacterial culture in the TDYE production medium. For optimizing pH, the medium was prepared by varying the pH from 4.0 to 12.0 at 1.0 unit interval. Optimum temperature for protease production was achieved by incubating the medium at 15, 25, 35, 45, 55 and 65°C. Agitation speed was determined by incubating the bacterial culture at static condition (zero rpm) and shaking conditions in a range of 50–200 rpm interval. Effect of varying inoculum percentage from 0.5% to 2% with 0.5% variation on protease production was determined. Similarly, for the investigation of optimal incubation time for protease production, the bacterial culture was inoculated in the medium and incubated for 96 hrs at 37°C. Protease production was determined at 24 hrs intervals.

Effect of carbon and nitrogen sources on protease production

The TDYE production medium was supplemented with different carbon sources (0.1%) such as sucrose, maltose, lactose, glycerol, soluble starch and galactose separately with dextrose as control and different nitrogen sources (1%) such as soya bean meal, casaminoacids, casein, ammonium chloride, ammonium sulphate and peptone separately with trptone as control to study their effect on protease production.

3 Results and Discussion:

3.1 Isolation and screening of protease producing bacteria

In the present study, a total number of 60 bacterial colonies from the soil of dairy farm industry have been isolated and screened for the protease production on Skim milk agar plates. 48 strains were identified as protease producers by zone of hydrolysis around the colonies and the activity of the strains which showed maximum hydrolysis was determined. In protease assay, DF showed highest production (254 U/ml) compared to other strains and this isolate is used for further optimization studies.

3.2 Identification of the selected strain

Phenotypic characterization

The selected colony DF appeared to be medium size, irregular, cloudy, shine, smooth, moist, raised, entire and opaque on nutrient agar medium. The bacterium is a gram positive rod, facultative, spore forming and arranged in 2-3 chains. When grown at different temperatures, the strain DF did not grow at temperature of 5°C and 10°C but showed growth between 20°C and 55°C. Growth was observed in media having initial pH between 4 and 12 and in media having NaCl concentrations between 2.5% and 5%. Biochemical characteristics of the culture DF are shown in table 1.

Table 1: Biochemical characteristics of strain DF

TESTS	RESULTS
Catalase test	positive
Oxidase test	positive
Nitrate test	negative
H2S test	negative
Urease test	negative
Indole test	negative
Methyl red test	negative
Voges proskauer test	negative
Citrate test	negative
Starch hydrolysis	negative
Gelatin Hydrolysis	positive
Casein hydrolysis	positive
Hemolysin	negative
Aminoacid and derivatives utilization:	
Arginine	negative
Lysine	negative
Ornithine	negative
Carbohydrate utilization:	
Starch	negative
Raffinose	negative
Sucrose	positive
Lactose	positive
Maltose	negative
Trehalose	negative
Cellobiose	negative
Melibiose	negative
Ribose	negative
Arabinose	negative
Xylose	negative
Rhamnose	negative
Dextrose	positive
Manose	negative
Galactose	negative
Fructose	negative
Aldonitol (ribitol)	negative
Manitol	negative
Sorbitol	negative
Dulcitol(galacitol)	negative
Inositol	negative
Myo-inositol	negative
Salicin	positive
Special tests:	
Esculin	positive
ONPG	positive

Molecular characterization:

The identity of the selected strain DF was confirmed by carrying out 16S rRNA gene sequencing. The sequence of the 16S rRNA gene was compared with GenBank entries, using BLAST programme and the sequence showed a similarity of 99% with the *Bacillus stratosphericus, Bacillus aerophilus, Bacillus altitudinis, Bacillus safensis*, and *Bacillus pumilus*. From the multiple sequence alignment data, length of the sequence was 1434 nucleotides with 1381 conserved and 53 variable nucleotides. It is inferred that the culture DF is showing affliation towards *Bacillus stratosphericus* with 90% boot strap confidence values [Fig 1] and hence the culture DF under study is conclusively confirmed as *Bacillus stratosphericus*.

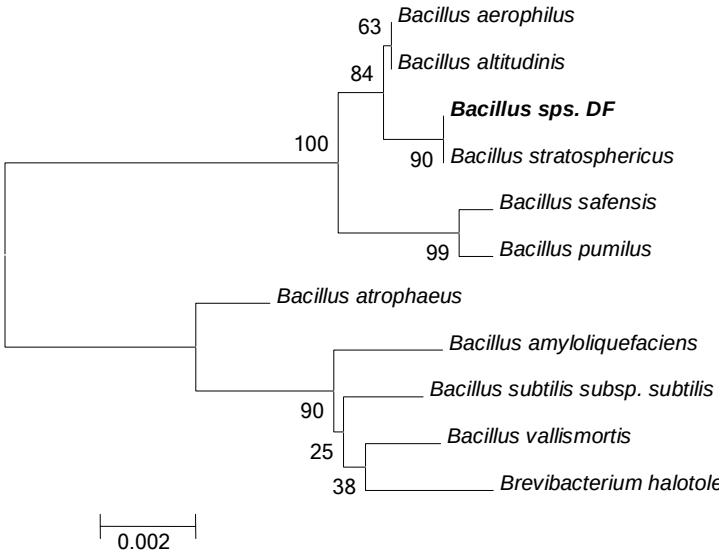

Fig 1: Phylogenetic relationship of strain DF (highlighted). The tree is constructed using the Neighbor-joining method.

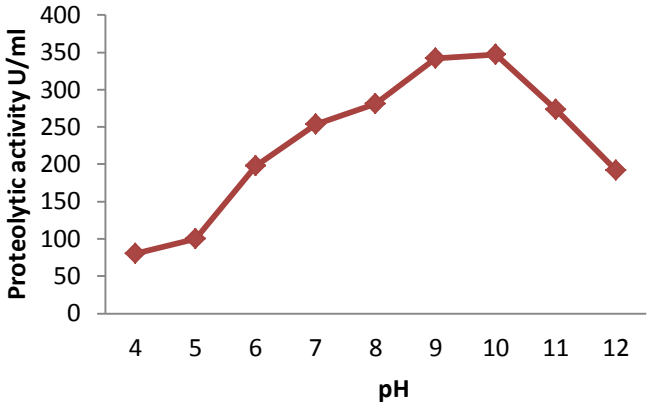

Fig. 2: Effect of initial pH on protease production in *Bacillus stratosphericus* DF

3.3 Optimization parameters

3.3.1 Effect of initial pH on protease production

Among the physical parameters, pH of the culture medium plays an important role by inducing physiological changes in microbes and their enzyme secretion. The obtained results demonstrated that though protease production was detected over a broad pH range from 4.0 to 12.0, maximum enzyme production (347 U/ml) was noted at alkaline pH 10 (Fig. 2). From the survey of literature, it can be seen that the optimum pH range of alkaline proteases is generally between pH 9 and 11 [13, 14] with few exception pH 11.5 [15, 16], pH 12-13[17]. A similar report of initial pH 10 was also studied in *Bacillus sp.* SSR1 [18].

3.3.2 Effect of incubation temperature on protease production

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The isolate produced protease at different temperatures from 15 to 55°C (Fig. 3) with maximum production at 35°C (352 U/ml). Either increase or decrease in temperature reduced protease production. The optimum temperature of 35°C was also reported in *B.pumilus* MK6-5 [19] for maximum protease production.

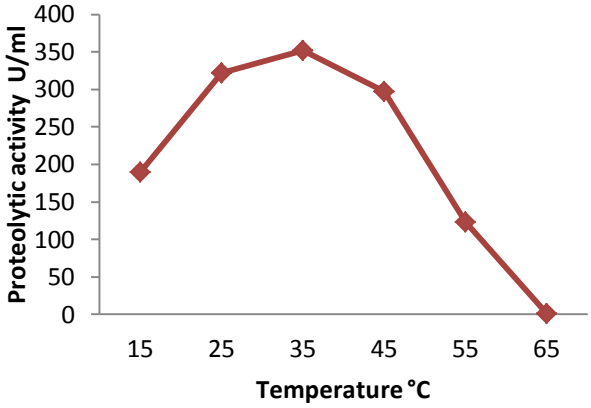

Fig. 3: Effect of temperature on protease production in *Bacillus stratosphericus* DF.

3.3.3 Effect of agitation speed on protease production

The effect of various agitation speeds was investigated for protease production; optimum production of protease (Fig. 4) was observed at the agitation speed of 150 rpm (381 U/ml). In the static condition, protease production was very low compared to shaking conditions (50, 100,150 and 200 rpm). *Bacillus sp*.SSR1 [20] and *Flavobacterium balustinum* P104 [21] showed optimum yield of protease production under the agitation speed of 150 rpm.

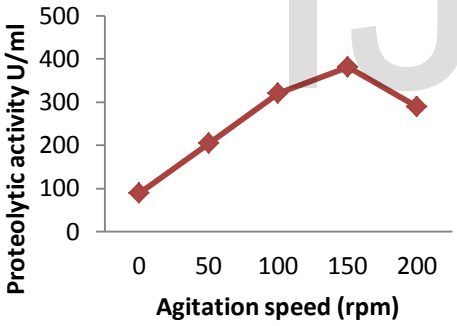

Fig. 4: Effect of agitation speed on protease production in *Bacillus stratosphericus* DF.

3.3.4 Effect of inoculum level on protease production

In the present study, the optimum inoculum level for protease production was observed (Fig. 5) at 1% [403 U/ml]. There was a reduction in protease production when inoculum size was reduced which may be due to insufficient number of bacteria, which would lead to reduced amount of enzyme production. Higher inoculum size may have resulted reduced dissolved oxygen and increased competition towards nutrients [5]. *Bacillus licheniformis TD4* [5] has been reported that 1% inoculum showed maximum enzyme production.

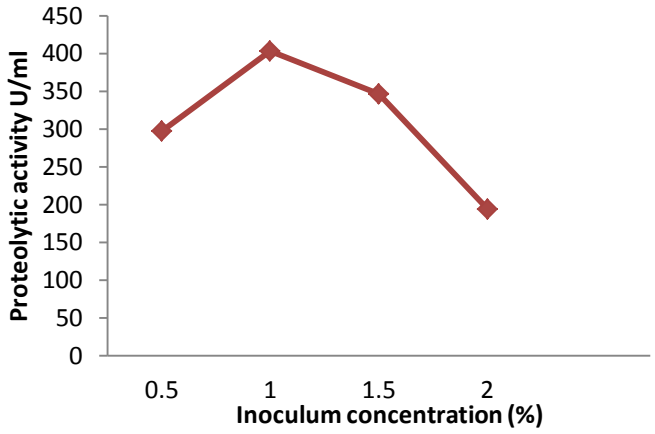

Fig. 5: Effect of inoculum level on protease production in *Bacillus stratosphericus* DF.

3.3.5 Effect of incubation period on protease production

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The enzyme production varies with incubation time. The results of the effect of incubation time on protease production (Fig.6) indicate that the protease production increased up to 48 hrs and later showed a decline. Maximum production of protease occurred at 48 hrs (492 U/ml). A similar observation has been reported in *Acaligenes faecalis* [22] and *B.licheniformis* ATCC 21415 [23].

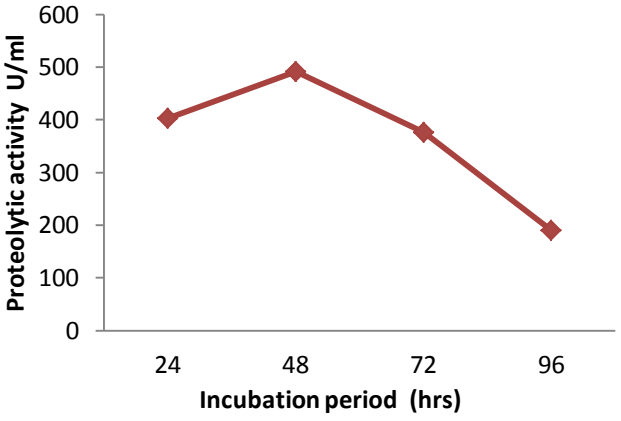

Fig. 6: Effect of Incubation period on protease production in *Bacillus stratosphericus* DF

3.3.6 Effect of carbon sources on protease production

Among various carbon sources used, protease production was highest in the medium containing lactose (563 U/ml), followed by maltose (506 U/ml) shown in (Fig.7). Less production of enzyme was recorded in the medium containing glycerol. A similar report for maximum protease production by lactose was observed in *B.brevis* MTCC B0016 [24] and in *Aspergillus flavus* IMI 327634 [25].

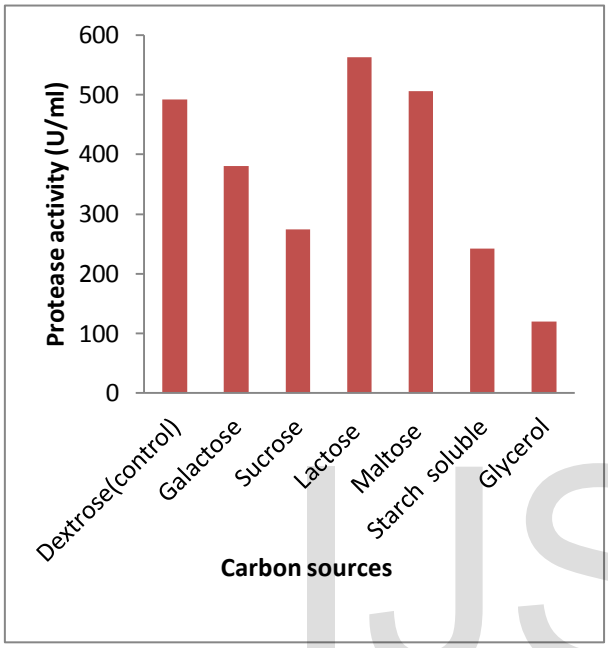

Fig. 7: Effect of carbon sources on protease production in *Bacillus stratosphericus* DF

3.3.7 Effect of nitrogen sources on protease production

Various nitrogen sources were investigated for protease production. High yield of protease production was observed (Fig. 8) in soya bean meal (586 U/ml). Soya bean meal was also reported in *Alcaligenes faecalis* [22], *B.brevis* MTCC B0016 [24].Both organic and inorganic nitrogen compounds were utilized by DF strain which shows the versatility of the bacteria utilizing a range of compounds.

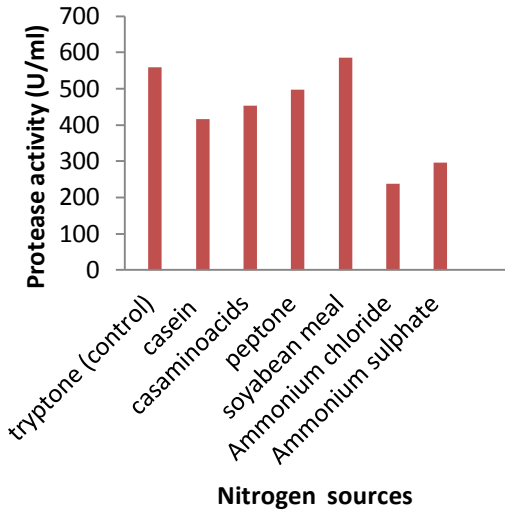

Fig. 8: Effect of nitrogen sources on protease production in *Bacillus stratosphericus* DF.

4 Conclusion

In the present study, we have isolated *Bacillus stratosphericus* from the collected soil sample and investigated the optimal media components for maximum protease production. The optimum pH, temperature, agitation speed, inoculum size, incubation period, carbon and nitrogen sources for protease production was determined as 10, 35°C, 150, 1%, 48 hrs, lactose and soya bean meal respectively. The protease activity was enhanced to 586 U/ml under the optimal culture conditions. To date, no report is available on isolation and production of protease by *Bacillus stratosphericus* and this is the first report on *B.stratosphericus*, producing an alkaline protease from dump yard of dairy form industry. Further studies are required to find out the potential applications of the alkaline protease obtained from this study.

Acknowledgement

The authors acknowledged the support from Department of Biochemistry, College of science, Gandhi Institute of Technology and Management, deemed to be University for providing the necessary research facilities.

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