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Screening Of A Halotolerant Bacillus Species For Protease Production From Kali Estuary

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Abstract

Uncontrolled anthropogenic activities like urbanization, industrialization, modern agricultural practices and habitat destruction are root cause for generation of pollutants in unprecedented levels. These pollutants are acting mutagens and teratogens causing environmental as well as health hazards. Bacteria as a result of its distribution in various habitats including extremophilic environments evolved their metabolic ability to utilize pollutants and release nonhazardous and useful products in to surroundings by productions of different classes enzymes like laccases, proteases, lipases, hydrolases, dehydrogenases and dehalogenases. In the present study, a mesohalotollerant (6-8%) Bacillus was isolated from Kali estuary by screening on skimmed milk agar media. The identified species was *Bacillus vietnamensis.* The protease activity was tested against different inducers like peptone, tryptone, milk, groundnut cake and pongamia cake. Among all substrates, peptone showed to be highest inducer of the protease and of the crude protease activity with peptone was showed to be 772.75 U/ml. estimated. The protease is expected to be highly promising for environment clean up, food industry and industrial applications.

Keywords: Protease, Bacillus, Mesohalotolerant, Kali, Sediment, Marine Biology Karwar

Introduction

Estuaries are dynamic ecosystems characterized by fluctuating salinity gradients. This unique environment fosters a diverse microbial community, including halotolerant microorganisms that have adapted to thrive in these brackish conditions. Among these, Bacillus species are of particular interest due to their remarkable resilience, diverse metabolic capabilities, and potential for producing industrially relevant enzymes. Bacillus species adapt to high salt environments [1, 2]. A key challenge for these microbes is maintaining osmotic balance across their cell membranes in the presence of high external salt concentrations. Halotolerant bacteria employ various strategies to counter this challenge. One approach involves the accumulation of compatible solutes (osmolytes) within the cell. These small, water-soluble molecules, such as glycine betaine and proline, help balance the intracellular and extracellular osmotic pressure, preventing cell dehydration [3]. Additionally, halotolerant bacteria often modify the composition of their cell membranes by incorporating specific fatty acids that maintain membrane fluidity and functionality under high salt conditions [4]. Proteases, a class of enzymes that break down proteins into smaller peptides and amino acids, are of particular interest due to their diverse industrial applications. Halotolerant proteases produced by Bacillus spp. offer several advantages. Firstly, their

exceptional stability in saline environments makes them ideal for processes involving fluctuating salt concentrations, a hallmark of estuarine ecosystems [5]. Secondly, these enzymes may exhibit unique properties due to their adaptation to changing salinity. Proteases produced by Bacillus spp. in estuaries play a crucial role in nutrient cycling within this vital ecosystem [6]. By breaking down organic matter from various sources, including dead organisms and detritus, these enzymes release essential amino acids and nitrogen compounds. These nutrients become readily available for other organisms in the estuarine food web, promoting ecosystem health and productivity. Protease is an enzyme helps in proteolysis. An enzymes are biocatalysts playing an important role in metabolic and biochemical reactions. Proteases from microorganisms which are important in industries including detergent industry, leather industry and pharmaceuticals. The present study was aimed to isolate industrially important protease producing Bacillus from Kali Estuary Uttara Kannada Karnataka.

Materials and Methods

Sample Collection: Sediment samples were collected from Kali Estuary (14°50'11"N & 74°09'59"E), Uttara Kannada District Karnataka, during low tide. The samples were collected aseptically in sterile polythene bags and then processed in lab.

Figure 1: Map Showing Sunkeri Kali Estuary

Sample Analysis: Bacterial spores were isolated through a heat treatment process. One gram of sediment sample was suspended in nine millilitres of distilled water in a sterilized test tube and also incubated at 80°C for one hour. After the incubation period, the treated samples were inoculated onto skimmed milk agar plates. This heat treatment method was employed to selectively isolate spores from the environmental samples, which are resistant to high temperatures and can germinate to form bacterial colonies on the agar plate.

Isolation of Protease producing Bacillus: The isolated spores through heat treatment was (0.1 ml) inoculated on Skimmed Milk Agar plates and incubated at 30^0C for 24- 48 hours. The colonies showing zone of inhibition on skimmed milk agar were further purified for the morphological, biochemical and molecular identification. For the identification of bacteria different types of tests were carried out like Gram's staining, motility, catalase, oxidase, MR VP, Indole production, citrate, carbohydrate fermentation test for glucose, sucrose, lactose, manitol, maltose, arabinose , ONGP test, Nitrate reduction etc, salt tolerance test (0%, 3%, 6%, 8% and 10%) and urease test. These tests were followed from the scheme for identification of Bacillus to species level given by Fritze, D. (2002) [7]. 16S rRNA sequencing of Bacillus species was amplified using 27F and 1492R primers. Then the sequence was blasted using NCBI.

Protease Production: Protease activity was determined using different substrate like casein, peptone, tryptone, milk, groundnut cake and pongamia cake. O.1 ml of supernatant sample is taken in test tube and 1ml of 1% Substrate and 0.9 ml of distilled water and incubated at room temperature for 1hour. The reaction was terminated by adding 4ml of 5% TCA, allowed it to settle without disturbing for 1hour. Then the contents were centrifuged at 5000rpm for 10min. 1ml of clear supernatant was taken to estimate protein by Lowry's method. Take 1ml of supernatant sample add 4.5ml Na_2Co_3 and incubate at room temperature for 10 mins, then add 0.5ml 1N Folin Ciocalteu's reagent. The test tubes were incubated at dark for 30min. The optical density of the samples was measured at 660nm. The activity is calculated as units/ml enzyme using following formula.

$$\text{Units/mL Enzyme} = \frac{(\mu\text{mol tyrosine equivalents released}) \times (11)}{(0.1) \times (60) \times (2)}$$

Results

The screening of protease producing salt tolerant Bacillus from the Sediments of Kali Estuary Uttara Kannada was carried out, 10 different bacterial colonies were isolated, out of which one bacterial colony showing the highest activity was selected for further biochemical and molecular analysis. A distinct pale yellow to yellow colored bacterial colony was identified as a potential protease producer. The bacteria was found to be gram-positive, and positive to other test like Malonate test, Voges Proskuer's test, Citrate test, ONGP test, Nitrate reduction test, Catalase test, Arginine test, Sucrose test, Mannitol test, Glucose test, Arabinose test and Trehalose test. PCR amplification of the 16S rDNA yielded a product of 1.4 KB, and sequencing showed a 97.72% sequence similarity with *Bacillus vietnamensis.* A distance tree based on the blast results also clustered the query sequence with *Bacillus vietnamensis*

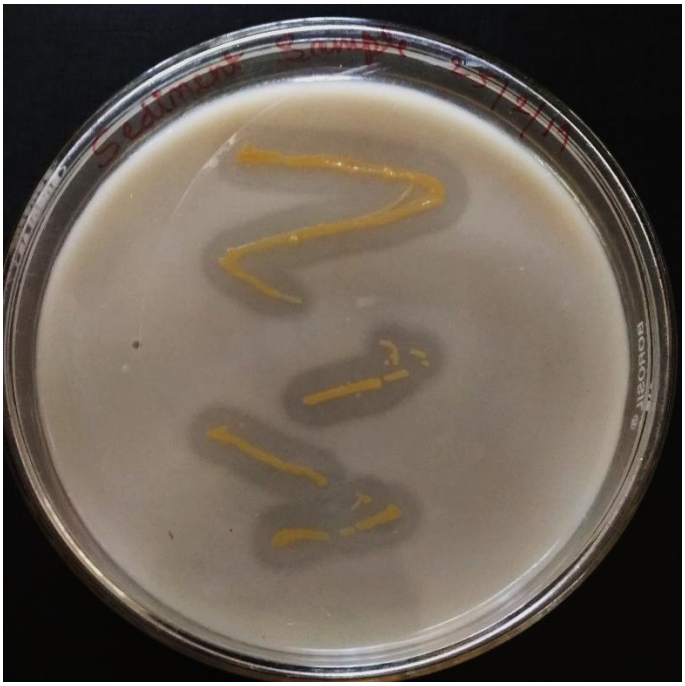

Figure 2 Bacillus species showing Zone of Inhibition

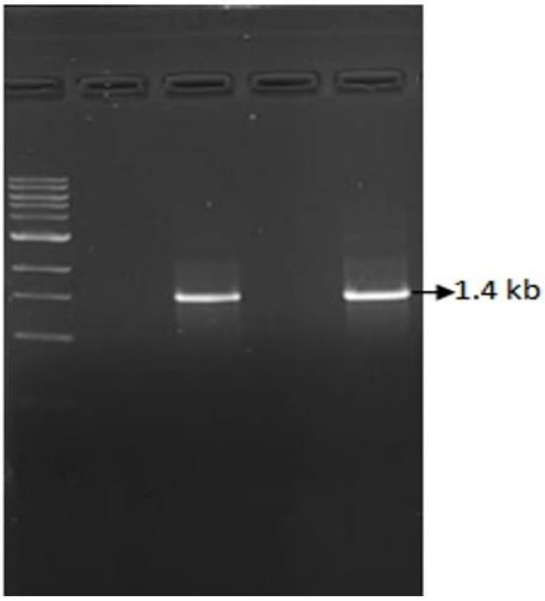

Figure 3 PCR product showing 1.4kb bands

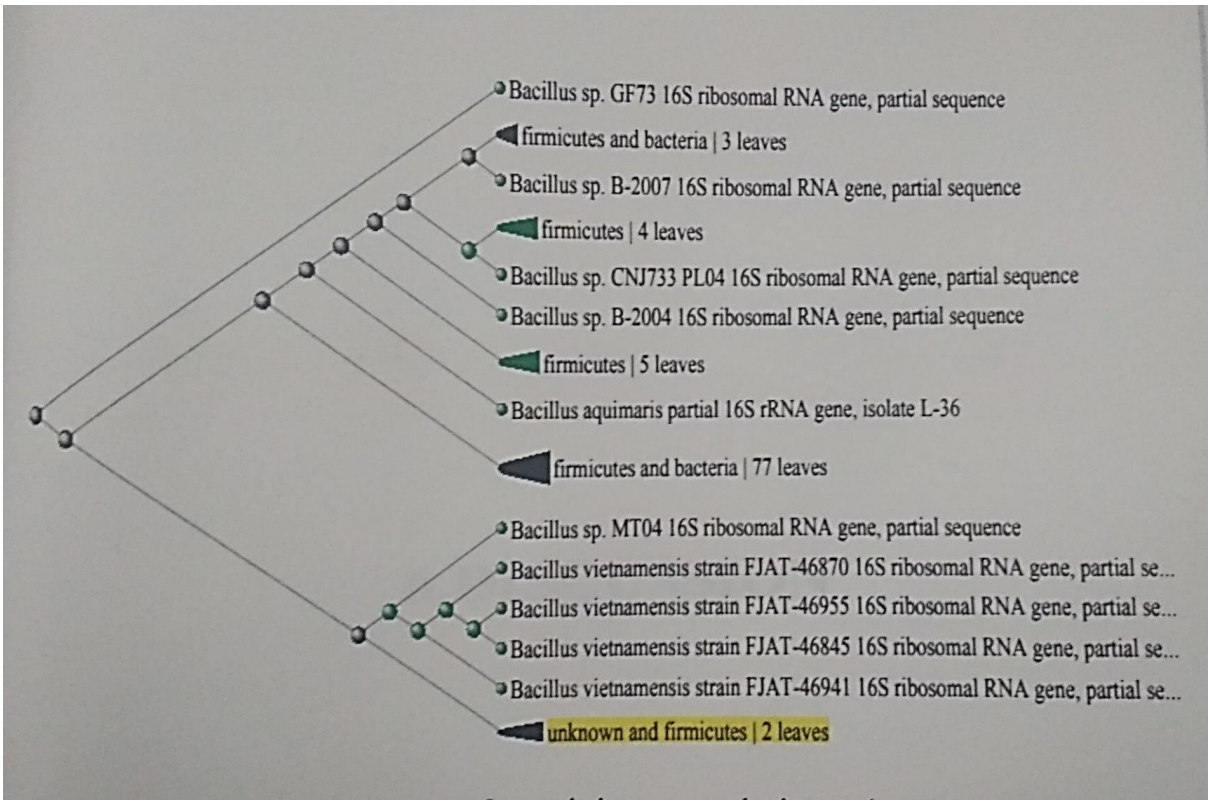

Figure 4 Distance tree based on fast minimum evolution using BLAST data

Estimation of Protease Activity: Protease activity was tested with different inducer like peptone, tryptone, milk, groundnut cake and *pongamia* cake. Among all substrates, peptone showed to be highest inducer of the protease and of the crude protease activity with peptone was showed to be 772.75U/ml was the highest inducer of protease activity (Fig.5).

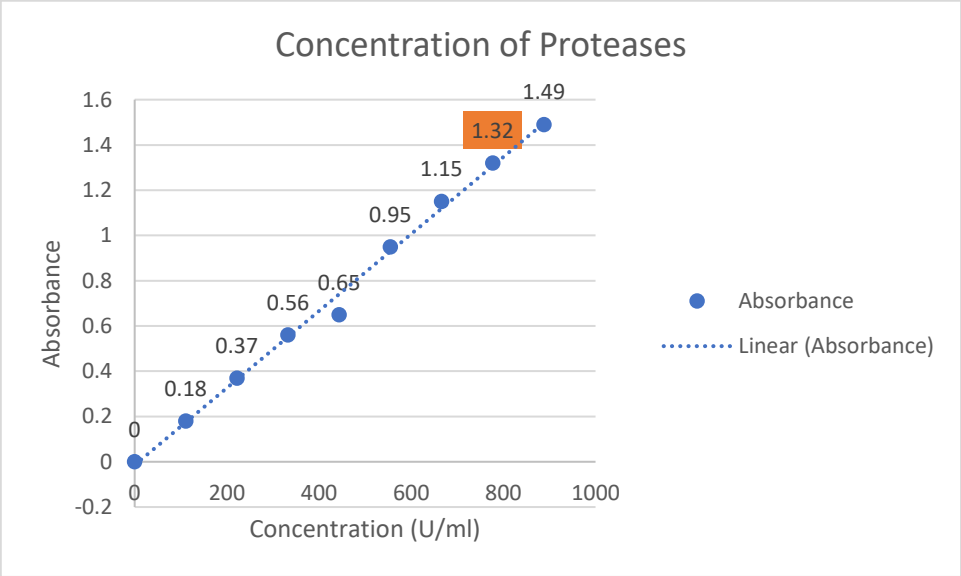

Figure 5 Graph showing Protease Activity

Conclusion

The present study isolated and identified a mesohalotolerant *Bacillus vietnamensis* strain from the Kali estuary with promising potential protease production. Peptone was confirmed as the most effective inducer for protease activity, reaching a remarkable level of 772.75 U/ml. This indicates the suitability of the strain for environment cleanup, food industry, and other industrial applications. Future research need to focus on purifying and characterizing the enzyme, investigating its optimal reaction conditions (temperature, pH, stability), and

exploring its specific applications in bioremediation or enzyme-based products. Additionally, studying the mechanism of protease induction under stressed conditions could provide valuable insights into the strain's adaptation strategies and further optimization of production.

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