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SCREENING AND CHARACTERIZATION OF PROTEASE PRODUCING HALOPHILIC BACTERIA FROM SALTPAN AREA VEDARANYAM, TAMIL NADU

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ABSTRACT

This study was focused on screening and characterization of protease producing bacterial strain from saltpan located in vedaranyam. The isolated potential bacterial strain was identified as Bacillus firmus by various staining, biochemical tests includes 16S rRNA sequencing method. The effects of various environmental factors on the production of protease were studied. The organism showed the significant growth and enzyme production at pH 9.0 and temperature range at 37ºC. The protease enzyme was analyzed and purified by using ammonium sulphate precipitation method and membrane dialysis. The protease enzyme liberated from these strains showed better stability and activity in pH 8.0 and at temperature 40˚C suggesting that enzyme used in antibacterial activity and pharmaceutical applications.

KEY WORDS

Saltpan soil, Halophilic bacteria, 16S rRNA, Protease enzyme, Bacillus firmus

INTRODUCTION

Halophilic bacteria are well adapted to saturated NaCl concentrations with several halophils growing optimum environments above 25–30% NaCl (Tapingkae et al., 2010). They have been widely measured as a rich source of useful for salt-stable enzymes (LitchWeld, 2011). The globe was enclosed with 73% of the sea water that contains 2.7% of common salt. The various enzyme derived from marine bacteria and fungi have enzymatic activity and can effectuate several biochemical reactions with different enzymes. Especially, several hydrolytic enzymes possessed by halophilic microorganisms and are capable of functioning beneath conditions that cause precipitation and denaturation of most proteins (Manikandan & Senthilkumar, 2017).

Halophiles are extremophilic microorganisms that succeed in environments with very high concentrations of salt and inhabited in hypersaline environments all over the world (Gutierrez et al., 2002), that producing enzymes are active and stable in extremely saline conditions and retain catalytic proficiency at very low water activity (Eichler, 2001). They are having negatively charged amino acid on the solvent-exposed outsides of the protein help of adapted to the environmental pressure (DasSarma & DasSarma, 2015).

However, their biotechnological prospects haven't been extensively exploited. additionally, halophilic bacteria produce a wide variety of extracellular salt-tolerant hydrolytic enzymes consistent to amylases, proteases, and xylanases that have diverse possible usages in different extents like food technology, feed additives and chemical industries (Niehaus et al., 1999) (Pandey et al., 2000) (Collins et al., 2005) (Oztetik & Cakir, 2014) The moderately halophilic *Bacillus* sp are isolated from various saltpan areas, estuarial water, salt lakes, sea ice and water (Agnew et al., 1995) (Arahal et al., 2000).

Species of the genus *Bacillus* were isolated from aquatic and terrestrial environments, extremely alkaline and acid waters, and salt lakes (Hsu et al., 2013). The bacterium *Bacillus* sp. L21 and *Bacillus licheniformis* can produce proteolytic enzymes that are alkali-resistant and halotolerant (Tari et al., 2006).

The halophilic enzymes are exhibited some biochemical characters and stabilize enzymes under the extreme conditions of the high amount of alkalinity (Marhuenda-Egea, 2002). These chemicals stay dynamic and stable in high salt condition and some are thermotolerant and alkaliphilic (de et al., 2009).

In distinction, moderate halophiles accumulate the protoplasm high amounts of specific organic osmolytes, that perform as osmoprotectants, providing diffusion balance whereas meddling with the standard metabolism of the cell (Camacho et al., 2009). The in-depth biochemical selection and easy genetical manipulation of these enzymes could generate new ones for numerous biotechnological applications (Xiong et al., 2007). The impartial microorganism has a generally low thermotolerance contrasted with alkalic proteases (Rao et al., 1998). The microorganism is the most potential resource of proteolytic enzyme. Few strains of microbe that can produce protease are *Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus subtilis, Bacillus thuringiensis* and *Pseudomonas* (Pt, Satria, & Asa, 2012).

The basic principle of probiotic is the utilization of microorganism ability to break or disentangle carbohydrate chains, protein, and fat because of the special enzymes owned by the microbe to break the chains. Protease is a proteolytic enzyme that catalyzes the severance of peptide bond into oligopeptide and amino acid. The application of protease in biotechnology can be found in the detergent industry, leather industry, an additive in the food industry, pharmacy, agriculture, and fisheries (DasSarma & DasSarma, 2015) (Delgado-García et al., 2012). Protease enzymes used in tannery industries have vast applications. Seeking a potential candidate for efficient protease production has emerged in recent years (Marimuthu Anandharaj *et al*., 2015).

Few commercial proteolytic enzymes that were successfully purified are alcalase from *Bacillus licheniformis*, esperase from *Bacillus lentus*, a bio feed pro from *Bacillus licheniformis*, another one that is subtilisin from *Bacillus alcalophilus* (Gupta et al., 2002). The halophilic medium components especially the nitrogen source and fermentation conditions greatly influence the growth of microbes, and physicochemical factors such as pH, temperature, NaCl, and inoculum size (Kumar et al., 2012). Optimization of growth conditions required for halophilic protease production by conventional methods (Cazetta et al., 2007). Such restrictions can be overcome by using response surface methodology (RSM) which is routinely used for optimization studies in several biotechnological and industrial processes (Khuri & Mukhopadhyay, 2010). To optimize medium components using statistical tools for enhancing the halophilic protease production and yield from Halophilic bacterium. The optimized conditions were then applied to scale up the halophilic protease production with a view to facilitating its application in the various field.

MATERIALS AND METHODS

Study area

Vedaranyam is a village situated in Nagapattinam district of Tamil Nadu, India. The geographical coordinates latitude and longitude of Vedaranyam is 10.3717° N, 79.8511° E respectively.

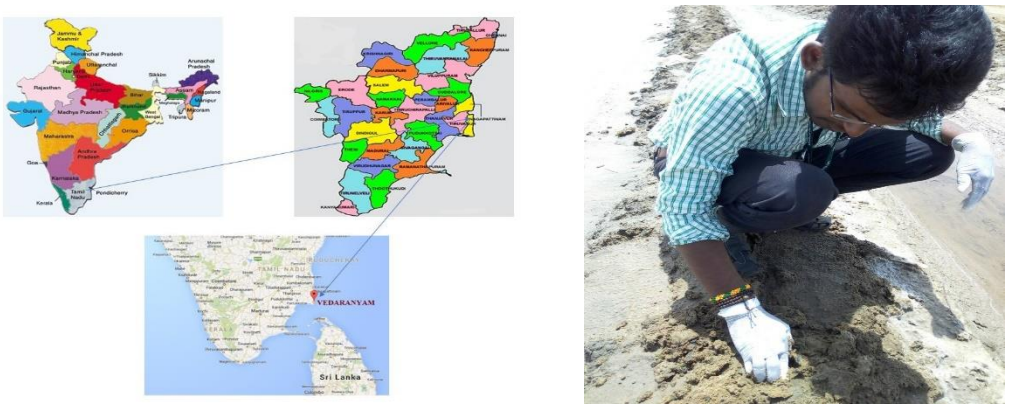
Sample collection

The saltpan soil samples were collected at the depth of 10 cm, 20 meters near the saltpan side in Vedaranyam Taluk, Nagapattinam District (Fig. 1). The soil sample was collected and transported to laboratory using in sterile polythene bags and stored in a refrigerator for further use (Lopez et al., 1956).

Physiochemical parameters of soil sample

The saltpan soil samples were analysed by physiochemical parameters such as colour, pH, salinity, alkalinity, electrical conductivity (EC), permeability, humidity, nitrogen, phosphate, potassium, lime status, sodium, magnesium, calcium, zinc, fluoride, mercury, silver, selenium, and chromium were estimated by using standard analytical methods. The physical and chemical environment is important for many of the processes that take place both in biotic and abiotic environments (Patadia, 2015).

Figure.1: Sample collection and study area



Isolation and screening of halophilic bacterial strain

The sterilized polythene bags were utilized for the saltpan soil samples collection from Vedaranyam. Then 2gram of soil sample was weighed and suspended in 100 ml of distilled water. The contents were serially diluted up to 10^{-7} dilution factor. From each dilution, 0.1 ml was taken and inoculated on sterile Halophilic Agar media - M590 (pH 8.0) with using spread plate technique (Bergey et al., 1974). After that, the inoculation processes the plates were incubated at 37ºC for 2-3 days. The pure cultures were obtained by repeated streaking. The protease enzyme producing bacteria were screened by using Gelatin Agar medium (pH 9.0), which contained 9g/l: gelatin 30g, peptone 10g, and NaCl 100g. The pure cultures were separately named as Vedaranyam strain-1,2,3, etc., That strains are simply named VE. The VE-2 strain has a maximum around the clear zone vs colonies in diameter on Gelatin Agar media. Selected as a potential producing protease enzyme and was characterized based on grams staining and biochemical tests.

Enzyme production

The isolated pure culture was inoculated into the protease production medium and incubated at 37˚C for 24-48 hours on a rotary shaker. At the end of fermentation period, the culture medium was centrifuged at ten thousand revolutions per minute for a quarter-hour to get the crude extract that was used as an enzyme source and also the protease activity was assayed. (Wende et al., 2010)

Protease assay

The screening of protease-producing bacteria isolated from same basin soil samples by serial dilution method. Applicable dilutions were spread by using L-rod, on skim milk agar media containing following composition that including peptone (0.1 %, w/v), NaCl (0.8 %, w/v), agar (2.0 %, w/v), and skim milk (10 %, w/v) and incubated at 37°C for 2 days. After that period, individual colonies were inoculated in agar slants for further uses, a pure culture maintained in agar stabs and glycerol stocks. These cultures were used to screen the potential protease production, all pure cultures could be spread on the same medium. The clear zone around colonies was indicating casein hydrolysis, it was considered as the confirmation of proteolytic activity (Fig. 2). Totally, 8 strains were produced clear zone around the colonies, and the VE-2 strain has the highest activity, it was selected for further study. Morphological and physiological characteristics of the VE-2 were observed according to the standard protocols (Smibert et al., 1994).

Figure.2: *Bacillus firmus* on a halophilic agar plate

Identification of efficient protease producing bacteria

Morphological characterization

Macroscopic observation of colony morphology and pigmentation, microscopic characterizations were carried out based on the staining method.

Biochemical tests

Biochemical characterization was carried out by different tests were including IMViC tests, oxidase test, catalase test, urease test, nitrate reductase, carbohydrate fermentation, gelatin liquefaction, H_2S production, casein hydrolysis, starch hydrolysis and lipid hydrolysis (Siddharthan Nagarajan et al., 2017).

Identification by 16S rRNA Sequencing and Phylogenetic Analysis

The isolated bacteria VE-2 strain was further confirmation by 16S rRNA gene sequencing through the standard protocol (Smibert et al., 1994). DNeasy Blood and Tissue kit (Qiagen, USA) were used for genomic DNA isolation. The one-day bacterial cultures used for the DNA isolation method. Universal bacterial primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA from genomic DNA. PCR reaction comprising 35 cycles, including initial denaturation (94°C for 5 min), denaturation (94°C for 1 min), annealing (56°C for 30s), and elongation (72°C for 1.5 min). The final PCR products were purified by using the QIAquick PCR purification kit (QIAGEN, India). Purified PCR products were sequenced by using an automated gene sequencer (ABI prism 3730 DNA analyzer, USA). The resulting sequence was searched for sequence similarity in the GenBank database. The alignment of the partial 16S rRNA sequence was performed by using the basic local alignment search tool (BLASTn). The phylogenetic tree was constructed by using Mega software version 6.0. Isolated strain was identified as *Bacillus firmus* from saltpan area Vedaranyam. using 16S rRNA based molecular technique which is the available online data base of NCBI having GenBank accession number – MH306651.

RESULT AND DISCUSSION

Physiochemical Parameters of soil sample

Physiochemical parameters of saltpan soil samples were analyzed by Omega Laboratories (Analytical testing & Research Centre), Namakkal. The soil sample was dark brown color and high alkalinity. The physiochemical characters are listed in (Table.1). According to (Noorjahan, 2014), reported the pH range 9.14±0.0187 indicated the pH of the saltpan soil was found to be alkaline.

Table.1: Physiochemical parameters of saltpan soil sample

S.No	Parameters	Unit	Test Results
1.	Color (Hazen units)	-	Black
2.	pH Value at 35°C	-	9.10
3.	Salinity	mmhos/cm	1.120
4.	Acidity	mg/kg	05.95
5.	Electrical/Specific Conductivity	mmhos/cm	1.165
6.	Permeability	Mm/hr.	12.80
7.	Humidity	g/m^3	25.10
8.	Nitrogen	%	00.49
9.	Phosphate	%	02.94
10.	Potassium	%	00.62
11.	Lime status	%	08.90
12.	Sodium	%	00.16
13.	Magnesium	%	00.67
14.	Calcium	%	01.55
15.	Zinc	%	00.28
16.	Fluoride	%	00.45
17.	Mercury	%	00.06
18.	Silver	%	00.04
19.	Selenium	%	00.15
20.	Chromium	%	00.16

Isolation and Screening of Halophilic Bacteria

The samples collected from vedaranyam Salt pan. The samples were serially diluted upto 10^{-7} dilution each dilution 0.1 ml was taken and inoculated on sterile halophilic agar. After the incubation observe the halophilic bacterial growth (Fig. 2). The pure cultures were obtained by repeated streaking.

Isolation and Identification of Protease producing Bacteria

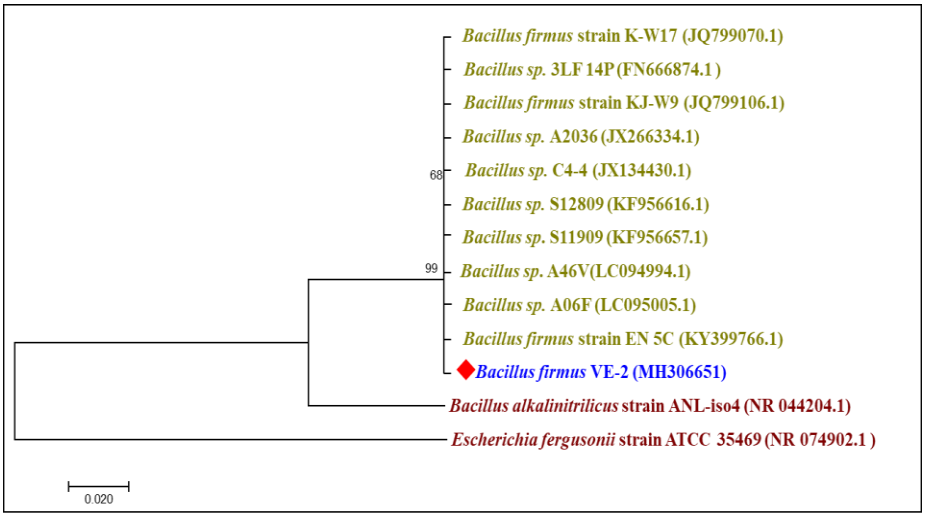
In this present study, the proteolytic bacterial strains were isolated from saltpan soil. Isolated bacterial strains were screened for protease producing ability on skim milk agar. Among 8 isolates (VE1, VE2, VE3, VE4, VE5. VE6, VE7, and VE8) only 5 isolates showed maximum zone around the colonies on skim milk agar indicates the protease production. Individual colonies were purified through repeated streaking on fresh agar plates. All positive protease producing colonies were cultivated in Casein liquid medium for 3 days, and protease activity was measured (Fig. 3). The strain VE-2 exhibited higher proteolytic activity compared with others were selected for further studies.

Figure.3: Protease Assay Skim Milk Agar

Identification and Molecular Characterization of VE-2 strain

The bacterial strain VE-2 is a Gram-positive, rod-shaped, facultatively anaerobic bacterium and motile. The strain was oxidase-positive, and it could grow well at pH 9.0 – 11 (optimal pH 8) and at 30–45°C (optimal 37°C). Based on Bergey's Manual of Determinative Bacteriology, these physiochemical experiment results suggested that this VE-2 strain belongs to the *Bacillus* family. The 16s rDNA sequencing and phylogenetic analysis revealed that the VE-2 is affiliated with genus *Bacillus* and closely related to *Bacillus firmus* (99 % identity), and the closest neighbors were found to be *Bacillus alkalinitrilicus* (97 % identity). The genomic G+C content of VE-2 strain was found to be 47.82 mol%. The 16s rRNA sequence was deposited in the NCBI database under the accession number of MH306651 (Fig. 4).

Figure.4: Evolutionary relationships of taxa

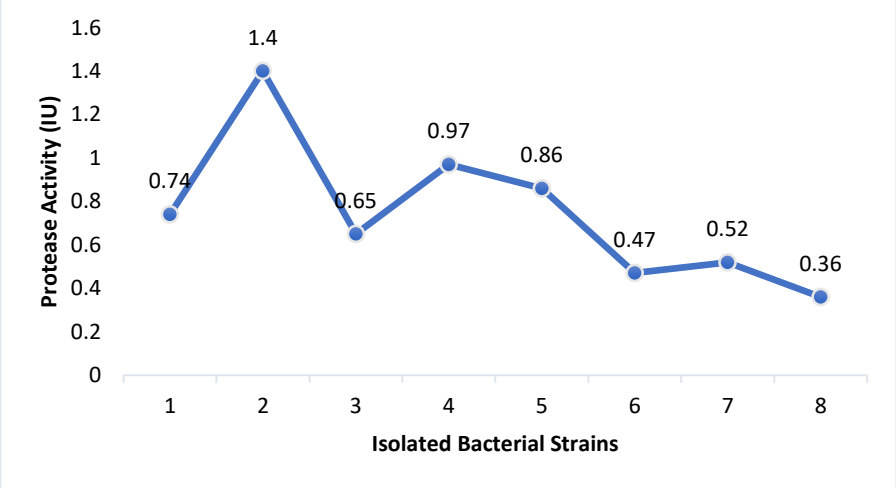


The evolutionary history was inferred using the Neighbor-Joining method (M, 1987). The optimal tree with the sum of branch length = 195.21679618 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 560 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Estimation of protease activity

The objective of the present investigation was to select the bacterial strains with a high level of protease producing ability (Fig.5). To achieve the aims, we have selected during the initial screening, a total number of 8 different bacterial strains were isolated on skim milk agar medium. The five isolates were checked for a quantitative test of protease production. The maximum protease activity was attained after 48 h by isolate VE-2. It was found that maximum production occurred at end of the exponential phase. Other each strain showed high production of protease, isolates VE-1, VE-3, VE-4, VE-5, VE-6. The lowest enzymatic activity was observed by isolates VE-7, VE-8 with enzyme activity respectively. Consequently, the greatest protease producing strain was recognized as a protease maker and it was taken to facilitating exploratory examinations and biochemical test. Correspondingly (Alnahdi, 2012) proposed the five isolates were checked for a quantitative trial of extracellular protease in a fluid medium and the greatest protease activity was accomplished after 72 h.

Figure.5: Estimation of Protease activity



Identification of proteolytic bacterial strain

The identification of the selected isolate was characterized based on their morphological, cultural, physiological and biochemical properties and is presented in proteolytic bacterial isolates respectively. The isolated VE-2 strain was gram-positive and this strain showed a non -motile, catalase, nitrate reduction, gelatin and casein hydrolysis (Table.2). The isolated proteolytic strain was identified and confirmed as *Bacillus firmus* based on the 16S rRNA sequencing.

Table.2: Biochemical characterization of the isolated bacterial strain

S. No.	Characteristics	Results
1.	Gram Staining	+ve
2.	Shape	Rod
3.	Motility	Non-motile
4.	Spore formation	Spore
5.	Oxygen requirement	Facultative anaerobic
6.	Indole test	+ve
7.	Methyl red test	-ve
8.	Voges-Proskauer test	-ve
9.	Citrate test	+ve
10.	Catalase test	+e
11.	Nitrate reductase	+ve
12.	Casein hydrolysis	+ve
13.	Gelatin hydrolysis	+ve
14.	Starch hydrolysis	+ve

Applications of isolated protease enzyme

These organisms producing enzymes have antibacterial activity which is checked by using some clinical isolated microorganisms. Compounds from microorganisms that can get by under outrageous pH could be especially helpful for applications under exceptionally acidic or very antacid response conditions. Be that as it may, one of the striking properties of acidophilic and alkaliphilic microorganisms is their capacity to keep up an unbiased pH inside, thus the intracellular proteins from these microorganisms don't should be adjusted to extraordinary development conditions. In any case, this does not represent extracellular proteins, which need to work in low or high pH conditions because of acidophilus and alkaliphiles, individually. Proteases, amylases, lipases and different chemicals that are impervious to and dynamic at high pH and high chelator centralizations of present-day cleansers are attractive. This has provoked the screening of alkaliphilic microorganisms and Archaea for their capacity to deliver such chemicals. By these methods, a few valuable proteins have just been distinguished and gotten. Mixes of homology-based PCR and movement screening have been connected to screen for and recognize antacid proteases in a gathering of thermos acidophilic archaeal and bacterial strains disconnected from hot conditions (Van den Burg, 2003).

CONCLUSION

The present characterization of eight isolated halophilic bacterial isolates was utilizing to produce secondary metabolites of the protease enzyme. This discovered by antimicrobial protein from halophilic bacteria that bacteria producing proteins are a potential application in the field of biological control of pathogenic bacteria. Further biochemical and molecular characterization after complete purification will be undertaken.

Conflict of Interests

There is no conflict of interests amongst authors regarding this publication.

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