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Investigations and Characterization of Alkaline Protease-Producing Fermentibacillus sp. RSCVS-HS3

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The study's goal was to investigate and characterize alkaline protease-producing Fermentibacillus sp. RSCVS-HS3. The bacterium Fermentibacillus sp. RSCVS-HS3 was isolated from Vindhya region (Rewa division) of Madhya Pradesh of central India. It grew on casein-containing media at pH 12 and protease activity was found positive. . The enzyme was in a stable state in its crude form at 50? and pH 12, indicating thermal stability and the alkaline nature of the enzyme. It was rod-shaped, long, filamentous, gram-positive bacterium and was positive for casein hydrolysis. Based on NCBI BLAST and Phylogenetic Analysis of 16s rRNA, it was identified as Fermentibacillus sp. RSCVS-HS3. This is probably the first study to date, stating the alkaline protease synthesis from any Fermentibacillus species. It was closest to Fermentibacilolus polygoni IEB3 but in contrast to this, the identified bacterium showed casein hydrolysis. This is probably the first study to date, stating the alkaline protease synthesis from any Fermentibacillus species.

Keywords: Alkaline protease; Bacteria; Characterization; Fermentibacillus; Identification; Soil.

Enzymes have been known to have important roles in a variety of processes for a long time. Their presence can be traced back to ancient civilizations, when microbial enzymes were used in baking, alcohol manufacture, brewing, and cheese production, among other things1. However, with increasing knowledge of enzyme manufacturing and purification from various sources, the number of applications of enzymes has expanded dramatically since then. Microorganisms are known to secrete both extracellular and intracellular enzymes commercially and more than 3000 different kinds of microbial extra cellular enzymes have been reported2.

Enzymes have become an alternative to chemical catalysts. They not only improve efficiency but also ensures the sustainability of industrial operations3. Proteases make up the majority of the industry's enzyme market, accounting for around 60% of it commercially4. Since the development of enzymology, microbial proteases have received the greatest attention5.

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More than two-third of the world's commercial protease sales come from them6. These enzymes have garnered interest not only because they play a crucial role in metabolic catalytic activities in living things, but also because they are widely used in industry7. They have uses in a variety of industries, including food, detergent, bakery, leather, pharma, newborn formulae, and so on, due to appealing properties such as ease of manufacture, temperature tolerance, and the ability to work at a variety of pH ranges8. Proteases may be either exopeptidase or endopeptidase enzymes based on the terminal or middle site of catalysis in proteins respectively. Based on the occurrence of amino acid residue at the active site and catalytic mechanisms, protease enzymes are classified into important groups:

1. Serine proteases
2. Cysteine proteases
3. Aspartic proteases
4. Metallo-proteases.

They can also be classified as alkaline proteases, neutral proteases, and acidic proteases.

Among the several types of proteases (acidic, neutral, and alkaline), alkaline proteolytic enzymes are the most frequently used industrial enzyme because of their comparably high efficiency, activity, and durability at high pH9. Alkaline proteases have been identified from a wide range of sources and are generated by a broad spectrum of microorganisms, such as bacteria, yeasts, moulds, and human tissues5.

Bacteria, on the other hand, were selected because they grow quickly under a variety of nutritional and physiochemical conditions, require less space, are easy to maintain, and are accessible for genetic operations2. Bacillus, Pseudomonas, Streptomyces, Micrococcus, and many other bacteria also have been found to generate alkaline proteases. With over 35% of all microbial enzyme sales, Bacillus sp. has been the most significant source of hydrolytic enzymes enzyme production10,11. After the first enzyme, 'Carlsberg,' derived from Bacillus licheniformis, was commercially offered as additives in detergents in the 1960s, other Bacillus bacteria-derived catalysts have been identified and reported12. Although these alkaline protease enzymes are found to be stable at high pH and temperatures, the vast majority of them are unsuitable for use in detergent matrices.

A recent trend is the exploration of extremophile microorganisms that inhabit unusual habitats such as freezing hot springs, arctic waters, saline waters or extremely alkaline or acidic habitats. The enzymes isolated from them are more likely to have extreme qualities that make them suitable candidates commercially.

The search for microbial of novel proteolytic enzyme properties, such as suitable specificity and consistency over a broad range of pH, heat, and action retainment in the presence of ions, wetting agents, and chemical agents, among many other items, that can be used in a diverse array of scientific and commercial applications, has been sparked by significant advancements in agricultural production, industry, and biotechnology13. The protease enzymes generated by bacteria in the natural environment are often unstable at high temperatures4, despite numerous efforts at physicochemical remedies, bioengineering, and gene-shuffling techniques. In this context, Extremophiles, which are known for manufacturing protease enzymes under extreme physicochemical conditions, have received a lot of attention. There are limited studies documenting the alkaline proteases isolated from these bacteria, and just a few of them have been reported further for commercial usage14. As a result, there is an ongoing need to find new and improved strains of these microorganisms that generate alkaline proteases in higher titers and with superior properties.

The exploration of microorganisms from underexplored natural habitats can be another way of obtaining novel proteases. Biodiversity richness also represents an invaluable resource for discovering novel microbes. Novel microorganisms may yield novel enzymes with new combinations of properties, e.g., alkaline proteases with halophilic and/or thermotolerant properties.

In the present study, Vindhya region of Madhya Pradesh, India was investigated for such extremophile microorganisms. Actual sites for soil samples included agriculture field soil of village Hatwa, in Sihawal tehsil of district Sidhi, Badi Pul Dhobi Ghat locality of Huzur tehsil in Rewa District headquarter, Forest soil of 'Mukundpur

Zoo and Tiger Breeding Centre" in Amarpatan tehsil of Satna district. Soil of Vindhya region is known to be rich in lime stones, minerals etc. indicating alkaline nature of the soil. Due to this fact, many cement companies have established their factories in this region. Also, the region is very rich in animals, plants and microbe's diversity. Two national parks are established in the region: Sanjay National Park in Sidhi district and Bandhavgarh National Park in Umariya district. Beside these, many national sanctuaries are also established here. The exploration of plant and animal diversity has progressed to a significant extent in this region but a systematic study on diversity of microbes is very scanty. Alkali rich soils may contain alkali tolerant bacterial species which may be strong prospects for alkaline protein lytic strains.

The study's aim was to discover alkaline protease-producing bacterial strains from alkaline soil samples obtained in Madhya Pradesh, India. There has never been a study like this done on the soil of this area before. This is the one of few reports of such kind of alkaline proteases being produced by a Fermentibacillus species.

MATERIALS AND METHODS

Chemicals

HiMedia, India, and Fisher Scientific, USA, provided all of the chemicals and reagents used in the creation of the medium, screening, protease assay, and phenotypic identification.
HiMedia, India, furthermore offered:
1. dNTP Mix,
2. Taq Polymerase
3. Bacterial Genome DNA Isolation Kit.

The primers for said study were given by Bengaluru, India-based Eurofins Genomics.

Collection of samples

In the Vindhya area of Madhya Pradesh, India, soil samples were gathered from several alkaline regions in order to isolate bacteria that produce alkaline proteases. A sterile spatula was used to gather samples from a 2-centimeter depth of the surface. They were first tested with pH strips for high alkaline pH before being put into a sterile polybag. Actual sites for soil samples included: (a) Agriculture field soil of village Hatwa, in Sihawal tehsil of district Sidhi, which is known to be highly alkaline locally such that people use the soil for hair washing etc. (b) Badi Pul Dhobi Ghat locality (Cloth washing area) of Huzur tehsil in Rewa District headquarter (c) Forest soil of Mukundpur Zoo and Tiger Breeding Centre in Amarpatan tehsil of Satna district; all were from Vindhya region (Rewa division) of Madhya Pradesh, India. These samples had pH values of 9, 10, and 7.0, respectively.

Medium

Isolation was done on CPYA (Casein, Peptone, Yeast extract, Agar) medium (pH 12.0)15,16, which was also utilized to test for protease activity and crude enzyme synthesis. Medium (1 L) was prepared by making three separate solutions initially. The composition of these solutions was as such-
Solution A (in 0.01 N NaOH solution (400 mL)
1. casein (5.0 g)
Solutions B (In 595 mL distilled water):
1. peptone (5.0 g),
2. yeast extract (2.0 g),
3. NaCl (5.0 g),
4. K2HPO4 (1.0 g),
5. MgSO4.7H2O (0.2 g),
6. CaCl2 (0.1 g), and
7. Agar (20 g)
Solution C (in 20 mL distilled water)
1. 0 g Na2CO3.

Then, to avoid casein coagulation due to high pressure, these solutions were autoclaved separately. Solution A and Solution B were combined aseptically, and pH 12.0 was maintained by adding Solution C drop by drop.

Isolation and screening

Agar plate serial dilution was used for isolation. The 10-1-10-3 diluted sample solution (0.1mL) was evenly distributed over CPYA medium (pH 12.0) in the incubator, where it was incubated for 24 hours at 37°C. The colonies that developed were purified by repeatedly streaking an isolated single bacterial colony on brand-new CPYA medium plates. Pure isolates were streaked on CPYA media plates17,18 and incubated for approximately 24 to 48 hours at 37? to assess the generation of alkaline protease. Plates were then loaded with a 10% Trichloroacetic Acid (w/v) solution (TCA) and left to incubate for 30 minutes. The protease activity was used to determine the size of the proteolysis zone, which was seen as a clear transparent zone surrounding the colony.

Enzyme assay and quantification of alkaline protease activity

Isolates with a hydrolysis zone in the good to the excellent range were chosen and employed for production under submerged conditions. A tiny volume of bacterial culture was inoculated into Erlenmeyer flasks (150 mL) using CPYA broth (50 mL, pH 12.0). These were incubated for 48 hours at 37?. The fermented broth was then centrifuged for 10 minutes at 7000 × g. The enzyme assay was performed on thus obtained cell-free crude filtrate.

The crude filtrate was checked for extracellular protease activity on CPYA plates19 and determination of alkaline protease activity was done by a modified protease activity assay6 with casein as a substrate. Casein (1% w/v) solution was made in 50 mM potassium phosphate buffer (pH 12), by adjusting the pH with NaOH or HCl. Aliquots of 1.0 mL of casein solution were equilibrated at 50? for 15 min in tubes. The assay was commenced by adding 0.2 mL of crude enzyme filtrate in the test reaction mixture but not in reaction mixtures which are to be used as blanks and all the tubes were incubated at 50? temperature for 20 min. Consequential tyrosine liberation due to protease activity during this incubation time was measured and compared among different isolates. Trichloro Acetic acid was used as termination of enzyme reaction by addition of the 2 mL of 10% (TCA) solution in test and blank reaction mixtures both. Now 0.2 mL of crude enzyme filtrate is also added in blank reaction mixtures to account for the absorbance value of the crude enzyme itself and ensure that the final volume in each tube is equal. Then reaction mixture was allowed to stand for 30 min. After 30 min incubation, the reaction mixtures were centrifuged at 7000 × g for 10 min. One mL supernatant of these and one mL of differently diluted tyrosine standard solutions were added with 5 mL of 2% Na2CO3 (alkaline solution) in test tubes and incubated for 10 min. To the above solution 0.5 mL of Folin-Ciocalteu's reagent working solution (made by mixing one volume of the stock solution and two volumes of distilled water) was added and tubes were incubated for 30 min in dark. The color due to reaction with reagent was measured at 750 nm. The L-tyrosine standard stock solution was prepared to have final concentration of 100 mg /mL. This solution was diluted further in different tubes appropriately with distilled water to make the tyrosine standard curve which was used to interpolate released tyrosine concentration from unknown samples. The amount of enzyme that liberated 1g tyrosine per ml per minute under the conditions described above was defined as one unit of protease activity.

Phenotypic characterization

Phenotypic characterization of the strain was performed following the standard schemes given in Bergey's Manual of Determinative Bacteriology20.

Identification using molecular approach

DNA Isolation, PCR Amplification and DNA Sequencing

Using a genomic DNA isolation kit, genomic DNA was extracted from the isolate with the highest potential for generating alkaline proteases. Using universal primers, the 16s rRNA gene was successfully amplified. After that, amplified DNA was submitted to Ahmadabad, Gujarat's "Genexplore Diagnostics and Research Centre Pvt. Ltd." for DNA sequencing.

Identification

The resulting DNA sequence was compared to the NCBI 16s rRNA gene database using BLAST (Fig. 1) (National Centre for Biotechnology Information). Using the MEGA7 programme and the highest likelihood option21, a phylogenetic tree was created using related bacterial 16s rRNA gene sequences from NCBI. The Neighbor-Joining method22 was used to infer the evolutionary history.

The most promising one was identified using 16S rRNA homology analysis and phylogenetic tree analysis. The isolate's 16s rRNA gene sequence was submitted to Genbank (https://www.ncbi.nlm.nih.gov/genbank).

RESULTS

Isolation and screening

A high proportion of isolates were collected and tested for alkaline protease production. Colonies with various characteristics that developed on CPYA media plates were isolated and utilized to solubilize casein on CPYA media. Whereas many colonies appeared on isolation plates from Badi pul Dhobi ghat (DH) locality of Rewa and Agriculture field soil of Hatwa (HS), Sidhi, very few bacterial colonies appeared

on isolation from Forest soil (FS) samples of Mukundpur Zoo and Tiger breeding center (Fig. 2). A total number of 6 isolates: Isolates no. HS1, HS2, HS3 (from Hatwa, Sidhi); DH11, DH12 and DH31 (from Badi pul Dhobi ghat Rewa) that produced a clearing zone in the excellent to good range were chosen for future research (Fig 3). Among all the isolates, HS3 had the largest zone of clearances of 2.2 cm, followed by HS1, HS2, DH31, DH11, DH12 which produced 1.8 cm, 1.7 cm, 1.6 cm, 1.4 cm, 1.2 cm zone of clearance respectively (Table 1).

Enzyme assay and Quantification of alkaline protease activity

After confirming the extracellular protease production of cell free crude extract on solid media, crude filtrates were subjected to enzyme assay in submerges condition. Isolate no. HS1, HS2, HS3, DH11, DH12 and DH31 crude enzyme filtrates have 0.027U /mL, 0.025 U /mL, 0.039 U /mL, 0.014 U /mL, 0.008 U /mL, 0.023 U /mL protease activity, respectively. Isolate no. HS3 produced highest protease activity of 0.039 U /mL among all and selected for further study. Table 2 shows the amount of tyrosine released from casein and equivalent proteases units.

Phenotypic characterization

All of the isolates had small, spherical, and regular colonies. Isolate no. HS1, HS2, HS3, DH11, DH12, and DH31 had creamy white, off white, white, creamy white, creamy white, creamy white, and off-white colonies, respectively. All of

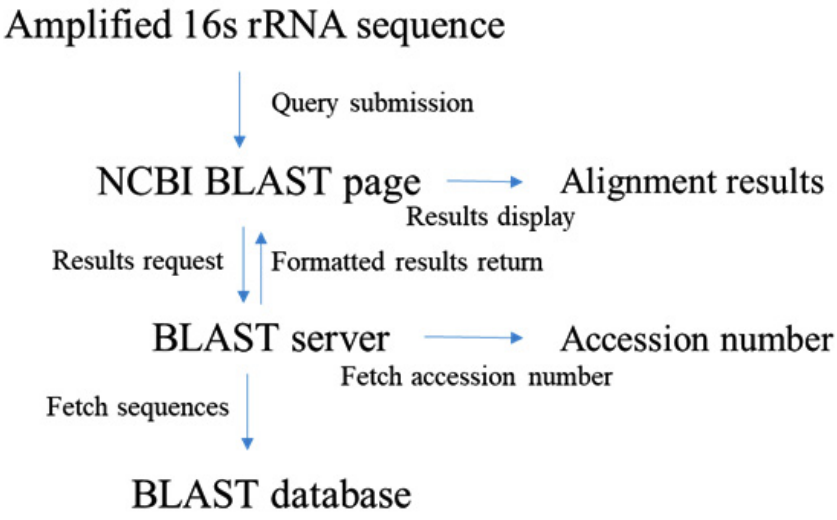

Fig. 1. Blast Flowchart for 16s rRNA homology analysis

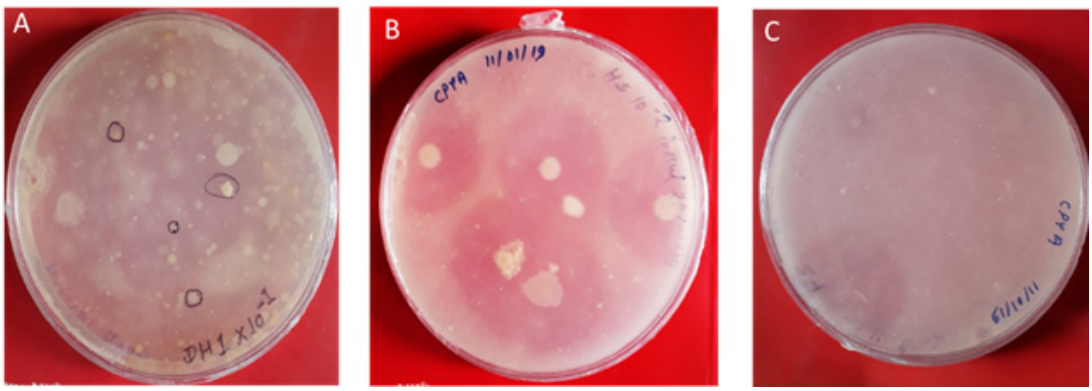

Fig. 2. Primary screening of soil samples on CPYA medium having pH 12

the isolates were long, filamentous, rod-shaped, and Gram positive in gramme staining (Fig.4, Table 1). Morphological and biochemical characters of strain RSCVS-HS3 is given in Table 3. The organism is motile, gram-positive, endospore-forming, and rod-shaped. It showed positive results for catalase, oxidase tests, casein hydrolysis and negative for nitrate reductase and starch hydrolysis. It could produce acids from L-arabinose.

Identification using a molecular approach

DNA Isolation, PCR Amplification and DNA Sequencing

DNA was extracted from the most potent isolate, HS3, and PCR amplification was accomplished using the 357F forward primer, which had the sequence CTCCTACGGGAGGCAGCAG, and the 1391R reverse primer, which had the sequence GACGGGCGGTGTGTRCA. The following steps were performed in the PCR reaction: 1 cycle of initial denaturation at 95? for 5 minutes; 35 cycles of the following steps: Denaturation at 95? for 30 seconds, Annealing at 49? for 30 seconds, Elongation at 72? for 1:30 minutes, and one cycle of Final Extension at 72? for 10 minutes23 (Fig. 5).

Identification

The 16S rRNA gene homology was used to molecularly identify isolate no. HS3. The 16S

Table 1. Zone of Hydrolysis and Phenotypic characters of alkaline protease enzyme producing selected isolates

S. No.	Isolate No.	Color of colony	Gram reaction	Cell shape	Zone of hydrolysis (in cm)
1	HS1	Creamy white	Positive	Rod	1.8
2	HS2	Off white	Positive	Rod	1.7
3	HS3	White	Positive	Rod	2.2
4	DH11	Creamy white	Positive	Rod	1.4
5	DH12	Creamy white	Positive	Rod	1.2
6	DH31	Off white	Positive	Rod	1.6

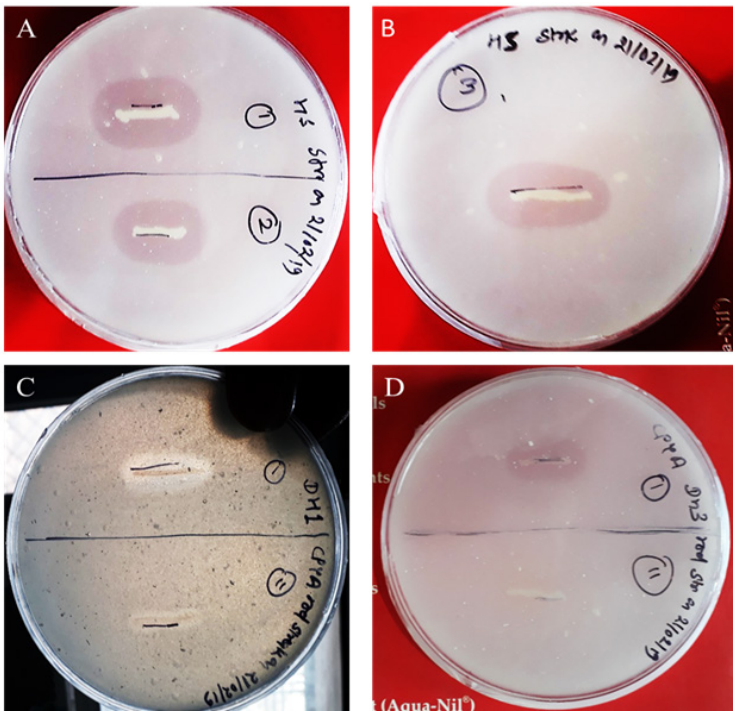

Fig. 3. Zone of clearance produced by selected isolates

rRNA sequence of isolate no. HS3 had 95 percent similarity with the sequence of Fermentbacillus polygoni IEB3 after investigation with the nucleotide BLAST programme (Accession number Genebank: NR149287.1). With closely related 16s rRNA gene sequences from other bacillus species, a phylogenetic tree was generated using the MEGA7 programme (Fig. 6). On the basis of both, 16S rRNA homology analysis and phylogenetic tree analysis with the reference strain, the isolate no. HS

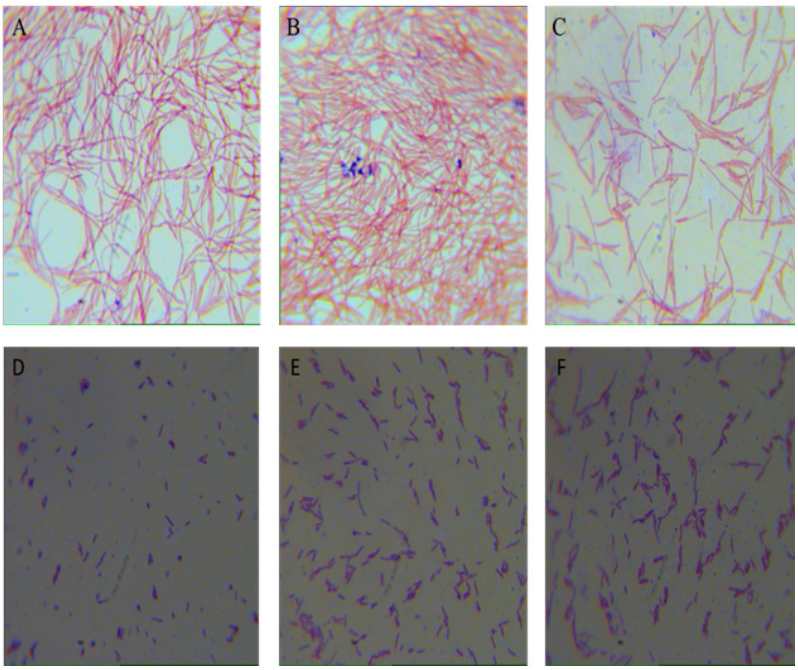

Fig. 4. Gram stain images of selected isolates with binocular microscope.

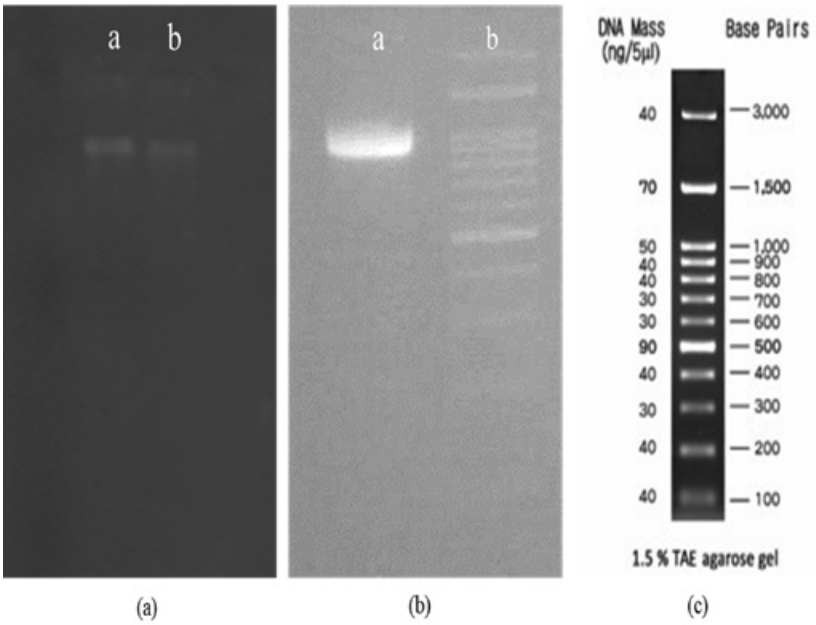

Fig. 5. Electrophoregram of isolated whole genome DNA and amplified 16s rRNA gene of Isolate no. HS3.

3 was identified as Fermentibacillus sp. RSCVS-HS3. The 16s rRNA sequence was submitted to the Genbank database with the accession number MT279752 (https://www.ncbi.nlm.nih.gov/genbank).

DISCUSSION

A large number of bacterial isolates were screened for alkaline protease production. Whereas many colonies appeared on isolation plates from Badi pul Dhobi ghat (DH) locality of Rewa and Agriculture field soil of Hatwa (HS), Sidhi, very few bacterial colonies appeared on isolation from Forest soil (FS) samples of Mukundpur Zoo and Tiger breeding centre (pH 7). It was interesting to note that alkaliphilic bacteria were also obtained from soil having neutral pH, as of forest soil of Mukundpur Zoo and Tiger breeding centre and agriculture field soil of Hatwa, Sidhi. Comparatively less in number or all most none of bacterial colonies appeared on isolation at such high alkaline pH 1224 on CPYA solid media even from alkaline soil samples. No microbial colony, other than bacteria, appeared even after 24 to 36 hours incubation25. Since fungi are known for being slow growing, thus no fungal colonies formed from the sample sites. Colonies with varying features were isolated and tested for protein degradation on CPYA medium plates. Pure culture was obtained out of which only some were able to hydrolyze casein protein26. Isolate no. HS3 produced highest

Table 2. Alkaline protease activity of culture filtrate of selected isolates.

Isolate No.	Tyrosine released (mg /mL)	Enzyme unit (mmol /mL/min)
HS1	6.1	0.027
HS2	5.7	0.025
HS3	8.8	0.039
DH11	3.2	0.014
DH12	1.8	0.008
DH31	5.2	0.023

Table 3. Morphological and biochemical characterization of RSCVS-HS3

S. No.	Characters	Results
1	Morphology	Rods
2	Spore forming	+
3	Motility	+
4	Gram	+
5	Catalase test	+
6	Oxidase test	+
7	Nitrate reduction test	-
8	Casein hydrolysis	+
9	Starch hydrolysis	-
10	Sucrose	-
11	Mannitol	-
12	L-Arabinose	+

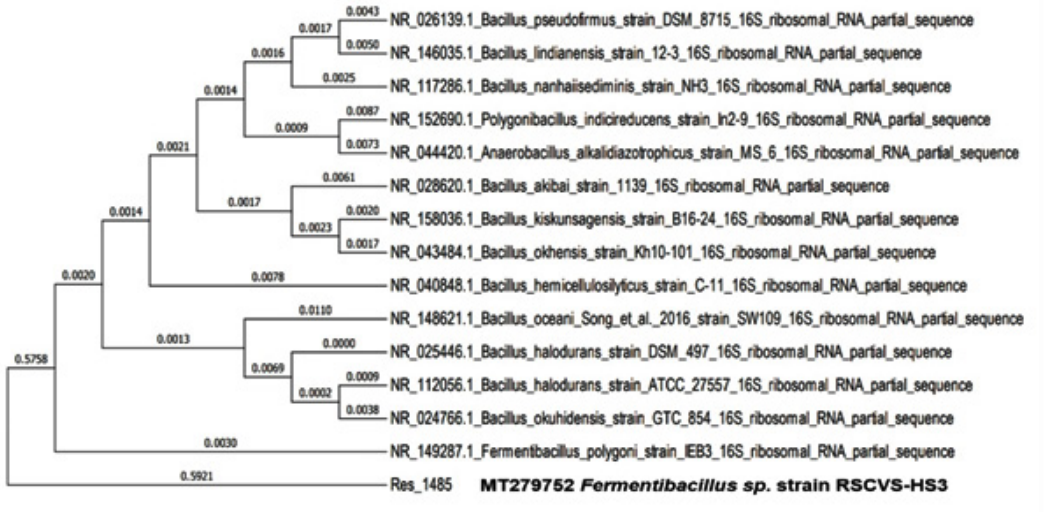

Fig. 6. Phylogenetic analysis of Fermentibacillus Sp. RSCVS-HS3, on the basis of similar 16s rRNA sequences

protease activity among all at high temperature of 50? and at pH 12 indicating its thermal stability and alkaline nature similar to many other alkaline bacillus species. The most potent alkaline protease producing isolate no. HS3, upon NCBI BLAST and Phylogenetic Analysis27 was identified as Fermentibacillus sp. RSCVS-HS3 because the closest similar reference bacteria, Fermentibacillus polygoni IEB3 showed 95.50 % similarity but was earlier reported to be negative to casein hydrolysis28 whereas it showed casein protease activity. It also showed positive results for catalase, oxidase tests and negative for nitrate reductase and starch hydrolysis. It could produce acids from L-arabinose. Fermentibacillus sp. RSCVS-HS3 exhibited phenotypic similarities to several other alkaline bacillus species, including being long, rod-shaped in morphology, gram-positive, white color colony, motile and spore-forming capabilities. The alkaline protease produced from it was extracellular like from many other alkaline proteases producing bacillus species, as was checked on solid media and by enzyme assay in submerged condition by calculating crude enzyme activity 0.039 micro mol /mL/min1,7,24,29,30. It was showing protease activity even at enzyme assay incubation temperature of 500C and at pH 12 indicating thermal stability and alkaline nature.

CONCLUSION

The study describes alkaline protease producing Fermentibacillus sp. RSCVS-HS3 (Genebank: MT279752) isolated from agriculture field soil of Vindhya region (Rewa division) of state of Madhya Pradesh, India. At pH 12, the bacterium was able to thrive. It demonstrated extracellular alkaline protease activity, and the raw enzyme filtrate was stable at 50? and pH 12, suggesting the enzyme's heat stability and alkaline nature. It was shown to be closely related to other alkali bacillus species. however, it was most closely related to Fermentibacillus polygoni IEB3 phenotypically and phylogenetically. But in contrast to closest reference bacteria Fermentibacillus polygoni IEB3, it showed casein protease activity. This is probably the first report stating alkaline protease production from Fermentibacillus sp. RSCVS-HS3 and also the first, reporting any alkaline protease producing bacterium from alkaline soil samples of Vindhya region Madhya Pradesh, India. Further refining of the enzyme manufacturing method and physio-chemical study of the enzyme might lead to the identification of the bacteria as a viable alkaline protease industrial possibility.

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Conflict of Interest

The authors declare no conflict of interest.

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