Bacillus halodurans RSCVS-PF21 an alkaline protease producing bacteria from poultry farm soil

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Abstract
The aim of the study was to investigate potent alkaline protease producing indigenous bacterial strains from alkaline soils of Vindhya region of Madhya Pradesh, India. Accordingly, isolation and screening of alkaline protease producing alkaliphilic bacteria was performed from different habitats of Vindhya region of Madhya Pradesh, India. Selection and identification of promising alkaline protease producing isolates, enzyme activity assay and partial characterization of crude alkaline protease filtrate were done.

Many alkaline proteases producing bacterial isolates were isolated from poultry farm soils of Vindhya region, Madhya Pradesh, India. Out of several proteolytic isolates, most potent new bacterial strain was identified as Bacillus halodurans RSCVS-PF21 (Genbank Accession No. MT279908) based on phenotypic, biochemical and molecular characterizations. Crude alkaline protease was stable and effective at high temperature. It was also highly stable in different organic solvents, so it can be used in many industrial processes.

Keywords: *Bacillus halodurans* RSCVS-PF21, Alkaline proteases, Bacteria, Poultry farm soil.

Introduction

Proteases (EC 3.4) comes under class of hydrolases of enzyme classification, that hydrolyses proteins into smaller polypeptides or single amino acids. They are the most dominant group of enzymes from the industry point of view, constituting about sixty percent of the total enzyme market[44]. Proteases from microorganisms have been the most widely explored enzymes since the advent of enzymology[40]. Microorganisms have been known to produce both intracellular and extracellular proteases commercially. They account for approximately two-third share of the total commercial protease sale around the globe[6].

Enzymes have gained attention not only due to their critical role in metabolic activities in the organisms but also due to their wide applications in industries[39] such as food, detergent, bakery, leather, pharma, infant formulas etc. due to their features like production ease, thermal stability and wide pH range applications[16]. Alkaline proteases (EC.3.4.21-24, 99) are the most frequently used industrial enzyme among all due to their comparatively high activity and greater stability at high pH[18]. Alcalase, Esperase, Savinase, Biofeed pro, Durazym, Protosol, Novozyme, Subtilisin, Maxatase, Maxacal are some brand names of alkaline protease used for different purposes such as in detergent industry, silk degumming, textile, food, feed etc.[7] Alkaline proteases have been isolated from diverse sources. It is believed that extremophile microorganisms produce more stable alkaline proteases.

Extremophilic microorganisms live in extreme ecological niches such as low or high temperatures, extremes of pH (pH 0-3 or 9-12), high pressures, high salt concentrations (5-30%)[34]. The proteases produced by microbes present in normal environment are usually unstable under extreme conditions, even with several attempts of physicochemical treatments, protein engineering and gene-shufflings[44]. Microorganisms living in extreme habitats are believed to produce proteases which are stable in those situations[44] but limited information is available reporting such microorganisms[41]. Novel microorganisms may yield novel enzymes with new combinations of properties e.g. alkaline proteases with halophilic and/or thermotolerant properties.

Temperature has a strong influence over the stability and catalytic activity of alkaline protease enzyme. Bacterial alkaline proteases have optimum temperatures of 50-60^{0}C, however, higher temperature has also been reported for some bacterial alkaline proteases e.g. *Bacillus licheniformis* MP1 at 70^{0}C[26] and *Bacillus* sp. MLA 64 at 95^{0}C[32]. In contrast, some alkaline proteases have optima at lower temperatures (37-40^{0}C)[42].

Alkaline proteases are known to be active over a wide range of pH (6-12). The pH optima of these usually lie within pH 9-11[29]. Alkaline proteases from Bacillus spp. have optimal pH values usually at pH 9-10. However, there are also reports on alkaline proteases with higher optimal pH values e.g. *Streptomyces* sp. at pH 11.5[47] and *Bacillus clausii* at pH 12[27].

Enzyme inhibition studies give crucial insights into the nature of enzyme action. Effects of various inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), ethylene-diamine tetraacetic acid (EDTA), di-isopropyl fluorophosphate (DFP), 1,10-phenanthroline, ethylene glycol tetraacetic acid (EGTA) and iodoacetic acid (IAA) have been studied to determine the class of the protease. Effect of organic solvents was reported by many studies. Aksoy et al[3] studied extracellular serine protease enzymes produced by *Thermoactinomyces* strains. Kumar and Bhalla[30] reported microbial proteases in peptide synthesis.

Both found that proteases could be used in ester synthesis considering their stability with organic solvents.

Alkaline proteases have high commercial potential accounting for 25% of the world global enzyme market[28]. These have applications in various sectors such as detergent, food and feed, pharmaceutical, leather, paper and pulp industries, textile, in silver recovery from photographic plates and in waste treatment.

Material and Methods

Chemicals: All the chemicals and reagents used in the study were obtained from HiMedia, India and Fisher Scientific, USA.

Collection of Soil Sample, Medium, Isolation and Screening of bacteria: Alkaline soil samples for the isolation of potent alkaline protease producing bacteria were collected from Government Kukkut Palan Kendra (poultry farm) near Chirahula temple, in district headquarters of Rewa of Madhya Pradesh, India. pH of soil samples was in range of 10-11. CPYA solid media was used for isolation and screening and CPYA broth was used for crude enzyme production and enzyme activity analysis[10].

Enzyme Production and Alkaline Protease Activity Analysis: Isolates producing clear zone around the colony in the range of good and excellent were selected for enzyme production under submerged conditions. The resulting cell-free crude filtrate was further used for enzyme assays. Alkaline protease production from these isolates was examined by radial diffusion assay method. The enzyme activity was calculated as relative enzyme activity (REA).

Enzyme Assay: The universal protease activity assay with casein as substrate[6] was followed with modifications to determine the protease activity of the crude proteases from potential protease producing bacterial strains. Casein (1% w/v) solution was made in 50 mM potassium phosphate buffer (pH 12). The pH of buffer was adjusted with NaOH or HCl. Aliquots of 1 ml of casein solution were equilibrated at 50ºC for 15 min in tubes. The assay was commenced by adding of 0.2 ml of crude enzyme filtrate in test reaction mixture but not in reaction mixtures to be used as blanks. All the tubes were incubated at 50ºC 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 for termination of enzyme reaction by addition of 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 to 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% Na_2CO_3 (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 distil water) was added and tubes were incubated for 30 min in dark. The colour due to reaction with reagent was measured at 750 nm.

L-tyrosine standard stock solution was prepared having final concentration of 100 µg/ml: Weigh 0.1000 g, L-tyrosine and dissolve in 60 ml hydrochloric acid (1 mol/L). The solution was transferred into a 100 ml volumetric flask and the hydrochloric acid solution (1 mol/L) was added to a total volume of 100 ml. This gave a solution of L-tyrosine of 1 mg/ml. Then mix 10.00 ml of L-tyrosine solution (1 mg/ml) with the hydrochloric acid solution (0.1 mol/L) in a 100 ml volumetric solution. Add the hydrochloric acid solution (0.1 mol/L) to a total volume of 100 ml to give the standard solution of L-tyrosine (100 µg/ml).

This solution was diluted further (to have 0, 20, 40, 60, 80 and 100 µg/ml tyrosine concentrations) in different tubes with distilled water to make the tyrosine standard curve which was used to determine released tyrosine concentration from unknown samples. One unit of protease activity was defined as the amount of enzyme that released 1µmol tyrosine per ml per minute under the conditions described above. The enzymatic activity was determined with the micro moles of tyrosine released using the formula:

$$\text{Units/ml (Enzyme Activity)} = \frac{(\mu\text{mol tyrosine equivalents released}) \times (\text{Total volume (in mol) of assay})}{(\text{Volume of Enzyme (in ml) used}) \times (\text{Time of assay (in min)}) \times (\text{Volume (in ml) used in Colorimetric Determination})}$$

Phenotypic Characterization of Bacteria: Phenotypic characterization of the strain was performed following the standard schemes given in the Bergey's Manual of Determinative Bacteriology[22].

DNA Isolation from Bacteria: Total genomic DNA was isolated from the most promising alkaline protease producing isolate, using HiPer Bacterial genomic DNA extraction kit.

PCR Amplification and DNA Sequencing: After DNA isolation, amplification of 16s rRNA gene with 16s rRNA universal primers was done by PCR[10]. Thus, amplified 16srRNA gene was further used for DNA sequencing. 16s rRNA gene PCR amplification and sequencing was performed by Genexplore Diagnostics and Research Centre Pvt. Ltd. Ahmadabad, Gujrat, India.

Molecular Characterization: The 16s rDNA sequence was subjected to Blast analysis against 16s rRNA gene database

in NCBI (National Centre for Biotechnology Information). A phylogenetic tree was constructed with related bacterial 16s rRNA gene sequences from NCBI using MEGA7 program by maximum likelihood option[31]. The evolutionary history was obtained using the Neighbor-Joining method. Based on 16S rRNA homology and phylogenetic tree analysis, the most promising isolate was identified. 16s rRNA gene sequence of the isolate was submitted to Genbank database (https://www.ncbi.nlm.nih.gov/genbank)[10].

Effect of Physiochemical Factors on Crude Alkaline Protease Activity and Stability: The effect of pH, temperature, organic solvents and surfactants on crude alkaline protease, was investigated according to Cui et al[12] with slight modifications.

Effect of pH on Crude Alkaline Protease Activity and Stability: The range of pH 7.0–12.0 was selected to observe the optimal pH of the crude protease activity at 50^0C for 20 min with 1% casein as a substrate. 200 mM sodium phosphate buffer was used for pH 7.0–8.0 and 200 mM glycine-NaOH buffer was used for pH 9.0–12.0. The influence of pH on cruse protease stability was also determined. For this, crude alkaline protease filtrate was incubated in advance in different pH buffers of 7.0–12.0 for 60 min at 50^0C. The incubations were then taken to measure the residual activity at 50^0C at pH 12.0.

Effect of Temperature on Crude Alkaline Protease Activity and Stability: The range of temperature 20^0C–70^0C was selected to observe the optimal temperature of the crude protease activity at pH 12.0 for 20 min with 1% casein as a substrate. Thermal stability of the protease was also investigated. The crude protease was incubated for 60 min at different temperatures in advance (20, 30, 40, 50, 60 and 70^0C). These incubations were then taken to examine the residual enzyme activity at 50^0C and pH 12.0. The unheated protease was considered as 100%.

Effect of Organic Solvents and Surfactants on Crude Alkaline Protease Activity: The effect of various organic solvents on crude enzyme activity was examined. The crude enzyme was incubated in 100 mM glycine-NaOH buffer system (pH 12.0) in advance with 25% (v/v) of several organic solvents (isopropanol, methanol and acetone) and shaken in incubator at 150 rpm for 20 min at 50^0C. The incubations were then taken to determine the residual activity under enzyme assay conditions. The protease activity was regarded as controls without any organic solvents.

The effects of 5 mM surfactant SDS and 1% (v/v) oxidizing agent H_2O_2 on crude protease activity were investigated by incubating these with crude enzymes in advance at 50^0C for 20 min. The incubations were then taken to determine the residual enzyme activity under enzyme assay conditions. The protease activity was regarded as 100% without SDS or H_2O_2.

Results and Discussion

Enzyme Assay and Quantification of Alkaline Protease Activity: After confirming the extracellular protease production of crude extract on CPYA solid media, crude filtrates of selected isolates were subjected to enzyme assay. Table 1 shows the amount of tyrosine released from casein and equivalent proteases units. Isolate no. PF11, PF 13, PF21 crude enzyme filtrates have 0.027U/ml, 0.025 U/ml, 0.036 U/ml protease activity respectively. Isolate no. PF21 produced highest protease activity of 0.036 U/ml among all and was selected for further study. It was showing good protease activity even at enzyme assay incubation temperature of 50^0C at pH 12 indicating its thermal stability and alkaline nature. Contesini et al[11], Daoud et al[13] and Yakul et al[46] have also reported bacterial alkaline protease activity at high temperatures and at high alkaline pH.

Phenotypic and Biochemical Characterization of RSCVS-PF21 (Isolate no. PF21): Morphological and biochemical characters of bacterial strain RSCVS-PF21 (Isolate no. PF21) are given in table 2. The organism is Gram positive, endospore forming, rod shaped and motile. It showed positive results for catalase, oxidase, NR (Nitrate reduction) test, MR (Methyl red) test, gelatine liquefaction, casein and starch hydrolysis tests and negative for citrate utilization and VP (Voges-Proskauer) test.

It could produce acids from glucose, sucrose, mannitol and arabinose. The phenotypic and biochemical features of the organism agreed with the description of the *Bacillus*, as given in Bergey's Manual of Determinative Bacteriology[22]. Thus, based on phenotypic characteristics, RSCVS-PF21 was putatively identified as a *Bacillus*.

Molecular Identification: According to Chauhan and Mishra[10], the molecular identification of isolate no. PF21 was done using 16S rRNA gene homology. DNA was isolated and about 1112 bp consensus sequence was obtained after amplification of 16s rRNA gene with 357F and 1391R universal primers and DNA sequencing of amplified product (Figure 1).

Table 1
Alkaline protease activity of culture filtrate of selected bacterial isolates from poultry farm soil.

S.N.	Isolate No.	Tyrosine released (μg/ml)	Enzyme unit (μmol/min/ml)
1	PF11	6.1	0.027
2	PF13	5.7	0.025
3	PF21	8.1	0.036

Table 2
Morphological and biochemical characterization of RSCVS-PF21

S.N.	Character	Result
1	Morphology	Rods
2	Spore forming	+
3	Motility	+
4	Gram	+
5	Catalase test	+
6	Oxidase test	+
7	Methyl red test	+
8	Voges-Proskauer Test	-
9	Nitrate reduction test	+
10	Citrate utilization test	-
11	Gelatin hydrolysis	+
12	Casein hydrolysis	+
13	Starch hydrolysis	+
14	D-Glucose	+
15	Sucrose	+
16	D-Mannitol	+
17	L-Arabinose	+

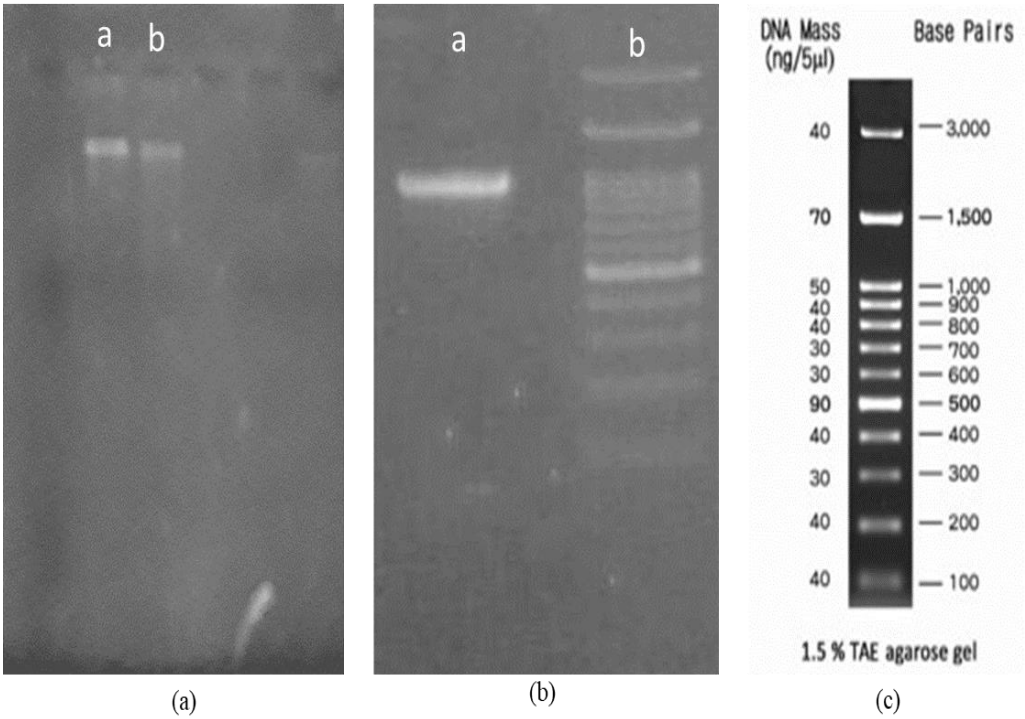


Figure 1: Agarose Gel Electrophoresis of isolated genome DNA and amplified 16s rRNA gene of Isolate no. PF21. (a) Isolated genome DNA of Isolate no. PF21. Both lane a and lane b, denotes the genome DNA of Isolate no. PF21, (b) 16s rRNA gene amplification with 357F forward and 1391R reverse, Universal 16s rRNA primers. Lane a: Amplified 1112 bp PCR product; Lane b: HiMedia 100bp DNA ladder, (c) HiMedia 100bp DNA ladder image with band size.

According to Chauhan and Mishra[10], the molecular identification of isolate no. PF21 was done using 16S rRNA gene homology. A phylogenetic tree was constructed using MEGA7 program with closely related 16s rRNA gene sequences of other bacillus species (Figure 2). Based on both, 16S rRNA homology and phylogenetic tree analysis, the isolate no. PF21 was identified as *Bacillus halodurans* RSCVS-PF21. 16s rRNA gene sequence was submitted to Genbank database (https://www.ncbi.nlm.nih.gov/genbank) with accession no. MT279908. It was found by analysis with nucleotide BLAST tool that 16S rRNA sequence of isolate no. PF21 had 95.64 % identity with the sequence of *Bacillus halodurans* DSM497 (Genebank Accession number: NR025446).

Even though *Bacillus halodurans* DSM497 was not reported to produce alkaline protease[8,15,35] some other identical bacteria were *Bacillus halodurans* strain ATCC 27557 (95.13 %) and *Bacillus okuhidensis* strain GTC 854 (95.03 %), *Fermentibacillus polygoni* strain IEB3 (94.02 %), *Bacillus nanhaiisediminis* strain NH3 (94.02 %). However recently some *Bacillus halodurans* strains were reported to produce alkaline proteases[13,46]. Phenotypically also *Bacillus halodurans* RSCVS-PF21 was related with many other alkaline proteases producing bacillus species, having long, rod shaped, Gram positive, white colony, motile and spore forming characteristics.

Effect of pH on Activity and Stability of the Crude Alkaline Protease from *Bacillus halodurans* RSCVS-PF21: The influence of pH on crude filtrate protease activity of strain *Bacillus halodurans* RSCVS-PF21 (Figure 3a) was examined with the pH range from 7.0 to 12.0 at 50^{0}C. The crude protease from *Bacillus halodurans* RSCVS-PF21 showed high activity in a broad pH range of 9.0–11.0 and the optimal pH was about 10.0. The relative activity of the crude enzyme was 60% at pH 11.0 and 40% at pH 12.0.

Results showed that crude protease was alkaline protease because it showed high enzyme activity in alkaline condition. Alkaline proteases from Bacillus spp. have optimal pH values usually at pH 9-10, e.g. *B. subtilis* PE-11 (pH 10)[2], *Bacillus laterosporous* (pH 9)[5], *Bacillus sp*. B001 (pH 10)[14] and *B. licheniformis* KBDL4 (pH 10)[36]. Alkaline proteases from other bacteria such as *Pseudomonas aeruginosa*[37,38], *Halogeometricum borinquense*[45], Streptomyces *gulbargensis* and *Streptomyces albidoflavus*[1] also showed optimum activity at pH 9-10. However, there are also reports on alkaline proteases with higher optimal pH values e.g. *Streptomyces* sp. (pH 11.5)[47] and *Bacillus clausii* (pH 12)[27]. *Subtilisin carlsberg*, a commercial detergent enzyme, produced by *Bacillus licheniformis* has also shown maximum activity at pH values of 8-10.5[23]. High enzyme activity in alkaline conditions is a significant element of nearly all detergent enzymes[4]. This was also described by Hadj-Ali et al[21], Li et al[33], Daoud et al[13] and Yakul et al[46].

The activity of the crude enzyme from *Bacillus halodurans* RSCVS-PF21 (Figure 3b) was very stable at pH between 7.0 and 12.0 (˜80%). This was in accordance to study by Tsai[43] et al.1988 and Jaouadi[25] et al. 2008, which reported that protease in detergents require broad pH stability (9.0–11.0). From above discussion, it was clear that the crude enzyme produced by strain *Bacillus halodurans* RSCVS-PF21 might be useful in the detergent industry as well as in other industrial applications, which require high alkaline pH stability of enzymes.

Effect of Temperature on Activity and Stability of the Crude Alkaline Protease from *Bacillus halodurans* RSCVS-PF21: The results showed that the crude alkaline protease from *Bacillus halodurans* RSCVS-PF21(Figure 4a) was active from 20 to 70^{0}C temperature with an optimal temperature at ~ 40^{0}C. The relative activities of crude alkaline protease from strain *Bacillus halodurans* RSCVS-PF21 were about ~88% and ~84% at 50 and 60^{0}C respectively.

The low optimum temperature of crude filtrate, was in accordance with lower optimum temperatures (37-40^0 C) of alkaline proteases reported by Chakrabarti et al[9], Gupta et al[19] and Tremacoldi et al[42].

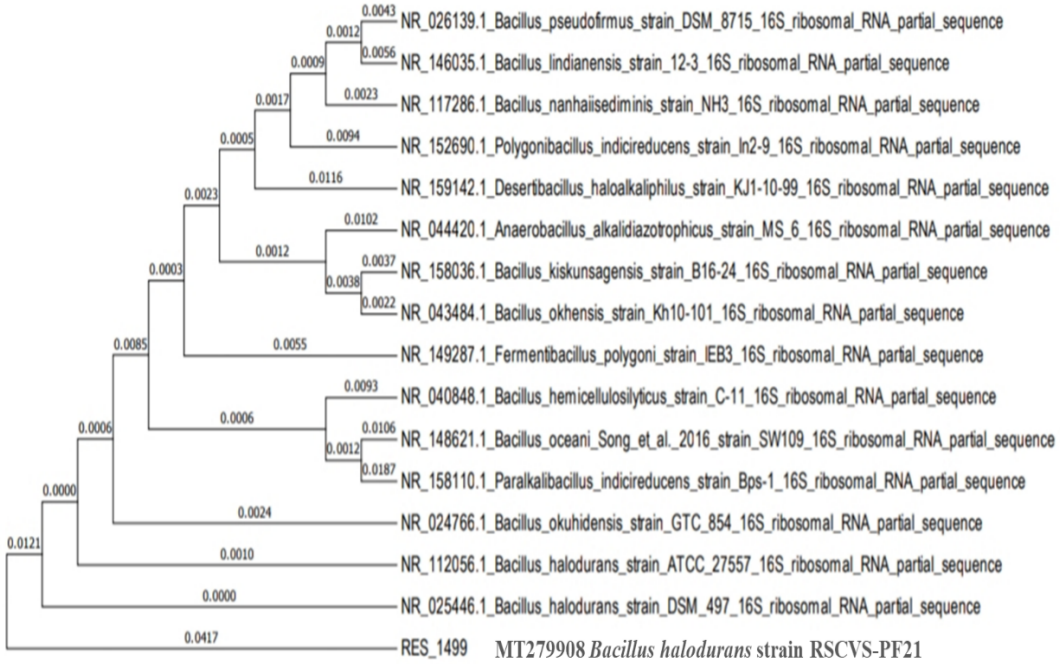
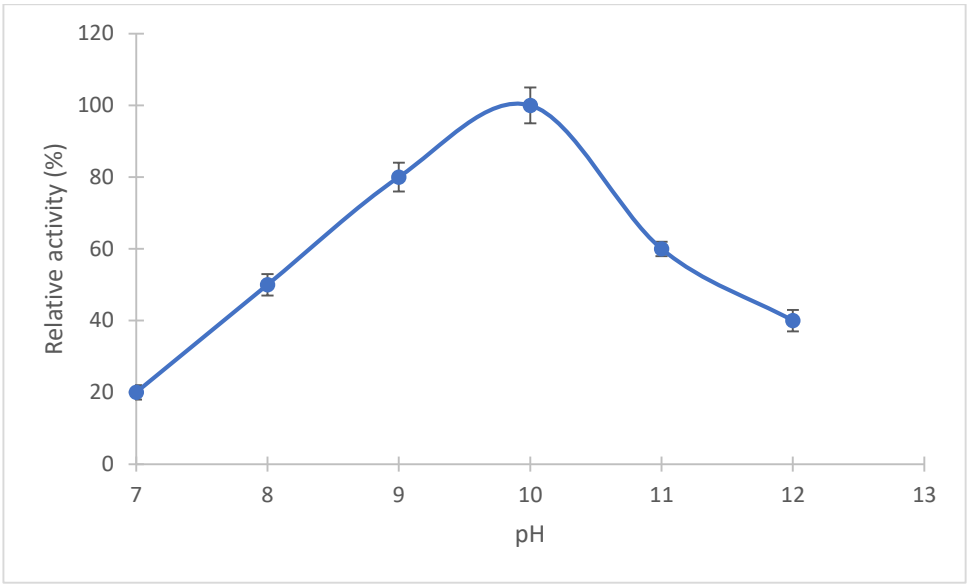
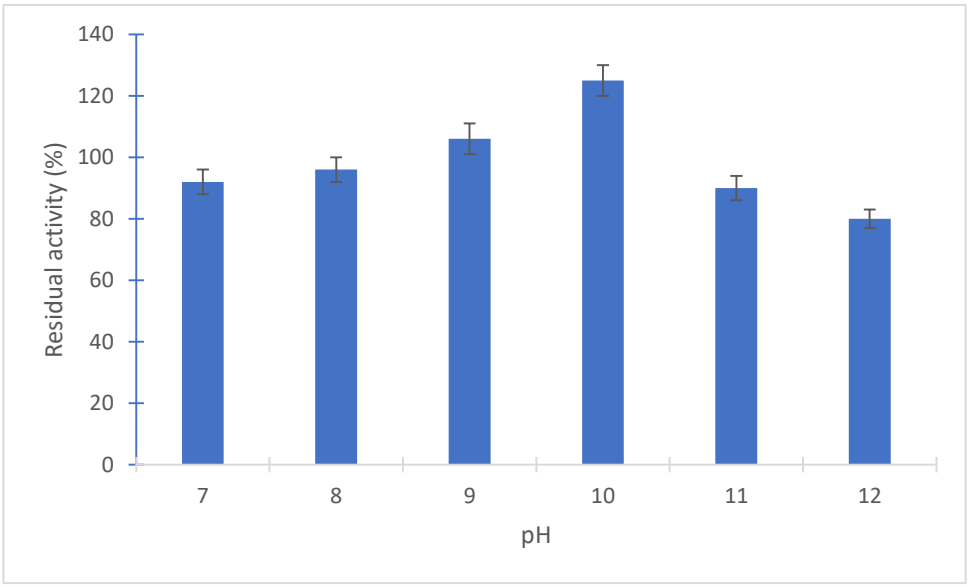


Figure 2: Phylogenetic analysis of *Bacillus halodurans* RSCVS-PF21, based on 16s rRNA sequences of other related species.



(a)



(b)

Figure 3: The effect of pH on (a) activity and (b) stability of the crude alkaline protease from *Bacillus halodurans* RSCVS-PF21. (a) The crude enzyme activity was tested in different pH buffer solutions at 50^{0}C. The crude enzyme activity was maximum at pH 10.0 which was regarded as 100%. (b) The crude protease stability against pH was tested after the crude enzyme was incubated in advance in different buffer solutions for 60 min at 50^{0}C. The residual crude enzyme activity was tested at 50^{0}C and pH 12.0. The crude enzyme activity was regarded as 100% without incubating.

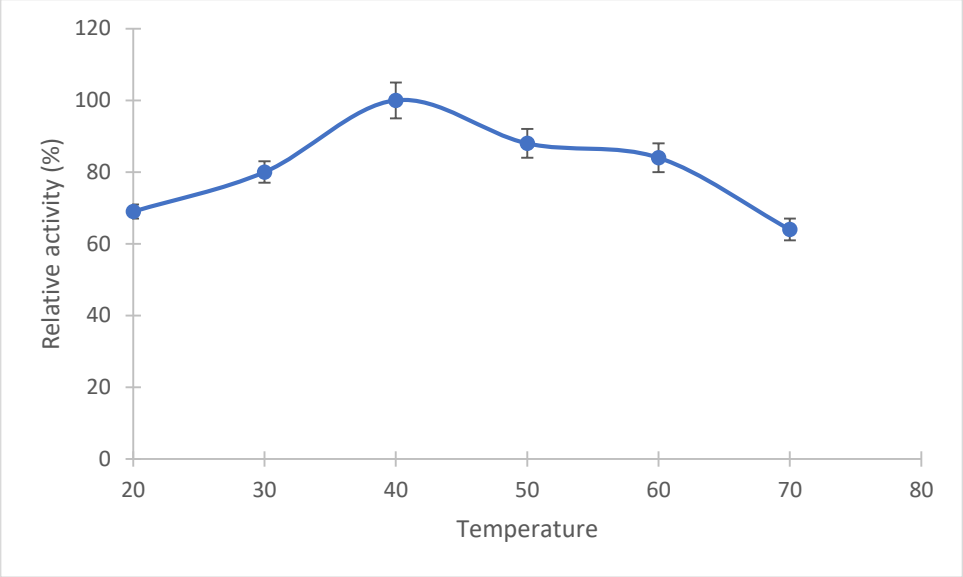
However, the optimal temperature of the protease was reported 60^{0}C from *B. pumilus*[28], *Bacillus subtilis* PE-11[2], *Bacillus mojavensis*[6], *Bacillus cereus* BG1[17] and *Bacillus* sp.[14] Higher temperature optima have also been reported for some bacterial alkaline proteases e.g. *Bacillus licheniformis* MP1 (70^{0}C)[26] and *Bacillus* sp. MLA 64 (95^{0}C)[32]. High alkaline protease activity at high temperature was also reported recently, by Daoud et al[13], Razzaq et al[39] and Yakul et al[46].

Thermostability investigations of the crude protease from *Bacillus halodurans* RSCVS-PF21 (Figure 4b) indicated that the crude protease enzymes were stable within temperatures 50^{0}C after 60 min incubation. The enzymes remained fully active after 60 min incubation at 50^{0}C. Even at 60 and 70^{0}C, the residual protease activity from *Bacillus halodurans* RSCVS-PF21 was 72% and 66% respectively. It showed that crude enzyme could be used under moderate to high heating conditions.

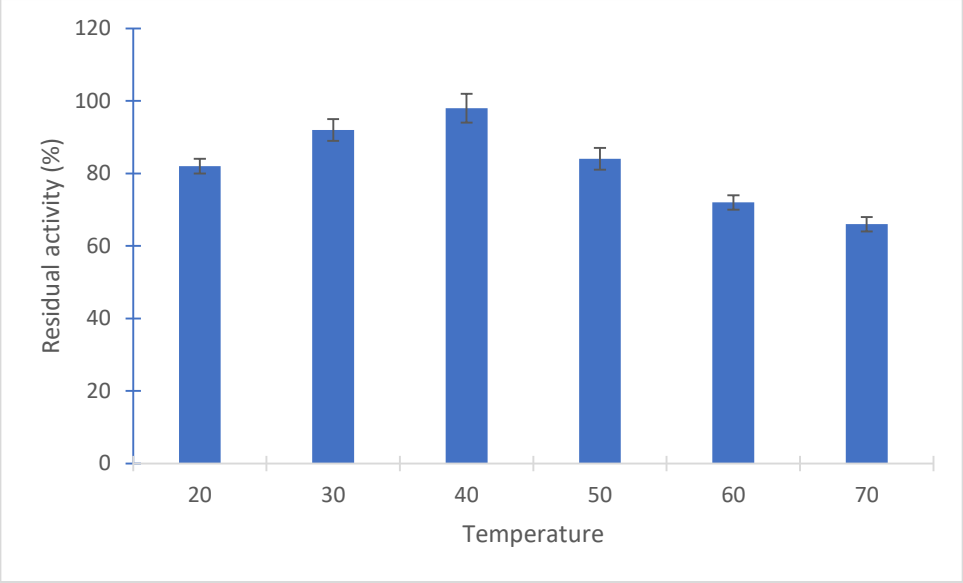
Hutadilok-Towatana et al[24], Haddar et al[20]and Deng et al[14] also reported thermal stability of alkaline proteases at higher temperatures. Thermal stability is a superior quality for enzyme application in laundry detergent formulations[19].

Effect of Surfactant and Organic Solvents on Activity of Crude Alkaline Protease from *Bacillus halodurans* RSCVS-PF21: A good protease used in detergent industry must be effective in wash performance and it should be stable and compatible with other detergent components such as oxidizing agents, surfactants etc.[29]

The crude protease filtrates from *Bacillus halodurans* RSCVS-PF21 were preincubated at 50^0C for 20 min existing with SDS and H_2O_2 and the residual enzyme activity was determined at 50^0C and pH 12.0 (Table 3). Crude enzyme was found to be very stable with the SDS at 5 mM. This differed from the study by Hadj-Ali et al[21] who reported that inhibition of enzyme activity with SDS was a common characteristic of serine alkaline proteases. Oxidizing agents H_2O_2 at 1% (v/v) showed a moderate inhibition. Although, oxidizing agents and surfactants had been reported to destabilize several alkaline proteases, the crude enzymes from *Bacillus halodurans* RSCVS-PF21 seemed very stable when these detergent additives were present. The influences of enzyme activity by different organic solvents were determined at 25% concentration at 50^0C and pH 12.0. The crude enzyme activities of *Bacillus halodurans* RSCVS-PF21 (Table 3) were enhanced by methanol and acetone while it was almost not affected by isopropanol.



(a)



(b)

Figure 4: Effect of temperature on the (a) activity and (b) stability of the crude protease from *Bacillus halodurans* RSCVS-PF21. (a) The activity of the protease enzyme was tested at various temperatures (20–70^0C). The crude enzyme activity was maximum at 40^0C regarded as 100%. (b) The crude protease stability against temperature was examined after these were incubated under temperatures between 20 and 70^0C for 60 min.
The residual activity was tested under the standard assay conditions. The enzyme activity incubating was considered as 100% without incubation.

Table 3
Effect of Surfactant and Organic Solvents on activity of crude alkaline protease from *Bacillus halodurans* RSCVS-PF21

S.N.	Additives	Concentration	Activity (%)
1	None	-	100
2	SDS	5mM	90 +/- 1
3	H_2O_2	1 % (v/v)	85 +/- 1.2
4	Methanol	25 % (v/v)	120 +/- 2.2
5	Isopropanol	25 % (v/v)	102 +/- 1.5
6	Acetone	25 % (v/v)	115 +/- 1.2

It can be concluded that the crude enzyme produced by *Bacillus halodurans* RSCVS-PF21 was highly stable in these organic solvents. Kumar and Bhalla[30] and Aksoy et al[3] have also reported high stability of alkaline proteases with organic solvents. This property can be used in ester synthesis.

Conclusion

Many alkaline proteases producing bacterial isolates were isolated from soils of different habitats of different localities of Vindhya region, Madhya Pradesh, India. It was found that very few bacterial colonies appeared on pH 12 on CPYA solid media. No fungal colony appeared even after 24 to 36 h incubation on pH 12. Only few bacterial isolates were able to hydrolyse casein. Out of several proteolytic isolates new bacterial strain was identified as *Bacillus halodurans* RSCVS-PF21 (Genbank accession no. MT279908, based on phenotypic, biochemical and molecular characterizations.

This could produce extracellular alkaline proteases, which showed high crude enzyme activity even at pH 12 and 50^0C temperature. Crude enzymes had very high pH optima (10-11) and were stable and active on broad pH range (7-12) (>80 % residual activity). It showed optimum temperature at 40^0C and was stable and active even beyond 50^0C temperatures (> 60 % residual activity). Crude protease was also stable in presence of surfactants and oxidizing agents which was an attractive feature for their potential use as a detergent additive. It was also highly stable in different organic solvents, so it can also be used in ester synthesis.

Thus, from above it is clear that novel alkaline proteases producing bacterial strain investigated from Vindhya region, M.P., India can be potential candidates in industrial applications which require protease activity in extreme conditions. The leads obtained in this investigation will form a valuable baseline database for future researchers in the region.

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