British Microbiology Research Journal
15(2): 1-14, 2016, Article no.BMRJ.26065
ISSN: 2231-0886, NLM ID: 101608140

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Characterization of Detergent-compatible Alkaline Protease from *Bacillus agaradhaerens* MTCC 9416

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Authors' contributions

This work was carried out in collaboration between all authors. Author NP contributed to the conception, design and coordination of this study along with analysis of results, phylogenetic analysis literature searches and drafting the manuscript. Author KM participated in the interpretation of data, helped in the statistical analysis, phylogenetic analysis and in editing the manuscript. Author PR assisted in editing the manuscript and discussion of results. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/26065
Editor(s):
(1) Ana Cláudia Coelho, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Fabio Tonissi Moroni, Universidade Federal de Uberlândia, Brazil.
(2) Mohamed Adel Ali Hassan, Genetic Engineering and Biotechnology Research Institute (GEBRI), Alexandria, Egypt.
(3) Omrane Belhadj, University of Tunis El Manar, Tunisia.
Complete Peer review History: http://sciencedomain.org/review-history/14962

Original Research Article

Received 31st March 2016
Accepted 26th May 2016
Published 8th June 2016

ABSTRACT

Aim: The present work deals with the purification and characterization of an alkaline protease produced by an alkalophilic bacterium, *Bacillus agaradhaerens* and establishment of its suitability as detergent additive.
Methodology: Bacterial isolates producing alkaline protease were screened from diverse samples *viz*. soil, sewage and industrial effluents by enrichment culture technique. The taxonomic status and molecular characterization of the bacterium showing maximum alkaline protease activity was determined. The alkaline protease produced by the organism was purified its molecular size was determined by gel permeation chromatography. The purified enzyme was studied for its feasibility as detergent additive.
Results: The bacterium under study was identified as *Bacillus agaradhaerens* by CSIR Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited with an accession number

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MTCC 9416. The genotypic characterization of the 16S ribosomal DNA gene was performed and the sequence was submitted to NCBI under the name *Bacillus agaradhaerens* strain nandiniphanse5 (NCBI Accession No: JN703504). The alkaline protease with a molecular weight of approximately 25 kDa, demonstrated optimum activity at 55℃ and pH 10.5, stability in pH range 7.0 to 12.0. The enzyme exhibited increased thermostability in presence of 25 mM $CaCl_2$, enhanced activity in presence of chlorides of Ca^{2+}, Mg^{2+}, K^{+}, Co^{2+} and Mn^{2+}. The protease exhibited highest degradation of casein followed by gelatin as compared to other protein substrates. The kinetic parameters were estimated to be 77.82 U/ml (V_{max}) and 6.66 mg/ml (K_m) using casein as substrate. The alkaline protease was also checked for its blood stain removal ability. The thermostable alkaline protease retained its activity in presence of detergent components with desired level stability and compatibility and therefore has a potential to be used commercially in the detergent industry.
This is the first report on characterization of detergent-compatible alkaline protease from *Bacillus agaradhaerens.*

Keywords: Bacillus agaradhaerens; alkaline protease; characterization; detergent compatibility.

1. INTRODUCTION

Managing microorganisms and harnessing them for the production of commercially important products is one of the most rapidly developing fields of research. Alkalophiles are unique microorganisms which occur naturally in alkaline habitats and have great potential for microbiological and biotechnological applications associated with their enzymes. The industrial enzymes produced naturally by alkalophilic microorganisms represent a major part of the global enzymes market and their production is rapidly increasing [1]. Most alkalophilic bacilli produce various alkaline enzymes, like proteases, amylases, xylanases, pullulanases, and cellulases [2]. Since enzymes produced by alkalophiles are active in the alkaline pH range, they are found to be most suitable in detergent formulations [3]. Proteases, amylases, lipases, cellulases, oxidases and peroxidases are added to detergents where they catalyze the breakdown of chemical bonds on the addition of water. To be suitable, they must be active under thermophilic (60℃) and alkalophilic (pH 9–11) conditions, as well as in the presence of the various components of detergents [4].

Alkaline proteases possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment and silver recovery [5-7]. Since their introduction as detergent additives, in 1914, the main industrial application of alkalophilic proteases has been in the detergent industry [8].

Alkaline proteases from high yielding strains of *Bacillus* have been studied extensively. Many species of *Bacillus* including- *B. subtilis, B. mojavensis, B. clausii* and *B. circulans* have been explored for alkaline protease production [9-12]. The proteases from these strains, having pH optima in the range 9–12 and good thermostability, have found commercial applications in detergents, in abating and dehairing of leather [13,6]. The search for new species of microbes having the ability to produce industrially important enzymes with novel properties is a continuous process.

The main reason for selecting natural enzymes from alkalophiles is their long term stability in detergent products; give a quicker and more reliable product and stability in the presence of detergent additives such as bleach activators, softeners, bleaches and perfumes. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Alkaline Protease Producing Bacterium

Bacterial isolates producing alkaline protease were screened from diverse samples *viz*. soil, sewage and industrial effluents by enrichment culture technique. The samples were inoculated in Horikoshi's broth medium, pH 10.0 [14] containing (w/v) glucose; 1.0%, peptone; 0.5%, yeast extract; 0.5%, KH_2PO_4; 0.1%, $MgSO_4$; 0.02%, Na_2CO_3; 1.0% (separately sterilized); followed by isolation on Horikoshi agar medium (pH 10.0). The morphological and cultural characteristics of the isolates obtained on Horikoshi's M-I (pH 10.0) agar plates were studied. Individual bacterial colonies

were evaluated for their proteolytic activity by measuring the zone of casein hydrolysis on milk agar medium (pH 10.0). The bacterium showing a maximum zone of hydrolysis was selected for further study. The isolate was maintained on Horikoshi medium slants having pH 10.0 and stored at 4℃.

2.1.1 Identification

The taxonomic status of the bacterium showing maximum protease activity was identified following the criteria laid down by Bergey's Manual of Systematic Bacteriology [15]. The identification was further confirmed by Microbial Type Culture Collection Center and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, (IMTECH), Chandigarh, India.

2.1.2 Molecular identification

The 16S ribosomal DNA sequencing of the isolate was performed by National Center for Cell Sciences (NCCS), Pune, India. The purified PCR product of 16S rDNA was sequenced using ABI DNA sequencer. The sequence data was aligned with publicly available sequences and analyzed to reach the identity. Phylogenetic tree was constructed using Neighbor-Joining method [16] and evolutionary relationships of taxa were inferred. Bootstrap values were calculated from multiple resamplings of the sequence dataset, which are the basis for multiple tree topologies. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Evolutionary analyses were conducted in MEGA6.06 [17].

2.2 Production and Purification of Alkaline Protease

The composition of production media selected after optimization consisted of (w/v) starch; 2.0%, casein; 3.0%, potassium dihydrogen phosphate; 0.375%, magnesium sulphate; 0.02% and (v/v) molasses; 2.0 ml, pH 10.0. The production medium was inoculated with 1% (v/v) inoculum; approximately 4.8 x 10^8 cells/ml, 0.5 MacFarland scale [18] and incubated at 30℃ for 48 h, on an orbital shaking incubator at 200 rpm. After incubation, the culture broth was centrifuged at 10,000 rpm for 20 min at 4℃. The supernatant was used as the crude enzyme.

2.2.1 Alkaline protease assay and protein estimation

The method used for protease assay was a modification of Hagihara and Anson methods [19,20] using casein as a substrate at a concentration of 0.65% (w/v) in 50 mM glycine-NaOH buffer, pH 10.5. One alkaline protease unit was defined as the enzyme amount that could produce 1 µg of tyrosine in 1 min at 55℃ under the defined assay conditions. The amount of tyrosine was determined using the standard curve of tyrosine. Protein concentration was estimated as per Lowry et al. [21] using bovine serum albumin (BSA) as a standard.

2.2.2 Purification of alkaline protease

Maximum precipitation of the enzyme was shown with ammonium sulphate at 80% saturation [22]. The protein precipitates were collected by centrifugation at 10,000 rpm for 20 min in cooling centrifuge at 4℃. The pellet of precipitates obtained was dissolved in minimal volume of 50 mM glycine-NaOH buffer, pH 10.5 and dialyzed against the same buffer for 24 h with two changes of the same buffer. The partially purified enzyme solution was preserved at -20℃. The dialyzed partially purified enzyme obtained from ammonium sulphate precipitation was further purified by gel filtration using Sephadex (Sigma G-200, granule size 40 to 120 µm bed volume, 15 to 20 ml per gram). The column was equilibriated with 25 mM Tris/HCl buffer (pH 8.0) and eluted with the same buffer containing 150 mM NaCl. The partially purified enzyme sample (1 ml) was gently applied over column bed and eluted in fractions of 2 ml each. UV absorption was taken at 280 nm to find out protein content in each fraction and fractions of highest O.D. were pooled together and were checked for enzyme activity and total protein. Total protein was determined by the method of Lowry et al. [21] using bovine serum albumin (BSA) as the standard.

2.3 Molecular Weight Determination by SDS-PAGE and Zymography

SDS-PAGE was performed according to Laemmli [23] with 5% and 10% separating gel at 100 V. Proteins bands were visualized by Coomassie brilliant blue R staining (0.06 g% Coomassie brilliant blue R 250, 8% methanol, 2% glacial acetic acid) for 2 h and the excess stain was removed by overnight treatment with destaining solution (40% methanol and 10% glacial acetic acid). Molecular weight of the enzyme was sized with the help of protein molecular weight markers. (Bangalore Genei). The protease profile was revealed by zymogram, using gelatin as copolymerized substrate [24].

2.4 Effect of Temperature on Alkaline Protease Activity and Stability

The effect of temperature on the enzyme activity was determined by performing the enzyme assay at temperatures ranged from 25-85℃ and pH 10.5 for 10 min.

The thermostability was determined by performing the standard assay after incubating the enzyme system at various temperatures (25℃, 35℃, 45℃, 55℃, 65℃, 75℃ and 85℃) for 60 min in the absence and presence of 25 mM $CaCl_2$ (enzyme : $CaCl_2$, 1:1). The percent residual activity was determined.

2.5 Effect of pH on Alkaline Protease Alkaline Activity

The enzyme assay was performed at pH values in the range of 7.0 to 12.0. The pH was adjusted using the following buffers: 0.05 M phosphate buffer (pH 7.0, 7.5), 0.05 M Tris HCl buffer (pH 8.0, 8.5), 50 mM glycine-NaOH buffer (pH 9.0 to 12.0). The standard enzyme assays were performed at the each pH values at 55℃ and the enzyme activity was expressed as percent relative activity by considering enzyme activity at optimum pH as 100%.

2.6 Substrate Specificity of Alkaline Protease

The alkaline protease was assayed at 55℃ and pH 10.5 in the presence of various protein substrates including 0.65 g% each of casein, yeast extract, egg albumin, blood, gelatin, meat extract, bovine serum albumin and raw milk. In the alkaline protease assay, casein (Hammarsten) in the enzyme system was replaced by the substrates mentioned above. The enzyme activity was expressed as enzyme units/ml.

2.7 Determination of K_m Value and V_{max}

The protease assay was carried out using different concentrations (0.5-5.0 mg/ml) of casein in 50 mM glycine-NaOH buffer (pH 10.5) at 55℃ for 10 min. The K_m value and V_{max} of alkaline protease was calculated from Lineweaver - Burk plot [25].

2.8 Effect of Metal Ions and Chelator (EDTA) on Enzyme Activity

The enzyme was pre-incubated for 1 h at 55℃ with 1 mM solutions of different metal ions i.e. K^+(KCl), Co^{2+} ($CoCl_2$), Ca^{2+}($CaCl_2$), Mg^{2+}($MgCl_2$), Mn^{2+}($MnCl_2$), 10 mM of concentration of EDTA (in 1:1 ratio). The residual activity was determined by performing enzyme assay and was expressed as percentage of activity without chemical agents, considered as 100%.

2.9 Effect of Detergent Additives on the Enzyme Activity

The enzyme activities were assayed in presence of various detergent additives (like sodium perborate, SDS, optical brightener) and expressed in term of residual activity (%) considering control as 100%. The control was without any additive.

2.9.1 Effect of sodium perborate

The enzyme was incubated in presence of various concentrations of the bleaching agent sodium perborate (0.1- 0.5 g%) in 1:1 ratio at 55℃ for 1 h. The percent residual activity of the enzyme was determined by performing enzyme assay.

2.9.2 Effect of SDS

Sodium dodecyl sulphate (SDS) acts as a surfactant in detergent. The enzyme was incubated in the presence of SDS (0.1 - 0.5 g%) for 1 h at 55℃ in 1:1 ratio. The percent residual activity of the enzyme was determined by performing enzyme assay.

2.9.3 Effect of optical brightener (Ranipal)

The enzymes were incubated with 10.0% -70.0 mg% concentrations of optical brightener (Ranipal) in 1:1 ratio for 1 h at 55℃. The percent residual activity of the enzyme was determined by performing enzyme assay.

2.10 Compatibility with Commercial Detergents

In order to evaluate the commercial application of enzyme, it was analyzed for compatibility with commercial detergents available in the market. The detergent brands used were Ariel® (Procter and Gamble Home Products Ltd, Mumbai, India), Rin®, Surf excel® (Hindustan Unilever Limited-Mumbai, India), Tide® (Procter and Gamble Home Products Ltd, Mumbai, India), Henko® (Henkel Spic, India), Wheel® and Ezee® (Godrej Consumer Products Ltd, Mumbai, India). The detergents were diluted in distilled water to a final concentration of 7 mg/ml to stimulate washing conditions. The indigenous enzyme in the detergent was

deactivated by heating at 100℃ for 10 min. Enzyme solution was incubated with the deactivated detergents at 55℃ for 30 min. The residual activity was determined under assay conditions and compared with control samples incubated at 55℃ for 30 min without any detergent. The enzyme activity of control was taken as 100%. Similar experiment was performed to determine the compatibility of the enzyme with commercial detergents in presence of 25 mM $CaCl_2$. The enzyme activity of control sample (enzyme + 25 mM $CaCl_2$, 1:1) was taken as 100%.

2.11 Application of Alkaline Protease as Detergent Additive in Blood Stain Removal

Small pieces of white cotton cloth (5 cm x 5 cm) were stained with blood and air dried for 7 days. The stained pieces of cloth were taken in separate flasks as- 100 ml distilled water + stained cloth, 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml), 100 ml distilled water + stained cloth + 2 ml enzyme solution, 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml) + 2 ml enzyme solution. After incubation for 15 min, the cloth pieces were taken out and dried. Untreated cloth pieces stained with spots were taken as control.

2.12 Statistical Analysis

The data are presented as means of at least three independent experiments, each selected experiment had a minimum of three replicates of each sample. Statistical significance among levels was evaluated using one way analysis of variance (ANOVA) in Excel Add-in Analysis ToolPak. The means were compared by ANOVA at 5% significance level. The regression analysis was done using Minitab 16 statistical software [26] at 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Alkaline Protease Producing Bacterium

Amongst the 19 alkalophilic isolates showing proteolytic activity, the bacterial isolate which gave maximum alkaline protease activity was considered for further study. The culture was identified based on morphological, cultural and biochemical characteristics as well as molecular approach following 16S rDNA sequencing. The organism was confirmed as *Bacillus agaradhaerens* by Microbial Type Culture Collection Center and Gene Bank (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, India and deposited under accession number MTCC 9416. After the sequence characterization, the nucleotide sequence was submitted to NCBI GenBank under the name *Bacillus agaradhaerens* strain nandiniphanse5, with an accession number JN703504. *Bacillus agaradhaerens* is one among the nine new species of alkalophilic *Bacillus* proposed by Nielsen et al. [27]. A phylogenetic tree was constructed from 16S rDNA sequences of members of genus *Bacillus* using Neighbour Joining method [16]. In the neighbour-joining tree, the sequences form a distinct lineage, with alkalophilic *Bacillus* species as the closest relatives. Phylogenetic construction of *Bacillus agaradhaerens* strain nandiniphanse5 against other species of *Bacillus* is shown in Fig. 1. The dataset *Bacillus agaradhaerens* strain nandiniphanse5 consisted of 770 bp (100%) is parsimony informative. The matrix was competently and manually aligned. Coding gaps as binary characters, missing data had no effect on the topology. The 100% bootstrap consensus tree is shown (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

3.2 Purification of Alkaline Protease of *Bacillus agaradhaerens*

Starch casein molasses medium supported maximum enzyme production, the alkaline protease activity was 435 U/ml for *Bacillus agaradhaerens.* The crude, extracellular enzyme produced by the alkalophilic bacterium was partially purified by ammonium sulphate with maximum precipitation obtained at 80% saturation of ammonium sulphate. Further purification was performed by GPC (Gel Permeation Chromatography) treatment after which alkaline protease was purified 22.98 folds with 14.9% recovery. The purification results are summarized in Table 1.

3.3 SDS PAGE and Molecular Weight Determination

SDS-PAGE method is widely used to monitor protein purification and to size proteins. The molecular weight of alkaline protease produced by *B. agaradhaerens* was nearly 25 kDa (Fig. 2A). The purified enzyme was homogenous showing a single band. The zymogram analysis revealed a single band of proteolytic activity on

the gelatin–zymogram gel system, which corresponds to the single band obtained on SDS-PAGE (Fig. 2C). An alkaline protease from *Bacillus clausii* GMBAE 42 with a molecular weight 26.5 kDa was reported by Kazan et al. [30]. Similar reports of alkaline proteases produced by *Bacillus* sp were cited by [11,12] who have reported molecular weight as 28 kDa and 27.5 kDa respectively.

3.4 Effect of Temperature on Alkaline Protease Activity

The relationship between enzyme activity and temperature is statistically significant with a *P* value of 0.0000 (<0.05). An 89.93% variation in enzyme activity can be accounted for by the cubic regression model for effect of temperature (Table 2). The optimum temperature of alkaline protease obtained from *Bacillus agaradhaerens* was 55℃ (Fig. 3a). The enzyme was found to be thermostable and retained about 45% of residual activity at 85℃ for 60 min (Fig. 3b). Similar results were described for other *Bacillus* optimum temperature for protease from *Bacillus subtilis* PE-11 [9] and *Bacillus* sp. GOS-2 [31] was 60ºC. The enzyme of the present study had a higher thermal stability demonstrating its suitability in detergent formulations.

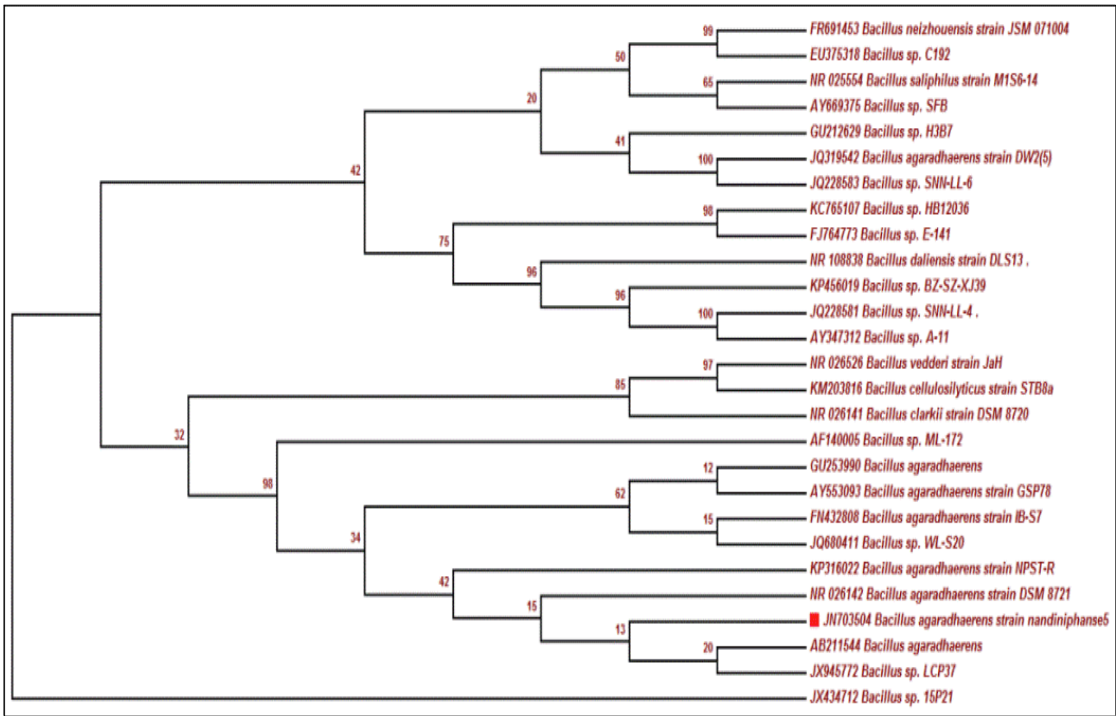

Fig. 1. Neighbour-joining tree for rDNA sequence types

Unrooted phylogenetic tree, constructed using the neighbour-joining method [16] and based on 16S rDNA sequences, showing the relationships of the identified alkalophilic bacterial strain and closely related members of Bacillus species. The sum of branch length of the optimal tree was 0.17518476. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [28]. The evolutionary distances were computed using the Maximum Composite Likelihood method [29] using MEGA6.06 [17] and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences of genus Bacillus. There were a total of 709 positions in the final dataset.

Table 1. Summary of purification of alkaline protease of *Bacillus agaradhaerens*

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield of activity (%)
Cell free extract	33520	9400	3.56	1	100
Ammonium sulphate precipitation (80%)	6978	474	14.72	4.13	20.81
GPC-200	4990	61	81.80	22.98	14.88

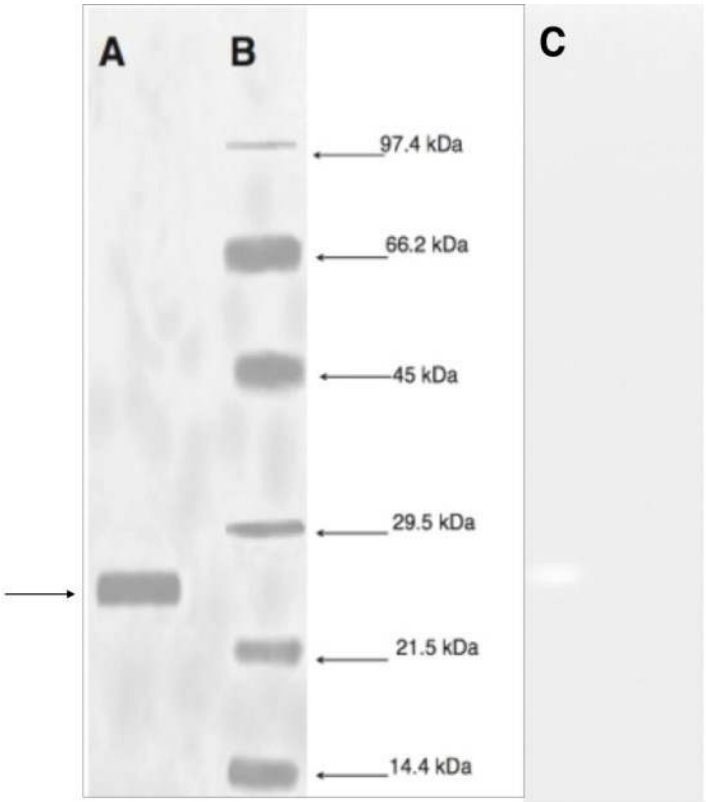

Fig. 2 SDS PAGE of the purified alkaline protease
SDS PAGE performed according to Laemmli's method [23] using 5% and 10% separating gel at 100 V. Proteins bands visualized by Coomassie brilliant blue R stain.
Lane A- Purified alkaline protease of *B. agaradhaerens* (indicated by arrow)
Lane B - Molecular mass markers (lysozyme:14.4 kDa; trypsin inhibitor:21.5 kDa; carbonic anhydrase:29.5 kDa; ovalbumin:45 kDa; BSA: 66.2 kDa; phoshorylase B: 97.4 kDa).
Lane C - Zymography of purified proteases. Samples were electrophoresed under non-denaturing conditions in 10% polyacrylamide with 0.1% gelatin.

Presence of 25 mM $CaCl_2$ increased the thermostability of the enzyme (Fig. 3b). Adinarayana et al. [9] and Patel et al. [32] have reported similar findings. Alkaline proteases produced from fungal isolates have also found to exhibit thermostability [33]. The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained by the salting out of the hydrophobic residues by Ca^{2+} in the protein, thus causing the adoption of a compact structure [34].

3.5 Effect of pH on Alkaline Protease Enzyme Activity

The relationship between enzyme activity and pH is statistically significant with a *P* value of 0.037 (<0.05). A positive correlation (+0.63) was reflected between pH and enzyme activity by regression analysis (Table 2) indicating that enzyme activity would increase with increase in pH although a decline was seen above pH 10.5 (Fig. 4) which may be due to denaturation. A 92.95% variation in enzyme activity (U/ml) can be accounted for by the quadratic regression model. These findings are in accordance with several earlier reports showing pH optima of 10.0-10.5 for protease [9]. The important detergent enzymes, subtilisin Carlberg and subtilisin Novo or BPN also showed maximum activity at pH 10.5 [35]. Several reports are cited in which pH optima were between pH 10.0 to 11.0 [10-12,32]. All detergent compatible enzymes are alkaline in nature, with high pH optima because the pH of laundry detergents is generally in the range of pH 9-12.

3.6 Substrate Specificity of Alkaline Protease

The enzymes could hydrolyze all tested substrates, the efficiency of substrate utilization was highest for casein and the lowest for meat

extract the order is casein. Gelatin, blood, milk, yeast extract, albumin, BSA and meat extract (Table 3). The dirt on the cloth may consist of different types of proteins separately or in combinations. Since the enzyme under study has the ability to hydrolyze various protein substrates, it can be a candidate of choice in detergent formulations for protein stain removal.

Table 2. One way ANOVA and Regression analysis of various parameters affecting enzyme activity

No.	Experimental analysis	ANOVA		Regression analysis		
		F value	F critical	Model	R^2 (adjusted)	*P* value
1	Effect of temperature on enzyme activity	0.00009	3.55	Cubic	89.93	0.000
2	Effect of temperature on enzyme stability (in presence of $CaCl_2$)	0.28	3.55	Cubic	89.94	0.000
3	Effect of temperature on enzyme stability (in absence of $CaCl_2$)	0.136	3.55	Cubic	89.60	0.000
4	Effect of pH on enzyme activity activity	0.009	3.00	Quadratic	92.95	0.037
5	Effect of substrates on enzyme activity	0.004	3.46	Cubic	47.91	0.001
6	Effect of metal ion on enzyme activity	0.024	4.00	Linear	09.89	0.281
7	Effect of Sodium perborate on enzyme activity	0.249	4.00	Linear	91.07	0.008
8	Effect of SDS on enzyme	0.471	3.00	Linear	90.21	0.000
9	Effect of optical brightener on enzyme activity	0.300	4.00	Linear	96.39	0.000
10	Effect of detergent on enzyme activity	0.005	4.00	Linear	68.05	0.014

One way ANOVA performed using Excel Add-In Tool Pack and Regression analysis performed at 95% confidence interval by using Minitab 16 statistical software

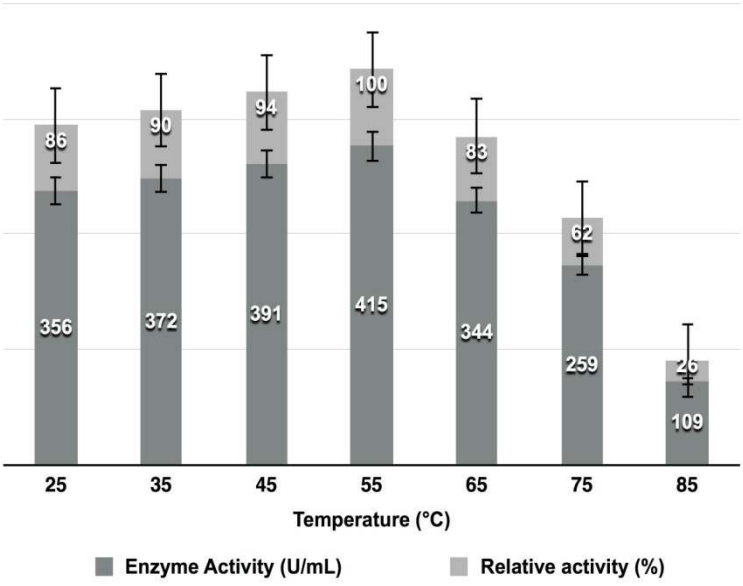

Fig. 3a Effect of temperature on alkaline protease activity of *Bacillus agaradhaerens* at pH 10.5 using casein as substrate

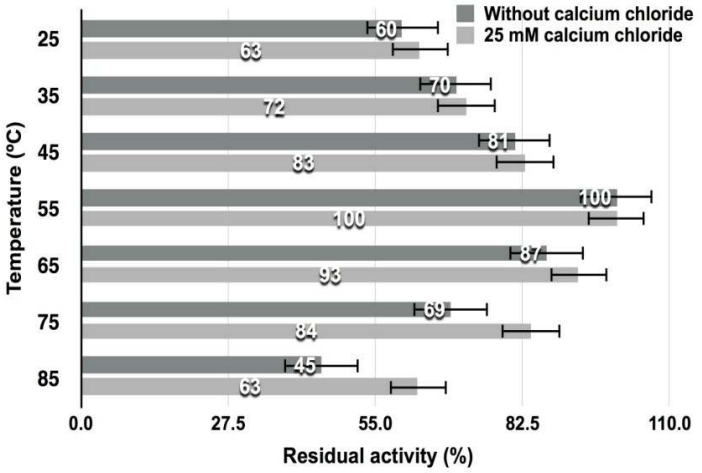

Fig. 3b Effect of temperature on stability of purified alkaline protease activity of *B. agaradhaerens* at pH 10.5 using casein as substrate with incubation for 60 minutes in presence and absence of $CaCl_2$

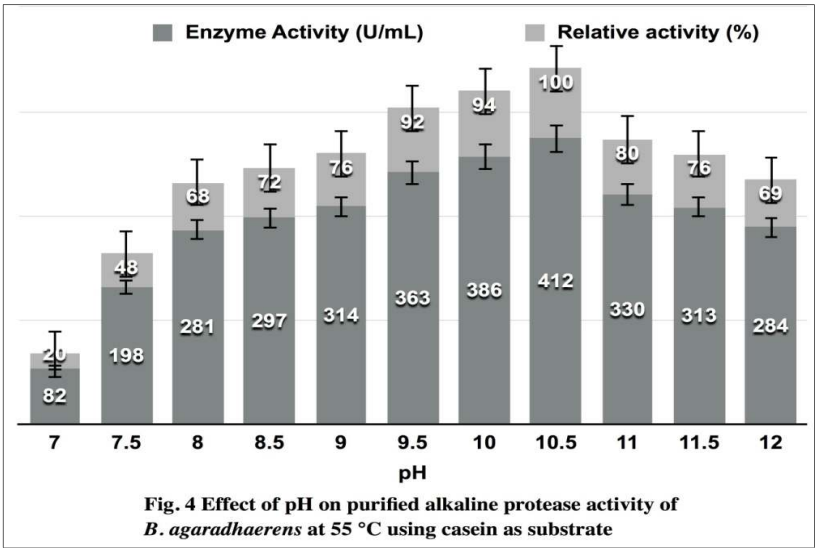

Fig. 4 Effect of pH on purified alkaline protease activity of *B. agaradhaerens* at 55 °C using casein as substrate

3.7 Determination of K_m and V_{max}

K_m and V_{max} value of the purified alkaline protease calculated from Lineweaver-Burk plot was 6.66 mg/ml and 77.82 U/ml respectively. The K_m value of a purified protease obtained by Mushtaq et al. [36] was 7.0 mg/ml, using casein as substrate. A lower K_m indicates higher substrate affinity of the enzyme and is desirable for efficiency.

3.8 Effect of Metal Ions and Chelator (EDTA) on Enzyme Activity

When the influence of various metal ions on enzyme activity was tested, $CaCl_2$ and $MgCl_2$ had a high stimulatory effect on the alkaline protease. $CoCl_2$ and $MnCl_2$ increased the enzyme activity slightly, followed by KCl. This indicates that metal ions are required for the activity of the enzyme (Table 3). Sathishkumara et al. [37] have also reported stimulation in activity of an alkaline protease produced by *Bacillus subtilis* GA CAS8, in presence of Mg^{2+} and Ca^{2+}. Adinarayan et al. [9] have cited the thermostability of alkaline protease in presence of metal ions like Ca^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+}. These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures. The stimulation of

Table 3. Effect of substrates, metal ions, EDTA, sodium perborate, SDS and optical brightener on alkaline protease activity

Substrates (0.65 g%)	Enzyme activity (U/ml)	Metal ions (1mM) & EDTA (10 mM)	Residual activity (100%)	Sodium perborate (g%)	Residual activity (100%)	SDS (g%)	Residual activity (100%)	Optical Brightener (mg%)	Residual activity (100%)
Casein	414±1.52	Control	100±0	Control	100±0	Control	100±0	Control	100±0
Gelatin	396±2.6	$CoCl_2$	112±3.6	0.1	100±7	0.1	100±5	10	99.5±3.5
Meat extract	295±3.21	$CaCl_2$	176±3	0.2	86±6	0.15	107±5	20	98±1
Albumin	328±4.36	$MgCl_2$	157±3.6	0.3	73.5±5	0.2	115±6	30	93±2.08
Milk	385±2.00	$MnCl_2$	109±4.5	0.4	73.5±4	0.25	119.8±4	40	90±2.64
Yeast extract	343±1.00	KCl	123±5.3	0.5	62±5	0.3	123±5	50	87.5±3.6
Blood	361±3.05	EDTA	89±4.3			0.35	122.7±5	60	82±3.7
BSA	347±3.78					0.4	122.7±5	70	78.7±2.64
						0.45	133.6±4		
						0.5	133.6±5		

Data is represented as mean ± standard error (SE) for three replicates

fungal alkaline protease produced by *Aspergillus* species by Ca^{2+} and Mg^{2+} has also been described [33].

The enzyme was relatively stable in presence of the chelating agent, EDTA, indicating its suitability as a detergent enzyme, since EDTA is used in detergent formulation as a water softener [10]. When pre-incubated in presence of 10 EDTA for 1 h, 89% activity was retained (Table 3). Seifzadeh et al. [38] have reported an alkaline protease produced by *Bacillus* sp. GUS1 which retained its activity up to 90% in the presence of 1 mM EDTA and more than 80% in the presence of 5 mM and 10 mM of the chelator. Similarly, a laundry detergent compatible alkaline protease produced by *Bacillus* species Y showed profound increase in activity in presence of EDTA [39].

3.9 Compatibility of Alkaline Protease with Detergent Additives

3.9.1 Effect of sodium perborate

The alkaline protease produced by *Bacillus agaradhaerens* retained more than 82% of its activity in presence of 0.5 g% of sodium perborate at 40℃ for 1 h (Table 3). The alkaline protease produced from *Bacillus* sp. JER02 [40] retained 93% of its initial activity, after 1 h of incubation at room temperature with sodium perborate (1%, v/v). Joo et al. [41] have also reported a bleach-resistant alkaline protease produced by a *Bacillus* sp.

3.9.2 Effect of SDS on alkaline protease activity

As depicted in Table 3, the activation of alkaline protease in presence of SDS indicates the enzyme may be used as an effective additive in detergents. The negative charges of the SDS react with positive charges of calcium and magnesium present in the hard water/wash water thereby deactivating them [3]. About 133.6% activity is shown in presence of 0.5 g% of SDS for 1 h at 40℃. This shows that SDS is possibly an activator of the enzyme, increasing its activity. This stability in presence of SDS of alkaline protease produced by our isolate, *Bacillus agaradhaerens*, is in accordance to literature cited for other *Bacillus* species *viz*. *Bacillus mojavensis*, *Bacillus clausii, Bacillus* sp. GUS1 [10,11,38].

3.9.3 Effect of optical brightener on alkaline protease activity

When the effect of optical brightener was studied, it was found that the activity of *Bacillus agaradhaerens* alkaline protease gradually decreased with increase in the concentration of optical brightener, Ranipal (Table 3). About 78.7% of residual activity was retained in presence of 70 mg% of Ranipal. Sevalkumar et al. [31] have reported an extracellular alkaline protease produced by *Bacillus* GOS-2 which was stable in presence of optical brightener.

3.10 Compatibility with Commercial Detergents

A good detergent enzyme is expected to be stable in presence of commercial detergents. As shown in Fig. 5, the *Bacillus agaradhaerens* alkaline protease, after 30 min of incubation at 55℃ in presence of commercial detergents, retained 93% and 94% of the original activity in case of Surf Exel® and Tide®, respectively and 84% of the original activity in case of Henko®. It almost retained all its relative activity in presence of Rin® which was nearly equal to that of control and 98% of its relative activity in presence of Ariel®. However, the residual activity in presence of other detergents *viz*. Wheel® and Ezee® decreased to 65% and 73% respectively. In presence of 25 mM $CaCl_2$, an increase in relative activity was recorded with all detergents. All proteases are stabilized in presence of certain levels of Ca^{2+} ions [42]. Therefore, 100-1000 ppm of Ca^{2+} is added to liquid detergents that contain protease. The detergent compatibility of alkaline protease of *Bacillus* species has been reported [13,39]. The stability of alkaline protease with detergents at 60℃ in presence of 10 mM $CaCl_2$ as a stabilizer has been cited by Adinarayana et al. [9]. Alkaline proteases of fungal origin have also been reported to show stability in presence of detergents [33].

3.11 Application of Alkaline Protease as Detergent Additive in Blood Stain Removal

It was found that among the different conditions of washing tested, the mixture of alkaline protease and detergent was able to significantly remove blood stain from white cotton fabric (Fig. 6). Blood stain removal was improved by addition of alkaline protease to detergent solution, as reported by Adinarayana et al. [9] and Selvakumar et al. [31].

3.12 Statistical Significance

Statistical analysis was performed using one way ANOVA in Excel Add-in Analysis ToolPak and Minitab 16 statistical software [26]. One way ANOVA was used to analyze the mean values in various experiments and understand the applicability of model by studying the F values which were found sufficiently less than the F critical values (Table 2). The regression analysis was done using Minitab 16 software at 95% confidence interval where the *P* values <0.05 reflected the significance of results (Table 2).

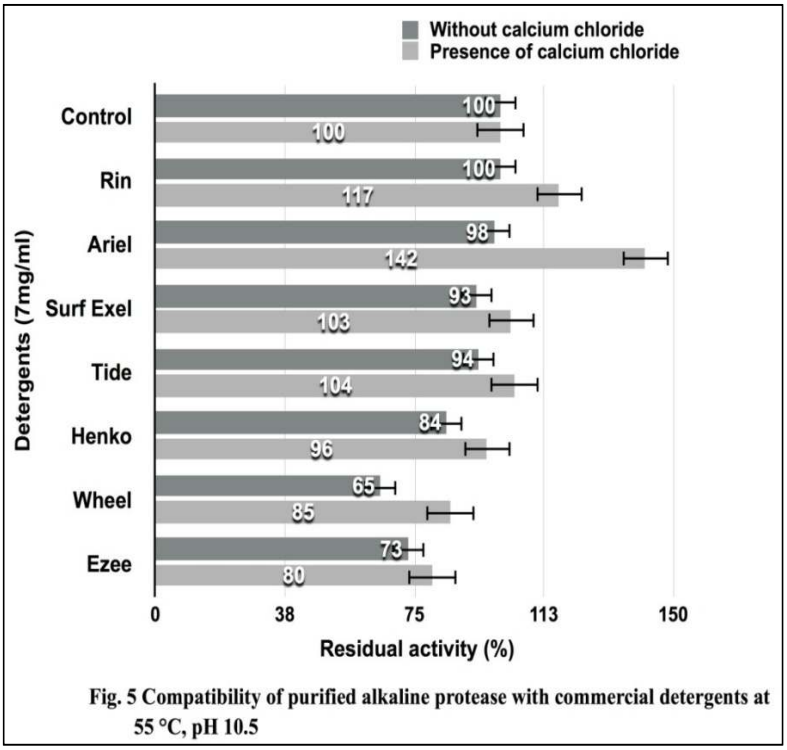

Fig. 5 Compatibility of purified alkaline protease with commercial detergents at 55 °C, pH 10.5

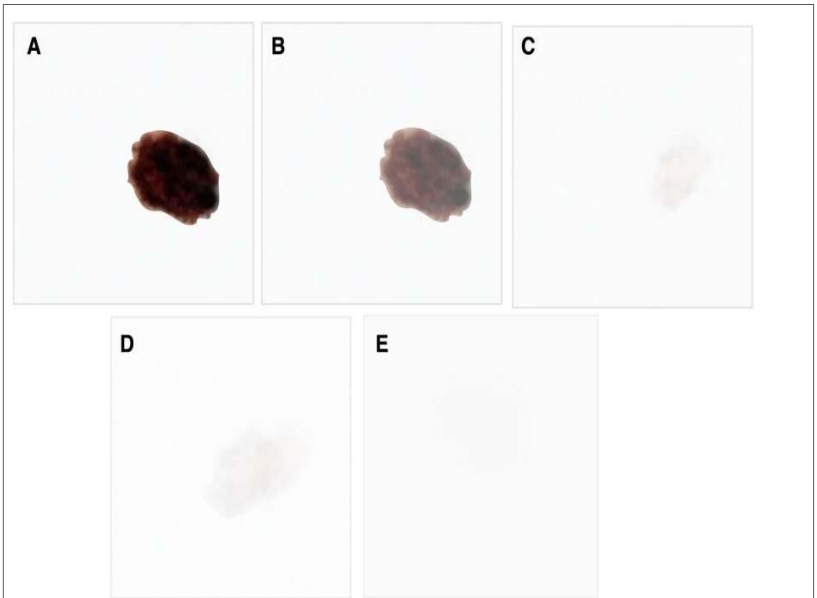

Fig.6 Wash performance test:Destaining efficiency of alkaline protease produced by *B. agaradhaerens*
A-Control, untreated blood stained cloth
B-Blood stained cloth treated with distilled water
C-Blood stained cloth treated with distilled water and detergent
D-Blood stained cloth treated with distilled water and enzyme
E-Blood stained cloth treated with distilled water, enzyme and detergent

4. CONCLUSION

The characterization of protease produced by the alkalophilic bacterium, *Bacillus agaradhaerens* (MTCC 9416) was undertaken to establish its suitability in detergent formulation. The organism produced a thermostable and alkali-stable protease which showed affinity for various protein substrates with stability in presence of high pH and temperature values. It was compatible and stable in presence of detergent additives. These properties suggest that the alkaline protease in the present study can be commercially used as a constituent of detergent formulations.

ACKNOWLEDGEMENTS

The financial assistance provided by UGC, CRO, Bhopal, India, for performing the present work is deeply acknowledged. The authors are thankful to Dr. Yohesh Shouche, NCCS, Pune, India, for performing the 16S rDNA sequencing of the isolate. We also express our thanks to the Management and Principal of PMB Gujarati Science College, Indore, India, for providing instrumental and technical support. Our special thanks to Mr. Sandeep Fattepurkar, Director, New Multivision, Solapur, India, for assistance in graphical and statistical analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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