Research article

Production and characterization of an organic solvent activated protease from haloalkaliphilic bacterium *Halobiforma* sp. strain BNMIITR

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ABSTRACT

An unusual haloalkaliphilic bacterium known as *Halobiforma* sp. strain BNMIITR, which was noticed to produce an extracellular alkaline protease, was found in a soil sample from Northern India's Sambhar Lake. On the generation of protease, the effects of dietary elements including nitrogen and carbon sources, amino acids, and growth conditions like temperature and pH were investigated. When low-cost agricultural by-products were employed as nitrogen sources, the manufacturing of enzymes was significantly boosted. In the present study, protease production was enhanced by 2.94 fold and 2.17 fold. By solvent precipitation and Hydrophobic interaction chromatography (HIC) on Phenyl Sepharose 6 Fast Flow matrix, the enzyme was purified 31.67 fold. It was determined that the apparent molecular mass was 21 kDa. The pH range where the enzyme was most stable was 6.0–12.0, with a temperature of 50 °C as optimum. When there was alkaline earth metals and heavy metals, protease was discovered to be active. It was evident that the enzyme was a serine type of protease because it was active in the presence of a variety of surfactants, oxidizing and reducing chemicals, and phenylmethylsulfonyl fluoride (PMSF) completely inhibited activity. Enzyme exhibited a wide range of substrate specificity. Amazingly, enzyme remained stable both in polar and nonpolar solvents. The most interesting aspect of this enzyme is enhanced activity in polar solvents like dimethylformamide (DMF) and dimethyl sulfoxide (DMSO). It was discovered that the protease was stable and compatible with a number of widely available detergents.

1. Introduction

On a global scale, the industrial enzymes market reached around 6.8 billion USD in 2017, with projections indicating a potential increase to 10 billion USD by the year 2024 [1]. Among hydrolases, proteases are the most commercially exploited (60 %) enzyme. Wide applications of protease in leather, food, ultrafiltration membrane cleaning, pharmaceuticals, meat tendering, detergent and silver extraction from used X-ray film had already been reported in literature [2,3]. Medically, protease serves as an agent for treatment of inflammation, pain, wound healing and arthritis [4].

Normally proteases work in aqueous conditions but if organic solvents are present they catalyze the synthesis of esters and peptides.

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Co-solvent systems (water and an organic solvent that is miscible with water) are the most common type of organic solvent reaction system, 2) A biphasic system (water and an organic solvent that is water insoluble), 3) anhydrous organic solvent system [5]. To keep the synthesis reactions simple and affordable, an enzyme that can withstand organic solvents is preferred. Application of various protein engineering approaches has increased the stability and activity of enzymes in organic solvents. If the enzyme is already resistant to organic solvents, no further tempering is required.

Peptide and ester synthesis reactions take place in the co-solvent system but at higher solvent concentration enzyme activity reduces it is due to the interplay of the solvents at the interface between enzymes and water molecules. Recently it has been observed that greener solvents like ionic liquid can also replace as reaction media for enzymatic bio-catalysis [6–8]. As a novel strategy, deep eutectic solvents (DESs) are emerging as viable substitutes for traditional organic solvents [9].

Halophiles require salt for both the activity and stability of their enzymes because they are adapted to flourish in high-salt environments. High-salt environments resemble environments with chemical solvents. Thus, solvent stability of halophilic enzymes seems to be a generic distinctive trait that makes them potentially useful in non-aqueous enzymology [10,11].

Halophilic proteins not only tolerate salt, but they also function at high temperatures and pH levels. The highest activity of a protease from a moderately halophilic *Bacillus* sp. strain was found at pH optimum 9.0, t1/2 190 min at 60 °C, and 1 % (w/v) NaCl. The protease displays stability in both polar and nonpolar liquids at high concentrations [12]. A protease isolated from *Bacillus* sp. TSA5 strain optimum activity was found at 80 °C and pH 8.5 [13]. For potential application, protease should be stable in organic solvent, detergent and oxidizing agent.

Halophilic Archaea are adapted to hypersaline environments and show a slow growth rate which is a limiting factor for their industrial applications. Nutritional factors like nitrogen and carbon sources, amino acids and culture conditions such as pH, temperature are known to affect the protease production [2]. Thus optimization of media components and culture conditions are necessary to achieve maximum protease production.

In the present study a highly solvent tolerant and activated enzyme from *Halobiforma* sp. strain BNMIITR has been purified and characterized.

2. Methods

2.1. Organism, growth condition and medium composition

In a soil sample retrieved from the Sambhar lake in Sambhar, Rajasthan (North India), the isolate BNMIITR was discovered. A complex halophilic medium with the following composition was incubated at 45 °C with the purified isolate while rotating at 180 rpm. NaCl: 250 g/L; KCl: 2 g/L; MgSO$_4$.7H$_2$O: 20 g/L; tri-sodium-citrate: 3 g/L; casamino acid: 7.5 g/L; agar: 20 g/L; MnCl$_2$.4H$_2$O: 0.36 mg/L; FeSO$_4$.7H$_2$O: 1.6 mg/L [14]. With the help of 1 M potassium hydroxide (KOH), the pH of the medium was raised to 9.0.

2.2. Protease production

The growing medium mentioned above served as the initial site of protease production. When there are casamino acids, very little protease synthesis was seen. In order to avoid using casamino acid in the production medium, it was also discovered that yeast extract had a negligible inhibitory effect on the development of protease. This meant that the manufacturing medium for yeast extract was also changed. The production of protease was then improved on a medium containing (g/L): 1 % casein protein, 45 °C, 180 rpm rotational shaking, 180 g/L NaCl, 2 g/L KCl, 20 g/L MgSO$_4$.7H$_2$O, 3 g/L tri-sodium citrate, 0.36 mg/L MnCl$_2$.4H$_2$O, and 1.6 mg/L FeSO$_4$.4H$_2$O. With the help of 1 M potassium hydroxide (KOH), the pH of the production medium was raised to 10. The crude enzyme source was the cell-free supernatant. The cell-free supernatant served as the crude enzyme source. The medium was optimized one parameter at a time due to its complexity.

2.3. Assay for protease activity

Using the culture supernatant as a source of protease, the culture was centrifuged at 10,000×*g* for 10 min at 4 °C. Using casein as the substrate, the proteolytic activity of the crude enzyme was assessed with modified Kunitz method [15]. In brief, 0.8 mL of the casein solution (50 mM tris buffer, pH 9.0, and 1 % casein in the final reaction mixture) and 0.2 mL of the crude protease were mixed together, and the mixture was then kept at 45 °C for 30 min. 1.0 mL of 10 % trichloroacetic acid (TCA) was incorporated into the mixture to halt the reaction, and the mixture was then left to stand at room temperature. The supernatant was separated after 15 min by centrifugation at 12,000×*g* for 15 min. Tyrosine was used as the standard to measure the absorbance spectrophotometrically at 280 nm. Before adding the enzyme, 1.0 mL of TCA was included to the blank. The quantity of protease needed to generate 1 g of tyrosine per min was used to define one unit of protease activity.

2.4. Production improvement

2.4.1. Salinity, pH, and temperature effects

The impact of NaCl on protease manufacturing was discovered by cultivating the strain BNMIITR in a NaCl range of 2–5 M in the production medium having an initial pH of 9.0. At 45 °C and 180 rpm, a rotatory incubator shaker was used for the incubation process. Effects of various salts such as KCl, sodium sulfate (Na$_2$SO$_4$), sodium phosphate (Na$_2$PO$_4$), sodium nitrate (NaNO$_3$) and sodium acetate

(CH$_3$COONa) on protease production were also evaluated similarly. Growing the isolate at an initial pH range of 7–11 with 3 M NaCl at 45 °C in the production medium allowed researchers to find out the impact of pH on the generation of enzyme. To ascertain the effect of temperature on protease generation, strain BNMIITR was grown in a temperature range of 30–55 °C with a production medium that had an initial pH of 10.0 and 3 M NaCl.

2.4.2. Influence of various carbon and nitrogen sources on the development of proteases

Tri sodium citrate from the production medium was substituted with a 1 % (w/v) concentration of several carbon sources to find out the consequences of carbon sources on protease synthesis. Glucose, fructose, sucrose, lactose, galactose, glycerol, mannitol, starch, xylose, trisodium citrate, and inexpensive agricultural byproducts like wheat bran and wheat flour were employed as carbon sources. By substituting casein from the production medium with various sources of nitrogen with a 1 % (w/v) concentration, the influence of nitrogen sources on protease generation was examined. Inorganic nitrogen sources like ammonium nitrate (NH$_4$NO$_3$), ammonium chloride (NH$_4$Cl), ammonium sulfate (NH$_4$)$_2$SO$_4$, and urea, as well as inexpensive agricultural byproducts like soybean meal, soybean husk, chickpea flour, and chickpea husk, were employed to research how they affected the generation of proteases. Incubation was carried out at 45 °C, pH 10 and 3 M NaCl on a rotatory incubator shaker (180 rpm).

2.4.3. The influence of various amino acids on the generation of Enzyme

Everyone is aware that amino acids catabolically suppress the synthesis of proteases. By adding extra amino acids to the medium, it was investigated how amino acids affected the manufacturing of proteases (at concentrations of 0.1, 0.5 and 1 %). Incubation was carried out at 45 °C, pH 10 and 3 M NaCl on a rotatory incubator shaker (180 rpm).

2.5. Enzyme purification

One mL of 96 h-grown culture (in complicated medium) was inoculated into a 100 mL (500-mL) Erlenmeyer flask of production medium with the exception that tri-sodium citrate was swapped out for xylose. The manufacturing medium's initial pH was set to 10 using 1 M KOH. Centrifugation of culture at 12,000×g for 20 min at 4 °C produced 2 L of supernatant. Using the Pall advanced separation system (Manual centramate system) and 10 kDa membrane cassettes (Pall omega 10 kDa T series), 2 L of crude enzyme were reduced to 200 mL.

Concentrated enzyme was precipitated by pre chilled acetone. First, concentrated enzyme was combined with pre-chilled acetone up to 40 % (v/v), which was then stored at −20 °C for an hour. A precipitate was obtained by centrifugation of the mixture at 8000×g for 10 min, however, this precipitate was rejected since it contained unwanted additional proteins. Now supernatant was again precipitated with gradual addition of pre chilled acetone up to 80 % saturation and kept at −20 °C for 1 h and the precipitate was collected. In 50 mL of 50 mM tris buffer pH 9.0 with 1.2 % NaCl, the precipitate was dissolved. The solution was further concentrated to 5 mL using centrifugal concentrators (Amiconultra Millipore 10 kDa) in a centrifuge at 5000×g and 4 °C.

A further purification process using HIC was performed on the concentrated enzyme. Gravity flow was used to purify the HIC. Two mL of concentrated enzyme solution were placed onto a Phenyl Sepharose 6 Fast Flow column (1 cm 24 cm bed volume) before it was equilibrated with 60 mL of tris buffer pH 9.0 containing 25 % NaCl. The column was washed with 50 cc of equilibrating solution until no more protein could be seen in the washings. For subsequent elution, It was done using a linear gradient of NaCl (20 %–0 %) in a 50 mM tris buffer at pH 9.0 (20 %–0 %). The enzyme was eluted at 11-10 % NaCl in tris buffer 50 mM pH 9.0. Protease-active fractions were combined for further characterization.

Pooled fractions were concentrated using centrifugal concentrators (Amiconultra Millipore 10 kDa) in a centrifuge at 5000×g and 4 °C. The concentrated protein sample was than buffer exchanged with 50 mM tris buffer pH 9.0 and 1.2 % NaCl (40 mL) using centrifugal concentrators (Amiconultra Millipore 10 kDa) in a centrifuge at 5000×g and 4 °C. Buffer exchange with 50 mM tris buffer pH 9.0 containing 1.2 % (12 g/l) NaCl was repeated three times.

After HIC it was discovered that the enzyme loses 80–90 % of its activity when there is no NaCl and needs a minimum of 1–2% NaCl to retain its activity, the enzyme was purified. Thus, for all subsequent tests, The enzyme was employed in a solution containing 50 mM tris buffer, 1.2 % NaCl(12 g/l) at 9 pH.

2.6. Polyacrylamide gel electrophoresis

Following Laemmli's 1970 protocol [16], the Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was executed.

Casein zymography was performed following Venugopal *et. Al.* [17] protocol with minor changes. The purified enzyme sample was applied in non-reducing conditions with a running buffer containing 20 mM NaCl. After electrophoresis, the gel was transferred to a 50 mM tris buffer with 1.2 % NaCl (12 g/l) pH 9.0 and incubated at 45 °C for 12 h. Stained with 0.1 % Coomassi, this procedure was carried out according to Laemmli's method [16] casein was co-polymerized with 10 % polyacrylamide gel at a final concentration.

2.7. Characterization of purified enzyme

Casein (final concentration in reaction mix: 1.0 %) was used as the substrate for the characterization of the purified protease glycine NaOH buffer (50 mM pH 10.0) and at 45 °C.

2.7.1. Choosing the ideal pH and temperature

Casein (final concentration in the reaction mixture: 1 % ie 1 g/100 mL), 50 mM buffers of KH_2PO_4 (pH 6.0–7.5), Tris (pH 8.0–9.5), and glycine NaOH (pH 10–12), and 45 °C for 30 min were used to evaluate the isolated enzyme activity. Relative activities were computed based on the enzyme's activity at 10.0 pH (optimum pH was discovered to be 10), after activities had been obtained. Temperature effects on the purified enzyme were investigated by incubating it with substrate solution (final 1 % concentration in glycine NaOH buffer, 50 mM at 10.0 pH) for 30 min at various temperatures (20–70 °C). Activities related to the topic were shown. The enzyme's activity at 50 °C was calculated as 100 %. Enzyme was very unstable at 50 °C, thus further characterization was done at 45 °C.

2.7.2. Determination of substrate specificity

To investigate the substrate specificity of the purified protease from *Halobiforma* sp. strain BNMIITR, we used substrates such as casein, bovine serum albumin (BSA), and gelatine. In brief, 0.2 mL of purified enzyme and 0.8 mL of substrate blend (glycine NaOH buffer, 50 mM at 10.0 pH; final substrate concentration in reaction mix: 1 %) were combined, and the cocktail was incubated at 45 °C for 30 min.

According to Skovgaard et al. [18]. the extracellular protease from *Halobiforma* sp. BNMIITR was tested for its elastase activity using N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide as substrate.

Esterase activity of enzymes was tested by employing *p*-nitrophenyl acetate (pNPA) as substrate according to Oceguera-Cervantes et al. [19].

2.7.3. Metal ions impact on enzyme activity

Using glycine NaOH buffer (pH 10) and various metals at concentrations of 5 and 10 mM to measure the enzyme's activity, the impact of various metal ions on the purified enzyme of *Halobiforma* sp. BNMIITR (in Tris buffer 50 mM with 1.2 % NaCl (12 g/l) at 9.0 pH) was investigated. In accordance with the procedure for the typical enzyme assay, activities were evaluated. The enzyme's activity was calculated as absolute (control) when no metal was present.

2.7.4. Thermal stability of the enzyme

By varying the temperature at which the enzyme was kept in tris buffer 50 mM with 1.2 % NaCl at 9.0 pH, (50 °C, 45 °C, 30 °C and 20 °C) for 24 h, the stability of the enzyme was ascertained. Samples were collected on a regular basis. Relative activities were calculated and initial activity was taken as 100 %.

2.7.5. Additives effects on the activity of enzyme

Protease activity was investigated at 45 °C in glycine NaOH buffer at 10 pH with various additives like serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF), EDTA and phenanthroline at 5 mM and 10 mM concentrations, cationic detergent Cetyl-trimethylammonium bromide (CTAB) anionic anionic detergent SDS and neutral detergents like Triton X-100, Tween-20, odium cholate, Tween-40, Tween-80 and sodium deoxycholate at 0.1 and 0.5 % concentrations, reducing agents as Dithiotheritol (DTT), oxidizing agents hydrogen peroxide, Ammonium persulphate (APS) at 5 and 10 mM concentration. Activities were assessed using the common enzyme assay.

2.7.6. Effects of organic solvents on protease activity

Impact of several solvents were studied by determining enzyme activity at 45 °C and 10 pH with various non polar solvents like toluene, xylene, n-heptane butanol, dichloromethane, n-hexane, octane and polar solvents like DMF, DMSO, methyl alcohol, ethyl alcohol, isopropanol, at 10 %, 30 % and 50 % (v/v) concentrations. In accordance with the procedure for the typical enzyme assay, activities were recorded. The enzyme's activity was calculated as absolute (control) when no solvent was present.

2.7.7. Stability of proteases in organic solvents

1 mL of Protease was incubated with 1 mL of organic solvents at 30 °C and 45 °C while being constantly shaken at 180 rpm. Samples were taken out after 24 and 48 h. The usual technique was followed to determine residual protease activity. As a control, the enzyme was incubated without any solvent.

2.8. Compatibility of the enzyme with detergents

Trading detergents including Rin, Ariel, Surf excel, Wheel and Tide were incubated with one mL of the enzyme at 1.0 % w/v in Tris buffer (50 mM with 1.2 % (12 g/l) NaCl at 9.0 pH) to determine the enzyme's compatibility with detergents. When residual activity was tested 4 h later, the activity without detergent was assumed to be 100 %.

3. Result and discussion

Halobiforma sp. strain BNMIITR was identified as such by Gupta et al. [20] based on pigmentation, antibiotic sensitivity, biochemical and physiological characterization, NaCl-dependent growth and 16S rRNA gene sequencing. After 96 h, the peak protease production was seen. The following culture conditions and nutritional parameters were optimized to increase the amount of proteases produced by *Halobiforma* sp. strain BNMIITR.

3.1. Effect of salinity

A wide range of salinities (2–5 M) can support the growth of *Halobiforma* sp. strain BNMIITR. The strain exhibited no growth at NaCl concentrations below 1.8 M. When the impact of salinity on protease synthesis was investigated, it was discovered that 3 M NaCl produced the highest levels of 40.84 ± 0.5957 U/mL and 749.50 ± 12.01 U/mg of specific activity (Supplementary Fig. 1). At the shake flask level, impacts of various salts on protease generation were also evaluated. In the presence of KCl, growth was very minimal and no protease synthesis was seen. NaNO$_3$ and CH$_3$COONa activity were, respectively, 5.0125 ± 0.72478, 8.2958 ± 0.00589, and 6.6041 ± 0.736 U/mL when there was Na$_2$PO$_4$ Na$_2$SO$_4$ inhibits the growth of the isolate. Thus, at a concentration of 3 M, NaCl was discovered to be the salt that produces proteases the best (Table 1).

3.2. Impact of pH and temperature

Halobiforma sp. strain BNMIITR can grow in a broad range of initial pH (6- 11), pH works as a critical factor in microbial processes. Maximum protease activity (48.75 ± 0.3871 U/mL and specific activity 864.50 ± 19.99 U/mg) was found when initial pH was 10 (Supplementary Fig. 2).

Like pH, temperature also works as a critical factor in microbial processes. By altering the physical characteristics of the cell membrane, temperature has an impact on the formation of protease. Enzyme production was discovered to be maximum at 45 °C (Supplementary Fig. 3).

3.3. Impact of various carbon sources on the synthesis of proteases

The synthesis of enzymes is regulated by both sources of nitrogen and carbon. Table 1 summarizes the influences of various carbon sources on the generation of proteases. Isolate was able to utilize all carbon sources used. Maximum production (activity 75.28 ± 1.69 U/mL and specific activity 1284.86 ± 20.76 U/mg) was found when xylose was used as a carbon source (Table 1). When low cost agricultural byproducts, such as wheat bran and wheat flour (14.81 ± 1.30 and 22.77 ± 2.28 U/mL, respectively), were utilized as the production medium, activity was decreased. When compared to the initial production medium (3 M NaCl, pH 9.0, 45 °C, and the presence of casein and tri sodium citrate as nitrogen and carbon sources), protease generation in the current study increased by 1.71

Table 1
Impact of Various salts Nitrogen and carbon sources on protease synthesis from isolate *Halobiforma* sp. strain BNMIITR.

Substrate supplied	Enzyme activity in U/ml	Specific activity U/mg
Salt 3 M		
NaCl	40.84 ± 0.59	749.50 ± 12.01
KCl	0	0
Na$_2$SO$_4$	0	0
Na$_2$PO$_4$	5.012 ± 0.724	50.90 ± 6.01
NaNO$_3$	8.29 ± 0.0058	298.85 ± 11.19
CH$_3$COONa	6.60 ± 0.736	76.21 ± 4.38
Carbon Source (1 % 1 g/100 mL)		
Glucose	51.79 ± 2.35	908 ± 19.04
Fructose	58.24 ± 3.47	927 ± 23.92
Sucrose	54.60 ± 4.12	900.22 ± 29.39
Lactose	56.57 ± 3.87	985.92 ± 26.46
Galactose	64.73 ± 1.49	1027.78 ± 14.64
Glycerol	51.88 ± 0.79	853.95 ± 13.72
Mannitol	55.29 ± 2.24	933.98 ± 13.63
Starch	49.89 ± 0.64	900.77 ± 16.49
Xylose	75.28 ± 1.69	1284.86 ± 20.76
Trisodium citrate	45.68 ± 0.43	861.86 ± 20.49
Citric acid	49.62 ± 0.58	881.64 ± 24.04
Wheat flour	14.81 ± 1.30	183.95 ± 14.32
Wheat bran	22.77 ± 2.28	22.77 ± 2.28
Nitrogen Source (1 %1 g/100 mL)		
NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$, NaNO$_3$,NH$_4$Cl, Casamino acid, Soy bean casein digest and Urea	0	0
Peptone	2.28 ± 0.12	71.82 ± 1.33
Yeast extract	5.90 ± 0.40	176.47 ± 4.89
Casein	70.22 ± 1.78	$1307.08. \pm 7.08$
Skimmed milk	64.89 ± 2.88	$1024.91 \pm 2 8.58$
Gelatin	52.62 ± 1.56	1104.42 ± 6.33
Chick pea flour	184 ± 7.65	2203.22 ± 14.04
Chick pea husk	96.47 ± 2.78	1453.22 ± 15.80
Soy bean flour	123.08 ± 2.82	1631.27 ± 6.94
Soy bean husk	66.51 ± 0.94	950 ± 10.74
Soy Peptone	19.75 ± 1.44	223.24 ± 14.49

*Protein quantification was carried out using the dye binding method, and caseinolytic activity was tested at 45 °C and pH 9.0 for 30 min.

fold at 3 M NaCl, pH 10, casein and xylose as nitrogen and carbon sources. Glucose is known to catabolically repress the production of many enzymes including protease [21] but in the current research, it was found that all the sugars have positive effects on protease production. When lactose from *Halobacillus karajensis* was present, the highest protease production was seen [22].

3.4. Impact of various nitrogen sources on the development of proteases

Table 1 summarizes how nitrogen sources affected the synthesis of proteases. The inclusion of inorganic nitrogen sources in the production medium entirely hindered the development of proteases. When using organic nitrogen sources as yeast extract and peptone, very poor output was seen. Considerable protease production was observed only when proteins were used as nitrogen sources,

Table 2
Impact of various amino acids at varying concentrations (0.1, 0.5, and 1 %) on the production of proteases by the isolate *Halobiforma* sp. strain BNMIITR. (Positive control without amino acid).

Amino acid	Enzyme activity in U/ml	Specific activity U/mg	Relative Enzyme activity in U/ml	Relative Specific activity U/mg
Positive Control	70.44 ± 2.87	1288.06 ± 19.32	100 %	100 %
Glutamate				
0.1 %	68.73 ± 1.70	1232.44 ± 32.30	97.57 %	95.68 %
0.5 %	47.55 ± 1.89	1007 ± 13.74	67.34 %	78.19 %
1 %	16.48 ± 1.01	297.33 ± 11.7	23.39 %	23.08 %
Asparagine				
0.1 %	75.18 ± 4.40	1252.04 ± 18.08	106.72 %	97.20 %
0.5 %	80.42 ± 2.18	1394.73 ± 24.74	114.16 %	108.28 %
1 %	78.47 ± 1.43	1387.27 ± 37.65	111.39 %	107.70 %
Lysine				
0.1 %	16.05 ± 1.19	332.47 ± 21.92	22.78 %	25.81 %
0.5 %	10.81 ± 0.94	253.27 ± 7.61	15.346 %	19.66 %
1 %	7.65 ± 0.53	174.02 ± 9.21	10.86 %	13.51 %
Proline				
0.1 %	81.86 ± 3.09	1394.44 ± 22.38	116.21 %	108.25 %
0.5 %	79.10 ± 1.20	1359.14 ± 20.70	112.29 %	105.51 %
1 %	71.73 ± 3.17	1278.34 ± 16.21	101.78 %	99.24 %
Threonine				
0.1 %	17.02 ± 1.03	457.65 ± 18.60	24.16 %	35.53 %
0.5 %	9.8 ± 0.94	303.66 ± 15.81	13.91 %	23.57 %
1 %	2.28 ± 0.11	81.99 ± 1.91	3.23 %	6.36 %
Tyrosine				
0.1 %	70.07 ± 1.72	1311.86 ± 17.75	99.47 %	101.84 %
0.5 %	63.5 ± 2.3	1132.79 ± 37.5	90.14 %	87.94 %
1 %	42.06 ± 0.19	813.22 ± 7.01	59.71 %	63.13 %
Tryptophane, Methionine	0	0	0	0
Arginine				
0.1 %	60.97 ± 3.28	1193.18 ± 27.11	86.55 %	93.97 %
0.5 %	35.49 ± 0.81	553.51 ± 15.4	50.38 %	42.97 %
1 %	5.522 ± 0.39	104.48 ± 7.9	7.83 %	8.11 %
Glycine				
0.1 %	51.73 ± 1.57	1086.05 ± 35.18	73.44 %	84.31 %
0.5 %	8.019 ± 0.10	212.60 ± 6.6	11.38 %	16.50 %
1 %	0	0	0	0
Histidine				
0.1 %	24.96 ± 1.11	527.45 ± 15.97	35.43 %	40.94 %
0.5 %	12.90 ± 1.00	288.69 ± 26.22	18.31 %	22.41 %
1 %	0	0	0	0
Alanine				
0.1 %	54.96 ± 0.48	1041.13 ± 19.90	78.023 %	80.82 %
0.5 %	41.66 ± 1.87	814.50 ± 36.03	59.14 %	63.23 %
1 %	39.33 ± 0.65	716.29 ± 25.20	55.83 %	55.60 %
Aspartic Acid				
0.1 %	64.20 ± 1.96	1151.73 ± 30.60	91.14 %	89.41 %
0.5 %	61.56 ± 1.01	1130.43 ± 9.07	87.39 %	87.76 %
1 %	50.57 ± 1.36	936.23 ± 21.52	71.79 %	72.68 %
Phenylalanine				
0.1 %	21.91 ± 2.67	457.52 ± 32.22	31.10 %	35.51 %
0.5 %	9.520 ± 0.10	254.88 ± 5.02	13.51 %	19.78 %
1 %	0	0	0	0
Serine				
0.1 %	32.18 ± 2.15	645.14 ± 24.44	45.68 %	50.08 %
0.5 %	25.21 ± 0.67	512.87 ± 1.98	35.78 %	39.81 %
1 %	15.40 ± 0.08	341.16 ± 4.45	21.86 %	26.48 %

*Protein quantification was carried out using the dye binding method, and caseinolytic activity was tested at 45 °C and pH 9.0 for 30 min.

which was 70.22 ± 1.78 U/mL in the presence of casein. So it can be concluded that the protease was inducible in nature. Protease production was considerably increased when low costagro industrial byproducts like soybean husk, soybean flour, chickpea flour and chickpea husk were used as nitrogen sources. Protease activity was found to be 123.08 ± 2.82 U/mL and 184 ± 7.65 U/mL in the presence of soybean flour and chickpea flour respectively (Table 1). The only limitation of using low-cost agro-industrial byproducts was delayed growth and delayed production of enzymes. When there was casein maximum production was achieved within four days but when low-cost agro-industrial byproducts were used maximum production was achieved after 8–10 days. In the present study, the protease production was enhanced by 2.94 fold and 2.17 fold when chickpea flour and soybean flour were used as nitrogen sources as compared to theinitial production medium (3 M NaCl, pH 9.0, 45 °C and when there was tri sodium citrate and casein as carbon and nitrogen source). Thus this study shows that nitrogen source has a more prominent effect on protease production as compared to carbon source.

In the present time, efforts were made to reduce the cost of industrial protease production by using cheap raw materials. India is a country that has huge agro-industrial residues. So it is economical to use such cheap agro-industrial residues as sources of nitrogen and carbon for the creation of desired products. Sonleither [23] has reported that soy meal, lactose, sucrose, and starch can be used as good sources for industrial protease production. Protease production by strain BNMIITR was noticed to be very low when there was yeast extract and peptone but it was considerably increased in the presence of casein and other proteins which showed that enzyme was inducible in nature. Casein was found as best inducer in many bacterial strains as in *Bacillus licheniformis* MIR [24]. Inorganic nitrogen sources also reported to inhibit enzyme production and similar outcomes were seen in the most present research as well. The inclusion of inorganic nitrogen sources did not result in the formation of any proteases in *Halobacterium* sp. and *Chromohalobacter* sp. TVSP101 [25,26].

3.5. Effect of various amino acids on protease production

Amino acids can catabolically repress protease generation [27]. Amino acids reported to repress the expressions of genes encoding proteases and transporters [28]. Protease production in *Halobiforma* sp. strain BNMIITR was probably inhibited by positively charged amino acids and was not affected by negatively charged amino acids but concentration above 0.1 % had negative effects on the production.

All aromatic amino acids except tyrosine at 0.1 % concentration had negative effects on protease production. Phenylalanine at 1 % and tryptophan and methionine at 0.1 % concentration completely inhibited the protease production. Phenylalanine was found to completely inhibit protease generation in *Streptomyces clavuligerus* [29]. In the present study, glycine was found to completely inhibit protease production at 1 % concentration. Activities were 80.83 %, 63.23 %. 55.61 % in the presence of 0.1, 0.5 and 1 % alanine whereas it was 50.08 %, 39.81 %, 26.48 % in the presence of 0.1, 0.5 and 1 % serine (Table 2).

3.6. Purification of the enzyme

Two litres of the crude enzyme was concentrated to 200 mL by using Pall advanced separation system with 10 kDa membrane cassettes which brought about 2-fold purification. Following acetone precipitation, the precipitate was dissolved in 50 mL of a tris buffer with 1.2 % sodium chloride at 9.0 pH. The precipitated enzyme was further concentrated to 5 mL using centrifugal concentrators (Amiconultra Millipore 10 kDa) and 8.03 fold purification was obtained after this step.

Solvent precipitation is the most commonly used method for haloarchaeal protease purification, extracellularular protease of *Natrialba magadii* was purified using ethanol precipitation [30] and of *Salinivibrio* sp. strain AF-2004 using acetone precipitation [31].

Because non-polar interactions between the matrix and protein are frequently increased when there are high salt concentrations,

Table 3
Purification of extracellular protease from *Halobiforma* sp. BNMIITR. Protease activity and specific activity profile at different steps of purification and characterization of the enzyme.

| Purification of the enzyme | | | | | | |
Purification step	Volume (ml)	Total protein in mg	Specific activity (U/mg protein)	Purification fold	Total activity units	Yield in %
Culture medium supernatant	2000	109.1	1292.52	1	140000	100 %
Protein concentrator (10 kD)	200	40.6	2700.55	2.0	109800	78.42 %
Acetone precipitation	50	3.7	10380.81	8.034	38683	27.63 %
HIC	80	0.36	40919.55	31.67	148712	10.62 %

Enzyme characterization						
Optimum	pH 10			Optimum temperature 50 °C		

| **Substrate specificity** | | |
Substrate	Enzyme activity in U/ml	Relative Activity in %
Casein	158.52 ± 3.59	100 %
Bovine Serum Albumin (BSA)	37.788 ± 2.71	23.83 %
Gelatin	3.59 ± 0.65	2.26 %
N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Elastase)	58.32 ± 0.190	36.79 %
Paranitrophenylactetate (Esterase)	47.5 ± 2.63	26.65 %

HIC has been demonstrated to be a successful method for the purification of halophilic proteins [32].

The concentrated enzyme was further purified by HIC. Total four fractions were obtained and protease activity was assessed in pooled fractions (Supplementary Fig. 4). Fraction one was found to have protease activity. Specific activity of the protease after HIC was 40919.55 U/mg and after this stage, an overall 31.67 fold purification was accomplished with a final yield of 10.62 %. Results of all purification steps have been summarized in Table 3.

Purified enzyme was exchanged with a 50 mM tris buffer at 9.0 pH, with salt concentration ranging from (0–10 %). It can be concluded from the results that enzymes require minimum 1–2 % NaCl to maintain it's activity thus in all further studies, the enzyme was buffer exchanged with tris buffer 50 mM with 1.2 % NaCl, 9.0 pH after purification.

3.7. Casein zymography and SDS PAGE

As mentioned in the materials and techniques section, casein zymography and SDS PAGE were carried out. By using SDS PAGE, all protein samples from various purification processes were examined. Purified enzyme after HIC was found to be a monomer and showed a single band whose molecular weight corresponds to 21 kDa (Fig. 1). This was further confirmed by casein zymography. Many serine proteases have been reported from haloarchaea with molecular mass in a range of 40–66 kDa [33], A moderately halophilic bacterium *Pseudoalteromonas ruthenica* developed a halo protease CPI of 38.0 kDa [34].

3.7.1. Optimization of pH and temperature

The enzyme was demonstrated to function throughout a broad pH range of 6–12, with 10 being the ideal pH (Supplementary Fig. 5). Protease from *Halobiforma* sp. BNMIITR can function at a wide range of temperatures, with 50 °C showing the highest activity (Supplementary Fig. 6). With an optimal pH of 10.0 and a temperature of 55 °C, *Geomicrobium* sp. EMB2 protease was stable over the pH range of 6.0–12.0 [32]. The most active form of an external serine protease was found in the *Halogeometricum borinquense* strain TSS101 [35], a very halophilic archaebacteria. Enzymes were discovered to be unstable at 50 °C thus further characterization was done at 45 °C.The current research outlines the extraction of a novel protease from the TSA5 strain. Analysis of the 16SrRNA sequence indicated a similarity to *Bacillus* species. The protease exhibited its highest activity at a pH of 8.5 and a temperature of 80 °C [3].

3.7.2. Determination of substrate specificity

With the use of several substrates like casein, BSA, and gelatine, the purified enzyme's protease activity was examined. Extracellular protease activity of *Halobiforma* sp. N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide was used as a substrate to measure BNMIITR. Esterase activity of enzymes was tested by using *p*-nitrophenyl acetate (pNPA) as a substrate. Results of substrate specificity have been summarized in Table 3. Enzymes were found to have maximum activity with casein (100 %) followed by BSA 23.83 % and very less activity was observed with gelatine (2.26 %). Besides having caseinolytic activity, it also had esterase and elastase activities which are rarely reported in proteases which make the enzyme efficient for further industrial application. Esterase and elastase relative activities were 26.65 % and 36.79 % respectively (100 % as caseinolytic activity).

An extracellular protease of *Halobacterium halobium* (ATCC 43214) was found to have esterase activity [36]. The serine protease from *Bacillus pumilus* demonstrated detergent stability comparable to its 100 % caseinolytic activity. Additionally, in Comparative Biochemical Studies (CBS), this protease displayed esterase and elastase activities at levels of 75 % and 26 %, respectively [37].
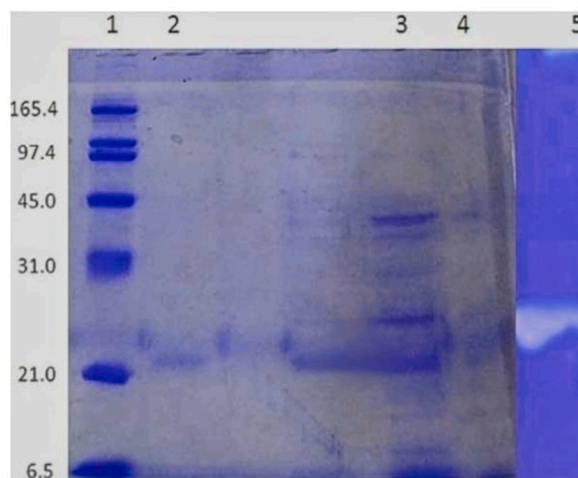

Fig. 1. Extracellular protease from isolate *Halobiforma* sp. BNMIITR was electrophoresed on sodium dodecyl sulfate polyacrylamide gel, Lane 1 containing a wide-range protein molecular weight marker (BIORAD). Purified protease is shown in Lane 2 after HIC, and protease after acetone precipitation is shown in Lane 3. Shown in lane 5 is a casein zymogram.

3.7.3. Impact of metal ions on enzyme activity

Activity of extracellular protease of *Halobiforma* sp. BNMIITR remained unaffected by barium, calcium, copper and cobalt when used at 1 mM concentration but activity was reduced to 77.93 %, 89.42 %, 94.57 % and 78.47 % respectively when used at 5 mM concentration. Activity showed a considerable decrease in the presence of nickel and zinc. The relative activities were 79.62 % and 52.63 % when there was nickel and zinc at 1 mM concentration and 37.00 % and 40.31 % when there was nickel and zinc at 5 mM concentration. Activity remained unaffected by manganese even when used at 5 mM concentration. Iron and magnesium have stimulatory effects on extracellular protease of *Halobiforma* sp. strain BNMIITR. The estimated relative activities were 114.15 % and 105.46 % when there was 1 mM iron and magnesium respectively whereas they were 102.99 % and 111.17 % when there was 5 mM iron and magnesium respectively (Fig. 2).

At doses of 1 mM and 5 mM, Ni^{+2} reduced the activity of a protease from a haloalkaliphilic *Bacillus* sp. by 75 % and 50 %, respectively, while Mn^{+2}, Zn^{+2}, and Mg^{+2} had no effect on the activity. However, Ca^{2+} increased activity [38]. A protease from *Salinivibrio* sp. strain AF-2004, a moderately halophilic bacteria, was activated by Ca^{2+} and Mg^{2+}, but its activity was decreased by 66 % when there was 5 mM zinc and by 16 % when there was 5 mM nickel [39].

These studies support the present finding that the activity of protease of *Halobiforma* sp. was inhibited by Zn^{2+}, enhanced by Mg^{2+} whereas Co^{2+}, Ba^{2+}, Ca^{2+} had no significant effects. Inhibitions of protease by Zn^{2+} and heavy metal like Ni^{2+} have also been reported [40]. Thus the enzyme in the present study showed resistance towards alkaline earth metal and heavy metals like iron, manganese, copper and cobalt but was sensitive to nickel and zinc.

3.7.4. The enzyme's thermo stability

The stability of an enzyme is a crucial factor in its industrial application. Many techniques have been created to keep the enzymes stable at high temperatures. Enzyme was very unstable at 50 °C and retained only 22.91 and 0.84 % activities after 2 and 6 h of incubation. Relative activities at 45 °C were 84.08 %, 63.744 %, 45.33 % and 37.20 % after 2, 6, 12 and 24 h of incubation (Supplementary Fig. 7). The enzyme preserved more than 83 % of its activity at 30 °C after 24 h of incubation, compared to more than 96 % at 20 °C.

3.7.5. Impact of additives on enzyme activity

Activity of extracellular protease of strain BNMIITR remained unaffected by Tween-20, Tween-40, Tween-80 at 0.1 and 0.5 % concentrations whereas it was slightly decreased by Triton X-100 to 90.90 % at 0.5 % concentration. Activity was decreased when there was CTAB, SDS, sodium cholate and sodium deoxycholate and relative activities were 93.93 %, 48.30 %, 83.50 % and 88.50 % at 0.1 % concentration and 63.63 %, 37.73 %, 59.79 % and 65.57 % at 0.5 % concentration respectively. Activities were reduced to 66.36 %, 50.50 %, and 65.95 %, 54.51 % when there was 5 and 10 mM of phenanthroline and EDTA respectively (Fig. 3).

5 mM PMSF totally reduced the activity, demonstrating that it was a serine protease. EDTA blocks the majority of serine proteases. Triton X-100, H_2O_2, Tween-80, Tween-40, and Tween-20 had no impact on the protease activity of the *Halobiforma* sp. strain BNMIITR, whereas CTAB, SDS, phenanthroline, EDTA, sodium cholate, and sodium deoxycholate inhibited it. DTT, on the other hand, enhanced it. When there was 1 mM EDTA, 10 mM EDTA, and 0.1 % SDS, the activity of a protease (extracellular and serine type) from the halophilic archaea *Halogeometricum borinquense* strain TSS101 was decreased to 65 %, 38 %, and 53 %, respectively [41]. CTAB, SDS, Triton X-100, and Tween-80 had no impact on the *Geomicrobium* sp. EMB2 extracellular serine protease's ability to function [32]. The activity of a protease from *Natrialba magadii* increased to 210 % when there was 10 mM DTT after being increased to 166 % when there was 1 mM DTT [42].

3.7.6. Protease activity and stability in organic solvents: effects of organic solvents

Effects of several non-polar solvents, such as toluene, n-hexane, n-heptane, xylene, butanol, dichloromethane, and octane, on pure
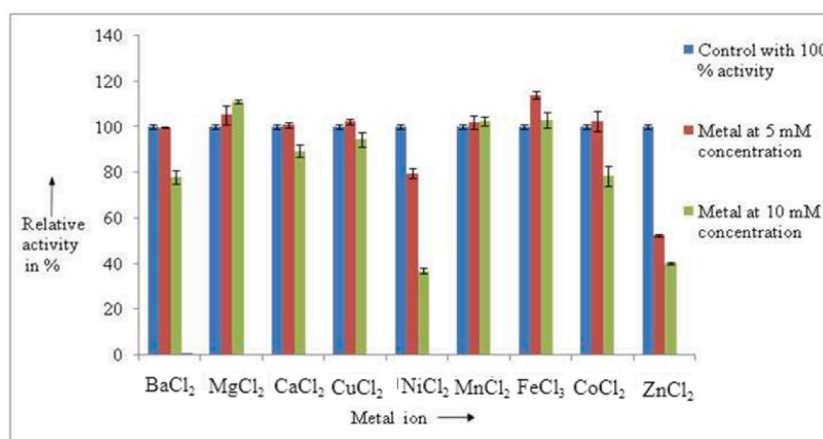

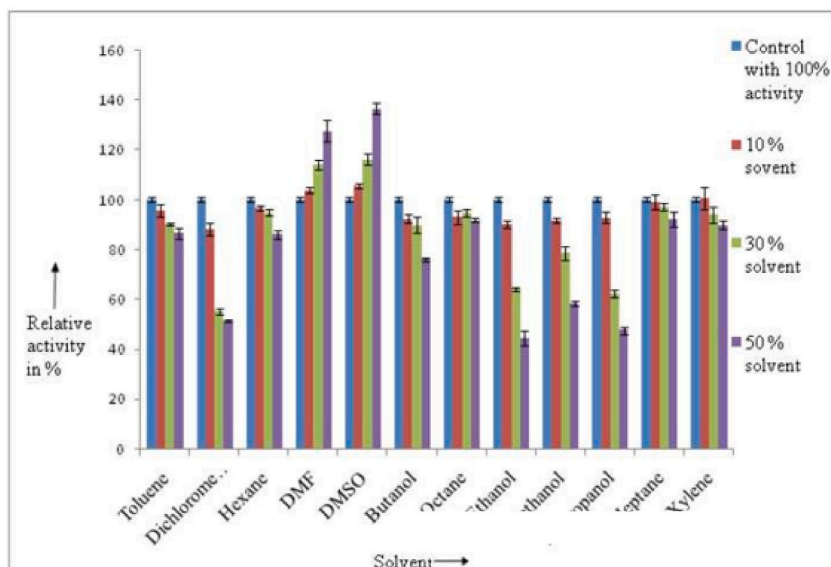**Fig. 2.** Impact of metal ions on the enzyme.

Fig. 3. Impact of various additives on the enzyme.

enzyme at 45 °C in glycine NaOH buffer (pH 10) and polar solvents as DMF, DMSO, methyl alcohol, ethyl alcohol, isopropanol, at 10 %, 30 % and 50 % (v/v) were checked. The relative activities were presented after determining the activities. Activity of extracellular protease of *Halobiforma* sp. BNMIITR remained unaffected by polar solvents at 10 % v/v concentration, but activity was reduced to 64.22 %, 78.70 %, and 62.39 % when there was 30 % methanol, ethanol and isopropanol respectively and to 44.40 %, 58.49 %, 47.60 % when there was 50 % methanol, ethanol and isopropanol respectively. Enzyme retained more than 86 % activity when there was nonpolar solvents (toluene, hexane, heptane, xylene) even at 50 % concentration. Activities were reduced to 92.45 %, 89.93 %, 76.05 % in the presence of 10, 30 and 50 % butanol respectively whereas it was 88.22 %, 55.13 %, 51.48 % when there was10, 30 and 50 % dichloromethane respectively. Interestingly activities were increased to 103.91 %, 114.17 % and 127.59 % when there was10, 30 and 50 % DMF respectively and 105.60 %, 116.33 % and 136.78 % in the presence of 10, 30 and 50 % DMSO respectively (Fig. 4).

The solvent activation in protease has rarely been reported. Some enzymes show enhancement in activity when there are organic solvents (solvent activation), a lipase from *P. aeruginosa* activated when there were polar solvents such as 2-propanol [43]. A protease from *Bacillus cereus* showed higher activity due to the solvent activation by nonpolar solvents. When there is solvent, enzymes remain unaffected to the denaturation by water with formation of multiple hydrogen bonds [44]. An exploration was conducted on a recently identified peptidase named SAPV, derived from a moderately halophilic *Virgibacillus natechei* sp. nov., strain FarDT. The enzyme rSAPV
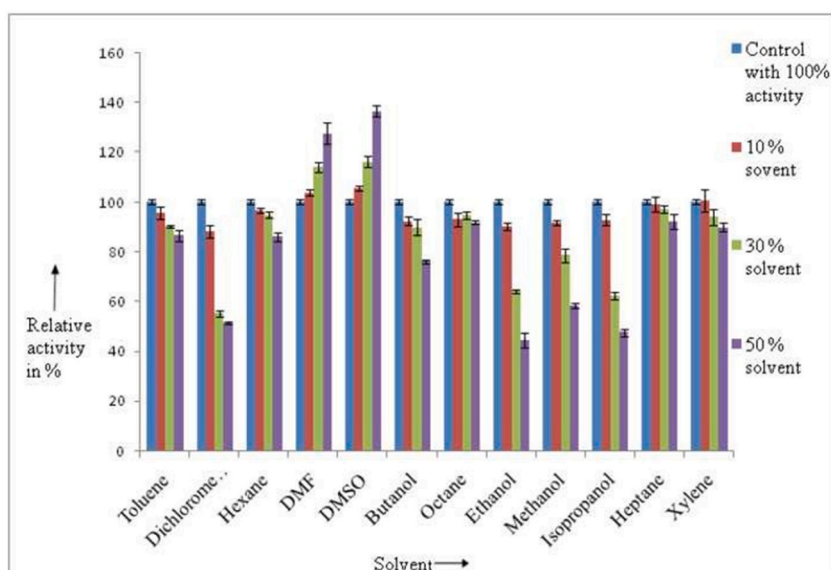

Fig. 4. Impact of organic solvents on enzyme.

was found to possess inherent stability and demonstrated elevated peptidase activities when exposed to organic solvents [45]. An alkaline protease from strain K of *Pseudomonas aeruginosa* that is stable in organic solvents was found to be activated by water immiscible solvents. Organic solvent has low dielectric constant in comparison to water thus helps to form stronger intra-protein electrostatic interactions [46]. An elastase from strain K was activated by both water miscible and water immiscible solvents, 17 % enhancement of the activity was observed by DMSO [47]. The enzyme in the current study showed very high activity in presence of DMF and DMSO. This property of enzyme can be further explored in organic synthesis.

The enzyme exhibited activity even after 48 h of incubation at 30 °C and was remarkably stable in both nonpolar and polar solvents (Fig. 5). When 50 % polar and nonpolar solvents were present, *Halobiforma* sp. strain BNMIITR's extracellular protease remained very stable. It retained more than 88 % activity in polar and non polar solvents after 24 h of incubation at 30 °C in comparison to control after 24 h. After 48 h at 30 °C, activities are recorded as 50.87 %, 65.02 %, 68.69 %, 55.93 %, 68.96 %, 67.23 %, 68.70 %, 67.62 %, 72.50 % and 72.15 % when there was buffer, ethanol, methanol, isopropanol, DMF, DMSO, xylene, dichloromethane, hexane and heptane respectively when compared to initial activity of enzyme (Tris buffer 50 mM 1.2 % NaCl 9.0 pH) at zero h.

After 24 h of incubation at 45 °C enzyme was found to have 30.64 %, 41.26 %, 37.12 %, 65.42 %, 69.28 %, 62.26 %, 51.35 %, 70.77 % and 61.66 % (Table 4.) when there was ethanol, methanol, isopropanol DMF, DMSO, xylene, dichloromethane, hexane and heptane respectively when compared to initial activity of enzyme (Tris buffer 50 mM 1.2 % NaCl 9.0 pH l) at zero h. Thus this study shows that presence of polar solvents like DMF and DMSO not only activates but it also improves the stability of the enzyme. A protease from *Salinivibrio* sp. strain AF-2004 which had considerably better stability when there were organic solvents [39]. Consequently, the protease's characteristic that, when present with organic solvents, is more stable than aqueous, is unique. Haloalkaliphilic archaeon (*Natrialba magadii*) protease After 24 hof incubation at 30 °C, demonstrated improved stability in the presence of DMF and DMSO, but activity in water miscible solvent was much lower [48].

Structure of a protein is maintained by a balance between hydrogen interactions Vander waal forces and electrostatic interactions. In aqueous system water forms weak bonds with the enzyme which bring about the destabilization of protein. When there were organic solvents, aqueous activity minimized thus stability of the protein when there was organic solvent was increased [5]. The study examines alkaline proteases that can withstand solvents, originating from haloalkaliphilic bacteria, actinobacteria, and archaea found in diverse saline environments along the coast of Gujarat, India. Enzymes that can withstand organic solvents have been reported from many genera such as *Pseudomonas, Bacillus, Enterobacter, Rhodococcus* and *Geomicrobium* [32,49]. Most of the enzymes get destabilized in organic solvents because organic solvents disrupt the aqueous shield surrounding the enzyme [5]. A protease from *Bacillus* sp. was reported to be activated up to 10 and 30 % in the presence of 5 and 10 % isopropanol and DMF, respectively.

The majority of halophilic proteases have been discovered to be active and stable under high salinity conditions, making them suitable for usage in organic solvent systems. It was discovered that a serine protease from *Chromohalobacter* sp. retained 100 % of its activity when there was 10 % (v/v) DMSO but lost 25 % in the presence of 10 % (v/v) DMF [50]. A protease of halophilic *Bacillus* sp. showed enhanced activity in polar solvents but activity was highly decreased in nonpolar solvents by more than 80 % in 50 % toluene after 24 h [51]. A protease from marine *Saccharopolyspora* sp. A9 had more than 70 % activity in non-polar solvents but activity was 21–23 % in polar solvents after 48 h [52].

Organic solvent compatible proteases may play an important role in marine industry by reducing biofouling, thus organic solvent
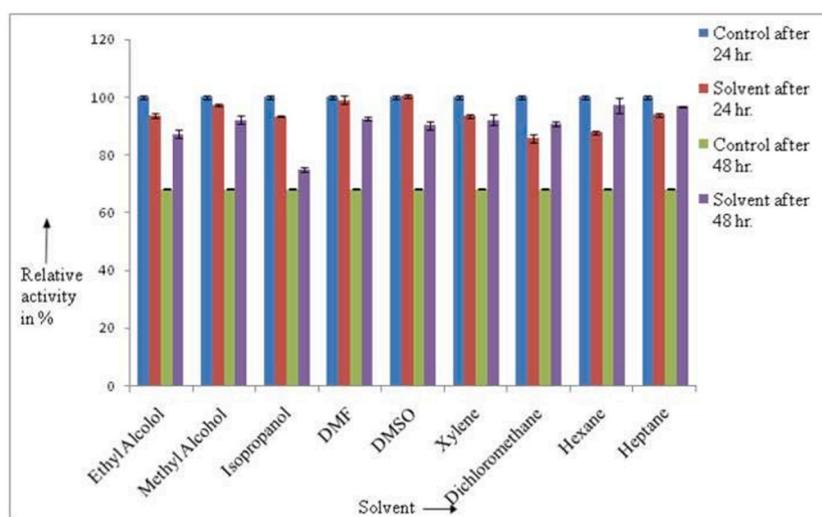

Fig. 5. Impact of organic solvents on enzyme. After 24 h (100 %), relative activities were estimated using the aqueous phase (1 ml enzyme + 1 ml 50 mM Tris buffer pH 9.0, including 1.2 % NaCl).
*Figs. 1–5 Enzyme (in 50 mM Tris buffer pH 9.0, including 1.2 % NaCl) activity was examined for 30 min at 45 °C and pH 10 in a buffer containing 50 mM glycine and NaOH. Control (100 % is shown by the blue bar). The values shown are the mean minus the standard deviation, for n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tolerant and stable enzymes are industrially more desirable [4].

3.8. Detergent compatibility of the enzyme

After 4 h of incubation with the branded detergents Tide, Ariel, Wheel, Rin, and Surf excel (1.0 % w/v), it was discovered that the protease was stable and compatible with detergents since it preserved more than 95 % of its activity. Table 4 provides a summary of the findings. Further applications for this characteristic include detergent compositions. A recently developed serine alkaline protease, identified as SAPGB and derived from *Gracilibacillus boraciitolerans* strain LO15 exhibited notable resilience to various organic solvents and displayed exceptional compatibility with detergents [53]. An alkaline protease with thermos stability, obtained from *Caldicoprobacter guelmensis,* has the potential for utilization as an additive in detergent formulations, specifically for enhancing cleaning effectiveness [54].

Cellulase and protease enzymes are recognized constituents within laundry detergent formulations, playing a role in the chemical breakdown of stains and enhancing the overall cleaning efficacy on laundered fabrics [55]. Elucidates on laundry detergent compositions wherein the synergy between a protease enzyme and a modified polyamine cotton soil release agent leads to improved cleaning and soil release advantages [56]. Outlines that the incorporation of a lipase enzyme, in conjunction with a proteolytic enzyme and a surfactant, is purported to yield benefits in terms of removing dingy soil and maintaining whiteness. The protease enzymes, as described, contribute in the range of 0.005–0.1 [57].

4. Conclusion

The enzyme described in this work demonstrated exceptional stability in both polar and non-polar solvents. The level of activity was extremely high when non-polar solvents were present. At 30 °C for 48 h, it remained stable in both polar and nonpolar solvents. The solvents utilized in trans-esterification reactions catalyzed by proteases are DMF and DMSO. When there was DMF and DMSO, the majority of the identified proteases lost their activity. The protease in the current study was activated when there was DMF and DMSO and retained more than 65 % of initial activity in the presence of 50 % DMF and DMSO at 45 °C after 24 h and more than 57 % of initial activity in the presence of 50 % DMF and DMSO at 30 °C after 48 h, demonstrating its potential for use in chemical synthesis.

The protease in the current study is remarkably stable when coupled with nonpolar solvents like xylene, a crucial component of paints. Additionally, this protease is suited for use in saline environments since it needs less NaCl to function and its activity is boosted by Mg^{+2} while being unaffected by Ca^{+2}. The majority of solvent-stable proteases exhibit decreased activity in artificial seawater as a result of NaCl, Mg^{+2}, and Ca^{+2} inhibition. The majority of halophilic proteins cannot function in such an environment because they need high salt concentrations.

Microbial biofouling starts with the creation of a conditional layer that has marine environment unicellular microorganisms adhered to it. In the current investigation, the enzyme displayed a wide range of substrate specificity. As a result, it can break down a variety of proteins and lipoproteins and be applied to the production of marine paint. It was discovered that the enzyme presented in the current study was 95–100 % compatible with conventional detergents and was active in the presence of diverse surfactants, oxidizing, and reducing agents. Further applications for the extracellular protease of Halobiforma sp. BNMIITR include detergent formulation. The distinctive physical characteristics of our enzyme underscore its potential value in commercial applications, particularly in the formulation of laundry detergents and as antifouling agent. Further investigations are recommended to explore and validate its suitability for broader commercial use.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Meenu Gupta: Writing – review & editing, Writing – original draft, Investigation. **Bijan Choudhury:** Supervision, Project administration. **Naveen Kumar Navani:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Meenu Gupta reports administrative support was provided by J. D Womens College Patna. The author declared that, having no conflict of interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25084.

Table 4

The stability of an enzyme in the presence of organic solvents After 24 h at 45 °C.

Effects of organic solvents on protease stability		
Organic solvent	Enzyme activity in U/ml after 24 h at 45 °C	Relative activity in % in comparison to initial 0 h activity
Control	61.86 ± 0.54	44.19 %
Ethyl alcohol	42.90 ± 0.83	30.64 %
Methyl alcohol	57.76 ± 0.76	41.26 %
Isopropanol	51.97 ± 3.0	37.12 %
DMF	91.59 ± 2.82	65.42 %
DMSO	96.99 ± 0.55	69.28 %
Xylene	81.17 ± 1 .49	62.26 %
Dichloromethane	71.89 ± 3.90	51.35 %
Hexane	99.09 ± 0.43	70.77 %
Heptane	86.32 ± 3.34	61.66 %
Detergent compatibility of the enzyme at 45 °C after 4 h		
Commercial detergent	**Enzyme activity in U/ml after 4 h at 45 °C**	**Relative activity in %**
Control	186.30 ± 3.175	100 %
Surf Exel	180.66 ± 2.59	96.96 %
Rin	194.13 ± 3.19	104.20 %
Nirma	191.87 ± 2.43	102.98 %
Wheel	188.05 ± 2.77	100.93 %
Tide	178.80 ± 1.60	95.97 %
Ariel	187.62 ± 3.64	100.70 %

*Experiments were done in 50 mMTris buffer pH 9.0 and 1.2 % NaCl.The values shown are the mean minus the standard deviation, for n = 3. Relative activity was estimated using the enzyme's detergent compatibility at 45 °C after 4 h and the aqueous phase (1 ml enzyme + 1 ml 50 mMTris buffer pH 9.0, including 1.2 % NaCl).

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