



## Research article

Enhanced catalytic activity of *Bacillus aryabhatai* P1 protease by modulation with nanoactivatorAnupama P. Pathak<sup>a</sup>, Mukundraj G. Rathod<sup>b</sup>, Megha P. Mahabole<sup>c</sup>, Rajendra S. Khairnar<sup>c,\*</sup><sup>a</sup> School of Life Sciences (DST-FIST Phase-I & UGC-SAP DRS-II Sponsored School), Swami Ramanand Teerth Marathwada University, Nanded 431606, Maharashtra, India<sup>b</sup> Department of Biotechnology & Bioinformatics (U.G. & P.G.), Yeshwant College of Information Technology (BT & BI) Parbhani (affiliated to S.R.T.M. University, Nanded), Maharashtra, India<sup>c</sup> School of Physical Sciences, Swami Ramanand Teerth Marathwada University, Nanded 431606, Maharashtra, India

## ARTICLE INFO

## Keywords:

Bioinformatics  
 Biotechnology  
 Microbiology  
 Proteins  
 Biochemistry  
 Alkaline protease  
 Alkaliphiles  
*Bacillus aryabhatai*  
 Nanoactivator  
 Nanobiotechnology  
 Nanoceramics

## ABSTRACT

In the developing area of modern nanobiotechnology, the research is being focused on enhancement of catalytic performance in terms of efficiency and stability of enzymes to fulfill the industrial demand. In the context of this interdisciplinary era, we isolated and identified alkaline protease producer *Bacillus aryabhatai* P1 by polyphasic approach and then followed one variable at a time approach to optimize protease production from P1. The modified components of fermentation medium (g/L) were wheat bran 10, soybean flour 10, yeast extract 5, NaCl 10, KH<sub>2</sub>PO<sub>4</sub> 1, K<sub>2</sub>HPO<sub>4</sub> 1 and MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 (pH 9). The optimum alkaline protease production from P1 was recorded 75 ± 3 U/mg at 35 °C and pH 9 after 96 h of fermentation period. Molecular weight of partially purified P1 alkaline protease was 26 kDa as revealed by SDS-PAGE. Calcium based nanoceramic material was prepared by wet chemical precipitation method and doped in native P1 protease for catalytic activity enhancement. Catalytic activity of modified P1 protease was attained by nanoactivator mediated modulation was more by 5.58 fold at pH 10 and 30 °C temperature. The nanoceramic material named as nanoactivator, with grain size of 40–60 nm was suitable to redesign the active site of P1 protease. Such types of modified proteases can be used in different nanobiotechnological applications.

## 1. Introduction

A wide range of microorganisms from extreme habitats is known for producing novel and robust biocatalysts, extremozymes [1]. Alkaline protease is one of the groups of commercially important extremozymes that catalyzes the hydrolysis of peptide bonds in protein molecules. Protease constitute at least 60% of the total industrial enzyme market where they are widely used in several industrial sectors such as laundry detergent, food, pharmaceutical, chemical, leather and silk apart from waste treatment [2, 3, 4, 5, 6]. It has been well-documented that proteases can efficiently hydrolyze proteins to biopeptides, which have excellent antioxidants, anti-inflammatory and anti-microbial activities and may help to prevent various chronic diseases such as obesity and cardiovascular diseases [7]. Alkaline proteases are the major content of enzyme based cleaning agents which are used to remove stains of grass, blood, egg and human sweat from cloths. Subtilopeptidase A is an alkaline protease based optical cleaner which is used for cleaning of eye

contact lenses. Alkaline proteases are also used for degumming of silk, desensitization of milk proteins, selective delignification of hemp and dehairing by hydrolyzing hair follicular proteins [2, 8, 9, 10]. These enzymes, owing to their characteristic nature of aiding digestion, have the potential to contribute to the development of value-added products [11]. This has created an increasing attention toward the exploitation of exotic microbial strains for the production of alkaline proteases from novel sources [9, 11]. With the continuous search of an efficient alkaline protease producer, *Bacillus* genus has found to be the highest potent producer of Alcalase™, Esparase™, Savinase™ and Subtilisin Carlsberg™ for a commercial purpose [2, 12, 13, 14]. However the demand for potent active proteases with good stability over a range of temperature, pH, mineral ions and various organic solvent continue to initiate the search for novel proteolytic enzymes [14, 15].

The increasing interest and escalating demand of alkaline proteases for industrial processes have spurred the search for new or improved properties in proteases by using some imminent technologies.

\* Corresponding author.

E-mail address: [profkhairnar@gmail.com](mailto:profkhairnar@gmail.com) (R.S. Khairnar).

Traditionally, the production of alkaline protease was optimized by using statistical approaches like OVAT, Plackett-Burman design, response surface methodology and Taguchi. Later on, the innovations are being made in the naturally occurring enzymes utilizing various tools of r-DNA technology and protein engineering, which allows editing and redesigning peculiar residues of the enzymes for its better fitment into the process [1]. Recently, with the progress in technology, it has become possible to modify native (natural) enzyme by doping nanoactivator to obtain a better catalytic efficiency with improved functional features [16]. With the development of nanotechnology, a major impact on materials science has been noticed. The production of nanomaterials has gained considerable attention to catalysis, adsorption and optical applications, particularly when the biomaterials are involved [17, 18]. In this regards, nano-hydroxyapatite bioceramic (nano-HAp) is attracting interest as a bio-nanomaterial in biomedical and healthcare field and in wastewater treatment [19, 20]. Nano-hydroxyapatite bioceramic is robust in nature and its outstanding properties like biocompatibility, bioactivity, osteoconductivity, non-toxicity, non-inflammatory are conducive for a variety of versatile applications that include bone tissue engineering, bone void fillers for orthopedic, traumatology, spine, maxillofacial and dental surgery, orthopedic and dental implant coating, restoration of periodontal defects, edentulous ridge augmentation, endodontic treatment like pulp capping, repair of mechanical furcation perforations and apical barrier formation, fillers for reinforcing restorative glass ionomer cement (GIC) and restorative composite resin, desensitizing agent in post teeth bleaching, remineralizing agent in toothpastes, early carious lesions treatment and delivery of drug and gene [18, 21, 22]. Thus, it exhibits excellent biocompatibility with various kinds of cells and tissues, making it an ideal candidate for tissue engineering, orthopedic and dental applications [23, 24].

Beside aforementioned applications, nano-hydroxyapatite bioceramic based composites are used as a filter aid for absorbing and decomposing automotive pollutant carbon monoxide and removal of fluoride through an ion-exchange mechanism [25, 26]. Recently nano-hydroxyapatite is used in catalysis and protein separation; however, its many unrevealed applications are under investigation into various multidisciplinary teams of researchers [27, 28, 29].

In present investigation, isolation and partial characterization of efficient alkaline protease was carried out. The production was carried out using a variety of synthetic and cheaply available carbon and nitrogen sources. This manuscript describes an eco-friendly approach to optimize alkaline protease production from efficient producer. The novel part of our work is the dynamic features of calcium metal based nano-HAp-bioceramic material, named as nanoactivator, which was synthesized from hydroxyapatite ceramic (HAp) by wet chemical precipitation method. It was used then for modification of native alkaline protease in order to enhance catalytic activity of protease. Nano-HAp is resistant to extremely high temperature and pH. It is therefore not only compatible additive but can be used to enhance resistance and catalytic efficiency of alkaline protease in every application. Hence the subject is certainly, timely and scientifically important.

## 2. Materials and methods

### 2.1. Materials used

The solvents, sugars, staining dyes and other chemicals were used of analytical grade and purchased from either HiMedia or SD-fine Chem. Ltd. Mumbai. The instruments viz. colony counter (HiMedia, Mumbai), compound microscope (Olympus Corporation, Japan), cooling centrifuge machine (Remi, Mumbai), bacteriological incubator (Kumar Corporation, Mumbai), orbital shaking incubator (CIS 24BL, Remi, Mumbai), digital pH meter and thermometer (Systronics), refrigerator (Godrej), spectrophotometer (Shimadzu Corporation), Weighing balance, SDS-PAGE assembly (Genei, Bangalore) and micropipette (Genei, Bangalore) were used to perform necessary experiments.

### 2.2. Methodology

#### 2.2.1. Isolation and screening

In April 2011, poultry farm waste contaminated soil samples were collected in pre-sterilized cotton bags and transported to the laboratory after ~2 h. The samples were immediately processed for further analyses. 1 g of composite soil sample was mixed with 10 mL of distilled water and pH was measured. The diluted stock samples were then inoculated in pre-sterilized Horikoshi and Akiba broth (pH 10) and incubated at 30 °C and 120 rpm speed for 72 h. The enriched samples were further spread on Horikoshi and Akiba agar plates to obtain isolated colonies [30]. Isolated colonies were further spot inoculated on alkaline casein-yeast extract-peptone (CYP) agar plates containing (g/L) casein 10, peptone 5, yeast extract 1, K<sub>2</sub>HPO<sub>4</sub> 1, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2, CaCl<sub>2</sub> 0.1, Agar 25 (pH 9). These plates were incubated at 30 °C for 48 h. After incubation, amido black solution was poured on each grown culture to observe either presence or absence of a zone of proteolysis [31]. A colony showing the largest zone of proteolysis was designated as P1 and the pure culture of P1 further used for alkaline protease production.

#### 2.2.2. Alkaline protease production and partial purification

Horikoshi-I medium (composition in g/L: glucose 10, peptone 5, yeast extract 5, K<sub>2</sub>HPO<sub>4</sub> 1, KH<sub>2</sub>PO<sub>4</sub> 1, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2) (pH 10) was inoculated with 5% inoculum of P1 isolate containing  $12 \times 10^7$  cfu/mL and incubated in orbital shaking incubator at 30 °C and 120 rpm for 72 h. Partially purified enzyme was obtained by centrifuging fermented broth at 4 °C and 10,000 rpm speed followed by precipitation with 60% saturated ammonium sulfate. The precipitate was further dissolved in 0.2 M Glycine-NaOH buffer and samples were dialyzed against the same buffer (pH 10). Partially purified alkaline protease was stored at 4 °C for further investigation [3, 4].

#### 2.2.3. Enzyme activity assay and molecular weight determination of P1 protease

The catalytic activity of P1 protease was assayed by incubating 1 mL of crude P1 protease with 1 mL of 1% buffered casein (0.2 M Glycine-NaOH buffer, pH 10.0). The reaction mixture was incubated for 10 min and after incubation the reaction was terminated by adding 3 mL of 0.44 M Trichloro acetic acid. The absorbance was measured by using an UV/VIS spectrophotometer at 280 nm. One unit activity was defined as the amount of enzyme required to liberate  $1 \mu\text{mol min}^{-1} \text{mL}^{-1}$  of tyrosine, under the assay conditions [3, 4, 32]. The total protein content of crude P1 protease was determined using BSA as standard [33]. Molecular weight of partially purified P1 protease was determined using the method as described by Laemmli [3, 34].

#### 2.2.4. Preliminary identification of P1 isolate

Morphological and microscopic characters, sugar utilization pattern and enzyme profile of P1 were recorded. P1 was subjected for Gram staining and micrometry to determine its Gram-stain reaction and cell size respectively [35]. P1 was further inoculated in modified alkaline Schaeffer medium and spore staining was performed after incubation of 15 days [30, 36]. The sugar utilization pattern was determined by inoculating pure culture of P1 in basal medium containing adonitol, arabinose, cellobiose, dextrose, fructose, galactose, glycerol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose individually. The extracellular enzyme profile was determined by inoculating P1 in medium containing substrate viz. starch (20 g/L), gelatin (10 g/L), cellulose (5 g/L), urea, tween-80 (10 g/L) and pectin (5 g/L) individually [31]. Catalase, oxidase and IMViC tests were also carried out as per the standard procedures. The alkaline SIM agar was used to determine motility and H<sub>2</sub>S production. Effect of pH on the growth of P1 was determined using a universal buffer of various pH values ranging from pH 6–13. Effect of temperature on growth of P1 was determined by incubating culture at various temperatures from 25–75 °C. Effect of NaCl

concentration on growth of P1 was determined at various NaCl concentrations of 0–10%. Effect of incubation period on growth of P1 was determined by observing its growth rate up to 120 h [3,4,35,36,37,38,39].

### 2.2.5. Ribotyping

Ribotyping is a molecular technique that uses information about 16S rRNA gene sequence which provides accurate grouping of organism hence it is considered a powerful tool for the rapid identification and characterization of bacterial species [40]. In this context, P1 was subjected for 16S rRNA molecular analysis. DNA was isolated by cell lysis method and 16S rDNA was amplified in a thermocycler using a pair of primers, forward: (27f) AGAGTTTGATCMTGGCTCAG and reverse: (1492r) TACGGYTACCTTGTTACGACTT. The amplified 16S rDNA PCR product was sequenced using an automated sequencer (Applied Bio-Systems, USA). Sequence similarity search was performed for the identified 16S rRNA partial gene sequence with online bioinformatics search tool BLAST [17, 18]. The phylogenetic analysis of 16S rRNA gene sequence of the P1 along with the sequences retrieved from the Genebank was carried out with MEGA 6.06 using the neighbor-joining method and 1,000 bootstrap replicates [41]. P1 was processed for deposition at National Centre for Microbial Resource formerly known as the Microbial Culture Collection, NCCS, Pune after biochemical and molecular identification [12, 42, 43].

### 2.2.6. Optimization of physicochemical factors for maximum alkaline protease production

**2.2.6.1. Optimization of physical factors and salinity of medium.** Horikoshi-I medium [30] was used as a basal medium for optimization of physicochemical parameters for maximum alkaline protease production by using one variable at a time approach (OVAT) in a submerged fermentation system. The optimum factor achieved once in a step was fixed subsequently for the next step of the investigation. Production of alkaline protease from P1 was evaluated at pH range 8–12, temperature range 25–65 °C with increments of 1 pH unit and 10 °C respectively. Effect of incubation period on alkaline protease production from P1 was determined by observing enzyme production up to 120 h. Growth of P1 and production of P1 protease was determined at 0–6% NaCl concentrations and 60–180 rpm agitation speed [3, 4].

**2.2.6.2. Optimization of carbon source.** Effect of different carbon sources on growth and production of P1 protease was evaluated by replacing glucose (10 g/L) in basal medium, with various carbon sources viz. arabinose, cellobiose, fructose, galactose, lactose, maltose, mannitol, mannose and starch and low cost agro industrial residues viz. wheat bran, rice bran and wheat flour. Protease production in the presence of glucose was considered as control [3, 4].

**2.2.6.3. Optimization of nitrogen source.** Various nitrogen sources viz. beef extract, sodium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, urea, yeast extract, tryptone, gelatin and skimmed milk, protein rich cheap agricultural materials viz. soybean flour and chickpea flour, and agro-industrial residues viz. corn seed meal and cottonseed meal were used in replacement with peptone (5 g/L) to study their effect on growth and production of P1 protease. Protease production in the presence of peptone was considered as control [3, 4].

### 2.2.7. Bulk production and partial purification of P1 protease

Bulk production of P1 protease was carried out in an Erlenmeyer flask (capacity 1 L) containing modified Horikoshi-I medium (MHM) composed of wheat bran 5 g, soybean flour 5 g, NaCl 5 g, KH<sub>2</sub>PO<sub>4</sub> 0.5 g, K<sub>2</sub>HPO<sub>4</sub> 0.5 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g and distilled water 500 mL (pH = 9). 5% inoculum of P1 isolate containing 12 × 10<sup>7</sup> cfu/mL was added in MHM. The

production of alkaline protease was carried out at 35 °C and 120 rpm agitation speed for 96 h. After completion of the production period, the fermented broth was centrifuged at 10,000 rpm for 10 min and the cell free supernatant was collected. Enzyme precipitation was carried out by using the ammonium sulfate precipitation method (30–80% saturation). The highest catalytically active fraction of enzyme precipitate was collected and dissolved in 50 mM NaCl, glycine-NaOH buffer (200 mM, pH 10), carbonate-bicarbonate buffer (200 mM, pH 10), Tris-HCl buffer (200 mM, pH 10), KCl-NaOH buffer (200 mM, pH 10), tap water and distilled water individually. The most catalytically active fraction was dialyzed and dissolved in the same kind of buffer [3, 4].

### 2.2.8. Characterization of P1 protease: Part-I

**2.2.8.1. Determination of kinetic parameters.** Different concentrations of casein substrate (5–20 mg/mL) were prepared in 0.2 M glycine-NaOH buffer (pH 10) and used to determine the optimum substrate concentration for catalytic activity of P1 alkaline protease.  $K_m$  and  $V_{max}$  values were calculated after constructing Lineweaver-Burk plot [44]. Concentration of casein at which maximum enzyme activity obtained was considered to be 100% and relative enzyme activity was calculated. The effect of various modifiers such as metal ions, activators, inhibitors, chelators, solvents, detergents and other selected effectors was evaluated on P1 protease activity [3, 4, 45, 46, 47, 48, 49].

**2.2.8.2. Substrate specificity.** Catalytic activity of partially purified P1 alkaline protease was assayed using various substrates by mixing 1 mL of P1 protease and 1 mL of assay buffer containing 10 mg/ml gelatin, BSA and feather meal individually and the protease activity was determined. The casein dependent protease activity was considered as a control for calculating percent relative activity [3, 4, 50].

**2.2.8.3. Determination of optimum pH and temperature of P1 protease.** The optimum pH of P1 protease was determined at pH range 3–13 using universal buffer [51]. The optimum temperature for catalytic activity was determined by assaying enzyme reactions to various temperatures ranging from 10–80 °C [4, 17].

### 2.2.9. Synthesis of nano hydroxyapatite bioceramic (nano HAp)

The nanoceramic selected for modification of P1 protease was calcium hydroxyapatite [Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>]. This nanoceramic was synthesized by a wet chemical precipitation method in which calcium nitrate tetrahydrate (Ca(NO<sub>3</sub>)<sub>4</sub>H<sub>2</sub>O) and di-ammonium hydrogen phosphate ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) were used as precursors. The molar concentrations of the above materials were maintained so as to have the ratio of Ca/P of 1.67 (10:6). The precipitation was carried out by the drop-wise addition of 0.6 M di-ammonium hydrogen phosphate into 1 M calcium nitrate tetrahydrate solution, under constant stirring of 150 rpm at 80 °C for 4 h, followed by aging for about 24 h. The white precipitates were then washed 3–4 times with milliQ water to remove the rest of the precursors, and dried in a hot air oven at 100 °C. The dried sample was further annealed at 1000 °C for phase formation. AFM and XRD analyses of the preparation phase were performed for checking crystalline nature and finding grain size [52, 53, 54, 55, 56, 57].

### 2.2.10. Nanoceramic based optimum modification in P1 protease

**2.2.10.1. Modification of P1 protease by doping different concentration of nano HAp.** Modification was carried out by incubating crude P1 protease with different concentrations of selected nanoceramic (2–10 mg/mL) in the shaking incubator at 30 °C for 2 h. After incubation alkaline protease activity assay was performed as mentioned earlier. The activity of P1 protease in the absence of nanoceramic was considered to be 100% (control) and residual (remaining) activities were calculated. The

optimum concentration of nanoceramic required to make necessary modifications was recorded [16, 58].

**2.2.10.2. Modification of P1 protease by doping nano HAp under different incubation period.** The time required for optimum modification in P1 protease was evaluated by using pre-determined optimum concentration of nanoceramic and changing the pre-incubation period as 1, 3, 6 and 9 h. Catalytic performance of modified P1 protease was assayed. The activity of P1 protease without pre-incubation with nanoceramic was considered to be 100% (control) and residual activities were calculated [16].

### 2.2.11. Characterization of modified P1 protease: Part-II

**2.2.11.1. Effect of pH and temperature on modified P1 protease activity and hydrolysis of protein substrates.** Optimum pH and temperature for catalytic activity was determined by replacing native P1 protease with modified P1 protease in assay conditions as mentioned previously. Hydrolysis of different protein substrates (10 mg/ml) viz. gelatin, BSA and feather meal by the modified P1 protease was also attempted under the assay conditions. Casein dependent activity was considered to be 100% and relative enzyme activities were calculated [16, 58].

### 2.2.12. Statistical analysis

Data are reported as means  $\pm$  standard deviations based on the experiments performed in triplicates (n = 3). Mean values were considered for graphical representation of data. In figures, the data points are presented with error bars of standard deviations. MS-Excel 2013 software was used for statistical analysis [3, 4].

## 3. Results and discussion

### 3.1. Isolation and screening

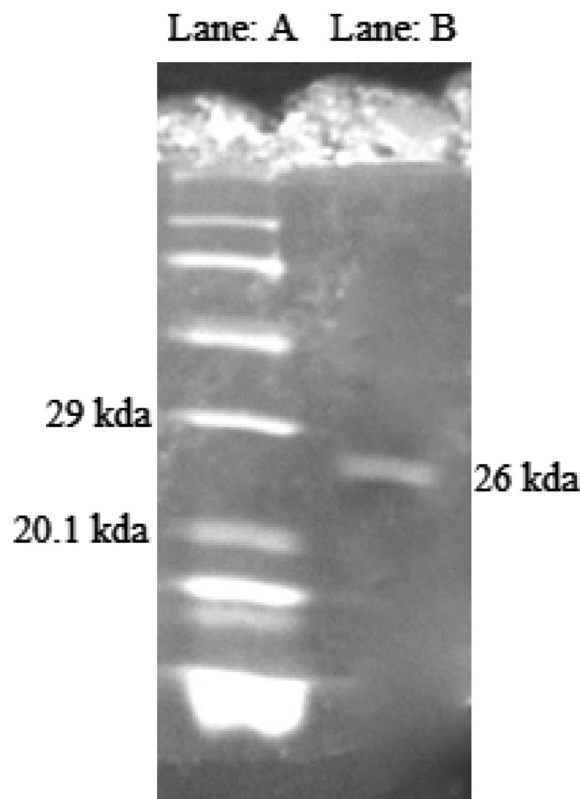
pH of the collected poultry farm waste soil sample was 8.0. After enrichment of samples, 40 microbes were isolated. These isolates were plated individually on alkaline CYP agar plates (casein 1% w/w). Ten isolates showed zone of proteolysis. Out of ten protease producers, an efficient alkaline protease producer was selected for further investigation and designated as P1.

### 3.2. Alkaline protease production, partial purification and molecular weight determination

P1 has produced 17 U/mL of alkaline protease at pH 10.0 and 30 °C after an incubation period of 72 h. The protein content of partially purified P1 protease was determined as 2 mg/mL and specific enzyme activity was calculated 8.5 U/mg. SDS-PAGE analysis revealed that the molecular weight of partially purified P1 alkaline protease was 26 KDa (Figure 1). Many researchers have recorded the molecular weights of alkaline proteases in the range of 15–30 kDa with few exceptions as 31.6, 33, 36 and 45 kDa [11]. Molecular weights of partially purified alkaline proteases from *Halomonas venusta* LAP515, *Brachy bacterium* sp. LAP214, *Bacillus pseudofirmus* LAP220, and *Brevibacterium casei* LAP223 were recorded as 20.1, 14.3, 29, and 43 KDa respectively [3].

### 3.3. Preliminary identification

A colony of P1 was appeared white and round without any pigmentation. Colony size was 3 mm with smooth surface and raised elevation. P1 was Gram-stain positive and 3  $\mu$ m long and 1  $\mu$ m wide thick rod. Cells of P1 were motile and spore former. Dextrose, fructose, lactose, sucrose, mannitol, maltose, xylose, arabinose, galactose, glycerol, ribose, inulin and salicin were utilized by P1 however cellobiose, sorbitol, mannose, adonitol, mellibiose, raffinose, rhamnose and trehalose were not utilized. It has utilized trisodium citrate as a sole source of carbon and showed



**Figure 1.** SDS-PAGE analysis of partially purified P1 alkaline protease. Lane A: Standard broad-range protein molecular weight markers GeNei™ Bioscience, Cat. No. PMWB 623110475001730. Lane B: Partially purified P1 protease (26 KDa). The unedited and non-adjusted full image of the gel has been provided in the supplementary file.

negative results of indole, MR and VP tests. P1 was positive for catalase and oxidase tests and negative for H<sub>2</sub>S production test. P1 has hydrolyzed casein, starch and gelatin and produced extracellular caseinase, amylase and gelatinase however cellulose, urea, tween-80 and pectin were not hydrolyzed. P1 has shown growth at pH range 8–11 with an optimum at pH 9. P1 could not grow at pH 6, 7 and 12. P1 has shown growth at temperature range 20–50 °C with optimum at 30 °C. P1 showed growth in the range of 0–5% NaCl concentration. Optimum incubation period for the growth of P1 was recorded 24 h.

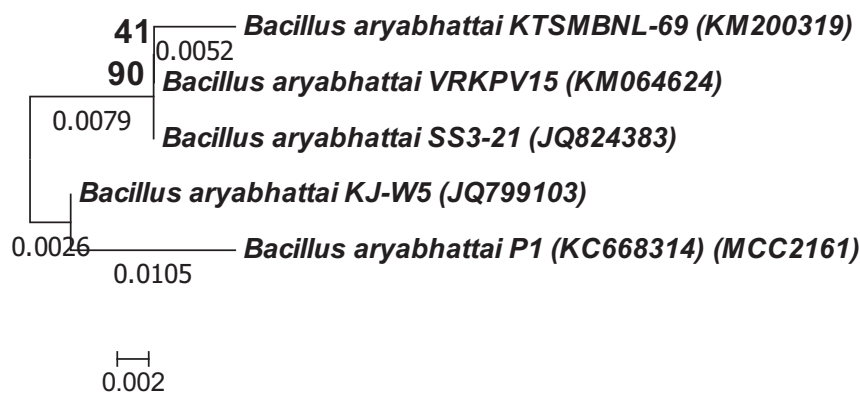
### 3.4. 16S rRNA based identification of strain P1

The sequenced PCR product of 1582 bp length was subjected to BLAST analysis. The sequence similarity analysis of 16S rDNA sequence has shown its maximum identity of 98% with *Bacillus aryabhatai* PSB57; therefore we decided to designate P1 as *Bacillus aryabhatai* and the sequence data was submitted in NCBI Genebank under the accession number KC668314. The result of phylogenetic analysis showed distinct clustering of the isolate and confirmed the results of the sequence similarity analysis (Figure 2). *Bacillus aryabhatai* P1 was deposited under accession number MCC2161 in National Centre for Microbial Resource at National Center for Cell Science (NCCS), Pune, Maharashtra.

### 3.5. Optimization of physicochemical factors for maximum alkaline protease production

#### 3.5.1. Effect of pH on P1 protease production

Environmental factors such as temperature, pH, and incubation period considerably influence microbial metabolism up to a large extent. These factors are important to promote, stimulate, enhance and optimize

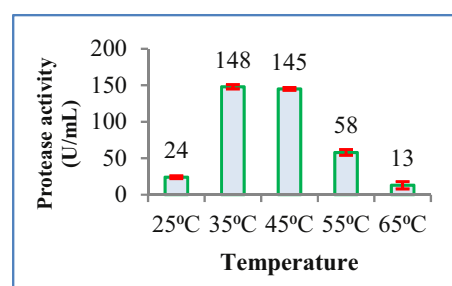


**Figure 2.** Evolutionary relationship and phylogenetic affiliation of *Bacillus aryabhatai* P1 (isolated from poultry farm waste samples) with its closely related species from previously reported various habitats viz. poultry litter (KM200319), plastic waste dumped marine soil, Marakannam Beach, Tamil Nadu (KM064624), Arabian sea coast (JQ799103) and sago industry waste sample (JQ824383). For detailed information, Genbank accession numbers are given in parenthesis.

the production of proteases [59]. From Figure 3, it was observed that P1 protease production was reached maximum up to 147 U/mL at pH 9, then protease production was decreased at pH 10–12. In comparison to this, the optimum temperature and pH for protease production from *Bacillus aryabhatai* K3 was found at 35 °C and 8 respectively [60].

### 3.5.2. Effect of temperature on P1 protease production

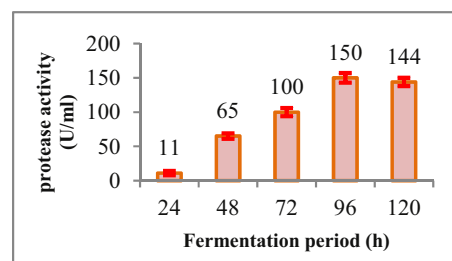
Temperature is a critical parameter that has to be controlled and varied from organism to organism for maximum cell growth and enzyme production. The optimum temperature requirement reported for alkaline protease production by different microorganisms differs widely from 20–80 °C [59]. From Figure 4, it was observed that the production of P1 protease was greatly increased up to 148 U/mL at 35 °C, and then marginally decreased at 45 °C. A sharp decrement in P1 protease production was observed at 55–65 °C.



**Figure 4.** Effect of temperature (25–65 °C) on P1 protease production. Fermentation was carried at 25–65 °C and pH 9 for 72 h (120 rpm). Crude P1 protease assayed individually and the highest 148 U/mL activity was recorded when fermentation was carried at 35 °C.

### 3.5.3. Effect of incubation period on P1 protease production

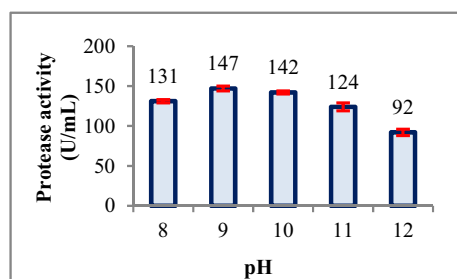
Protease production by *Bacillus aryabhatai* P1 was increased gradually after 24 h, and reached a maximum up to 150 U/mL after 96 h of incubation as seen in Figure 5. In a study, the optimum incubation period for protease production by *B. licheniformis* and *Bacillus coagulans* has been reported as 96 h [61]. Generally, the incubation period required for the optimum protease production by bacteria or fungus may be as long as 48 h to 9 days [59].



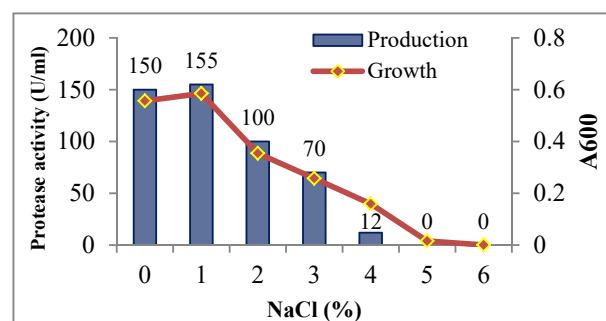
**Figure 5.** Effect of fermentation period on P1 protease production. Fermentation was carried for 24–120 h, pH 9 and 35 °C (120 rpm). The highest 150 U/mL activity P1 protease recorded after 96 h of production period.

### 3.5.4. Effect of salinity of medium on P1 protease production

P1 protease activity was increased and recorded 155 U/mL in the next step of optimization of production when the medium was supplemented with 1% NaCl. Further increment in NaCl content resulted decrement in P1 protease production as illustrated in Figure 6. Comparable results were obtained for alkaline-saline protease production from *Bacillus luteus*



**Figure 3.** Effect of pH on P1 alkaline protease production. Fermentation was carried out at pH range 8–12 and 30 °C for 72 h (120 rpm). Crude P1 protease assayed individually and the highest 147 U/mL activity recorded when pH of fermentation medium was adjusted to 9.



**Figure 6.** Growth and P1 protease production at different concentrations of NaCl (0–6%) in fermentation medium (Fermentation conditions: pH 9, 35 °C temperature, 96 h incubation period and 120 rpm agitation speed).

H11, where protease production increased with increasing salinity (1–9% NaCl, m/V) and reached its maximum at 4–5% NaCl at pH 9 [62]. NaCl is a mild chaotrope. Its property is to change the 3D-confirmation of macromolecules like protein, lipid and nucleic acid. The degree of change in confirmation is dependent on the concentration of chaotropic agent added [63]. Thus, at 1 % concentration of NaCl in the medium, the confirmation of P1 protease could be modified slightly and that might cause increment in catalytic performance of P1 protease. This could be one of the plausible reasons for retaining the maximum activity of P1 protease at 1% NaCl.

### 3.5.5. Effect of agitation speed on P1 protease production

Agitation rates have been shown to affect protease in various strains of bacteria [64]. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks and also affect the nutrient availability [65]. In the present investigation, P1 protease has shown maximum 155 U/mL protease activity at 120 rpm agitation speed (Figure 7). At 180 rpm speed protease activity was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed. High agitation rates could also damage bacterial cells, so that reduction of protease producers will result in decreased protease production [64, 66]. Agitation speed of 0 and 60 rpm affected the growth of the P1 considerably. At these agitation rates, insufficient aeration and nutrient uptake, perhaps caused the inability of bacteria to grow efficiently. In a similar study a notable increment in the protease production with the agitation rate of 180 rpm was reported. It was also revealed that a decrease in the agitation rate drastically lowered the total protease yield [64].

### 3.5.6. Effect of carbon sources on protease production

Out of thirteen different carbon sources tested wheat bran has supported the highest production of P1 protease (440.12 U/mL) followed by wheat flour, rice bran, glucose, mannitol, lactose, starch, galactose, fructose, mannose, maltose, arabinose and cellobiose as depicted in Figure 8. A considerable amount of P1 protease activity viz. 400.33 and 350 U/mL were also seen with wheat flour and rice bran, respectively. The easily available substrate wheat bran is found to be more promising for protease production [67]. Selection of low cost substrates like agricultural residues made the production process very economical as the cost effective media components always reduce industrial enzyme production cost. In the present study *B. aryabhatai* P1 not only utilized successfully the complex agricultural residues, but induced considerably high enzyme production. However, this is in contrast to a report which showed the highest protease production by *Bacillus* sp. B18 with simple organic carbon source like glucose instead of complex agricultural residues [45]. In general, the microbes employed for fermentation processes prefer to utilize the simplest organic nutrient sources. The 40% of cost in the production process is harnessed by the substrates; hence, it is obligatory to formulate media with cost-effective components [68]. Proteases

derived from the *Bacillus* sp. have been exploited by various optimization studies [69].

### 3.5.7. Effect of nitrogen sources on protease production

*B. aryabhatai* P1 grew fast and protease production was greatest (480.45 U/mL at 96 h) in medium containing soybean flour. This was the 9.6% increment in protease activity as compared to control (peptone). Considerable amounts of proteases were produced when casein and chickpea flour substrates used (in each ~475 U/mL). Complex organic nitrogen sources viz. soybean flour, casein, skimmed milk, chickpea flour, corn seed meal and cottonseed meal have shown significant increment in growth and P1 protease production as compared to simple inorganic nitrogen sources (Figure 9). This affirms the value of using agricultural waste materials as fermentation substrates for significant production of protease. Utilization of low price nitrogen source like agricultural residues must be an efficient criterion for economic production of industrial enzymes [70]. Substrate composition can have a major impact on protease production in submerged fermentation [71]. The substrate must have a carbon and nitrogen content appropriate for the fermentation [72]. Substantial carbohydrate content in medium stimulates the expression of hydrolytic enzymes [71]. Porto et al. [73] reported high protease production by *Streptomyces clavuligerus* growing on soybean flour medium and Sankareswaran et al. [74] reported cost effective production of alkaline protease with groundnut oil cake, rice bran, wheat bran, coconut oil cake, and gingelly oil cake.

### 3.6. Bulk production and partial purification of P1 protease

The alkaline protease enzyme which has been produced from *Bacillus aryabhatai* P1 purified through three steps of purification. The steps involved in partial purification of the alkaline protease produced by strain P1 and the acquired data are summarized in Table 1. 60% saturation rate of ammonium sulfate has shown the maximum catalytic activity of P1 alkaline protease. The crude enzyme (cell free supernatant) in ammonium sulfate precipitation (60%) increased the specific activity from 61.05 to 65.98 U/mg and the initial 100% yield of crude enzyme became 97.64%. Thereafter, dialysis was performed in 200 mM glycine-NaOH buffer to remove residual salts and non-protein compounds of ammonium precipitation. Amongst all tested buffers, relative activity of P1 protease fraction was the highest in 200 mM glycine-NaOH buffer (pH 10) as depicted in Figure 10. Dialysis causes diffusion of salt and non-protein molecules diffuse into the solution (buffer) which has a lower pressure passing through the cellophane dialysis bag [75]. A consistent increase in purification fold and specific activity at each step was observed. After dialysis, P1 protease resulted in a 1.23-fold increase, with 75 U/mg specific activity and 83.36% yield. Ramachandran and Arutselvi reported that the partially purified alkaline protease from *Nomuraea rileyi* retained 1.825 U/mg specific activity and 3.5% yield with a 3.64 purification fold [76].

### 3.7. Characterization of P1 protease: Part-I

P1 protease activity has been demonstrated under broad temperature and pH range moreover, in the presence of surfactants, solvents, chelators and commercial detergents. The results of the characterization of partially purified alkaline protease *Bacillus aryabhatai* P1 are discussed as follows:

#### 3.7.1. Determination of kinetic parameters

Optimum casein concentration for catalytic activity of P1 alkaline protease was 15 mg/mL (Figure 11).  $K_m$  and  $V_{max}$  values of P1 protease were calculated as 7.69 mg/mL and 250 U/mg respectively. In previous studies, we have reported  $K_m$  and  $V_{max}$  values of partially purified alkaline protease from *Bacillus alcalophilus* LW8 as 24 mg/mL and 1000 U/mg respectively [4]. Thakur et al. have reported  $K_m$  and  $V_{max}$  values of

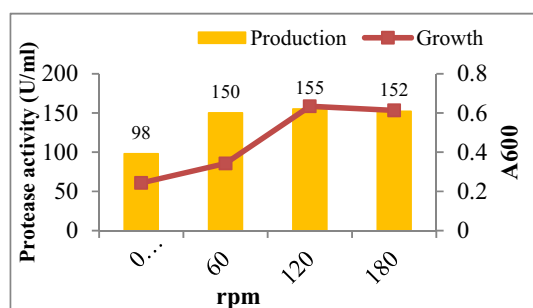
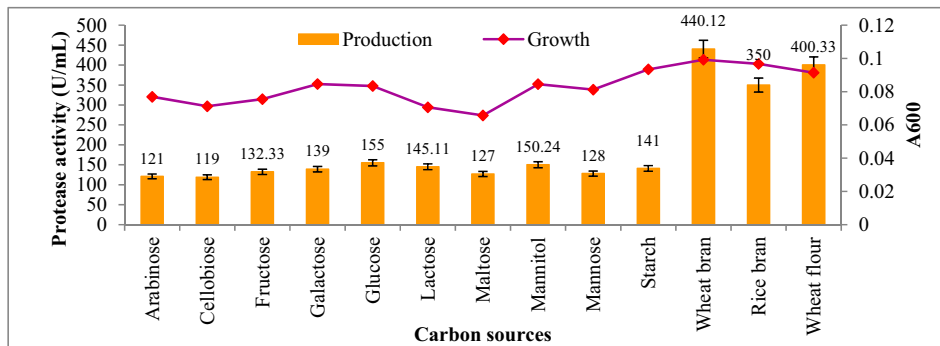
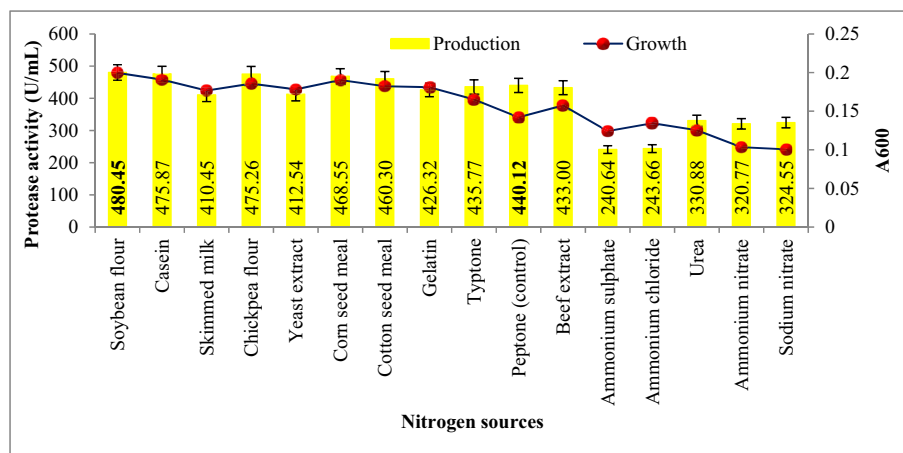


Figure 7. Growth and P1 protease production at different agitation speed 0–180 rpm (Fermentation conditions: Horikoshi-I medium containing 1% NaCl at pH 9, 35 °C temperature, 96 h incubation period).



**Figure 8.** Effect of carbon sources on growth and production of P1 alkaline protease. Fermentation conditions: 1% NaCl in Horikoshi-I medium, pH = 9, 35 °C, 120 rpm and 96 h incubation period.



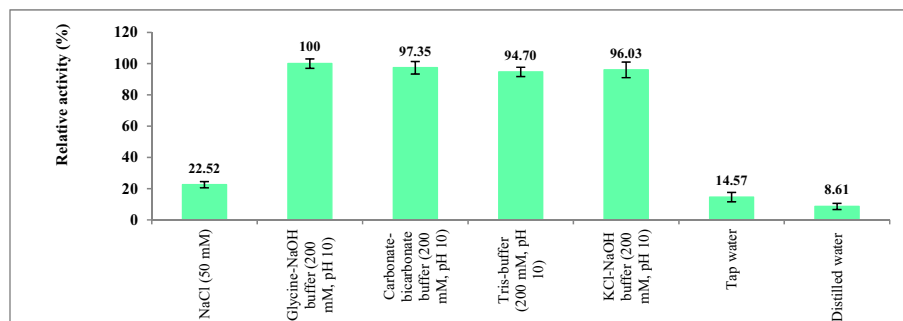
**Figure 9.** Growth and P1 protease production evaluation by replacing the nitrogen source peptone with selected organic, inorganic and complex nitrogen sources at 5 g/L concentration. Fermentation conditions: Horikoshi-I medium (in g/L) containing NaCl 10, and wheat bran 10; pH = 9, 35 °C, 120 rpm and 96 h incubation period. Protease production in presence of peptone was treated as control.

**Table 1.** Partial purification steps of alkaline protease from *Bacillus aryabhatai* P1.

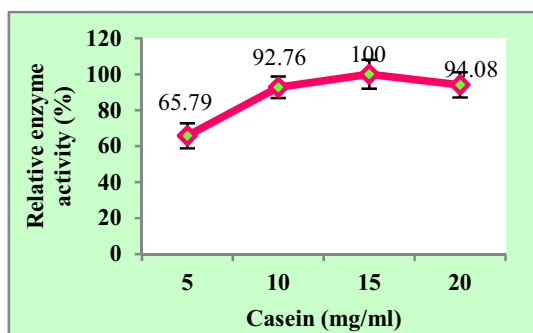
Preparation of enzyme fractions	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Cell free supernatant	480.45 ± 3.21	7.87 ± 0.22	61.05	1.00	100.00
Enzyme precipitate	469.13 ± 1.37	7.11 ± 0.11	65.98	1.08	97.64
Dialysate	400.50 ± 2.27	5.34 ± 0.03	75.00	1.23	83.36

Bulk production was carried out in 500 mL Modified Horikoshi-I medium under optimized culture conditions.

The enzyme was precipitated by ammonium sulfate (60 % saturation), dissolved in 200 mM glycine-NaOH buffer (pH = 10) and dialyzed by using dialysis membrane-70 (HiMedia, Mumbai). The alkaline protease activity assay was performed at each step of purification.



**Figure 10.** Effect of various buffers on catalytic activity of P1 protease. Fractions of precipitated enzymes were dissolved in various buffers and assayed for alkaline protease activity to find out the suitable buffer for dialysis.



**Figure 11.** Effect of substrate concentration on catalytic activity of P1 alkaline protease. Maximum P1 protease activity was recorded at 15 mg/mL of casein.

purified alkaline proteases from mutant *Bacillus* sp. as 0.167 mg/ml and 4761.9 mg/ml/min respectively [77].

### 3.7.2. Effect of metal ions on partially purified P1 protease activity

Residual (remaining) activities of partially purified P1 alkaline protease in the presence of 1 mM and 5 mM metal chlorides are depicted in Figure 12. The residual activity of partially purified P1 alkaline protease in the presence of 1 mM metal chlorides was the highest with  $Mn^{2+}$  (113.60%) followed with  $Ca^{2+}$  (105.44%),  $K^+$  (104.08%),  $Na^+$  and  $Mg^{2+}$  (each 102.04%) and  $Zn^{2+}$  and  $Fe^{3+}$  (each 100.68%). Therefore, these metal cations were found to be positive modifiers for P1 protease activity at 1 mM concentration. On the other hand, at the same concentration  $Ba^{2+}$  (98.63%),  $Ag^{2+}$  (95.23%),  $Cu^{2+}$  (91.83%),  $Hg^{2+}$  (70.06%) and  $Pb^{2+}$  (61.90%) were found to be negative modifiers as the decrement in residual activity of P1 protease was recorded.

The residual activity of partially purified P1 alkaline protease in the presence of 5 mM metal chlorides was the highest with  $Mn^{2+}$  (111.56%) followed with  $Ca^{2+}$  (110.88%),  $Mg^{2+}$  (108.84%),  $K^+$  (105.44%) and  $Na^+$  (100%). Therefore, these metal cations were found to be positive modifiers for P1 protease activity at 5 mM concentration. On the other hand, at the same concentration  $Fe^{3+}$  (88.43%),  $Cu^{2+}$  (88.43%),  $Zn^{2+}$  (85.03%),  $Ag^{2+}$  (82.99%)  $Ba^{2+}$  (70.74%),  $Hg^{2+}$  (41.97%) and  $Pb^{2+}$  (47.61%) were found to be negative modifiers as the decrement in residual activity of P1 protease was recorded. Stimulatory effects of  $Ca^{2+}$  and  $Mg^{2+}$  were also observed for protease produced by *Bacillus firmus* CAS 7 [71]. These cations facilitate interaction between the catalytic site of the protease enzyme and substrate [78]. This strategy necessitates metallic ions as cofactors.  $Ca^{2+}$ ,  $Mg^{2+}$  and  $Mn^{2+}$  defend by stabilizing enzyme structure and maintain active conformation of enzyme at elevated temperature and pH [71, 79, 80]. Inhibitory effect  $Cu^{2+}$ ,  $Zn^{2+}$  and  $Fe^{3+}$  reported by us is in agreement with the report of Farhadian et al. [81].

$Pb^{2+}$  and  $Hg^{2+}$  are considered to be universal inhibitors (negative modifiers) of enzyme activity [70, 82]. In contrast, we noted 48% and

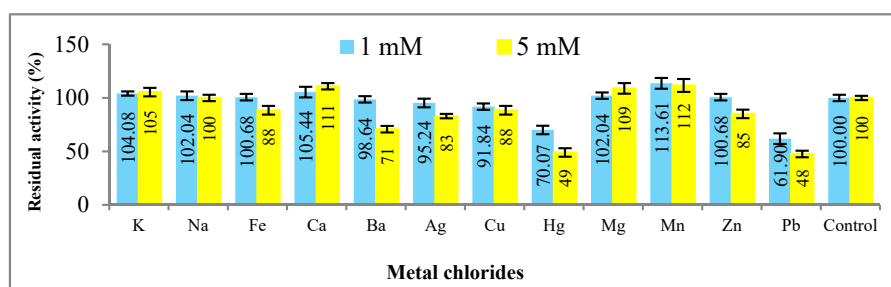
49% residual activity of P1 protease with 5 mM  $Hg^{2+}$  and  $Pb^{2+}$  respectively.  $Hg^{2+}$  is known to react with protein thiol groups (converting them to mercaptides) as well as histidine and tryptophan residues, which inactivates the enzyme [71, 83]. More than a quarter of all known enzymes require the presence of metal atoms for full catalytic activity. Metal ions can influence the activity of enzymes by multiple ways: they may accept or donate electrons to activate electrophiles or nucleophiles; they themselves act as electrophiles; they may mask the effect of nucleophiles to prevent unwanted side reactions; they may bring together enzyme and substrate by means of co-ordinate bonds and may hold the reacting groups in the required three-dimensional orientation; they may simply stabilize the catalytically active conformation of the enzyme [70, 84].

### 3.7.3. Effect of selected effectors on partially purified P1 protease activity

Residual activities of partially purified P1 alkaline protease in the presence of selected effectors such as chelators, inhibitors and denaturants are depicted in Figure 13. Significant relative enzyme activity (96.55%) of P1 protease was observed in the presence of EDTA (5 and 10 mM) which is known as a specific inhibitor of metallo-type proteases. The activity of P1 protease in the presence of chelating agent EDTA is advantageous for its use as a detergent additive in the process of detergent formulation, where alkaline proteases are commonly added and chelating agents are included to overcome the problem of water hardness [8, 85]. Complete inhibition of P1 protease activity was observed in the presence of PMSF (10 mM), DTT (10 mM) and urea (5 mM). PMSF is one of the serine protease inhibitors. PMSF is known to sulphonate the essential serine residue in the active site of the protease, resulting in a total loss of enzyme activity [86]. This inhibition profile suggested that the alkaline protease produced from *Bacillus aryabhatai* P1 belongs to the family of serine proteases. Many of the *Bacillus*-derived alkaline proteases reported so far, belong to the class of serine proteases [8]. Catalytic activity of P1 protease was increased in the presence of 2-mercaptoethanol (5 mM), tween 20 (1%) and tween 80 (1%) by 3.45, 1.38, 2.76% respectively. Enhancement of protease activity with 2-mercaptoethanol, tween 20 and tween 80 was reported by Suberu et al. [86], Mothe and Sultanpuram [87] and Sarker et al. [88]. The activity of P1 protease was slightly reduced by 4.14% and 16.55% in the presence of anionic surfactant SDS at 1 and 10% concentration respectively. Activity of protease from *Bacillus subtilis* B22 was reduced by 29% in the presence of 5% SDS [71]. SDS is a protein denaturant and therefore commercial detergents are formulated with SDS [89]. The enzyme to be added as detergent additive must be SDS-stable to achieve excellent wash performance [90]. P1 protease retained 55.17 and 35.86% activity in the presence of 1 and 2%  $H_2O_2$ . In contrast, Joo et al. reported alkaline protease from *Bacillus clausii* I-52 which was stable at 10%  $H_2O_2$  [91].

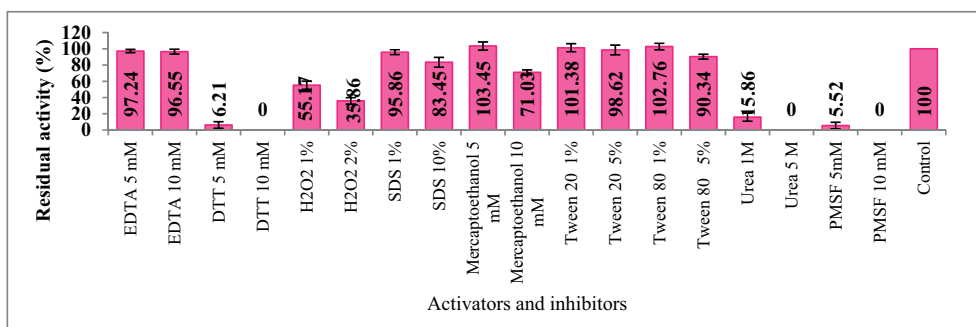
### 3.7.4. Effect of selected solvents on partially purified P1 protease activity

There is a great industrial demand for the organic solvent tolerant proteases for application in the synthesis of useful products in the

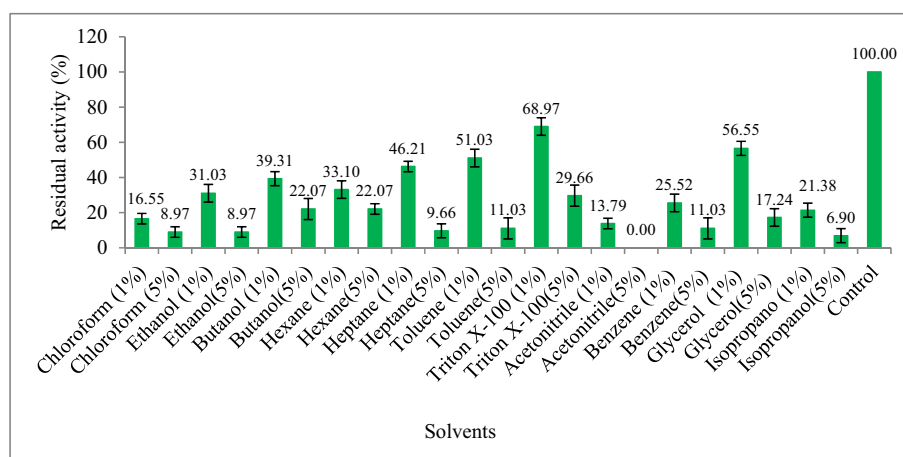


**Figure 12.** Effect of metal ions on catalytic activity of P1 alkaline protease. P1 protease was pre-incubated for 1 h with selected metal chloride solution. Thereafter, the enzyme was assayed. P1 protease activity (original activity) calculated in absence of selected metal chloride was considered as 100% (control) and residual (remaining) activities (%) were calculated as displayed in the shaded vertical bars.





**Figure 13.** Effect of various effectors on catalytic activity of P1 alkaline protease. P1 protease was pre-incubated for 1 h in each selected effector individually. The P1 protease activity assayed in the absence of test effectors was considered to be control (100%) and residual (remaining) activities (%) were calculated as displayed in the shaded vertical bars.



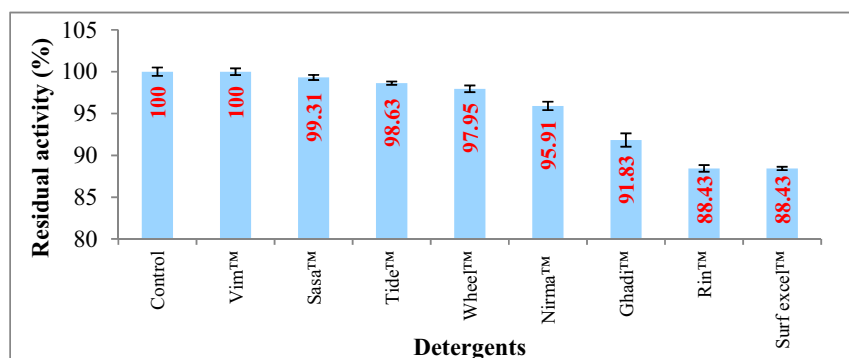
**Figure 14.** Effect of solvents on catalytic activity of P1 alkaline protease. P1 protease was pre-incubated for 1 h in each selected solvent individually. The P1 protease activity assayed in the absence of test solvent was considered as control (100%) and residual (remaining) activities (%) were calculated as displayed on the shaded vertical bars.

presence of organic solvents [71]. Tolerance of partially purified P1 protease to solvents at 1 and 5% (v/v) is shown in Figure 14. The effect of different organic solvents on stability revealed that the P1 protease activity had a moderate inhibitory effect with 1% Triton-X-100 (68.97 %), glycerol (56.55%) and toluene (51.03%), whereas, strong inhibitory effect was found with 5% isopropanol (6.90%), chloroform and ethanol (each 8.97%), heptanes (9.66%) and toluene and benzene (each 11.03%). 5% acetonitrile completely inhibited the P1 protease activity. Solvents can be highly toxic and enzymes can lose activity due to reduction in structural and hydrophobic interactions. The stability of protease in the presence of organic solvents can be attributed to a larger number of negatively charged acidic amino acids compared to basic

amino acids in the enzymes. The negative charges maintain stability by forming a hydrated ion network with cations [71, 92]. Replacement of some water molecules in an enzyme with organic molecules sometimes stabilizes the structure of the enzyme [80]. A protease from *B. pumilus* 115b has been inactivated by toluene and benzene [93]. The alkaline proteases with stability in organic solvents were reported earlier by many researchers [71, 77, 80, 87].

**3.7.5. Effect of detergents on partially purified P1 protease activity**

The partial purified protease was compatible with all the commercial detergents used and the residual activity was above 88% in all the detergents. The bars show the protease activity in the respective



**Figure 15.** Effect of detergents on activity of P1 alkaline protease. Detergent solutions were boiled for 1 h to deactivate by denaturing the endogenous enzymes and then, diluted to 10 mg/mL. P1 protease was pre-incubated for 1 h in each selected detergent fraction and then assayed for protease activity. Under similar incubation conditions, the enzyme activity of a control (devoid of any detergent) was taken as 100% and residual (remaining) activities (%) were calculated as displayed in the shaded vertical bars.

commercial detergents available in the market as depicted in Figure 15. The highest activity of P1 protease was noted in presence of Vim™ (100%), whereas more than 95% activity was retained in presence of Sasa™, Tide™, Wheel™, Ariel™ and Nirma™; moreover more than 88% activity was retained with Ghadi™, Rin™ and Surf excel™. It has been reported that the alkaline protease produced from *B. licheniformis* RP1 retained 95% of its initial activity with Ariel followed by Axion (94%) and then Dixan (93.5%) when pre-incubated with detergents at 40 °C for 1 h [94]. Ramkumar et al. observed more than 91% residual activity of alkaline protease from *B. licheniformis* NK with Ariel followed by Bahar, Tide and Bonux at 1 mg/mL concentration of commercial detergents [95]. Many researchers have diluted the commercial detergents at 7 mg/mL concentration and then used in the compatibility assay [96, 97, 98, 99]; however, we have used 10 mg/mL concentration of detergent solutions and recorded almost similar type of results, this could suggest the tolerance of P1 protease at elevated concentrations of detergents. Therefore, P1 protease could be used as an excellent bio-additive to the detergents.

### 3.7.6. Effect of various substrates on partially purified P1 protease activity

Substrate specificity is a characteristic feature of alkaline proteases and any alkaline protease with a broad range of substrate specificity can be recommended for industrial applications [11]. The relative activity of P1 alkaline protease was determined in the presence of 1% (w/w) gelatin, albumin and feather meal as 45, 25 and 3.12% respectively. Here casein dependent activity was considered to be 100%. The results suggested that P1 protease is able to digest selected protein substrates, albeit to different extents, could convert them into small peptides and amino acids, implying that this alkaline protease has a broad range of substrate specificity. The protein hydrolysates obtained from casein, soy protein and whey protein are useful in the formulation of hypoallergenic infant food. Further, they can also be useful in the fortification of fruit juices and in preparation of high protein therapeutic diets [95].

### 3.7.7. Effect of different assay pH on partially purified P1 protease activity

P1 protease was remarkably active in a broad pH range of 7–11. The highest (optimum) catalytic activity of P1 protease was recorded at pH 9. Considerable activity of P1 protease was recorded at pH 8 (90.38%), whereas moderate activity was recorded at pH 10 (62.69%), pH 11 (64.62%) and pH 7 (61.54%). The broad pH range of protease activity, its optimum activity at higher pH values and the stability for a longer duration at alkaline pH suits this enzyme highly attractive for detergent industry, since laundry detergents generally operate at a pH of 7–11 and other industrial processes involving alkaline processes. Apart from detergent industries, alkaline proteases are also utilized in the formulation of household dish washing and body cleaning sanitizers, preparation of protein hydrolysates, dehairing and bating of skin and hides during a leather processing [95, 100].

### 3.7.8. Effect of different assay temperature on partially purified P1 protease activity

P1 protease was greatly active at a broad temperature range of 30–70 °C. The highest (optimum) catalytic activity of P1 protease was recorded at 50 °C, whereas noticeable activity was found at 60 °C (96.67%), 40 °C (85%), 70 °C (76.67%) and 30 °C (53.33%). P1 protease has retained ~20% activity at 80, 20 and 10 °C. Optimum pH and temperature values of various alkaline proteases have been reviewed and reported by many researchers [2, 3, 4, 5, 6, 11, 69].

## 3.8. Synthesis of nano hydroxyapatite bioceramic (nano HAp)

Nano HAp bioceramic was synthesized and its AFM image has been depicted in Figure 16. The smallest particle grain size of nanoceramic as revealed by XRD was confirmed to be 40–60 nm. AFM is used widely to collect data onto various mechanical, functional and electrical properties at the nanoscale as well as for topography (surface) studies [101]. In our

previous report, similar type of nano HAp (40–60 nm) was synthesized and well-studied with AFM and scanning electron microscopy [16]. Mukhopadhyay et al. have used HAp nanoparticles (<200 nm) for the improvement in thermostability and activity of pectate lyase from *Bacillus megaterium* [102], whereas the copper oxide nanospheres (<350 nm) were used for the enhancement in thermostability, pH stability and dye degrading activity of a bacterial laccase [103]. This implies the importance of the size and type of nanomaterials to be used in nano-enzymology studies.

## 3.9. Nanoceramic based optimum modification in P1 protease

P1 protease was modified in two ways to achieve maximum enhancement in catalytic activity. The results of modification of partially purified P1 protease are discussed as follows:

### 3.9.1. Modification of P1 protease by doping different concentration of nano HAp

P1 protease activity was greatly increased with the inclusion of 2–10 mg/mL nanoHAp and found to be the highest at 4 mg/mL as depicted in Figure 17. This was a 2.2 fold increment in the original activity of P1 protease. Therefore the nano HAp material was named as nanoactivator based on its extraordinary enhancement in the catalytic activity of P1 protease. We have reported previously the same concentration of nano-activator for necessary modification in *B. licheniformis* UN1 amylase [16]. In previous reports, maximum pectate lyase activity was recorded with the inclusion of 8.8 µg/ml [102] and 10.5 µg/ml [58] concentration of hydroxyapatite nanoparticles, whereas the peak laccase activity was reported with the inclusion of 0.22 mg/mL of Cu<sub>2</sub>O nanoparticles [103].

### 3.9.2. Modification of P1 protease by doping nano HAp under different incubation period

The pre-incubation period of 1–9 h was found to be highly significant as the P1 protease activity was curiously enhanced with the inclusion of 4 mg/mL nanoactivator. There was no increment in protease activity without pre-incubation with nanoactivator (control). The highest (175%) activity was recorded after 1 h of pre-incubation period followed by 3 h (170%), 6 h (167.50%) and 9 h (164.43%) with the inclusion of nano-activator (Figure 18). 1 h of the pre-incubation period of enzyme with nanoactivator (4 mg/mL) under constant shaking was found adequate to achieve its peak conformational level for biocatalysis. Further extension in the pre-incubation period could affect on the conformational level of the enzyme in continuous shaking condition. A similar type of work was reported previously where pre-incubation of *B. licheniformis* UN1 amylase with nanoactivator was carried out at 70 °C at 2 random slots of 2 and 12 h [16]; however Mukhopadhyay et al. have not assessed the effect of varying pre-incubation period on pectate lyase and laccase activity [102, 103].

## 3.10. Characterization of modified P1 protease: Part-II

Modified P1 protease as reported in section 3.9.1 and 3.9.2 was further used to demonstrate its activity under broad assay temperature and pH range; moreover hydrolysis of various protein substrates are discussed as follows:

### 3.10.1. Effect of different assay pH on modified P1 protease activity

Nanoactivator mediated, modified P1 alkaline protease has shown outstanding enhancement in catalytic efficiency in comparison with the native P1 alkaline protease. Here native enzyme refers to the unmodified enzyme. Interestingly, the profile of the activity was very different for the two systems. Native protease has shown its optimum catalytic activity at pH 9, whereas modified P1 proteases showed at pH 10. This kind of shift in the optimum activity of the both systems is highly noticeable in the present work as depicted in Figure 19. Catalytic efficiency of modified P1 protease was extremely enhanced by 5.67 fold at pH 5 followed by 5.58

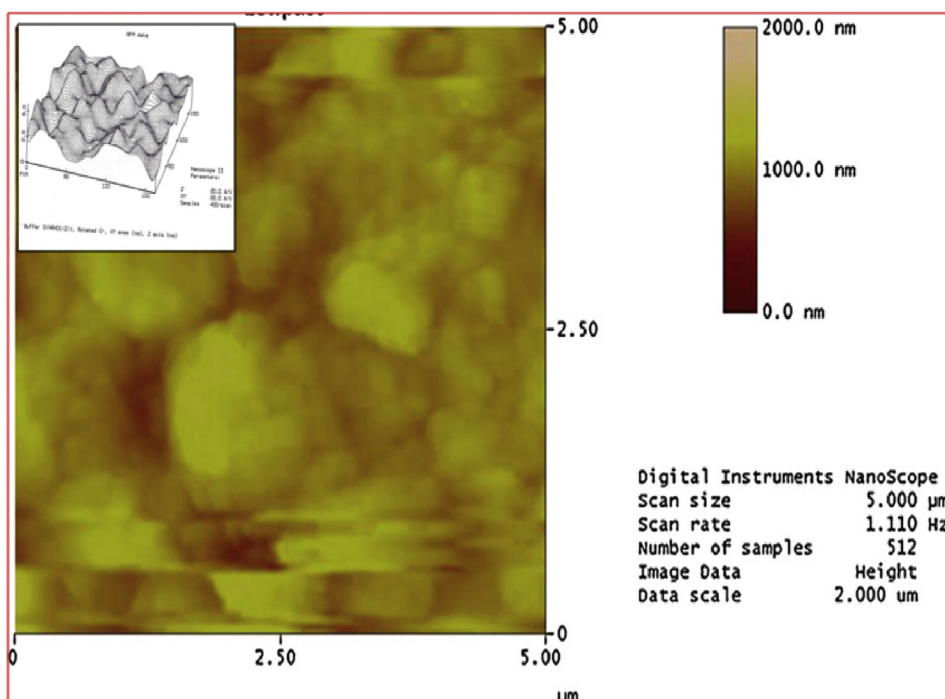


Figure 16. Atomic force microscopic image of selected nanoactivator material.

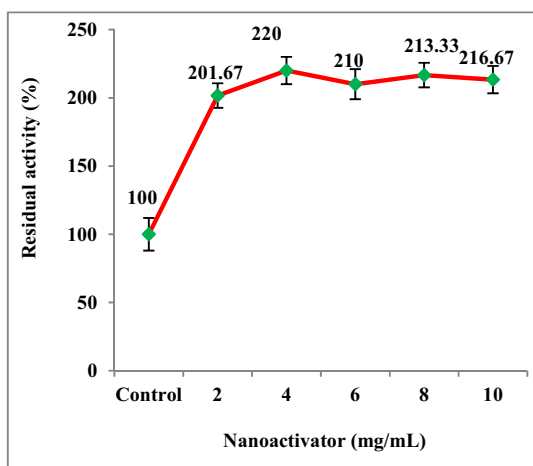


Figure 17. The effect of varying concentration of nanoactivator powder required for necessary modification in native P1 protease at pH 10 and 30 °C. The activity of P1 protease in absence of nanoactivator was considered as 100% (control) and residual (remaining) activities were calculated.

fold at pH 10 and 5.11 fold at pH 12. Further, effective enhancement in the modified P1 protease activity was recorded at pH 4 (3.63 fold) and pH 11 (3.24 fold). Considerable enhancement in the modified P1 protease activity was recorded at pH 6 (2.95 fold) followed by pH 13 (2.78 fold), pH 9 (2.5 fold), pH 8 (2.47 fold), pH 7 (2.01 fold) and pH 3 (2 fold) when compared with native enzyme.

3.10.2. Effect of different assay temperature on modified P1 protease activity

An extraordinary enhancement in the modified P1 protease activity was recorded when tested at 30-70 °C assay temperatures in comparison with the activity of native enzyme as depicted in Figure 20. The profile of the activity was very different for the two systems as seen in section 3.10.1. Native protease has shown its optimum catalytic activity at 50 °C, whereas modified P1 proteases showed at 30 °C. This kind of versatility of P1 protease suggests its applicability at high and moderate

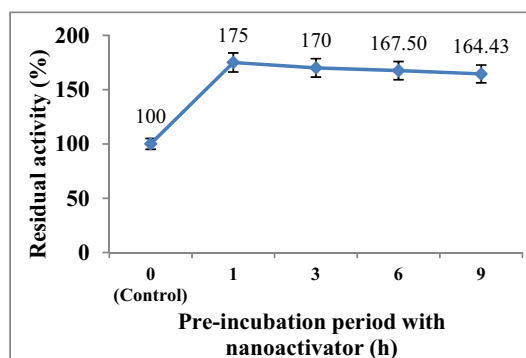


Figure 18. Evaluation residual activities by pre-incubating crude P1 protease (pH = 10) with 4 mg/mL nanoactivator for 0–9 h at 30 °C and 120 rpm. The activity of P1 protease without pre-incubation with nanoactivator was considered as 100% (control) and residual (remaining) activity of each system was determined as described in Section 2.2.3.

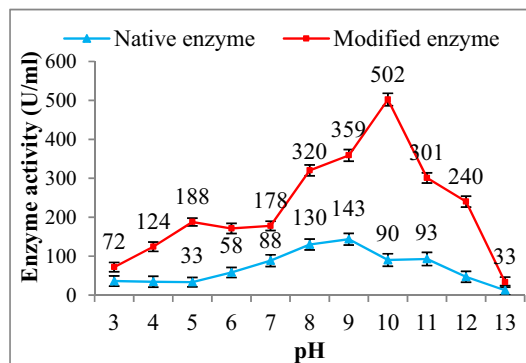
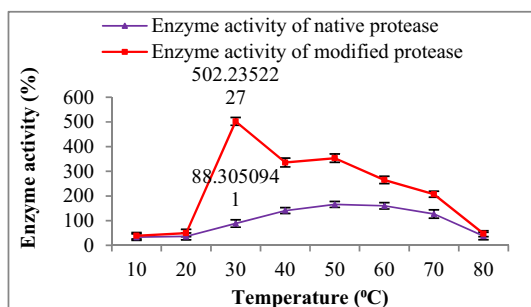


Figure 19. Effect of pH on native and modified P1 alkaline protease. Native protease (90 U/mL at pH 10 and 30 °C) was modified as reported in section 3.9.1 and 3.9.2, and then assayed for protease activity as described in section 2.2.3 at a broad range of pH 3–13 using the universal buffer.



**Figure 20.** Effect of temperature on native and modified P1 alkaline protease. Native protease (88.31 U/mL at pH 10 and 30 °C) was modified as reported in section 3.9.1 and 3.9.2, and then assayed for protease activity as described in section 2.2.3 at a broad range of pH 3–13 using the universal buffer.

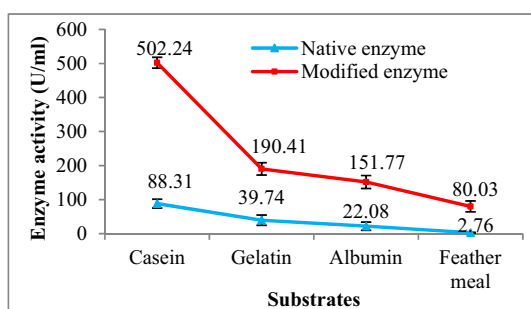
temperatures. Catalytic efficiency of modified P1 protease was greatly enhanced by 5.69 fold at 30 °C followed by 2.38 fold at 40 °C and 2.13 fold at 50 °C. Further, considerable enhancement in the modified P1 protease activity was recorded at 60 °C (1.65 fold) followed by 70 °C (1.63 fold), 20 °C (1.38 fold), 80 °C (1.25 fold), and 10 °C (1.17 fold) when compared with native enzyme.

### 3.10.3. Hydrolysis of various protein substrates by modified P1 protease

Modified P1 protease has effectively hydrolyzed different protein substrates as compared to the native enzyme (Figure 21). Modified P1 protease hydrolyzed feather meal by 29 fold and albumin by 6.87 fold than the native protease. Hydrolysis of casein and gelatin were enhanced by 5.69 and 4.79 fold respectively, when native protease was replaced with modified P1 protease under the assay conditions.

### 3.11. Effect of nanoactivator

Effect of nanoactivator and similar types of nanomaterials in biocatalysis has been well-demonstrated previously, although very few, but precise reports are available with some plausible explanations as discussed here [16, 27, 28, 58, 102, 103]. We have previously reported that at high temperatures, Ca, the principal component of nanoactivator participates in achieving correct configuration of thermostable enzymes and thereby increases enzyme activity [16]. It has been seen that, calcium has an important role in folding and stabilization of protease in alkaline condition. This has been supported by the experimentation on *Pseudomonas aeruginosa* alkaline protease [104]. Properties of nanomaterials such as large surface-to-volume ratios, high surface reaction activity, high catalytic efficiency, and strong protein adsorption ability could lead to improved activity. Enzyme stability increases with nano-scaled supports with possible modulation of the catalytic specificity [102, 103, 105, 106, 107]. In a study, the enzyme with nanoparticle



**Figure 21.** Substrate specificity of native and modified P1 alkaline protease. Native protease (88.31 U/mL at pH 10 and 30 °C) was modified as reported in section 3.9.1 and 3.9.2, and then assayed for protease activity as described in section 2.2.3 by replacing casein with the other selected substrates in the buffer.

pre-incubation enhanced enzyme-substrate affinity and lowered the activation energy compared to that of untreated enzyme [102]. Dutta et al. evaluated the chaperon like activity of nano hydroxyapatite based on entropy–enthalpy compensation profile of extracellular pectate lyase [58].

Nanoactivator helped in redesigning the active sites and might promote binding of enzyme-substrate ligand that could result in increased catalytic activity of modified P1 protease.

Thus, the presence of nanoactivator appeared to confer enhanced activity of protease. However, the question why a nanoactivator formulation of enzyme is more effective in enhancing the activity as compared to native enzyme needs further molecular level investigation.

## 4. Conclusions

*Bacillus aryabhatai* P1 was alkaliphilic and belonged to phylum firmicutes. Optimized production and partial purification of P1 protease was carried out. P1 protease exhibited 75 U/mg specific activity and 83.36% yield. Excellent enhancement in catalytic activity of P1 alkaline protease was observed at pH 10 and 30 °C after nanoactivator mediated modifications. The capability of nanoactivator mediated, modified P1 protease to hydrolyze casein, gelatin, albumin and feather meal was also remarkably increased. The nanoactivator and modulated enzymes suggest their use for different nanobiotechnological applications. Besides, the modified enzyme may serve as a model system and may provide new insights about the molecular basis of alkali-stability and may pave the way for modifying enzymes for specific applications.

## Declarations

### Author contribution statement

Anupama P. Pathak: Conceived and designed the experiments; Performed the experiments, Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mukundraj G. Rathod, Megha P. Mahabole, Rajendra S. Khairnar: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Competing interest statement

The authors declare no conflict of interest.

### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2020.e04053>.

## Acknowledgements

The authors are thankful to Dr. Udhav V. Bhosle, Honorable Vice-Chancellor of S.R.T.M. University, Nanded (India) for providing infrastructure and necessary facilities and National Centre for Microbial Resource, NCCS, Pune for accepting *Bacillus aryabhatai* P1 under general deposition.

## References

- [1] M. Baweja, L. Nain, Y. Kawarabayasi, P. Shukla, Current technological improvements in enzymes toward their biotechnological applications, *Front. Microbiol.* 7 (2016) 965.

- [2] A.P. Pathak, M.G. Rathod, A review on alkaline protease producers and their biotechnological perspectives, *Indian J. Mar. Sci.* 47 (6) (2018) 1113–1119.
- [3] M.G. Rathod, A.P. Pathak, Optimized production, characterization and application of alkaline proteases from taxonomically assessed microbial isolates from Lonar soda lake, India, *Biocatal. Agric. Biotechnol.* 7 (2016) 164–173.
- [4] M.G. Rathod, A.P. Pathak, Wealth from waste: optimized alkaline protease production from agro-industrial residues by *Bacillus alcalophilus* LW8 and its biotechnological applications, *J. Taibah Univ. Sci.* 8 (2014) 307–314.
- [5] M.G. Rathod, A.P. Pathak, Data on optimized production and characterization of alkaline proteases from newly isolated alkaliphiles from Lonar soda lake, India, *Data brief* 8 (2016) 863–866.
- [6] A.P. Pathak, M.G. Rathod, A report on thermostable alkaline protease producing bacteria from a terrestrial thermal spring, *Indian J. Mar. Sci.* 44 (2015) 1104–1111.
- [7] L. Zheng, X. Yu, C. Wei, L. Qiu, C. Yu, Q. Xing, Y. Fan, Z. Deng, Production and characterization of a novel alkaline protease from a newly isolated *Neorospira crassa* through solid-state fermentation, *LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.)* 122 (2020) 108990.
- [8] H. Genckal, C. Tari, Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats, *Enzym. Microb. Technol.* 39 (2006) 703–710.
- [9] M. Venugopal, A.V. Saramma, An alkaline protease from *Bacillus circulans* BM15, newly isolated from a mangrove station: characterization and application in laundry detergent formulations, *Indian J. Microbiol.* 47 (2007) 298–303.
- [10] S. Yang, L. Zhai, L. Huang, D. Meng, J. Li, Z. Hao, Z. Guan, Y. Cai, X. Liao, Mining of alkaline proteases from *Bacillus altitudinis* W3 for desensitization of milk proteins: their heterologous expression, purification, and characterization, *Int. J. Biol. Macromol.* 153 (2020) 1220–1230.
- [11] C.G. Kumar, H. Takagi, Microbial alkaline proteases: from a bioindustrial viewpoint, *Biotechnol. Adv.* 17 (1999) 561–594.
- [12] M.G. Rathod, A.P. Pathak, Efficient decolorization of textile dyes by alkaline protease producing bacterial consortia, *Indian J. Mar. Sci.* 47 (7) (2018) 1468–1477.
- [13] A.P. Pathak, M.G. Rathod, Production and characterization of thermostable gelatinase from *Bacillus globisporus* isolated from Unkeshwar hot spring of Maharashtra, *Indian J. Mar. Sci.* 46 (9) (2017) 1883–1888.
- [14] N.A. Al-Dhabi, G.A. Esmail, A.K.M. Ghilan, M.V. Arasu, V. Duraipandian, K. Ponnuragan, Characterization and fermentation optimization of novel thermo stable alkaline protease from *Streptomyces* sp. Al-Dhabi-82 from the Saudi Arabian environment for eco-friendly and industrial applications, *J. King Saud Univ. Sci.* 32 (1) (2020) 1258–1264.
- [15] M. Baweja, P.K. Singh, A. Sadaf, R. Tiwari, L. Nain, S.K. Khare, P. Shukla, Cost effective characterization process and molecular dynamic simulation of detergent compatible alkaline protease from *Bacillus pumilus* strain MP27, *Process Biochem.* 58 (2017) 199–203.
- [16] R.S. Khairnar, M.P. Mahabole, A.P. Pathak, Nanoactivator mediated modifications in thermostable amylase from *Bacillus licheniformis*, *Indian J. Biochem. Biophys.* 19 (2012) 468–471.
- [17] G. Wei, P.X. Ma, Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering, *Biomaterials* 25 (19) (2004) 4749–4757.
- [18] N. Kantharia, S. Naik, S. Apte, M. Kheur, S. Kheur, B. Kale, Nano-hydroxyapatite and its contemporary applications, *J. Dent. Res. Sci. Develop.* 1 (1) (2014) 15–19.
- [19] A. Fihri, C. Len, R.S. Varma, A. Solhy, Hydroxyapatite: a review of syntheses, structure and applications in heterogeneous catalysis, *Coord. Chem. Rev.* 347 (2017) 48–76.
- [20] V.N. Narwade, M.P. Mahabole, K.A. Bogle, R.S. Khairnar, Waste water treatment by nanoceramics: removal of lead particles, *Int. J. Eng. Sci. Innov. Technol.* 3 (3) (2014) 324–329.
- [21] V. Uskokovic, D.P. Uskokovic, Nanosized hydroxyapatite and other calcium phosphates: chemistry of formation and application as drug and gene delivery agents, *J. Biomed. Mater. Res. B* 96 (1) (2011) 152–191.
- [22] M.P. Ferraz, F.J. Monteiro, C.M. Manuel, Hydroxyapatite nanoparticles: a review of preparation methodologies, *Biom. J.* 2 (2004) 74–80.
- [23] M. Okada, T. Furuzono, Hydroxylapatite nanoparticles: fabrication methods and medical applications, *Sci. Technol. Adv. Mater.* 13 (2012), 064103.
- [24] A.H. Dewi, I.D. Ana, The use of hydroxyapatite bone substitute grafting for alveolar ridge preservation, sinus augmentation, and periodontal bone defect: a systematic review, *Heliyon* 4 (2018), e00884.
- [25] K. Pandi, N. Viswanathan, Synthesis of alginate bioencapsulated nano-hydroxyapatite composite for selective fluoride sorption, *Carbohydr. Polym.* 112 (2014) 662–667.
- [26] M. Nasr-Esfahani, S. Sekri, Alumina/TiO<sub>2</sub>/hydroxyapatite interface nanostructure composite filters as efficient photocatalysts for the purification of air, *React. Kinet. Mech. Catal.* 107 (1) (2012) 89–103.
- [27] A. Mekki-Berrada, S. Bennici, J.P. Gillet, J.L. Couturier, J.L. Dubois, A. Auroux, Fatty acid methyl esters into nitriles: acid–base properties for enhanced catalysts, *J. Catal.* 306 (2013) 30–37.
- [28] D. Stosic, S. Bennici, S. Sirotnin, C. Calais, J.L. Couturier, J.L. Dubois, A. Auroux, Glycerol dehydration over calcium phosphate catalysts: effect of acidic–basic features on catalytic performance, *Appl. Catal. A-Gen.* 447 (2012) 124–134.
- [29] (Last accessed on 11/04/2020), <https://www.fluidinova.com/hydroxyapatite-properties-uses-and-applications>.
- [30] K. Horikoshi, T. Akiba, Alkaliphilic Microorganisms: a New Microbial World, Springer Publisher, New York, 1982.
- [31] M.G. Rathod, A.P. Pathak, Evaluation of the enzymatic profile of microbial isolates from Lonar Soda Lake, *Indian J. Mar. Sci.* 46 (1) (2017) 116–124.
- [32] S.S. Yang, J.Y. Wang, Protease and amylase production of *Streptomyces rimosus* in submerged and solid state cultivations, *Bot. Bull. Acad. Sin. (Taipei)* 40 (1999) 259–265.
- [33] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [34] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [35] K.R. Aneja, Experiments in Microbiology, Plant Pathology and Biotechnology. fourth ed. New Delhi, 2007.
- [36] P.H.A. Sneath, S. Mair, M.E. Sharpe, J.G. Holt, Bergey's Manual of Systematic Bacteriology, first ed. Baltimore, 1984.
- [37] P.D. Vos, G.M. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.H. Schleifer, W.B. Whitman, Bergey's Manual of Systematic Bacteriology, second ed. 3, Springer, Dordrecht, Heidelberg, London, New York, 2009.
- [38] S.G. Dastager, R. Mawlanakar, P. Mua, A. Verma, S. Krishnamurthi, N. Joseph, Y.S. Shouche, *Bacillus encimensis* sp. nov. isolated from marine sediment, *Int. J. Syst. Evol. Microbiol.* 65 (2015) 1421–1425.
- [39] V.V. Sonalkar, R. Mawlanakar, V.V. Ramana, N. Joseph, Y.S. Shouche, S.G. Dastager, *Bacillus filamentosus* sp. nov., isolated from sediment sample, *Anton. Leeuw. J. G.* 107 (2015) 433–441.
- [40] J.E. Clarridge, Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases, *Clin. Microbiol. Rev.* 17 (2004) 840–862.
- [41] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, *Mol. Biol. Evol.* 30 (2013) 2725–2729.
- [42] A. Sharma, Y.S. Shouche, Microbial culture collection (MCC) and international depository authority (IDA) at National Centre for cell science, Pune, *Indian J. Microbiol.* 54 (2013) 129–133.
- [43] O. Prakash, Y.S. Shouche, K. Jangid, J.E. Kostka, Microbial cultivation and the role of microbial resource centers in the omics era, *Appl. Microbiol. Biotechnol.* 97 (2013) 51–62.
- [44] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, *J. American Chem. Soc.* 56 (1934) 658–666.
- [45] N. Fujiwara, A. Masui, T. Imanaka, Purification and properties of the highly thermostable alkaline protease from an alkalophilic and thermophilic *Bacillus* sp. *J. Biotechnol.* 30 (1993) 245–256.
- [46] B. Bhunia, D. Dutta, S. Chaudhuri, Extracellular alkaline protease from *Bacillus licheniformis* NCIM-2042: improving enzyme activity assay and characterization, *Eng. Life Sci.* 11 (2011) 207–215.
- [47] B. Bhunia, A. Dey, Statistical approach for optimization of physicochemical requirements on alkaline protease production from *Bacillus licheniformis* NCIM 2042, *Enzym. Res.* 22 (2012) 1–13.
- [48] J. Renganathan, J. Shanmugam, A. Balumuri, S. Sundaram, Characterization of thermo stable serine alkaline protease from an alkaliphilic strain *Bacillus pumilus* MCAS8 and its applications, *Appl. Biochem. Biotechnol.* 168 (2012) 1849–1866.
- [49] S.K. Rai, A.K. Mukherjee, Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04, *Bioresour. Technol.* 100 (2009) 2642–2645.
- [50] A.P. Pathak, M.G. Rathod, Exploration of a hot spring for thermostable protease producers, *J. Microbiol. Biotechnol. Food Sci.* 7 (2) (2019) 101–109, 2019.
- [51] D.A. Ellis, A new universal buffer system, *Nature* 191 (1961) 1099–1100.
- [52] V.N. Narwade, R.S. Khairnar, V. Kokol, In situ synthesized hydroxyapatite–cellulose nanofibrils as biosorbents for heavy metal ions removal, *J. Polym. Environ.* 26 (5) (2018) 2130–2141.
- [53] V.N. Narwade, R.S. Khairnar, V. Kokol, In-situ synthesised hydroxyapatite-loaded films based on cellulose nanofibrils for phenol removal from wastewater, *Cellulose* 24 (11) (2017) 4911–4925.
- [54] V.N. Narwade, M.P. Mahabole, K.A. Bogle, R.S. Khairnar, Waste water treatment by nanoceramics: removal of lead particles, *Int. J. Eng. Sci. Innov. Technol.* 3 (3) (2014) 324–329.
- [55] V.N. Narwade, R.S. Khairnar, Cobalt adsorption on the nano-hydroxyapatite matrix: isotherm and kinetic studies, *B. Pol. Acad. Sci. Tech.* 65 (2) (2017) 131–137.
- [56] S.R. Anjum, V.N. Narwade, K.A. Bogle, R.S. Khairnar, Graphite doped Hydroxyapatite nanoceramic: selective alcohol sensor, *Nano-Structure. Nano-Object.* 14 (2018) 98–105.
- [57] S. Begum, V.N. Narwade, D.I. Halge, S.M. Jejurikar, J.W. Dudge, S. Muduli, M.P. Mahabole, K.A. Bogle, Remarkable photocatalytic degradation of Remazol Brilliant Blue R dye using bio-photocatalyst 'nano-hydroxyapatite', *Mater. Res. Express* 7 (2) (2020), 025013.
- [58] N. Dutta, A. Mukhopadhyay, A.K. Dasgupta, K. Chakrabarti, Nanotechnology enabled enhancement of enzyme activity and thermostability: study on impaired pectate lyase from attenuated *Macrophomina phaseolina* in presence of hydroxyapatite nanoparticle, *PLoS One* 8 (5) (2013), e63567.
- [59] K.M. Sharma, R. Kumar, S. Panwar, S. Vats, A. Kumar, Microbial alkaline proteases: optimization of production parameters and their properties, *J. Genet. Eng. Biotechnol.* 15 (1) (2017) 115–126.
- [60] K.M. Sharma, R. Kumar, S. Vats, A. Gupta, Production, partial purification and characterization of alkaline protease from *Bacillus aryabhattai* K3, *Int. J. Adv. Pharm. Biol. Chem.* 3 (2) (2014) 290–298.
- [61] S. Asokan, C. Jayanthi, Alkaline protease production by *Bacillus licheniformis* and *Bacillus coagulans*, *J. Cell Tissue Res.* 10 (1) (2010) 2119.
- [62] A. Kalwasinska, U. Jankiewicz, T. Felfoldi, A. Burkowska-But, M. Swiontek Brzezinska, Alkaline and halophilic protease production by *Bacillus luteus* H11 and its potential industrial applications, *Food Technol. Biotechnol.* 56 (4) (2018) 553–561.

- [63] R. Sinha, S.K. Khare, Protective role of salt in catalysis and maintaining structure of halophilic proteins against denaturation, *Front. Microbiol.* 5 (2014) 165.
- [64] A. Akhavan Sepahy, L. Jabalameli, Effect of culture conditions on the production of an extracellular protease by *Bacillus* sp. isolated from soil sample of Lavizan Jungle Park, *Enzym. Res.* 2011 (2011) 1–7.
- [65] W.C.A.D. Nascimento, M.L.L. Martins, Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz. J. Microbiol.* 35 (1–2) (2004) 91–96.
- [66] L.P. Geok, C.N.A. Razak, R.N.Z.A. Rahman, M. Basri, A.B. Salleh, Isolation and screening of an extracellular organic solvent-tolerant protease producer, *Biochem. Eng. J.* 13 (1) (2003) 73–77.
- [67] A. Razzaq, S. Shamsi, A. Ali, Q. Ali, M. Sajjad, A. Malik, M. Ashraf, Microbial proteases applications, *Front. Bioeng. Biotech.* 7 (2019) 1–20.
- [68] H.S. Joo, C.G. Kumar, G.C. Park, K.T. Kim, S.R. Paik, C.S. Chang, Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*, *Process Biochem.* 38 (2002) 155–159.
- [69] M. Baweja, R. Tiwari, P.K. Singh, L. Nain, P. Shukla, An alkaline protease from *Bacillus pumilus* MP 27: functional analysis of its binding model toward its applications as detergent additive, *Front. Microbiol.* 7 (2016) 1195.
- [70] B.K. Bajaj, G. Jamwal, Thermostable alkaline protease production from *Bacillus pumilus* D-6 by using agro-residues as substrates, *Adv. Enzym. Res.* 1 (2) (2013) 1–7.
- [71] P. Elumalai, J.M. Lim, Y.J. Park, M. Cho, P.J. Shea, B.T. Oh, Agricultural waste materials enhance protease production by *Bacillus subtilis* B22 in submerged fermentation under blue light-emitting diodes, *Bioproc. Biosyst. Eng.* (2020) 1–10.
- [72] R.J.S. de Castro, H.H. Sato, Synergistic effects of agroindustrial wastes on simultaneous production of protease and  $\alpha$ -amylase under solid state fermentation using a simplex centroid mixture design, *Ind. Crop. Prod.* 49 (2013) 813–821.
- [73] A.L.F. Porto, G.M. Campos-Takaki, J.L. Lima Filho, Effects of culture conditions on protease production by *Streptomyces clavuligerus* growing on soy bean flour medium, *Appl. Biochem. Biotechnol.* 60 (2) (1996) 115–122.
- [74] M. Sankareswaran, S. Anbalagan, P. Prabhavathi, Optimization of production of an extracellular alkaline protease by soil isolated *Bacillus* species using submerged and solid-state fermentation with agricultural wastes, *Afr. J. Microbiol. Res.* 8 (2014) 8872–8877.
- [75] Y. Junaidi, A. Pertiwinigrum, Y. Erwanto, N.A. Fitriyanto, Semi purification and identifications molecule protein weigh of alkaline protease enzyme from *Bacillus cereus* LS2B, *Int. J. BioSci. Biotechnol.* 9 (3) (2017) 89–99.
- [76] N. Ramachandran, R. Arutselvi, Partial purification and characterization of protease enzyme from *Nomurea rileyi*, *Int. J. Pharma Sci. Res.* 4 (9) (2013) 3460.
- [77] N. Thakur, A. Kumar, A. Sharma, T.C. Bhalla, D. Kumar, Purification and characterization of alkaline, thermostable and organic solvent stable protease from a mutant of *Bacillus* sp. *Biocatal. Agric. Biotechnol.* 16 (2018) 217–224.
- [78] R. Hadjidj, A. Badis, S. Mechri, K. Eddouaouda, L. Khelouia, R. Annane, M. Hattab, B. Jaouadi, Purification, biochemical, and molecular characterization of novel protease from *Bacillus licheniformis* strain K7A, *Int. J. Biol. Macromol.* 114 (2018) 1033–1048.
- [79] N. Mokashe, A. Chaudhari, U. Patil, Optimal production and characterization of alkaline protease from newly isolated halotolerant *Jeotgalicoccus* sp. *Biocatal. Agric. Biotechnol.* 4 (2) (2015) 235–243.
- [80] B. Ghorbel, A. Sellami-Kamoun, M. Nasri, Stability studies of protease from *Bacillus cereus* BG1, *Enzym. Microb. Technol.* 32 (5) (2003) 513–518.
- [81] S. Farhadian, A. Asoodeh, M. Lagzian, Purification, biochemical characterization and structural modeling of a potential htrA-like serine protease from *Bacillus subtilis* DR8806, *J. Mol. Catal. B Enzym.* 115 (2015) 51–58.
- [82] R. Fontes, J. Meireles Ribeiro, A. Sillero, Inhibition and activation of enzymes. The effect of a modifier on the reaction rate and on kinetic parameters, *Acta Biochim. Pol.* 47 (1) (2000) 233–257.
- [83] M. Kecha, S. Benallaoua, J.P. Touzel, R. Bonaly, F. Duchiron, Biochemical and phylogenetic characterization of a novel terrestrial hyperthermophilic archaeon pertaining to the genus *Pyrococcus* from an Algerian hydrothermal hot spring, *Extremophiles* 11 (1) (2007) 65–73.
- [84] V. Khajuria, K. Sharma, P. Sathia, K. Razdan, S. Singh, B.K. Bajaj, Production of a detergent-compatible alkaline protease from *Bacillus cereus* K-3, *J. Mater. Environ. Sci.* 6 (8) (2015) 2089–2096.
- [85] H. Lund, S.G. Kaasgaard, P. Skagerlind, L. Jorgensen, C.I. Jorgensen, M. Van de Weert, Protease and amylase stability in the presence of chelators used in laundry detergent applications: correlation between chelator properties and enzyme stability in liquid detergents, *J. Surfactants Deterg.* 15 (3) (2012) 265–276.
- [86] Y. Suberu, I. Akande, T. Samuel, A. Lawal, A. Olaniran, Cloning, expression, purification and characterisation of serine alkaline protease from *Bacillus subtilis* RD7, *Biocatal. Agric. Biotechnol.* 20 (2019) 101264.
- [87] T. Mothe, V.R. Sultanpuram, Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus*, 3 *Biotech* 6 (1) (2016) 53.
- [88] P.K. Sarker, S.A. Talukdar, P. Deb, S.A. Sayem, K. Mohsina, Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003, *SpringerPlus* 2 (1) (2013) 506.
- [89] K.H. Maurer, Detergent proteases, *Curr. Opin. Biotechnol.* 15 (4) (2004) 330–334.
- [90] R. Oberoi, Q.K. Beg, S. Puri, R.K. Saxena, R. Gupta, Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. *World J. Microbiol. Biotechnol.* 17 (2001) 493–497.
- [91] H.S. Joo, C.G. Kumar, G.C. Park, S.R. Paik, C.S. Chang, Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties, *J. Appl. Microbiol.* 95 (2) (2003) 267–272.
- [92] D. Jain, I. Pancha, S.K. Mishra, A. Shrivastav, S. Mishra, Purification and characterization of haloalkaline thermoactive, solvent stable and SDS-induced protease from *Bacillus* sp.: a potential additive for laundry detergents, *Bioresour. Technol.* 115 (2012) 228–236.
- [93] R.N.Z.R.A. Rahman, S. Mahamad, A.B. Salleh, M. Basri, A new organic solvent tolerant protease from *Bacillus pumilus* 115b, *J. Ind. Microbiol. Biotechnol.* 34 (7) (2007) 509–517.
- [94] J. Singh, N. Batra, R.C. Sobti, Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1, *Process Biochem.* 36 (8–9) (2001) 781–785.
- [95] A. Ramkumar, N. Sivakumar, A.M. Gujarathi, R. Victor, Production of thermotolerant, detergent stable alkaline protease using the gut waste of *Sardinella longiceps* as a substrate: optimization and characterization, *Sci. Rep.* 8 (1) (2018) 1–15.
- [96] W.C.A.D. Nascimento, M.L.L. Martins, Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergent, *Braz. J. Microbiol.* 37 (3) (2006) 307–311.
- [97] F.N. Niyonzima, S.S. More, Purification and characterization of detergent-compatible protease from *Aspergillus terreus* gr. 3 *Biotech* 5 (1) (2015) 61–70.
- [98] M.Q. Lam, N.N.N. Mut, S. Thevarajoo, S.J. Chen, C. Selvaratnam, H. Hussin, H. Jamaluddin, C.S. Chong, Characterization of detergent compatible protease from halophilic *Virgibacillus* sp. CD6, 3 *Biotechol.* 8 (2) (2018) 104.
- [99] M.O. Benmrad, S. Mechri, N.Z. Jaouadi, M.B. Elhouli, H. Rekek, S. Sayadi, S. Bejar, N. Kechaou, B. Jaouadi, Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom *Pleurotus sajor-caju* strain CTM10057 with industrial interest, *BMC Biotechnol.* 19 (1) (2019) 43.
- [100] S. Kamal, S. Rehman, H.M. Iqbal, Biotechnological valorization of proteases: from hyperproduction to industrial exploitation—a review, *Environ. Prog. Sustain. Energy* 36 (2) (2017) 511–522.
- [101] S. Vahabi, B.N. Salman, A. Javanmard, Atomic force microscopy application in biological research: a review study, *Iran. J. Med. Sci.* 38 (2) (2013) 76.
- [102] A. Mukhopadhyay, A.K. Dasgupta, D. Chattopadhyay, K. Chakrabarti, Improvement of thermostability and activity of pectate lyase in the presence of hydroxyapatite nanoparticles, *Bioresour. Technol.* 116 (2012) 348–354.
- [103] A. Mukhopadhyay, A.K. Dasgupta, K. Chakrabarti, Thermostability, pH stability and dye degrading activity of a bacterial lactase are enhanced in the presence of Cu<sub>2</sub>O nanoparticles, *Bioresour. Technol.* 127 (2013) 25–36.
- [104] L. Zhang, J.F. Conway, P.H. Thibodeau, Calcium-induced folding and stabilization of the *Pseudomonas aeruginosa* alkaline protease, *J. Biol. Chem.* 287 (6) (2012) 4311–4322.
- [105] I. Lynch, K.A. Dawson, Protein-nanoparticle interactions, *Nano Today* 3 (1–2) (2008) 40–47.
- [106] L. Chronopoulou, G. Kamel, C. Sparago, F. Bordini, S. Lupi, M. Diociaiuti, C. Palocci, Structure–activity relationships of *Candida rugosa* lipase immobilized on poly(lactic acid) nanoparticles, *Soft Matter* 7 (6) (2011) 2653–2662.
- [107] R. Konwarh, N. Karak, S.K. Rai, A.K. Mukherjee, Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase, *Nanotechnology* 20 (22) (2009) 225107.