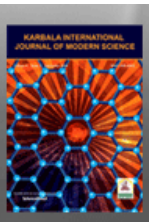

Karbala International Journal of Modern Science

Volume 8 | Issue 4 Article 10

Potential use of proteolytic bacteria Paenibacillus dendritiformis (BT7) isolated from Batu tannery effluents for the detergent industry

Chandran Masi
Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University,, chandran.chandran@aastu.edu.et

Degafneh Tadesse
Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University,

Abate Ayele
Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University

Potential use of proteolytic bacteria Paenibacillus dendritiformis (BT7) isolated from Batu tannery effluents for the detergent industry

Abstract

This research was aimed at identifying a bacterium that can produce alkaline proteases. As a result, bacteria that produce proteases were isolated from Batu tannery effluents, tested for protease synthesis on skim milk agar plates, and validated with a protease assay. Microscopic and molecular phylogenetic analyses identified Paenibacillus dendritiformis (BT7) as the bacterial isolate with the highest alkaline protease production. The isolate's maximum enzyme production was obtained by 2% inoculum size, 40°C temperature, 9.0 pH, and a 48-hour incubation time with production media components such as glucose, casein, MgCl2, and 2% NaCl. The maximal enzyme activity was 270 U/mL under all optimum culture conditions. Concentrated ammonium sulfate precipitation (75%) and dialysis were employed to obtain a cell-free, partially purified protease. The specific activity of the dialysate, which accounts for 3% of the enzyme yield, was discovered to be 134 U/mL. The partially purified protease was used for application in blood stain removal. It was studied and found that the alkaline protease resistance under stringent conditions is very stable with bleach detergent. Also, this enzyme could clean blood-stained fabrics. This study shows that the alkaline protease from Paenibacillus dendritiformis - BT7 could be used in various ways in the detergent industry that are good for the environment.

Keywords

Alkaline protease, Bloodstain, Batu tannery, Detergent, Paenibacillus dendritiformis

RESEARCH PAPER

Potential Use of Proteolytic Bacteria *Paenibacillus dendritiformis* (BT7) Isolated from Batu Tannery Effluents for the Detergent Industry

Chandran Masi [a,b,*], Degafneh Tadesse [a], Abate Ayele [a,b]

[a] Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, P.O. Box 16417, Addis Ababa, Ethiopia
[b] Center of Excellence, Bioprocess and Biotechnology, Addis Ababa Science and Technology University, P.O. Box 16417, Addis Ababa, Ethiopia

Abstract

This research was aimed at identifying a bacterium that can produce alkaline proteases. As a result, bacteria that produce proteases were isolated from Batu tannery effluents, tested for protease synthesis on skim milk agar plates, and validated with a protease assay. Microscopic and molecular phylogenetic analyses identified *Paenibacillus dendritiformis* (BT7) as the bacterial isolate with the highest alkaline protease production. The isolate's maximum enzyme production was obtained by 2% inoculum size, 40 °C temperature, 9.0 pH, and a 48-h incubation time with production media components such as glucose, casein, $MgCl_2$, and 2% NaCl. The maximal enzyme activity was 270 U/mL under all optimum culture conditions. Concentrated ammonium sulfate precipitation (75%) and dialysis were employed to obtain a cell-free, partially purified protease. The specific activity of the dialysate, which accounts for 3% of the enzyme yield, was discovered to be 134 U/mL. The partially purified protease was used for application in blood stain removal. It was studied and found that the alkaline protease resistance under stringent conditions is very stable with bleach detergent. Also, this enzyme could clean blood-stained fabrics. This study shows that the alkaline protease from *P. dendritiformis* - BT7 could be used in various ways in the detergent industry that are good for the environment.

Keywords: Alkaline protease, Bloodstain, Batu tannery, Detergent, *Paenibacillus dendritiformis*

1. Introduction

Protease is a protein-degrading enzyme that catalyzes the breakdown of protein molecules into peptides and amino acids [1,2]. Protease (EC: 3.4.21−24, 99) is one of the most versatile commercial enzymes, with around 2% of all genes in higher organisms coding for proteases [2,3]. Protease is one of the three main industrial enzyme families, accounting for 65% of global enzyme trade. It's employed in a variety of ecologically favourable and safe industrial products [4]. Proteases are essential for cell growth and development and can be present in all microorganisms. It's also being researched as a bioactive catalyst in biological processes that are alive [5,6]. People who study proteases think about the pH range in which they work best. Proteases can be acidic, alkaline, or neutral [7].

Proteases are categorized as exo-peptidases or endopeptidases depending on the site of action on the polypeptide chain [8]. Exopeptidases catalyze and cleave the terminal peptide bonds surrounding the amino and carboxyl termini of the substrate only at the end of the polypeptide chain. Exopeptidases are enzymes that only operate on polypeptide chains at their ends. Depending on whether they function at the N or C terminus, they are classified as amino (or) carboxypeptidases [9,10]. A protease

Received 21 May 2022; revised 15 August 2022; accepted 19 August 2022.
Available online 10 November 2022

* Corresponding author at: Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, P.O. Box 16417, Addis Ababa, Ethiopia.
E-mail address: chandran.chandran@aastu.edu.et (C. Masi).

https://doi.org/10.33640/2405-609X.3267

known as an aminopeptidase separates individual amino acids from a peptide's amino-terminal end. Carboxyl-terminal peptidases, on the other hand, are enzymes that cut peptide chains at their carboxyl terminus [8].

Even though plants and animals generate proteases, microbial sources are preferred because they can meet the world's ever-increasing demand. Bacteria represent a promising source of protease enzymes for billions of dollars in international trade [2]. Microbial proteases are known for their versatility in pH, temperature, and substrate type. Although proteases have been studied in a variety of microorganisms, including bacteria, fungi, and viruses, *Bacillus* species (bacteria) constitute the most prevalent source for industrial protease applications. [11,12]. *Bacillus* is an important and relevant source of industrial alkaline proteases, and it is likely the sole commercialized species for alkaline protease synthesis [13]. Proteases must be active and stable in industrial applications, including extremes in temperature, pH, inhibitors, and oxidizing agents [14]. If potential enzymes are used in industrial applications, they must be processable and stable for long periods [15].

Leather, textiles, food, medicines, detergents, and the photographic film industry are all industries that use proteases [16]. The laundry detergent industry requires effective, environmentally sustainable, and cost-effective techniques for undesired protein breakdown [12,17]. Skin trimming keratin waste, flesh waste, chrome shaving waste, and buffing trash are all examples of proteinaceous solid waste generated by the leather industry [18]. Proteinaceous stains such as blood, food, and grass stains can be removed with alkaline proteases included in detergent formulations. Several fermentation factors, including medium, nitrogen, carbon sources, pH, temperature, inoculum size, agitation, and incubation duration, can be tuned to maximize the output of industrially essential enzymes [17,19]. For future enzyme uses, new alkaline protease enzymes must be developed. Also, bacteria-produced enzymes found in effluents increase the relevance of converting industrial waste into valuable enzymes, particularly alkaline proteases [20]. The purpose of this research is to find, screen, optimize, and characterize alkaline protease-producing bacteria from leather industry effluent that can be used in detergents.

2. Materials and methods

2.1. Sample collection and isolation of bacterial strains

The soil and water samples were collected using sterile plastic bags and bottles at the Batu tannery in Addis Ababa, Ethiopia. The samples were then placed in an icebox and sent to Addis Ababa Science and Technology University's industrial biotechnology laboratory. Finally, samples were maintained in the refrigerator at −4 °C until they were used. To isolate bacteria, soil and wastewater samples were serially diluted and dispersed on nutrient agar supplemented with 1% casein and kept at 37 °C overnight at pH 9.0 [3,21,22]. Using sterile distilled water containing 0.85% NaCl, serial dilutions up to 10^{-8} were conducted. After that, a glass spreader was used to spread 0.1 mL of each dilution aseptically on nutrient agar plates. The plates were incubated overnight at 37 °C in an incubator. Colonies were evaluated after 24 hours of incubation, and various growth patterns of bacteria were detected on each plate.

2.2. Screening for protease-producing bacteria

On skimmed milk agar, the pure colonies were tested (SMA). The fresh culture was spot injected on skimmed milk agar (g/l) with pH 9.0 (using phosphate buffer) and incubated at 37 °C for 24 hours. [23,24].

2.3. Molecular identification of potent proteolytic bacterial isolates

16S rRNA gene sequencing was also used for molecular identification. Two universal primers for 16S rRNA gene amplification were utilized to get amplified PCR results. The forwarding primer 27F had the sequence AGAGTTTGATCMTGGCTCAG, while the reverse primer 1492R had the sequence GGTTACCTTGTTACGACTT. An ABI 3730xl sequencer was used to sequence the 16s rRNA gene. In India, BioEdit7.2 was used to align the 16S rRNA gene sequences. BLAST-NCBI was used to analyze the sample sequences [6,25]. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA) was used to match the sequences to 16S rRNA sequences accessible in public databases, and the gene was identified at the generic level. The CLUSTAL-X Multiple Sequence Alignment Program was used to align the isolated isolates' 16S rRNA sequences with sequences from related species obtained from Gene-Bank (Strasburg, France). PHYLIP will be utilized for the phylogenetic analysis, and phylogenetic trees were generated using the neighbor-joining approach with the Tree View tool. A bootstrap investigation was used to ensure that the branching pattern was constant [25].

2.4. Optimization of cultural conditions for protease production

On enzyme synthesis and protein content, the impacts of various media nutrient composition and physical variables were investigated. After 24 hours of incubation in the culture medium at various temperatures (i.e., 25, 30, 35, 40, 45, 50, 55, and 60 °C), the influence of temperature on the generation of alkaline proteases was assessed. The medium was prepared by adjusting the pH from 6.0 to 12.0 at a 1.0 unit interval to optimize pH. Similarly, the bacterial isolate BT7 was inoculated in the protease production medium and incubated for 12, 24, 48, 72, and 96 hours for an optimal incubation period. The effect of changing the inoculum size from 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% v/v at a 0.5% v/v difference on protease production was investigated [26].

Carbon sources include galactose, fructose, sucrose, starch, maltose, lactose, cellulose (0.5% w/v), and nitrogen sources like $NaNO_3$, NH_4Cl, KNO_3, Casein, Yeast Extract, Peptone, and Ammonium Acetate (1% w/v) were also studied. The cell-free supernatants were used to detect the protease enzyme's specific activity after 24 hours of incubation [7]. The effect of salt concentration, NaCl, on protease synthesis was investigated by adding various concentrations of NaCl to the production media. The experiment was conducted separately at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% NaCl in the carbon and nitrogen sources, optimum basal medium injected with 2% of 24 h culture broth [25]. Different metal ions' inhibitory activity on protease synthesis was also investigated. Zinc chloride, Calcium chloride, Ferric chloride, Manganese sulphate, Mercuric chloride, Magnesium chloride, Copper sulphate and Zinc sulphate were among the metal ions used. Each was added to an optimum basal medium, which included hydrocarbon, carbon and nitrogen sources, at a concentration of 0.2% while maintaining a pH of 9.0. After 48 hours of incubation, the effect was assessed [26].

2.5. Production, extraction, and partial purification of crude enzyme

Bacterial cultures were inoculated in nutrient broth and incubated at 37 °C and 120 rpm for 48 hours. The basal medium was used to produce enzymes (glucose 10.0g, peptone 10.0g, K_2HPO_4 1.0g, $MgSO_4$ 0.2g, Na_2CO_3 5.0g, distilled water 1000 ml). 0.1N NaOH and 0.1N HCl were used to alter the pH of the medium to 9.0. The medium was autoclaved for 15 minutes at 15 lbs/inch2 pressure (121 °C) and inoculated with 10 ml of overnight seed culture; flasks holding 1000 ml of medium were incubated at 37 °C for 48 hours at 120 rpm in a rotary shaker incubator [27]. After 48 hours, the culture is centrifuged at 10,000 rpm for 10 min. The clear supernatant was used as the crude enzyme. This crude enzyme was precipitated with ammonium sulphate precipitation (75% saturation), and the enzyme precipitate obtained was centrifuged at 10,000 g for 10 min. The collected supernatant followed by dialysis was utilized to get a partially pure cell-free protease. Finally, crude enzyme and partially purified enzyme were analyzed to determine the protease activity and protein concentration.

2.6. Assay for proteolytic activity

The activity of alkaline proteases was determined using a modified version of the method published by Takami et al. [28]. According to this method, equilibrium was attained by incubating 0.25 mL of glycine (6.01 g): NaCl: NaOH buffer (50 mM, pH 10.5) with 2.5 ml of 0.6% of casein powder dissolved in the same buffer at 30 °C. A 0.25 mL aliquot of the enzyme solution was added to this mixture and incubated for 20 minutes. TCA solution (2.5 mL) was added. After 10 minutes, the entire mixture was centrifuged for 15 minutes at 5000 RPM. The supernatant was then combined with 2.5 ml of 0.5M Na_2CO_3 and 0.5 ml of Folin-phenol Ciocalteu's solution after 30 minutes. The optical densities of the solutions at 660 nm of the sample blanks were measured using a spectrophotometer (Biochrom, England). One unit of alkaline protease activity was defined as the enzyme quantity necessary to liberate 1 μg of tyrosine per minute under the experimental conditions. A standard curve was generated by utilizing tyrosine solutions (0–100 g/ml). The results of each enzyme test were done triplicate, and the mean values were recorded [29,30].

2.7. Assay for total protein

Using Lowry's technique, the samples' total protein content was ascertained. Bovine Serum Albumin (BSA) (1 mg/ml) was the protein standard utilized.

2.8. Blood stain removal

According to Aftab et al. [31], the protease activity of an isolate as a detergent additive (555, Ethiopia) was studied, and the image was analyzed using image j software. As a result, six pieces of white cotton cloth, each measuring 4 cm × 4 cm, were stained with blood, as shown below.

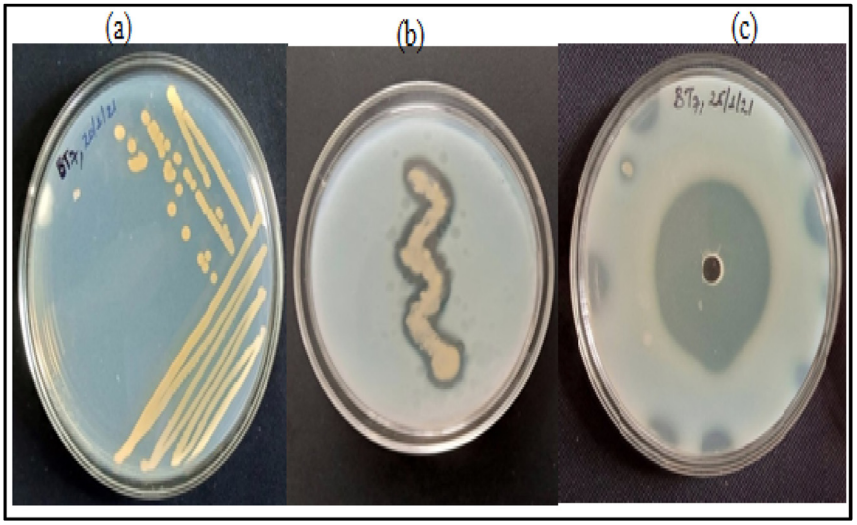

Fig. 1. (a) Bacterial isolate subculture, (b) Isolate screening in 1% skimmed milk agar, and (c) Secondary screening for potential bacteria isolates (Zone of protein hydrolysis).

(a). In Petri dishes, a blood-stained towel was immersed in 10 ml of distilled water (Negative Control)
(b). In Petri dishes, a blood-stained towel was dipped in 5 ml of distilled water and 5 ml of enzymes.
(c). In Petri dishes, a blood-stained cloth was dipped in 2.5 mL distilled water and 7.5 mL isolated enzyme solution.
(d). In Petri dishes, a blood-stained towel was immersed in a 10 ml isolate enzyme solution.
(e). In Petri plates, a blood-stained cloth was dipped in 5 ml of enzyme solution and 5 ml of detergent.
(f). In Petri dishes, a blood-stained cloth was immersed in 10 ccs of detergent (Positive control).

The blood-stained clothing was then incubated in Petri dishes for 10 minutes at 37 °C. Finally, a blood-stained cloth was used as a control that had not been subjected to these treatments [29].

3. Results and discussions

3.1. Isolation and screening of microorganisms that produce alkaline proteases

Three separate tannery waste samples were obtained, and bacteria were isolated using serial dilution and spread plate procedures. The samples on nutrient agar yielded a total of 53 distinct bacterial strains. All isolates were primarily screened for protease production on the skim milk agar plate method shown in Fig. 1. Among 53 isolates, 20 isolates showed a significant protein hydrolysis zone (Table 1). Khan et al. [32] used soil samples to isolate 118 isolates with proteolytic activity on skim milk agar plates. The results could be because the sample came from a variety of places, including tanneries, soap factories, garden soil, and soil compost. More positive isolates are obtained as a result of larger sample size and a larger number of sampling sites [10]. Those isolates were subjected to secondary screening, i.e., well-zone diffusion using culture

Table 1. Proteolytic isolates and their respective clear zones of protein hydrolysis in mm.

S. NO	Proteolytic isolates	Protein hydrolysis zone in mm	S. NO	Proteolytic isolates	Protein hydrolysis zone in mm
1	BT1	25	11	BT 25	25
2	BT4	24	12	BT 28	21
3	BT5	17	13	BT 31	14
4	BT7	30	14	BT33	15
5	BT12	19	15	BT 34	21
6	BT 14	17	16	BT 39	20
7	BT 16	18	17	BT 42	19
8	BT 18	20	18	BT 45	14
9	BT 20	21	19	BT 48	19
10	BT 23	15	20	BT 53	24

Table 2. Proteolytic isolates and their Protease activity in U/ml.

S. No	Proteolytic isolates	Protease activity (U/ml)
1	BT1	180 ± 0.36
2	BT4	172 ± 0.42
3	BT7	220 ± 0.18
4	BT25	182 ± 0.74
5	BT53	185 ± 0.08

supernatant, and, accordingly, five isolates showed a significant protein hydrolysis zone (BT1, BT4, BT7, BT25, and BT53). Finally, we selected the BT7 isolate due to the highest zone of protein hydrolysis (30 mm) (Table 1) and protease activity (220 ± 0.18U/ml) (Table 2) for further studies. Out of 188 bacterial isolates, Hamza [33] evaluated 36 alkaline protease-positive isolates, with five isolates with higher clear zones being researched further.

3.2. *Identification of potent proteolytic bacterial isolates*

The maximum alkaline protease-producing strain BT7 was selected depending on the highest zone of

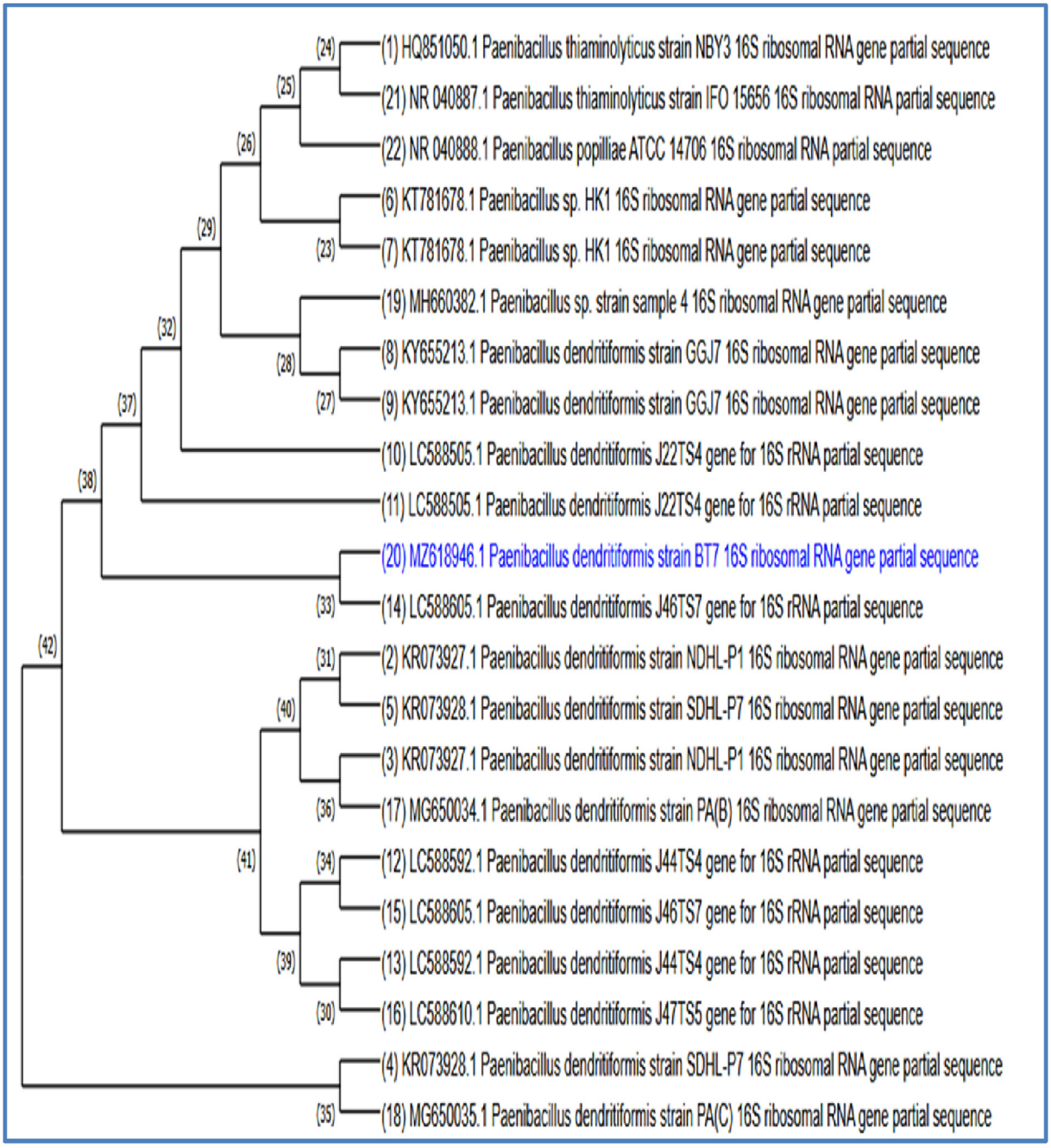

Fig. 2. Phylogenetic tree of Paenibacillus dendritiformis -BT7.

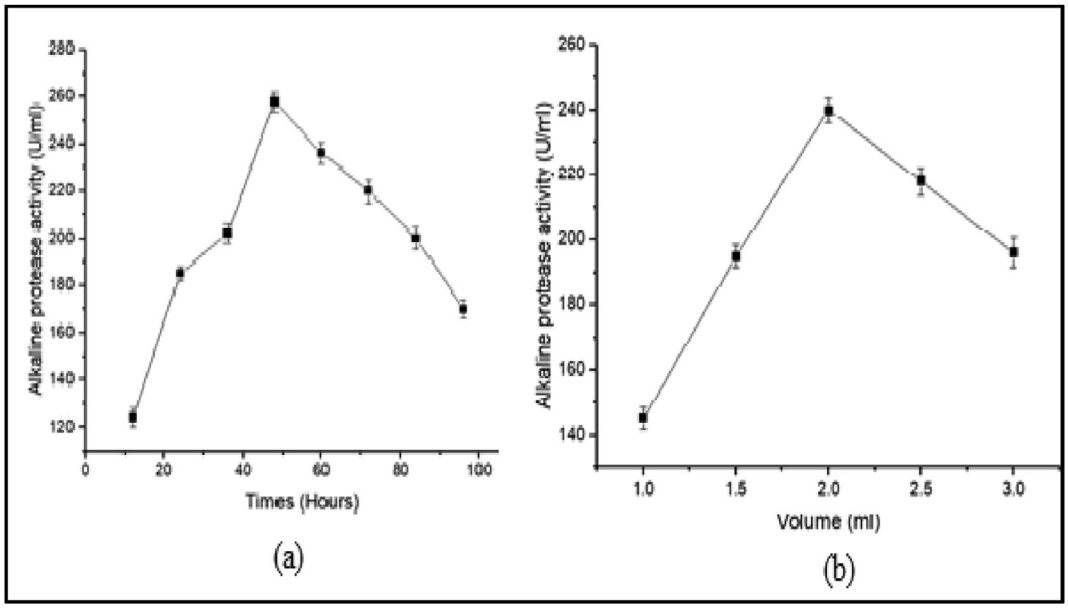

Fig. 3. (a) Effect of incubation time, and (b) Inoculum size on alkaline protease Production.

protein hydrolysis (30 mm) and protease activity (220 U/ml). This strain was selected for further use. *Paenibacillus* isolates BT7 produced medium colonies with circular edges, convex elevation, and an entire boundary (*Bacillus* spp). Endospore development and the ability to grow and produce enzymes optimally at elevated temperatures are two physiological and phenotypic features. BT7 was also a rod-shaped gram-positive bacterium [34]. Using a 1% agarose gel and a 1.5 Kb DNA ladder, the amplified 16S rRNA gave a 1500 bp DNA fragment an intact band. In addition, the 16S rRNA gene analysis of isolate BT7 indicated the highest sequence similarity (99%) to the genus *Paenibacillus*, indicating that it belongs to the *Bacillus* species. Isolate BT7 has a high association with *Paenibacillus dendritiformis*, according to phylogenetic relationship analysis using the neighbor-joining method (Fig. 2), which is 99.9% identical.

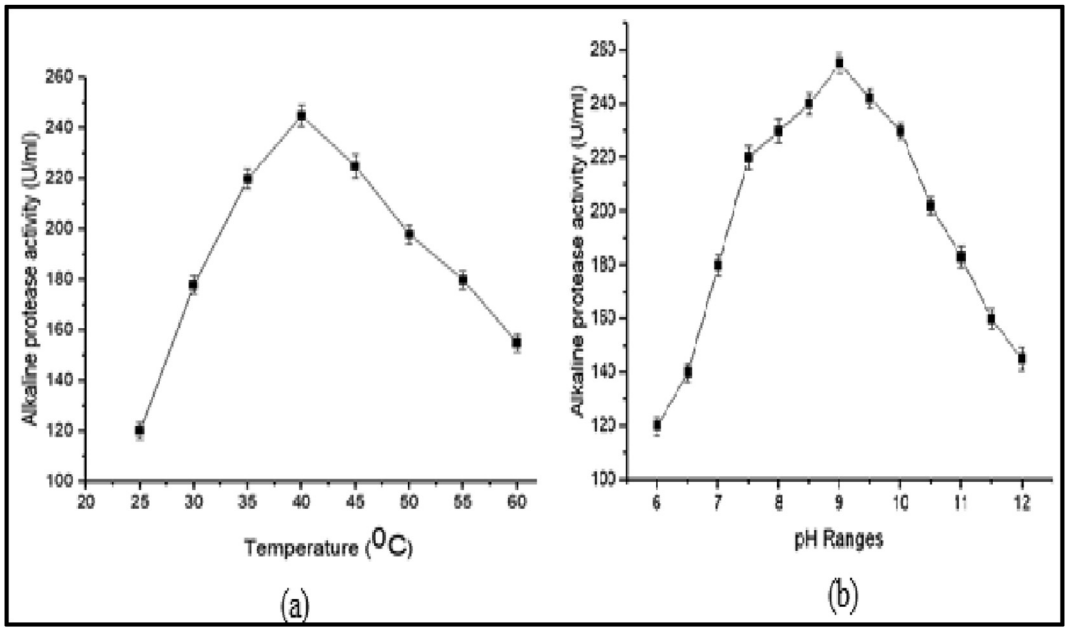

Fig. 4. (a) Effect of temperature (b) pH on alkaline protease production.

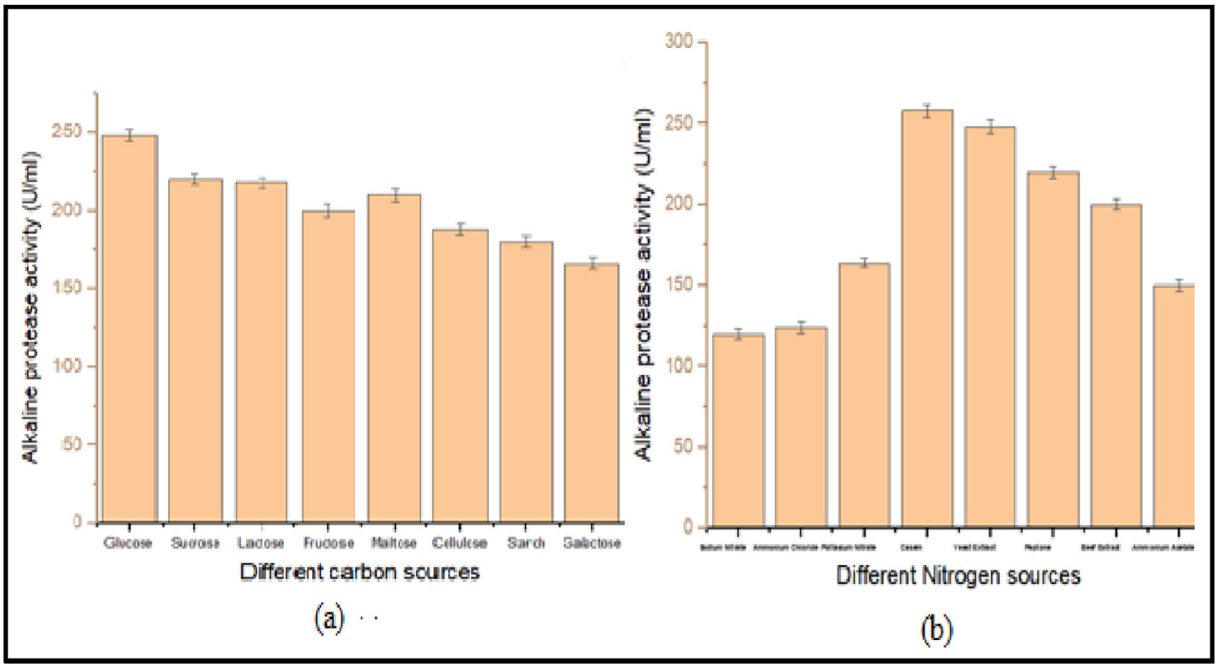

Fig. 5. (a) Effect of different carbon sources, (b) Different nitrogen sources on alkaline protease production.

The selected *P. dendritiformis*-BT7 for media optimization, mass production, and detergent industry application is illustrated below:

3.3. Media optimization of protease production conditions

Optimization factors such as incubation time, inoculum size, temperature, pH, sodium chloride concentration, different carbon sources, metal ions, and nitrogen sources that may influence the production of protease enzyme were optimized in the media for maximum activity of *P. dendritiformis*-BT7.

3.3.1. Effect of incubation time on alkaline protease production

The effects of incubation time (12–96 h) on the isolate *P. dendritiformis* - BT7's growth and protease

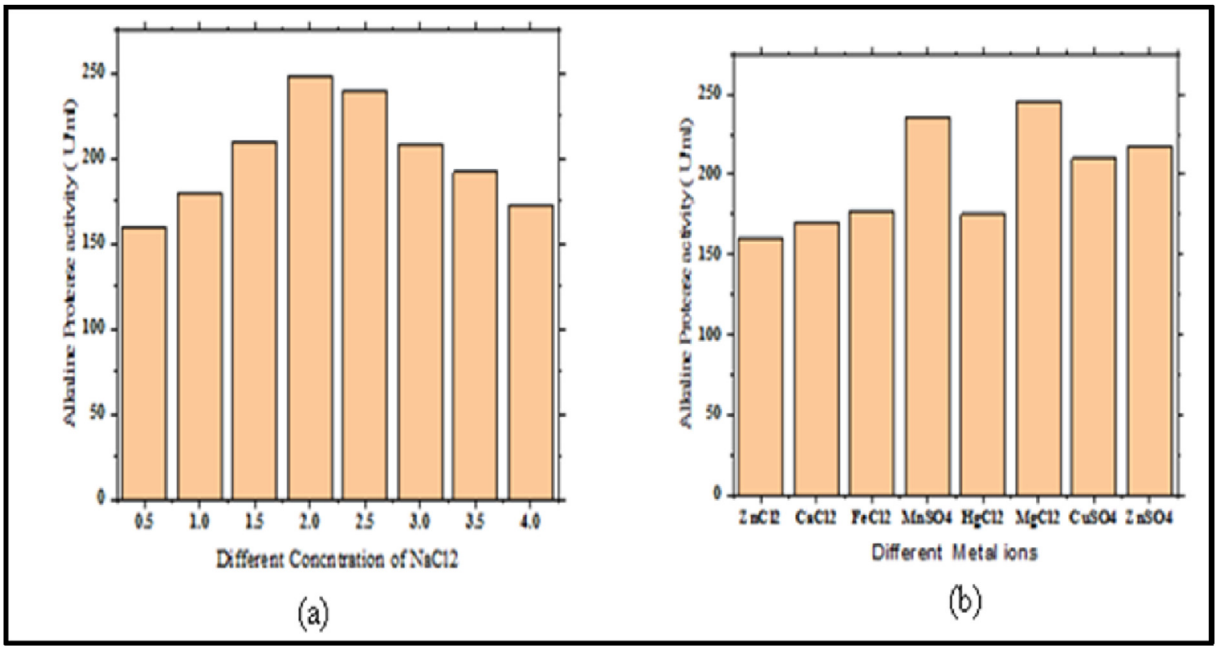

Fig. 6. (a) Effect of salt concentration; (b) Effect of metal ions on alkaline protease production.

Table 3. Summary of the partial purification of an alkaline protease.

Purification technique	Volume (mL)	Protein (mg/mL)	Total protein (mg)	Alkaline Protease activity (U/mL)	Total activity (U)	Specific activity (U/mL)	Yield (%)
Culture supernatant	500	0.35	175	270	13500	77.14	99
Ammonium Sulphate precipitation (75%) and dialysis	20	2.2	44	295	5900	134	3%

production were investigated in this study. In this investigation, 48 hours of incubation was the optimal time for maximum protease production (Fig. 3a). These results have shown a similar observation to Chu [35]. He worked on a *Bacillus* sp. strain that made a protease that could work for 48 hours at a time.

3.3.2. *Effect of inoculum size on alkaline protease production*

The alkaline protease production was optimized using different inoculum sizes ranging from 1% to 3% v/v of *P. dendritiformis*-BT7. At a 2% v/v inoculum size, the protease activity in organisms increased to a maximum of 240 ± 0.43 U/mL as the inoculum size was raised (Fig. 3b). These results were the following: Odu & Akujobi [36], reported that a 2% inoculum size gives higher protease production. These findings follow Nadeem et al. [37], who found that a 2% v/v inoculum size of *Bacillus licheniformis* N-2 culture resulted in the highest enzyme activity. The generation of alkaline protease decreased as the inoculum size grew larger. There may be less food or enzymes that break down when there are a lot of bacteria in the mix. These results reveal less growth and production of alkaline protease [38,39].

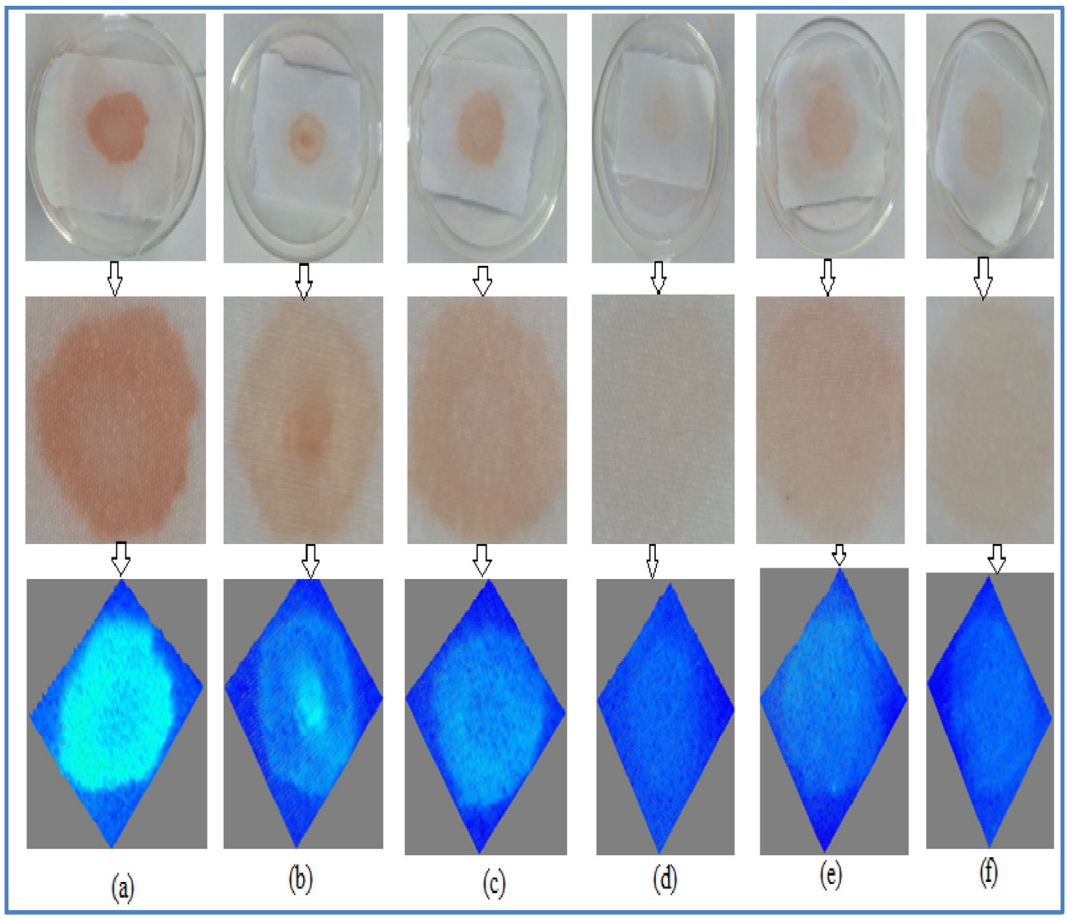

Fig. 7. The compatibility of alkaline protease with commercial detergents and its efficacy in blood stain removal (a) Negative control water (10 mL), (b) Water (5 mL) with Enzyme (5 mL), (c) Water (2.5 mL) with Enzymes (7.5 mL), (d) Enzymes (10 mL), (e) Enzymes (5 mL) with Detergent (5 mL), (f) Positive control Detergents (10 mL).

3.3.3. Effect of temperature on alkaline protease production

The temperature has been identified as one of the most important parameters influencing bacteria's growth and ability to produce alkaline protease enzymes. As a result, isolate BT7 was grown under different conditions (25, 30, 35, 40, 45, 50, 55, and 60 °C). *P. dendritiformis*-BT7produce protease best at 35–45 °C in this study (Fig. 4a), with the maximum growth at 40 °C (242 ± 0.32 U/mL). In addition, Fig. 5 shows that protease enzyme production occurred between 35 °C and 45 °C, with a maximal activity of 220 ± 0.14 U/mL and 222 ± 0.38 U/mL, respectively. Results that agreed with earlier findings were reported for other proteases. For protease from *Bacillus pumilus* D3 [39], *Bacillus cereus* SU12 [40], and *Bacillus* sp. For example, in SSR1 [13], the optimal temperature was 40 °C.

3.3.4. Effect of pH on alkaline protease production

All enzymatic processes and the movement of various components through the cell membrane are affected by the pH of the culture. The molecular basis of pH altering bacterial metabolism in the culture broth, on the other hand, is unknown. Because the medium pH value affects the proton motive force in chemiosmosis, relative metabolic efficiency may be high in the optimum pH range [16]. Changes in pH significantly affected the bacteria isolate's growth and activity. The pH change was found to play an important role in the bacteria's growth and protease activity. At pH 9, the greatest growth and protease production were seen (Fig. 4b). Similar results were reported with *Bacillus cereus* strain AT [41]; *Bacillus subtilis* [42]; *Bacillus* sp. SP-5 [43]; *Bacillus* sp. Cab44 [33]; *Bacillus* sp. [44]; *Bacillus* sp [45]. and *Bacillus subtilis* [46].

3.3.5. Effect of carbon sources on alkaline protease production

Glucose, sucrose, lactose, fructose, maltose, cellulose, starch, and galactose were used to examine the effects of various carbon sources on cell growth and protease production. Isolate *Paenibacillusdendritiformis*-BT7 grew well on most of the carbon sources used in this study. Glucose has the highest protease activity among the carbon sources, with 248 ± 0.72 U/mL, followed by sucrose with 228U/ml (Fig. 5a). *Bacillus circulans* and *Bacillus licheniformis* N-2 both make the most protease when fed glucose [47,48].

3.3.6. Effect of nitrogen sources on alkaline protease production

The growth and protease production of isolate *P. dendritiformis*-BT7 were studied using several nitrogen sources such as $NaNO_3$, NH_4Cl, KNO_3, casein, yeast extract, peptone, and ammonium acetate. Isolate BT7 grew the fastest and produced the most protease (258 ± 0.78 U/mL) when casein was used as a nitrogen source, followed by yeast extract (Fig. 5b). For growth and metabolism, various organisms require various nitrogen supplies. Suleiman et al. [7] found that supplementing bacteria with 1% (w/v) casein enhanced their protease output. Protease production was increased by adding 1% (w/v) casein in *Bacillus* sp. Y. [49]. At 34.57 U/mg, *Bacillus pseudofirmus* SVB1 makes more alkaline protease when casein is present [50].

3.3.7. Effect of salt concentrations on alkaline protease production

Figure 6a indicates that the isolate *P. dendritiformis*-BT7 could grow in a wide range of NaCl concentrations from 0.5 to 4%, with optimum growth and protease production activity (248 ± 0.32 U/mL) at 2% NaCl (Fig. 6a). and appreciable activity at 1.5 and 2.5%. The result was in line with Divakar et al. [51] reports, with the maximum protease production of 2% (NaCl) by *Thermoactino mycesthalpophilus*.

3.3.8. Effect of metal ion concentrations on alkaline protease production

For the isolate *P. dendritiformis*-BT7, the highest level of protease production (245 ± 0.54 U/mL) was observed in the presence of Mg^{2+} (Fig. 6b). Hg^{2+}, Zn^{2+}, and Ca^{2+} won't affect enzyme production, but the other metal ions tested exhibited a decrease in protease production with a complete absence in the presence of Zn^{2+}. According to Jellouli et al. [52], adding Mg^{2+} to the protease from *Bacillus licheniformis* MP1 increased enzyme activity by about 13%. Magnesium deficiency in the culture broth decreases glycolysis because several glycolytic

Table 4. Bloodstain removal of protease activity.

Stain	Control Water (10 ml)	Water (5 ml) + enzymes (5 ml)	Water (2.5 ml) + enzymes (7.5 ml)	Enzymes (10 ml)	Enzymes (5 ml) + Detergent (5 ml)	Detergent (10 ml)
	(a)	(b)	(c)	(d)	(e)	(f)
Blood	*	**	***	****	**	***

* = Poor removal of the blood stain; ** = Good removal of the blood stain.
*** = Very good removal of the blood stain; **** = Excellent removal of the blood stain.

enzymes require it as a cofactor [37]. In addition, the cations protect the enzyme from being damaged by heat and are important for keeping the enzyme in an active state at high temperatures [14].

3.4. Partial purification of alkaline protease

The alkaline protease was produced in 1000 mL conical flasks containing the basal medium with media-optimized parameters. The pH of the medium was adjusted to 9.0 after 48 hours at 37 °C. The crude protease sample was precipitated with 75% saturated ammonium sulphate followed by dialysis. In the crude extract, the enzyme activity (protease) was 270 ± 0.18 U/mL and 175 mg/mL. After ammonium sulphate and dialysis (partially purified enzyme), the enzyme activity (protease) was 295 ± 0.45 U/mL, and 44 mg/mL (below Table 3). According to Hakim et al. [11], the partially purified protease of bacterial isolates was significantly active over 7.0–12.0 pH, with the highest activity at pH 9. Temiz et al. [53] studied the 20–40% ammonium sulphate precipitate for partial purification of alkaline protease, which gave the maximum specific activity.

3.5. The effectiveness of alkaline protease in removing blood stains and its compatibility with commercial detergents

In terms of potential industrial applications, we looked at the enzyme's compatibility with common detergents. With the commercial laundry detergents tested, the isolated protease showed substantial action. The enzyme was incubated for 1 hour with industrial laundry detergents, and it was shown that it retained up to 85–99% of its activity under these harsh circumstances [30,54]. While the protease alone was efficient in removing blood spots from fabrics, the best results were obtained when the protease was used in conjunction with a commercial laundry detergent (Fig. 7 and Table 4).

Because detergent enzymes must act on protein substrates bound to solid surfaces, this key feature makes them appealing as hard surface cleaner additives. Proteinaceous stains, common in laundry, may also benefit from their use. Alkaline proteases' effectiveness in removing blood stains from cotton cloth [34,38]. Proteases can be used in detergents to help cut down on the amount of detergent used, which can help cut down on global pollution levels [55].

4. Conclusion

The current study found that *P. dendritiformis* (BT7), a bacterium isolated from the Batu tannery, is a good source of enzymes for a variety of industrial uses. In the current research, *P. dendritiformis* (BT7) alkaline protease was isolated, screened, identified, optimized, characterized, and purified. At pH 9.0, 2% inoculum volume, 40 °C temperature, and 48 hours of incubation, this alkaline protease displayed maximum activity. Furthermore, after being partially purified, the alkaline protease showed the highest enzyme activity. As a result, *P. dendritiformis* BT7's alkaline protease could be a suitable enzyme for ecologically friendly detergents. As a result, it was recommended that the detergent industry use these microorganisms.

Author contributions

Concept: Degafneh Tadesse and Chandran Masi, Methodology: Degafneh Tadesse, Chandran Masi, and Abate Ayele. Data analysis: Degafneh Tadesse, Chandran Masi, and Abate Ayele. Project administration: Chandran Masi, Supervision: Chandran Masi, Writing: Degafneh Tadesse, Original draft: Degafneh Tadesse, Review: Abate Ayele Editing: Chandran Masi, Final approval of the article: The published version of the manuscript has been read and approved by all authors.

Data availability statement

The data described in this work are openly available in [repository name, e.g., NCBI] at [Doi: https://www.ncbi.nlm.nih.gov/nuccore/MZ618946.1/], reference number [GenBank: MZ618946.1].

Funding

This project funded by the Directorate of Research and Technology Transfer at Addis Ababa Science and Technology University, provided internal research for this study (Ref No: IBC 06/2011).

Institutional review board statement

"Not Applicable" for studies not involving humans or animals.

Informed consent statement

"Not Applicable" for studies not involving humans.

Conflicts of interest

The author claims that the publishing of this paper has no conflicts of interest. Plagiarism, informed permission, misconduct, data fabrication and/or falsification, duplicate publishing and/or

submission, and redundancy were some of the ethical concerns the authors addressed.

Acknowledgements

For their cooperation with this study, we would like to thank our President, Vice President, College Dean, Head of Department, and Lab Coordinator in the Department of Biotechnology, College of Biological and Chemical Engineering.

References

[1] F. Matkawala, N. Sadhana, K. Anil, N. Anand, Enhanced production of alkaline protease by *Neocosmospora* sp. N1 using custard apple seed powder as inducer and its application for stain removal and dehairing, Biocatal. Agric. Biotechnol. 21 (2019) 101–310, https://doi.org/10.1016/j.bcab.2019.101310.

[2] H. Mushtaq, A. Jehangir, S.A. Ganai, S. Farooq, B.A. Ganai, R. Nazir, Biochemical characterization and functional analysis of heat-stable high potential protease of *Bacillus amyloliquefaciens* strain HM48 from soils of Dachigam National Park in Kashmir Himalaya, Biomolecules 11 (2021) 117, https://doi.org/10.3390/biom11010117.

[3] C. Masi, C. Chandramohan, M.F. Ahmed, Immobilization of the magnetic nanoparticles with alkaline protease enzyme produced by *Enterococcus hirae* and *Pseudomonas aeruginosa* isolated from dairy effluents, Braz. Arch. Biol. Technol. 60 (2017) 1–18, https://doi.org/10.1590/1678-4324-2017160572.

[4] C. Zhou, H. Zhou, D. Li, H. Zhang, H. Wang, F. Lu, Optimized expression and enhanced production of alkaline protease by genetically modified *Bacillus licheniformis* 2709, Microb. Cell Fact. 19 (2020) 1–13, https://doi.org/10.1186/s12934-020-01307-2.

[5] H.P. Jadhav, M.S. Sonawane, M.H. Khairnar, R.Z. Sayyed, Production of alkaline protease by rhizospheric *Bacillus cereus* HP_RZ17 and *Paeni bacillusxylanilyticus* HP_RZ19, Environ. Dev. Sustain. 3 (2020) 5–13. https://DOI:10.1007/s42398-020-00096-z.

[6] T. Shafique, J. Shafique, S. Zahid, M. Kazi, O. Alnemer, A. Ahmad, Screening, selection and development of *Bacillus subtilis* apr-IBL04 for hyper production of macromolecule alkaline protease, Saudi. J. Biol. Sci. 28 (2021) 1494–1501, https://doi.org/10.1016/j.sjbs.2020.11.079.

[7] A.D. Suleiman, A. Rahman, H. MohdYusof, F. MohdShariff, N.A. Yasid, Effect of cultural conditions on protease production by a thermophilic *Geobacillus thermoglucosidasius* SKF4 isolated from Sungai Klah hot Spring Park, Malaysia, Molecules 25 (2020) 2609, https://doi.org/10.3390/molecules25112609.

[8] N. Thakur, M. Goyal, S. Sharma, D. Kumar, Proteases: industrial applications and approaches used in strain improvement, Biological Forum–An International Journal 10 (2018) 158–167. https://www.researchgate.net/profile/Dinesh-Kumar 231/publication/330661548.

[9] I. Danilova, M. Sharipova, The practical potential of *Bacilli* and their enzymes for industrial production, Front. Microbiol. 11 (2020) 1782, https://doi.org/10.3389/fmicb.2020.01782.

[10] C. Masi, G. Gemechu, M. Tafesse, Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent, Ann. Microbiol 71 (2021) 1–11, https://doi.org/10.1186/s13213-021-01631-x.

[11] A. Hakim, F.R. Bhuiyan, A. Iqbal, T.H. Emon, J. Ahmed, A.K. Azad, Production and partial characterization of dehairing alkaline protease from *Bacillus subtilis* AKAL7 and *Exiguo bacteriumindicum* AKAL11 by using organic municipal solid wastes, Heliyon 4 (2018) e00646, https://doi.org/10.1016/j.heliyon.2018.e00646.

[12] H. Setegn, Chandran Masi, Mesfin Tafesse, Isolation and characterization of pectinase-producing bacteria (*Serratia marcescens*) from avocado peel waste for juice clarification, BMC Microbiol 22 (2022) 1–15, https://doi.org/10.1186/s12866-022-02536-8.

[13] J. Singh, N. Batra, R.C. Sobti, Serine alkaline protease from a newly isolated *Bacillus* sp, SSR1, Process Biochem. 36 (2001) 781–785, https://doi.org/10.1016/S0032-9592(00)00275-2.

[14] K.M. Sharma, R. Kumar, S. Panwar, A. Kumar, Microbial alkaline proteases: optimization of production parameters and their properties, J. Genet. Eng. Biotechnol. 15 (2017) 115–126, https://doi.org/10.1016/j.jgeb.2017.02.001.

[15] J. Chapman, A.E. Ismail, C.Z. Dinu, Industrial applications of enzymes: recent advances, techniques, and outlooks, Catalysts 8 (2018) 238, https://doi.org/10.3390/catal8060238.

[16] 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. Sci. 3 (2014) 290–298. https://www.ijapbc.com/files/12-3216.pdf.

[17] B. Asha, M. Palaniswamy, Optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil, J. Appl. Pharm. Sci. 8 (2018) 119–127, https://doi.org/10.7324/JAPS.2018.8219.

[18] J. Ahmad, T.A. Ansari, Alkaline protease production using proteinaceous tannery solid waste, J. Petrol. Environ. Biotechnol. 4 (2013) 136, https://doi.org/10.4172/2157-7463.1000136.

[19] C. Masi, Immobilization of *Pseudomonas aeruginosa* in different matrices for the production of alkaline protease, Int. J. Recent Technol. Eng. 9 (2020) 956–959. https://doi:10.35940/ijrte.F9573.059120.

[20] G. Gemechu, C. Masi, M. Tafesse, G. Kebede, A review on the bacterial alkaline proteases, J. Xidian Uni. 14 (2020) 632–634, https://doi.org/10.37896/jxu14.11/022.

[21] A. Gessesse, B.A. Gashe, Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake, Biotechnol. Lett. 19 (1997) 479–481. https://DOI: 10.1023/a:1018308513853.

[22] M.S. Rahman, M.R. Islam, O.K. Mondol, M.S. Rahman, F. Sabrin, U.S. Zohora, Screening of protease producing bacteria from tannery wastes of leather processing industries at Hazaribag, Bangladesh, Jahangirnagar Univ. J. Biol. Sci. 7 (2018) 23–34, https://doi.org/10.3329/jujbs.v7i1.37970.

[23] K. Manorma, S. Sharma, H. Singla, K. Kaunda, M. Kaur M, Screening and isolation of protease producing bacteria from rhizospheric soil of apple orchards from Shimla District (HP), India, Int. J. Curr. Microbiol. Appl. Sci. 6 (2017) 249–255, https://doi.org/10.20546/ijcmas.2017.605.030.

[24] C. Suganthi, A. Mageswari, S. Karthikeyan, M. Anbalagan, A. Sivakumar, K.M. Gothandam, Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments, J. Genet. Eng. Biotechnol. 11 (2013) 47–52, https://doi.org/10.1016/j.jgeb.2013.02.002.

[25] S. Sankaralingam, M.V. Sundaram M, M. Chardrasekar, T. Shankar, D. Prabu, N.Y. Kumar, Solid state fermentation of protease production by newly isolated *Bacillus lexus*, Int. J. Adv. Sci. Res. 4 (2014) 66–78. http://www.rspublication.com/ijst/index.html.

[26] S. Wang, X. Lin, X. Huang, L. Zheng, D.S. Zilda, Screening and characterization of the alkaline protease isolated from PLI-1, a strain of *Brevibacillus* sp. collected from Indonesia's hot springs, J. Ocean Univ. China. 11 (2012) 213–218, https://doi.org/10.1007/s11802-012-1845-6.

[27] C. Masi, E. Balaji, J. Vigneshwar, C. Chandramohan, M.F. Ahmed, N. Parthasarathy, Isolation and production of proteolytic enzyme by bacterial strains by using agrowates as substrate, Asian J. Chem. 26 (2014) 2182–2184, https://doi.org/10.14233/ajchem.2014.16394.

[28] H. Takami, T. Akiba, K. Horikoshi, Production of extremely thermo stable alkaline protease from *Bacillus* sp. no. AH101, Appl. Microbiol. Biotechnol. 30 (1989) 120–124, https://doi.org/10.1007/BF00263997.

[29] R.P. Singh, P.N. Jha, Characterization and optimization of alkaline protease production from *Bacillus licheniformis* HSW-16 isolated from Sambhar salt lake, Int. J. Appl. Sci. Biotechnol. 3 (2015) 347–351, https://doi.org/10.3126/ijasbt.v3i2.12757.
[30] A.S. Ibrahim, Y.B. Elbadawi, M.A. El-Tayeb, K.S. Al-Maary, D.A.F. Maany, S.S.S. Ibrahim, A.A. Elagib, Alkaline serine protease from the new halotolerant alkaliphilic Salipaludi *bacillus agaradhaerens* strain AK-R: purification and properties, 3 Biotech 9 (2019) 1–11, https://doi.org/10.1007/s13205-019-1928-9.
[31] S. Aftab, S. Ahmed, S. Saeed, S.A. Rasool, Screening, isolation and characterization of alkaline protease producing bacteria from soil, Pak. J. Biol. Sci. 9 (2006) 2122–2126, https://doi.org/10.3923/pjbs.2006.2122.2126.
[32] P.S. Patil, S. Sabale, A. Devale, Isolation and characterization of protease producing bacteria from rhizosphere soil and optimization of protease production parameters, Int. J. Curr. Microbiol. Appl. Sci. 2 (2015) 58–64.
[33] T. Hamza, Isolation and screening of protease producing bacteria from local environment for detergent additive, Am. J. Life Sci. 5 (2017) 116–124, https://doi.org/10.11648/j.ajls.20170505.11.
[34] A.P. Pathak, K.B. Deshmukh, Alkaline protease production, extraction and characterization from alkaliphilic *Bacillus licheniformis* KBDL4: a Lonar soda lake isolate, Indian J. Exp. Biol. 50 (2012) 569–576. http://nopr.niscair.res.in/handle/123456789/14487.
[35] W.H. Chu, Optimization of extracellular alkaline protease production from species of *Bacillus*, J Ind Microbiol Biotechnol 34 (2007) 241–245, https://doi.org/10.1007/s10295-006-0192-2.
[36] N.N. Odu, C.O. Akujobi, Protease production capabilities of *Micrococcus luteus* and *Bacillus* species isolated from abattoir environment, Int J Microbiol Res 2 (2012) 127–132, https://doi.org/10.5923/j.microbiology.20120205.03.
[37] M. Nadeem, J.I. Qazi, S. Baig, Optimization of process parameters for alkaline protease production by *Bacillus licheniformis* N-2 and kinetics studies in batch fermentation, Turk. J. Biol. 32 (2008) 243–251. https://journals.tubitak.gov.tr/biology/vol32/iss4/3.
[38] S. Chakraborty, Z.F. Joy, A. Haque, A. Iqbal, S. Akhter, P.K. Sarker, M.A. Sayem, Optimization of production and partial characterization of cellulase and protease enzymes from *Aeromonashydrophila* ASM-S32, J. Adv. Biotechnol. Exp. Ther. 2 (2019) 103–113.
[39] B. Ozcelik, P. Aytar, S. Gedikli, E. Yardõmcõ, F. Çalõşkan, A. Çabuk, Production of an alkaline protease using *Bacillus pumilus* D3 without inactivation by SDS, its characterization and purification, J. Enzyme Inhib. Med. Chem. 29 (2014) 388–396, https://doi.org/10.3109/14756366.2013.788503.
[40] S. Umayaparvathi, S. Meenakshi, M. Arumugam, T. Balasubramanian, Purification and characterization of protease from *Bacillus cereus* SU12 isolated from *oyster Saccostreacucullata*, Afr. J. Biotechnol. 12 (2013) 5897–5908, https://doi.org/10.5897/AJB2013.12805.
[41] P. Vijayaraghavan, S. Lazarus, S.G.P. Vincent, De-hairing protease production by an isolated *Bacillus cereus* strain AT under solid-state fermentation using cow dung: biosynthesis and properties, Saudi J. Biol. Sci. 21 (2014) 27–34, https://doi.org/10.1016/j.sjbs.2013.04.010.
[42] N.S. Nisha, J. Divakaran, Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from seawater, Afr. J. Biotechnol. 13 (2014) 1707–1713. https://doi: 10.5923/j.microbiology.20120205.03.
[43] P. Sinha, R.K. Singh, R. Srivastva, R. Sharma, S.P. Tiwari, Characterization and optimization of alkaline protease enzyme produced by soil borne bacteria, Trends Life Sci 2 (2013) 2319–4731.
[44] A. AkhavanSepahy, L. Jabalameli, Effect of culture conditions on the production of an extracellular protease by *Bacillus* sp. isolated from soil sample of Lavizan Jungle Park, Enzyme Res 7 (2011), https://doi.org/10.4061/2011/219628.
[45] A. Khusro, One Factor at A Time-based optimization of protease from poultry associated *Bacillus licheniformis*, J. Appl. Pharm. Sci. 6 (2016) 88–95, https://doi.org/10.7324/JAPS.2016.60315.
[46] G. Pant, A. Prakash, J.V.P. Pavani, S. Bera, G.V.N.S. Deviram, A. Kumar, A.R. Prasuna, Production, optimisation and partial purification of protease from *Bacillus subtilis*, J. Taibah Univ. Sci. 9 (2015) 50–55, https://doi.org/10.1016/j.jtusci.2014.04.010.
[47] O. Nwokoro, O.H. Ekwem, U. Oyeagu, Extracellular alkaline protease production by *Bacillus licheniformis* in submerged fermentation, Annals. Food Sci. Technol. 18 (2017) 468–477. https://www.researchgate.net/profile/Ogbonnaya-Nwokoro/publication/352736844.
[48] D. Vaishnav, J. Suthar, T. Oza, G. Dave, N. Sheth, G. Sanghvi, A statistical approach for the enhanced production of thermostable alkaline protease showing detergent compatibility activity from *Bacillus circulans*, Biocatal. Biotransformation. 32 (2014) 151–160, https://doi.org/10.3109/10242422.2014.913579.
[49] S. Srividya, Influence of process parameters on the production of detergent compatible alkaline protease by a newly isolated *Bacillus* sp, Y, Turk. J. Biol. 35 (2011) 177–182. https://dergipark.org.tr/en/pub/tbtkbiology/issue/11705/139777.
[50] S. Sen, V.D. Veeranki, B. Mandal, Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1, Ann. Microbiol. 59 (2009) 531–538, https://doi.org/10.1007/BF03175142.
[51] G. Divakar, M. Sunitha, P. Vasu, P. Ellaiah, Optimization of process parameters for alkaline protease production under solid-state fermentation by *Thermoaction mycesthalpophilus* PEE 14, Indian J. Biotechnol. 5 (2006) 80–83. http://nopr.niscair.res.in/handle/123456789/5554.
[52] K. Jellouli, O. Ghorbel-Bellaaj, H.B. Ayed, L. Manni, R. Agrebi, M. Nasri, Alkaline-protease from *Bacillus licheniformis* MP1: purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization, Process Biochem 46 (2011) 1248–1256, https://doi.org/10.1016/j. procbio.2011.02.012.
[53] H. Temiz, N.S. Ustun, S. Turhan, U. Aykut, Partial purification and characterization of alkaline proteases from the Black Sea anchovy (*Engraulisencrasicholus*) digestive tract, Afr. J. Biotechnol. 12 (2013) 56–63, https://doi.org/10.5897/AJB12.2900.
[54] T. Manavalan, A. Manavalan, S. Ramachandran, K. Heese, Identification of a novel thermostable alkaline protease from *Bacillus megaterium*-TK1 for the detergent and leather industry, Biology 9 (2020) 472, https://doi.org/10.3390/biology9120472.
[55] B. Bhunia, A. Dey, Statistical approach for optimization of physiochemical requirements on alkaline protease production from *Bacillus licheniformis* NCIM 2042, Enzyme Res 2012 (2012) 905804, https://doi.org/10.1155/2012/905804.